Methanosphaerula palustris gen. nov., sp. nov., a hydrogenotrophic methanogen isolated from a minerotrophic fen peatland

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Peatlands are important sources of CH₄ emissions to the atmosphere and molecular surveys have identified a diverse, but mainly uncultured, euryarchaeal community in them. Characterization of a strain, E1-9c^T, associated with uncultured group E1, from a minerotrophic fen is reported. Cells were regular cocci, usually found in pairs, that stained Gram-positive and were resistant to lysis by 0.1 % SDS. Multiple flagella were observed, but motility was not observed in wet mounts. Optimal growth was obtained at moderate temperatures (28–30 °C) and slightly acidic pH (5.5). Total Na⁺ and NaCl were only tolerated at concentrations less than 100 mM and 0.5 %, respectively, and Na₂S concentrations above 0.1 mM were inhibitory. H₂/CO₂ and formate were the only methanogenic substrates used by E1-9c^T; formate concentrations above 50 mM were inhibitory for growth. Vitamins, coenzyme M and acetate (4 mM) were required for growth and the doubling time was about 19 h. Phylogenetic analysis of the 16S rRNA gene and inferred McrA amino acid sequences showed that E1-9c^T represented an independent lineage within the order *Methanomicrobiales*. Physiological and phylogenetic comparisons with different members of the order supported classification of E1-9c^T in a new genus in the *Methanomicrobiales*. The name *Methanosphaerula palustris* gen. nov., sp. nov. is proposed; strain E1-9c^T (=ATCC BAA-1565^T =DSM 19958^T) is the type strain of *Methanosphaerula palustris*.

Peat-forming wetlands or peatlands (Gore, 1983) store nearly 30 % of all terrestrial carbon (Gorham, 1991) and are estimated to contribute around 20 % (115 Tg) of total annual CH₄ emissions to the atmosphere (Cicerone & Oremland, 1988). Hence, the methanogenic archaea inhabiting these ecosystems are of importance, particularly since the CH₄ concentration in the atmosphere has more than doubled in the past 200 years (Cicerone & Oremland, 1988). Surveys of 16S rRNA gene sequences from acidic and neutral pH peatlands have shown a broad diversity of euryarchaeal sequences (Cadillo-Quiroz *et al.*, 2006, 2008; Galand *et al.*, 2003; Kotsyurbenko *et al.*, 2004). The majority of sequences are not closely related to cultured strains, but instead form independent clusters, suggesting the existence of novel euryarchaeal groups. Two of these clusters, named groups E1 and E2 in the

Methanomicrobiales (Cadillo-Quiroz *et al.*, 2006), have been shown to be highly abundant in clone libraries and by terminal restriction fragment length polymorphism (T-RFLP) analysis of samples from acidic bogs and near-neutral pH fen peatlands (Basiliko *et al.*, 2003; Cadillo-Quiroz *et al.*, 2006, 2008).

Several phylogenetic analyses have shown that E1- and E2-related sequences form independent branches within the *Methanomicrobiales* (Basiliko *et al.*, 2003; Cadillo-Quiroz *et al.*, 2006, 2008; Galand *et al.*, 2003; Hales *et al.*, 1996). The order *Methanomicrobiales* is diverse and its members exhibit great morphological and physiological variability (Dubach & Bachofen, 1985). This order comprises the families *Methanomicrobiaceae*, *Methanocorpusculaceae* and *Methanospirillaceae* (Boone *et al.*, 2001; Garcia *et al.*, 2000). It also includes three genera that are not placed within any family: the genus *Methanocalculus*, which is listed as a *genus incertae sedis* (Boone *et al.*, 2001), with four reported species (Lai *et al.*, 2002, 2004; Mori *et al.*, 2000; Ollivier *et al.*, 1998), the proposed genus '*Candidatus Methanoregula*', with one species, '*Candidatus Methanoregula boonei*', isolated from an acidic bog (Bräuer *et al.*, 2006a, b), and the recently described genus *Methanolinea*, with one species, *Methanolinea tarda*, isolated from sewage sludge (Imachi *et al.*, 2008).

Abbreviations: TEM, transmission electron microscopy; T-RFLP, terminal restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *mcrA* gene sequences of strain E1-9c^T are EU156000 and EU296536, respectively.

