Methanosarcina flavescens sp. nov., a methanogenic archaeon isolated from a full-scale anaerobic digester

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A novel, strictly anaerobic, methanogenic archaeon, strain E03.2T, was isolated from a full-scale biogas plant in Germany. Cells were non-motile sarcina-like cocci, occurring in aggregates. Strain E03.2T grew autotrophically on H2 plus CO2, and additionally cells could utilize acetate, methanol, mono-, di- and trimethylamine as carbon and energy sources; however, growth or methanogenesis on formate was not observed. Yeast extract and vitamins stimulated growth but were not mandatory. The optimal growth temperature of strain E03.2T was approximately 45 °C; maximal growth rates were obtained at about pH 7.0 in the presence of approximately 6.8 mM NaCl. The DNA G+C content of strain E03.2T was 41.3 mol%. Phylogenetic analyses based on 16S rRNA gene and mcrA sequences placed strain E03.2T within the genus Methanosarcina. Based on 16S rRNA gene sequence similarity strain E03.2T was related to seven different species of the genus Methanosarcina, but most closely related to Methanosarcina thermophila TM-1T. Phenotypic, physiological and genomic characteristics indicated that strain E03.2T represents a novel species of the genus Methanosarcina, for which the name Methanosarcina flavescens sp. nov. is proposed. The type strain is E03.2T (= DSM 100822T = JCM 30921T).

Members of the genus Methanosarcina are of ecological importance because they are more metabolically versatile than other methanogenic archaea. While most methanogens employ only the hydrogenotrophic pathway for primary energy metabolism (methanogenesis), which involves reduction of CO2 to methane using molecular hydrogen (or sometimes formate) as electron donor, many species of the genus Methanosarcina also use methylated compounds such as methanol or methylamines via the methylotrophic pathway, and acetate via the aceticlastic pathway, as carbon and energy sources [reviewed by Rother (2010) and Thauer et al. (2008)]. Members of the genus Methanosarcina can be found in many different anaerobic environments such as marine and freshwater sediments, soils, and digestive tracts of warm-blooded animals. Recent isolates were derived from diatomaceous shale formations and from a subsurface sulphurous lake (Ganzert et al., 2014; Shimizu et al., 2015).

Beside their ecological importance, members of the genus Methanosarcina also play an important role in wastewater treatment and anaerobic digestion. Strains of Methanosarcinaarkeri and Methanosarcina mazei, which are now important model organisms (Kohler & Metcalf, 2012), were isolated from anaerobic sewage sludge (Bryant & Boone, 1987; Mah et al., 1978; Mah, 1980; Mah & Kuhn, 1984). Since aceticlastic methanogenesis is a key step of anaerobic digestion, members of the genus Methanosarcina often represent dominant species of the archaeal communities in biogas plants (De Vrieze et al., 2012; Illmer et al., 2014; Yu et al., 2014). Methanosarcina thermophila was originally isolated from a laboratory-scale anaerobic digester (Zinder et al., 1985), but detailed characterization of a Methanosarcina isolate from a full-scale biogas plant was, to our knowledge, thus far not reported. Characterization of micro-organisms involved in the biogas process offers the possibility to aid our understanding of anaerobic digestion and, ultimately, to optimize it. Also, correlating the characteristics of isolates with knowledge from the GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene and partial mcrA sequence of strain E03.2T are KT828541 and KT828542, respectively. The draft genome sequence of strain E03.2T was deposited in the GenBank database under accession number LKAZ00000000.

Three supplementary figures, a supplementary table and a detailed method for genome sequencing are available in the online Supplementary Material.
culture-independent approaches, e.g. of sequencing-based descriptions of the biocenosis, could identify indicator organisms relevant during process disruptions, as was already shown for *M. thermophila*-related organisms (Kampmann et al., 2014).

