***Methanoculleus palmolei* sp. nov., an irregularly coccoid methanogen from an anaerobic digester treating wastewater of a palm oil plant in North-Sumatra, Indonesia**

Gerhard Zellner,¹ Paul Messner,² Josef Winter³ and Erko Stackebrandt⁴

Author for correspondence: Gerhard Zellner. Tel: +49 89 3187 2579. Fax: +49 89 3187 3361.
e-mail: gerhard.zellner@gsf.de

¹ GSF – Forschungszentrum für Umwelt und Gesundheit, Institut für Hydrologie, Ingolstädter Landstraße 1, D-85764 Neuherberg, Germany

² Zentrum für Ultrastrukturforschung und Ludwig-Boltzmann-Institut für Molekulare Nanotechnologie, Universität für Bodenkultur, A-1180 Vienna, Austria

³ Institut für Ingenieurbiologie und Biotechnologie des Abwassers, D-76128 Karlsruhe, Germany

⁴ DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

Strain INSLUZ^T (= DSM 4273^T) was isolated from a biogas-producing bioreactor treating wastewater of a palm oil mill on North-Sumatra (Indonesia). Cells of strain INSLUZ^T were highly irregularly coccoid, 1.25–2.0 µm in diameter, had a cell envelope consisting of the cytoplasmic membrane and an S-layer of hexagonally arranged glycoprotein subunits with an *M_r* of 120 000, and were flagellated (motility was not observed). Cells were mesophilic and grew most rapidly at 40 °C on H₂/CO₂, formate, 2-propanol/CO₂, 2-butanol/CO₂ and cyclopentanol/CO₂ to give methane. Tungstate promoted growth on H₂/CO₂ with acetate as the solely required organic medium supplement. The G+C content of DNA was 59 mol% (*T_m* method) and 59.5 mol% (HPLC method). 16S rDNA analysis revealed a phylogenetic relationship to *Methanoculleus* species; the name *Methanoculleus palmolei* sp. nov. is therefore proposed for strain INSLUZ^T (= DSM 4273^T).

Keywords: methanogens, *Methanoculleus palmolei* sp. nov., methanogenic bioreactor, palm oil wastewater treatment, phylogeny

INTRODUCTION

Irregularly coccoid methanogens occur in various habitats all over the world. However, there are only a few reports on the presence of irregularly coccoid methanogens in wastewater reactors (Zellner & Winter, 1987a; Schnürer *et al.*, 1994; Zellner *et al.*, 1987, 1989b, 1990, 1991), despite the high diversity of methanogenic subpopulations of anaerobic wastewater reactors (Raskin *et al.*, 1994; Zellner *et al.*, 1997). All irregularly coccoid methanogens isolated from bioreactors belong to the genera *Methanoculleus* or *Methanocorpusculum* within two distinct families, the *Methanomicrobiaceae* and the *Methanocorpusculaceae*, respectively (Boone & Xun, 1987;

Ferguson & Mah, 1983; Maestrojuán *et al.*, 1990; Ollivier *et al.*, 1985, 1986; Xun *et al.*, 1989; Zabel *et al.*, 1985; Zellner *et al.*, 1989b, 1990).

A highly polluting wastewater is produced by palm oil mills which is 100 times more recalcitrant than domestic wastewater. Worldwide, 6.2 × 10⁶ t palm oil and 15.4 × 10⁶ t palm oil effluent are produced per annum (Ma & Ong, 1986). Nothing is known about the microbial populations adapted to the anaerobic treatment of this specific type of wastewater.

In this paper, the isolation and characterization of a hydrogenotrophic, methanogenic strain, strain INSLUZ^T, are reported; the strain was isolated from a methanogenic wastewater bioreactor treating palm-oil-mill effluent in North-Sumatra (Indonesia). Some characteristics of the secondary alcohol dehydrogenase activity of the unclassified strain INSLUZ^T have been described previously (Bleicher *et al.*, 1989). Features of

Abbreviation: PAS stain, periodic acid-Schiff stain.

The EMBL accession number for the 16S rDNA sequence of strain INSLUZ^T reported in this paper is Y16382.

strain INSLUZ^T matched those of the genus description of *Methanoculleus* (Boone *et al.*, 1993) and 16S rDNA analysis revealed its phylogenetic relationship to *Methanoculleus* species.

