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Methanogenesis from Methanol at Low Temperatures by a Novel Psychrophilic Methanogen, "*Methanolobus psychrophilus*" sp. nov., Prevalent in Zoige Wetland of the Tibetan Plateau▽†

Guishan Zhang,[1,2] Na Jiang,[2] Xiaoli Liu,[1] and Xiuzhu Dong[1]*

State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China,[1] and Graduate School, Chinese Academy of Sciences, Beijing 100049, People's Republic of China[2]

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The Zoige wetland of the Tibetan plateau is at permanent low temperatures and is a methane emission heartland of the plateau; however, cold-adaptive methanogens in the soil are poorly understood. In this study, a variety of methanogenic enrichments at 15°C and 30°C were obtained from the wetland soil. It was demonstrated that hydrogenotrophic methanogenesis was the most efficient type at 30°C, while methanol supported the highest methanogenesis rate at 15°C. Moreover, methanol was the only substrate to produce methane more efficiently at 15°C than at 30°C. A novel psychrophilic methanogen, strain R15, was isolated from the methanol enrichment at 15°C. Phylogenetic analysis placed strain R15 within the genus *Methanolobus*, loosely clustered with *Methanolobus taylorii* (96.7% 16S rRNA similarity). R15 produced methane from methanol, trimethylamine, and methyl sulfide and differed from other *Methanolobus* species by growing and producing methane optimally at 18°C (specific growth rate of 0.063 ± 0.001 h^{-1}) and even at 0°C. Based on these characteristics, R15 was proposed to be a new species and named "*Methanolobus psychrophilus*" sp. nov. The K_m and V_{max} of R15 for methanol conversion were determined to be 87.5 ± 0.4 μM and 0.39 ± 0.04 mM h^{-1} at 18°C, respectively, indicating a high affinity and conversion efficiency for methanol. The proportion of R15 in the soil was determined by quantitative PCR, and it accounted for 17.2% ± 2.1% of the total archaea, enumerated as 10^7 per gram of soil; the proportion was increased to 42.4% ± 2.3% in the methanol enrichment at 15°C. This study suggests that the psychrophilic methanogens in the Zoige wetland are likely to be methylotrophic and to play a role in methane emission of the wetland.

Wetlands in cold areas, such as permafrost and tundra, contribute a large proportion of global methane emission, since about 75% of the Earth's biosphere is cold (3). The Zoige wetland of the Tibetan plateau is such a wetland. Although it is located in a low-latitude region (33°56′N, 102°52′E), its average annual temperature is around 1°C due to the high altitude (3,400 to 3,600 m). Previous studies estimated the annual methane emission in the Tibetan plateau to be in the range of 0.56 to 1 Tg (8, 9), and the Zoige wetland and the headstreams of the Yangtze and Yellow rivers could be the CH_4 emission flux heartlands of the plateau (17). Chen et al. (4) recently reported a mean methane emission rate of 14.45 mg CH_4 m^{-2} h^{-1} (0.17 to 86.78 mg CH_4 m^{-2} h^{-1}) for the Zoige wetland covered by two dominant vegetations (*Carex muliensis* and *Eleocharis valleculosa*) during summer, indicating that psychroadaptive methanogens could be active in the wetland soil. However, no studies were conducted to characterize the methanogen populations and the methanogenesis pathways in this cold wetland.

Studies on methane production in cold terrestrial ecosystems, using radiolabeled methanogenic precursors and stable isotope signatures, indicated that the acetoclastic methanogenesis pathway was strongly prevalent (11, 12, 32, 41), possibly due to psychroactive homoacetogenesis supplying extra acetate in addition to fermentation-derived acetate (22, 23). Methanogen population structure analysis in boreal wetlands, such as a Siberia peat bog, based on unculturable approaches also showed that *Methanosarciaceae*, *Methanomicrobiaceae*, and rice cluster II presented as the dominant group (23). A study on methanogenic community shifting in a cellulose-fermenting enrichment of paddy soil in response to different temperatures showed that acetoclastic methanogenesis was the predominant metabolic type, with *Methanosaeta* members dominating at 15°C and *Methanosarcina* dominating at 30°C (36). However, no culture-based analyses of methanogenic metabolic types and methanogen populations were reported for cold wetlands. In this study, a novel psychrophilic methylotrophic methanogen, which could be prevalent in Zoige wetland soil, was isolated using methanol as a substrate, and its methanogenesis from methanol at low temperatures was characterized as well.