Strain E03.2\textsuperscript{T} was isolated from a biogas sludge sample obtained in March 2013 from a full-scale commercial biogas plant (536 kW\textsubscript{el}) located near Cologne (Germany). The plant was fed with maize silage and cattle manure as well as dry poultry faeces (785 : 221 : 141 t of fresh mass) and operated at a temperature of 40 °C and at a pH between 7.7 and 8.2 (adjusted with calcium bicarbonate to prevent acidification). Samples were taken in plastic bottles, sealed and immediately transported to the laboratory at 4 °C.

All manipulations were conducted under strictly anaerobic conditions, either in an anaerobic glove box (Coy Laboratory Products) containing an atmosphere of N\textsubscript{2}/CO\textsubscript{2}/H\textsubscript{2} (79.9 : 20 : 0.1, by vol.) or by applying anaerobic techniques (Balch et al., 1979). Unless otherwise noted, basal liquid (BL) medium (Kern et al., 2015), prepared under an N\textsubscript{2}/CO\textsubscript{2} (80 : 20, v/v) atmosphere, was used. Unless indicated otherwise, the final pH of the medium was 7.2.

For the enrichment of methanogenic archaea, biogas sludge was diluted 10\textsuperscript{-1} and 10\textsuperscript{-2} in anaerobic BL medium and 100 μl diluted sludge was spread onto agar plates consisting of BL medium with methanol (62.5 mM) and solidified with 1 % (w/v) Difco agar (Becton Dickinson). After incubation for 21 days in anaerobic jars, pressurized with 50 kPa N\textsubscript{2}/CO\textsubscript{2}/H\textsubscript{2}S (79.9 : 20 : 0.1, by vol.), at 40 °C, colonies were picked for purification by streak plating onto solid BL medium with methanol as substrate. Additionally, sodium 2-mercaptoethanesulfonate (coenzyme M; 0.71 mM) was added to the medium to stimulate growth of methanogens and 1.0 mg ml\textsuperscript{-1} ampicillin and kanamycin were added as bacterial growth inhibitors. Streak plating was repeated until uniform colonies of the isolates were obtained. One of these isolates was designated E03.2\textsuperscript{T} and characterized further.

Single colonies of isolate E03.2\textsuperscript{T} were used to inoculate 5 ml BL medium with 62.5 mM methanol as growth substrate and incubated at 40 °C with slight agitation. Purity of the E03.2\textsuperscript{T} culture was confirmed by fluorescence microscopy and by PCR amplification using the bacteria-specific primers Eubac27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGGTATCAGAC-3’) (DeLong, 1992). Cell morphology of isolate E03.2\textsuperscript{T} was visualized by phase-contrast microscopy with an Axio Imager.M1 (Zeiss) equipped with a HBO 100 light source, and with fluorescence microscopy using the shift-free excitation filter set 18 (Zeiss) for assessing fluorescence of the methanogenic cofactor F\textsubscript{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 two 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).

Colonies of isolate E03.2\textsuperscript{T} on agar plates were dark yellow and had a ‘wart-like’ appearance with irregular shape and a rough surface. In liquid culture, cells of isolate E03.2\textsuperscript{T} were present as irregular cocci in multicellular aggregates (Fig. 1a, c), with a strong F\textsubscript{420} fluorescence (Doddema & Vogels, 1978) (Fig. 1b), which is typical for species of the genus *Methanosarcina*. The cell aggregates were large enough to be visible by eye (Fig. S1, available in the online Supplementary Material). Despite adapting strain E03.2\textsuperscript{T} to grow in BL medium containing 50 mM MgCl\textsubscript{2}, 4 mM CaCl\textsubscript{2} and 0.4 M NaCl, or to grow in marine high-salt medium (Metcalf et al., 1996), the strain never grew as single cells, as was shown previously for five other species of the genus *Methanosarcina* (Sowers et al., 1993). Motility of strain E03.2\textsuperscript{T} could not be observed and no intracellular structures were visible.

