Methanosarcina horonobensis sp. nov., a methanogenic archaeon isolated from a deep subsurface Miocene formation

Satoru Shimizu,¹ Rahul Upadhye,¹ Yoji Ishijima¹ and Takeshi Naganuma²

Correspondence

Satoru Shimizu
satoru.shimizu@h-rise.jp

¹Horonobe Research Institute for the Subsurface Environment, Northern Advancement Center for Science and Technology, Horonobe-cho, Teshio-gun, Hokkaido 098-3221, Japan

²Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan

A methanogenic organism, designated strain HB-1^T, from the domain Archaea was isolated from groundwater sampled from a subsurface Miocene formation located in Horonobe, Hokkaido, Japan. The strain grew on methanol, dimethylamine, trimethylamine, dimethylsulfide and acetate but not on monomethylamine, H₂/CO₂, formate, 2-propanol, 2-butanol or cyclopentanol. Cells were Gram-reaction-negative, non-motile, irregular cocci that were 1.4–2.9 µm in diameter and occurred singly or in pairs. The strain grew at 20–42 °C (optimum 37 °C), at pH 6.0–7.75 (optimum pH 7.0–7.25) and in 0–0.35 M NaCl (optimum 0.1 M). The G+C content of the genomic DNA was 41.4 mol%. 16S rRNA gene sequencing revealed that the strain was a member of the genus *Methanosarcina* but that it clearly differed from all recognized species of this genus (93.1–97.9% sequence similarity). The phenotypic and phylogenetic features of strain HB-1^T indicate that it represents a novel species of the genus *Methanosarcina*, for which the name *Methanosarcina horonobensis* sp. nov. is proposed. The type strain is HB-1^T (=DSM 21571^T =JCM 15518^T =NBRC 102577^T).

To date, few methanogens have been isolated from deep terrestrial subsurface environments other than oilfields (Christian *et al.*, 2005); these include *Methanobacterium subterraneum*, isolated from deep granitic groundwater (Kotelnikova *et al.*, 1998); *Methanolobus zinderi*, from a deep coal seam (Doerfert *et al.*, 2009); and *Methanolobus profundi*, from a gas field (Mochimaru *et al.*, 2009).

The Wakkanai Formation is a Miocene structure in northernmost Japan. It consists of layers of siliceous mudstone (Fukusawa, 1987). Palaeo-seawater and methane are trapped in the formation (Iwatsuki *et al.*, 2009). Previously, we used 16S rRNA gene sequences to characterize the community structure of resident methanogens by extracting DNA from water taken from 288.7–644.1 m below ground level from the Horonobe area of the formation. Our results indicated that the archaeal 16S rRNA gene libraries were dominated by methanogen clones that were mostly related to members of the genera *Methanoculleus* and *Methanosarcina* (Shimizu *et al.*, 2006). However, to our knowledge, no methane-producing strains

have yet been isolated from the formation for further characterization.

In this study, we describe the characteristics of a methanogenic archaeon, designated strain HB-1^T, which was sampled from an aquifer at the Wakkanai Formation.

A groundwater sample was collected on 15th January 2005 from the Wakkanai Formation by using previously described methods (Shimizu *et al.*, 2006). The sample was extracted 288.7–303.0 m below ground level from survey borehole HDB-6, which was drilled by the Japan Atomic Energy Agency (JAEA) in the Horonobe area.

Groundwater samples were inoculated into modified DSM 120 medium (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium120.pdf), in which the concentrations of L-cysteine-HCl·H₂O and Na₂S·9H₂O were changed to 0.5 g l⁻¹, and incubated at 37 °C in an N₂/H₂/CO₂ (80:10:10, v/v/v) atmosphere. The enrichment culture, which actively produced methane, was periodically transferred to fresh medium. After 14 days of incubation on solid medium, shiny, convex, clear and yellowish colonies were formed. Colonies were circular, with diameters of 0.5–1.0 mm and had entire margins.

An epifluorescence microscope (BX-51; Olympus) was used to perform phase-contrast and autofluorescence

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HB-1^T is AB288262.

