

Anaeromicrobium sediminis gen. nov., sp. nov., a fermentative bacterium isolated from deep-sea sediment

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Abstract

A novel anaerobic, mesophilic, heterotrophic bacterium, designated strain DY2726D^T, was isolated from West Pacific Ocean sediments. Cells were long rods (0.5–0.8 µm wide, 4–15 µm long), Gram-positive and motile by means of flagella. The temperature and pH ranges for growth were 25–40 °C and pH 6.5–9.0, while optimal growth occurred at 37 °C and pH 7.5, with a generation time of 76 min. The strain required sea salts for growth at concentrations from 10 to 30 g l⁻¹ (optimum at 20 g l⁻¹). Substrates used as carbon sources were yeast extract, tryptone, glucose, cellobiose, starch, gelatin, dextrin, fructose, fucose, galactose, galacturonic acid, gentiobiose, glucosaminic acid, mannose, melibiose, palatinose and rhamnose. Products of fermentation were carbon dioxide, acetic acid and butyric acid. Strain DY2726D^T was able to reduce amorphous iron hydroxide, goethite, amorphous iron oxides, anthraquinone-2,6-disulfonate and crotonate, but did not reduce sulfur, sulfate, thiosulfate, sulfite or nitrate. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain DY2726D^T was affiliated to the family *Clostridiaceae* and was most closely related to the type strains of *Alkaliphilus transvaalensis* (90.0% similarity) and *Alkaliphilus oremlandii* (89.6%). The genomic DNA G+C content was 33.4 mol%. The major cellular fatty acids of strain DY2726D^T were C_{16:1}, C_{14:0} and C_{16:0}. On the basis of its phenotypic and genotypic properties, strain DY2726D^T is suggested to represent a novel species of a new genus in the family *Clostridiaceae*, for which the name *Anaeromicrobium sediminis* gen. nov., sp. nov. is proposed. The type strain of *Anaeromicrobium sediminis* is DY2726D^T (=JCM 30224^T=MCCC 1A00776^T).

The deep-sea environment represents the largest continuous ecosystem on our planet. Studies of microbial diversity based on cultivation-dependent methods conducted on deep-sea sediments sample have suggested that *Gammaproteobacteria*, *Firmicutes* and *Actinobacteria* are the most frequently identified groups [1–4]. Anaerobic bacteria in such sediments can rapidly hydrolyse organic matter [5].

The family *Clostridiaceae* was the first of the 19 families within the order *Clostridiales* to be described [6], and it contains a large proportion of anaerobic fermentative micro-organisms, including the genera *Clostridium*, *Alkaliphilus* and other related genera [7, 8]. Some species of this family have been frequently isolated from marine anaerobic environments, including *Clostridium bryantii* from sea sediments [9], *Clostridium lortetii* from Dead Sea sediments [10], *Clostridium oceanicum*

from marine sediments collected off the Atlantic and Pacific coasts of tropical South America [11], *Clostridium halophilum* DSM 5387^T from marine hypersaline sediments [12], *Caloranaerobacter azorensis* MV1087^T from hydrothermal sulfide samples [13], *Caminicella sporogenes* AM1114^T and *Clostridium caminithermale* DVird3^T from deep-sea hydrothermal vent chimney sample [8, 14] and *Wukongibacter baidiensis* DY30321^T from a sample of mixed hydrothermal sulfides [15]. In this study, an anaerobic fermentative bacterial strain from deep-sea sediment sample was enriched and isolated. Strain DY2726D^T is proposed as a representative of a novel species of the new genus *Anaeromicrobium* within the family *Clostridiaceae*.

Strain DY2726D^T was isolated from a deep-sea sediment sample collected at a depth of 5445 m in the west Pacific Ocean

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Abbreviation: AQDS, anthraquinone-2,6-disulfonate.

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The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain DY2726D is KJ137882.

One supplementary figure is available with the online Supplementary Material.

(154.1° E 16.0° N) in July 2012, during the cruise DY125–27 of R/V *Hai Yang Liu Hao*. The sample was collected using a deep-sea multi-corer. One subsample was immediately used to inoculate (at 1 : 10, w/v) a sterile liquid medium referenced as FRPFO on board, prepared anaerobically and kept under an atmosphere of highly purified 100 % nitrogen. The FRPFO medium contained (concentrations in g l⁻¹, unless stated otherwise): peptone, 10; sea salts (Sigma), 30; PIPES, 6.05; cysteine-HCl, 0.5; resazurin, 1 mg; and amorphous Fe(III) oxyhydroxide (pH 7.0, 50 mM) as an electron acceptor. Enrichment cultures were performed at 25 °C without shaking. One strain, designated DY2726D^T, was not able to form colonies in solidified medium with 1.5 % agar or 0.2 % gelrite, and was purified by three repeated dilution-to-extinction series [16]. This strain was isolated and its purity was confirmed by microscopic observations and by cloning and sequencing of ten 16S rRNA clone genes. It was referenced as strain DY2726D^T.

