J.Natn.Sci.Foundation Sri Lanka 2023 51 (1): 149 - 158
DOI: http://dx.doi.org/10.4038/jnsfsr.v51i1.10779

RESEARCH ARTICLE

Environmental Microbiology

Insights into the ecological roles of assembling genomes for stimulated methanogenic archaea *Methanoculleus* in coal seams

BJ Liu and Y Li *

State Key Laboratory of Mining Response and Disaster Prevention and Control in Deep Coal Mines, Anhui University of Science and Technology, Huainan City, Anhui Province, 232001, China.

Submitted: 08 November 2021; Revised: 23 August 2022; Accepted: 27 January 2023

Abstract: Biogenic coalbed methane is produced by biological processes mediated by the synergistic interaction of microbial complexes in coal seams. However, the comprehensive ecological role of methanogenic archaea in biogenic coalbed methane production remains poorly understood. Here, we study the metagenome assembled genomes (MAGs) of *Methanoculleus* from coal seams, which were stimulated by minimal salts plus yeast media in anaerobic conditions. The *Methanoculleus* genus accounted for the highest proportion of archaea (80.4 ± 2.8%) once the CH_4 concentration in the headspace increased to 15.0 ± 2.6% on the 50th day. The *Methanoculleus* MAGs were closely related to *M. thermophiles*; even so, 30 genes were detected in MAGs which were lacking in the genomes of *M. thermophiles* ATCC 33837. A deeper look at the metabolic pathway showed several metabolic pathways, including methanogenesis, glycolysis, urea cycle, TCA cycle and sulphur reduction. The CO_2 and acetate were the primary carbon sources of these cells for the methanogenesis pathway. Glycolysis and sulphate reduction processes were the main processes for providing acetate. In addition, the cells had a variety of other functions, including nitrogen fixation and hydrogen production. Overall, this study enabled a better understanding of the ecological roles of *Methanoculleus* for biogenic methane in coal seams by combining bioinformatic techniques.

Keywords: Metabolic pathway, metagenome assembly, methanogenesis, sulphate reduction.

INTRODUCTION

Coalbed methane (CBM) is an important medium for transforming global energy utilisation. The harm to the environment caused by this simple organic matter is much lower than that caused by the exploitation of fossil energy, including oil and coals (Ritter *et al.*, 2015; McLeish *et al.*, 2021). CBM mainly includes thermogenic and biogenic processes in its formation. A staggering proportion (20-40%, even higher under favourable biogeologic conditions) of global methane reserves are biogenic, being mediated by the synergistic interaction of microbial complexes (Thielemann *et al.*, 2004; Faiz & Hendry, 2006; Rathi *et al.*, 2019). Therefore, the biogenic coalbed methane formation mechanism and development have gradually attracted more attention.

To our knowledge, the formation of biogenic coalbed methane is a very complex process which involves a series of functional groups, including hydrolysing and fermenting bacteria, hydrogen and acetogen producing bacteria, and methane-producing archaea (Wang *et al.*, 2018; Vick *et al.*, 2019a). The researchers on coal seam microorganisms mainly focused on the abundance and activity of methanogenic archaea, besides microbial diversity (Szafranek-Nakonieczna *et al.*, 2018; Plyatsuk *et al.*, 2020), because these methanogenic groups are the drivers of the final step in the degradation of organic matter in coal seams into methane, converting CO_2, H_2, acetate, formate, or other simple compounds in the coal seams into methane (Vick *et al.*, 2019a).

* Corresponding author (liyang20130104@163.com; https://orcid.org/0000-0002-8946-3962)

The methanogens that exist in coal seams are characterised by a variety of metabolic strategies, including strict hydrogenotrophs (such as *Methanobacterium* and *Methanoregula*), acetotrophs (*e.g.*, *Methanothrix*), and formate utilisers (*e.g.*, *Methanoregula*). However, the methanogenic archaea that drive the secondary biogenic CBM are diverse in coals from different regions. *Methanosarcina* were dominant archaea in the pristine Pniówek coal due to their metabolic flexibility (Pytlak *et al.*, 2020). *Methanosaeta* and *Methanobacterium* were dominant groups in the coals from the Konin Basin (Bucha *et al.*, 2020). *Methanobacterium* and *Methanosarcina* were dominant in the southern Qinshui Basin (Li *et al.*, 2020). However, the understanding of these methanogens has been limited to what they are and how to stimulate them. The ecological function of these archaea has not been thoroughly explored, which helps understand the formation mechanism of biogenic CBM.