Plots showing the effects of sodium ion concentration on growth of strain E1-9c^T and formate utilization by strain E1-9c^T are available as supplementary material with the online version of this paper.

'*Candidatus Methanoregula boonei*' is a member of the E2 group and was isolated using low-ionic-strength media and reducing agents other than Na_2S to avoid inhibition of growth (Bräuer *et al.*, 2006b). Recently, the isolation of a novel methanogen, designated strain E1-9c^T, using the same culture medium coupled to T-RFLP analysis as a screening tool (Cadillo-Quiroz *et al.*, 2008) has been reported. Strain E1-9c^T was isolated from a minerotrophic fen in upstate New York (42° 21' N 76° 28' W) where sequences belonging to the E1 group were diverse and abundant (Cadillo-Quiroz *et al.*, 2008). In our previous paper, conditions that enriched and allowed for isolation of a member of the E1 group, using T-RFLP analysis as a selection and tracking tool, were reported. Culture purity was evaluated by culture techniques, phase-contrast microscopy, fluorescent *in situ* hybridization, T-RFLP analysis and clone library analysis and strain E1-9c^T was proposed as the first member of a novel genus within the *Methanomicrobiales*. In this report, a more detailed evaluation of the morphological, physiological and phylogenetic characteristics of strain E1-9c^T is presented and the novel species *Methanosphaerula palustris* gen. nov., sp. nov., represented by strain E1-9c^T, is described.

PM2 medium, a modified version of PM1 medium (Bräuer *et al.*, 2006b), was used. It contains the following components (mg l⁻¹): 1.5 KCl, 13.6 KH₂PO₄, 26.8 NH₄Cl, 0.024 CoCl₂·6H₂O, 0.075 ZnCl₂, 0.019 H₃BO₃, 0.024 NiCl₂·6H₂O, 0.024 Na₂MoO₄·2H₂O, 1.3 FeCl₂·4H₂O, 0.026 MnSO₄·4H₂O, 1.6 MgSO₄, 2.4 CaCl₂·2H₂O, 0.009 CuSO₄·5H₂O, 3.5 AlK(SO₄)₂·12H₂O and 3.7 disodium EDTA. Medium was prepared under a 70 % N₂/30 % CO₂ atmosphere as described previously (Bräuer *et al.*, 2006b) using the modified anaerobic technique of Hungate (Sowers & Noll, 1995). Several hours before inoculation, sterile anaerobic additions were made to the basal medium with the following final concentrations: 1.0 mM titanium (III) nitrilotriacetate [7.2 ml 1 M Tris/HCl adjusted to pH 8, 4.8 ml 0.5 M sodium nitrilotriacetate and 0.55 ml 15 % titanium (III) chloride (Fluka)]; 10 mM MES (filter-sterilized 1 M stock solution adjusted to pH 7.5); 0.5 mM coenzyme M (2-mercapthoethanesulfonic acid); 0.4 mM sodium acetate; 1 % (v/v) vitamin solution (Balch *et al.*, 1979); and 0.04 mM Na₂S·9H₂O (filter-sterilized 20 mM stock solution). The final liquid volumes in the tubes were ~5 ml and H₂/CO₂ (80 %/20 %; 70.7 kPa) was added to the headspace unless otherwise specified. Cultures were incubated on a gyratory shaker at 30 °C and 200 r.p.m. and all evaluations were done for over 29 days.

Cell morphology and motility were examined by phase-contrast microscopy with a Nikon Eclipse E600 microscope. Gram staining and determination of susceptibility to lysis in the presence of SDS were done as described previously (Boone & Whitman, 1988). SDS tests were performed at final concentrations of 0.1–5.0 % (w/v) and lysis was determined by microscopic observations. Negative-staining, ultrathin-sectioning transmission elec-

tron microscopy (TEM) was performed as described elsewhere (Firtel *et al.*, 1995) using 2 % uranyl acetate (pH 6.5) and a Phillips Technai 12 Biotwin electron microscope equipped with a Gatan Multiscan model 791 camera and Digital Micrograph software. Photos were taken at 100 kV. Flagella observations were done with low uranyl acetate staining (0.2 %) and images were enhanced digitally using the Photoshop software by a differential adjustment of the brightness and contrast of cell body and flagella versus background surface.

