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EXPERIMENTAL ARTICLES

Methanocalculus natronophilus sp. nov., a New Alkaliphilic Hydrogenotrophic Methanogenic Archaeon from a Soda Lake, and Proposal of the New Family *Methanocalculaceae*

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Abstract—A mesophilic hydrogenotrophic methanogenic archaeon, strain Z-7105^T, was isolated from the bottom sediments of a collector in the vicinity of a soda lake Tanatar II (Altai, Russia). The cells were motile, irregular cocci 0.2–1.2 μm in diameter. The organism was an obligate alkaliphile, growing within a pH range from 8.0 to 10.2, with the optimum at pH 9.0–9.5. It was obligately dependent on carbonates, growing at 0.5 to 1.6 M total carbonates with the optimum at 0.7–0.9 M. Sodium ions were also obligately required at concentrations from 0.9 to 3.3 M Na^+ (optimum at 1.4–1.9 M). The organism was halotolerant, but Cl^- ions were not required. Hydrogen and formate were used as electron donors. Acetate was required for anabolism. The DNA G+C content was 50.2 mol %. According to the results of its 16S rRNA gene sequence analysis, the isolate belonged to the genus *Methanocalculus*, being the first known alkaliphilic member of this genus. Its similarity to the neutrophilic and halotolerant *Methanocalculus* species (*M. halotolerans, M. taiwanensis, M. pumilus*, and *M. chunghsingensis*) was 98.2–97.1%, which is within the interspecific range for this genus. The level of DNA–DNA hybridization between strain Z-7105^T and the *Methanocalculus* type species *M. halotolerans* DSM 14092^T was 32%. The genus *Methanocalculus*, including the new isolate and the previously described species, is distant from other genera of methanogens (<90% 16S rRNA gene similarity). Based on significant phenotypic differences and the results of phylogenetic analysis, including DNA–DNA hybridization, it is proposed to assign strain Z-7105^T (=DSM 25006^T, =VKM B-2765^T) to the new species *Methanocalculus natronophilus* sp. nov., and to incorporate the genus into the new family *Methanocalculaceae* fam. nov.

Keywords: soda lakes, alkaliphiles, methanogens, hydrogenotrophs, archaea, *Methanocalculus natronophilus, Methanocalculaceae*

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Unlike thalassic lakes, soda lakes have high carbonate alkalinity, which determines their hydrochemistry and high pH values. The hypothesis of G.A. Zavarzin that considers the alkaliphilic microbial communities of modern soda lakes to be analogues of Proterozoic microbiota [1, 2] has been developed and well-grounded in his laboratory over the last two decades, and the results of these studies were summarized recently [3–7].

It is believed that in the early biosphere, in the absence of other electron acceptors, the leading role at final stages of organic matter degradation belonged to the low-potential reduction processes driven by hydrogenotrophic anaerobic organisms for which the electron acceptor was CO_2 [6]. Two metabolic pathways are possible under such conditions: hydrogenotrophic acetogenesis and methanogenesis. Both processes could have been important as a hydrogen sink in Precambrian soda lake ecosystems under conditions of reduced sulfur cycle. In the course of our studies aimed at the isolation of anaerobes of these groups, we managed to isolate the first extremely natronophilic, lithotrophic, hydrogenotrophic, homoacetogenic bacterium *Fuchsiella alkaliacetigena* gen. nov., sp. nov. from sediments of the highly mineralized Tanatar soda lakes (Altai, Russia) [8]. The next task was to reveal hydrogenotrophic alkaliphilic methanogenic archaea that grow under conditions of high mineralization. The growth of such methanogens was demonstrated in situ in samples from both low-mineralized [9, 10] and highly mineralized [11] soda lakes. However, so far, only one alkaliphilic methanogen has been described, namely *Methanobacterium alcaliphilum* [12], isolated from the alkaline Wadi Natrun (Egypt) reservoir; it has a growth optimum of 0.3 M Na^+ and the upper growth limit of 0.7 M Na^+. Taking Mongolian soda lakes as an example, it was shown that methanogenesis from hydrogen is not limited by high salt concentrations (165–390 g/L) [11]. However, this

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fact has not been explained due to the absence of isolates of hydrogenotrophic methanogens capable of growth under conditions of high mineralization.

In the present work, we describe the first hydrogenotrophic alkaliphilic representative of the genus *Methanocalculus*, isolated from a soda lake and capable of growth under conditions of high mineralization, having a growth optimum of 1.4–1.9 M Na^+ and an upper limit of growth at 3.3 M Na^+.