Several researchers have attempted to stimulate the production potential of biogenic coalbed methane through various methods, including nutrients (Jones *et al.*, 2010; In't Zandt *et al.*, 2018), exogenous strains (Wang *et al.*, 2016) and other chemical means (Beckmann *et al.*, 2019; Webster *et al.*, 2019). In this study, it was found out that some methanogens can be stimulated and enriched by nutrients. Furthermore, we successfully constructed the metagenome assembled genomes (MAGs) of the foremost methanogenic archaea, *Methanoculleus* sp., from the metagenome of microbial communities in coal seams, using bioinformatics methods. By inferring the metabolic remodelling of these organisms from high-quality MAGs, the potential ecological functions of these organisms in coal seams are further explored. This study greatly expands our current understanding of methanogenic archaea *Methanoculleus* sp. in coal seams.

MATERIALS AND METHODS

Sample collection, culturing, DNA extraction, and sequencing

Coal samples were collected from 1412A coal working face of Zhangji Coal Mine (116°29′ E, 32°46′ N) in Huainan Mining Industry Co., Ltd., China. The coal samples at a distance of 15 m from the coal wall were taken by a coring rig (ZYD-3200S, Φ 108 mm), with a length of about 80-100 mm. The coal samples were surface-stripped with a sterile knife, placed in sterilised vacuum bags, and transported to the laboratory using an ice cooler. The maceral group composition (vitrinite, inertinite and exinite) in coal was analyzed based on ISO 7404-3: 2009, methods for the petrographic analysis of coals – Part 3: Method of determining maceral group composition, MOD. The proximate analysis indicators, including air dried basis moisture (M_{ad}), air dried basis ash (A_d), and dry ash-free volatiles (V_{daf}), were measured by an industrial analyzer (SDTGA8000, Sande, Changsha, China). The air dried basis total sulphur ($S_{t,d}$) was analyzed by an automatic coal sulphur meter (TKCL-6000, Tianke, Hebi, China). The basic properties of the coal sample were as follows: vitrinite 63.50%, inertinite 25.40%, exinite 11.10%, M_{ad} 1.93%, A_d 10.77 %, V_{daf} 33.36 %, $S_{t,d}$ 0.61%.

To stimulate the methane producing capacity of the coal, the coal samples were cultured in anaerobic conditions in minimal salts plus yeast media (MSY) that consisted of NH_4Cl 0.1 g/L, $K_2HPO_4 \cdot 3H_2O$ 0.4 g/L, $MgCl_2 \cdot 6H_2O$ 0.1 g/L, yeast extract 1 g/L, and trace element solution 1.0 mL/L (Li *et al.*, 2008). The coal samples with medium were cultured using 500 mL sterile culture bottles at 37 °C, the headspace air in the sterile culture bottles was replaced with high purity nitrogen and sealed with butyronitrile plugs.

To continuously monitor the methane production of the coal samples, the CH_4 concentration in the headspace of the sterile culture bottles was measured with a gas chromatograph equipped with a thermal conductivity detector (TCD) and a TDX-01 packed column using N_2 as a carrier gas. The temperatures of the detector, injector, and oven were 100, 105, and 60°C, respectively. The methane content in the headspace air increases slowly after 40 days, thus we collected samples on the 50th day.

After culturing, total DNA was extracted with the FastDNA® SPIN Kit for soil (MP Biomedicals, Cleveland, OH, USA) according to the manufacturer's instructions, each of the coal samples was subjected to metagenomic sequencing. Metagenomic sequencing was conducted with an Illumina Hiseq4000 by LC-Bio Technology Co., Ltd. (Hangzhou, China).