MATERIALS AND METHODS

Soil sampling. The sampling site is located in the Zoige wetland (33°56′N, 102°52′E; altitude, 3,430 m) of the Tibetan plateau, where *Carex muliensis* and *Eleocharis valleculosa* grow as the dominant vegetation. The average soil temperatures are between 6°C and 15°C from June to September at a soil depth of 10 to 30 cm (37), the pH value is 7.0 to 7.5, and the NaCl concentration is around 40 mM. The rhizosphere soil of *Eleochalis valleculosa* at a depth of 20 to 30 cm was sampled at the end of June 2005. The soil samples were put into sterile serum bottles, which were sealed with butyl rubber stoppers and then kept in a cool box during transportation.

* Corresponding author. Mailing address: State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, No. 3A, Datun Road, Chaoyang District, Beijing 100101, People's Republic of China. Phone and fax: 86 10 6480 7413. E-mail: dongxz@sun.im.ac.cn.

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Methanogen strains, media, and enrichment of methanogenic cultures. *Methanolobus oregonensis* DSM5435^T, *Methanolobus taylorii* DSM9005^T, *Methanolobus vulcani* DSM3029^T, *Methanolobus bombayensis* DSM7082^T, *Methanobacterium formicicum* DSMZ1535^T, *Methanosarcina barkeri* DSMZ800^T, and *Methanomethylovorans hollandica* DSMZ15978^T were purchased from DSMZ (Braunschweig, Germany). Strain R15 and *Methanomethylovorans hollandica* Z1 were isolated from the rhizosphere soil of *Eleochalis valleculosa* in this study.

Prereduced basal medium was prepared as described previously (40), but with rumen fluid and titanium solution omitted. The medium was dispensed into screw-cap tubes sealed with butyl rubber stoppers, and the gas phase for routine cultivation was N_2 unless indicated otherwise.

The methanogenic cultures were enriched in the basal medium with the addition of each of the methanogenic substrates to a final concentration as follows: 20 mM of acetate, formate, methanol, and trimethylamine and 1.01×10^5 Pa H_2-CO_2 (80:20) in the headspace. A mixture of ampicillin (final concentration, 2 mg/ml) and kanamycin (final concentration, 1 mg/ml) was added to inhibit bacterial growth (21). One gram of soil sample was inoculated into 5 ml of medium inside an anaerobic box (Forma anaerobic system 1029), and subsequent transfers were done with syringes and needles. The enrichments with each substrate were incubated at either 15°C or 30°C. CH_4 production was followed for all enrichments during the incubation period, and the methanogenesis rates were calculated from the linear range of the methane accumulation curve against time of incubation (days). The data reported are averages for triplicate experiments.

Isolation and physiology of psychrophilic methylotrophic methanogen. The methanogenic enrichment on methanol at 15°C was 10-fold serially diluted in the basal medium containing 20 mM methanol, and then the Hungate rolling tube technique was performed. Colonies that produced fluorescence under UV light at a wavelength of 420 nm (Nikon Diaphot) were picked for further purification. The purity of cultures was examined periodically by monitoring cell morphology under a normal bright-field microscope and colony homogeneity, as well as the absence of growth in rich media such as peptone-yeast extract-glucose broth.

Cell morphology was examined by using light microscopy (Olympus BH-2 microscope) as well as electron microscopy (Hitachi H-600A microscope). For electron microscopy studies, cells were negatively stained with uranyl acetate.

Substrate utilization was tested by measuring methane production of the isolate growing in basal medium supplemented with the tested substrates. Requirements for growth factors were determined by measuring growth in the medium, omitting one of the components in each test, including vitamins, yeast extract, peptone, etc. The pH range for growth was estimated by cultivating the strain in methanol medium with various pH values, adjusted with 10% (wt/vol) NaOH or HCl. The growth temperature range was measured by cultivating the strain in methanol medium in a water bath with a temperature controller and in an ice-water mixture as 0°C. To determine NaCl tolerance, 0 to 1,500 mM NaCl was added to the methanol medium. Specific growth rates were calculated from the linear range of the growth curve determined with the optical density at 550 nm (OD_{550}) according to the method described by Franzmann et al. (13).