When determining the effect of total sodium on growth of strain E1-9c^T, the background sodium content from the different components of the medium (~15 mM) was added to NaCl values. Substrate utilization tests were performed in duplicate under both N₂/CO₂ and H₂/CO₂ atmospheres. The latter condition was used to verify that the substrate was not inhibitory. The following substrates were tested at a final concentration of 10 mM: sodium acetate, trimethylamine, 2-propanol, methanol, ethanol, 2-butanol, sodium propionate, sodium butyrate and sodium formate. Formate utilization tests also included treatments with or without the addition of sodium selenite (5 µM) and tests at pH 6.4. Antibiotic susceptibility tests were performed by adding anaerobic filter-sterilized stocks of penicillin, ampicillin, vancomycin, tetracycline, bacitracin, kanamycin, spectinomycin, rifampicin and chloramphenicol (each at 100 µg ml⁻¹ final concentration). Both sodium and antibiotic sensitivity tests were done under a H₂/CO₂ atmosphere for over a month. Treatments were monitored for CH₄ production with a flame ionization detector as described previously (Cadillo-Quiroz *et al.*, 2006). Growth rates were calculated from methane production from the exponential portion of the methane accumulation curve (Ni & Boone, 1991).

The *mcrA* gene was amplified using the ME1 and ME2 primer set as described previously (Hales *et al.*, 1996). Phylogenetic analyses of 16S rRNA gene sequences was carried out using ARB software (Ludwig *et al.*, 2004) and the latest release (January 2007) of the 'greengenes' database (DeSantis *et al.*, 2006). The 16S rRNA gene sequence alignment was exported from ARB using an 'Archaeal' nucleotide base frequency filter that included positions with more than 50 % invariance (1140 valid columns) to avoid possible treeing artefacts. Phylogenetic trees were constructed by Bayesian analyses. The approximation of posterior probabilities was done with MRBAYES version 3.0 (Ronquist & Huelsenbeck, 2003) using four-chain Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) analysis. Trees and model parameter values were sampled from a target distribution generated when chains converged. Bayesian consensus trees were built with a burnout of 300 and posterior probabilities were calculated. Tree topology was confirmed using maximum-likelihood and neighbour-joining methods with Olsen evolutionary distance correction as implemented in the ARB software (Ludwig *et al.*, 2004). The presented tree represents the most frequently observed topology across

various phylogenetic analyses. For *mcrA* deduced amino acid sequences, sequences were aligned using CLUSTAL_X and analysed using MRBAYES under similar conditions to those described above. For this analysis, 236 aa residues were compared for the McrA tree.

The genomic DNA G+C content of E1-9c^T was calculated by a thermal denaturation fluorimetric method using the Bio-Rad iCycler iQ real-time PCR system as described previously (Gonzalez & Saiz-Jimenez, 2002). The G-nome kit (Qbiogene) was used to isolate genomic DNA from strain E1-9c^T and the following cultures: '*Candidatus* Methanoregula boonei' 6A8 (G+C content 54.5 mol%; S. Bräuer, personal communication), *Escherichia coli* K-12 (50 mol%) and *Pseudomonas aeruginosa* PAO1 (66 mol%). Additionally, DNA from *Micrococcus luteus* (G+C content 72 mol%) and *Clostridium perfringens* (27 mol%) was obtained from commercial sources (Sigma-Aldrich). Thermal denaturation was performed with approximately 2.5 µg DNA from the isolate and standards. Thermal conditions consisted of a ramp from 55 to 90 °C at 0.5 °C min⁻¹. Fluorescent DNA melting curves were

generated in triplicate. The DNA G+C content for strain E1-9c^T was calculated using a linear regression analysis of melting temperatures against the DNA G+C content of the standards.

