Further expansion of methane metabolism in the Archaea

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Abstract

The recent discovery of key methane-metabolizing genes in the genomes from the archaeal phyla *Bathyarchaeota* and *Verstraetearchaeota* has expanded our understanding of the distribution of methane metabolism outside of the phylum *Euryarchaeota*. Here, we recovered two near-complete crenarchaeotal metagenome-assembled genomes (MAGs) from circumneutral hot springs that contain genes for methanogenesis, including the genes that encode for the key methyl-coenzyme M reductase (MCR) complex. These newly recovered archaea phylogenetically cluster with *Geoarchaeota* (deep lineage of archaeal order *Thermoproteales*), and the MCR-encoding genes clustered with the recently reported methanogens within the *Verstraetearchaeota*. In addition, genes encoding hydroxybutyryl-CoA dehydratase were identified in the newly recovered methanogens, indicating they might carry out the β-oxidation process. Together, our findings further expanded the methane metabolism outside the phylum *Euryarchaeota*.

Introduction

Methane-metabolizing archaea play a key role in the global carbon cycle as they are
major contributors to the formation or oxidation of methane (Reeburgh 2007; Thauer
et al., 2008). The recent discoveries of predicted methanogenic archaea in the phyla
Bathyarchaeota (Evans *et al.,* 2015) and *Verstraetearchaeota* (Vanwonterghem *et al.,*
2016) challenged our original hypothesis that methane metabolism originated from the
phylum *Euryarchaeota* (Gribaldo and Brochier-Armanet 2006), indicating the current
phylogenetic distribution of methanogens or archaeal methanotrophs will be expanded.
The methyl-CoM reductase (MCR) complex not only act as a key role in the
methanogenesis, but also activate methane to methyl-coenzyme M in anaerobic
methane oxidation process (Knittel and Boetius 2009; Orphan *et al.,* 2001). However,
a recently detected *Ca. Syntrophoarchaeum* organisms that was reported to catalyze
propane and butane oxidation via enzymes similar to methyl-coenzyme M reductase
(Laso-Perez *et al.,* 2016), indicating the MCR complex might not be limited to the
activation of methane in archaea. The similarity of *Ca. Syntrophoarchaeum* and
Bathyarchaeota MCR sequences suggests the *Bathyarchaeota* might play a role in
short-chain hydrocarbons oxidation. These results indicate the mechanisms associated
methane metabolism in archaea requires further study. In this study, culture-
independent metagenomics was used to recover previously unrecognized methane-
metabolizing archaea present in hyperthermal hot springs.

Recovery of crenarchaeotal genome with divergent MCR complex

In this study, we collected biomass from two different spring heads of Ulu Slim hot
spring (UShs), Malaysia. The temperatures of two spring heads were 89 °C (US89) and
80 °C (US80). A total of 43 metagenome-assembled genomes (MAGs) were recovered
from the co-assembled metagenome (US89, ~36 Gb; US80, ~36 Gb), including 1
Crenarchaeota-like (Cren_UShs, completeness of 93.4%) MAG, which contains genes
encoding for the MCR complex (*mcrABG*) and accessory proteins (*mcrCD*). One
additional *mcrABG* complex was detected from a contig (C_19902, 16.8 kb) that was
not recruited to any MAG. Using these newly recovered *mcrA* gene sequences, we

recovered several closely related *mcrA* sequences from two hot spring metagenomes:

Jinze hot spring (JZhs), Tengchong, China (Eloe-Fadrosh *et al.,* 2016) and Great boiling

hot spring (GBhs), Nevada, United States of America (Peacock *et al.,* 2013). Another

near complete (completeness of 99.0%) *Crenarchaeota*-like MAG that contains genes

that encode for a complete MCR complex in JZhs (Cren_JZhs, Supplementary Figure

S1 and Table S1) was recovered.

These newly recovered *mcrA* genes have a close relationship with homologues in

recently reported *Verstraetearchaeota* genomes (70-90% amino acid (aa) identity).

Phylogenetic analyses of the McrA amino acid sequences confirmed the newly

recovered *mcrA* gene sequences are basal to the *Verstraetearchaeota* homologues and

form a cluster distant to the *Euryarchaeota*, *Bathyarchaeota* and methanotrophic McrA

clades (Fig. 1a). The close relationship with *Verstraetearchaeota* was also confirmed

by the phylogenetic analysis of McrB (Supplementary Figure S2) and McrG

(Supplementary Figure S3). However, the low average genomic amino acid identity

(AAI, 45.9-48.4%, Supplementary Table S2) and variable co-located gene loci (Fig. 1b)

between the newly recovered MAGs and *Verstraetearchaeota* genomes suggest

Cren_UShs and Cren_JZhs are distinct to *Verstraetearchaeota*. A genome tree (Fig. 2a)

constructed using 56 concatenated ribosomal proteins (RPs) of 704 archaeal genomes

place the Cren_UShs and Cren_JZhs genomes adjacent to the *Geoarchaeota* (Kozubal

et al., 2013) which is a deep lineage of the archaeal order *Thermoproteales* (Guy et al.,

2014). Furthermore, the tetra-nucleotide word frequency pattern confirms the

Cren_UShs and Cren_JZhs are divergent from members in the phylum

Verstraetearchaeota (Fig. 2b and 2c). These phylogenetic and genome attributes

confirm that the newly recovered MAGs are previously unrecognized crenarchaeotal

archaea that are likely associated with methane-metabolism.