MATERIALS AND METHODS

The source of isolation. As the material for enrichment and isolation of methanogenic archaea, we used samples taken by V.V. Kevbrin in the field season of year 2007 from bottom sediments of Tanatar soda lakes (Altai, Russia) and from the collectors in the vicinity of these lakes . The samples differed in their total mineralization (65–235 g/L), determined by high content of sodium carbonates, but had similar pH values (10–10.5). Strain Z-7105 was isolated from sample 15-6 taken from the southern part of a collector in the vicinity of the soda lake Tanatar II with pH 10.4 and total mineralization of 60 g/L.

Enrichment cultures and cultivation conditions. To reveal anaerobic hydrogenotrophic microorganisms that use CO_2 as an electron acceptor, the samples were inoculated into cultivation medium simulating the hydrochemical composition of the water of the studied lakes (total mineralization of 10 and 21%). Culture medium with a mineralization of 21% contained the following (g/L): KH_2PO_4, 0.2; $MgCl_2 \cdot 6\ H_2O$, 0.1; NH_4Cl, 0.25; KCl, 0.2; NaCl, 94.8; Na_2CO_3, 100.2; $NaHCO_3$, 15.0; $Na_2S \cdot 9H_2O$, 0.5; pH 10.15. The same medium diluted twofold was used as the medium with 10% mineralization.

Enrichment cultures were grown in hermetically closed 100-mL vials with hydrogen as the gas phase and 20 mL of medium, which was inoculated with 1 mL of sediment samples. The incubation was at 35°C. The growth of the two groups competing for hydrogen—methanogens and acetogens—was judged from methane and acetate formation measured after 30 days of incubation.

The main medium for isolation of methanogens contained the following (g/L): KH_2PO_4, 0.2; $MgCl_2 \cdot 6H_2O$, 0.1; NH_4Cl, 0.25; KCl, 0.2; NaCl, 15.7; Na_2CO_3, 68.0; $NaHCO_3$, 38.0; $CH_3COONa \cdot 3H_2O$, 0.2; $Na_2S \cdot 9H_2O$, 0.5; Bacto (BD) yeast extract, 0.05, or vitamin solution [13], 10 mL/L; trace element soulution [14], 1 mL/L; pH 9.5. Hydrogen (100% in the gas phase) or sodium formate (5 g/L) were used as electron donors.

Pure culture, designated Z-7105^T, was obtained by tenfold dilutions in liquid medium under hydrogen in the presence of 200 mg/L of vancomycin.

Preparation of media and cultivation were carried out under strictly anaerobic conditions under an atmosphere of hydrogen or nitrogen (if formate was used as the substrate).

Physiological characteristics. The dependences of methanogenesis on pH, temperature, and mineralization were determined on medium with 5 g/L of formate, each in two replicate series of Hungate tubes.

The pH dependence was determined at 35°C on the main medium containing 70 g/L of $NaHCO_3$ and 30 g/L of NaCl and devoid of Na_2CO_3. The pH was adjusted to the required values by the addition of 12 M HCl or of 12 M NaOH. By the end of the experiment, the pH values shifted insignificantly, no more than by 0.2 units towards acidic values. The requirement for NaCl, dependence on mineralization, and the optimal concentrations of salts were determined as described previously [8]. The dependence on temperature was determined in the range of 10–50°C with a step of 5°C, using the main medium with carbonates devoid of NaCl and with a total mineralization of 1.73 M Na^+.

Sensitivity to antibiotics was determined by the addition to the medium of sterile concentrated antibiotic solutions to final concentrations of 0.2 g/L.

The substrates tested for their suitability for methanogenesis were added to the main medium from sterile concentrated solutions before inoculation to final concentrations of 10 mM (trimethylamine, *n*-propanol, and iso-propanol), 20 mM (acetate), and 40 mM (formate, methanol, pyruvate, and butanol). Hydrogen oxidation was tested with 100% hydrogen in the gas phase.

Analytical methods. Methane and hydrogen were determined on a Crystal 5000.2 gas chromatograph (Chromatec, Russia). Separation was carried out in a 1-m glass column filled with 5A molecular sieve (Serva, Germany).

Morphology studies. Living cells were examined under a ZETOPAN phase-contrast microscope (Reichert, Austria). Thin sections and cells stained with 1% phosphotungstic acid for flagella visualization were examined under a JEM-100C electron microscope (Japan).

DNA analysis. The DNA G+C content was determined from thermal denaturation/reassociation curves, and the DNA–DNA hybridization level was determined by the optical reassociation method as described previously [15] using a Cary 100 Bio spectrophotometer (Varian, United States) at a heating rate of 0.5°C/min. *Methanocalculus halotolerans* SEBR 4845^T (=DSM 14092^T), employed in DNA–DNA hybridization, was obtained from the German Collection of Microorganisms (DSMZ).