METHODS

Media, media preparation and cultivation. Basal media for enrichment cultures and isolation procedures were prepared as previously described (Zellner & Winter, 1987a). Medium I (Balch *et al.*, 1979), which was modified by omission of yeast extract and peptone (modified medium I) or WHP medium (modified medium I supplemented with 1 µM sodium tungstate (WHP medium), was used to cultivate strain INSLUZ^T and reference methanogens (Zellner *et al.*, 1990). All other strains were cultivated in complex medium as previously described (Zellner & Winter, 1987a). Substrates and additives were added from anaerobic, sterile stock solutions as indicated. Cells were grown on H₂/CO₂ (80:20, v/v, 300 kPa) in 120 ml serum bottles on a rotary shaker (150 r.p.m.) at the temperatures indicated. For testing the growth pH range, the pH value of the medium was adjusted by addition of 10% (v/v) HCl prior to inoculation. The pH increased during growth as a result of consumption of CO₂ during methanogenesis. H₂/CO₂ was refilled in a fed-batch mode; the produced methane, however, was not replaced. For mass cultivation, cells were grown on H₂/CO₂ (80:20, v/v) with a flow rate of 50 l h⁻¹ in a 12 l Biostat S fermenter (B. Braun, Biotech International).

Source and habitat of the isolate. Strain INSLUZ^T (= DSM 4273^T) was isolated from a biogas reactor of a palm oil plant on North-Sumatra (Indonesia), which was operated at 55 °C.

Enrichment and isolation of the strain. Despite the operation of the palm-oil-mill effluent-treating digester at 55 °C, no methanogens could be enriched on H₂/CO₂ (80:20, v/v) in WHP medium (pH 7.0) incubated at 50, 60, 70 or 80 °C. However, if incubated at 37 °C, autofluorescent, irregular coccoid methanogens grew on H₂/CO₂. This culture was serially diluted into WHP medium and the highest dilution showing growth of the fluorescent, irregularly coccoid strains was again serially diluted and 0.1 ml dilutions were streaked on agar plates with WHP medium plus 2.5% (w/v) Oxoid agar (Unipath). All manipulations were performed in an anaerobic chamber (Coy Laboratory Products) under a gas atmosphere of N₂/H₂ (95:5, v/v). The plates were transferred into a stainless steel anaerobic jar (Balch *et al.*, 1979), pressurized with 300 kPa H₂/CO₂ (80:20, v/v) and incubated at 37 °C. After 20 d incubation, the gas pressure had reduced to 150 kPa indicating gas consumption. The anaerobic jar was then transferred into the anaerobic chamber, and colonies were picked and injected into 120 ml serum bottles supplied with 20 ml WHP medium under H₂/CO₂ (80:20, v/v, 300 kPa) and incubated at 37 °C. This procedure resulted in the isolation of strain INSLUZ^T; purity was tested as described previously (Boone & Whitman, 1988).

Analyses. Volatile fatty acids, alcohols, hydrogen and methane were analysed by GC (Zellner & Winter, 1987a, c).

Light microscopy. Epifluorescence microscopy of methanogens was carried out as described previously (Zellner *et al.*, 1991).

Electron microscopy and S-layer studies. Micrographs of thin-sectioned or freeze-etched cell preparations were taken on a Philips EM 301 electron microscope at 80 kV and the whole-cell protein pattern of strain INSLUZ^T was analysed by SDS-PAGE with Coomassie blue or periodic acid-Schiff (PAS) staining (Messner & Sleytr, 1992; Sleytr *et al.*, 1993; Zellner *et al.*, 1989a, b).

Polyamine analysis. The polyamine patterns of the cells were determined after extraction, derivatization with dansyl chloride and separation by HPLC (Zellner & Kneifel, 1993).

Antigenic fingerprinting. The antigenic fingerprint of strain INSLUZ^T was determined with a panel of antibody S-probes of methanogenic reference strains as described previously (Macario & Conway de Macario, 1983, 1985).

