Methermicoccus shengliensis gen. nov., sp. nov., a thermophilic, methylotrophic methanogen isolated from oil-production water, and proposal of *Methermicoccaceae* fam. nov.

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A thermophilic, methylotrophic methanogen, strain ZC-1^T, was isolated from the Shengli oilfield, China. Cells of strain ZC-1^T were motile cocci, 0.7–1.0 μm in diameter and always occurred in clusters of two to four cells. Lysis-susceptibility experiments and analysis of transmission electron micrographs of strain ZC-1^T suggested the presence of a proteinaceous cell wall. Strain ZC-1^T used methanol, methylamine and trimethylamine as substrates for methanogenesis. Optimal growth, with a doubling time of around 5 h, occurred at pH 6.0–6.5, 65 °C, 0.3–0.5 M NaCl and 0.05–0.20 M $MgCl_2$. The DNA G+C content of this organism was 56 mol%. Analysis of 16S rRNA gene sequence and the inferred amino acid sequence of the *mcrA* gene of strain ZC-1^T indicated that it is related specifically to members of the family *Methanosaetaceae* (90.6 and 76.6 % sequence similarity, respectively). However, strain ZC-1^T failed to grow with acetate as substrate for methanogenesis, which is a special characteristic of the family *Methanosaetaceae*. Based on these phenotypic and phylogenic characteristics, strain ZC-1^T is proposed to represent a novel genus and species, for which the name *Methermicoccus shengliensis* gen. nov., sp. nov. is proposed. The type strain is ZC-1^T (=CGMCC 1.5056^T=DSM 18856^T). *Methermicoccaceae* fam. nov. is also proposed.

The order *Methanosarcinales* comprises two families, *Methanosarcinaceae* and *Methanosaetaceae*. All described methanogens that dismutate methyl compounds and ferment acetate belong to the order *Methanosarcinales*. Among them, most methylotrophic methanogens are found in the family *Methanosarcinaceae*; the sole exception is the genus *Methanosphaera* of the family *Methanobacteriaceae* (Biavati *et al.*, 1988), members of which grow with methanol plus H_2. The family *Methanosarcinaceae* currently includes eight genera: *Methanosarcina*, *Methanococcoides*, *Methanohalobium*, *Methanohalophilus*, *Methanolobus*, *Methanosalsum*, *Methanimicrococcus* and *Methanomethylovorans* (Garrity & Holt, 2001; Jiang *et al.*, 2005; Sprenger *et al.*, 2000). *Methanimicrococcus blatticola* is unusual in the family because it requires both a methyl compound and H_2, similar to members of the genus *Methanosphaera* (Sprenger *et al.*, 2000). Members of the family *Methanosaetaceae* that ferment acetate as the sole source of energy include *Methanosaeta concilii* (Patel & Sprott, 1990), *Methanothrix thermophila* (Boone & Kamagata, 1998; Kamagata & Mikami, 1991) and *Methanosaeta harundinacea* (Ma *et al.*, 2006).

Work in our laboratory has focused on the study of anaerobes, especially methanogens, of petroleum reservoirs. Several hydrogenotrophic and methylotrophic methanogens have been isolated and identified in previous studies. Here, we describe a novel thermophilic, methylotrophic methanogen, strain ZC-1^T.

Strain ZC-1^T was isolated from oil-production water of block L801 in Shengli oilfield, where the oil reservoir is located 1680–1800 m below the sea floor and has a pressure of 9.24 MPa. The *in situ* temperature of the reservoir ranges from 75 to 80 °C and the total mineralized degree is 9794 mg l^{-1}. Crude oil was recovered by injecting seawater and then aerobic microbial cultures. Enrichments were performed with anaerobic medium in vials sealed with butyl-rubber stoppers and aluminium caps according to Ollivier *et al.* (1997), modified by reducing the

Abbreviation: HS-coM, coenzyme M.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *mcrA* gene sequences of strain ZC-1^T are DQ787474 and EF026570, respectively.

