# *Methanocalculus pumilus* sp. nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site

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A mesophilic hydrogenotrophic methanogen, strain MHT-1[T], was isolated from the leachate of a sea-based site for solid waste disposal (the port of Osaka, Japan). Strain MHT-1[T] was found to be an irregular coccus and was able to use $H_2/CO_2$ and formate as energy sources. Acetate was required for growth. The optimum temperature and pH for growth were 35 °C and 6·5–7·5, respectively. Strain MHT-1[T] was resistant to high concentrations of several heavy metals such as $CdCl_2$ and $CuSO_4$. The G+C content of the DNA was 51·9 mol%. Analysis of the 16S rRNA gene revealed that the isolate was a member of the genus *Methanocalculus* but distinct from its nearest neighbour, *Methanocalculus halotolerans*, there being a sequence similarity of 98·9%. DNA–DNA hybridization analysis revealed 51% relatedness with the DNA of *M. halotolerans* strain SEBR 4845[T]. The optimum NaCl concentration was 1·0%, whereas the optimum in *M. halotolerans* was 5·0%. A new species, *Methanocalculus pumilus*, is proposed for strain MHT-1[T]. The type strain is MHT-1[T] (= DSM 12632[T] = JCM 10627[T]).

## INTRODUCTION

The number of waste-disposal sites is increasing. Except for the surface layer and the area near the point where pipes are installed for releasing gases, the sites are usually anaerobic. The gases generated by microbial decomposition contain a large amount of methane, which is obviously produced from the reclaimed wastes (Zinder, 1993). This observation indicates that methanogens play an important role for mineralizing organic matter in the sites (Takamizawa *et al.*, 1994; Qian & Barlaz, 1996). In spite of public regulations, wastes at the disposal sites contain heavy metals (Ham *et al.*, 1992; Kock *et al.*, 1989; Oleszkiewicz & Sharma, 1990). For example, the sampling site chosen for this study (i.e. the sea-based site for the disposal of solid waste, Osaka Port, Japan) handles reclaimed wastes (mostly incineration ash)

containing large amounts of heavy metals (Cu, 32 mmol kg$^{-1}$; Pb, 9 mmol kg$^{-1}$; Zn, 199 mmol kg$^{-1}$) (Takamizawa *et al.*, 1987, 1991). This evidence suggests that there is a possibility of heavy-metal-tolerant methanogens existing in waste-disposal sites. Here, we describe the isolation and characterization of a methanogen from a heavy-metal-contaminated disposal site.

## METHODS

**Source of organism.** *Methanocalculus halotolerans* SEBR 4845[T] was purchased from Oregon Collection of Methanogens (OCM 470[T]).

**Sampling site.** Leachate samples were taken from pipes (25 cm in diameter), used for monitoring sinkage and water quality, at the Osaka North Port Sea-Based Solid-Waste-Disposal Site in Japan. The site was located in the sea and was an artificial island composed of northern and southern sections surrounded by sea walls (Takamizawa *et al.*, 1987, 1991). At the time of sampling, the southern section was being filled with waste mainly consisting of incineration ash.

The *in situ* temperature was 43–57 °C and the pH was approximately 8·0.

**Enrichment and isolation.** The media were prepared according to the method described by Sowers & Schreier (1995). The basal medium contained ($l^{-1}$): 0·75 g $K_2HPO_4$, 0·75 g $KH_2PO_4$, 0·90 g $NH_4Cl$, 0·36 g $MgCl_2.6H_2O$, 0·50 g $Na_2S.9H_2O$, 0·50 g cysteine.HCl, 20·0 g NaCl, 2·0 g yeast extract (Oriental Yeast), 2·0 g trypticase peptone (BBL), 0·001 g resazurin, 10 ml trace-element solution (Lobo & Zinder, 1988) and 10 ml DSM 141 vitamin solution (Sowers & Schreier, 1995). Twenty millilitre vials (containing 10 ml medium) sealed with butyl-rubber stoppers and aluminium caps were used throughout the study. After the basal medium (without reductants) containing growth substrates was autoclaved, filtered solutions (0·22 μm membrane filter) of $Na_2S.9H_2O$, cysteine.HCl and alcohol (when necessary) were supplied. $Na_2CO_3$ solution was also added directly in stoppered vials for adjustment of the initial pH.

Primary enrichments were initiated by inoculating leachate samples into the sterilized basal medium containing ($l^{-1}$) 5·0 g sodium formate, 5·0 g sodium acetate and 10 ml methanol, as growth substrates, under an $N_2$ atmosphere (101·29 kPa). Incubation was performed at 30, 50 and 70 °C (pH 7·5).

Methanogens were isolated using the serial dilution method. Ampicillin (1000 μg ml$^{-1}$) or streptomycin (100 μg ml$^{-1}$) was employed as a selective inhibitor of eubacteria in all of these experiments (Whitman *et al.*, 1992). After purification, isolates were kept in the medium without antibiotic.

