Methanobacterium aggregans sp. nov., a hydrogenotrophic methanogenic archaeon isolated from an anaerobic digester

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A novel, strictly anaerobic, hydrogenotrophic methanogen, strain E09F.3T, was isolated from a commercial biogas plant in Germany. Cells of E09F.3T were Gram-stain-negative, non-motile, slightly curved rods, long chains of which formed large aggregates consisting of intertwined bundles of chains. Cells utilized $H_2 + CO_2$ and, to a lesser extent, formate as substrates for growth and methanogenesis. The optimal growth temperature was around 40 °C; maximum growth rate was obtained at pH around 7.0 with approximately 6.8 mM NaCl. The DNA G+C content of strain E09F.3T was 39.1 mol%. Phylogenetic analyses based on 16S rRNA and mcrA gene sequences placed strain E09F.3T within the genus Methanobacterium. On the basis of 16S rRNA gene sequence similarity, strain E09F.3T was closely related to Methanobacterium congoense C$^T$ but morphological, physiological and genomic characteristics indicated that strain E09F.3T represents a novel species. The name Methanobacterium aggregans sp. nov. is proposed for this novel species, with strain E09F.3T ($= DSM 29428^T = JCM 30569^T$) as the type strain.

The family Methanobacteriaceae belongs to the order Methanobacteriales and is represented by four genera including Methanobacterium. Members of the genus Methanobacterium are widespread in anaerobic habitats such as freshwater sediments, gastrointestinal tracts of animals, and anaerobic digesters (Boone, 2001). Recent isolates came from northern peatlands (Cadillo-Quiroz et al., 2014) and from a subsurface lake sediment (Schirmack et al., 2014). All species of the genus Methanobacterium described so far grow by reduction of carbon dioxide to methane using molecular hydrogen as the electron donor (hydrogenotrophic methanogenesis) (Boone, 2001). Some isolates can also use secondary alcohols (Cadillo-Quiroz et al., 2014; Godsy, 1980) instead of $H_2$, or grow with formate as the sole source of carbon and energy (Bryant & Boone, 1987; Joulian et al., 2000; Shcherbakova et al., 2011).

Species of the genus Methanobacterium usually occur as curved, crooked or straight rods, long to filamentous (Boone, 2001). The formation of cell clusters is common for species of the genus Methanosarcina but not for members of the genus Methanobacterium. An exception is the thermophilic Methanobacterium thermaggregans (Blotevogel & Fischer, 1985). More than 20 species of the genus Methanobacterium have so far been isolated from various anaerobic habitats. Although members of the genus are prevalent in biogas plants (Klocke et al., 2007; Krakat et al., 2010; Leclerc et al., 2004; Stantscheff et al., 2014), which suggests their importance for biogas production, only Methanobacterium congoense, Methanobacterium formicicum, and Methanobacterium beijingense originated from anaerobic digesters (Cuzin et al., 2001; Ma et al., 2005; Stantscheff et al., 2014). In this work, isolation and characterization of a new hydrogenotrophic, mesophilic Methanobacterium strain from a biogas plant in Germany is described.

Strain E09F.3T was isolated in Dresden (Germany) from a biogas sludge sample obtained in August 2013 from a commercial biogas plant located near Cologne (Germany). The anaerobic digester was operated at 40 °C and at a pH between 7.7 and 8.2. Maize silage, cattle manure and dry poultry faeces were the main feeding substrates. Samples were taken in sealed plastic bottles and stored at 4 °C. All manipulations were conducted under strictly anaerobic conditions, either in an anaerobic glove box (Coy) containing an atmosphere of $N_2/CO_2/H_2$ (78 : 18 : 4, by vol.) or by applying anaerobic techniques as described elsewhere (Balch & Wolfe, 1976). Unless otherwise noted, basal liquid medium, prepared under an $N_2/CO_2$ (80 : 20, v/v) atmosphere, was used in all experiments and contained (g l$^{-1}$ demineralized water): KH$_2$PO$_4$, 0.5; NH$_4$Cl, 0.4; MgCl$_2$6H$_2$O, 0.4; CaCl$_2$2H$_2$O, 0.05; NaCl, 0.4; MOPS, 4.2; NaHCO$_3$, 3.9; 1-cysteine hydrochloride, 0.5; Na$_2$S, 0.5; resazurin, 0.001; trace element solution, 0.1 % (v/v); vitamin solution, 0.2 %
Single, slightly curved rods were non-motile, 2.0 to 2.5 m long and 0.2 to 0.5 m in diameter. Single cells showed strong fluorescence at a wavelength of 420 nm, which is mainly due to cofactor F_{420} (Doddema & Vogels, 1978).