Morphological characteristics of the cells were determined by light microscopy (CX21; Olympus) and transmission electron microscopy (JEM-1230; JEOL). Cells of strain DY2726D^T were regular long rods (0.5–0.8 μm in width, 4–15 μm in length), motile by means of flagella (Fig. S1a, available in the online Supplementary Material). Cells appeared mainly singly. Spores were never observed, even in the late stationary phase of growth or after heat stimulation (80 °C, 10 min). Cells stained Gram-negative using the Gram stain kit (Beijing Solarbio Microorganism Reagent Co.; Guangdong Huankai microbial Sci and Tech Co.) with control strains under aerobic and anaerobic conditions. Although the KOH reaction was negative, cells were identified as Gram-positive type [17]. Transmission electron micrographs of thin sections showed that cells of strain DY2726D^T had a thin peptidoglycan cell wall (Fig. S1b). However, the whole genome sequence showed that strain DY2726D^T lacked some characteristics of Gram-negative bacteria, such as the lipid A biosynthesis pathway and Type I, II, III, IV and V secretion systems. We identified strain DY2726D^T as a Gram-positive bacteria. The Gram stain reaction may have incorrectly grouped some strains of clostridia, eubacteria and bifidobacteria as staining Gram-negative or Gram-variable [18]. For these species, additional methods and genome analysis are necessary to make an identification.

Physiological characterization of the novel isolate was carried out in FRPFO medium dispensed anaerobically in 50 ml vials sealed with butyl-rubber stoppers, and reduced with 0.05 % (w/v) sterile cysteine-HCl solution just before inoculation. Unless otherwise stated, methods were carried out anaerobically under an atmosphere of N₂ (100 %, 1 bar), and incubation was performed in the dark at 37 °C and pH 7.0. Growth was routinely monitored by direct cell counting using a modified Thoma chamber (depth 10 μm). Growth rates were calculated using linear regression analysis of eight points along the linear portions of the growth curves and logarithmically transformed [14]. Determination of the temperature range for growth was tested over the range 4–50 °C (4, 15, 20, 25, 28, 30, 35, 37, 40, 45 and 50 °C). Growth was observed from 25 to

40 °C, with maximum growth rate at 35–37 °C. The pH range for growth was tested from an initial pH 4.0 to pH 10.0, at 37 °C, in basal medium buffered and adjusted to the required pH (initial pH at 20 °C) with MES (pH 4.0–6.0), PIPES (pH 7.0–8.0), HEPES (pH 8.0–9.0) or AMPSO (pH 8.0–9.0), and Gly-NaOH (pH 9.0–10.0). Growth was observed from pH 6.5 to 9.0 and the optimum pH for growth was 7.5. Salt tolerance was tested at 37 °C in FRPFO medium prepared with various concentrations of NaCl (0–100 g l⁻¹, 5 g l⁻¹ intervals) and various concentrations of sea salts (Sigma; 0–100 g l⁻¹, 5 g l⁻¹ intervals). Strain DY2726D^T required sea salts and grew at concentrations ranging from 10 to 30 g l⁻¹, with an optimum at 20 g l⁻¹. Under optimal growth conditions, the shortest generation time was 76 min. Compared with its closest relatives, the temperature range for growth of strain DY2726D^T was similar to those for the genus *Alkaliphilus*, but its pH range for growth was neutral (Table 1).

Strain DY2726D^T was a chemo-organoheterotrophic bacterium, utilizing complex organic compounds including peptone, tryptone and yeast extract. The ability of the isolate to use single carbon sources for growth was tested in triplicate in Hungate culture tubes and Biolog AN microplates in anaerobic jars under optimal growth conditions. The following substrates were utilized for growth of strain DY2726D^T: starch, gelatin, cellobiose, dextrin, D-fructose, L-fucose, D-galactose, D-galacturonic acid, D-glucosaminic acid, gentiobiose, D-glucose, D-glucose 6-phosphate, D-mannose, melibiose, 3-methyl D-glucose, palatinose, L-rhamnose, α-ketobutyric acid and α-ketovaleric acid. However, lactate, malate, maltose, sucrose, tartrate, trehalose, acetic acid, formic acid, fumaric acid, glyoxylic acid, malic acid, succinic acid and alaninamide were not utilized. The strain was unable to grow on amino acid pairs (alanine and glycine; alanine and proline) via the Stickland reaction [19]. The major fermentation products of glucose, determined by GC (QP2010; Shimadzu), were carbon dioxide, acetic acid and butyric acid.