Metagenomic assembly and genome binning

The adapters and duplicated sequences for the raw sequencing data were removed by Trimmomatic. In addition, low quality reads with length < 50 bp and/or average phred value < 20 in a 4 bp sliding window were trimmed at both ends. The high-quality reads were assembled using Metahit (v1.2.8) with the parameter "-k 21, 29, 39, 59, 79, 99, 119, 141". All the high-quality reads were mapped onto assembled scaffolds by BBMap with the parameters "minid = 0.97, local = t". The coverage information of scaffolds was computed by the script jgi_summarize_bam_contig_depths, and these scaffolds were binned into metagenome assembled genomes (MAGs) by MetaBAT2 with the parameters "-m 1500". In addition, the genome completeness, potential contamination and strain heterogeneity of each MAG was evaluated by CheckM.

Analyses of genome bins

The binned genome was submitted in RAST for annotation and classification. Taxonomic affiliations of MAGs were determined with GTDB-Tk (Chaumeil *et al.*, 2020). Besides, the IQ-TREE was used to create a phylogenomic maximum-likelihood tree with the alignment of concatenated protein sequences derived from single copy marker genes retrieved from GTDB-Tk. In addition, the phylogenetic tree of protein coding sequences of desulphoferrodox in FeS_4 iron-binding domain-containing protein, putative sulphite reductase, and archaeal distant homology with glucose-6-phosphate isomerase were obtained from the reconstructed genome. The tree was constructed using MEGA-X by neighbour-joining tree and bootstrap method based on multiple protein sequence alignment (MUSCLE). The reference protein sequences were downloaded from NCBI.

RESULTS AND DISCUSSION

Binning results and identification

Genomic reconstruction of metagenomes is of great significance for understanding functional microorganisms (Hua *et al.*, 2019) in coal seams. Methanogenic archaea genomes assembled from metagenomes are helpful in revealing the biogeochemistry (Anantharaman *et al.*, 2016) of coal seam and their importance to the formation of biogenic methane. In this study, it was not possible to obtain all functional microbes in coal seam through nutrient stimulation. But the implementation of nutrient addition is conducive to understanding the methanogens with enrichment potential in the study area, which plays an important role in directly applying microorganisms to production. In this study, the CH_4 concentration in the headspace were increased to 15.0 ± 2.6% at the 50th day, which showed that a certain amount of methane was generated by stimulating the microbial activity of coal seam with nutrients. The *Methanoculleus* genus accounted for the highest proportion of archaea (80.4 ± 2.8%). In addition, 3 high-quality *Methanoculleus* MAGs (completeness > 90%, contamination < 5%) were successfully reconstructed from metagenomes that were sequenced from coal samples, after binning from the metahit generated assembly file (Table 1). From the GTDB-Tk analysis and phylogenetic tree based on the single copy marker genes (Figure 1), these three bins were highly related to *Methanoculleus thermophilus* ATCC 33837 (GenBank GCA_001571405.1). The binned genomes showed 61.3% GC content, and MAGs were classified as high quality with sizes ranging from 2.18 to 2.39 Mbp. Both rRNAs and tRNAs are detectable in MAGs. The results indicated that such *Methanoculleus* sp. were the main methanogens that are easily stimulated by nutrients in the study area. Coal seams are important underground anaerobic habitats in the world. The main methanogenic archaea in such anaerobic habitats differ greatly due to the difference of coal forming age (Liu *et al.*, 2019), pre coal material (Wang *et al.*, 2018), sedimentary environment (Tanikawa *et al.*, 2018) and microbial sources (Vick *et al.*, 2019b; Li *et al.*, 2021). *Methanosarcina* were dominant archaea in the pristine Pniówek

Table 1: Summary statistics of the *Methanoculleus* bins reconstructed from coal samples.

	Comp. (%)	Cont. (%)	GC	Contig N50 (Kbp)	Bases (Mbp)	No. of contigs	No. of CDS	No. of features	No. of tRNA	No. of rRNA
NL_1.7	97.71	2.614	0.613	238.83	2.39	16	2490	2739	49	3
NL_2.6	97.71	2.614	0.613	240.94	2.39	16	2498	2742	49	3
NL_3.1	96.40	2.614	0.613	151.64	2.18	33	2303	2526	50	4

Note: Comp. - completeness, Cont. - contamination

coal due to their metabolic flexibility (Pytlak *et al.*, 2020). *Methanosaeta* and *Methanobacterium* were dominant groups in the coals from the Konin Basin (Bucha *et al.*, 2020). *Methanobacterium* and *Methanosarcina* were dominant in the southern Qinshui Basin (Li *et al.*, 2020).