For scanning electron microscopy, cells were fixed with 2.5 % glutaraldehyde in phosphate buffer (57.5 mM; pH 7.2) for 2 hours. After conducting an ethanol series and critical-point drying (Bal-Tec) gold coating was performed with a sputter coater K550 (Emitech). Images were taken with a Supra 40VP scanning electron microscope (Zeiss). The majority of E09F.3\textsuperscript{T} cells were present in long, extensively intertwined chains forming large aggregates (Fig. 1b). The fact that cells were slightly curved and grew in chains appeared to facilitate formation of these large intertwined ‘bundles’. It was not possible to disaggregate strain E09F.3\textsuperscript{T} and growth in aggregates was observed under all cultivation conditions. The ability of E09F.3\textsuperscript{T} to grow on H_{2} + CO_{2} and its cell morphology indicated its affiliation with the genus Methanobacterium. However, no mesophilic species of the genus Methanobacterium has been reported to grow in large and stable aggregate of intertwined cell filaments. Only for the thermophilic Methanobacterium thermoggre-gans the tendency for formation of aggregates has been described (Blotevogel & Fischer, 1985).

To analyse the taxonomic rank of isolate E09F.3\textsuperscript{T} in detail, a nearly complete fragment (1408 bp) of the 16S rRNA gene of strain E09F.3\textsuperscript{T} was amplified from genomic DNA by PCR using the primers Arch21F (5’-TCCGCTTACCTTGTTACGACTT-3’) and Univ-1517-a-A-21 (5’-ACGGCTACCTTGTTACGACTT-3’; Weisburg et al., 1991). Also, a large fragment (1118 bp) of the mcrA gene (encoding the alpha subunit of methyl-coenzyme M reductase; Nölling et al., 1996) was amplified using a forward primer (5’-GACCTCCTCGGTGTTACAGACTT-3’; Simankova et al., 2003) and a reverse primer (5’-TTCATTGGCCTAGTGTTAGGT-3’; Luton et al., 2002). PCR products were sequenced by GATC Biotech (Cologne, Germany) employing the BigDye Terminator Cycle Sequencing protocol (Applied Biosystems). Phylogenetic trees of the (nearly complete) 16S rRNA gene and of the mcrA gene sequences were reconstructed using the maximum-likelihood method with MEGA 6.06 based on a Geneious 7.1.7 CLUSTAL W alignment.

Phylogenetic analysis using the nearly complete 16S rRNA gene (KP006499) confirmed that strain E09F.3\textsuperscript{T} belongs to the genus Methanobacterium (Fig. 2). The isolate is most closely related to Methanobacterium congolense C\textsuperscript{T} (98.5 % sequence similarity; NR028175). The next relative is Methanobacterium paludis SWANI\textsuperscript{T} (97.1 % sequence similarity; CP002772). Comparison of the mcrA genes confirmed these relationships (data not shown). The 16S rRNA gene sequence similarity to the nearest relative, Methano- bacterium congolense is close to the recommended threshold similarity of 98.7–99 % to distinguish microbial species (Stackebrandt & Ebers, 2006), which made comparison of the genomic characteristics of E09F.3\textsuperscript{T} with those of Methanobacterium congolense C\textsuperscript{T} by DNA–DNA

For enrichment, biogas sludge was diluted 1 : 10 in basal liquid medium and 50 µl of the dilution was added to serum flasks and incubated at 40 °C in an incubator (Infors HT Minitron) with slight agitation (40 r.p.m.). Growth of methanogenic archaea in the enrichment was assessed microscopically by increase of F_{420} fluorescence (Infors HT Minitron) with slight agitation (40 r.p.m.). Serum flasks and incubated at 40 °C in an incubator (C in anaerobic jars pressurized to 100 kPa H_{2}/CO_{2}/H_{2}S (79.9 : 20 : 0.1, by vol.).

Colonies were ochre in colour with a rough surface and were visible after at least 14 days of cultivation on agar plates. Single colonies were used to inoculate 5 ml of the dilution was added to 25 ml in serum flasks (100 ml nominal volume) or 5 ml in Balch tubes (27 ml nominal volume). Unless indicated otherwise, the final pH of the medium was 7.2.