DNA isolation and 16S rRNA gene amplification and sequencing. DNA was isolated using a modified Birnboim–Doly method [16] and the Wizard technology (Promega, United States). For PCR and further sequencing of the PCR products, a universal primer system was used [17].

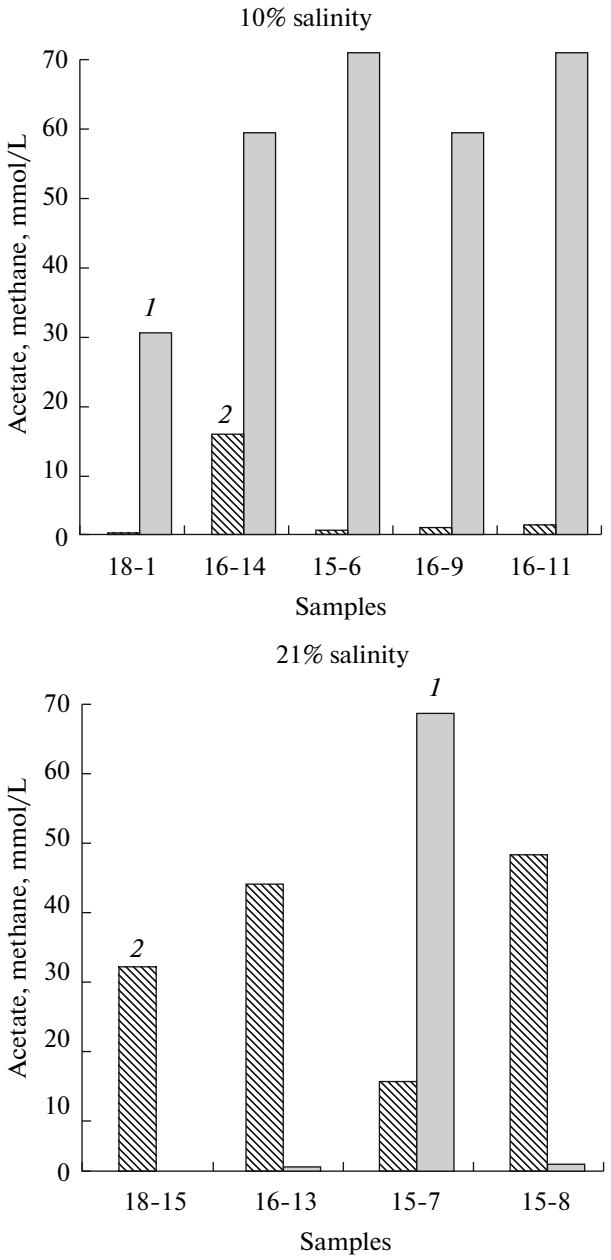

Fig. 1. Comparison of the processes of hydrogenotrophic methanogenesis (*1*) and acetogenesis (*2*) in samples from Tanatar reservoirs.

The primer pair used for the amplification of the full-size 16S rRNA gene was 1492r (5'-TACGGYTACCTTGTTACGACTT-3') and 8fa (5'-TCCGGTTGATCCTGCCGG-3').

DNA sequencing was performed by the Sanger method [18] using the BigDye Terminator v. 3.1 Sequencing Kit (Applied Biosystems Inc., United States) on an ABI PRIZM 3730 DNA Analyzer (Applied Biosystems Inc.) according to manufacturer's recommendations. Both internal and external primers were used for sequencing, and it was performed in both directions. The obtained sequence spectrograms were edited using Chromas v. 1.45 software package (http://www.techelysium.com.au./chromas.html).

Phylogenetic analysis of the 16S rRNA gene. The search for closely related species was carried out using the BLAST online tool (http://blast.ncbi.nlm.nih.gov/blast) at the NCBI site (http://www.ncbi.nlm.nih.gov). The required nucleotide sequences were retrieved from the GenBank/EMBL/DDBJ database at the NCBI site. The alignment of the sequences and subsequent phylogenetic tree construction was carried out using the SeaView 4.4.0 software package [19]. The phylogenetic tree was constructed using the neighbor-joining procedure (BioNJ) and an algorithm for paralinear distances (LogDet), which takes into account unequal rates of nucleotide substitutions [20].

Deposition of the nucleotide sequences. The obtained 16S rRNA gene sequence of strain Z-7105^T has been deposited in GenBank under accession number JX966306.

RESULTS

Enrichment cultures. Initial inoculation of the samples into the culture media with 21 and 10% mineralization led to utilization of carbon dioxide as an electron acceptor and hydrogen as an electron donor by two groups of anaerobes which competed for hydrogen: hydrogenotrophic methanogens and acetogens (Fig. 1). At 10% mineralization, methanogenesis prevailed in all of the studied samples taken from the Tanatar reservoirs. At 21% mineralization, homoacetogenesis prevailed in most samples, indicating the presence of extremely alkaliphilic homoacetogens isolated and characterized by us previously [8].