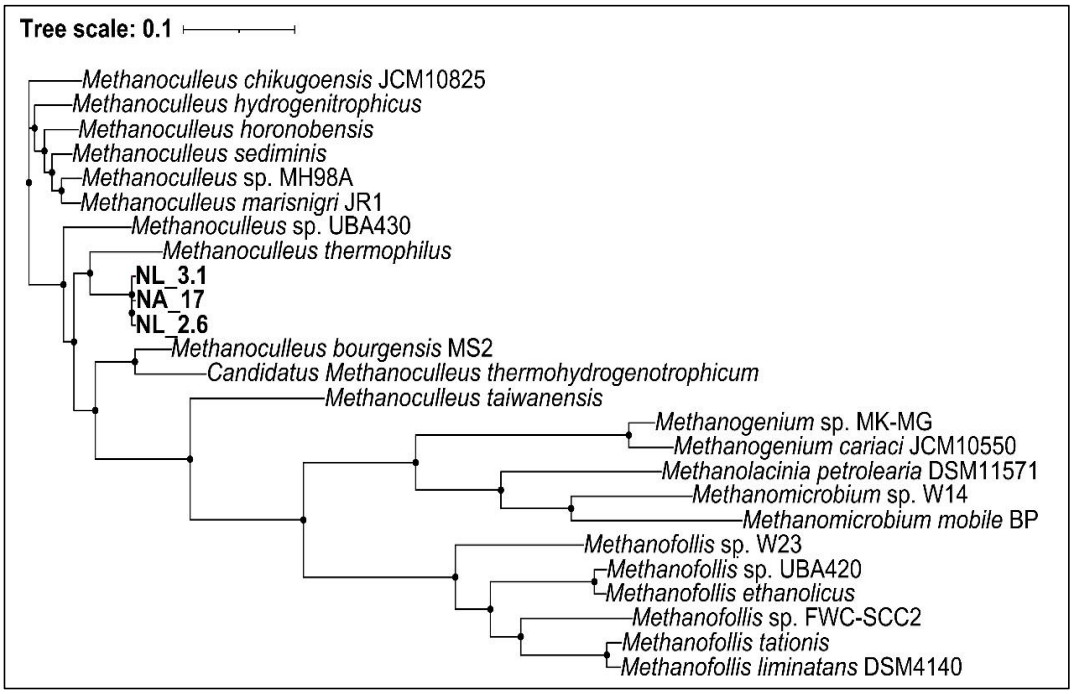

Figure 1: Phylogenetic tree of *Methanoculleus* MAGs. The phylogenetic tree was analyzed by GTDB-Tk (v1.3.0) based on their genome sequences and visualized by itol (*https://itol.embl.de/*).

Main metabolic pathway

From RAST annotation it was mined that the MAGs possessed the essential metabolic genes required for a normal archaeal cell to flourish in the anaerobic environments. The genome of MAGs showed the genes in the category of carbon, nitrogen and sulphur metabolism. The archaea showed transport and utilisation of multiple amino acid, tryptophan, biotin, carbohydrate, and inorganic acid (Supplementary Data 1-3). A deeper look at the metabolic pathway showed several metabolic pathways including methanogenesis, glycolysis, urea cycle, TCA cycle, and sulphur reduction (Figure 2). The CO_2 was the main carbon source for the methanogenesis pathway in these cells. Besides, acetate could also be utilised to produce CH_4 via serine metabolism.