Three supplementary figures are available with the online version of this paper.

microscopy of cells in the exponential phase. The resulting images revealed that cells were irregular cocci, 1.4–2.9 µm in diameter and occurred singly or in pairs (Supplementary Fig. S1a, b, available in IJSEM Online). No motility was observed under phase-contrast microscopy and no flagella were seen when negatively stained cells were observed using transmission electron microscopy (JEM-2000EX; JEOL) (Supplementary Fig. S1c). Cells of strain HB-1^T were found to be Gram-reaction negative and lysed in a 0.01 % (w/v) SDS solution.

To determine growth conditions and substrate utilization patterns, samples were cultured using DSM 120 medium modified as before, but with the concentration of NaCl changed to 0.1 M. The headspace was filled with H₂/CO₂ (80:20, v/v; 200 kPa) to examine the utilization of H₂/CO₂ as a methanogenic substrate or N₂/CO₂ (80:20, v/v) to study the utilization of other substrates. The pH of the medium was adjusted by adding HCl or NaOH solution. Cultures were incubated in triplicate under each set of conditions. A UVmini-1240 spectrophotometer (Shimadzu) was used to measure OD₆₆₀ in order to ascertain growth rates. Strain HB-1^T grew at 20–42 °C and pH 7.0 with an optimum growth temperature of 37 °C (Supplementary Fig. S2a). No growth occurred within 2 months at 15 °C or 45 °C. The optimum pH for growth was 7.0–7.25 (the initial pH of the culture medium), although growth was observed at pH 6.0–7.75; no growth was observed within 2 months at pH 5.5 or 8.0 (Supplementary Fig. S2b). Strain HB-1^T grew in the presence of 0–0.35 M NaCl, with an optimum concentration of 0.1 M (Supplementary Fig. S2c). Furthermore, growth was observed in the presence of 0–0.75 M MgSO₄, with an optimum concentration of 0–0.2 M (Supplementary Fig. S2d). No growth occurred within 2 months in medium supplemented with 0.4 M NaCl or 0.8 M MgSO₄.

Strain HB-1^T used (20 mM each) methanol, dimethylamine, trimethylamine, dimethylsulfide and acetate as methanogenic substrates. The strain exhibited good growth (0.14, 0.09 and 0.07 h⁻¹, respectively) on methanol, trimethylamine and dimethylamine but very slow growth on dimethylsulfide and acetate. Growth and methane formation were not observed on H₂/CO₂ (80:20, v/v; 200 kPa) or on (20 mM each) monomethylamine, formate, 2-propanol, 2-butanol or cyclopentanol. Growth of the strain was stimulated by Bacto yeast extract and Bacto casitone (BD). Furthermore, vitamins and trace minerals were required for growth in the DSM 120 medium.

The sensitivity of strain HB-1^T to (100 µg l⁻¹ each) antibiotics such as ampicillin, penicillin G, vancomycin, kanamycin and tetracycline was tested at 37 °C and pH 7.0. Strain HB-1^T was resistant to all antibiotics except tetracycline, with which no growth occurred within 2 months.

Cells of strain HB-1^T were harvested in late exponential phase and used for DNA isolation according to previously described methods (Marmur, 1961). Crude DNA was

purified according to the method of Hamamoto & Nakase (1995). HPLC with an LC-10A component (Shimadzu) was used to determine the G+C content of the DNA (Katayama-Fujimura *et al.*, 1984); this was found to be 41.4 ± 0.4 mol% (mean ± SD, n=3).