Determination of methanol conversion kinetics of strain R15. Cells of "*Methanolobus psychrophilus*" R15 in middle log phase were collected by centrifugation inside the anaerobic box and resuspended in 5 ml phosphate-buffered saline (pH 7.2). Methanol was then added to the cell suspension to final concentrations in the range of 60 μM to 800 μM and incubated at 18°C. The total amount of cell protein was measured by Bradford assay (2). Methanol consumption and methane production were measured at 10- to 30-min intervals for up to 5 h. The conversion rate (V) at each methanol concentration (S) was calculated from the linear range of the degradation curves, and then V_{max} was estimated. The K_m constant was calculated according to the Monod equation [$V = V_{max}S/(K_m + S)$] (6).

Analytical techniques. Methanol and methane were measured with a model GC-14B gas chromatograph (Shimadzu) equipped with a flame ionization detector and a C_{18} column as described previously (42). The temperature parameters were set as follows: for methanol determination, the column temperature was 150°C, the injector temperature was 170°C, and the detector temperature was 250°C; and for methane determination, the column temperature was 50°C, the injector temperature was 80°C, and the detector temperature was 100°C. Under these conditions, the detection limitation was 20 μM for methanol and 1.6 μM for methane.

DNA extraction and purification. DNAs from strain R15 and methanogenic enrichment cultures were extracted using a modified cetyltrimethylammonium bromide method as described previously (44), with the following modifications. Cells were suspended in 750 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) containing 7.5% Chelex (Sigma), 0.05 EDTA (pH 7.0), 2% sodium dodecyl sulfate, and 200 μg of proteinase K. After removal of the Chelex layer by centrifugation, 100 μl of 5 M NaCl and 80 μl of cetyltrimethylammonium bromide (10% [wt/vol] in 0.7 M NaCl) were added, and the mixture was incubated at 65°C for 30 min. DNAs were purified exactly as described previously (44).

Determination of DNA base composition and DNA-DNA hybridization. The G+C content of the DNA was determined by the thermal denaturation method (27), using *Escherichia coli* K-12 as a reference. DNA-DNA hybridization was determined for the initial reassociation rate at 67°C according to the method of Owen and Pitcher (29). The determinations were performed using a DU 800 spectrophotometer (Beckman).

Amplification and sequencing of 16S rRNA gene and phylogenetic analysis. The 16S rRNA gene of strain R15 was amplified with the universal archaeal primer 21F (7) and the prokaryotic primer 1541R (33), as described previously (15). Purified PCR products of about 1,400 bp were cloned into the pUCm-T vector and sequenced by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

The 16S rRNA gene sequence of strain R15 was submitted to GenBank to search for similar sequences by use of the BLAST algorithm. The best-matching sequences were retrieved and aligned, and similarity analysis was performed by CLUSTAL X (35). Phylogenetic trees were constructed using the neighbor-joining method implemented in MEGA 4.0 (34). The topologies of the resultant trees were evaluated by bootstrap analysis (10) based on 1,000 resamplings.

Quantification of strain R15 in the soil by quantitative real-time PCR. The principle of quantitative PCR (qPCR) is described elsewhere (30). PCRs were carried out on an ABI Prism 7000 sequence detection system (Applied Biosystems). All qPCR reactions were performed in eight-strip PCR tubes (Axygen), and the reaction signals were generated by binding of SYBR green to double-stranded DNA.

Real-time PCR primers were designed based on a strain-specific region of the strain R15 16S rRNA gene (EF202842), using Primer Premier 5.0, Oligo 6.0, and Premier Express 2.0. The designed primers, R15F (5′-GCTACACGCGGGCT ACAATGA-3′) and R15R (5′-AATTTAGGTTCGAACACGGCATGAA-3′), were at positions 1161 to 1181 and 1382 to 1406, respectively, and were applied in quantitative real-time PCR (246-bp amplicon) to quantify the biomass of strain R15 in the in situ soil and methanogenic enrichments. The specificity of the primers was verified by no amplification from the DNAs of *Methanobacterium formicicum* DSMZ1535^T, *Methanosarcina barkeri* DSMZ 800^T, and *Methanomethylovorans hollandica* DSMZ 15978^T or the clones of the 16S rRNA library constructed for Zoige wetland soil covered by *Eleochalis valleculosa* vegetation (43). Primers Arc787 and Arc1059 (38) were used to quantify the biomass of total archaea (273-bp amplicon).