concentrations of $MgCl_2 . 6H_2O$ and $CaCl_2 . 2H_2O$ to 3 and 0.14 g l^{-1}, respectively. The pH was adjusted to 7.0 by using 10 M KOH before autoclaving, and $Na_2S . 9H_2O$ and $NaHCO_3$ were injected from sterile stock solutions to final concentrations of 0.03 and 0.5 %, respectively, before inoculation. The gas phase consisted of H_2/CO_2 (80/20, v/v) at a pressure of 200 kPa. After incubation at 55 °C in the dark for 2 weeks, a large amount of methane was detected. Fresh inoculum (5 ml) was then transferred anaerobically into a new bottle of sterile M141 medium (DSMZ, 1993) with methanol (120 mmol l^{-1}) under H_2/CO_2 (4/1, v/v). Ampicillin (1 mg ml^{-1}) was added to inhibit the growth of non-methanogenic organisms. Positive cultures were diluted serially and single colonies were obtained by the Hungate roll-tube method (Hungate, 1969). The purity of isolates was checked by phase-contrast microscopy after cultivation in an enriched medium and identified medium in the absence of antibiotics (Zhang & Zhao, 1987; Zhao *et al.*, 1986).

An Olympus BH-2 phase-contrast microscope and Olympus OM-2 camera were used routinely to observe cells. An Amray-1000B scanning electron microscope and a Hitachi-600VI transmission electron microscope were used to observe the microstructure of strain ZC-1^T (Lai & Chen, 2001; Mikucki *et al.*, 2003). Gram reaction was determined and susceptibility tests were performed as described by Boone & Whitman (1988), except that the Gram reaction was determined in PBS buffer. Strain ZC-1^T occurred in clusters of two to four cells. Cysts formed during the early exponential phase, but dispersed at stationary phase (Fig. 1a, b). Cells were motile cocci, 0.7–1.0 μm in diameter (Fig. 1b, c). Most cells lysed during the Gram-staining procedure; the residual cells stained positive. Cells also lysed in SDS (0.01 %, w/v), demineralized water and TE buffer after 30 min at room temperature, but they did not lyse in PBS (0.2 M, pH 7.4) or NaCl solution (0.9 %, w/v). These results were similar to those of cells with a proteinaceous wall; the presence of such a cell wall for strain ZC-1^T was confirmed by electron microscopy of ultrathin sections (Fig. 1d). Strain ZC-1^T formed colonies after around 20 days incubation at 60 °C, at which time the surface colonies in agar roll tubes were about 1–5 mm in diameter, yellow, smooth, circular and convex with entire edges. Colonies fluoresced blue–green under UV light.

To investigate potential substrates used by strain ZC-1^T, sodium formate (20 mM), sodium acetate (20 mM), trimethylamine (20 mM), monomethylamine (20 mM), ethanol (20 mM), dimethylsulfide (5, 10 and 40 mM), propan-2-ol (10 mM), isobutanol (10 mM), butan-2-ol (10 mM), H_2/CO_2 (80/20, 200 kPa) and H_2/CO (70/30, 200 kPa) were tested. Utilization of substrates was determined in M141 medium by monitoring methane production. Requirements for specific growth factors were tested in M141 medium. Trace element and vitamin solutions were prepared as described by Balch *et al.* (1979). Potential growth-stimulating compounds were tested in basal medium [KCl, 0.34 g; NH_4Cl, 0.25 g; K_2HPO_4, 0.2 g; NaCl, 24 g; $MgCl_2 . 6H_2O$, 10.2 g; yeast extract (Oxoid), 2 g; resazurin, 0.001 g; cysteine/$HCl . H_2O$, 0.5 g; distilled water, 1 l; pH approx. 7.0], with addition of sludge fluids (5 ml) or coenzyme M (HS-coM; 0.0025 g) as appropriate. Cells of strain ZC-1^T were strictly anaerobic and used only methanol, methylamine and trimethylamine as substrates for methanogenesis. No growth or methanogenesis was observed on sodium formate, sodium acetate, ethanol, dimethylsulfide, secondary