Determination of DNA G+C content. The G+C content (mol%) of strain INSLUZ^T was determined by the thermal denaturation (*T_m*) method, direct quantification with HPLC (Zellner *et al.*, 1989b), and UV spectroscopy according to the methods of Ulitzur (1972). Isolation and purification of DNA and HPLC determination of the G+C content (mol%) was performed according to the method of Zellner *et al.* (1989b). Calf thymus DNA with a G+C content of 42 mol% was used as a reference for the *T_m* method. The value obtained by UV spectroscopy was calculated from the quotients of the extinctions at different wavelengths [240/280, 240/275, 240/270, 245/280, 245/275 and 245/270 (Ulitzur, 1972)].

16S rDNA sequence determination and analysis. Extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA, and sequence analysis of the purified PCR products were carried out as described previously (Rainey *et al.*, 1996) and the sequence reaction mixture was electrophoresed using a model 373A automated DNA sequencer (Applied Biosystems). To determine the closest relatives of strain INSLUZ^T, its phylogenetic position was analysed using the ARB database (Strunk & Ludwig, 1995). Fine resolution of relatedness between INSLUZ^T and its closest relatives was performed using the ae2 editor (Maidak *et al.*, 1994). A phylogenetic dendrogram was reconstructed from corrected similarity values (Jukes & Cantor, 1969) using the treeing algorithm of De Soete (1983). Bootstrap values were determined using the PHYLIP package (Felsenstein, 1993). The accession numbers of the 16S rDNAs of reference strains were as follows: *Methanoculleus thermophilicus* DSM 2373^T, M59129; *Methanoculleus marisnigri* DSM 1498^T, M59134; *Methanoplanus limicola* DSM 2279^T, M59143; *Methanomicrobium mobile* DSM 1539^T, M59142; *Methanogenium organophilum* DSM 3569^T, M59131; *Methanogenium cariaci* DSM 1497^T, M59130; *Methanospirillum* DSM 864^T, M60880; and *Methanocorpusculum parvum* DSM 3823^T, M59147. The sequences of *Methanoculleus bourgensis* DSM 3045^T, *Methanoculleus olentangi* DSM 2772^T, *Methanocorpusculum labreanum* DSM 4855^T and *Methanogenium tationis* DSM 2702^T were obtained from the Ribosomal Database Project (Maidak *et al.*, 1994). The incorrect orthographies of *Methanoculleus bourgense* and *Methanoculleus thermophilicus* were, according to Rule 61 of the Bacteriological Code (Lapage *et al.*, 1992), corrected to *Methanoculleus bourgensis* (corrig.) and *Methanoculleus thermophilus* (corrig.).

RESULTS

Morphology of strains

Cells of strain INSLUZ^T stained Gram-negative and were highly irregular cocci, 1.25–2.0 µm in diameter. The cells were not seen to be motile but they were flagellated (Fig. 1b).

S-layer lattice

Addition of 1% (w/v) SDS led to lysis of cells of strain INSLUZ^T indicating a cell envelope with a proteinaceous cell wall. The cell envelope of strain INSLUZ^T consisted of a cytoplasmic membrane and an S-layer of hexagonally arranged glycoprotein subunits (Fig. 1a, b). The centre-to-centre spacing of the morphological units of the S-layer lattice was 15.2 nm. The apparent M_r of the glycoprotein subunits, as indicated by a positive PAS staining reaction, was 120 000 (Fig. 1c). The S-layer lattice and composition of strain INSLUZ^T were compared with those of the other species of the genus *Methanoculleus* (Table 1). All irregular *Methanoculleus* species studied so far possess hexagonal S-layer lattices consisting of glycoprotein subunits with M_r values of 101 000–138 000. The M_r of the S-layer glycoprotein of strain INSLUZ^T was within this range.

Antigenic fingerprinting

The antigenic fingerprint of strain INSLUZ^T was determined with a panel of antibody S-probes for the following reference methanogens (Macario & Conway de Macario, 1983, 1985): *Methanospirillum hungatei* JF1^T; *Methanococcus vannielii* SB^T; *Methanococcus*

voltae PSv^T; *Methanoculleus marisnigri* JR1^T; *Methanogenium cariaci* JR1c^T; *Methanomicrobium mobile* BP^T; *Methanobrevibacter smithii* ATCC 35061^T; *Methanococcus maripaludis* JJ^T; *Methanoplanus limicola* M3^T; and *Methanococcus thermolithotrophicus* SN1^T. No antigenic relationship between strain INSLUZ^T and any of the methanogenic reference strains, recognized by the antibody S-probes, was detected.