In the special coal seam environment of this study area, *Methanoculleus* sp. could use CO_2 and acetate as the main metabolic substrates for methanogenesis. Among the metabolites of methanogenesis, CO_2 and acetate were the main precursors in coal seams. Beckmann *et al*. (2019) found that acetate is the primary energy carrier in the Sydney Basin coal reservoir. In addition, most biogenic CBM reservoirs are produced by CO_2 reduction (Zhang & Liang, 2017). In this study, the glycolysis pathway was still the main energy source of these methanogens in coal seams under anaerobic conditions. This pathway was an important process for *Methanoculleus* sp. to obtain acetate. In the process of glycolysis or the modified version, glucose-6-phosphate isomerase plays a central role in sugar metabolism of all the cells (Hansen *et al.*, 2001). And the archaeal distant homologue of glucose-6-phosphate isomerase was deemed as the first characterised archaeal member of the glucose-6-phosphate isomerase superfamily (Rudolph *et al.*, 2004), showing its importance in the archaeal metabolism. In addition, Rudolph *et al*. (2004) suggested that such genes might also be obtained from bacteria.

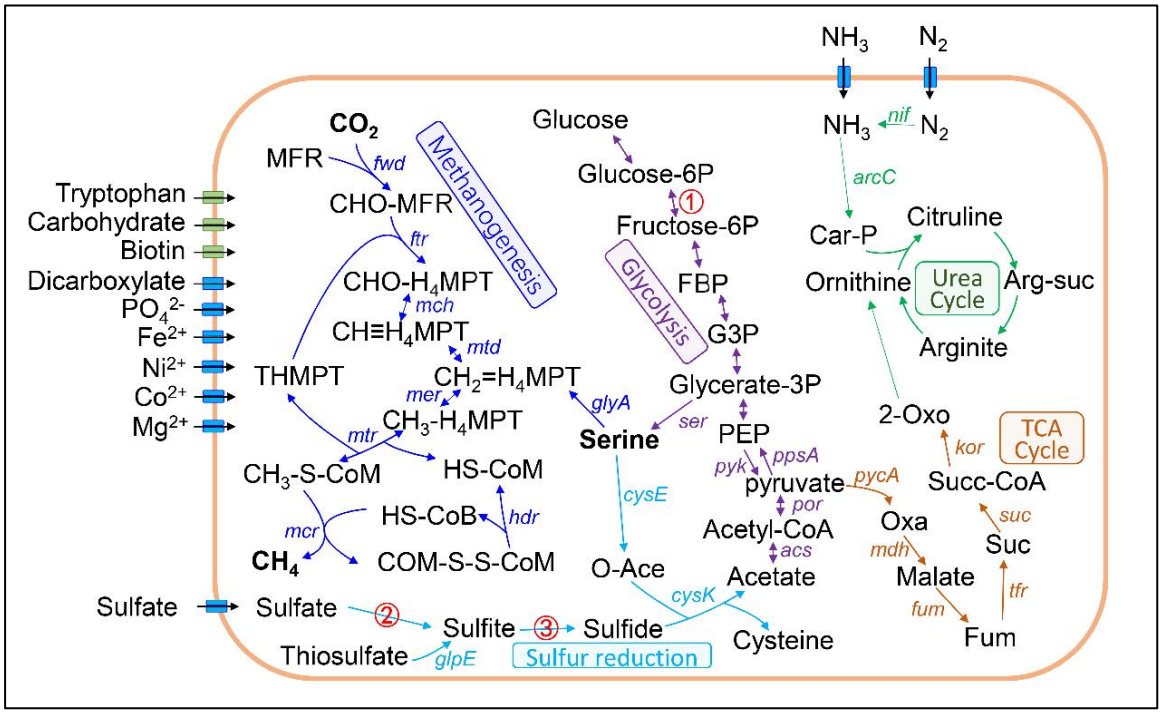

Figure 2: Overview of metabolic potentials in *Methanoculleus* MAGs. The significant major pathways related to metabolisms of carbon, nitrogen and sulphur, flagellar and motor protein, and various transporters in MAG groupings. Detailed gene information associated with above mentioned pathways was recorded in Supplementary Data 1-3. MFR: methanofuran; FBP: fructose-1,6-bisphosphatase; G3P: glyceraldehyde-3P; PEP: phosphoenolpyruvate; O-Ace: O-acetyl-L-serine; Car-P: carbamoyl-P; Arg-suc: arginosuccinate; 2-Oxo: 2-oxoglutarate; Succ-CoA: succinyl-CoA; Suc: succinate; Fum: fumarate; Oxa: oxaloacetate.