pUCm-T plasmids containing the complete 16S rRNA genes of strain R15 (EF202842) and *Methanomethylovorans hollandica* Z1 (EF174501) were used as quantitative calibrators for *Methanolobus psychrophilus* R15 and total archaea, respectively. The DNA preparations were quantified by a NanoDrop ND-1000 UV-Vis spectrophotometer, and the copy numbers of the 16S rRNA gene were calculated according to the equation described by Yu et al. (39). The plasmids were then 10-fold serially diluted from 10^8 to 10 16S rRNA molecules/ml and used to generate calibration curves of 16S rRNA copies for strain R15 (see Fig. S1A in the supplemental material) and total archaea (see Fig. S1B in the supplemental material).

Each qPCR mixture contained 2× SYBR green master mix (Applied Biosystems), 1 μl DNA template prepared as described above, 100 nM of each primer, and double-distilled H_2O to a final volume of 25 μl. The PCR was initiated at 50°C for 2 min to optimize AmpErase uracil-*N*-glycosylase activity, followed by denaturation at 95°C for 10 min and 40 cycles of amplification as follows: 95°C at 30 s, 57°C at 40 s, and 72°C at 40 s. Fluorescence data were collected during the elongation step. The reactions were all performed in triplicate.

Nucleotide sequence accession number. The 16S rRNA GenBank accession number for strain R15 is EF202842.

RESULTS

Enrichment of psychroactive methanogens from Zoige wetland soil. In total, five types of methanogenic enrichments were obtained by using Zoige wetland soil as an inoculant. Table 1 shows that the highest methanogenesis rate at 30°C was determined for the H_2-CO_2 enrichment, while the highest rate at 15°C was determined for the methanol enrichment. Moreover, the methanogenesis rate derived from methanol at 15°C (32.1 ± 2.6 μM h^{-1}) was two times higher than that at 30°C (14.8 ± 3.2 μM h^{-1}), although the methanogenesis rate

TABLE 1. CH_4-producing rates of enriched Zoige wetland soil incubated at 15°C and 30°C

Substrate (concn)	CH_4 production rate ($\mu M\ h^{-1}$)	
	15°C	30°C
H_2-CO_2 (1.01×10^5 Pa)	7.0 ± 1.2	16.7 ± 2.3
Formate (20 mM)	8.6 ± 0.8	5.2 ± 1.1
Trimethylamine (20 mM)	10.3 ± 1.4	10.4 ± 2.7
Acetate (20 mM)	12.0 ± 2.2	7.2 ± 2.8
Methanol (20 mM)	32.1 ± 2.6	14.8 ± 3.2

from acetate appeared to be higher at 15°C than at 30°C as well. This suggested that the dominant psychroactive or psychrophilic methanogens inhabiting the Zoige wetland could prefer methanol over other substrates for methanogenesis.

Isolation and characterization of psychrophilic methanogen strain R15. The methanol methanogenic enrichment at 15°C was subcultured in the same medium to isolate the psychrophilic methanogens. Single colonies were observed in Hungate rolling tubes after cultivation at 15°C for 60 days. The colonies were white circular colonies of 1.5 to 2.0 mm in diameter and emitted green fluorescence at 420 nm. The purified strain assigned as methanogen R15 was oval (0.9 to 1.0 by 1.0 to 1.2 μm), with two flagella inserted at the end of the cell (Fig. 1A). Cells were also coated by a capsule-like material and frequently formed loose aggregates (Fig. 1B).

Both phenotypic and genetic characteristics of strain R15 met the minimal criteria for the genus *Methanolobus*, which include producing CH_4 from methanol, trimethylamine, and methyl sulfide but not from H_2-CO_2, acetate, or formate. R15 also required NaCl for growth, and better growth occurred in a medium supplemented with 15 to 300 mM NaCl, with the

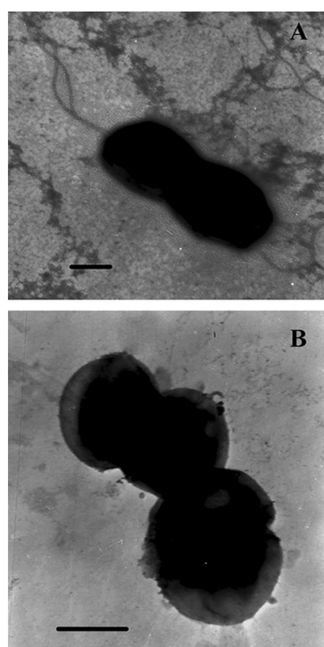

FIG. 1. Electron micrographs of *Methanolobus psychrophilus* R15 growing in 20 mM methanol at 18°C. (A) Cell with two polar flagella; (B) cell aggregate. Bars = 0.5 μm.