Substrates for growth and methanogenesis

Growth and methane production of strain INSLUZ^T were only observed with H₂/CO₂, formate, 2-propanol/CO₂, 2-butanol/CO₂ and cyclopentanol/CO₂ as substrates. Maximal OD₅₇₈ and methane production levels (per 20 ml modified medium I containing 60 mM acetate and 1 µM sodium tungstate obtained in 120 ml serum bottles) were as follows: H₂/CO₂, OD₅₇₈ 1.00, 4187 µmol methane; formate, 0.07, 323 µmol; 2-propanol/CO₂, 0.15, 285 µmol; 2-butanol/CO₂, 0.07, 120 µmol; and cyclopentanol/CO₂, 0.06, 420 µmol. No stimulation of growth on formate was observed by supplementation with 0.5 µM SeO₃²⁻ (OD₅₇₈ 0.06, 280 µmol methane). Growth of strain INSLUZ^T and methanogenesis were not observed on acetate, methanol, ethanol, 1-propanol, 2-pentanol, 2,3-butanediol, dimethylamine or lactate.

Culture conditions

The optimal growth temperature of strain INSLUZ^T was about 40 °C with the shortest doubling time of 13.5 h (Fig. 2). No growth was obtained below 21 °C and above 51 °C, despite the fact that the habitat of strain INSLUZ^T was a bioreactor operated at 55 °C.

Fig. 1. (a) Electron micrographs of a thin section of a cell of strain INSLUZ^T. Bar, 0.1 µm. (b) Electron micrograph of a freeze-etched preparation of strain INSLUZ^T showing the hexagonal arrangement of the S-layer glycoproteins and a piece of a flagellum (marked F). Bar, 0.1 µm. (c) SDS-PAGE of cell extracts of strain INSLUZ^T. Lanes: 1, M_r standards; 2, Coomassie blue-stained; and 3, PAS-stained.

<i>Methanoculleus</i> strain	DSM no.	Lattice constant (nm)*	$10^{-3} \times M_r$ of protein subunit	Reference
<i>M. palmolei</i> INSLUZ ^T	4273 ^T	15.2	120	This study
<i>M. marisnigri</i> JR1 ^T	1498 ^T	ND	138	Zellner <i>et al.</i> (1990)
<i>M. olentangyi</i> RC/ER ^T	2772 ^T	15.4	132	Zellner <i>et al.</i> (1990)
<i>M. bourgensis</i> MS2 ^T	3045 ^T	15.4	101	Zellner <i>et al.</i> (1990)
<i>M. thermophilus</i> CR-1 ^T	2373 ^T	ND	130	Zabel <i>et al.</i> (1985)
<i>M. thermophilus</i> UCLA	2624	ND	130	Zabel <i>et al.</i> (1985)
<i>M. thermophilus</i> Ratisbona	2640	ND	130	Zabel <i>et al.</i> (1985)

Detailed description of Figure 1: The graph plots Generation time (h) on the vertical axis against Temperature (°C) on the horizontal axis. The vertical axis has major tick marks at 10, 30, 50, 70, and 90. The horizontal axis has major tick marks at 20, 40, and 60. A smooth, U-shaped curve is drawn through several data points. The data points are represented by open circles. The curve reaches its minimum generation time (approximately 14 hours) at a temperature of about 40 °C. As the temperature deviates from this optimum in either direction, the generation time increases significantly.

Temperature (°C)	Generation time (h)
25	40
32	22
35	16
38	14
40	14
42	22
45	22

Phylogenetic tree showing the relationships between various species based on 16S rDNA sequences. The scale bar indicates 5%.