Cells of E1-9c^T were regular cocci, 0.5–0.8 µm in size and stained Gram-positive. The cells were mostly associated in pairs and planes of division were commonly visible (Fig. 1a). Other cocci in the *Methanomicrobiales*, such as those of strains of the genus *Methanogenium*, tend to be irregular and do not form pairs (Table 1). *Methanofollis aquaemaris* N2F9704^T forms regular cocci when in the exponential phase that change to irregular cocci in the stationary phase (Lai & Chen, 2001). Cultures of E1-9c^T did not show such a change in morphology. Under phase-contrast microscopy, E1-9c^T cells had a dark centre surrounded by a transparent outer layer; cells were autofluorescent when exposed to near-UV light, indicating the presence of F₄₂₀, a fluorescent coenzyme often present in high concentrations in methanogens (Garcia, 1990). The cell envelope was resistant to lysis with 0.1 % SDS although, at concentrations above 2 %, some evidence of envelope damage was observed.

Fig. 1. TEM of cells of strain E1-9c^T. (a) Negative staining showing the regular coccus morphology. Bar, 500 nm. (b) Negative staining with low uranyl acetate addition and digital enhancement (see text) to allow contrast for the observation of multiple flagella (F). Bar, 500 nm. (c–f) Ultrathin sectioning microscopy showing several intracellular details such as S-layer (SL), cytoplasmic membrane (CM), division plane of the cell (DP), tubular-like S-layer-connecting structures (T) and unknown intracellular structures (US). Bars, 500 nm (c, e), 200 nm (d) and 100 nm (f).

Table 1. Physiological characteristics of strain E1-9c^T and other representative coccoid and closely related methanogens belonging to the order *Methanomicrobiales*

Strains: 1, E1-9c^T (data from this study); 2, '*Candidatus* Methanoregula boonei' 6A8 (Bräuer *et al.*, 2006a); 3, *Methanolinea tarda* NOBI-1^T (Imachi *et al.*, 2008); 4, *Methanospirillum hungatei* JF-1^T (Balch *et al.*, 1979; Ferry *et al.*, 1974); 5, *Methanoculleus palmolei* INSLUZ^T (Zellner *et al.*, 1998); 6, *Methanofollis aquaemaris* N2F9704^T (Lai & Chen, 2001); 7, *Methanocorpusculum aggregans* MSt^T (Ollivier *et al.*, 1985); 8, *Methanoplanus petrolearius* SEBR 4847^T (Ollivier *et al.*, 1997); 9, *Methanocorpusculum parvum* XII^T (Zellner *et al.*, 1987); 10, *Methanocalculus halotolerans* SEBR 4845^T (Ollivier *et al.*, 1998). All strains used H₂/CO₂ as methanogenic substrate. ND, No data available.

Character	1	2	3	4	5	6	7	8	9	10
Source	Rich fen	Acidic bog	Sewage sludge	Sewage sludge	Anaerobic digester	Fish pond	Anaerobic digester	Oil well	Anaerobic digester	Oil well
Cell shape*	RC	TR, IC	TR, F	S	IC	RC, IC	IC	IC	IC	IC
Growth pH										
Optimum	5.7	5.1	7	6.6–7.4	6.9–7.5	6.5	6.5–7.0	7.0	6.8–7.5	7.6
Range	4.8–6.4	4.50–5.55	6.7–8.0	ND	6.5–8.0	6.3–8.0	6.0–8.0	5.3–8.2	ND	7.0–8.4
Growth temperature (°C)										
Optimum	30	35–37	50	30–37	40	37	37	37	37	38
Range	14–35	10–40	35–55	ND	22–50	20–43	25–40	28–43	25–44	25–45
NaCl concentration for growth (%)										
Optimum	<0.2	ND	0	ND	ND	0.5	ND	1–3	0–4.7	5
Range	0–0.5	ND	0–1.5	ND	ND	0–6	0–2.5	0–5	ND	0–12.5
Minimum generation time (h)	19	44	144	17	13.5	63	8	10	8	12
DNA G + C content (mol%)	58.9	54.5	56.3	45	59	ND	52	50	49	55
Utilization of:										
Formate	+	–	+	+	+	+	+	+	+	+
2-Propanol	–	–	–	ND	+	–	ND	+	+	–
2-Butanol	–	–	–	ND	+	–	ND	ND	+	–
Acetate requirement for growth	+	+	+	+	ND	–	+	+	+	+

*F, Filaments; IC, irregular cocci; RC, regular cocci; S, spirilla; TR, thin rods.