**Physiological characteristics.** $H_2/CO_2$, sodium formate (20 mM), sodium acetate (20 mM), sodium pyruvate (20 mM), glucose (10 mM), trimethylamine (5 mM), methanol (20 mM), ethanol (5 mM), 1-propanol (5 mM), 2-propanol (5 mM), 1-butanol (5 mM) and 2-butanol (5 mM) were evaluated as growth substrates. Unless otherwise specified, preparation of media was based on the method described above. Twenty millilitre vials containing 10 ml medium were flushed with $N_2/CO_2$ (80 : 20, v/v; 101·29 kPa). When $H_2/CO_2$ was used as the substrate, the gas phase of 20 ml vials containing 5 ml medium was replaced with $H_2/CO_2$ (80 : 20, v/v; 101·29 kPa). After inoculation (10 %, v/v, of medium), the vials were incubated statically or in a shaker at 160 r.p.m. (when $H_2/CO_2$ was used). In the experiment for testing the acetate requirement, to prevent carry-over from the former culture, the cells were harvested by centrifugation at 4 °C, washed anaerobically with sterile phosphate buffer (pH 7·5) containing $Na_2S.9H_2O$ (0·5 g l$^{-1}$) by flushing with oxygen-free $N_2$ and suspended in the basal medium (which was free of trypticase peptone and yeast extract). This procedure was repeated three times; the washed cells obtained were inoculated into the medium, and $H_2/CO_2$ was used as the energy source in the absence of yeast extract and trypticase peptone.

Growth was monitored by measuring methane production or turbidity at 660 nm, using 20-ml vials that fit into a spectrophotometer. The specific methane production rate ($\mu$) was calculated from the exponential production phase (Kadam *et al.*, 1994).

**Gas chromatography.** Methane was analysed by GC (GC-4 equipped with TCD and a Unibead C 60/80 column; Shimadzu) (Zabel *et al.*, 1984).

**Microscopy.** An Olympus AX80 microscope was used for routine observation. Autofluorescence of cells was observed in a Olympus BHS-RFCA microscope with a UV lamp and filters (20UG-1 and 17L420). Transmission electron micrographs were taken using an electron microscope (model H-7000; Hitachi) operated at 75 kV. Samples were prepared according to the protocol of Franzmann *et al.* (1997), with slight modifications; cells at late-exponential phase were fixed directly in cacodylate buffer supplemented with 5 % glutaraldehyde. Cells were fixed overnight at 4 °C, collected by centrifugation and then fixed for 3 h in cacodylate-buffered 1 % osmium tetroxide. The cells were stained *en bloc* with 0·5 % aqueous uranyl acetate and embedded in resin. Ultrathin sections were prepared with a Reinchert ultramicrotome.

**Gram staining and susceptibility to lysis.** The Gram reaction and susceptibility tests were performed as described by Boone & Whitman (1988).

**DNA base composition and hybridization.** Cells were harvested from 500 ml culture by centrifugation at 4 °C. The cell pellet was suspended in Tris-EDTA buffer (pH 8·0) and subjected to three cycles of freezing–thawing. Proteinase K was then added at 0·075 mg ml$^{-1}$ with calcium chloride and SDS (final concentrations were 0·003 and 2·5 %, respectively) and the mixture was incubated for 3 h at 37 °C (Kim *et al.*, 1996). The DNA was purified by the method of Marmur (Marmur, 1961) and suspended in Tris-EDTA buffer (pH 8·0). The purified DNA was hydrolysed with P1 nuclease from the DNA GC Kit (Yamasa Shoyu) followed by alkaline phosphatase treatment (Kamagata & Mikami, 1991). The G + C content was determined by HPLC (SCL-6B; Shimadzu). Separation was achieved at 40 °C by using a flow rate of 1 ml min$^{-1}$, a column of Shim-pack CLC-ODS (Shimadzu) and 5 % methanol in 10 mM phosphate buffer (pH 3·5) as the mobile phase. Each deoxyribonucleoside was detected by determining the absorbance at 260 nm and an equimolar mixture of four deoxyribonucleosides was used as the standard (Yamasa Shoyu).

Genetic relatedness was investigated by slot-blot DNA–DNA hybridization with a random-prime labelling system and [(α-$^{32}$P]dCTP (Amersham Pharmacia Biotech) (Jeanthon *et al.*, 1998). Target DNA (500 ng) denatured by 0·8 M NaOH was slotted on to a nylon membrane (Bio-Rad) and the labelled DNA was reassociated in a solution containing 50 % formamide, 0·12 M $Na_2HPO_4$, 0·25 M NaCl, 7 % SDS and 1 mM EDTA. After incubation overnight at 43 °C, the membranes were washed with SSC containing 0·1 % SDS. Hybridization signals were detected by autoradiography. Triplicate tests were performed for each assay and self-hybridization of the probe with homologous target DNA was set to 100 %.

