Oligosphaera ethanolica gen. nov., sp. nov., an anaerobic, carbohydrate-fermenting bacterium isolated from methanogenic sludge, and description of Oligosphaeria classis nov. in the phylum Lentisphaerae

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A mesophilic, obligately anaerobic, carbohydrate-fermenting bacterium, designated 8KG-4T, was isolated from an upflow anaerobic sludge blanket reactor treating high-strength organic wastewater from salted vegetable production processes. Cells of strain 8KG-4T were non-motile, spherical and 0.7–1.5 μm in diameter (mean, 1.0 μm). Spore formation was not observed under any culture conditions tested. The strain grew optimally at 37 °C (range for growth 25–40 °C) and pH 7.0 (range, pH 6.5–7.5), and could grow fermentatively on glucose, ribose, xylose, galactose and sucrose. The main end products of glucose fermentation were acetate, ethanol and hydrogen. Organic acids, alcohols and amino acids were not utilized for growth. Yeast extract was not required for growth. Nitrate, sulfate, thiosulfate, elemental sulfur, sulfite and Fe(III) nitrilotriacetate were not used as terminal electron acceptors. The G+C content of the genomic DNA was 61.1 mol%. 16S rRNA gene sequence analysis revealed that the isolate represented a previously uncultured lineage at the subphylum level within the phylum Lentisphaerae known as ‘WWE2 subgroup I’. The major cellular fatty acids were anteiso-C15 : 0, iso-C16 : 0, C16 : 0 and anteiso-C17 : 0. Respiratory quinones were not detected. The most abundant polar lipid of strain 8KG-4T was phosphatidylethanolamine. A novel genus and species, Oligosphaera ethanolica gen. nov., sp. nov., is proposed to accommodate strain 8KG-4T (=JCM 17152T = DSM 24202T = CGMCC 1.5160T). In addition, we formally propose Oligosphaeria classis nov. and the subordinate taxa Oligosphaerales order nov. and Oligosphaeraceae fam. nov.

Members of the phylum Lentisphaerae are widely distributed in nature, such as ocean (Giovannoni & Stingl, 2005), faecal samples (Zoetendal et al., 2003; Ley et al., 2008; Yildirim et al., 2010), anaerobic sludge (Riviére et al., 2009; Schlüter et al., 2008), sulfide- and sulfur-rich springs (Elshahed et al., 2007), marine sediments (Polymenakou et al., 2009) and landfill leachate (Limam et al., 2010). Two major lineages within the phylum have been previously identified at the subphylum level (subgroups I and II). Subgroup II contains the orders Lentisphaerales and Victivallales containing the genera Lentisphaera and Victivallis, respectively, each of which contains a single cultured species. Subgroup I, referred to as ‘WWE2 subgroup I’ (Limam et al., 2010), contains a vast number of environmental rRNA gene sequences, the majority of which were retrieved from anoxic ecosystems such as anaerobic digester sludge (Riviére et al., 2009). However, no cultured strains representing the subgroup I have been described so far. Here, we describe a novel strain isolated from methanogenic wastewater treatment sludge and
propose a novel species belonging to WWE2 subgroup I within the phylum *Lentisphaerae*.

Strain 8KG-4T was originally obtained from the granular sludge of a mesophilic (35 °C) full-scale upflow anaerobic sludge blanket reactor treating a high-strength organic wastewater from salted vegetable production processes (Narihiro et al., 2009). The medium used for isolation and cultivation was prepared as described previously (Sekiguchi et al., 2000). Gently washed and homogenized sludge was serially diluted 10-fold in anaerobic liquid medium supplemented with low concentrations of glucose (0.1–1.0 mM) with pH 7.5. Growth of cells was observed in the 10⁻⁴ dilution tube after 2 months of incubation at 37 °C. Cells in the highest dilution were further purified by repeated serial dilution in glucose medium and then by the roll tube method (Hungate, 1969) with glucose agar (2%; Agar noble; Difco). Small and slow-growing light brown, lens-shaped colonies were formed after 3 weeks of incubation at 37 °C. The colonies were picked and transferred to liquid medium supplemented with 1 mM glucose and cells were observed after 2 weeks of anaerobic incubation at 37 °C, indicating the organism was a slow-growing bacterium compared to general heterotrophic anaerobes. This roll tube isolation step (transferring single colonies from solid medium to liquid medium) was repeated several times and a purified strain, designated strain 8KG-4T, was obtained. The purity of the culture was checked by microscopic observation.