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Fig. 1. Morphology of E09F.3\textsuperscript{T}. (a) Phase-contrast micrograph showing single rods, filaments and a part of an aggregate; bar, 10 µm. (b) Scanning electron micrograph showing intertwined cell-filaments and filaments consisting of rods; magnification, ×7860; bar, 1 µm.
hybridization (DDH) necessary. DDH was carried out by the identification service of the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany) as described by De Ley et al. (1970) under consideration of the modifications described by Huß et al. (1983). The DNA–DNA similarity between \textit{Methanobacterium congolense} DSM 7095\textsuperscript{T} (=C\textsuperscript{T}) and strain E09F.3\textsuperscript{T} was only 40.7 \pm 4.7 \% (mean \pm SD, two independent experiments). This value clearly distinguishes the two strains into two separate species based on the recommended threshold value of 70 \% DNA–DNA similarity (Wayne et al., 1987).

The mol\% G + C content of chromosomal DNA of E09F.3\textsuperscript{T} was determined by HPLC analysis (Mesbah et al., 1989; Tamaoka & Komagata, 1984) at the DSMZ to be 39.1 \pm 0.7 mol\% (mean \pm SD, two independent experiments). According to Boone (2001), a DNA G + C content between 32 and 61 mol\% is typical for the genus \textit{Methanobacterium}.

Gram staining was conducted as described by Gerhardt et al. (1994). In contrast to its closest relative, \textit{Methanobacterium congolense}, E09F.3\textsuperscript{T} stained Gram-negative. When exposed to 0.1 \% SDS (Boone & Whitman, 1988), most cells of E09F.3\textsuperscript{T} lysed within 10 min but were more stable in demineralized water, as lysis occurred only within 60 min (data not shown). In contrast, cells of \textit{Methanobacterium congolense} DSM 7095\textsuperscript{T} already lyse when exposed to 0.05 \% SDS (data not shown).

As an additional measure to distinguish isolate E09F.3\textsuperscript{T} from the closely related \textit{Methanobacterium congolense} C\textsuperscript{T}, their protein inventories were compared. To this end, cells from exponentially growing cultures were disrupted by sonification and cell debris was removed by centrifugation (13 000 \textit{g}, 10 min). Both strains were treated equally (regarding growth medium, growth phase when harvesting, SDS-solubilization of proteins). Samples of the soluble protein extract were analysed by SDS-PAGE followed by Coomassie staining (Fig. S1, available in the online Supplementary Material). The protein pattern was substantially different in the two strains, which corroborates the notion of them representing different species.

Since strain E09F.3\textsuperscript{T} grows in large aggregates, it was not possible to use optical density as a measure for biomass. Instead, a linear relationship between dry mass of cells and methane formation during exponential growth by following methane formation and dry biomass was established experimentally (data not shown). Methane was quantified with a GC-17A gas chromatograph (Shimadzu) equipped with a flame-ionization detector. The temperature of the Optima-5 column used (0.25 \mu m; Macherey-Nagel) was 130 °C with nitrogen as carrier gas at a flow rate of 34 ml min\textsuperscript{-1}. Injector and detector temperatures were 200 °C and 280 °C, respectively. Calibration and integration of peaks was carried out with GC solution analysis (Shimadzu) version 2.41. Dry biomass was determined by filtering cultures through a cellulose acetate membrane filter (0.2 \mu m; Satorius), drying of the filters at 70 °C for at least 10 h, and subsequent weighing, with uninoculated medium used for background correction.

Strain E09F.3\textsuperscript{T} grew with H\textsubscript{2} + CO\textsubscript{2} (150 kPa) as the sole carbon and energy source with a doubling time of 4.6 to 5.8 h. Chemolithoautotrophic growth is common for species of the genus \textit{Methanobacterium} (Boone, 2001). E09F.3\textsuperscript{T} could also use formate (100 mM) for methanogenesis and growth. The methane formation rate from H\textsubscript{2} + CO\textsubscript{2} (1.22 \pm 0.34 \mu mol h\textsuperscript{-1} ml\textsuperscript{-1} of culture) was higher than from formate (0.53 \pm 0.08 \mu mol h\textsuperscript{-1} ml\textsuperscript{-1}). With methanol (125 mM), 2-butanol (30–50 mM) + CO\textsubscript{2} (40 kPa) and 2-propanol (30–50 mM) + CO\textsubscript{2} (40 kPa) as substrates, neither methane formation nor growth was observed. Addition of yeast extract (0.1 to 0.3 \%) to the medium stimulated growth of strain E09F.3\textsuperscript{T}; addition of coenzyme M (0.61 mM) and doubling the amount of the vitamin solution showed no positive effect on growth. In the absence of the vitamin solution strain E09F.3\textsuperscript{T} could still grow, albeit somewhat more slowly, which demonstrated that none of the vitamins used are essential.

