

Characterization of *Methanosarcina mazei* JL01 Isolated from Holocene Arctic Permafrost and Study of the Archaeon Cooperation with Bacterium *Sphaerochaeta associata* GLS2^T †

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Abstract: A mesophilic methanogenic culture, designated JL01, was isolated from Holocene permafrost in the Russian Arctic. After long-term extensive cultivation at 15 °C, it turned out to be a tied binary culture of archaeal (JL01) and bacterial (*Sphaerochaeta associata* GLS2) strains. Strain JL01 was a strict anaerobe and grew on methanol, acetate, and methylamines as energy and carbon sources. Cells were irregular coccoid, non-motile, non-spore-forming, and Gram-stain-positive. Optimum conditions for growth were 24–28 °C, pH 6.8–7.3, and 0.075–0.1 M NaCl. Phylogenetic tree reconstructions based on 16S rRNA and concatenated alignment of broadly conserved protein-coding genes revealed 16S rRNA's close relation to *Methanosarcina mazei* S-6^T (similarity 99.5%). The comparison of whole genomic sequences (ANI) of the isolate and the type strain of *M. mazei* was 98.5%, which is higher than the values recommended for new species. Thus, strain JL01 (=VKM B-2370 = JCM 31898) represents the first *M. mazei* isolated from permanently subzero Arctic sediments. The long-term co-cultivation of JL01 with *S. associata* GLS2^T showed the methane production without any additional carbon and energy sources. Genome analysis of *S. associata* GLS2^T revealed putative genes involved in methanochondroitin catabolism.

Keywords: archaea; methanogens; permafrost; *Methanosarcina* sp.; *Sphaerochaeta associata*; metabolic cooperation

1. Introduction

Methanogens are unique and are the only group of microorganisms responsible for the generation of all of the Earth's biogenic methane. These archaea use very small range of substrates and they are highly adaptable to various harsh conditions, and even extreme environments [1]. Research on the Arctic permafrost revealed the presence of viable methanogenic archaea [2]. The use of cultivation methods made it possible to identify and describe new methanogenic species of the genera *Methanosarcina* and *Methanobacterium* in the Pliocene and Pleistocene permafrost, which are responsible for the formation of methane under extreme subzero conditions [3–5]. The results of the

metagenomic sequencing of two samples of permafrost sediments suggested that the composition of the archaeal community and the presence of methanogens in it, mainly represented by the *Methanosarcinaceae* and *Methanobacteriaceae* families, can be determined based on their permafrost origin [6]. Many methanogenes are known to be involved in tight metabolic and syntrophic associations [7]. Earlier, we isolated coccoid spirochaete *Sphaerochaeta associata* GLS2^T from the long-term stable methanogenic enrichment culture JL01. In this article, we show that *Methanosarcina* sp. JL01 produces methane in cooperation with saccharolytic bacterial partner *Sphaerochaeta associata* GLS2^T and without any carbon and energy sources added in the medium. Also, we present a more detailed characterization of strain JL01 [1], and prove that it is a representative of the first *Methanosarcina* strain isolated from permafrost.

2. Materials and Methods

2.1. Enrichment and Isolation

Methane-producing culture JL01 was isolated from the 2.0-m-deep Holocene permafrost sample that was collected in the Russian North East Arctic (70°06' N, 154°04' E) between the Lena delta and the mouth of the Kolyma river in 2000 [1]. A pure culture of the methanogenic strain was maintained in an anaerobic MSG medium containing (l⁻¹): 5.0 g NaCl, 0.2 g MgCl₂ × 6H₂O, 1.0 g NH₄Cl, 0.1 g CaCl₂ × 2H₂O, 1.5 g KH₂PO₄, 2.5 g K₂HPO₄ × 3H₂O, 1.0 g Casamino acids, 10 mL of vitamin solution (medium 141; DSMZ), and 10 mL of trace element solution (medium 141; DSMZ); 0.001 g of resazurine, 0.25 g of cysteine hydrochloride hydrate, 0.25 g of Na₂S × 9H₂O, and 4.0 g of sodium acetate trihydrate and kanamycin (150 mg L⁻¹). The strain purity was confirmed by light microscope examination of the absence of growth in MSG medium containing glucose (2 gL⁻¹) and peptone (2 gL⁻¹), from which the acetate and kanamycin were omitted.