Methane metabolism

KEGG orthology (KO) based analysis revealed the metabolic features of Cren_UShs

and Cren_JZhs are highly similar to the *Verstraetearchaeota* and *Bathyarchaeota* genomes and distant to euryarchaeotal methanogens and methanotrophs (Supplementary Figure S4). Also, these newly recovered MAGs share more gene orthologues with *Verstraetearchaeota* populations (V1-V4, 566) than with *mcr*-containing *Bathyarchaeota* populations (BA1 and BA2, 225) (Supplementary Figure S5a), which was in line with the phylogenetic analyses of McrA. Only 160 and 156 gene orthologues were found to be unique in Cren_UShs and Cren_JZhs genomes, respectively, when compared to V2-V4 representatives of the phylum *Verstraetearchaeota* (Supplementary Figure S5b), indicating some different metabolic pathways have potentially been adopted by this newly recovered crenarchaeotal methanogens.

In addition to the key genes coding for MCR complex (*mcrABG*), core methane metabolism pathway genes in Cren_UShs and Cren_JZhs (Fig. 2d) resemble the hyperthermophilic hydrogenotrophic methanogen *Methanopyrales kanderli* (Slesarev *et al.,* 2002). However, *mcr* gene and genome trees place these organisms basal to *Verstraetearchaeota* and other H_2-depdendent methylotrophs, suggesting these methylotrophs may have lost the ability for hydrogenotrophic methanogenesis (Borrel *et al.,* 2016), while the hyperthermophilic *Crenarchaeota*-like organisms have retained this function (Fig. 3). Most of genes encoding methyl-H_4MPT S-methyltransferase (Mtr) were not found in *Verstraetearchaeota* and *Bathyarchaeota*. In contrast, *mtrABCDEFGH* genes are all found in these two MAGs (Supplementary Table S3). The remaining genes for the hydrogenotrophic methanogenesis or anaerobic methane oxidation are identified in the present study, which are missing from the methane-metabolizing archaea within *Verstraetearchaeota* and *Bathyarchaeota.*

Genes involved in the reduction of heterodisulfide (CoM-SS-CoB) formed during the final step of methanogenesis are identified in these two MAGs. The heterodisulfide reductase (Hdr) and F_{420}-non-reducing hydrogenase (Mvh) for the conventional CoM-SS-CoB are found in Cren_UShs and Cren_JZhs, while genes of *mvhA* and *mvG* were

not identified in these two archaea genomes. Besides that, the genes for HdrD are in three copies in both Cren_UShs and Cren_JZhs, and two copies in each MAG are co-located with genes with flavin adenine dinucleotide-containing dehydrogenase (*glcD*), which is in line with the previous reported methane-metabolizing archaea within *Verstraetearchaeota* and *Bathyarchaeota*. The *glcD* coupled *hdrD*, therefore, may be adopted in these newly recovered archaea to reduce CoM-SS-CoB with carboxylate utilization (Evans *et al.,* 2015; Hocking *et al.,* 2014; Vanwonterghem *et al.,* 2016). In addition, homologues of the membrane-bound NADH-quinone oxidoreductase (Nuo), which might be the F_{420}-methanophenazine oxidoreductase (Fpo), are identified in Cren_UShs and Cren_JZhs and perform the re-oxidizing reduced ferredoxin. However, several subunits of fpo are not identified in these two MAGs (Supplementary Table S3) which might be complemented by energy-conserving hydrogenase B (Ehb) (Thauer *et al.,* 2008).