The ability of the novel isolate to use electron acceptors was tested by adding elemental sulfur (12 g l⁻¹), sulfate (20 mM), sulfite (1 mM), thiosulfate (20 mM), nitrate (10 mM), anthraquinone-2,6-disulphonate (AQDS, 2 mM), Fe(III) oxyhydroxide (pH 7.0, 50 mM), amorphous iron(III) oxide (pH 9.0, 50 mM), goethite (α-FeOOH, pH 12.0, 50 mM), Fe(III) citrate (20 mM), Fe(III) chlorite (20 mM), EDTA-Fe(III) (20 mM), fumarate (10 mM), crotonate (10 mM) or oxygen (0.05–0.5 %, v/v) to the medium. The respiratory activity was determined by measuring the turbidity of the cultures and the formation of reduced products of the electron acceptors (sulfide and ferrous iron) [20, 21]. The micro-organism's ability to reduce nitrate was determined by the phenol disulfonic acid method [22]. The disappearance of crotonate and its products was measured by HPLC [23]. When AQDS becomes reduced, the colour of AQDS changes from clear to orange [24]. Strain DY2726D^T was found to be strictly anaerobic. It could grow only by fermentation, and facultatively reduced AQDS, crotonate and insoluble forms of Fe(III), including Fe(III) oxyhydroxide

Table 1. Characteristics between strain DY2726D^T and its phylogenetically closest relatives

Strains: 1, DY2726D^T; 2, *Alkaliphilus transvaalensis* SAGM^T (data from [7]); 3, *Clostridium halophilum* DSM 5387^T (data from [12]); 4, *Clostridium camini-thermale* DVird3^T (data from [8]); 5, *Caminicella sporogenes* AM1114^T (data from [14]). Peptone utilization was positive for all strains. +, Positive; –, negative; ND, no data; w, weak growth.

Characteristic	1	2	3	4	5
Cell size (µm)	0.5–0.8×4.0–15.0	0.4–0.7×3.0–6.0	0.8–1.0×2.5–7.0	0.4–0.5×5.0–9.0	0.5–0.7×3.0–10.0
Temperature (°C) (optimum)	20–40 (37)	20–50 (40)	18–49 (41)	25–58 (45)	45–65 (55–65)
NaCl (g l ⁻¹) (optimum)	10–30 (20)	0–33 (5)	20–130 (80)	15–60 (30)	20–60 (25–30)
pH (optimum)	6.5–9.0 (7.5)	8.5–12.5 (10)	6.0–8.0 (7.4)	5.8–8.2 (6.6)	4.5–8.0 (7.5–8.0)
16S rRNA gene sequence similarity (%) to DY2726D ^T	100	90.0	89.2	88.3	87.9
Substrate utilization					
Cellobiose	+	–	+	–	–
Fructose	+	–	+	+	–
Galactose	+	–	ND	+	w
Products of glucose fermentation	CO ₂ , acetic acid, butyric acid	ND	Ethanol, lactate	Propionate	Acetate, butyric acid, ethanol, H ₂ , CO ₂
Electron acceptors					
S ⁰	–	+	ND	–	+
Thiosulfate	–	+	–	–	+
Stickland reaction	–	ND	+	+	–
DNA G+C content (mol%)	33.4	36.4	26.9	33.1	23.2–25.2
Isolation source	Deep-sea sediments (West Pacific Ocean)	Deep alkaline waters of a gold mine (South Africa)	Anoxic hypersaline sediments	Deep-sea hydrothermal vent sample (Atlantic Ocean Ridge)	Deep-sea hydrothermal vent sample (East-Pacific Rise)

(pH 7.0), amorphous iron(III) oxide (pH 9.0) and goethite (α-FeOOH; pH 12.0). The novel isolate was unable to reduce soluble forms of Fe(III) such as Fe(III) citrate, Fe(III) chlorite and EDTA-Fe(III). Sulfur compounds such as elemental sulfur (S⁰), sulfate, sulfite, thiosulfate and nitrate were not utilized as electron acceptors (Table 1).