- Methanoculleus bourgensis* (100)
- Methanoculleus olentangyi* (73)
- Strain INSLUZ^T (= DSM 4273^T) (80)
- Methanoculleus marisnigri* (100)
- Methanoculleus thermophilus*
- Methanogenium tationis*
- Methanoplanus limicola*
- Methanomicrobium mobile* (75)
- Methanogenium organophilum* (100)
- Methanogenium cariaci*
- Methanospirillum hungatei*
- Methanocorpusculum labreanum* (100)
- Methanocorpusculum parvum*

59 mol% determined via the thermal denaturation (T_m) method. The value obtained by UV spectrophotometry was 60.0 mol%.

Phylogenetic analysis

DNA G + C content

1114

The degree of similarity of species of *Methanoculleus* and members of neighbouring genera is significantly lower, ranging between 92 (*Methanogenium*) and 90% (*Methanoplanus*, *Methanomicrobium* and *Methanogenium*).

DISCUSSION

The distinct phylogenetic position of strain INSLUZ^T within the radiation of *Methanoculleus* species, based on 16S rDNA similarity of less than 97% with its neighbouring species, is the main argument to propose strain INSLUZ^T as the type strain of a new species in the genus *Methanoculleus*.

Other differences concerned the architecture of the cell envelope and the antigenic fingerprints. The S-layer composition and architecture of strain INSLUZ^T was in concordance with S-layers found for other species of the genus *Methanoculleus* (Zellner *et al.*, 1989b, 1990). The M_r of the S-layer glycoprotein subunits of strain INSLUZ^T was 120 000 and distinct from those of any other *Methanoculleus* strain studied so far (101 000 and 130 000–138 000), while a much broader range (90 000–155 000) was observed for irregular coccoid methanogens belonging to different genera (Zellner *et al.*, 1989a, b, 1990). The polyamine pattern of strain INSLUZ^T also resembled that of other *Methanoculleus* species (Zellner *et al.*, 1990; Blotevogel *et al.*, 1991). The comparison of these and further features, as well as the DNA G+C content (Table 2), support the distinct phylogenetic position of strain INSLUZ^T as a member of a new species of the genus *Methanoculleus* (Maestrojuán *et al.*, 1990).

Studies on the growth-promoting effect of tungstate and influence of medium variations indicated that tungstate could replace the requirement of *Methanoculleus palmolei* for complex medium components; this was observed for most irregular coccoid strains of the order *Methanomicrobiales* (Boone *et al.*, 1993). The

growth-promoting effect of tungstate on strain INSLUZ^T was less pronounced, as reported for other hydrogenotrophic methanogens (Zellner & Winter, 1987b).

The secondary alcohol-oxidizing strain INSLUZ^T contained a secondary alcohol dehydrogenase activity which was dependent on F_{420} . An F_{420} -dependence of the secondary alcohol dehydrogenase activity was also observed for *Methanoculleus marisnigri*, *Methanoculleus bourgensis*, *Methanogenium liminatans* and *Methanolacinia paynteri*, while *Methanocorpusculum* or *Methanobacterium* strains contained an NADP⁺-dependent secondary alcohol dehydrogenase activity (Bleicher *et al.*, 1989).

Description of *Methanoculleus palmolei* sp. nov.

Methanoculleus palmolei (palm.o.le'i. L. fem. n. *palma* palm; L. masc. n. *oleum* oil; *palmolei* from oil of the palm; a methane-producing bag-shaped archaeon derived from a bioreactor anaerobically treating palm-oil-processing wastewater).

Cells are coccoid methanogens, 1.25–2 µm in diameter. Acetate is required as growth factor and growth is stimulated by potassium and tungstate ions. Cells are mesophilic and grow optimally at 40 °C (range 22–50 °C) and at neutral pH values around pH 6.9–7.5 (pH range 6.5–8.0). Cells grow on H₂/CO₂, formate, 2-propanol/CO₂, 2-butanol/CO₂ and cyclopentanol/CO₂. No growth and no methane production is observed on acetate, methanol, ethanol, 1-propanol, 2-pentanol/CO₂, 2,3-butanediol, dimethylamine and lactate. A secondary alcohol dehydrogenase activity, dependent on F_{420} , oxidizes 2-propanol, 2-butanol, 2-pentanol and cyclopentanol, but not ethanol, 1-propanol, cyclohexanol, 2,3-butanediol or acetoin. The DNA G+C content is 59 mol% (T_m method) and 59.5 mol% (HPLC method). Strain INSLUZ^T (= DSM 4273^T) is the type strain.