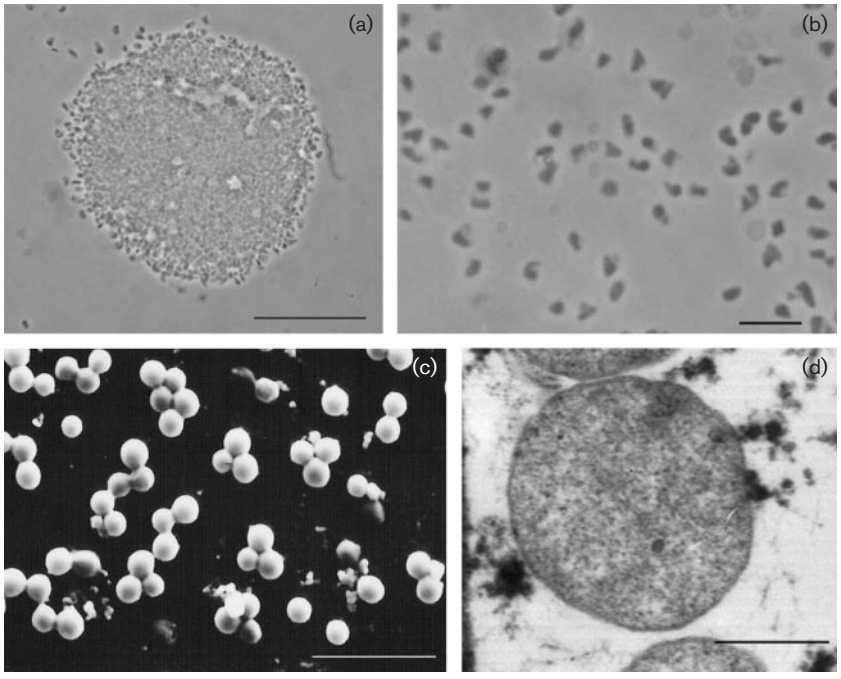

Fig. 1. (a, b) Phase-contrast micrographs of (a) cysts of strain ZC-1^T and (b) clusters of several cells of strain ZC-1^T. (c) Scanning electron micrograph showing regular cocci of strain ZC-1^T. (d) Transmission electron micrograph of ultrathin sections of strain ZC-1^T. Bars, 30 μm (a); 10 μm (b); 5 μm (c); 0.5 μm (d).

alcohols, H_2/CO_2 or H_2/CO. Strain ZC-1^T did not grow when $MgCl_2$ or both yeast extract and trypticase were removed from the M141 medium. Growth was much slower with trypticase than with yeast extract in basal medium (data not shown). The $MgCl_2$ concentration for good growth ranged from 0.05 to 0.20 M. Sludge fluid and HS-coM stimulated growth dramatically. After 156 h incubation, total methane production reached 0.41 mM with HS-coM, whereas the control reached just 0.04 mM. Cells also grew faster when sludge fluid was added (data not shown).

To investigate the sensitivity of strain ZC-1^T to antibiotics, kanamycin, streptomycin, ampicillin, chloramphenicol, rifampicin (all at 200 μg ml^{-1}) and erythromycin (125 and 500 μg ml^{-1}) were tested in basal medium with methanol (120 mM) at 65 °C. Growth was determined from total methane production. Growth of strain ZC-1^T was inhibited completely by chloramphenicol (200 μg ml^{-1}) and erythromycin (500 μg ml^{-1}); kanamycin (200 μg ml^{-1}) and erythromycin (125 μg ml^{-1}) caused partial inhibition of growth. The other antibiotics had no effects on the growth of strain ZC-1^T.

Effects of pH, temperature and NaCl concentration on strain ZC-1^T were determined in triplicate in basal medium with methanol (120 mM). Specific growth rates were measured according to Powell (1983). Gradient pH was adjusted according to Takai *et al.* (2002). Strain ZC-1^T grew between pH 5.5 and 8.0; no growth was observed at pH 5.0 or 8.5. The temperature range for growth was between 50 and 70 °C; growth was not observed at 45 or 75 °C. Strain ZC-1^T required 0.1–1.1 M NaCl for growth. Optimal growth, with a doubling time of around 5 h, occurred at pH 6.0–6.5, 65 °C, 0.3–0.5 M NaCl and 0.05–0.20 M $MgCl_2$.

