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

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Strain INSUZT (= DSM 4273T) was isolated from a biogas-producing bioreactor treating wastewater of a palm oil mill on North-Sumatra (Indonesia). Cells of strain INSUZT 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 $M$, of 120000, 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 palmoi sp. nov. is therefore proposed for strain INSUZT (= DSM 4273T).

Keywords: methanogens, Methanoculleus palmoi 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; Schnurer 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; Maestrojuan 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, $6.2 \times 10^8$ t palm oil and $15.4 \times 10^8$ 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 INSUZT, 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 INSUZT 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 INSUZT 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 \(\mu\)M 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 H\(_2/\)CO\(_2\) (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 CO\(_2\) during methanogenesis. H\(_2/\)CO\(_2\) was refilled in a fed-batch mode; the produced methane, however, was not replaced. For mass cultivation, cells were grown on H\(_2/\)CO\(_2\) (80:20, v/v) with a flow rate of 501 h\(^{-1}\) in a 121 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 H\(_2/\)CO\(_2\) (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 H\(_2/\)CO\(_2\). This culture was serially diluted into WHP medium and the highest dilution showing growth of the fluorescent, irregular-coccoid strains was again serially diluted and 0.1 ml dilutions were streaked on agar plates with WHP medium plus 2.5\% (w/v) Oxo-d agar (Unipath). All manipulations were performed in an anaerobic chamber (Coy Laboratory Products) under a gas atmosphere of N\(_2)/\)H\(_2\) (95:5, v/v). The plates were transferred into a stainless steel anaerobic jar (Balch et al., 1979), pressurized with 300 kPa H\(_2/\)CO\(_2\) (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 H\(_2/\)CO\(_2\) (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 aa2 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 350\(T\), 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 olenangyi DSM 2772\(T\), Methanocorpusculum labreanum DSM 4855\(T\) and Methanogenium tations DSM 2702\(T\) were obtained from the Ribosomal Database Project (Maidak et al., 1994). The incorrect orthographies of Methanoculleus bourgens 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\textsuperscript{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\textsuperscript{T} indicating a cell envelope with a proteinaceous cell wall. The cell envelope of strain INSLUZ\textsuperscript{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\textsubscript{r} of the glycoprotein subunits, as indicated by a positive PAS staining reaction, was 120000 (Fig. 1c).

The S-layer lattice and composition of strain INSLUZ\textsuperscript{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\textsubscript{r} values of 101 000–138000. The M\textsubscript{r} of the S-layer glycoprotein of strain INSLUZ\textsuperscript{T} was within this range.

Antigenic fingerprinting

The antigenic fingerprint of strain INSLUZ\textsuperscript{T} was determined with a panel of antibody S-probes for the following reference methanogens (Macario & Conway de Macario, 1983, 1985): Methanospirillum hungatei JF1\textsuperscript{T}; Methanococcus vannielii SB\textsuperscript{T}; Methanococcus voltae PSv\textsuperscript{T}; Methanoculleus marisnigri JR1\textsuperscript{T}; Methanogenium cariaci JR1\textsuperscript{e}; Methanomicrobium mobile BP\textsuperscript{T}; Methanolobus tindarius T3\textsuperscript{T}; Methanococcus maripaludis JJ\textsuperscript{T}; Methanoplanus limicola M3\textsuperscript{T}; and Methanococcus thermolithotrophicus SN1\textsuperscript{T}. No antigenic relationship between strain INSLUZ\textsuperscript{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\textsuperscript{T} were only observed with H\textsubscript{2}/CO\textsubscript{2}, formate, 2-propanol/CO\textsubscript{2}, 2-butanol/CO\textsubscript{2} and cyclopentanol/CO\textsubscript{2} as substrates. Maximal OD\textsubscript{578} 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: H\textsubscript{2}/CO\textsubscript{2}, OD\textsubscript{578} 1.00, 4187 μmol methane; formate, 0.07, 323 μmol; 2-propanol/CO\textsubscript{2}, 0.15, 285 μmol; 2-butanol/CO\textsubscript{2}, 0.07, 120 μmol; and cyclopentanol/CO\textsubscript{2}, 0.06, 420 μmol. No stimulation of growth on formate was observed by supplementation with 0.5 μM SeO\textsubscript{4}\textsuperscript{2−} (OD\textsubscript{528} 0.06, 280 μmol methane). Growth of strain INSLUZ\textsuperscript{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\textsuperscript{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\textsuperscript{T} was a bioreactor operated at 55 °C.
Table 1. S-layers of species of the genus Methanoculleus

All Methanoculleus strains had hexagonal S-layer architecture and glycoprotein subunits.