Our further work focused on the isolation of alkaliphilic hydrogenotrophic methanogens, which were most active at moderate mineralization (2.65 M Na^+, pH 10.0). After a series of transfers in the main cultivation medium with 0.05 g/L yeast extract under hydrogen, enrichment cultures of methanogens were obtained from several samples. Microscopy revealed predominance of angular cocci with a small admixture of rods. Sample 15-6, which demonstrated the most active methanogenesis (Fig. 1), was chosen for pure culture isolation.

Pure culture isolation. In spite of the addition of the antibiotic vancomycin, which affects bacteria but not archaea, a time-consuming procedure appeared to be necessary to separate the methanogenic culture from the bacterial component. Neither of the components produced colonies in agar medium in roll-tubes. The bacterial component was isolated in liquid glucose-containing medium and identified as a new representative of the order *Halanaerobiales: Halanaerobium* sp. strain Z-7106. It was resistant to a number of antibiot-

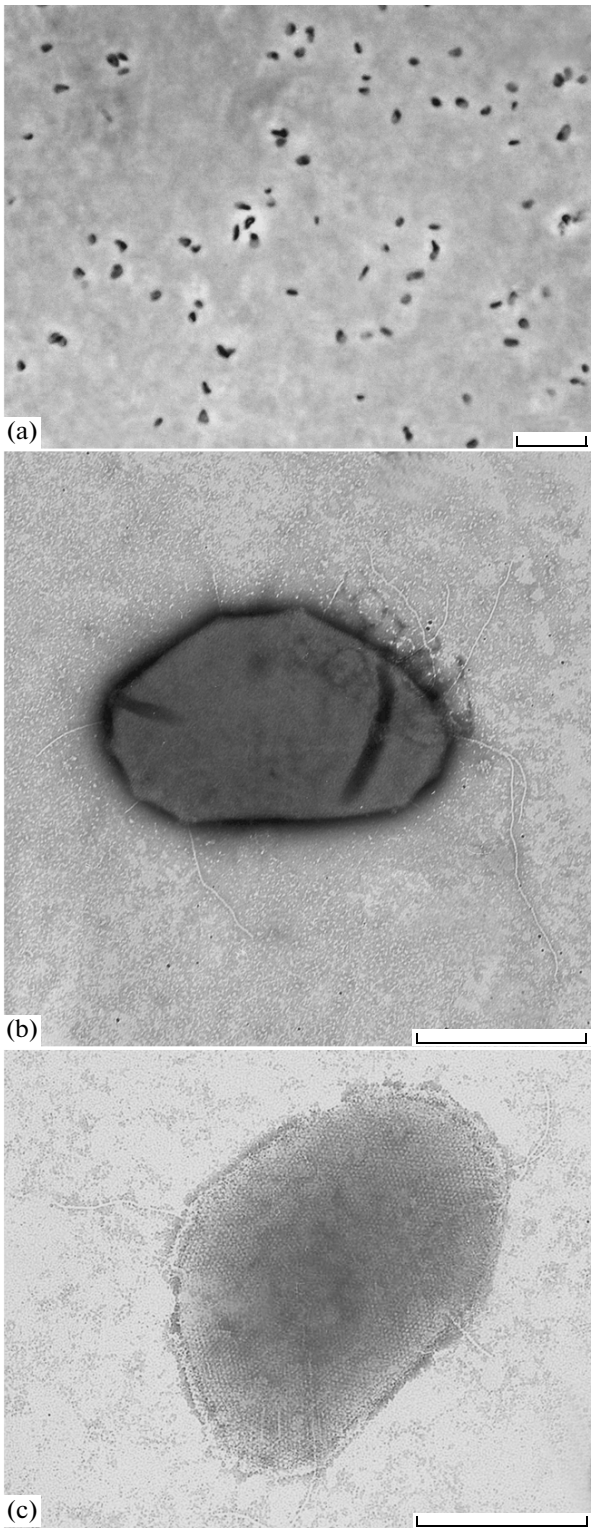

Fig. 2. Morphology of strain Z-7105^T cells: (a) cells under light microscope, phase contrast; bar, 5 μm; (b) cells with flagella, negative staining with phosphotungstic acid, electron microscope; bar, 0.5 μm; (c) cells with the cell wall S-layer; bar, 0.5 μm.