2.2. Phenotypic, Physiological and Biochemical Characterization

All morphological and physiological characteristics of strain JL01 were determined according to the minimal standards for the description of new taxa of archaea [8]. The cell morphology was examined using a Lumam I-2 microscope (LOMO, Russia) in phase contrast mode using 90 × 15 magnification and a JEM-100 electron microscope (JOEL, Japan). The methanogen isolate was investigated for the growth that occurred at pH 4–9, temperatures of 0–50 °C, and sodium chloride requirements of 0–0.5 M. To determine the utilization of different carbon substrates as an energy source, a basal MSG medium supplemented with one of the tested substrate was used: 20 mM of propionate, 20 mM of butyrate, H₂/CO₂ (80:20, 1.5 atm), 100 mM of methanol, 50 mM of formate, 50 mM of acetate, 20 mM of ethanol, 20 mM of 2-propanol, 20 mM of 2-butanol, 50 mM of methylamine, 50 mM of dimethylamine, and 50 mM of trimethylamine. All tests were performed in two replicates and confirmed by two transfers. The incubation was performed over 10 days. The growth was estimated by measuring the concentration of methane in the gas phase using gas chromatography [8]. The effect of the antibiotics on the growth of strain JL01 was determined using the following antibiotics: (mg L⁻¹) chloramphenicol (10), bacitracin (10), polymyxin (10), vancomycin (2000), erythromycin (1000), kanamycin (2000), and penicillin G (2000).

2.3. Cooperation Study

A cooperation between *Methanosarcina* sp. JL01 and *S. associata* GLS2^T (VKM B-2742^T) was established in anaerobic MSG medium for methanogenic archaea by inoculating 15% (v/v) of archaeal strain pre-grown on the same medium with methanol and 10% (v/v) of bacterial strain grown on SM medium with xylose [9]. For comparison, archaeal strain was grown on MSG medium with acetate and methanol. There was also a variation with *Methanosarcina* sp. JL01, *S. associata* GLS2^T, and methanol. The experiment was carried out in duplicate at 28 °C. Methane and acetate were monitored immediately after inoculation, then after 1, 3, 9, and 16 days using gas chromatography.

2.4. Molecular and Phylogenetic Analyses

Extraction and purification of genomic DNA were carried out by a modified Marmur method [10]. The 16S rRNA gene was amplified by PCR using the universal archaeal primers F21-41 [11] and Arch1386r [12]. The A subunit of methyl coenzyme M reductase gene (*mcrA*) was amplified using the MCRf and MCR1R primer pair [13]. The G+C content of DNA and DNA-DNA relatedness tests were performed spectrophotometrically.

2.5. Genomes Analysis

The genome sequence of strain JL01 was determined in the Centre for Genomic Regulation (Barcelona, Spain) and deposited in NCBI under accession number CP029709. The sequencing and annotation of *S. associata* GLS2^T genome were carried out at the U.S. Department of Energy Joint Genomic Institute within the project Genomic Encyclopedia of Type Strains, Phase III project (Contract No. DE-AC02-05CH11231). The GLS2^T whole genome shotgun project (WGS) is available in the GenBank database under the accession number FXUH01000000.

3. Results and Discussions

3.1. Enrichment and Pure Culture Isolation

The initial incubation of the Holocene permafrost sample [1] with acetate resulted in enrichment culture JL01 producing methane at 15 °C. This enrichment contained a stable contaminating bacterial partner identified as *S. associata* GLS2^T [9], which coexisted well with methanogen for a long time on the medium under conditions that were optimal for the methanogen. The concomitant bacterium was resistant to various antibiotics but the methanogenic strain JL01 was purified by repeated transfers. Strain JL01 formed yellow grainy colonies 1 mm in diameter after 21 days of cultivation. The cells formed small aggregates on the bottom of the cultivation bottles when grown in liquid cultures.