<table>
<thead>
<tr>
<th>Methanoculleus strain</th>
<th>DSM no.</th>
<th>Lattice constant (nm)*</th>
<th>10⁻³ × M₅ of protein subunit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. palmolae INSLUZᵀ</td>
<td>4273ᵀ</td>
<td>15:2</td>
<td>120</td>
<td>This study</td>
</tr>
<tr>
<td>M. marisnigri 1R1ᵀ</td>
<td>1498ᵀ</td>
<td>ND</td>
<td>138</td>
<td>Zellner et al. (1990)</td>
</tr>
<tr>
<td>M. olentangyi RC/ERᵀ</td>
<td>2772ᵀ</td>
<td>15:4</td>
<td>132</td>
<td>Zellner et al. (1990)</td>
</tr>
<tr>
<td>M. bourgensis MS²ᵀ</td>
<td>3045ᵀ</td>
<td>15:4</td>
<td>101</td>
<td>Zellner et al. (1990)</td>
</tr>
<tr>
<td>M. thermophilus CR-1ᵀ</td>
<td>2373ᵀ</td>
<td>ND</td>
<td>130</td>
<td>Zabel et al. (1985)</td>
</tr>
<tr>
<td>M. thermophilus UCLA</td>
<td>2624</td>
<td>ND</td>
<td>130</td>
<td>Zabel et al. (1985)</td>
</tr>
<tr>
<td>M. thermophilus Ratisbona</td>
<td>2640</td>
<td>ND</td>
<td>130</td>
<td>Zabel et al. (1985)</td>
</tr>
</tbody>
</table>

*Centre-to-centre spacing of hexagonally arranged protein subunits of the S-layer. ND, Not determined.

Fig. 2. Effect of temperature on growth of strain INSLUZᵀ on H₂/CO₂ in WHP medium.

Fig. 3. Dendrogram showing the phylogenetic position of strain INSLUZᵀ within the genus Methanoculleus. The sequences of some phylogenetically neighbouring taxa were used to place the genus within the family Methanomicrobiaceae. Bootstrap values (expressed as percentages of 500 replications) of 70% or more are indicated at the branch points. Bar, 5% sequence divergence.

The optimal pH for growth was 6.9-7.5 (pH range 6.5-8.0). Growth of strain INSLUZᵀ on H₂/CO₂ was slightly promoted by tungstate and potassium ions (not shown).

Polyamines

Polyamine analysis of strain INSLUZᵀ revealed the following polyamine pattern (μmol (g dry wt)⁻¹): putrescine, 52.64; sym-homospermidine, 11-21; and spermine, 0.03. 1,3-Diaminopropane, norspermidine, spermidine and norspermine were below the detection limit of 0.02 μmol (g dry wt)⁻¹.

DNA G + C content

The G + C content of DNA of strain INSLUZᵀ was 59-5 mol% determined by HPLC (with a standard deviation of three determinations of ±01 mol%) and 59 mol% determined via the thermal denaturation (Tₘ) method. The value obtained by UV spectrophotometry was 60-0 mol%.

Phylogenetic analysis

The almost complete primary structure of the 16S rDNA of strain INSLUZᵀ was sequenced (1431 nt). The phylogenetic position indicated that strain INSLUZᵀ was a member of the genus Methanoculleus. Phylogenetically, strain INSLUZᵀ clusters between the species pair Methanoculleus bourgensis DSM 3045ᵀ and Methanoculleus olentangyi DSM 2772ᵀ, (97.7 and 97.5% 16S rDNA similarity, respectively), and Methanoculleus marisnigri (97.5% similarity). Methanoculleus thermophilus is slightly less related, showing a range of 16S rDNA similarities of 95.8-96.7% to the other members of the genus (Fig. 3).
The degree of similarity of species of Methanolobus 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\(^\dagger\) within the radiation of Methanolobus species, based on 16S rDNA similarity of less than 97% with its neighbouring species, is the main argument to propose strain INSLUZ\(^\dagger\) as the type strain of a new species in the genus Methanolobus.

Other differences concerned the architecture of the cell envelope and the antigenic fingerprints. The S-layer composition and architecture of strain INSLUZ\(^\dagger\) was compared and influence of medium variations indicated that the DNA G+C content (Table 2), support the distinct phylogenetic position of strain INSLUZ\(^\dagger\) as a member of a new species of the genus Methanolobus (Maestroquain et al., 1990).

Studies on the growth-promoting effect of tungstate and influence of medium variations indicated that tungstate could replace the requirement of Methanolobus palmei for complex medium components; this was observed for most irregular cocoid strains of the order Methanomicrobiales (Boone et al., 1993). The growth-promoting effect of tungstate on strain INSLUZ\(^\dagger\) was less pronounced, as reported for other hydrognotrophic methanogens (Zellner & Winter, 1987b).