Cell morphology was examined under a fluorescent microscope (BX50F; Olympus). Transmission electron microscopy was performed with a Hitachi H-7000 transmission electron microscope as described previously (Sekiguchi et al., 2003). The Gram-staining reaction was performed by the method of Hucker (Doetsch, 1981). Cells were non-motile, spherical and 0.7–1.5 μm in diameter (Fig. 1a). Motility was examined microscopically in various growth phases. Spore formation was not observed under any culture conditions (for example, aeration or pasteurization of the inoculum). Cells were Gram-stain-negative. Electron microscopy demonstrated that strain 8KG-4T possessed a Gram-negative cell wall (Fig. 1b).

The physiological characteristics of strain 8KG-4T were examined as described previously (Sekiguchi et al., 2000). Growth was determined at 20–55 °C (at intervals of 5 °C), at pH 5.0–8.5 (at intervals of 0.5 pH unit; 37 °C) and with 0–3.0 % (w/v) NaCl (at intervals of 0.5 %). The growth of cells was evaluated based on the increase in optical density (OD) at 400 nm and the production of hydrogen. Unless otherwise indicated, the organism was cultured anaerobically (N₂/CO₂; 80 : 20, v/v) at 37 °C without shaking. Aerobic growth was tested in a medium containing 2 mM glucose under aerobic conditions without reducing agents. Strain 8KG-4T grew anaerobically on 2 mM glucose medium after 8 weeks of incubation at 25–40 °C (optimum, 37 °C), at pH 6.5–7.5 (optimum, approximately pH 7.0) and with 0–1.0 % (w/v) NaCl, but did not grow at temperatures below 25 °C or above 40 °C. The isolate was a strictly anaerobic organism: it could not grow in the presence of oxygen (20 %, v/v, in the gas phase) nor after N₂/CO₂ purging only (without reducing agents). Growth and hydrogen production were observed with the following substrates (5 mM each): glucose, ribose, xylose, galactose and sucrose. Yeast extract was not required for or did not stimulate growth. None of the following substrates supported the growth of strain 8KG-4T in pure culture or co-culture with *Methanospirillum hungatei* DSM 864T (1–5 mM unless otherwise specified): yeast extract (0.1%), tryptone (0.1%), pyruvate, crotonate, lactate, H₂/CO₂ (1 atm., 80/20, v/v) plus acetate, formate, propionate, butyrate, iso-butyrate, benzoate, hydroquinone, phenol, malate, fumarate, succinate, glycerol, methanol, ethanol, 1-butanol, 1-propanol, maltose, arabinose, fructose, man- nose, raffinose, starch (1 g l⁻¹), pectin, citrate, Casamino acids, L-glutamate, serine, cysteine, threonine, glycine, 2-oxoglutarate, alanine, leucine, isoleucine, valine, aspartate, proline, methionine, phenylalanine, histidine, asparagine, glutamine, arginine and lysine. In medium supplemented...
with glucose, the major end products were acetate, hydrogen and ethanol (1 mol glucose was converted to approximately 0.7 mol acetate, 1.3 mol ethanol and 2.2 mol hydrogen; electron recovery 103 %) (Fig. 2). The effect of glucose concentration on the growth of strain 8KG-4T in pure culture and co-culture with M. hungatei were evaluated on 2, 4, 5, 6, 8, 10, 15 and 20 mM glucose media in duplicate. In this experiment, the growth rate of cells was evaluated based on the increase in OD at 400 nm and the production of hydrogen (or methane for the co-cultures). The growth of pure culture on low concentrations of glucose was more stable than that on high concentrations of glucose, i.e. glucose concentrations above 10 mM completely inhibited the growth of the strain in pure culture, but not co-culture with the hydrogenotrophic methanogen M. hungatei. Pure cultures with >6 mM glucose showed relatively long lag times for growth and only one pure culture in duplicate cultures exhibited this growth. Low concentrations of glucose (<5 mM) supported the growth of both duplicate cultures. However, the growth rate of pure culture on low glucose media (<5 mM) was 0.5-times lower than that of co-culture on the same media. Strain 8KG-4T did not use any of the following electron acceptors within 4 weeks of incubation with glucose (mM): nitrate (5), sulfate (5), thiosulfate (2), elemental sulfur (5), Fe(III) nitritotriacetate (2) and sulfite (1).