In addition to forming dark yellow colonies on agar plates, cell aggregates of strain E03.2\textsuperscript{T} in liquid culture were also dark yellow, but only when grown on methylated compounds (methanol and methylamines); under these conditions, even the culture supernatant sporadically appeared yellow (Fig. S1), which indicated secretion of (a) coloured compound(s). However, no growth condition could be defined to date, which would predictably lead to this phenotype. Future efforts will have to aim at identifying cultivation conditions, which lead to predictable pigment secretion, at unravelling the identity of the compound(s), and at characterizing its/their biological role.

The fact that strain E03.2\textsuperscript{T} uses methanol as a growth substrate and forms cell aggregates indicated its affiliation with the genus *Methanosarcina*. To analyse the taxonomic rank of isolate E03.2\textsuperscript{T} in more detail, phylogenetic analyses were conducted. To this end, a nearly complete fragment (1409 bp) of the 16S rRNA gene sequence was amplified from genomic DNA by PCR using the primers Arch21F (5’-TCCGGTTGATCCTCGCCG-3’; Delong, 1992) and Univ-1517-a-A-21 (5’-ACGGCTACTTGGTTAGCAGTT-3’; Weissburg et al., 1991). Also, a large fragment (1150 bp) of the mcr\textsubscript{A} gene [encoding the alpha subunit of methyl-coenzyme M reductase (Nölling et al., 1996)] was amplified using a forward primer (5’-GACCTCCAAGTCAAGACCC-3’; Simankova et al., 2003) and a reverse primer (5’-TTGATGTGGTGGTGG-3’; Luot 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 16S rRNA gene sequences (reconstructed using the maximum-likelihood method with MEGA 6.06 software based on a Geneious 7.1.7 Clustal W alignment) confirmed that strain E03.2\textsuperscript{T} belongs to the genus *Methanosarcina* (Fig. 2). The isolate was most closely related to *M. thermophila* TM-1\textsuperscript{T} (98.9 % 16S rRNA gene sequence similarity; GenBank accession no. AB977357). The next closest relatives were *Methanosarcina siciliae* DSM 3028\textsuperscript{T} (98.6 %; FR733698), *Methanosarcina acetivorans* C2A\textsuperscript{T} (98.6 %; NC_003552), *Methanosarcina vacuolata* DSM 1232\textsuperscript{T} (98.5 %; FR733661) and *Methanosarcina barikeri* MST\textsuperscript{T} (98.5 %; AJ012094).
Comparison of the mcrA gene sequence (1096 bp) from isolate E03.2T with its nearest relatives confirmed M. thermophila TM-1T as the closest relative (97.3% mcrA sequence similarity; data not shown).

Because species of the genus Methanosarcina commonly share more than 98.7–99% 16S rRNA gene sequence similarity, which is the recommended threshold to distinguish microbial species, a genome analysis was conducted to address the phylogenetic rank of strain E03.2T. Genomic sequencing has the potential to measure genetic relatedness of prokaryotic species with great precision (Li et al., 2015), thus, the genome of strain E03.2T was sequenced (see Supplementary Material for method). From the draft genome sequence of strain E03.2T (GenBank accession no. LKAZ00000000), consisting of 3,268,085 bp assembled in 74 contigs (Table S1), a G+C content of 41.3 mol% was deduced, which is typical for the genus Methanosarcina (Table 1). The draft genome sequence of strain E03.2T and those of related species (Table 1) were used to perform genome-to-genome distance analyses with the help of the Genome-to-Genome Distance Calculator (GGDC, formula 2) from the Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Auch et al., 2010). The highest similarity value was obtained for M. thermophila TM-1T (51.3%), which confirmed the results based on the 16S rRNA gene sequence comparison but is clearly sufficiently low to allow classification of strain E03.2T as a novel species of the genus Methanosarcina.

When exponentially growing cells of strain E03.2T were exposed to 0.1% SDS (Boone et al., 1993), cells lost their

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**Fig. 1.** Morphology of strain E03.2T. Phase-contrast (a) and fluorescence (b) micrographs showing typical aggregates of cells grown in methanol. Fluorescence of the methanogenic cofactor F420 was assessed with Zeiss filter set 18. (c) Scanning electron micrograph (×5030 magnification) showing small aggregates of coccoid cells assembled into larger aggregates. Bars, 50 μm (a, b); 2 μm (c). A colour version of this figure can be found in the online Supplementary Material.