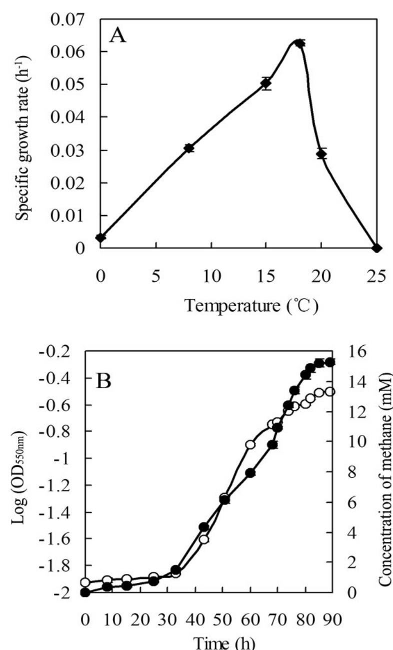

FIG. 2. Growth of *Methanolobus psychrophilus* R15 on 20 mM methanol. (A) Specific growth rates at various temperatures. Data are means for three replicates. (B) Curves of log OD_{550} (open circles) and methane production (filled circles) at 18°C. Data are means for three replicates. Standard deviations are shown.

optimal growth occurring at 200 to 250 mM NaCl but no growth occurring above 800 mM NaCl or below 5 mM NaCl (see Fig. S2 in the supplemental material). Biotin, but not thiamine or yeast extract, was essential to growth, although yeast extract stimulated growth. The G+C content of the chromosomal DNA (44.9 mol%) also fell in the range for the genus *Methanolobus* (39 to 46 mol%).

The growth and CH_4 production of strain R15 were further characterized. As shown in Fig. 2, R15 grew in the temperature range of 0°C to 25°C (Fig. 2A) and produced methane optimally at 18°C, with a specific growth rate of $0.063 \pm 0.001\ h^{-1}$ (Fig. 2B). Even at 0°C, the OD_{550} reached 0.304 after incubation of R15 for 60 days. In contrast, none of the described *Methanolobus* spp. grew exclusively below 25°C. DNA-DNA hybridization experiments also revealed the lower level of relatedness between strain R15 and other *Methanolobus* spp., and the homology between R15 and *M. vulcani* DSM3029^T, *M. bombayensis* DSM7082^T, *M. taylorii* DSM9005^T, and *M. oregonensis* DSM5435^T was 30.8%, 28.3%, 32.6% and 35.7%, respectively. Therefore, based on the differential characteristics listed in Table 2, combined with the DNA-DNA hybridization data, methanogen R15 could represent a novel *Methanolobus* species characterized by growing exclusively at low temperatures.

Phylogenetic affiliation of strain R15. To ascertain the phylogenetic affiliation of strain R15, the complete 16S rRNA gene (1,447 bp) was sequenced, and a phylogenetic tree was constructed based on a consensus length of 1,378 bp of the related 16S rRNA sequences (Fig. 3). Phylogenetic analysis placed strain R15 in the genus *Methanolobus*, with the highest 16S rRNA sequence similarity (96.7%) to *Methanolobus taylo-*

TABLE 2. Differential characteristics that distinguish *Methanolobus psychrophilus* R15 from described *Methanolobus* species