TEM of negatively stained cells (Fig. 1b) showed that cells of E1-9c^T possessed multiple flagella. Flagella were around 14 nm thick and could reach lengths of 8–12 µm. Flagella were readily lost under stress conditions such as high-speed centrifugation and oxic environments, perhaps explaining the absence of motility in light microscopy observations of wet mounts. An electron-dense cell wall about 30–70 nm thick was observed in thin sections (Fig. 1c–f), and negative staining of the cell surface did not show a pattern that was characteristic of outer S-layers (not shown). Most strains of the *Methanomicrobiales* are sensitive to lysis with dilute detergents because they possess a single S-layer cell wall, and strains with this type of cell wall commonly stain Gram-negative (Boone *et al.*, 1993). Electron microscopy, resistance to SDS lysis and Gram-positive staining observations suggest that the cell wall of E1-9c^T is different from a single S-layer and that this thick layer may play a role in adaptation to low-ionic-strength environments, analogous to the methanochondroitin layer in *Methanosarcina* species (Sprott & Beveridge, 1994). In thin sections, the cytoplasm of some cells apparently shrank away from the outer layer as a result of the osmotic

strength of the 2% glutaraldehyde fixative, and tubular structures were visible in the intervening space (T in Fig. 1c, e, f). It has been suggested that these tubular structures connect the S-layer to the cytoplasmic membrane (Sprott & Beveridge, 1994) and they are identical in appearance to those observed in *Methanosarcina mazei* (Robinson, 1986). Additionally, in many cells, an unknown structure was observed in the cytoplasm (US in Fig. 1c, d, f) resembling arrays of protein complexes, ~10 nm in diameter, that were less electron-dense than ribosomes. These structures do not resemble the 'methanochondrion' internal membrane systems, now believed to be preparation artefacts (Sprott & Beveridge, 1994). The potential protein complexes are similar in size to purified methylcoenzyme M methylreductase complexes (~10 nm) and smaller than F₄₂₀-reducing hydrogenase complexes (~15 nm) (Wackett *et al.*, 1987).

Our previous report (Cadillo-Quiroz *et al.*, 2008) showed that E1-9c^T is a mesophile with optimal growth at 28–30 °C and that it is mildly acidiphilic, with an optimum pH of about 5.5 and a narrow pH range of 4.8–6.4. Only a few

Fig. 2. Phylogenetic analysis of strain E1-9c^T and members of the order *Methanomicrobiales* based on 16S rRNA gene sequences (a) and deduced amino acid sequences from the *mcrA* gene (b). Accession numbers are shown in parentheses. Bar, 0.1 changes per sequence position.

other methanogens have been described with a pH optimum <5.5. '*Candidatus Methanoregula boonei*' 6A8, in the order *Methanomicrobiales*, isolated from an acidic (pH 4.5) bog peatland, is the most acidiphilic methanogen described to date, with optimal pH around 5.1 (Bräuer *et al.*, 2006a). *Methanobacterium espanolae*, isolated from pulp mill waste sludge, represents the next most acidiphilic reported methanogen, with optimum pH around 5.6 (Patel *et al.*, 1990). Strain E1-9c^T has a lower optimum pH than that of the fen from which it was isolated (pH 6.5).

The optimum total Na⁺ concentration for strain E1-9c^T was between 15 (background Na⁺ concentration in medium) and 35 mM; concentrations above 100 mM strongly inhibited growth (Supplementary Fig. S1, available in IJSEM Online). Strain E1-9c^T tolerated <0.5 % NaCl, a value lower than those found for many strains isolated from other non-marine sources (Table 1). The inhibitory concentration of total Na⁺ for E1-9c^T was nearly twice that of '*Candidatus Methanoregula boonei*' (S. Bräuer, personal communication). The difference in Na⁺ tolerance between E1-9c^T and '*Candidatus Methanoregula boonei*' could hypothetically reflect an adaptation to the difference in total Na⁺ concentration observed in the pore water of Michigan Hollow (~30 µM Na⁺) versus McLean Bog (~2 µM Na⁺) (Dettling *et al.*, 2007), the respective sources of each bacterium. E1-9c^T was also highly sensitive to Na₂S

concentrations. E1-9c^T required Na₂S as a sulfur source (Rajagopal & Daniels, 1986), but concentrations above 0.1 mM were inhibitory (Cadillo-Quiroz *et al.*, 2008). Na₂S is commonly used as a reducing agent at concentrations between 1 and 2 mM (Sowers & Noll, 1995).