Genomic DNA was isolated and purified by using the method of Marmur (1961) as modified by Jarrell *et al.* (1992), and its G+C content was determined by the thermal-denaturation method (Marmur & Doty, 1962), using a Beckman DU 800 spectrophotometer. *Escherichia coli* K12 DNA was used as a reference. The G+C content of the genomic DNA of strain ZC-1^T was 56 mol%, which was higher than those of members of the genus *Methanosarcina*, but similar to those of members of the genus *Methanosaeta*.

The protocol for PCR amplification was as follows: a single colony was dissolved in 100 μl sterile demineralized water for 10 min at 99 °C and centrifuged at 10 000 r.p.m. in a microfuge for 5 min. Supernatant (5 μl) served as the DNA template. The 16S rRNA and *mcrA* genes were amplified with a TaKaRa Thermocycler Dice TP600, using a TaKaRa 16S rDNA Bacterial Identification PCR kit. The primers for 16S rRNA gene amplification were the archaeon-specific primers 8F/1492R and 109F/915R (Banning *et al.*, 2005). The primers for *mcrA* gene amplification were ME1/ME2 (Hales *et al.*, 1996). PCR products were purified with a TaKaRa Agarose Gel DNA Purification kit version 2.0 and sequenced directly with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kit and an ABI PRISM 3730XL DNA sequencer. A partial 16S rRNA gene sequence (1346 bp) and the DNA (669 bp) and inferred amino acid sequences of the *mcrA* gene were obtained and compared with sequences deposited in GenBank by using the BLAST program (Altschul *et al.*, 1990), and then aligned with those of related methanogens in the order *Methanosarcinales* by using CLUSTAL_X software (Thompson *et al.*, 1997). Phylogenetic trees were constructed by using the neighbour-joining method in MEGA3.1 software (Kumar *et al.*, 2004). Bootstrap values were calculated after 1000 replications. The 16S rRNA gene sequence of strain ZC-1^T had 90.6 % similarity to that of *Methanosaeta harundinacea* 8Ac^T and 89.1 % similarity to those of *Methanohalobium evestigatum* Z-7303^T and *Methanomethylovorans hollandica* DMS1^T (Fig. 2). The inferred amino acid sequence of the *mcrA* gene of strain ZC-1^T showed 76.6 % similarity to that of *Methanothrix thermophila* PT^T and 70.3 % similarity to that of *Methanohalobium evestigatum* Z-7303 ^{T}(Fig. 3).

Most methylotrophic methanogens are in the family *Methanosarcinaceae*. Typically, these organisms are non-motile, mesophilic, irregular cocci that use methanol

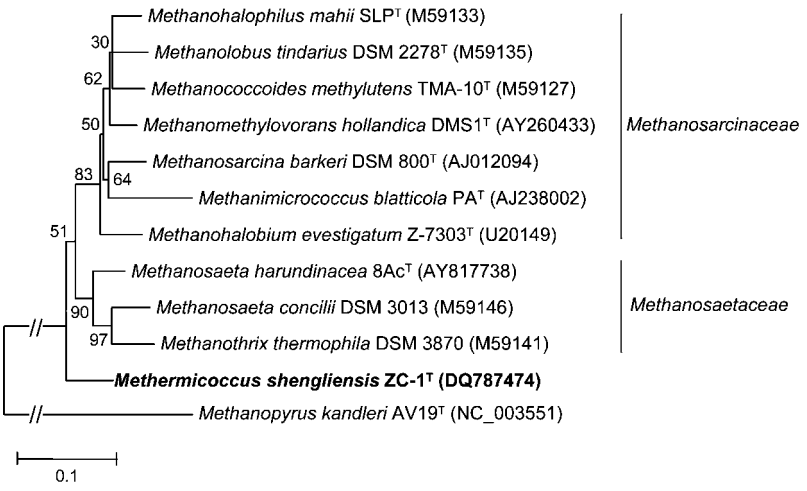

Fig. 2. Phylogenetic tree of 16S rRNA gene sequences of strain ZC-1^T and related strains in the order *Methanosarcinales*. The tree was constructed by using the neighbour-joining method in MEGA3.1 software, based on 1159 unambiguous bases and 1000 bootstrap replications. The sequence of *Methanopyrus kandleri* AV19^T (GenBank accession no. NC_003551) was used as the outgroup. Bar, 10 % estimated difference in nucleotide sequence.