Characteristic	Description or value					
	M. psychrophilus R15[T]	*M. tindarius* ATCC 35996[Ta]	*M. oregonensis* DSM 5435[b]	*M. taylorii* DSM 9005[c]	*M. vulcani* DSM 3029[d]	*M. bombayensis* DSM 7082[e]
Morphology						
Coccoid cells	+	+	+	+	+	+
Loose aggregates	+	+	+	+	+	−
Flagella	One or two polar	Monotrichous	−	−	−	−
Substrate						
Methyl sulfides	+	−	+	+	−	+
Growth factor requirement	Biotin	NR[f]	Biotin, thiamine	Biotin	Biotin	+
Growth						
Temp range/optimal temp (°C)	0–25/18	7–50/28	7–44/34	11–40/39	13–45/40	13–46/37
pH	6.0–8.0	5.5–8.0	7.4–9.5	7.0–8.6	5.5–8.0	6.0–8.5
NaCl (mM)	10–800	60–1,270	100–1,600	200–1,200	100–1,200	300–2,000
Methanol conversion rate (mM h^{-1})						
0°C	0.016	0	0[g]	0[g]	0[g]	0[g]
18°C	0.36	NR[f]	0.13[g]	0.099[g]	0.048[g]	0.057[g]
25°C	0.001	NR[f]	0.32[g]	0.24[g]	0.27[g]	0.37[g]
Mol% G+C of DNA	44.9	46	41	41	39	39

[a] Data are from reference 20.
[b] Data are from reference 25.
[c] Data are from reference 28.
[d] Data are from reference 18.
[e] Data are from reference 19.
[f] NR, not reported.
[g] This study.

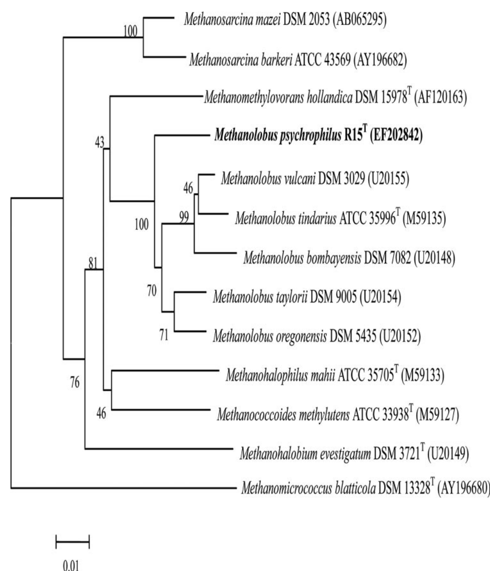

FIG. 3. Phylogenetic tree showing the position of strain R15 among other species of the genus *Methanolobus*. Based on a consensus 1,378 bp of the 16S rRNA gene, the tree was constructed by the neighbor-joining method. The topology of the tree was estimated by bootstraps based on 1,000 replications. The number on each branch node is the percentage supported by bootstraps. GenBank accession numbers of 16S rRNA sequences are given in parentheses. Bar, 1% sequence divergence.

rii. Therefore, a novel *Methanolobus* species, *Methanolobus psychrophilus*, was proposed based on the phylogenetic divergence and phenotypic differences.

Methanogenesis and kinetics of methanol conversion by strain R15 at low temperatures. Methanol conversion rates by growing cells of strain R15 were determined at a variety of low temperatures. The highest rate was determined at 18°C, with a maximal conversion rate of 0.36 ± 0.09 mM methanol h^{-1} (Fig. 4A), and about 16.5 mM CH_4 was formed from 22.7 mM of methanol. Strain R15 even degraded methanol at 0°C (Fig. 4B), although the conversion rate (0.016 ± 0.003 mM methanol h^{-1}) was quite low. In contrast, none of the *Methanolobus* species degraded methanol at temperatures below 7°C (Table 2).

By suspending the resting cells (81.4 μg cell protein/ml) of strain R15 in various concentrations of methanol and incubating them at 18°C, methanol conversion kinetics were determined. The conversion rate was measured as a K_m value of 87.5 ± 0.4 μM and a V_{max} value of 0.39 ± 0.04 mM h^{-1}, suggesting that strain R15 could efficiently produce CH_4 even from low levels of methanol. Stoichiometrically, 0.73 ± 0.019 mmol CH_4 was formed from 1 mmol methanol consumed by the resting cells of strain R15 at 18°C.