In these cells, methanogenesis was also associated with some important metabolic pathways including glycolysis and sulphur reduction. Glycerate-3P, the intermediate product of glycolysis, could also have produced serine through serine biosynthesis and eventually converted to CH_4. Sulphate could be reduced to sulphide, which could be forming acetate with O-acetyl-L-serine by cysteine synthase. In addition, several enzymes for reactions in these two processes were different from those for the general reactions in Kyoto encyclopaedia of genes and genomes (KEGG), such as archaeal distant homology with glucose-6-phosphate isomerase (D-glucose-6-phosphate <=> D-fructose-6-phosphate), desulphoferrodoxin FeS_4 iron-binding domain-containing protein (sulphate => sulphite) and putative sulphite reductase (sulphite => sulphide). The amino acid sequences in these enzymes were widely distributed in the *Methanoculleus* genus (Figure 3).

In this study, *cys* gene was the main sulphate reduction gene in MAG. Li *et al.* (2022) showed that sulphur reduction genes are mainly dominated by the *cys* genes, and also showed the core linking of the network co-occurrence of the C-N-S genes in coal seams. Besides, sulphates and other substances participate in reactions that convert organic substances into acetate (Matsuura *et al.*, 2021). The existence of these desulphurising *Methanoculleus* sp. may also favour coal degradation (Midgley *et al.*, 2010), because many desulphurizers could provide electron donors for the coal seam organic decomposition (Zhang *et al.*, 2015). Beckmann *et al.* (2019) considered that high sulphate concentrations did not prevent the growth of methanogenic archaea, but the sulphate-reducing bacteria had limited energy and competed with the methanogenic archaea. In this study, sulphate was also utilised by archaea through the certain sulphate reduction pathway. Several *cys* enzymes were used to synthesize sulphites and convert sulphates into sulphides; the existence of sulphate utilisation enhanced the archaeal ability to produce amino acids, such as cysteine and methionine (Tikariha & Purohit, 2019). This can also provide a small amount of acetate in the coal seams. The MAGs also had their specific genes for sulphate reduction including desulphoferrodoxin FeS_4 iron-binding domain-containing protein and putative sulphite reductase. Desulphoferrodoxin was first reported by Moura *et al.* (1990) in *Desulfovibrio desulphuricans* (ATCC 27774) and *Desulfovibrio vulgaris* (strain Hildenborough), suggesting its special sulphate reduction capacity.