3.2. Characterization of Novel Methanogenic Strain

The cells of strain JL01 were non-motile irregular cocci, 1.0–1.5 µm in diameter. They grouped into multicellular aggregates (Figure 1a). Cells were stained Gram-positive. The cell wall was 40–50 nm in width (Figure 1b). Some cells had electron dense inclusions.

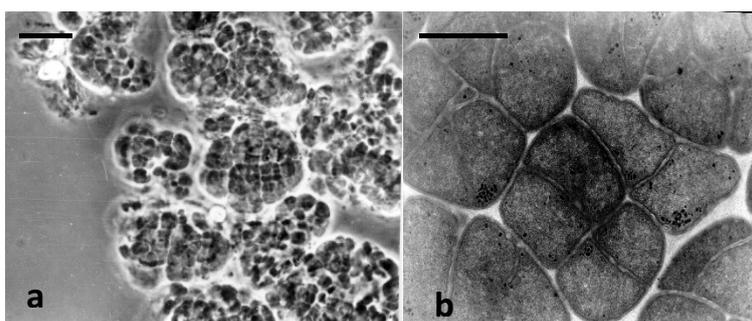


Figure 1. Micrographs of strains JL01 cells: (a)–phase-contrast microscope, bar 10 µm; (b)–ultra-thin section, bar 1 µm.

The strain JL01 was a strict anaerobe and grew on methanol, acetate, and methylamines as energy and carbon sources. The isolate did not utilize H₂/CO₂, formate, 2-propanol, 2-butanol, butyrate, ethanol, or propionate as single sources of carbon and energy. The addition of Casamino acids (1.0 g L⁻¹) to the medium stimulated growth on methanol and acetate. JL01 grew at temperatures between 10 and 37 °C (optimum at 24–28 °C), pH 5.5 and 8.5, (optimum at 6.8–7.3), and NaCl concentrations from 0.01 to 0.2 M (optimum at 0.075–0.1M). The strain was resistant to penicillin, vancomycin, erythromycin, and kanamycin. The addition of bacitracin slowed the growth of the strain.

3.3. Phylogenetic Analyses

A nearly complete fragment of 16S rRNA gene (1370 bp) and a fragment of *mcrA* gene (512 bp) were amplified from genomic DNA of the strain JL01. Phylogenetic analysis showed that the topology of the tree based on the *mcrA* does not match the topology of the phylogenetic tree based on the 16S rRNA genes. According the analysis of the 16S rRNA gene, the closest relatives to the strain JL01 were *M. mazei* S-6^T (99.5%) and *M. soligelidi* SMA-21^T (99.4%). Comparison of *mcrA* gene sequences showed that it was 99.4% identical to *M. horonobensis* strain HB-1^T, and was 93.1% and 96.2% identical to *M. mazei* and *M. solidgelidi*, respectively (Figure 2).

The G+C contents of DNA determined spectrophotometrically in strains JL01 and *M. mazei* S-6^T were 39.2 mol% and 42.3 mol%, respectively. The results of wet DNA-DNA hybridization indicated only 26.2 ± 2.7% relatedness (mean ± SD of 3 determinations) between strains JL01 and *M. mazei* S-6^T.