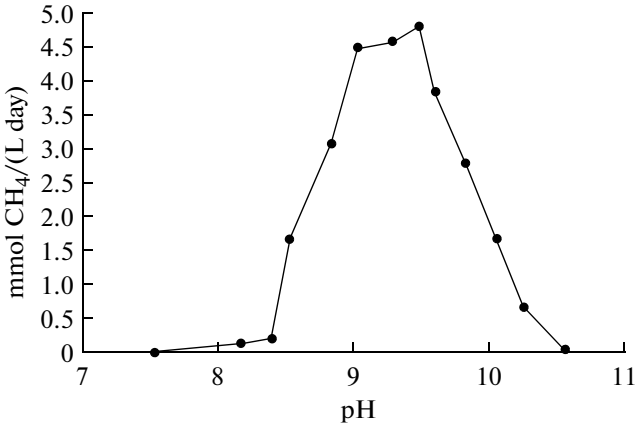

Fig. 3. pH dependence of methanogenesis by strain Z-7105^T.

ics and was able to grow at low concentrations of yeast extract. After we replaced yeast extract with acetate (0.2 g/L) and vitamins, we managed to isolate the archaeon, designated as strain Z-7105^T, by the method of tenfold dilutions in liquid medium under hydrogen. The purity of this methanogenic culture was confirmed microscopically and by the absence of growth in media with 1 g/L of yeast extract and trypticase or with 3 g/L of glucose, as well as by the partial sequencing of the 16S rRNA gene with the use of various primers.

Morphology. In the exponential phase of growth, cells of strain Z-7105^T were irregular angular cocci 0.2–1.2 μm in diameter, single, in pairs or in small aggregates (Fig. 2a). The variations in diameter were due to an asymmetric position of septa formed in the course of cell division (Fig. 2b). The cells were motile by means of peritrichous flagella (Fig. 2b).

The treatment of the cells with SDS (0.07%) resulted in their immediate lysis, suggesting the proteinaceous nature of the cell wall [21]. Negative staining of whole cells allowed us to visualize, on some of the cells, an S-layer consisting of hexagonal subunits located as a monolayer (Fig. 2c).

Growth characteristics. Strain Z-7105^T was strictly anaerobic and could grow only in the presence of reducing agents.

The strain appeared to be an obligate alkaliphile, growing within a pH range from 8.0 to 10.2 with the optimum at pH 9.0–9.5. At pH 7.5 and lower or at pH 10.5 and higher no growth occurred (Fig. 3). The strain was obligately dependent on carbonate ions but independent of chloride ions. Thus, the strain is a true alkaliphile and natronophile. To maintain alkalinity, sodium carbonates were necessary, which could not be replaced by an equimolar amount of NaCl with the addition of the Tris (pH 8.5) and CAPS (pH 9.7) organic buffers. Strain Z-7105^T grew at high alkalinity, up to a total carbonate concentration of 1.6 M, with

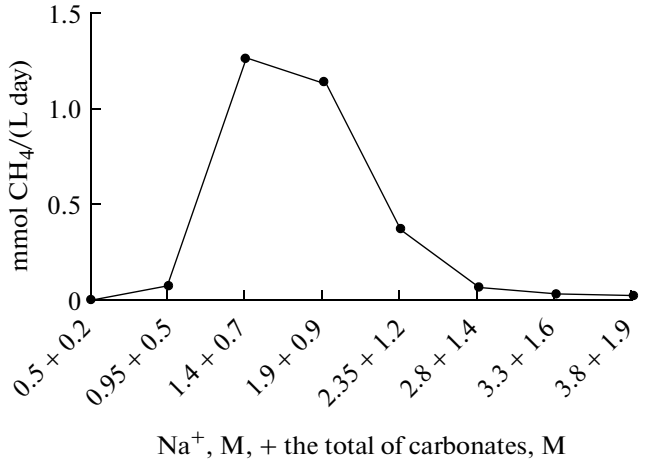

Fig. 4. Dependence of methanogenesis by strain Z-7105^T on mineralization of the medium (summarized molar concentration of sodium and carbonate ions).

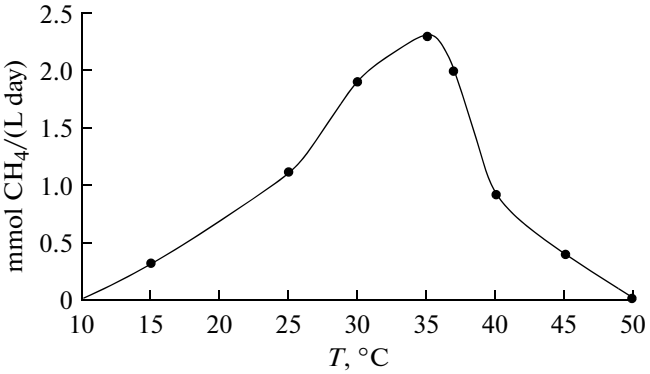

Fig. 5. Temperature dependence of methanogenesis by strain Z-7105^T.