**Phylogenetic analysis of 16S rRNA gene.** The 16S rRNA gene was amplified using the following primers: forward primer 5′-TCYGKTTGATCCYGSCRGAG-3′ (*Escherichia coli* positions 8–27) and reverse primer 5′-GGTTACCTTG-TTACGACTT-3′ (*E. coli* positions 1510–1492) (Alm *et al.*, 1996). The reaction mixture contained (in 100 μl): 10 pmol each primer, 250 μmol each dNTP, PCR buffer with 15 mM $MgCl_2$ and 1·25 U AmpliTaq Gold (Applied Biosystems). The PCR was performed as follows: 95 °C for 9 min, followed by 35 cycles consisting of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min. The PCR products were sequenced with an ABI 377 automated sequencer (Applied Biosystems). The following five primers were used for sequencing: 5′-GTGTTACCGCGGCGGCTGG-3′,
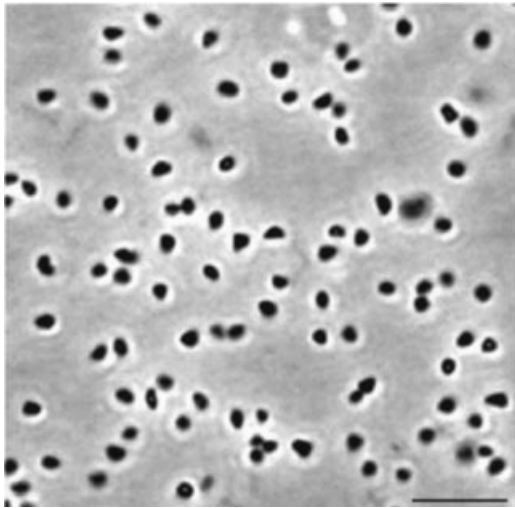
**Fig. 1.** Phase-contrast micrograph of strain MHT-1$^T$. Bar, 5 µm.

5′-ACTACCCGGGTATCTAATC-3′, 5′-AGTCAGGCA-ACGAGCGAGA-3′, 5′-TCTCGCTCGTTGCCTGACT-3′ and 5′-ACGGGCGGTGTGTGCAAG-3′ (*Methanobacterium thermoautotrophicum* ΔH, accession number X68720, sequence positions 377–359, 647–629, 933–952, 952–933 and 1257–1240, respectively). Sequences were compared with the BLAST program from the National Center for Biotechnology Information and the Ribosomal Database Project (Larsen *et al.*, 1996). Evolutionary distance tree sequence similarities were calculated with the CLUSTAL W program and the TREEVIEW package (Saitou & Nei, 1987; Thompson *et al.*, 1994).

**Effect of heavy metals.** To determine the heavy-metal sensitivity of isolates, the medium was supplemented with 1 mM CdCl$_2$, K$_2$CrO$_4$ or CuSO$_4$. Na$_2$S.9H$_2$O was removed from the medium to avoid a reaction with the metals, and only cysteine. HCl was used as a reductant. Growth and methanogenesis were not affected by the lack of Na$_2$S.9H$_2$O. Methane production was measured to determine the heavy-metal sensitivity.