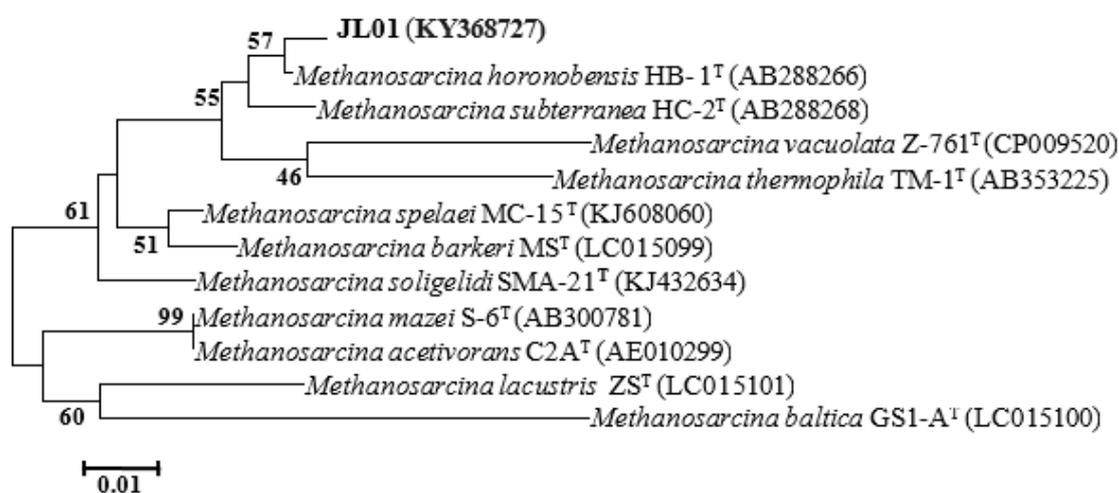


Figure 2. Phylogenetic tree based on *mcrA* gene sequences showing the relationship between the strain JL01^T and other members of the genus *Methanosarcina*. The numbers at the branch nodes indicate bootstrap percentages based on the neighbor-joining method (1000 replicates). Accession numbers are shown in parentheses.

Phylogenetic tree reconstructions based on 16S rRNA and concatenated alignment of broadly conserved protein-coding genes revealed 16S rRNA's close relation to *Methanosarcina mazei* S-6^T (Figure 3).

3.4. Genome Organization of Strain JL01

The genome assembly of strain JL01 contained 323 contigs, which were organized into 1 scaffold. The sequence total and ungapped lengths were 4,186,733 bp and 4,127,022 bp, respectively. The calculated G+C content was 41.59%, the contig N₅₀ value was 57,424 bp, and the L₅₀ was 21. The total count of genes was 3511, of which there were 3210 protein coding sequences and 229 pseudogenes. There were 72 RNA genes including 12 rRNA genes, 58 tRNA genes, and 2 ncRNA genes. The JL01 genome had no identified plasmids. It had 7 CRISPR regions predicted with RefSeq and 14 predicted with DFAST. The coding ratio was 73.4%. The genome of strain JL01 contained the key genes of the hydrogenotrophic acetoclastic, and methylotrophic pathways of methanogenesis. The strain lacked some genes encoding proteins involved in coenzyme M synthesis (EC 4.4.1.19, EC 3.1.3.71 and EC 1.1.1.337) and formate dehydrogenase (EC 1.17.1.9, EC 1.17.98.3 or EC 1.17.1.10) genes, which are responsible for the conversion of formate to CO₂ in the hydrogenotrophic pathway. The genome contained genes for nitrogenase and hydroxylamine reductase. The G+C contents of strains JL01 and *M. mazei* S-6^T were 41.6 mol% and 41.4 mol%, respectively. The comparison of whole genomic sequences (ANI) of the isolate and the type strain S-6 of *M. mazei* was 98.5%, which is higher than the values recommended for new species (95–96%).