Table 2. Phenotypic features of the species of the genus *Methanoculleus*

Data compiled from literature cited. NR, Not reported.

<i>Methanoculleus</i> strain	DSM no.	Cell size (µm)	Flagellation (number)/motility	Substrate*	Minimum doubling time (h)	Growth temp. (optimum/range; °C)	DNA G+C content (mol %) [†]	Reference
<i>M. palmolei</i> INSLUZ ^T	4273 ^T	1.25–2.0	+ / –	H, F, P, B	13.5	40/21–51	59.0 ² , 59.5 ³	This study
<i>M. marisnigri</i> JR1 ¹	1498 ^T	1.3	+(NR) / –	H, F, P, B ¹	NR	20–25/10–45	61.2 ¹	Romesser <i>et al.</i> (1979)
<i>M. olentangyi</i> RC/ER ^{†‡}	2772 ^T	1.0–1.5	– / –	H, F, (P), (B) ¹	10.9	37/30–45	54.4 ¹	Corder <i>et al.</i> (1983)
<i>M. bourgensis</i> MS2 ^{†‡}	3045 ^T	1.0–2.0	– / –	H, F, P, B ¹	18.0	37/25–55	59 ¹	Ollivier <i>et al.</i> (1986)
<i>M. thermophilus</i> CR-1 ^T	2373 ^T	1.0–1.3	– / –	H, F	2.5	55/37–65	59–60 ¹ , 57–59 ²	Rivard & Smith (1983)
<i>M. thermophilus</i> UCLA	2624	0.7–1.8	+(1) / +	H, F	1.8	55–60 / < 70	56–59 ⁷²	Ferguson & Mah (1983)
<i>M. thermophilus</i> Ratisbona	2640	0.9–1.5	+(1) / +	H, F ¹	3.4	58/30–60	57 ²	Zabel <i>et al.</i> (1985)
<i>M. thermophilus</i> TCI	3915	0.6–1.5	NR	H, F, P, B	NR	55/30–60	54.7 ²	Widdel <i>et al.</i> (1988)
<i>M. olentangyi</i> CBI ^T	6216 ^T	1.0	– / –	H, F	3.8	45/20–50	48.6 ²	Blotevogel <i>et al.</i> (1991)

* H, H₂/CO₂; F, formate; P, 2-propanol/CO₂; B, 2-butanol/CO₂. Substrates in parentheses are only weakly utilized. 1, Secondary alcohol utilization tested by Zellner & Winter (1987c).

[†] DNA G+C content determined by: 1, buoyant density; 2, thermal denaturation; or 3, HPLC.

[‡] *Methanoculleus olentangyi* and *Methanoculleus bourgensis* are apparently subjective synonyms (Xun *et al.*, 1989; Boone *et al.*, 1993).

ACKNOWLEDGEMENTS

The authors wish to thank Dr Helmut Kneifel (Jülich, Germany) for analysing the polyamine pattern of strain INSLUZ^T, Dr Everly Conway de Macario (Albany, NY) for the antigenic fingerprint, and Anja Hagelstein (DSMZ, Braunschweig, Germany) for growing the cells of strain INSLUZ^T for 16S rDNA analysis. We wish to thank Ulrike Mendrock for her help in the molecular work. This investigation was supported in part by grants of the Deutsche Forschungsgemeinschaft. Part of the work described here was carried out at the Institute of Microbiology of the University of Hannover, Hannover, Germany.