## RESULTS

### Enrichment and isolation

For primary enrichment, a 1 ml leachate sample from the pipe was inoculated into the medium containing formate, acetate and methanol as substrates. After 4 weeks incubation at 30, 50 and 70 °C, methanogenesis occurred at all three temperatures and significant growth of microscopic F$_{420}$-autofluorescent microbes was eventually observed at 30 and 70 °C. In the enrichment at 30 °C, irregular cocci that showed blue-green autofluorescence indicative of F$_{420}$ were abundant. They were able to grow on formate or H$_2$/CO$_2$ (data not shown). We tried to isolate an irregular coccus by the roll-tube method, but this was unsuccessful. Therefore, an attempt was made to isolate this methanogen by serial dilution using H$_2$/CO$_2$ as the substrate in the presence of ampicillin (1000 µg ml$^{-1}$). Of the serial dilution tubes, the culture receiving 10$^{-3}$ showed methanogenesis after 1 week and the culture
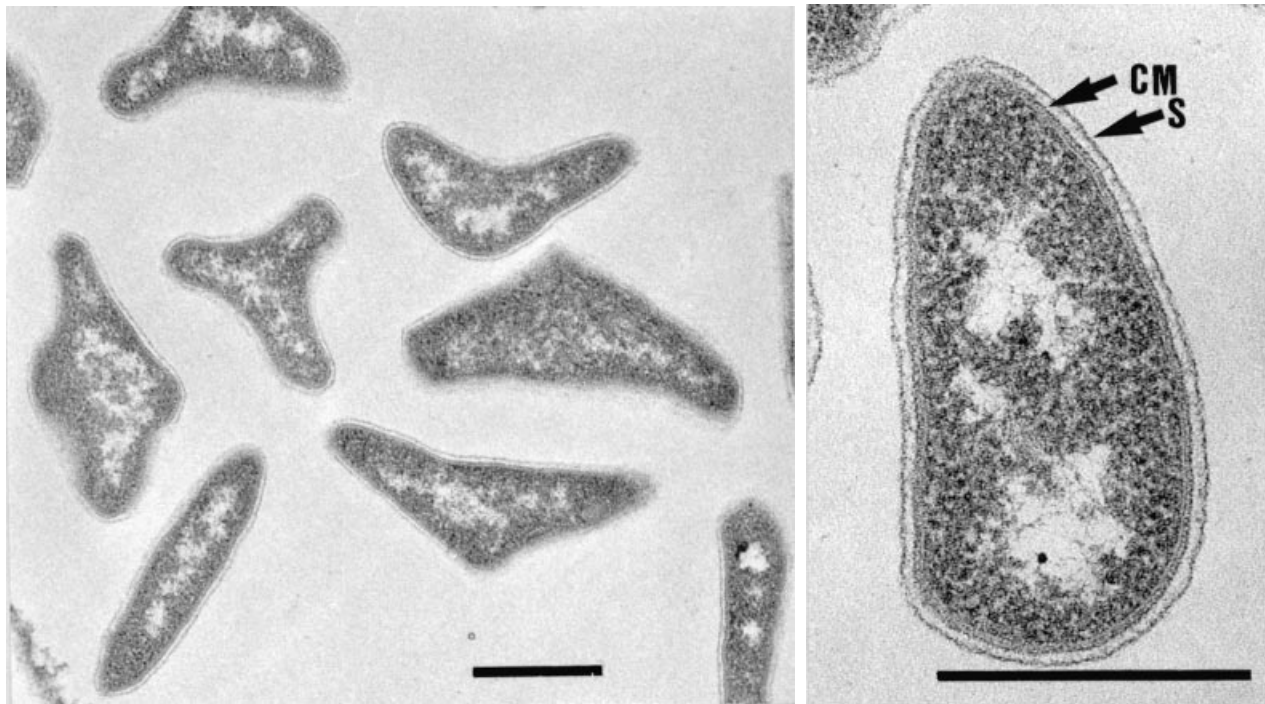


**Fig. 2.** Ultrathin sections of strain MHT-1$^T$. S, S-layer; CM, cytoplasmic membrane. Bar, 0·5 µm.

was subsequently serially diluted in the same medium. After the serial dilution experiment had been repeated more than 10 times, a mesophilic methanogen, designated as strain MHT-1$^T$, was obtained. The purity of the isolate was verified by microscopy and inoculation into basal medium containing various heterotrophic substrates with $N_2/CO_2$ (80:20, v/v; 101·29 kPa). No growth of contaminants was observed when the following substrates were used: 10 mM glucose, 10 mM maltose, 10 mM sucrose, 10 mM glycerol, 20 mM lactate, 20 mM lactate plus 20 mM $Na_2SO_4$, 2 g l$^{-1}$ polypeptone, 2 g l$^{-1}$ tryptone and 2 g l$^{-1}$ beef extract.

In the primary enrichment at 70 °C, a rod-shaped methanogen was dominant. Formate or $H_2/CO_2$ was also found to permit growth of the organism. This methanogen was isolated in pure culture by the same procedures as those used to isolate the mesophilic strain, except that streptomycin (100 μg ml$^{-1}$) was used instead of ampicillin. The isolate, designated as strain KHT-2 (JCM 10697), morphologically resembled members of the genus *Methanobacterium*; the phylogenetic analysis based on 16S rRNA sequence (accession number AB020530) revealed that the isolate possessed 100 % sequence similarity to *M. thermoautotrophicum*. Therefore, we focused on strain MHT-1$^T$ for further study.

## Morphology and cell structure

Strain MHT-1$^T$ was an irregular coccus, 0·8–1·0 μm in diameter (Fig. 1) and was non-motile under the microscope. Ultrathin sections of whole cells of the isolate revealed varied shapes. The cells possessed a cytoplasmic membrane surrounded by an S-layer-like surface layer (Fig. 2).

## Gram staining and susceptibility to lysis

Gram staining of the cells was negative in both the exponential and stationary growth phases. Susceptibility to lysis by detergent (SDS, 0·1 g l$^{-1}$) and by hypotonic conditions was observed.

## Physiological characteristics

**Growth properties.** We found that growth (turbidity at 660 nm) was tightly associated with methane production, so we determined the specific methane formation rate to accurately determine the optimum growth conditions. Moreover, cumulative methane produced in 3 d appeared to be a good indicator of growth, particularly with regard to identifiying the lag phase.