The secondary alcohol-oxidizing strain INSLUZ\(^\dagger\) 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 Methanolobus marisnigri, Methanolobus bourgisii, Methanogenium liminatans and Methanolacina panynteri, while Methanocorpusculum or Methanobacterium strains contained an NADP+-dependent secondary alcohol dehydrogenase activity (Bleicher et al., 1989).

**Description of Methanolobus palmei sp. nov.**

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

Cells are cocoid methanogens, 1-2.5–2 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 H\(_2\)/CO\(_2\), formate, 2-propanol/CO\(_2\), 2-butanol/CO\(_2\) and cyclopentanol/CO\(_2\). No growth and no methane production is observed on acetate, methanol, ethanol, 1-propanol, 2-pentanol/CO\(_2\), 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\(_\text{m}\) method) and 59.5 mol% (HPLC method). Strain INSLUZ\(^\dagger\) (DSM 4273\(^\dagger\)) is the type strain.

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

Data compiled from literature cited. NR, Not reported.

<table>
<thead>
<tr>
<th>Methanolobus strain</th>
<th>DSM no.</th>
<th>Cell size (μm)</th>
<th>Flagellation (number)/ mobality</th>
<th>Substrate*</th>
<th>Minimum doubling time (h)</th>
<th>Growth temp. (optimum/ range, °C)</th>
<th>DNA G + C content (mol%)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. palmei</em> INSLUZ(^\dagger)</td>
<td>4273(^\dagger)</td>
<td>1-2.5-2.0</td>
<td>+/–</td>
<td>H, F, P, B</td>
<td>13.5</td>
<td>30/21-51</td>
<td>59.0, 59.5</td>
<td>This study</td>
</tr>
<tr>
<td>M. marisnigri JRI(^\dagger)</td>
<td>1490(^\dagger)</td>
<td>1.5</td>
<td>+(+10%)</td>
<td>H, F, P, B</td>
<td>NR</td>
<td>20/20-10 45</td>
<td>61.2</td>
<td>Romesser et al. (1979)</td>
</tr>
<tr>
<td>M. marisnigri RCL(^\dagger)</td>
<td>2727(^\dagger)</td>
<td>1.5-1.5</td>
<td>–</td>
<td>H, F, P, (B)?</td>
<td>109</td>
<td>37/30-45</td>
<td>54.4</td>
<td>Corder et al. (1983)</td>
</tr>
<tr>
<td>M. bourgisii NZ112(^\dagger)</td>
<td>5047(^\dagger)</td>
<td>1.5-2.0</td>
<td>–</td>
<td>H, F, P, B</td>
<td>180</td>
<td>37/25-55</td>
<td>59</td>
<td>Oliver et al. (1986)</td>
</tr>
<tr>
<td>M. bourgisii CR1(^\dagger)</td>
<td>2373(^\dagger)</td>
<td>1.5-1.5</td>
<td>–</td>
<td>H, F</td>
<td>2.5</td>
<td>55/37-65</td>
<td>59–60%, 59.5</td>
<td>Rigard &amp; Smith (1983)</td>
</tr>
<tr>
<td>M. bourgisii UCLA</td>
<td>2374</td>
<td>0.7–1.8</td>
<td>+(+1)</td>
<td>H, F</td>
<td>1.8</td>
<td>55/60 &lt; 70</td>
<td>56-59%</td>
<td>Ferguson &amp; Mah (1985)</td>
</tr>
<tr>
<td>M. bourgisii R216</td>
<td>2600</td>
<td>0.9-1.5</td>
<td>+(+1)+</td>
<td>H, F</td>
<td>3.4</td>
<td>53/30-60</td>
<td>57</td>
<td>Zehbe et al. (1985)</td>
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<tr>
<td>M. bourgisii TCI</td>
<td>3915</td>
<td>0.6–1.5</td>
<td>NR</td>
<td>H, F, P, B</td>
<td>NR</td>
<td>55/30-60</td>
<td>54.7</td>
<td>Widell et al. (1988)</td>
</tr>
<tr>
<td>M. ebudicus mexicus CB1(^\dagger)</td>
<td>6216(^\dagger)</td>
<td>1.0</td>
<td>–</td>
<td>H, F</td>
<td>48</td>
<td>45/20-30</td>
<td>48.6</td>
<td>Blotevogel et al. (1991)</td>
</tr>
</tbody>
</table>


\(^\dagger\) DNA G + C content determined by: 1. buoyant density; 2. thermal denaturation; or 3. HPLC.

\(\dagger\) Methanolobus olefomangii and Methanolobus bourgisii are apparently subjective synonyms (Xun et al., 1989; Boone et al., 1993).
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