REFERENCES

- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Bleicher, K., Zellner, G. & Winter, J. (1989). Growth of methanogens on cyclopentanol/CO₂ and specificity of alcohol dehydrogenase. *FEMS Microbiol Lett* **59**, 307–312.
- Blotevogel, K.-H., Gahl-Janßen, R., Janssen, S., Fischer, U., Pilz, F., Auling, G., Macario, A. J. L. & Tindall, B. J. (1991). Isolation and characterization of a novel mesophilic, fresh-water methanogen from river sediment *Methanoculleus oldenburgensis* sp. nov. *Arch Microbiol* **157**, 54–59.
- 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.
- Boone, D. R. & Xun, L. (1987). Effects of pH, temperature, and nutrients on propionate degradation by a methanogenic enrichment culture. *Appl Environ Microbiol* **53**, 1589–1592.
- Boone, D. R., Whitman, W. B. & Rouvière, P. (1993). Diversity and taxonomy of methanogens. In *Methanogenesis: Ecology, Physiology, Biochemistry and Genetics*, pp. 35–80. Edited by J. G. Ferry. New York: Chapman & Hall.
- Corder, R. E., Hook, L. A., Larkin, J. M. & Frea, J. I. (1983). Isolation and characterization of two new methane-producing cocci: *Methanogenium olentangyi*, sp. nov., and *Methanococcus deltae*, sp. nov. *Arch Microbiol* **134**, 28–32.
- De Soete, G. (1983). A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621–626.
- Felsenstein, J. (1993). PHYLIP (phylogenetic inference package) version 3.5.1. Department of Genetics, University of Washington, Seattle, USA.
- Ferguson, T. J. & Mah, R. A. (1983). Isolation and characterization of an H₂-oxidizing thermophilic methanogen. *Appl Environ Microbiol* **45**, 265–274.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seeliger, H. P. R. & Clark, W. A. (editors) (1992). *International Code of Nomenclature of Bacteria*. (1990 Revision). Washington, DC: American Society for Microbiology.
- Ma, A. N. & Ong, A. S. H. (1986). Palm oil processing – new development in effluent treatment. *Water Sci Technol* **18**, 35–40.
- Macario, A. J. L. & Conway de Macario, E. (1983). Antigenic fingerprinting of methanogenic bacteria with polyclonal antibody probes. *Syst Appl Microbiol* **4**, 451–458.
- Macario, A. J. L. & Conway de Macario, E. (1985). Monoclonal antibodies of predefined molecular specificity for identification and classification of methanogens and for probing their ecological niches. In *Monoclonal Antibodies Against Bacteria*, pp. 213–247. Orlando, FL: Academic Press.
- Maestrojuán, G. M., Boone, D. R., Xun, L., Mah, R. A. & Zhang, L. (1990). Transfer of *Methanogenium bourgense*, *Methanogenium marisnigri*, *Methanogenium olentangyi*, and *Methanogenium thermophilicum* to the genus *Methanoculleus* gen. nov., emendation of *Methanoculleus marisnigri* and *Methanogenium*, a description of new strains of *Methanoculleus bourgense* and *Methanoculleus marisnigri*. *Int J Syst Bacteriol* **40**, 117–122.
- Maidak, B. L., Larsen, N., McCaughey, M. J., Overbeek, R., Olsen, G. J., Fogel, K., Blandy, J. & Woese, C. R. (1994). The Ribosomal Database Project. *Nucleic Acids Res* **22**, 3485–3487.
- Messner, P. & Sleytr, U. B. (1992). Crystalline bacterial cell-surface layers. *Adv Microb Physiol* **33**, 213–275.
- Ollivier, B. M., Mah, R. A., Garcia, J. L. & Robinson, R. (1985). Isolation and characterization of *Methanogenium aggregans* sp. nov. *Int J Syst Bacteriol* **35**, 127–130.
- Ollivier, B. M., Mah, R. A., Garcia, J. L. & Boone, D. R. (1986). Isolation and characterization of *Methanogenium bourgense* sp. nov. *Int J Syst Bacteriol* **36**, 297–301.
- Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. *Int J Syst Bacteriol* **46**, 1088–1092.
- Raskin, L., Poulsen, L. K., Noguera, D. R., Rittman, B. E. & Stahl, D. A. (1994). Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridisation. *Appl Environ Microbiol* **60**, 1241–1248.
- Rivard, C. J. & Smith, P. H. (1983). Isolation and characterization of a thermophilic marine methanogenic bacterium, *Methanogenium thermophilicum* sp. nov. *Int J Syst Bacteriol* **32**, 430–436.
- Romesser, J. A., Wolfe, R. S., Mayer, F., Spiess, E. & Walther-Mauruschat, A. (1979). *Methanogenium*, a genus of marine methanogenic bacteria and characterization of *Methanogenium cariaci* spec. nov. and *Methanogenium marisnigri* spec. nov. *Arch Microbiol* **121**, 147–153.
- Schnürer, A., Houwen, F. P. & Svensson, B. H. (1994). Mesophilic syntrophic acetate oxidation during methane formation by a triculture at high ammonium concentration. *Arch Microbiol* **162**, 70–74.
- Sleytr, U. B., Messner, P., Pum, D. & Sára, M. (1993). Crystalline bacterial cell surface layers. *Mol Microbiol* **10**, 911–916.
- Strunk, O. & Ludwig, W. (1995). ARB – a software environment for sequence data. Department of Microbiology, Technical University of Munich, Munich, Germany.
- Ulltutz, S. (1972). Rapid determination of DNA base composition by ultraviolet spectroscopy. *Biochim Biophys Acta* **272**, 1–11.
- Widdel, F., Rouvière, P. E. & Wolfe, R. S. (1988). Classification of secondary alcohol-utilizing methanogens including a new thermophilic isolate. *Arch Microbiol* **150**, 477–481.
- Xun, L., Boone, D. R. & Mah, R. A. (1989). Deoxyribonucleic acid hybridization study of *Methanogenium* and *Methanocorpusculum* species, emendation of the genus *Methanocorpusculum*, and transfer of *Methanogenium aggregans* to the genus *Methanocorpusculum* as *Methanocorpusculum aggregans* comb. nov. *Int J Syst Bacteriol* **39**, 109–111.
- Zabel, H. P., König, H. & Winter, J. (1985). Emended description of *Methanogenium thermophilicum*, Rivard and Smith, and