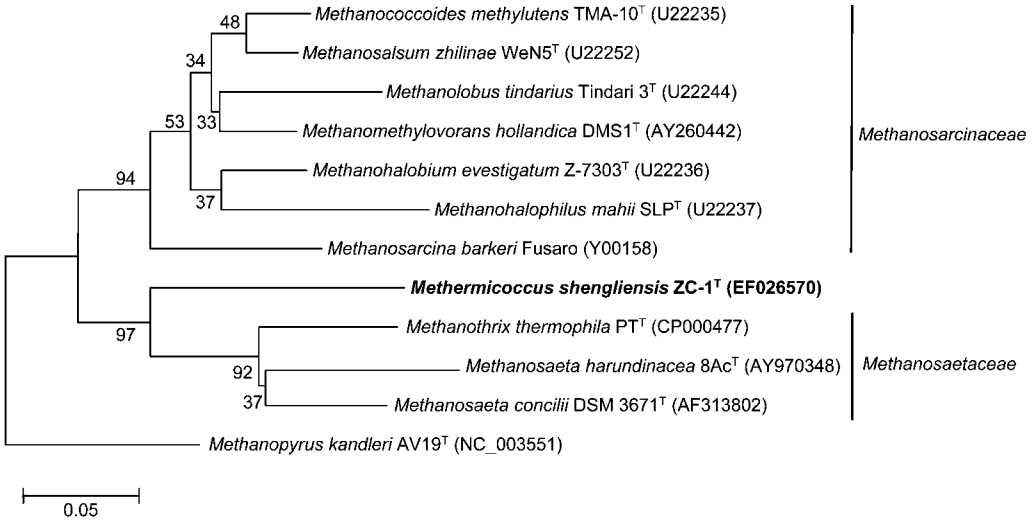

Fig. 3. Phylogenetic tree of inferred amino acid sequences (143 aa) of the *mcrA* gene of strain ZC-1^T and related strains in the order *Methanosarcinales*. The tree was constructed by using the neighbour-joining method in MEGA3.1 software with 1000 bootstrap replications. The sequence of *Methanopyrus kandleri* AV19^T (GenBank accession no. NC_003551) was used as the outgroup. Bar, 5 % estimated difference in nucleotide sequence.

and methylamines as substrates for methanogenesis. Some species also use H_2 and acetate for methanogenesis. Only a few thermophilic species in the family *Methanosarcinaceae* have been reported (Jiang *et al.*, 2005; Ollivier *et al.*, 1984; Touzel *et al.*, 1985; Zhilina & Zavarzin, 1987; Zinder & Mah, 1979). However, these species are all moderate thermophiles, with temperature optima equal to or below 55 °C. In contrast, strain ZC-1^T is the first reported methylotrophic methanogen with a temperature optimum as high as 65 °C. It can also survive at 70 °C. Phylogenetic analyses of the 16S rRNA and *mcrA* genes suggest that strain ZC-1^T is related more closely to the family *Methanosaetaceae* than the family *Methanosarcinaceae*. However, strain ZC-1^T does not ferment acetate, which is a special characteristic of the family *Methanosaetaceae*. In addition, 16S rRNA gene sequence similarities of 88–93 % are common for members of different families within the methanogens (Garrity & Holt, 2001). Based upon the low-level sequence similarity for the 16S rRNA gene with the most closely related methanogens and the morphological and physiological characteristics described in Table 1, we propose that strain ZC-1^T is a representative of a novel

Table 1. Physiological characteristics of strain ZC-1^T and related species in the order *Methanosarcinales*

Taxa: 1, strain ZC-1^T (data from this study); 2, *Methanosaeta harundinacea* 8Ac^T [data from Ma *et al.* (2006)]; 3, *Methanothrix thermophila* PT^T [data from Kamagata & Mikami (1991)]; 4, *Methanohalobium evestigatum* Z-7303^T [data from Zhilina & Zavarzin (1987)]; 5, *Methanomethylovorans hollandica* DMS1^T [data from Lomans *et al.* (1999)]. +, Positive; −, negative; ±, variable; ND, not determined; DMS, dimethylsulfide; MMA, methylamine; MT, methanethiol; TMA, trimethylamine; T_m, melting temperature.