Quantification of strain R15 in Zoige wetland soil and methylotrophic methanogenic community. Community DNAs were extracted from either the original soil sample or the enrichments with methanol or trimethylamine incubated at 15°C and 30°C for 60 days. By using a pair of strain R15-specific 16S rRNA primers and a pair of archaeal universal 16S

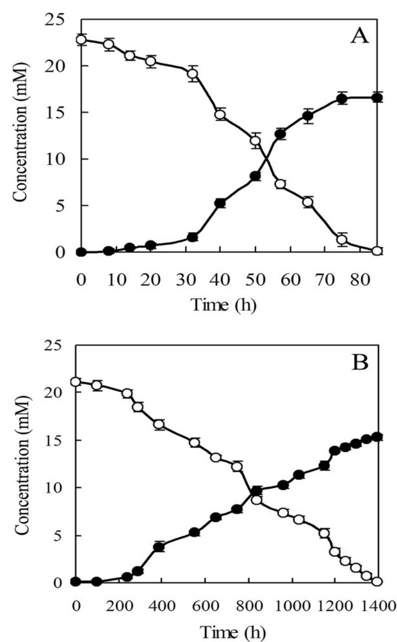

FIG. 4. Methanol degradation (open circles) and methane production (filled circles) by *Methanolobus psychrophilus* R15 growing cells at a methanol concentration of 20 mM, incubated at 18°C (A) and 0°C (B). Data are means for three replicates. Standard deviations are shown.

rRNA primers, the 16S rRNA gene copies of strain R15 and total archaea were determined to be 5.5×10^7 and 3.2×10^8 per gram of soil, respectively (Table 3). Thus, the cell mass of strain R15 could account for 17.2% ± 2.1% of the total archaea in the original soil sample. The 16S rRNA gene copies of strain R15 were increased 33- and 20-fold in methanol and trimethylamine enrichments, respectively, at 15°C, and the proportions among the total archaea in the methylotrophic enrichments were also elevated to 42.4% ± 2.3% and 31.2% ± 1.8%, respectively. However, the percentages of strain R15 in the total archaea decreased to 6.7% ± 1.1% and 3.8% ± 0.5%, respectively, in the two enrichments at 30°C.

DISCUSSION

With its location in a low-latitude region, the Zoige wetland receives intensive sunlight, with an annual sunlight time of 2,353.6 to 2,516.5 h (37), so cold-tolerant grasses such as *Carax* and *Eleocharis* grow vigorously. During the growing season, high CH_4 flux (14.45 mg m^{-2} h^{-1}) has been measured from the soil layer at a depth of 10 to 20 cm (4), where the roots of *Carex muliensis* and *Eleocharis valleculosa* can reach. Temperature is a determinative factor on the methanogen population in an ecosystem; generally, members of the *Methanosarciaceae* and *Methanomicrobiaceae* are prevalent, and acetoclastic methanogenesis dominates in the cold wetlands (11, 12, 23). The soil pH can be another limiting factor in determining the methanogen community and methanogenic pathways. Horn et al. (16) demonstrated that hydrogen was an important methanogenic precursor in acidic peat bogs, and hydrogenotrophic methanogens were enumerated to be 10^7 per gram of soil. Kotsyurbenko et al. showed that in a Siberian acidic peat bog (pH 4.2 to 4.8), acetoclastic and hydrogenotrophic methanogeneses operated closely, at a ratio of approximately 2:1, irrespective of the incubation temperature (23). The Zoige wetland is a neutral peatland with a pH value range of 7.0 to 7.5. As shown in this study, in contrast to the case for methanogens prevalent in acidic peat bogs, methylotrophic and acetoclastic methanogeneses are determined to be more robust at low temperatures in this neutral pH soil, although hydrogenotrophic methanogenesis can be comparable at 30°C. Furthermore, the novel psychrophilic *Methanolobus* strain R15 was isolated from a methylotrophic methanogenic enrichment obtained from the soil.

Strain R15 produces CH_4 from methanol efficiently at a low temperature (18°C), and even at 0°C, and has a high affinity for methanol (K_m of 87.5 ± 0.4 μM). All of these observations suggest that strain R15 could perform an effective methanol-derived methanogenesis in the Zoige wetland, as 0.48 to 2.6 mM methanol has been detected in the soil. Physiologically, strain R15 is also adapted to the physical-chemical environment of the Zoige wetland (neutral soil containing about 40 mM NaCl), as shown by its large number (10^7 per gram of soil, based on three or four 16S rRNA gene copies in *Methanosarcinales* [26]) and large proportion (about 17% of the total archaea) in the wetland. An even higher percentage (about 40%) of strain R15 was determined for the methylotrophic community at a lower temperature (15°C). Taken together, these data suggest that the methanogen R15 could be a predominant psychrophilic methanogen and function in methane emission in the cold Zoige wetland. Methanogen community structure in different wetlands could be correlated to the field vegetation types, which would either excrete unique compound mixtures or accumulate grass-specific biomass, with the latter providing methanogenic substrates via the multiple species involved in biodegradation relay. A linkage between vegetation type and archaeal community composition has been reported for Alaskan peatlands (31).