Other metabolic traits and environmental distribution

In addition to the methanogenic metabolism, genes for peptide, amino acid transporter and peptidase were found in Cren_UShs and Cren_JZhs, suggesting these organisms have potential for the degradation of extra-cellular peptide/amino acid (Fig. 3). It is similar to the members in the phylum *Verstraetearchaeota* that Cren_UShs and Cren_JZhs appears to be capable of using sugar to generate acetyl-CoA via the Embden-Meyerhof-Parnas (EMP) (Vanwonterghem *et al.,* 2016). Besides, the presence of genes encoding Acyl-CoA dehydrogenase, 4-hydroxybutyryl-CoA dehydratase and 3-hydroxyacyl-CoA dehydrogenase (Supplementary Table S4) suggests that β-oxidation process may be adopted in these newly recovered methanogens (Laso-Perez *et al.,* 2016). However, the genes for H_2-dependent methylotrophic methanogenesis that found in both *Bathyarchaeota* and *Verstraetearchaeota* populations are missing from Cren_UShs and Cren_JZhs genomes, indicating these *Crenarchaeota*-like methanogens conserve energy using the hydrogenotrophic methanogenesis. Comparing with the methanogens of the phyla *Verstraetearchaeota* and *Bathyarchaeota*, this study

is the first report of hyperthermophilic methanogens that were outside the phylum

Euryarchaeota, and genes encoding DNA reverse gyrase may afford thermal protection

for these two methanogens even at a low GC content (~44%) (Forterre 2002).

The microbial community of the US hot spring was dominated by crenarchaeotal

populations, while the newly recovered methanogenic archaea (Cren_UShs) only

accounted for 0.10% and 0.17% of the all recovered population genomes in US80 and

US89, respectively (Supplementary Figure S6). The environmental distribution of the

newly recovered methanogenic archaea we evaluated by public database searches for

mcrA genes. The best hits (>80% amino acid identity) to the Cren_UShs *mcrA* gene

sequence are mainly found in hot spring samples (Supplementary Table S5). The

number of identified *mcrA*-like sequences is as much as 1085 in one dataset of Jinze

hot spring, suggesting these novel archaea may play contribute to the methane cycling

in some thermophilic systems. One common trait of those samples that contain these

methanogens is the circumneutral condition.

In summary, the present study provided genomic evidence that methane metabolism

may be conducted in hyperthermophiles within *Crenarchaeota*. The genes for short-

chain hydrocarbon oxidation suggest these methane-metabolizing archaea may oxidize

short-chain hydrocarbon. However, the ecological roles of Cren_UShs and Cren_JZhs

are inferred by the genomic information, which need further physiological and omic

studies in the future.

Materials and methods

Sampling and sequencing

Water samples were collected from Ulu Slim hot spring in Malaysia (August 4, 2016)

at two different points, one was the main spring head (89°C) and another was the

secondary spring head (80°C). The US hot spring is a neutral hot spring with the highest

temperature of 104°C. For each sample a 1 L water sample of spring water at each

spring head was collected and *in-situ* filtered through a 0.45 μm hydrophilic PTFE

membrane (Whatman, USA). DNA was extracted from the filtered biomass using Fast

DNA Spin Kit for Soil (MP Biomedicals, France). DNA extracts from the 89°C (US89)

and 80°C (US80) hot spring water samples were sequenced on the Illumina HiSeq 4000

platform (Illumina, USA) using 150 bp paired-end reads with a 350 bp insert size.

Sequencing processing and metagenome binning

The US80 and US89 metagenomes were quality filtered and co-assembled using CLC's

de novo assembly algorithm (CLC GenomicsWorkbench v6.04, CLCBio, Qiagen) with

k-mer of 35 and minimum scaffold length of 1 kb, resulting in 47,532 scaffolds with

N50 of 5,337 bp, totaling 166.6 Mb. Metagenome assembled genomes (MAGs) were

recovered based on the coverage profile, *k*-mer signatures and GC content (Albertsen

et al., 2013). The completeness of recovered MAGs was assessed using CheckM (Parks

et al., 2015). All retrieved MAGs were firstly uploaded to Kyoto Encyclopedia of Genes

and Genomes (KEGG) GhostKOALA (Kanehisa *et al.,* 2016) for the preliminary

reconstruction of metabolic traits, and the MAG contained *mcrA* gene was submitted to

the Integrated Microbial Genomes Expert Review (IMG-ER) (Markowitz *et al.,* 2009)

system for genome annotation. The taxonomy of the putative methanogenic MAG was

evaluated by GenomeTreeTK v0.0.3 (https://github.com/dparks1134/GenomeTreeTK)

using classify_wf workflow. The relative abundance of recovered MAGs was estimated

based on the percentage of mapped reads of a given MAG to all the recovered MAGs,

which was normalized by the genome size of bins.

Data mining of newly recovered *mcrA* sequences

A total of 332 datasets were downloaded from public database that included

metagenomes from anaerobic digesters (78), hot springs (81) and hydrothermal vents

(222). Ublast (Edgar 2010) was used to screen these metagenome datasets (*e*-value of

1e-5 and accel of 0.5) against an *mcrA* sub-database which contains the newly

recovered and other reported *mcrA* sequences. The reads with a bitscore >50 and a top

hit to one of the newly recovered *mcrA* sequences were collected, and further filtered

with similarity to retain the hits with ≥80% similarity. Datasets that contains novel *mcrA*

gene sequences were subsequently assembled to recover new MAGs and *mcrA* genes.