To determine the optimum growth conditions, sodium formate (40 mM) was used as the substrate. The initial pH range for growth and methanogenesis was 5·5–9·0 for strain MHT-1$^T$, the optima being 6·5 and 7·0. Of the initial pH values, pH 7·0 and pH 7·5 gave high cumulative methane production in 3 d, whereas a
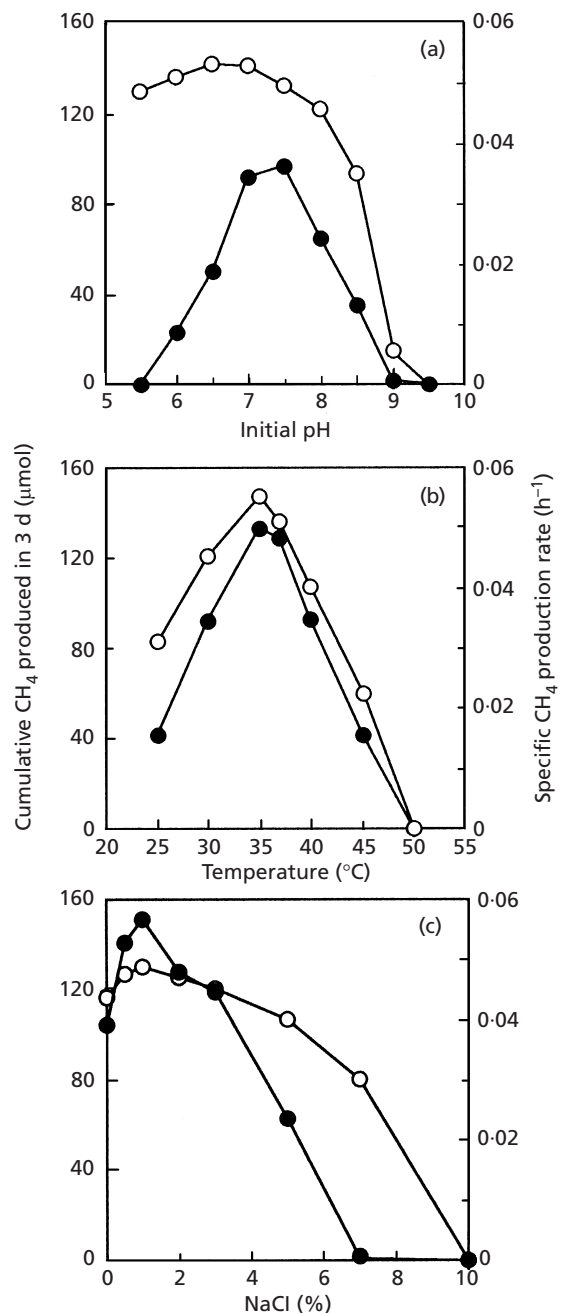


**Fig. 3.** Optimum growth conditions for strain MHT-1$^T$. Effect of (a) initial pH (37 °C and 2·0 % NaCl), (b) temperature (initial pH of 7·5 and 2·0 % NaCl) and (c) NaCl concentration (37 °C and initial pH of 7·5) on methane production. Values are means of triplicate cultures. Symbols: ○, specific CH$_4$ production rate; ●, cumulative CH$_4$ produced in 3 d.

significant lag phase was observed at pH values 5·5, 6·0, 8·5 and 9·0 (Fig. 3a). In view of these results, the optimum initial pH values were determined as 6·5 and 7·5 (Fig. 3a). The growth temperature for strain MHT-1$^T$ ranged from 25 to 45 °C, with an optimum at 35 °C (Fig. 3b). The patterns of the specific methane-production rate and the methane produced in 3 d were
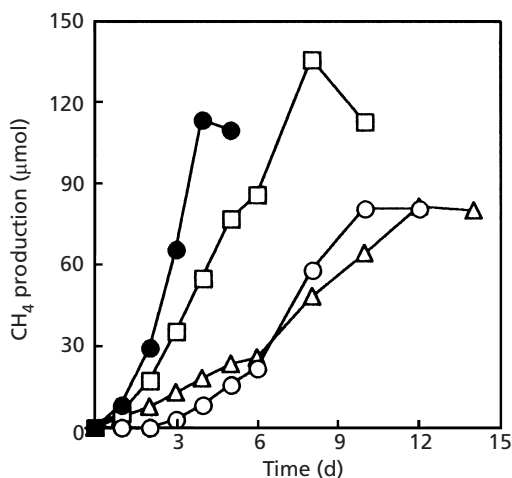
**Fig. 4.** Effect of heavy metals on CH$_4$ production of strain MHT-1$^T$. Symbols: ○, 1 mM CdCl$_2$; □, 1 mM K$_2$CrO$_4$; △, 1 mM CuSO$_4$; ●, without additions. H$_2$/CO$_2$ was used as the substrate and cysteine hydrochloride was used as the reducing agent. Values are means of triplicate cultures.

similar. The NaCl concentrations for growth ranged from 0 to 7·0% (w/v), with an optimum of 1·0% (evaluated from both cumulative methane production and the specific methane-production rate). A significant delay in growth was observed at 7·0% NaCl (Fig. 3c). The doubling time using formate as the energy source under optimum growth conditions was calculated to be 12 h, based on the specific methane-formation rate as well as the increase in turbidity (data not shown).