The 16S rRNA gene of strain HB-1^T was amplified using the primers A25F (5'-CYGGTTGATCCTGCCRG-3', where Y=C or T and R=A or G) and U1492R (5'-GGTTACCTTGTTACGACTT-3'), according to Dojka *et al.* (1998). The amplified PCR product was sequenced as previously described (Shimizu *et al.*, 2006), using the following sequencing primers: A25F (as above), U533F (5'-GTGCCAGCMGCCGCGGTAA-3', where M=A or C; Dojka *et al.*, 1998), 0922F (5'-GAAACTTAAKGAATTG-3', where K=G or T; Achenbach & Woese, 1995), 1100F (5'-GGCAACGAGCGMGACCC-3'; Lane, 1991), U1492R (as above), 1098Ra (5'-GGGTCTCGCTCGTTSCC-3', where S=C or G; Mikucki *et al.*, 2003), U907R (5'-CCGTCAATTCCTTTTAGTTT-3'; Dojka *et al.*, 1998), 536-519r (5'-GWATTACCGCGGCKGCTG-3', where W=A or T; Lane *et al.*, 1985), 368-353r (5'-AGKTTTCGCGCCTGCT-3'; Kotelnikova *et al.*, 1998) and 0112aR (5'-CCACGTGTTACTSAGC-3'; Achenbach & Woese, 1995).

The BLAST search tool was used to compare the nearly complete (1432 bp) 16S rRNA gene sequence of strain HB-1^T to sequences in the DNA database. Searches revealed that strain HB-1^T was most closely related to strains belonging to the genus *Methanosarcina* (93.1–97.9 % 16S rRNA gene sequence similarity) with highest similarity values between strain HB-1^T and *M. barkeri* MS^T (97.9 %) and *M. mazei* S-6^T (97.2 %), followed by *M. acetivorans* C2A^T (96.8 %), *M. lacustris* ZS^T (96.1 %), *M. thermophila* TM1^T (96.0 %), *M. frisia* C16^T (95.8 %), *M. sicilae* T4/M^T (94.7 %), *M. semesiae* MD1^T (94.7 %), *M. baltica* GS1-A^T (94.0 %) and *M. vacuolata* Z-761^T (93.1 %). Interestingly, the 16S rRNA gene sequence of strain HB-1^T was most similar (99.9 %) to that of uncultured clone HDBW-WA05 (accession no. AB237738), which had previously been obtained from deep subsurface groundwater of the Wakkanai Formation in the Horonobe area (Shimizu *et al.*, 2006).

Phylogenetic trees reconstructed using the neighbour-joining, maximum-parsimony and minimum evolution methods showed that strain HB-1^T clustered with members of the genus *Methanosarcina* and was most closely related to *M. mazei* S-6^T and *M. frisia* C16^T (Fig. 1 and Supplementary Fig. S3). *M. frisia* has been proposed as a synonym of *M. mazei* since DNA–DNA hybridization values between the type strains were 77 % similar and because the two species have a similar physiology (Maestrojuán *et al.*, 1992). Bootstrap analysis indicated a clear branching of strain HB-1^T from these species.

Strain HB-1^T had different phenotypic properties and substrate utilization characteristics to those of its closest phylogenetic relative, *M. mazei* and other related type strains (Table 1), as well as a lower tolerance of NaCl.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain HB-1^T and members of the genera *Methanosarcina* and related taxa. The tree was reconstructed using the neighbour-joining method. The two 16S rRNA gene sequences of *Methanospirillum hungatei* JF-1D^T (M60880) and *Methanococcus maripaludis* DSM2067^T (AF005049) were used to root the tree (not shown). Tree topology was evaluated by bootstrap analysis (based on 1000 replicates) with the neighbour-joining/maximum-parsimony/minimum evolution methods, using the MEGA 4.0 program package (Kumar *et al.*, 2008). Bar, 0.01 substitutions per nucleotide position.

Unlike *M. mazei*, strain HB-1^T was unable to use monomethylamine or H₂/CO₂; the other related type strains could also use monomethylamine. In addition to this, strain HB-1^T was tolerant of a much lower concentration of NaCl (0.35 M) than *M. mazei* (1.0 M) and other related type strains (≥ 0.6 M).

A SpectraFluor Plus (model F0129005; GENios) fluorescence, absorbance and luminescence instrument was used to quantify DNA–DNA hybridization values according to the method described by Ezaki *et al.* (1989). Strain HB-1^T

and its closest relative, *M. mazei* S-6^T, showed a relatedness value of 14 %. Even lower relatedness values were observed between strain HB-1^T and *M. barkeri* MS^T (13 %), *M. acetivorans* C2A^T (9 %), *M. vacuolata* Z-761^T (6 %) and *M. sicilae* T4/M^T (3 %). Genomic relatedness between strain HB-1^T and these type strains was too low to identify strain HB-1^T as belonging to any existing species of the genus *Methanosarcina*.