Determination of the whole-cell fatty acid composition was performed on cultures grown at 37 °C on YTG medium [1 g yeast extract, 1 g peptone, 2.5 g glucose, 30 g artificial sea salts, 6.05 g PIPES, 0.5 ml Wolf's vitamin solution (per litre distilled water: biotin 2 mg, folic acid 2 mg, pyridoxine-HCl 10 mg, thiamine-HCl.2H₂O 5 mg, riboflavin 5 mg, nicotinic acid 5 mg, D-Ca-pantothenate 5 mg, vitamin B12 0.10 mg, *p*-aminobenzoic acid 5 mg, lipoic acid 5 mg), 5 ml trace elements solution [25], 0.5 g cystein-HCl, 1 mg resazurin]. Cells were harvested at the end of the exponential growth phase (36 h of incubation). Fatty acids were extracted and analysed following the instructions of the Microbial Identification System operating manual [26]. The predominant fatty acids in strain DY2726D^T comprised C_{16:1} (42.0%), C_{14:0} (31.9%) and C_{16:0} (12.8%), significantly different from those of the closest related strain, *Alkaliphilus transvaalensis* SAGM1^T (51.6% iso-C_{15:0}, 12.2% iso-C_{17:0}, 9.2% iso-C_{15:1ω7c}, 7.2% iso-C_{17:1ω7c}) [7]. The quinones of strain DY2726D^T were extracted with the method described by Minnikin *et al.* [27]. No respiratory quinones were detected.

The G+C content of the genomic DNA of strain DY2726D^T was determined by HPLC, as described by Mesbah *et al.* [28]. The G+C content of strain DY2726D^T was 33.4 mol%. An almost-complete 16S rRNA gene sequence (1431 nt) was determined, using five primer pairs, and deposited in the NCBI database (accession no. KJ137882). The identification of phylogenetic neighbours was initially carried out using BLAST [29] and MEGA BLAST [30] against the database of type strains with validly published prokaryotic names [31]. A search of most similar 16S rRNA gene sequences was also performed with the web-based EzTaxon-e server [32, 33]. Alignment of all sequences was performed using the software CLUSTAL X (Version 2.3) [34] and phylogenetic trees were reconstructed using the neighbour-joining method with the software MEGA (Version 5.1) [35]. Bootstrap analysis was performed with 1000 replications to provide confidence estimates for tree topologies. The 16S rRNA gene sequence revealed that strain DY2726D^T was closely related to species in the family *Clostridiaceae* in the class *Clostridia*, with similarity values below 91.0%. The most closely related strain was *Alkaliphilus transvaalensis* SAGM1^T, with 90.0% 16S rRNA gene sequence similarity, followed by *Alkaliphilus oremlandii* OhILAs^T (89.6%), *Anaerobaculum carboniphilum* IRF19^T (89.6%), *Geosporobacter ferrereducens* IRF9^T (89.5%) and *Clostridium halophilum* DSM 5387^T (89.4%).

A phylogenetic tree of representative members in the class *Clostridia* was reconstructed from 16S rRNA gene sequences using 1301 homologous gene sequence positions (Fig. 1). In Fig. 1, compared with the *Alkaliphilus* lineage, strain DY2726D^T and related species (including *Clostridium halophilum* DSM 5387^T, *Clostridium caminithermale* DVird3^T and *Caminicella sporogenes* AM1114^T) were distributed in a separate lineage.

In conclusion, on the basis of the significant phylogenetic distance with its closest relatives (far below the threshold level of 94.5 % for the delineation of a new genus) [36], and with clear phenotypic differences with the closest neighbours (Table 1), we propose to identify strain DY2726D^T as the type strain of a novel species within a new genus, for which the name *Anaeromicrobium sediminis* gen. nov., sp. nov. is proposed.

DESCRIPTION OF ANAEROMICROBIUM GEN. NOV.

Anaeromicrobium (An.ae.ro.mi.cro'bi.um. Gr. pref. *an* not; Gr. n. *aer*, *aeros* air; N.L. neut. n. *microbium* microbe; N. L. neut. n. *Anaeromicrobium* an anaerobic microbe).

Cells are long rods; endospores are not observed; mesophilic, strictly anaerobic, chemo-organoheterotrophic and fermentative. The principal fatty acids are C_{16:1}, C_{14:0} and C_{16:0}. No respiratory quinones are detected. The DNA G+C content is approximately 33 mol%. 16S rRNA gene sequence comparisons place the genus in the domain Bacteria, within the class *Clostridia*, family *Clostridiaceae*.

DESCRIPTION OF ANAEROMICROBIUM SEDIMINIS SP. NOV.

Anaeromicrobium sediminis (se.di.mi'nis. L. gen. n. *sediminis* of a sediment).