the lower limit of 0.5 M, and it was obligately dependent on Na^+ ions, having a growth range of 0.9–3.3 M Na^+ and an optimum at 1.4–1.9 M (Fig. 4). The growth of strain Z-7105^T did not depend on NaCl, which could be excluded from the medium or replaced with Na_2SO_4, indicating strain Z-7105^T to be halotolerant. On the main carbonate medium (1.73 M Na^+ + 1.1 M total carbonates), the isolate grew within the range of 0–10% NaCl. The organism was mesophilic and grew in the temperature range from 15 to 45°C with the optimum at 35°C (Fig. 5).

Strain Z-7105^T could use only hydrogen or formate as energy source. Small quantities of sodium acetate (2 mM) were necessary for anabolic purposes. Yeast extract did not stimulate growth and could be replaced by vitamins. Under a nitrogen atmosphere, the strain produced no methane from acetate, methanol, trimethylamine, pyruvate, *n*-propanol, iso-propanol, or *n*-butanol after two months of incubation at 35°C.

Sensitivity to antibiotics. Strain Z-7105^T was resistant to ampicillin, benzylpenicillin, vancomycin, and kanamycin. The growth was completely suppressed by chloramphenicol and rifampicin and partially inhibited by novobiocin and streptomycin.

Phylogenetic analysis. The results of the search for closely related organisms allowed us to assign the new isolate to the genus *Methanocalculus* (Fig. 6). Currently, this genus includes four valid species with the following degrees of 16S rRNA gene similarity with the newly isolated strain: *M. halotolerans,* 97.8%; *M. pumilus*, 98.2%; *M. taiwanensis*, 98.0%; and *M. chunghsingensis*, 97.1%. These values are within the range of an interspecies similarity level for the representatives of this genus (98.2–99.1%) [22–24]. This interspecies level of 16S rRNA gene similarity within the genus *Methanocalculus* was confirmed by DNA–DNA hybridization between the species: the results were in the range of 10–51% [23, 24]. DNA–DNA hybridization level between strain Z-7105^T and the type strain of the genus *Methanocalculus*, *M. halotolerans* SEBR 4845^T (=DSM 14092^T) [25] was 32%, indicating the possibility of assigning strain Z-7105^T to a separate species. The DNA G+C content of strain Z-7105^T was 50.2 mol %.

DISCUSSION

The new methanogenic isolate is a strictly anaerobic hydrogenotrophic chemolithoheterotroph. The narrow range of the utilized substrates determines its role in the trophic system of the alkaliphilic microbial community as the role of a secondary anaerobe which uses hydrogen or formate produced by primary organotrophic anaerobes.

By its pH range and optimum and its requirements for carbonate and sodium ions, strain Z-7105^T corresponds to its ecological niche and can thrive in soda lakes with a high upper limit of total mineralization (up to 200 g/L) and high alkalinity (up to 1.6 M total carbonates). Among hydrogenotrophic methanogens, this strain is so far the only organism capable of growth at so high mineralization values. However, based on its pH and mineralization optima, strain Z-7105^T is to be referred to moderate alkaliphiles. It does not require sodium chloride but is tolerant to its presence and can be thus characterized as halotolerant natronophile.

Phenotypic properties of strain Z-7105^T are compared with those of *Methanocalculus* species in the table. In spite of the different isolation sources, which include a marine estuary, oil wells, and fresh water, the four species described previously are halotolerants and neutrophiles. They are similar in their ecophysiology and differ significantly from the strictly alkaliphilic strain Z-7105^T, which obligately requires carbonate and sodium ions and is an isolate from soda lakes, i.e., from an essentially different ecological niche differing from marine and freshwater reservoirs by hydrochemistry, high carbonate alkalinity, and pH values. Given