Genome and functional gene trees construction

A concatenated set of 56 archaeal ribosomal proteins (RPs) were used to construct the

genome tree. RPs were extracted from 704 publicly available archaeal genomes and the

two newly recovered *Crenarchaeota*-like MAGs using hidden Markov models (HMMs).

These amino acid sequences were aligned using MUSCLE (Edgar 2004), and a genome

tree constructed from the concatenated alignments using FastTree (v2.1.7) under the

WAG and GAMMA models (Vanwonterghem *et al.,* 2016). The tree was visualized and

decorated using iTOL(Letunic and Bork 2016), and rooted using the DPANN

superphylum as an outgroup.

The key genes for the enzymes involved in methanogenic metabolism, i.e., McrA,

McrB and McrG, were extracted from the newly recovered MAGs and searched against

the NCBI nr database (March, 2018) using Blastp (Camacho *et al.,* 2009) to identify

similar homologues. Complete amino acid sequences from the phyla *Bathyarchaeota*,

Methanomassiliicoccales, *Methanobacteriales*, *Methanococcales*, *Methanosarcinales,*

Methanocellales, *Methanomicrobialesand* and *Verstraetearchaeota* were used to

generate a phylogenetic tree construction using FastTree (v2.1.7). The functional gene

trees were rooted using the *Bathyarchaeota*.

Functional genes and genome comparison

Open reading frames (ORFs) for the contigs/scaffolds that contained *mcr* genes were

predicted into using Prodigal (Hyatt *et al.,* 2010) and compared using Blastp. The

genoPlotR (Guy *et al.,* 2010) R package of was used to visualize the gene loci co-

located with the *mcr* genes.

Metabolic comparison across archaeal genomes and the newly recovered MAGs were

performed based on the classified KO groups. Briefly, annotation results of all archaeal

genomes in the KEGG database (Release 80.1) were firstly collected and combined

with the four high-quality *Verstraetearchaeota* genomes (V1-V4) and the newly recovered *Crenarchaeota*-like MAGs, resulting in 167 archaeal genomes. The KO table was then summarized based on the KEGG (161 archaeal genomes) and IMG/ER annotation results (*Verstraetearchaeota* genomes and newly recovered MAG) and further used in a principle component analysis (PCA).

Tetranucleotide frequency-principal components analysis (TNF-PCA) across the newly recovered MAGs and *Verstraetearchaeota* population genomes was used to identify the taxa distance, a genome within the order *Thermoproteales* (*Thermofilum uzonense*) was selected as outgroup genome. The scaffolds/chromosome of selected genomes were shredded into sequences with length of 5 kb (4 kb overlapping). Nucleotide frequency of each sequence (>2 kb) was calculated with oligonucleotide word size of four. The summarized 4-kmer table of compared genomes was used in PCA analysis. The average amino acid identity was calculated by CompareM (https://github.com/dparks1134/CompareM).

Conflict of Interest

The authors declare no conflict of interest.

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Data availability.

The sequences have been deposited at the NCBI Sequence Read Archive under BioProject no. PRJNA449277.

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Figure 1. Phylogeny of mcrA gene and gene loci of the contigs/scaffolds that carry MCR complex. a, McrA amino acid sequences of newly recovered crenarchaeotal methanogens and reported methanogenic organisms were used to construct the phylogenetic tree. b, Homologous similarity was calculated using Blastp, the dotted black lines indicate continuity loci where unrelated genes are not displayed, and parallel double lines symbolize a break in locus organization (different contigs). MCR complex was colored in blue. Genes and noncoding regions are drawn to scale.

Figure 2. Genome comparison based on genome tree, sequence composition and distribution of genes involved in methane metabolism. a, Genome tree of 56 concatenated syntenic ribosomal proteins from 704 archaeal genomes, including two MAGs (Cren_UShs and Cren_JZhs) recovered in this study. b, Scaffolds/chromosome of selected genomes were shredded into sequences with length of 5 kb (4 kb overlapping). to perform the tetra-nucleotide word frequency PCA. c, Presence (blue)/absence (grey) of genes involved in methane metabolism and energy conservation for the newly recovered MAGs and reported Verstraetearchaeota (V1–V5) genomes. Annotation was based on the IMG/ER and KEGG GhostKOALA. Asterisks indicate not all subunits are identified in Cren_UShs and Cren_JZhs.

Figure 3. Reconstructed metabolism of the Cren_UShs and Cren_JZhs. The metabolic potentials of crenarchaeotal methanogens are primarily inferred based on the annotation results of IMG/ER. Genes found in both Cren_UShs and Cren_JZhs (black), only found in Cren_UShs (blue) are indicated, or missing from both genomes (gray). Dashed black arrows indicate genes that are missing or unknown in Cren_UShs and Cren_JZhs.