**Fig. 2.** Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1332 bp) showing the position of strain E03.2T and its closest relatives of the genus Methanosarcina, with Methanotrix concilii GP6T as the outgroup. The tree was reconstructed with bootstrap values based on 1000 replications; only values >50% are shown. GenBank accession numbers are given in parentheses. Bar, 0.02 evolutionary distance.
Table 1. Characteristics differentiating strain E03.2T from related members of the genus *Methanosarcina*

Strains: 1, E03.2T (data from this study); 2, *Methanosarcina thermophila* TM-1T (Zinder et al., 1985) 3, *Methanosarcina siciliae* DSM 3028T (Elberson & Sowers, 1997); 4, *Methanosarcina acetivorans* C2A1T (Sowers et al., 1984); 5, *Methanosarcina vacuolata* DSM 1232T (Zhilina & Zavarzin, 1987); 6, *Methanosarcina barkeri* MST (Maestrojuan & Boone, 1991); 7, *Methanosarcina spelaei* MC-15T (Ganzert et al., 2014); 8, *Methanosarcina horonobensis* HB-1T (Shimizu et al., 2011). All strains are positive for use of methanol as growth substrate but negative for use of formate. +, Positive or good growth; −, negative or no growth; ND, no data available.

<table>
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<td>Generation time (substrate)</td>
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<td>7–10 h (methanol)</td>
<td>5 h (methanol)</td>
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<td>ND</td>
<td>8 h (methanol)</td>
<td>5.1 ± 0.3 h (H₂ + CO₂)</td>
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<td>40 (15–42)</td>
<td>35–40 (15–48)</td>
<td>40 (20–45)</td>
<td>45 (25–50)</td>
<td>33 (0–54)</td>
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<td>6.5–6.8 (5.0–7.8)</td>
<td>6.5–7.0 (5.4–8.5)</td>
<td>7.5 (6.0–8.0)</td>
<td>7.0 (6.5–7.5)</td>
<td>6.6 (4.1–&gt;9.9)</td>
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<td>≤0.05</td>
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<td>&lt;0.2</td>
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<td>42</td>
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<td>41</td>
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<td>39–44</td>
<td>39</td>
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<td>26.2</td>
<td>25.8</td>
<td>ND</td>
<td>22.9</td>
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F_{420} fluorescence rapidly but the aggregates remained mostly intact. No lysis was observed with deionized water within 60 min. While loss of F_{420} fluorescence indicates lysis of the cells, stability of the aggregates is consistent with synthesis of a protective extracellular heteropolysaccharide (Sowers & Gunsalus, 1988).

As strain E03.2^T grew only in stable cell aggregates, it was not possible to monitor optical density as a measure for biomass, i.e. growth. Instead, methane formation and dry mass of cells during exponential growth was quantified as described by Kern et al. (2015) and a linear relationship between these two parameters during exponential growth was established experimentally (data not shown). Growth parameters were determined by averaging results from at least four independent cultures at 45 °C. During growth on H2 plus CO2, cultures were slightly agitated to facilitate mass transfer from the gaseous to the liquid phase. Strain E03.2^T grew with methanol (125 mM) as sole energy source with a generation time and growth rate of 9.68 ± 0.61 h and 0.074 ± 0.005 h^{-1}, respectively, which is typical for cytochrome-containing methanogens (Thauer et al., 2008). Stimulation of methanol-dependent growth was observed when yeast extract (0.1 %) or acetate (40 mM) was added.

Vitamins were dispensable for growth of strain E03.2^T further distinguishing it from *M. thermophila* TM-1^T, which has an obligatory requirement of for p-aminobenzoic acid (Murray & Zinder, 1985). Coenzyme M (0.71 mM), or a mixture of isobutyric acid, 3-methylbutanoic acid, 2-methylbutanoic acid, and valeric acid (each 5 mg ml^{-1}) had no apparent effect on growth.