When yeast extract and trypticase peptone were removed from the medium, very little growth was

observed on H$_2$/CO$_2$. However, growth recovered with the addition of acetate (0·5 g l$^{-1}$), indicating that acetate is required for growth (data not shown).

**Substrate utilization.** Strain MHT-1$^T$ used H$_2$/CO$_2$ and formate as the energy source. Growth and methanogenesis were never observed on acetate (20 mM), pyruvate (20 mM), glucose (10 mM), trimethylamine (5 mM), methanol (20 mM), ethanol (5 mM), 1-propanol (5 mM), 2-propanol (5 mM), 1-butanol (5 mM) or 2-butanol (5 mM) under N$_2$/CO$_2$ after 4 weeks incubation.

### Sensitivity to heavy metals

The effect of heavy metals on the growth of strain MHT-1$^T$ was determined (Fig. 4). Growth occurred in the presence of 1 mM CdCl$_2$, K$_2$CrO$_4$ or CuSO$_4$, although growth was slightly delayed by these heavy metals.

### 16S rRNA gene analysis

An almost complete 16S rRNA gene (1433 bases) was sequenced for strain MHT-1$^T$. Phylogenetic analysis demonstrated that strain MHT-1$^T$ is a close relative of *Methanocalculus halotolerans* SEBR 4845$^T$, having a sequence similarity of 98·9% (Fig. 5). However, these two methanogens were quite distant from any known methanogens (sequence similarity < 90·7%).

### DNA base composition and DNA–DNA hybridization

The G+C content of DNA from strain MHT-1$^T$ was 51·9 mol%. In DNA–DNA hybridization experiments, the DNA of the isolate exhibited 51% hybridization with the DNA of *M. halotolerans* SEBR 4845$^T$.
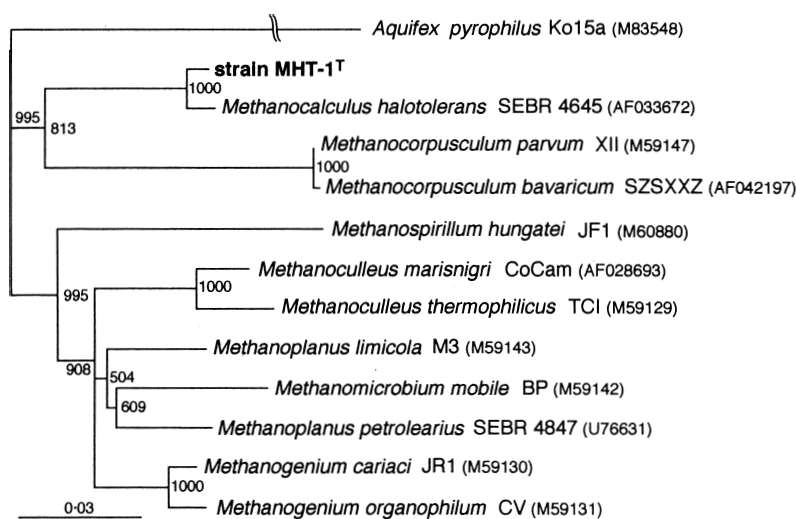


**Fig. 5.** Phylogenetic analysis of 16S rRNA genes showing the relationships among various methanogens. The significance of each branch is indicated by a bootstrap value. The accession number for each reference species is shown in parentheses. Bar, estimated substitutions per nucleotide position.

## DISCUSSION

A mesophilic irregular coccoid methanogen was isolated from the leachate in a waste-disposal site. Phylogenetic analysis indicated that strain MHT-1$^T$ is a close relative of *M. halotolerans*. One of the most interesting features is that these two methanogens form a cluster that is quite distinct from the other known methanogens (less than 90·7% similarity). *M. halotolerans* is a mesophilic hydrogenotrophic methanogen that was recently isolated from an oil-producing well (Table 1). Strain MHT-1$^T$ is very similar to *M. halotolerans* with respect to cell morphology, optimum temperature and pH for growth. However, the DNA similarity between strain MHT-1$^T$ and *M. halotolerans* was 51%. This value supports the view that the strain is a new species of the genus *Methanocalculus* (Boone & Whitman, 1988). Moreover, *M. halotolerans* grows optimally at 5% (w/v) NaCl and tolerates up to 12·5% NaCl (Ollivier *et al.*, 1998), whereas strain MHT-1$^T$ has an optimum of 1·0% NaCl and it could not grow at 10% NaCl.