For DNA G+C content determination, DNA was extracted and purified according to Kamagata & Mikami (1991). The G+C content was determined by HPLC (Shimadzu LC-6A) with a UV detector (Shintani et al., 2000). The DNA G+C content of strain 8KG-4T was 61.1 mol% (standard deviation 0.04 mol%). For quinone and fatty acid analyses, cells were harvested at exponential phase from cultures with glucose. The major cellular fatty acids were anteiso-C_{15:0} (22.0 %), iso-C_{16:0} (24.5 %), C_{16:0} (13.7 %) and anteiso-C_{17:0} (15.7 %). Quinone analysis was conducted according to Zhang et al. (2000) and no respiratory quinones were detected. Polar lipids were extracted and analysed by the methods of Tindall (1990) using two-dimensional TLC (silica gel 60 F254 plates, layer thickness 0.2 mm, no. 5554; Merck). The most abundant polar lipid of strain 8KG-4T was phosphatidylethanolamine. Several unknown phospholipids and aminophospholipids were also detected (Fig. S1, available in IJSEM Online).

For 16S rRNA gene sequencing, the genomic DNA was extracted according to the method of Hiraishi (1992). The 16S rRNA gene was amplified by PCR with Taq polymerase (Perkin Elmer) as described previously (Sekiguchi et al., 2000). The PCR primers used in the amplification were the bacterial domain universal primer 8F and the prokaryote universal primer 1490R (Weisburg et al., 1991). PCR products were sequenced directly on a Beckman CEQ-8000 DNA sequencer using a CEQ DTC Quick Start kit (Beckman Coulter). Sequence data were aligned using the ARB software package (Ludwig et al., 2004) and aligned data were corrected manually using the editing tool in the package. A phylogenetic tree based on 16S rRNA gene sequences was constructed by the neighbour-joining method (Saitou & Nei, 1987) in the ARB software package (Ludwig et al., 2004). Bootstrap resampling analysis (Felsenstein, 1985) for 1000 replicates was performed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods to estimate the confidence of tree topologies as described by Zhang et al. (2003) with slight modifications: i.e. the neighbour-joining and the maximum-parsimony trees were inferred using the PAUP* 4.0 package (Swofford, 2002), and the maximum-likelihood trees were made using the TREEFINDER program package (Jobb et al., 2004). A total of 1434 base pairs of the 16S rRNA gene from strain 8KG-4T was obtained. Phylogenetic analysis revealed that strain 8KG-4T was affiliated with WWE2 subgroup I of the phylum Lentisphaerae (Fig. 3). The most closely related microorganisms that have been cultured and characterized to date were Victivallis vadensis Cello™ (79.7 % 16S rRNA gene sequence similarity), an anaerobic bacterium isolated from a human faecal sample (Zoetendal et al., 2003), and Lentisphaera araneosa HTCC2155™ (79.1 % 16S rRNA gene sequence similarity), an aerobic, halophilic bacterium isolated from surface waters off the Oregon coast using dilution-to-extinction culture (Cho et al., 2004). To date, L. araneosa and V. vadensis are the only cultured species described for the phylum Lentisphaerae, and these species belong to another lineage, WWE2 subgroup II, which indicates the phylogenetic novelty of strain 8KG-4T at the class level.