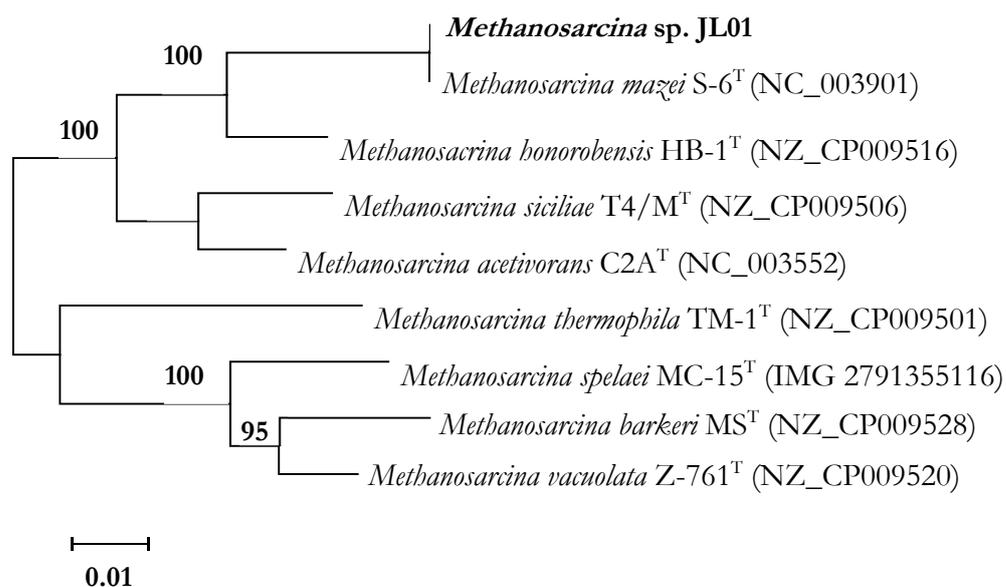


Figure 3. Maximum-likelihood phylogenetic tree of the strain JL01 based on a concatenated alignment of broadly conserved protein-coding genes (bootstrap = 1000). The sequence access numbers publicly available in Genbank are shown in parentheses. Evolutionary analyses were conducted in MEGA7.

3.5. Co-Cultivation *M. mazei* JL01 and *S. associata* GLS2^T

A cooperation study between *M. mazei* JL01 and *S. associata* GLS2^T was carried out in methanogenic medium by inoculating an archaeal strain grown with methanol and a bacterial strain grown on SM medium with xylose [9]. The results of co-cultivation on the mineral methanogenic medium showed that the CH₄ content in the presence of the bacterium was comparable (Figure 4) to that formed by the strain JL01. The bacterial strain formed acetate during growth on xylose (Figure 5). We suggest that GLS2 was able to ferment the components of methanochondroitin matrix as a substrate with the production of acetate.

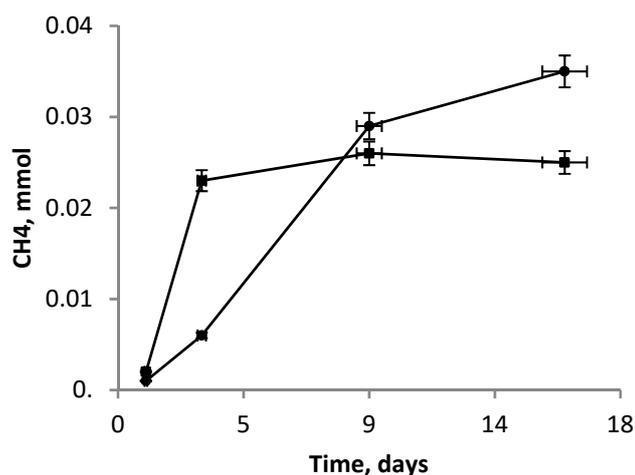


Figure 4. Methane formation by *M. mazei* JL01 with acetate (circle) and in cooperation with *S. associata* GLS2^T without any substrates (square). The temperature of cultivation was 28 °C.

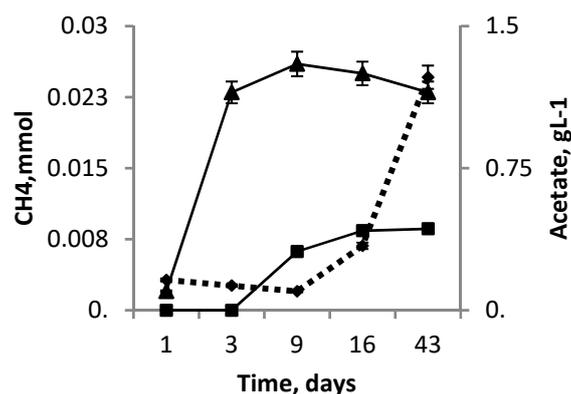


Figure 5. Methane formation by *M. mazei* JL01 (triangle) and acetate concentration (square) in a mixture with *S. associata* GLS2^T without substrate addition and acetate production (rhombus) by *S. associata* GLS2^T with xylose.