As previously mentioned, the incineration ash dumped in the waste-disposal site in which the strain was isolated contained large amounts of heavy metals. Thus we examined the effect of heavy metals on the growth of the isolate. Growth occurred in the presence of 1 mM $CdCl_2$, $K_2CrO_4$ or $CuSO_4$, although $CdCl_2$ and $CuSO_4$ were more inhibitory than $K_2CrO_4$. The sensitivity of *M. halotolerans* strain SEBR 4845$^T$ to these heavy metals was also tested under the same conditions and it was found that the strain produced only a trace amount of methane in the presence of 1 mM $CdCl_2$ and $CuSO_4$ (data not shown). To date, there are very few descriptions of resistance of methanogens to heavy metals. Kim *et al.* (1996) reported that

*Methanobacterium bryantii* MoH, *Methanobacterium formicicum* and *Methanococcus voltae* did not grow at all following the addition of 1 mM $CuSO_4$. However, *M. bryantii* BKYH, isolated from a copper-mining area, was able to grow in the presence of 1 mM $CuSO_4$. Our strain was also able to grow in the presence of 1 mM $CuSO_4$, suggesting that heavy-metal-containing habitats impose genetic acquisition of heavy-metal resistance. Heavy-metal-resistant methanogens may be common in such contaminated environments.

On the basis of its phenotypic and genotypic distinctiveness, as described above, we conclude that the isolate characterized here represents a new species of the genus *Methanocalculus*, for which we propose the name *Methanocalculus pumilus*.

### Description of *Methanocalculus pumilus* sp. nov.

*Methanocalculus pumilus* (pu′mi.lus. L. adj. *pumilus* little).

Cells are irregular cocci 0·8–1·0 μm in diameter and occur singly. Gram-negative. Susceptibility to lysis by SDS (0·1 g l$^{-1}$) and by hypotonic conditions is observed. Growth occurs between 25 and 45 °C, with an optimum at 35 °C. The pH range for growth is 5·5–9·0, with an optimum around neutral pH. Growth occurs at NaCl concentrations between 0 and 7%, with an optimum at 1·0%. The doubling time is approximately 12 h under optimum conditions. The substrates used for growth and methanogenesis are $H_2/CO_2$ and formate, but not acetate, pyruvate, glucose, trimethylamine, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol or 2-butanol. Acetate is required for growth. The G+C content of the DNA is 51·9 mol% (as determined by HPLC). The type strain

**Table 1.** Characteristics of *Methanocalculus pumilus* strain MHT-1$^T$ and *Methanocalculus halotolerans*

| Parameter | MHT-1$^T$ | *M. halotolerans*† |
|---|---|---|
| Collection no.* | DSM 12632$^T$ (= JCM 10627$^T$) | OCM 470$^T$ |
| Source | Leachate from waste-disposal site | African oil well |
| Temp. range (°C) | 25–45 | 25–45 |
| Optimum temp. (°C) | 35 | 38 |
| Initial pH range | 5·5–9·0 | 7·0–8·4 |
| Optimum initial pH | 6·5–7·5 | 7·6 |
| NaCl concn range (%) | 0–7 | 0–12·5 |
| Optimum NaCl concn (%) | 1·0 | 5·0 |
| Generation time (h) | 12 | 12 |
| G+C content (mol%) | 51·9 | 55 |
| Usable substrates | $H_2/CO_2$, formate | $H_2/CO_2$, formate |
| Nutritional requirement | Acetate | Acetate |

*Collections: DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; JCM, Japan Collection of Microorganisms; OCM, Oregon Collection of Methanogens.

† Data are from Ollivier *et al.* (1998).

is *Methanocalculus pumilus* MHT-1[T], which was isolated from leachate at a waste-disposal site in Osaka, Japan. Strain MHT-1[T] has been deposited in the DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 12632[T]) and the Japan Collection of Microorganisms (JCM 10627[T]).

## ACKNOWLEDGEMENTS

## REFERENCES

**Alm, E. W., Oerther, D. B., Larsen, N., Stahl, D. A. & Raskin, L. (1996).** The oligonucleotide probe database, *Appl Environ Microbiol* **62**, 3557–3559.

**Boone, D. R. & Whitman, W. B. (1988).** Proposal of minimal standards for describing new taxa of methanogenic bacteria, *Int J Syst Bacteriol* **38**, 212–219.

**Franzmann, P. D., Liu, Y., Balkwill, D. L., Aldrich, H. C., De Macario, E. C. & Boone, D. R. (1997).** *Methanogenium frigidum* sp. nov., a psychrophilic, H$_2$-using methanogen from Ace Lake, Antarctica, *Int J Syst Bacteriol* **47**, 1068–1072.