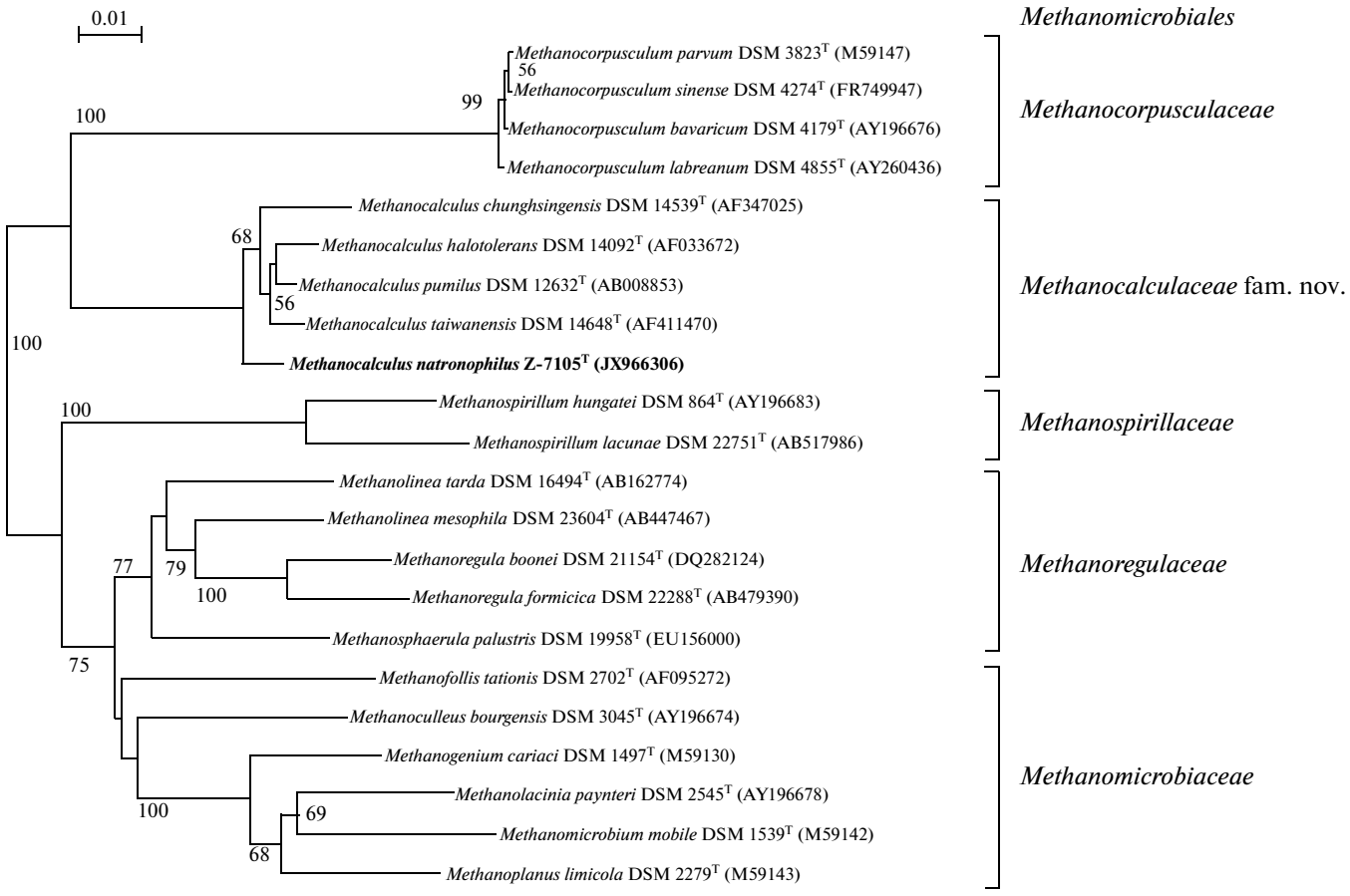

Fig. 6. Phylogenetic tree of representatives of the order *Methanomicrobiales* and the position of strain Z-7105^T within the order. Bootstrap values higher than 50% are shown. For all families except *Methanomicrobiaceae*, representatives of all valid genera and species are shown. For *Methanomicrobiaceae*, which includes numerous species, only type species of all genera are shown. The scale bar corresponds to 1 nucleotide substitution per 100 nucleotides.

the significant phenotypic differences, the newly isolated strain cannot be assigned to any known species of the genus *Methanocalculus*, which is confirmed by phylogenetic analysis and the results of DNA–DNA hybridization.

The new strain is the first alkaliphilic hydrogenotrophic representative of the genus *Methanocalculus* to be described in detail. However, strains with a high degree of 16S rRNA gene similarity (99.9%) to Z-7105^T had been isolated from Central Asia soda lakes [26] and alkaline Lonar Lake (India) [27], indicating wide geographical distribution of these hydrogenotrophic methanogenic archaea in soda lakes.

Phylogenetically, the genus *Methanocalculus* belongs to the order *Methanomicrobiales*, which includes four valid families (Fig. 6). However, this genus was not included in any of these families because of its remote phylogenetic position (<90% 16S rRNA gene similarity). The possibility has been noted of its placement into a new higher-rank taxon [28]. The closest representative of the family *Methanoregulaceae, Methanolinea mesophila* [29], has 89% 16S rRNA gene similarity with strain Z-7105^T, allowing the genus *Methanocalculus* to be assigned to a separate family.