Therefore, on the basis of the phenotypic and phylogenetic data, strain HB-1^T represents a novel species of the genus

Table 1. Characteristics of strain HB-1^T and species of the genus *Methanosarcina*

Strains: 1, HB-1^T (data from this study); 2, *M. mazei* S-6^T (Mah & Kuhn, 1984; Maestrojuán & Boone, 1991); 3, *M. frisia* C16^T (Blotevogel *et al.*, 1986; Maestrojuán *et al.*, 1992); 4, *M. sicilae* T4/M^T (Ni & Boone, 1991; Ni *et al.*, 1994); 5, *M. acetivorans* C2A^T (Sowers *et al.*, 1984); 6, *M. vacuolata* Z-761^T (Zhilina & Zavarzin, 1987; Maestrojuán & Boone, 1991); 7, *M. barkeri* MS^T (Bryant & Boone, 1987; Maestrojuán & Boone, 1991); 8, *M. thermophila* TM1^T (Zinder *et al.*, 1985; Zinder & Mah, 1979); 9, *M. lacustris* ZS^T (Simankova *et al.*, 2001); 10, *M. semesiae* MD1^T (Lyimo *et al.*, 2000); 11, *M. baltica* GS1-A^T (von Klein *et al.*, 2002). All strains were positive for utilization of methanol and trimethylamine. +, Positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Cell size (μm)	1.4–2.9	1.0–3.0	0.9–1.6	1.5–3.0	1.9	1.0–2.0	1.5–2.0	ND	1.5–3.5	1.4 ± 0.2	1.5–3.0
Gram stain	–	–	–	–	–	+	+	+	+	+	ND
Temperature range for growth (°C)	20–42	20–45	22–42	25–45	15–45	18–42	20–45	35–55	1–35	18–39	4–27
Optimum temperature (°C)	37	40–42	36	40	35–40	37–40	40–42	50	25	30–35	25
pH range for growth	6.0–7.75	5.5–8.5	6.5–7.2	6.0–7.7	5.5–8.0	5.0–7.0	5.0–8.0	5.5–8.0	4.5–8.5	6.2–8.3	4.0–8.5
Optimum pH	7.0–7.25	6.0–7.0	6.5–7.2	6.5–6.8	6.5–7.0	6.0	6.0–7.0	6.0–7.0	7.0	6.5–7.5	6.5
Tolerance of NaCl (M)	0–0.35	0.1–1.0	0.1–1.0	0–>1.7	0.1–1.0	0.1–0.6	0.1–0.8	ND	ND	0–1.0	0.2–1.2
Optimum NaCl for growth (M)	0.1	0.1–0.3	0.1–0.2	0.4–0.6	0.1–0.6	0.1–0.2	0.1	ND	ND	0.2–0.6	0.3–0.4
Utilization of:											
H ₂ /CO ₂	–	+	+	–	–	+	+	+	+	–	–
Monomethylamine	–	+	+	ND	+	+	+	+	+	+	+
Dimethylsulfide	+	ND	ND	+	ND	ND	ND	ND	–	+	–
Acetate	+	+	+	–	+	+	+	+	–	–	+
DNA G + C content (mol%)	41.4	42	38.2	42–43	41	36.3	38.8–43.5	42	43.4	ND	ND

Methanosarcina, for which the name *Methanosarcina horonobensis* sp. nov. is proposed.

Description of *Methanosarcina horonobensis* sp. nov.

Methanosarcina horonobensis (ho.ro.no.ben'sis. N.L. fem. adj. *horonobensis* belonging to Horonobe, the town in Hokkaido, Japan, from which the strain was isolated).