In terms of methanogenic substrates, E1-9c^T cultures used H₂/CO₂ and formate, but not acetate, trimethylamine, 2-propanol, methanol, ethanol, 2-butanol, propionate or sodium butyrate (all at 10 mM final concentration) as substrates for methanogenesis. With the exception of formate, none of these substrates were inhibitory during growth with H₂/CO₂. Formate additions (10 or 20 mM) caused a 3- to 4-week lag in growth in tubes also supplemented with H₂/CO₂ (Supplementary Fig. S2). Formate is commonly used by members of the order *Methanomicrobiales* (Boone *et al.*, 2001); however, formate can be toxic at concentrations <100 mM at low pH (<6) and, in some strains, formate dehydrogenase activity is dependent on the presence of selenium (Belay *et al.*, 1986; Jarrell & Kalmokoff, 1988; Sparling & Daniels, 1990). Therefore, additional experiments were performed to address the potential formate toxicity at low pH and the selenium requirement. At pH 5.7, strain E1-9c^T was able to use formate at low concentrations, as shown by the stoichiometric increase of methane following multiple additions of 10–20 mM formate (Supplementary Fig. S2).

Formate additions above 50 mM inhibited growth of strain E1-9c^T. Higher pH (6.4) or the addition of selenite (1 and 10 µM) did not improve the use of formate (Supplementary Fig. S2). The addition of 1 µM selenite accentuated the inhibitory effects of formate in tubes with H₂/CO₂ (Supplementary Fig. S2).

In addition to H₂/CO₂ as methanogenic substrate and mineral nutrients, E1-9c^T required vitamins, coenzyme M and acetate for growth. The use of acetate as a carbon source has been observed in many H₂/CO₂-utilizing methanogens (Balch *et al.*, 1979; Belay *et al.*, 1986; Bott *et al.*, 1985; Jarrell & Kalmokoff, 1988). For instance, *Methanospirillum hungatei* requires acetate at levels around 18 mM (Sprott & Jarrell, 1981) and acetate can contribute up to 65 % of total cellular carbon in *Methanothermobacter thermoautotrophicus* (Fuchs *et al.*, 1978). It was found that acetate concentrations around 4 mM were optimal for growth of E1-9c^T under H₂/CO₂ and that concentrations above 20 mM did not further stimulate growth (data not presented). E1-9c^T doubling times were ~30 h with 0.4 mM acetate (concentration used in the isolation of the strain) and ~19 h with 4 mM acetate under standard growth conditions of pH 5.7 and 30 °C.

Strain E1-9c^T was resistant to penicillin, ampicillin, vancomycin, tetracycline, bacitracin, kanamycin, spectinomycin and rifampicin (all at 100 µg ml⁻¹), but chloramphenicol inhibited growth.

The full 16S rRNA gene and intergenic transcribed sequence were determined for strain E1-9c^T and phylogenetic analysis showed that this strain was closely associated with environmental sequence subcluster 'c' of the E1 group (Cadillo-Quiroz *et al.*, 2008). The E1 group is an independent lineage within the order *Methanomicrobiales* (Cadillo-Quiroz *et al.*, 2006, 2008). Therefore, 16S rRNA gene and *mcrA*-based phylogenetic trees were constructed (Fig. 2) with predicted sequences of representative strains of the reported genera in this order. Sequences from the recently described methanogens 'Candidatus Methanoregula boonei' 6A8 (Bräuer *et al.*, 2006a) and *Methanolinea tarda* NOBI-1^T (Imachi *et al.*, 2008; Sakai *et al.*, 2007) were also included. Phylogenetic analysis based on 16S rRNA gene sequences showed that E1-9c^T was divergent from all other described genera within the *Methanomicrobiales* and possessed no more than 85–92 % sequence identity to strains of any other genus. The phylogenetic branching order in Fig. 2(a) indicates that *Methanospirillum*, and hence the family *Methanospirillaceae*, is the most closely related group (90 % identity). However, in terms of sequence identity, the closest relatives of strain E1-9c^T were *Methanolinea tarda* NOBI-1^T (94 % identity) followed by 'Candidatus Methanoregula boonei' 6A8 and *Methanoculleus palmolei* INSLUZ^T (both with 92 % identity). Phylogenetic analysis of amino acid sequences predicted from the *mcrA* gene supported strain E1-9c^T as an independent branch in the *Methanomicrobiales* although, when compared with the 16S