The influence of temperature, pH, NaCl and NH\textsubscript{4}Cl concentration on growth was determined from the specific methane formation rates using liquid basal medium with H\textsubscript{2} + CO\textsubscript{2} as substrate (Fig. S2). Temperature-dependence of growth was assessed from 25 to 50 °C at 5 °C intervals. The effect of NaCl was determined by adding 0 to 2 M NaCl (from sterile anaerobic stock) to the basal liquid medium. The background sodium content with no

\begin{figure}
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.png}
\caption{Phylogenetic tree (maximum-likelihood) showing the position of strain E09F.3\textsuperscript{T} and selected species of the genus \textit{Methanobacterium}. Based on 16S rRNA gene sequences (1408 bp). Bootstrap values (>50 \%) based on 1000 replications are shown. Accession numbers are given in parentheses. Bar, 0.005 evolutionary distance.}
\end{figure}
Table 1. Characteristics differentiating strain E09F.3T from strains of the genus *Methanobacterium*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain E09F.3T</th>
<th>Strain Methanobacterium congolense</th>
<th>Strain Methanobacterium swan1T</th>
<th>Strain Methanobacterium lacus</th>
<th>Strain Methanobacterium beijingense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Anaerobic digester (Germany)</td>
<td>Anaerobic digester (Congo)</td>
<td>Northern peat lands</td>
<td>Freshwater meromictic lake</td>
<td>Anaerobic digester (China)</td>
</tr>
<tr>
<td>Cell morphology</td>
<td>Aggregates, rarely in single cells</td>
<td>Rods, rarely in small aggregates</td>
<td>Rods</td>
<td>Long rods, filaments</td>
<td>Rods</td>
</tr>
<tr>
<td>Cell dimension (width x length)</td>
<td>0.2-0.4 x 2.0-15.0 μm</td>
<td>0.4-0.5 x 3.0-5.0 μm</td>
<td>0.6-1.5 x 2.8 μm</td>
<td>0.2-0.4 x 2.0-15.0 μm</td>
<td>0.8-1.5 x 2.8 μm</td>
</tr>
<tr>
<td>Gram staining</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Substrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pH</td>
<td>6.5 (5.0-8.6)</td>
<td>7.2 (5.9-8.2)</td>
<td>7.2 (5.9-8.2)</td>
<td>7.2-7.7 (6.5-8.6)</td>
<td>7.2-7.7 (6.5-8.6)</td>
</tr>
<tr>
<td>NaCl (mM)</td>
<td>≤50</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37 (25-50)</td>
<td>30 (14-40)</td>
<td>6.5 (5.0-8.6)</td>
<td>37 (25-50)</td>
<td>37 (25-50)</td>
</tr>
<tr>
<td>Optimal growth (range)</td>
<td>5-6 h</td>
<td>7.5 h</td>
<td>35-45 h</td>
<td>5.5-7 h</td>
<td>5.5-7 h</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>39.1 (HPLC)</td>
<td>39.5 (HPLC)</td>
<td>39.5 (HPLC)</td>
<td>35.7 (G)</td>
<td>39.1 (HPLC)</td>
</tr>
</tbody>
</table>

*Determined by HPLC analysis (HPLC), melting point analysis (HPLC), or genome sequencing (G).*

Despite a close relationship between strain E09F.3T and *Methanobacterium congolense*, the two differ substantially in morphological, physiological and genomic properties (Table 1). On the basis of its differences to *Methanobacterium congolense* and its unusual characteristic to grow in large cell aggregates, strain E09F.3T is described as a representative of a novel species of the genus *Methanobacterium*, and the name *Methanobacterium aggregans* sp. nov. is proposed.
Description of Methanobacterium aggregans sp. nov.

Methanobacterium aggregans (ag`gre.gans. L. part. adj. aggregans aggregating, aggregate-forming).

Cells are non-motile, slightly curved rods (0.2 to 0.5 × 2.0 to 2.5 µm) and stain Gram-negative. Colonies are coloured ochre with a rough surface. Utilizes both H₂ + CO₂ and formate for growth and methane formation. Optimal growth and methane formation at 40 °C and pH 6.7–7.0. Yeast extract and vitamins are not required for growth.

The type strain is E09F.3T (=DSM 29428T=JCM 30569T) and was isolated from a biogas sludge sample in Dresden, Germany. The DNA G+C content of the type strain is 39.1 ± 0.8 mol% (HPLC).

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References