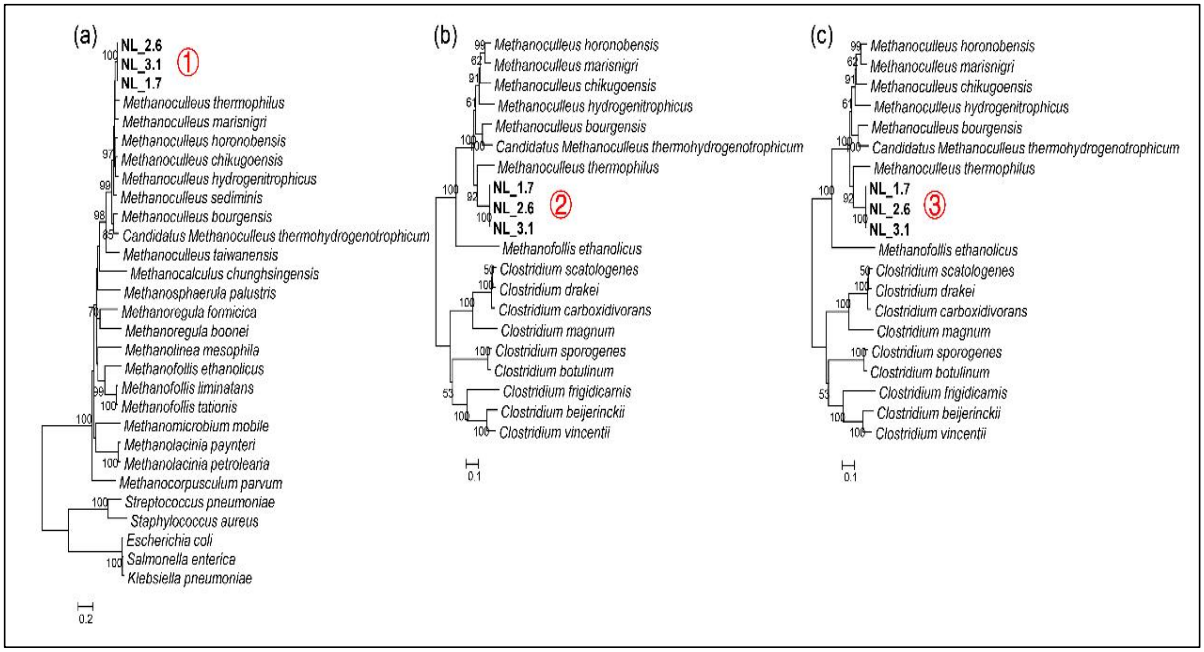

Figure 3: Phylogenetic analysis of protein coding sequences of desulfoferrodox in archaeal distant homology with glucose-6-phosphate isomerase (a), FeS_4 iron-binding domain-containing protein b) and putative sulfite reductase (c) from reconstructed genome. Bootstrap values were based on 1000 replicates, and only bootstrap values higher than 50% are shown.

In addition, the cells had a variety of other functions including nitrogen fixation and hydrogen production. Therein, [NiFe] hydrogenase nickel incorporation protein (*hypA*), [NiFe] hydrogenase nickel incorporation-associated protein (*hypB*) and [NiFe] hydrogenase metallocentre assembly protein (*hypCDE*) were found in MAGs. Ammonia could be used by archaea to synthesise glutamate, other amino acids and nucleotides (Tikariha & Purohit, 2019). Thus the presence of a well-regulated mechanism such as nitrogen fixation by the bacteria makes them suitable candidates for methanogenesis with low nitrogen availability. *Methanoculleus* MAGs could also provide sufficient metabolic substrates for methane production in coal seams by producing hydrogen. In this study, it was surprising to simultaneously detect the *hypABCDE* genes. These genes are encoding [NiFe] hydrogenase nickel incorporation proteins or metallocentre assembly proteins, which are essential for hydrogen production (Quemeneur *et al.*, 2011; Rai *et al.*, 2019). This also provides energy for other hydrogen-trophic methanogens.

Compare metabolic reconstruction

Comparing to the genomic data of *M. thermophilus* ATCC 33837 (GenBank GCA_001571405.1), 30 genes were detected in all the three MAGs but not in the comparison genomes (Table 2).These genes mainly associated with a variety of metabolisms (amino acids and derivatives, carbohydrates, fatty acids, lipids, isoprenoids, and protein metabolism) and environmental response (cell wall and capsule, stress response, and virulence, disease, and defense). Among them, most genes were homologous with *Methanoculleus* spp. (not *M. thermophilus*) and those homologies ranged from 67% to 95%. A portion of genes were homologous with other methanogens, such as the alcohol dehydrogenase (EC 1.1.1.1) homology with *Methanospirillum* sp. (homology 77%), type III restriction-modification system methylation subunit (EC 2.1.1.72) homology with *Methanocorpusculum labreanum* (homology 55%), and circadian clock protein KaiC homology with Candidatus *Methanoperedens* sp. BLZ2 (homology 64%). In addition, a few genes were homologous with some bacteria, such as arginase (EC 3.5.3.1) homology with *Phyllobacterium zundukense* (homology 74%) and N-acetylneuraminate synthase (EC 2.5.1.56) homology with *Tautonia sociabilis* (homology 74%).

Table 2: Genes in the all the three MAGs but not detected in standard strain *Methanoculleus thermophiles* ATCC 33837.