rRNA gene sequence analysis, some differences were observed in the branching order and order of sequence similarity with its closest relatives (Fig. 2). *mcrA* sequence analysis showed the following order in terms of the predicted amino acid sequence identities (%) to E1-9c^T: *Methanoculleus palmolei* INSLUZ^T (83 %) as the closest, followed by *Methanospirillum hungatei* JF-1^T (82 %) and then 'Candidatus Methanoregula boonei' 6A8 and *Methanolinea tarda* NOBI-1^T (both 81 %). In contrast to the 16S rRNA gene analysis, NOBI-1^T was more distantly related to E1-9c^T; however, the branching positions of strains NOBI-1^T, E1-9c^T and 6A8 were highly variable in the multiple analyses of predicted amino acid sequences of the *mcrA* gene, indicating that these sequences did not provide a robust phylogeny for these organisms.

A comparison of phenotypic (Table 1) and phylogenetic (Fig. 2) characteristics of strain E1-9c^T indicates its uniqueness among members of the order *Methanomicrobiales*, allowing us to propose it as the type strain of a novel species in a new genus, *Methanosphaerula palustris* gen. nov., sp. nov. It is proposed that this genus be a *genus incertae sedis*, temporarily not affiliated to any of the known families in this order. Phylogenetic analysis based on 16S rRNA gene sequences of members of the order *Methanomicrobiales* showed that the phylogenetic divergence of E1-9c^T from members of the other families (85–92 % identity) was close to the divergence levels observed among the families themselves (82–89 %), which suggests that E1-9c^T could represent a new family. It is expected that the more detailed genomic analysis of this strain presently under way and the isolation and description of additional related strains will allow a better evaluation of the taxonomy of these novel organisms within the *Methanomicrobiales*.

Description of *Methanosphaerula* gen. nov.

Methanosphaerula [Me.tha.no.sphae.ru'la. N.Gr. n. *methane* (from N. Gr. n. *meth(yl)* and chemical suffix *-ane*) methane; L. fem. n. *sphaerula* a small sphere; N.L. fem. n. *Methanosphaerula* small spherical methane-producer].

Cells are cocci and are often found in pairs. Methanogenic and strictly anaerobic members of the domain *Archaea*. Mesophilic and mildly acidiphilic; produce methane from H₂/CO₂ and formate. The type species is *Methanosphaerula palustris*.

Description of *Methanosphaerula palustris* sp. nov.

Methanosphaerula palustris (pa.lus'tris. L. fem. adj. *palustris* marshy, swampy or muddy, living in marshes).

Cells stain Gram-positive and do not lyse with 0.1 % SDS. Cells are 0.5–0.8 µm in diameter and autofluorescent when exposed to near-UV light. The optimum pH is about 5.5; growth occurs at pH 4.8–6.4. The temperature optimum is about 30 °C with growth occurring at 14–35 °C. Optimal

growth is observed in 15–35 mM total Na^+ , but no growth is observed in the presence of ≥ 100 mM total Na^+ or $\geq 0.5\%$ NaCl. Doubling time is about 19 h under optimal conditions. Methane is produced from H_2/CO_2 or formate, but not from ethanol, methanol, 2-propanol, 2-butanol, acetate, propionate or butyrate. Formate concentrations > 50 mM are inhibitory at pH 5.5. Vitamins, coenzyme M, acetate (4 mM) and low concentrations of Na_2S (< 0.08 mM) are required for growth.

The type strain is E1-9c^T (=ATCC BAA-1565^T =DSM 19958^T), isolated from minerotrophic fen peatland. The DNA G+C content of the type strain is 58.9 ± 2 mol%.

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