3.6. Genomic Data Support Possibility of the Cooperation

The genome analysis of *S. associata* GLS2^T showed a high number and diversity of carbohydrate transporters and a moderate diversity of carbohydrate-active enzymes (CAZy enzymes). The profile of CAZy enzymes encoded in genome of GLS2 exhibited peculiarities reflecting a particular adaptation of this bacterium towards usage of methanochondroitin matrix of methanosarcina. *S. associata* GLS2^T genome had genes for putative proteins which might be involved in chondroitin usage such as glucuronyl hydrolase (unsaturated chondroitin disaccharide hydrolase) (2 proteins SMP46638, SMP50557) and alginate lyase A1-III/heparinase II/III-like (3 proteins SMP46635, SMP50543, SMP40110). None of genes for putative unsaturated chondroitin disaccharide hydrolase and alginate lyase A1-III/ heparinase II/III-like were found in the genome of *S. halotolerans* 4-11^T isolated from formation water of a low-temperature petroleum reservoir and just single copies of respective genes were present in genomes of strains *S. globosa* Buddy^T and *S. pleomorpha* Grapes^T isolated from river sediments. GLS2^T genome contained genes for complete glycolytic and oxidative pentose phosphate pathways as well as for further metabolism of pyruvate into lactate (D-lactate dehydrogenase, SMP59869), acetate (acetate kinase, SMP48022), ethanol (acetaldehyde dehydrogenase/alcohol dehydrogenase AdhE, SMP46078), formate (formate C-acetyltransferase, SMP45624), and CO₂.

The JL01 genome had almost a complete pathway of cobalamin biosynthesis, in contrast to the GLS2^T genome which showed the presence of only four enzymes of the cobalamins “salvage pathway”.

JL01 and GLS2 genomic data pointed out complementary capabilities of partners in amino acids biosynthesis. JL01 had a complete tryptophan biosynthesis pathway beginning from chorismate, while GLS2 had 4 genes missing. At the same time, GLS2 had a complete set of genes in the shikimate pathway leading to chorismate, which is an important biochemical intermediate in biosynthesis of tryptophan, phenylalanine, tyrosine, and folates. The GLS2 genome showed the presence of complete histidine, threonine, cysteine, and leucine pathways, while the JL01 genome showed one to several missing steps in them.

Thus, the obtained data indicate highly complex interactions in a consortium of methanosarcina and GLS2. Genomic data point out the possibility of GLS2 to use components of methanochondroitin matrix and to produce acetate, a number of amino acids, and intermediates that could be used by methanosarcina, while methanosarcina in turn fixes molecular nitrogen and produces other biosynthetically complex compounds such as cobalamins and tryptophan. We hypothesize that JL01 and GLS2 demonstrate strict mutualistic multiple interactions by exchanging metabolites of high energy demand, which contribute to energy conservation under energy and nutrition-limited conditions in a permafrost environment.

4. Conclusions

Methanogens of *Methanosarcina* genus are of great ecological importance, as they are the only organisms fermenting acetate, methylamines, and methanol to methane, carbon dioxide, and ammonia (in case of methylamines). Since acetate is the precursor of 60% of all of the methane produced on earth, these organisms significantly contribute to the production of this greenhouse gas. Moreover, methanogenic archaea of the *Methanosarcinales* order is most often found among *Euryarchaeota* in an array of metagenomic data from samples of permanently frozen sediments such as permafrost [2]. The analysis of genomes of some bacteria of the *Sphaerochaeta* genus showed the absence of several genes coding for vitamin B₁₂ synthesis. Methanogenic archaea can produce its precursors [14] and can supply them to the bacterium. These facts explain the close cooperation of both GLS2^T and JL01 and allow us to assume that *M. mazei* JL01 takes advantage of the permafrost ecosystem from coexistence with a bacterial strain. We hypothesize that conditions of GLS2^T presence in the matrix of *Methanosarcina* exerted a selection pressure on the bacterial co-habitant, leading to the acquirement of necessary genes for the usage of plentiful carbohydrate, a main compound of the extracellular matrix of *Methanosarcina*. Subsequent lengthy co-cultivation experiments at subzero temperature will give us the final answer to this key question of the type of microbial activity that occurs in permafrost.

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Conflicts of Interest: The authors declare no conflict of interest.

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