Category	Gene	Homology with	% Homology
Amino acids and derivatives	Arginase (EC 3.5.3.1)	*Phyllobacterium zundukense*	74
	Delta-1-pyrroline-5-carboxylate dehydrogenase (EC 1.2.1.88)	*Methanomicrobiales archaeon* 53_19	86
Carbohydrates	Alpha-amylase (EC 3.2.1.1)	*Methanoculleus horonobensis*	85
	Glucoamylase (EC 3.2.1.3)	*Methanoculleus chikugoensis*	87
	1,4-alpha-glucan (glycogen) branching enzyme, GH-13-type (EC 2.4.1.18)	*Methanoculleus marisnigri*	91
Cell wall and capsule	N-acetylneuraminate synthase (EC 2.5.1.56)	*Tautonia sociabilis*	74
	Glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24)	*Methanoculleus bourgensis*	89
	dTDP-glucose 4,6-dehydratase (EC 4.2.1.46)	*Methanoculleus bourgensis*	84
	putative glycosyltransferase	*Methanoculleus bourgensis*	68
Clustering-based subsystems	Xanthosine/inosine triphosphate pyrophosphatase	*Methanoculleus bourgensis*	88
Cofactors, vitamins, Prosthetic Groups,	Alcohol dehydrogenase (EC 1.1.1.1)	*Methanospirillum* sp.	77
DNA metabolism	ATP-dependent DNA ligase (EC 6.5.1.1) LigC	*Candidatus Methanoculleus*	80
	Type III restriction-modification system methylation subunit (EC 2.1.1.72)	*Methanocorpusculum labreanum*	55
Fatty acids, lipids, and isoprenoids	(2E,6E)-farnesyl diphosphate synthase (EC 2.5.1.10)	*Methanoculleus marisnigri*	75
	Lycopene elongase (EC 2.5.1.-)	*Methanoculleus horonobensis*	89
	Phytoene dehydrogenase (EC 1.14.99.-)	*Methanoculleus hydrogenitrophicus*	94
	Phytoene synthase (EC 2.5.1.32)	*Methanoculleus sediminis*	86
	Dimethylallyltransferase (EC 2.5.1.1)	*Methanoculleus marisnigri*	75
	Octaprenyl diphosphate synthase (EC 2.5.1.90)	*Methanoculleus marisnigri*	75
Miscellaneous	DedA protein	*Methanoculleus* sp.	67
	Tungsten-containing aldehyde:ferredoxin oxidoreductase (EC 1.2.7.5)	*Methanoculleus* sp. MH98A	88
Nucleosides and Nucleotides	Xanthine permease	*Methanoculleus bourgensis*	75
Protein metabolism	Circadian clock protein KaiC	*Candidatus Methanoperedens* sp.	64
	DNA polymerase III alpha subunit (EC 2.7.7.7)	*Methanoculleus* sp.	71
	UDP-glucose 6-dehydrogenase (EC 1.1.1.22)	*Methanoculleus taiwanensis*	81
RNA metabolism	DNA-directed RNA polymerase subunit P (EC 2.7.7.6)	*Methanoculleus bourgensis*	95
stress response	Glycine betaine transporter OpuD	*Methanoculleus marisnigri*	92
	Superoxide dismutase [Mn/Fe] (EC 1.15.1.1)	*Methanoculleus marisnigri]*	90
Virulence, disease and defense	Transcriptional regulator, MerR family	*Methanoculleus chikugoensis*	81
	Multi antimicrobial extrusion protein	*Methanoculleus marisnigri*	87

CONCLUSION

Together these results showed the stimulated methanogenic archaea *Methanoculleus* and further expanded the ecological function of the functional groups in coal seams. The MAGs might be assigned to *M. thermophilus*, although their functions were somewhat different from that of the standard strain. *Methanoculleus* sp. could use CO_2 and acetate as the main metabolic substrate for methanogenesis. Glycolysis and sulphate reduction processes were the main processes for providing acetate, both of which differ from other general species. Overall, this study enabled a better understanding of the ecological roles of *Methanoculleus* for biogenic methane production, using a combination of bioinformatic techniques.

Acknowledgement

This study was funded by the Independent Research Fund of the State Key Laboratory of Mining Response and Disaster Prevention and Control in Deep Coal Mines in Anhui University of Science and Technology (SKLMRDPC20ZZ09), the Key Research and Development Projects in Anhui Province (202004a07020054), and the Natural Science Foundation of the Anhui Province Education Department (KJ2020A0322).

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