assignment of new isolates to this species. *Syst Appl Microbiol* **6**, 72–78.

Zellner, G. & Kneifel, H. (1993). Caldo-pentamine and caldo-hexamine in cells of *Thermotoga* species, a possible adaptation to the growth at high temperatures. *Arch Microbiol* **159**, 472–476.

Zellner, G. & Winter, J. (1987a). Analysis of a highly efficient methanogenic consortium producing biogas from whey. *Syst Appl Microbiol* **9**, 284–292.

Zellner, G. & Winter, J. (1987b). Growth promoting effect of tungsten on methanogens and incorporation of tungsten-185 into cells. *FEMS Microbiol Lett* **40**, 81–87.

Zellner, G. & Winter, J. (1987c). Secondary alcohols as hydrogen donors for CO₂-reduction by methanogens. *FEMS Microbiol Lett* **44**, 323–328.

Zellner, G., Vogel, P., Kneifel, H. & Winter, J. (1987). Anaerobic digestion of whey and whey permeate with suspended and immobilized complex and defined consortia. *Appl Microbiol Biotechnol* **27**, 306–314.

Zellner, G., Messner, P., Kneifel, H., Tindall, B. J., Winter, J. &

Stackebrandt, E. (1989a). *Methanolacinia* gen. nov., incorporating *Methanomicrobium paynteri* as *Methanolacinia paynteri* comb. nov. *J Gen Appl Microbiol* **35**, 185–202.

Zellner, G., Stackebrandt, E., Messner, P., Tindall, B. J., Conway de Macario, E., Kneifel, H., Sleytr, U. B. & Winter, J. (1989b). *Methanocorpusculaceae* fam. nov., represented by *Methanocorpusculum parvum*, *Methanocorpusculum sinense* spec. nov. and *Methanocorpusculum bavaricum* spec. nov. *Arch Microbiol* **151**, 381–390.

Zellner, G., Sleytr, U. B., Messner, P., Kneifel, H. & Winter, J. (1990). *Methanogenium liminatans* spec. nov., a new coccoid, mesophilic methanogen able to oxidize secondary alcohols. *Arch Microbiol* **153**, 287–293.

Zellner, G., Gevecke, M., Conway de Macario, E. & Diekmann, H. (1991). Population dynamics of biofilm development during start-up of a butyrate degrading fluidized-bed reactor. *Appl Microbiol Biotechnol* **36**, 404–409.

Zellner, G., Macario, A. J. L. & Conway de Macario, E. (1997). A study of three anaerobic methanogenic bioreactors reveals that syntrophs are diverse and different from reference organisms. *FEMS Microbiol Ecol* **22**, 295–301.