Cells are strictly anaerobic, gram-reaction-negative, irregular cocci that are 1.4–2.9 µm in diameter and lysed by 0.01 % (w/v) SDS. Grows on methanol, dimethylamine, trimethylamine, dimethylsulfide and acetate but not on monomethylamine, H₂/CO₂, formate, 2-propanol, 2-butanol or cyclopentanol. Cells grow at 20–42 °C (optimum 37 °C), at pH 6.0–7.75 (optimum pH 7.0–7.25), in 0–0.35 M NaCl (optimum 0.1 M) and with 0–0.7 M MgSO₄ (optimum 0–0.2 M).

The type strain, HB-1^T (=DSM 21571^T =JCM 15518^T =NBRC 102577^T), was isolated in Horonobe, Japan, from deep subsurface groundwater from a siliceous mudstone formation. The DNA G+C content of the type strain is 41.4 ± 0.4 mol%.

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References

- Achenbach, L. & Woese, C. (1995). 16S and 23S rRNA-like primers. In *Archaea: A Laboratory Manual: Methanogens*, pp. 521–523. Edited by K. R. Sowers & H. J. Schreier. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Blotevogel, K. H., Fischer, U. & Lüpkes, K. H. (1986). *Methanococcus frisius* sp. nov., a new methylo-trophic marine methanogen. *Can J Microbiol* **32**, 127–131.
- Bryant, M. P. & Boone, D. R. (1987). Emended description of strain MS^T (DSM 800^T), the type strain of *Methanosarcina barkeri*. *Int J Syst Bacteriol* **37**, 169–170.
- Christian, J., Olivier, N., Erwan, C. & Agnes, G. L. (2005). Hyperthermophilic and methanogenic archaea in oil fields. In *Petroleum Microbiology*, pp. 55–69. Edited by B. Ollivier & M. Magot. Washington: American Society for Microbiology.
- Doerfert, S. N., Reichlen, M., Iyer, P., Wang, M. & Ferry, J. G. (2009). *Methanolobus zinderi* sp. nov., a methylo-trophic methanogen isolated from a deep subsurface coal seam. *Int J Syst Evol Microbiol* **59**, 1064–1069.
- Dojka, M. A., Hugenholtz, P., Haack, S. K. & Pace, N. R. (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* **64**, 3869–3877.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Fukusawa, H. (1987). [The site of sedimentation of Late Neogene bedded siliceous rock in northern Hokkaido, Japan]. *J Geol Soc Japan* **93**, 37–55 (in Japanese with English abstract).
- Hamamoto, M. & Nakase, T. (1995). Ballistosporeous yeasts found on the surface of plant materials collected in New Zealand. *Antonie van Leeuwenhoek* **67**, 151–171.
- Iwatsuki, T., Ishii, E. & Niizato, T. (2009). Scenario development of long-term evolution for deep hydrochemical conditions in Horonobe area, Hokkaido, Japan. *J Geog* **118**, 700–716.
- Katayama-Fujimura, Y., Komatsu, Y., Kuraishi, H. & Kaneko, T. (1984). Estimation of DNA base composition by high performance liquid chromatography of its nuclease P1 hydrolysate. *Agric Biol Chem* **48**, 3169–3172.
- Kotelnikova, S., Macario, A. J. L. & Pedersen, K. (1998). *Methanobacterium subterraneum* sp. nov., a new alkaliphilic, eurythermic and halotolerant methanogen isolated from deep granitic groundwater. *Int J Syst Bacteriol* **48**, 357–367.
- Kumar, S., Nei, M., Dudley, J. & Tamura, K. (2008). MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9**, 299–306.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. New York: Wiley.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* **82**, 6955–6959.
- Lyimo, T. J., Pol, A., Op den Camp, H. J. M., Harhangi, H. R. & Vogels, G. D. (2000). *Methanosarcina semesiae* sp. nov., a dimethylsulfide-utilizing methanogen from mangrove sediment. *Int J Syst Evol Microbiol* **50**, 171–178.
- Maestrojuán, G. M. & Boone, D. R. (1991). Characterization of *Methanosarcina barkeri* MS^T and 227, *Methanosarcina mazei* S-6^T, and *Methanosarcina vacuolata* Z-761^T. *Int J Syst Bacteriol* **41**, 267–274.
- Maestrojuán, G. M., Boone, J. E., Mah, R. A., Menaia, J. A. G. F., Sachs, M. S. & Boone, D. R. (1992). Taxonomy and halotolerance of mesophilic *Methanosarcina* strains, assignment of strains to species, and synonymy of *Methanosarcina mazei* and *Methanosarcina frisia*. *Int J Syst Bacteriol* **42**, 561–567.
- Mah, R. A. & Kuhn, D. A. (1984). Transfer of the type species of the genus *Methanococcus* to the genus *Methanosarcina*, naming it *Methanosarcina mazei* (Barker 1936) comb. nov. et emend. and conservation of the genus *Methanococcus* (Approved List 1980) with *Methanococcus vannielii* (Approved List 1980) as the type species. *Int J Syst Bacteriol* **34**, 263–265.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Mikucki, J. A., Liu, Y., Delwiche, M., Colwell, F. S. & Boone, D. R. (2003). Isolation of a methanogen from deep marine sediments that contain methane hydrates, and description of *Methanoculleus submarinus* sp. nov. *Appl Environ Microbiol* **69**, 3311–3316.
- Mochimaru, H., Tamaki, H., Hanada, S., Imachi, H., Nakamura, K., Sakata, S. & Kamagata, Y. (2009). *Methanobolus profundus* sp. nov., a methylo-trophic methanogen isolated from deep subsurface sediments in a natural gas field. *Int J Syst Evol Microbiol* **59**, 714–718.
- Ni, S. S. & Boone, D. R. (1991). Isolation and characterization of a dimethyl sulfide-degrading methanogen, *Methanobolus siciliae* HI350, from an oil well, characterization of *M. siciliae* T4/M^T, and emendation of *M. siciliae*. *Int J Syst Bacteriol* **41**, 410–416.
- Ni, S., Woese, C. R., Aldrich, H. C. & Boone, D. R. (1994). Transfer of *Methanobolus siciliae* to the genus *Methanosarcina*, naming it *Methanosarcina siciliae*, and emendation of the genus *Methanosarcina*. *Int J Syst Bacteriol* **44**, 357–359.