Comparative phenotypic traits of V. vadensis Cello™, L. araneosa HTCC2155™ and strain 8KG-4T are shown in Table 1. These organisms share several common features such as spherical morphology and growth with carbohydrates. Strain 8KG-4T was similar to V. vadensis Cello™ in terms of fermenting glucose to ethanol. The major

Fig. 2. Degradation of glucose by pure culture of strain 8KG-4T.
physiological differences between strain 8KG-4T and \textit{V. vadensis} Cello\textsuperscript{T} were that strain 8KG-4T utilized a limited range of carbohydrates for growth (i.e., 5 mM each, glucose, ribose, xylose, galactose and sucrose), whereas \textit{V. vadensis} Cello\textsuperscript{T} utilization a wider variety (e.g., 10 mM each, cellobiose, fructose, galactose, glucose, lactose, lactulose, maltose, maltotriose, ribose, sucrose, xylose), and that strain 8KG-4T only grew on low concentrations of glucose (536), maltose, maltotriose, ribose, sucrose, xylose), and that cellobiose, fructose, galactose, glucose, lactose, lactulose, \textit{CelloT} were that strain 8KG-4T utilized a limited range of carbohydrates for growth (i.e., 5 mM each, glucose, ribose, xylose, galactose and sucrose), whereas \textit{CelloT} utilized a wider variety (e.g., 10 mM each, cellobiose, fructose, galactose, glucose, lactose, lactulose, maltose, maltotriose, ribose, sucrose, xylose), and that strain 8KG-4T only grew on low concentrations of glucose (10 mM), while \textit{V. vadensis} Cello\textsuperscript{T} was able to grow on high concentrations of glucose (10 mM).

Phylogenotypes within WWE2 subgroup I have been detected in abundance in anaerobic ecosystems such as anaerobic sludge digesters (Chouari et al., 2005; Riviere et al., 2009) and landfill leachate (Limam et al., 2010). Strain 8KG-4T was isolated from a methanogenic sludge, strongly suggesting that WWE2 subgroup I organisms greatly contribute to decomposition of carbohydrates in anaerobic food chains. The major reasons why WWE2 subgroup I organisms have not been previously isolated are speculated as follows: (i) the organisms are only capable of utilizing a very limited range of carbohydrates; (ii) they may preferably grow in environments with low levels of carbohydrates; and (iii) during enrichment of carbohydrate-utilizing organisms directly from environmental samples, many fast-growing organisms would outgrow relatively slow-growing organisms, hindering isolation of such organisms including our isolate. If these traits are taken into consideration, we could cultivate those previously uncultured organisms that are otherwise obscured by readily cultivable organisms.

On the basis of phylogenetic, genetic and physiological properties, it is evident that strain 8KG-4T represents a novel species of a novel genus of the phylum \textit{Lentisphaerae}. Therefore, the name \textit{Oligosphaera ethanolica} gen. nov., sp. nov. is proposed for strain 8KG-4T. The isolate is a member of WWE2 subgroup I within the phylum \textit{Lentisphaerae}, which forms a distinct lineage in the phylum (21% sequence divergence with other lineages). Members of this cluster are almost exclusively found in anaerobic habitats, ranging from anaerobic digester sludge and landfill leachate to the mammalian intestinal tract. On the basis of the clear monophyly of this cluster, we propose the class \textit{Oligosphaeracea} for all members of subgroup I. In addition, we propose the subordinate taxa \textit{Oligosphaerales} ord. nov. and \textit{Oligosphaeraceae} fam. nov.