**Ham, R. K., Hammer, V. A. & Boley, G. (1992).** Partitioning of elements by refuse processing, *J Environ Eng* **118**, 725–743.

**Jeanthon, C., L'Haridon, S., Reysenbach, A. L., Vernet, M., Messner, P., Sleytr, U. B. & Prieur, D. (1998).** *Methanococcus infernus* sp. nov., a novel hyperthermophilic lithotrophic methanogen isolated from a deep-sea hydrothermal vent, *Int J Syst Bacteriol* **48**, 913–919.

**Kadam, P. C., Ranade, D. R., Mandelco, L. & Boone, D. R. (1994).** Isolation and characterization of *Methanolobus bombayensis* sp. nov., a methylotrophic methanogen that requires high concentrations of divalent cations, *Int J Syst Bacteriol* **44**, 603–607.

**Kamagata, Y. & Mikami, E. (1991).** Isolation and characterization of a novel thermophilic *Methanosaeta* strain, *Int J Syst Bacteriol* **41**, 191–196.

**Kim, B.-K., De Macario, E. C., Nölling, J. & Daniels, L. (1996).** Isolation and characterization of a copper-resistant methanogen from a copper-mining soil sample, *Appl Environ Microbiol* **62**, 2629–2635.

**Kock, M., Schaffler, R., Sixl, W., Pichler-Semmelrock, F. P., Kosmus, W. & Marth, E. (1989).** Accumulation of heavy metals in animals. 3. Heavy metal contamination in small mammals at a waste disposal site, *J Hyg Epidemiol Microbiol Immunol* **44**, 536–541.

**Larsen, N., Olsen, J. G., Maidak, B. L., McCaughey, M. J., Overbeek, R. & Woese, C. R. (1996).** The Ribosomal Database Project, *Nucleic Acids Res* **24**, 82–85.

**Lobo, A. L. & Zinder, S. H. (1988).** Diazotrophy and nitrogenase activity in the archaebacterium *Methanosarcina barkeri* 227, *Appl Environ Microbiol* **54**, 1656–1661.

**Marmur, J. (1961).** A procedure for the isolation of deoxyribonucleic acid from microorganisms, *J Mol Biol* **3**, 208–218.

**Oleszkiewicz, J. A. & Sharma, V. K. (1990).** Stimulation and inhibition of anaerobic processes by heavy metals – a review, *Biol Wastes* **31**, 45–67.

**Olliver, B., Fardeau, M.-L., Cayol, J.-L., Magot, M., Patel, B. K. C., Prensier, G. & Garcia, J.-L. (1998).** *Methanocalculus halotolerans* gen. nov., sp. nov., isolated from an oil-producing well, *Int J Syst Bacteriol* **48**, 821–828.

**Qian, X. & Barlaz, M. A. (1996).** Enumeration of anaerobic refuse-decomposing micro-organisms on refuse constituents, *Waste Manag Res* **14**, 151–161.

**Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol Biol Evol* **4**, 406–425.

**Sowers, K. R. & Schreier, H. J. (1995).** Methanogens. In *Archaea: a Laboratory Manual*. Edited by F. T. Robb & A. R. Place. New York: Cold Spring Harbor Laboratory.

**Takamizawa, K., Yamamoto, O., Fukunaga, I., Inoue, Z. & Honda, A. (1987).** Removal of organics from leachate by the combined process of a facultative pre-treatment pond and an aerated lagoon at a sea-based solid waste disposal site, *Water Sci Technol* **19**, 101–107.

**Takamizawa, K., Yamamoto, O., Fukunaga, I., Inoue, Z. & Honda, A. (1991).** Design philosophy on leachate treatment facilities at a sea-based solid waste disposal site in Osaka, *Jpn Soc Waste Manag Experts* **2**, 25–32.

**Takamizawa, K., Shamoto, H., Horitsu, H., Kawai, K., Suzuki, T., Mori, T., Fukunaga, I. & Iida, H. (1994).** Distribution of sulfate-reducing bacteria at a sea-based solid waste disposal site and their role in solubilization of heavy metal ions in leachate, *Jpn Soc Waste Manag Experts* **5**, 89–97.

**Thompson, J. D., Higgins, D. J. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Res* **22**, 4673–4680.

**Whitman, W. B., Bowen, T. L. & Boone, D. R. (1992).** The methanogenic bacteria. In *The Prokaryotes*, 2nd edn, vol. 1, pp. 719–767. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer.

**Zabel, H. P., König, H. & Winter, J. (1984).** Isolation and characterization of a new coccoid methanogen, *Methanogenium tatii* spec. nov., from a solfataric field on Mount Tatio, *Arch Microbiol* **137**, 308–315.

**Zinder, S. H. (1993).** Physiological ecology of methanogens. In *Methanogenesis*. Edited by J. G. Ferry. New York: Chapman & Hall.