Cells are motile, long rods (0.5–0.8 μm in width, 4–15 μm in length). Gram-stain-negative but give a negative KOH test indicating a Gram-positive cell type. Cells grow in the temperature range 25–40 °C (optimum 37 °C), pH range 6.5–9.0 (optimum pH 7.5) and with sea salts concentrations of 10–30 g l⁻¹ (optimum 20 g l⁻¹). The shortest doubling time is 76 min under optimal growth conditions. It can utilize complex organic compounds, amino acids, sugars and organic acids including peptone, tryptone, yeast extract, cellobiose,

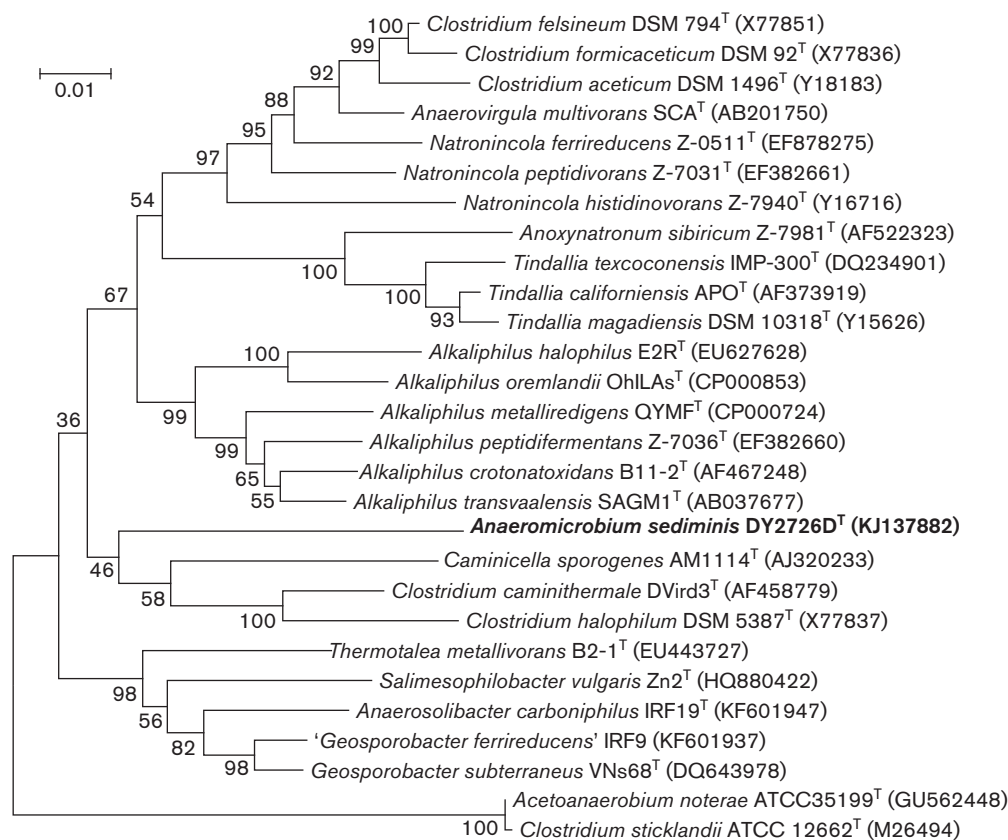


Fig. 1. Neighbour-joining phylogenetic tree based on a comparison of the 16S rRNA gene sequences (1301 bp, omitting unaligned regions) of strain DY2726D^T and its closest relatives within the family *Clostridiaceae*. GenBank accession numbers are given in parentheses. Bootstrap values (percentages) based on 1000 replications are shown at nodes. Bar, 0.01 nucleotide substitutions per nucleotide position.

dextrin, D-fructose, L-fucose, D-galactose, D-galacturonic acid, D-glucosaminic acid, gentiobiose, D-glucose, D-glucose 6-phosphate, D-mannose, melibiose, 3-methyl D-glucose, palatinose, L-rhamnose, α -ketobutyric acid and α -ketovaleric acid. Insoluble Fe(III) compounds, including amorphous Fe(III) oxyhydroxide (pH 7.0), amorphous iron (III) oxide (pH 9.0) and goethite (α -FeOOH, pH 12.0), can be reduced to Fe(II) while sulfur, sulfite, sulfate, thiosulfate and nitrate cannot be reduced.

The type strain, DY2726D^T (=JCM 30224^T=MCCC IA00776^T), was isolated from sediments of the West Pacific Ocean (154.1° E 16.0° N) at a depth of 5445 m. The DNA G+C content of the type strain is 33.4 mol%.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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