In this study, the highest methanogenesis rate was detected for methanol among the detected methanogenic substrates in

TABLE 3. 16S rRNA copy numbers for *Methanolobus psychrophilus* R15 and total *Archaea* in methylotrophic enrichments incubated for 60 days and in the original soil sample

Substrate (30 mM)	16S rRNA copy no. in enrichment at 15°C		% R15 in total *Archaea* at 15°C	16S rRNA copy no. in enrichment at 30°C		% R15 in total *Archaea* at 30°C
	R15 (10^7)	*Archaea* (10^8)		R15 (10^7)	*Archaea* (10^8)	
Methanol	185.2 ± 2.2	43.6 ± 2.4	42.4 ± 2.3	5.2 ± 0.4	7.8 ± 0.4	6.7 ± 1.1
Trimethylamine	112.4 ± 1.7	35.4 ± 3.2	31.2 ± 1.8	5.8 ± 0.7	15.3 ± 0.9	3.8 ± 0.5
In situ soil	5.5 ± 0.3	3.2 ± 0.4	17.2 ± 2.1			

Zoige wetland soil, and in a separate experiment, the highest most-probable-number quantification of methanol-utilizing methanogens was obtained (data not shown). Although the methanol level (0.48 to 2.6 mM) detected in Zoige wetland soil is not very high, it is much higher than those reported for other wetlands (5), suggesting that methanol-derived CH_4 can contribute more in Zoige wetland than in other ecosystems (5), which is probably attributable to the different vegetation. Although acetate has been demonstrated to contribute the largest proportion of CH_4 flux in paddy soil at relatively low temperatures (11) and in boreal permafrost (24), the methanol contribution to CH_4 flux in different types of wetlands may need to be reassessed, especially in cold terrestrial wetlands with luxuriant grasses, where methanol can be released from pectin and not many methylotrophic competitors exist, except for cohabiting homoacetogens.

So far, only a few psychrophilic methanogens have been isolated, and those were all isolated from Antarctic lakes, such as *Methanococcoides burtonii* (13) and *Methanogenium frigidum* (14). The description of the psychrophilic methanogen R15 in this work will contribute to the knowledge of the diversity of the cold-loving methanogenic archaeal family.

Currently, the genus *Methanolobus* is comprised only of members isolated from saline habitats and mesophilic growth (1). Upon the recruitment of strain R15, the genus description of *Methanolobus* has to be amended to include species that are both psychrophiles and mesophiles.

Description of the novel species *Methanolobus psychrophilus*. *Methanolobus psychrophilus* (psychrophilus. Gr. adj. *psychros*, cold; Gr. adj. *philus*, preferring; M. neut. adj. *psychrophilus*, preferring cold).

Cells are elliptical (0.9 to 1.0 by 1.0 to 1.2 μm), with one or two polar flagella, are wrapped by a capsule-like material, and frequently form loose aggregates. The strain is psychrophilic and grows most rapidly at 18°C, with no growth above 25°C. Methanol is the preferred methanogenic substrate; methylamine and methyl sulfide are used as well. H_2-CO_2, formate, and acetate are not utilized. It is slightly halophilic. Better growth occurs in a medium supplemented with 15 to 300 mM NaCl, optimal growth occurs with 200 to 250 mM NaCl, and no growth occurs with $<$5 mM or $>$800 mM NaCl. The pH range for growth is 6.0 to 8.0, and the optimum pH is 7.0 to 7.2. Yeast extract is not essential but stimulates growth. The G+C content of the DNA of strain R15 is 44.9 mol% (at melting temperature).

Methanolobus psychrophilus R15^T (JCM 14818^T; CGMCC 1.5060^T) is isolated from Zoige wetland soil, obtained from a permanently cold wetland located on the Tibetan plateau in southwestern China.

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