**Description of \textit{Oligosphaera} gen. nov.**

\textit{Oligosphaera} (Oli.go.spha'e.ra. Gr. adj. oligos little, not copious, scanty; L. fem. n. sphaera sphere; N.L. fem. n. Oligosphaera a spherical bacterium with limited nutritional properties).

Strictly anaerobic, non-spore-forming, non-motile, mesophilic, spherical cells. Able to ferment some sugars, but not fatty acids, alcohols or amino acids. The main end products...
from glucose fermentation are acetate, ethanol and hydrogen. Oxygen, nitrate, sulfate, sulfite, thiosulfate, elemental sulfur and Fe(III) nitrolotriacetate do not serve as electron acceptors for growth. The main fatty acids are anteiso-C_{15}:0, iso-C_{ 16}:0, C_{16}:0 and anteiso-C_{17}:0. Respiratory quinones are not detected. The most abundant polar lipid is phosphatidylethanolamine. The DNA G+C content of the type strain of the type species is 61.1 mol%. The type species is *Oligosphaera ethanolica*.

**Description of Oligosphaera ethanolica sp. nov.**

*Oligosphaera ethanolica* (e.tha.no’li.ca. N.L. n. ethanol ethanol; L. fem. suff. -ica suffix used with the sense of pertaining to; N.L. fem. adj. *ethanolica* belonging to ethanol, referring to ethanol, which is produced by the species).

Shows the following characteristics in addition to those given for the genus. Cells are 0.7–1.5 μm in diameter (mean 1.0 μm). Colonies on agar are light brown and circular after cultivation at 37 °C for 2–3 weeks. Grows at 25–40 °C (optimum, 37 °C), at pH 6.5–7.5 (optimum, approximately pH 7.0) and with 0–1.0% (w/v) NaCl. Yeast extract is not required for growth. Utilizes glucose, ribose, xylose, galactose and sucrose. Organic acids, alcohols and amino acids are not oxidized.

**Description of Oligosphaera ethanolica gen. nov., sp. nov.**

The type strain is 8KG-4^T (=JCM 17152^T =DSM 24202^T =CGMCC 1.5160^T), isolated from a mesophilic full-scale upflow anaerobic sludge blanket reactor treating wastewater from salted vegetable production processes. The DNA G+C content of the type strain is 61.1 mol%.

**Description of Oligosphaeraceae fam. nov.**

Oligosphaeraceae (O.li.go.spha.e.ra.e. N.L. fem. n. *Oligosphaera* type genus of the family; suff. -aceae ending to denote a family; N.L. fem. pl. n. *Oligosphaeraceae* family of the genus *Oligosphaera*).

The family is defined by phylogenetic analysis based on 16S rRNA gene sequences obtained from one cultured strain and a wide range of uncultivated bacteria retrieved mainly from mesophilic anaerobic digester sludge and landfill leachate. Gram-negative. Spores are not formed. Grows under strictly anaerobic conditions. Chemo-organotrophs. The type genus is *Oligosphaera*.

**Description of Oligosphaerales ord. nov.**

Oligosphaerales (O.li.go.spha.e.ri.ales. N.L. fem. n. *Oligosphaera* type genus of the order; suff. -ales ending to denote an
order; N.L. fem. pl. n. Oligosphaerales order of the genus Oligosphaera).

The description is the same as for the family Oligosphaeraceae. The order contains the family Oligosphaeraceae. The type genus is Oligosphaera.

Description of Oligosphaeria classis nov.

Oligosphaeria (O. li.go.spha.e’ria) N.L. fem. n. Oligosphaera type genus of the type order of the class; suff. -ia ending to denote a class; N.L. pl. neut. n. Oligosphaeria class of the order Oligosphaerales).

The description is the same as for the family Oligosphaeraceae. The class contains the order Oligosphaerales and the family Oligosphaeraceae. The type order is Oligosphaerales.

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