- Shimizu, S., Akiyama, M., Ishijima, Y., Hama, K., Kunimaru, T. & Naganuma, T. (2006). Molecular characterization of microbial communities in fault-bordered aquifers in the Miocene formation of northernmost Japan. *Geobiology* **4**, 203–213.
- Simankova, M. V., Parshina, S. N., Tourova, T. P., Kolganova, T. V., Zehnder, A. J. B. & Nozhevnikova, A. N. (2001). *Methanosarcina lacustris* sp. nov., a new psychrotolerant methanogenic archaeon from anoxic lake sediments. *Syst Appl Microbiol* **24**, 362–367.
- Sowers, K. R., Baron, S. F. & Ferry, J. G. (1984). *Methanosarcina acetivorans* sp. nov., an acetotrophic methane-producing bacterium isolated from marine sediments. *Appl Environ Microbiol* **47**, 971–978.
- von Klein, D., Arab, H., Völker, H. & Thomm, M. (2002). *Methanosarcina baltica*, sp. nov., a novel methanogen isolated from the Gotland Deep of the Baltic Sea. *Extremophiles* **6**, 103–110.
- Zhilina, T. N. & Zavarzin, G. A. (1987). *Methanosarcina vacuolata* sp. nov., a vacuolated methanosarcina. *Int J Syst Bacteriol* **37**, 281–283.
- Zinder, S. H. & Mah, R. A. (1979). Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H₂-CO₂ for methanogenesis. *Appl Environ Microbiol* **38**, 996–1008.
- Zinder, S. H., Sowers, K. R. & Ferry, J. G. (1985). *Methanosarcina thermophila* sp. nov., a thermophilic, acetotrophic, methane-producing bacterium. *Int J Syst Bacteriol* **35**, 522–523.