In addition to methanol, strain E03.2^T grew on mono- (50 mM), di- (50 mM) and trimethylamine (50 mM) as methylotrophic substrates (data not shown). In contrast to the closest relative *M. thermophila* TM-1^T, strain E03.2^T grew with H2 plus CO2 (150 kPa) as the sole carbon and energy source, but did so non-exponentially, due to the limitations of mass transfer from the gaseous to the liquid phase, which is common for hydrogenotrophic methanogens. Notably, cell aggregates during growth on H2 plus CO2 were much smaller than during growth on methanol, probably to facilitate access of cells to the gaseous (and poorly soluble) electron donor; also, cells never appeared pigmented under this condition. Strain E03.2^T also grew with acetate (80 mM) as the energy source with a doubling time of 20.4 ± 2.6 h. Formate (73.5 mM) did not support growth of strain E03.2^T.

The influence of temperature, pH, and NaCl was determined from methane formation during exponential growth on methanol (Fig. S2). The apparent optimal growth temperature for strain E03.2^T was approximately 45 °C, above which growth was impaired rather significantly (Fig. S2a). The optimum temperature for growth of strain E03.2^T is around the operating temperature of the anaerobic digester from which it was isolated (40 °C), but growth and methane formation was still substantial between 50 and 53 °C, which highlights the similarity of strain E03.2^T to *M. thermophila* (Table 1). The effect of NaCl on growth of strain E03.2^T was determined from 6.8 mM (basal NaCl content of BL medium) to 1.0 M (Fig. S2b). With 0.2 M NaCl in the medium, the growth rate was decreased by 50%, and raising the amount of NaCl further led to increased impairment of methane formation, which was completely abolished at 0.8 M NaCl. However, after adaptation to high-salt medium, which contains 0.4 M NaCl (Metcalf et al., 1996), growth of strain E03.2^T was comparable to growth under low-salt conditions. Osmoadaptation of *Methanosarcina* is a well-documented phenomenon (Lai et al., 1991; Müller et al., 2005) and this capacity may help strain E03.2^T to thrive at varying osmolarities caused by variations in substrate composition in the anaerobic digester. The effect of pH on growth of strain E03.2^T was tested from 5.0 to 8.5. The pH of the culture medium was measured before, during and after growth experiments and remained stable. The apparent optimal pH for growth and methanogenesis of isolate E03.2^T was between 7.0 and 7.5, which is characteristic for methanogenic archaea (Whitman et al., 2006). At pH 8.0 (the biogas plant from which strain E03.2^T originated is kept at pH 8.0), strain E03.2^T still grew near-optimally (Fig. S2c). This observed tolerance contrasts that of *Methanobacterium aggregans*, which was isolated from the same digester (Kern et al., 2015). At pH values above 8.5 or below 5.5, no growth of strain E03.2^T could be observed.

Based on phylogenetic, genomic and physiological characteristics, strain E03.2^T represents a novel species of the genus *Methanosarcina*, for which the name *Methanosarcina flavescens* sp. nov. is proposed.

**Description of Methanosarcina flavescens sp. nov.**


Cells are non-motile, irregular cocci with a diameter of 1.0–2.0 μm. Colonies are dark yellow, have a ‘wart-like’ appearance and are of irregular shape with a rough surface. Growth and methane formation occurs autotrophically with H2 plus CO2, methylotrophically with methanol, mono-, di-, and trimethylamine, and with acetate. Yeast extract and vitamins are not required, but stimulate growth. Optimal growth occurs at 45 °C and around pH 7.0.

The type strain is E03.2^T (=DSM 100822^T=JCM 30921^T) and was isolated from a biogas sludge sample in Dresden, Germany. The DNA G+C content of the type strain is 41.3 mol% (genome sequencing).

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