Based on the results of phylogenetic and phenotypic analyses, we propose to assign strain Z-7105^T to a new species of the genus *Methanocalculus, Methanocalculus natronophilus* sp. nov., within the family *Methanocalculaceae* fam. nov., which we propose for the species of the genus *Methanocalculus* (Fig. 6).

Description of *Methanocalculus natronophilus* sp. nov.

Methanocalculus natronophilus (na.tro.no.phi'lus. L. adj. *natronophilus* liking soda).

The cells are irregular angular cocci 0.2–1.2 μm in diameter, single, in pairs, or in small aggregates, motile by means of peritrichous flagella. Cell division is asymmetrical, by septum formation. Strictly anaerobic. Obligately alkaliphilic, with a pH growth range of 8.0–10.2 and an optimum at pH 9.0–9.5. Obligately natronophilic, growing on carbonate-bicarbonate medium at 0.9–3.3 M Na^+ (optimum, 1.4–1.9 M) and 0.5–1.6 M total carbonates (opti-

Comparison of the characteristics of the new isolate, valid species of the genus *Methanocalculus*, and the phylogenetically closest genus *Methanolinea mesophila*

Characteristics	Z-7105^T	*M. halotolerans* SEBR 4845^T [25]	*M. taiwanensis* P2F9705^T [22]	*M. pumilus* MHT-1^T [23]	*M. chunghsingensis* K1F9705b^T [24]	*Methanolinea mesophila* TNR^T [29]
Size, μm	0.2–1.2 (cocci)	0.8–1.0 (cocci)	0.8–1.4 (cocci)	0.8–1.0 (cocci)	1.0–1.6 (cocci)	2.0– 6.5 × 0.3 (rods)
Motility	+ (peritrich)	+ (peritrich)	–	–	–	–
Dependence on carbonates	+	–	–	–	–	–
Dependence on Na^+, limits (optimum), M	0.94–3.3 (1.4–1.9)	0–2 (0.85)	0–0.5 (0.2)	0–1.2 (0.2)	0–2 (0.2)	–
Temperature, limits (optimum), °C	15–45 (35)	25–45 (38)	28–37 (37)	24–45 (35)	20–45 (37)	20–40 (37)
NaCl concentration limits, %	0–10*	0–12	0–3	0–7	0–12	0–2.5
pH, limits (optimum)	8.0–10.2 (9–9.5)	7.0–8.4 (7.6)	6.3–8.3 (6.8)	6.5–7.5 (ND)	5.8–7.7 (7.2)	6.5–7.4 (7.0)
Utilization of H_2/CO_2 and formate	+	+	+	+	+	+
Acetate requirement for growth	+	+	+	+	+	+
Growth stimulation by yeast extract	–	+	+	+	+	+
DNA G+C content, mol %	50.2	55	ND	51.9	50.8	56.4
Habitat	Soda lake (Altai, Russia)	Oil well (Alsace, France)	Estuary (Taiwan)	Solid waste disposal site (Osaka, Japan)	Marine fishpond (Taiwan)	Rice field soil (Taiwan)

* In the presence of 1.1 M total carbonates.

mum, 0.7–0.9 M). Halotolerant. Cl^- ion is not required, but growth occurs at 0–10% NaCl in the presence of 1.1 M total carbonates. Mesophilic, growing at 14–45°C with the optimum at 35°C. Methane production occurs from $H_2 + CO_2$ and formate. Acetate is required for growth as the carbon source. Yeast extract does not stimulate growth and can be replaced with vitamins. Acetate, methanol, trimethylamine, pyruvate, *n*-propanol, iso-propanol, and *n*-butanol cannot be the substrates for methanogenesis.

The DNA G+C content is 50.2 mol %.

The habitat is bottom sediments of soda lakes. The type strain was isolated from bottom sediments of a collector in the vicinity of soda lake Tanatar II (Altai, Russia).

The type strain is Z-7105^T (=VKM B-2765^T, =DSM 25006^T).

Description of *Methanocalculaceae* fam. nov.

Methanocalculaceae (Me.tha.no'cal.cu.la'ce.ae. N.L. neut. n. *Methanocalculus* the type genus of the family; the suffix *aceae* denotes a family; N.L. fem. pl. n. *Methanocalculaceae* the family of the genus *Methanocalculus*).

The family includes morphologically irregular angular pebble-like motile or nonmotile cocci with strictly anaerobic chemolithoheterotrophic metabolism. The substrates for catabolism are $H_2 + CO_2$ and formate. Acetate is required for anabolism. Mesophiles. Neutrophiles or alkaliphiles. Halotolerants or natronophiles. Belong to the order *Methanomicrobiales* of the phylum *Euryarchaeota* of the *Archaea* domain. The type genus of the family is *Methanocalculus*.

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