Characteristic	1	2	3	4	5
Morphology	Coccoid (0.7–1.0 μm)	Rod-shaped (0.8–1.0 × 3.0–5.0 μm)	Rod-shaped (0.8 × 3.0 μm)	Irregular spheroid bodies in small aggregates	Irregular coccoid (1.0–1.5 μm)
Gram stain	+	±	−	ND	−
SDS sensitivity	Lysed in 0.01 %	Lysed in 1 %	Not lysed in 0.01 %	ND	Not lysed in 0.1 %
Substrate(s) for methanogenesis	Methanol, MMA, TMA	Acetate	Acetate	MMA, methanol	Methanol, MMA, TMA, DMS, MT
Optimal growth conditions					
Temperature (°C)	65	34–37	55	30–40	34–37
NaCl concentration (M)	0.3–0.5	ND	ND	2–4	0–0.04
pH	6.0–6.5	7.2–7.6	6.7	7–8	6.5–7.0
DNA G+C content (mol%)	56 (T_m)	55.7 (T_m)	52.7 (HPLC)	37 (T_m)	34.4 (T_m)
Source	Oil-production water	Anaerobic digester	Anaerobic digester	Sediments of saline lagoons in Sivash	Eutrophic pond sediment

family within the order *Methanosarcinales*. The novel genus and species *Methermicoccus shengliensis* gen. nov., sp. nov. and the family *Methermicoccaceae* fam. nov. are proposed to accommodate strain ZC-1^T.

Description of *Methermicoccaceae* fam. nov.

Methermicoccaceae (Me.ther′mi.coc.ca′ce.ae. N.L. masc. n. *Methermicoccus* type genus of the family; suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Methermicoccaceae* the family of the genus *Methermicoccus*).

Small, thermophilic cocci. Methanol, methylamine and trimethylamine are used as substrates for methanogenesis. The definition of the family is determined primarily by phylogenetic analyses of the 16S rRNA gene. The DNA G+C content is >50 mol%. The family currently contains a single genus.

The type genus of the family is *Methermicoccus*.

Description of *Methermicoccus* gen. nov.

Methermicoccus (Me.ther.mi.coc′cus. N.L. masc. n. *Methermicoccus* arbitrary name referring to a small, thermophilic, methane-producing coccus).

Small cocci with a mean diameter of 0.7 μm. Growth occurs at temperatures up to 70 °C; no growth occurs below 50 °C. Methanol, methylamine and trimethylamine are used as substrates for methanogenesis. Mg^{2+} is required for growth. The DNA G+C content is >50 mol%.

The type species of the genus is *Methermicoccus shengliensis*.

Description of *Methermicoccus shengliensis* sp. nov.

Methermicoccus shengliensis (shen.gli.en′sis. N.L. masc. adj. *shengliensis* pertaining to Shengli oilfield, where the type strain was isolated).

Cells are small, motile cocci with a diameter of 0.7–1.0 μm. Cells occur in clusters of two to four cells, and sometimes form cysts during the exponential phase. Cells are lysed in SDS (0.01 %, w/v) and H_2O, but not in PBS (0.2 M) or NaCl solution (0.9 %, w/v). HS-coM stimulates cell growth dramatically. Only methanol, methylamine and trimethylamine are used as substrates for methanogenesis. Fast growth occurs at pH 6.0–6.5 (range for growth, pH 5.5–8.0), 65 °C (range for growth, 50–70 °C), 0.3–0.5 M NaCl (range for growth, 0.2–1.1 M) and 0.05–0.20 M $MgCl_2$. The DNA G+C content is 56 mol%.

The type strain, ZC-1^T (=CGMCC 1.5056^T=DSM 18856^T), was isolated from oil-production water of Shengli oilfield, China.

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