Anoxybacter fermentans gen. nov., sp. nov., a piezophilic, thermophilic, anaerobic, fermentative bacterium isolated from a deep-sea hydrothermal vent

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A novel piezophilic, thermophilic, anaerobic, fermentative bacterial strain, designated strain DY22613^T, was isolated from a deep-sea hydrothermal sulfide deposit at the East Pacific Rise (GPS position: 102.6° W 3.1° S). Cells of strain DY22613^T were long, motile rods (10 to 20 μm in length and 0.5 μm in width) with peritrichous flagella and were Gram-stain-negative. Growth was recorded at 44–72 °C (optimum 60–62 °C) and at hydrostatic pressures of 0.1–55 MPa (optimum 20 MPa). The pH range for growth was from pH 5.0 to 9.0 with an optimum at pH 7.0. Growth was observed in the presence of 1 to 8 % (w/v) sea salts and 0.65 to 5.2 % (w/v) NaCl, with optimum salt concentrations at 3.5 % for sea salts and at 2.3 % for NaCl. Under optimal growth conditions, the shortest generation time observed was 27 min (60 °C, 20 MPa). Strain DY22613^T was heterotrophic, able to utilize complex organic compounds, amino acids, sugars and organic acids including peptone, tryptone, beef extract, yeast extract, alanine, glutamine, methionine, phenylalanine, serine, threonine, fructose, fucose, galactose, gentiobiose, glucose, mannose, melibiose, palatinose, rhamnose, turanose, pyruvate, lactic acid, methyl ester, erythritol, galacturonic acid and glucosaminic acid. Strain DY22613^T was able to reduce Fe(III) compounds, including Fe(III) oxyhydroxide (pH 7.0), amorphous iron(III) oxide (pH 9.0), goethite (α-FeOOH, pH 12.0), Fe(III) citrate and elementary sulfur. Products of fermentation were butyrate, acetate and hydrogen. Main cellular fatty acids were iso-$C_{15:0}$, iso-$C_{14:0}$ 3-OH and $C_{14:0}$. The genomic DNA G+C content of strain DY22613^T was 36.7 mol%. Based on 16S rRNA gene sequence analysis, the strain forms a novel lineage within the class *Clostridia* and clusters with the order *Haloanaerobiales* (86.92 % 16S rRNA gene sequence similarity). The phylogenetic data suggest that the lineage represents at least a novel genus and species, for which the name *Anoxybacter fermentans* gen. nov., sp. nov. is proposed. The type strain is DY22613^T (=JCM 19466^T=DSM 28033^T=MCCC 1A06456^T).

Abbreviations: AQDS, 9,10-anthraquinone-2,6-disulfonate; S°, elemental sulphur.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain DY22613T is KC794015.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.

Molecular inventories revealed a wide diversity of thermophilic prokaryotes at deep-sea hydrothermal vents, only some of which have been cultivated (Miroshnichenko & Bonch-Osmolovsaya, 2006). Some thermophiles of the class *Clostridia* have been isolated from deep-sea vents, including representatives of the genera *Caloranaerobacter* (Wery *et al.*, 2001), *Caminicella* (Alain *et al.*, 2002), *Tepidibacter* (Slobodkin *et al.*, 2003; Urios *et al.*, 2004), *Caldanaerobacter* (Fardeau *et al.*, 2004) and *Clostridium*. Two species of the genus *Clostridium* have also been isolated from deep-sea vents, namely, *Clostridium caminithermale* (Brisbarre *et al.*, 2003) and *Clostridium tepidiprofundi* (Slobodkina *et al.*, 2008). Recently, one novel species, *Vallitalea pronyensis*, has been isolated from a shallow hydrothermal vent chimney (Ben Aissa *et al.*, 2014). These six genera fall into the class *Clostridia*, a highly polyphyletic class of obligate anaerobes. Most of them ferment carbohydrates to acetate, ethanol, H_2 and CO_2. At the time of writing, the class *Clostridia* encompasses four orders including *Clostridiales*, *Halanaerobiales*, *Natranaerobiales* and *Thermoanaerobacterales* and the suborder *Eubacteriineae* (Rainey, 2009). The orders *Haloanaerobiales* and *Natranaerobiales* were created to accommodate halophilic anaerobes (Rainey *et al.*, 1995; Mesbah *et al.*, 2007). The order *Thermoanaerobacterales* is polyphyletic, encompassing species able to survive in environments of extreme elevated temperature (Hogan, 2010). The order *Clostridiales* is highly polyphyletic, not a natural group, with diverse clades. We describe in this report the characterization of a novel piezophilic, anaerobic, thermophilic, fermentative bacterium (designated strain DY22613^T) isolated from a deep-sea hydrothermal vent environment. On the basis of the physiological and phylogenetic evidence presented, we propose a novel genus, *Anoxybacter* gen. nov., to accommodate this micro-organism.

Strain DY22613^T was isolated from hydrothermal sulfides collected in July 2011 at a depth of 2891 m at the East Pacific Rise (GPS position: 102.6° W 3.1° S), during the DY125-22 cruise of R/V *Da Yang Yi Hao*. Sulfide samples were collected using a benthic seabed grab and stored hermetically in sealed sterile vials. Samples were transported at 4 °C to the laboratory. A sample composed of hydrothermal chimney fragments bearing polychaete tubes and tube worms was chosen to perform enrichment cultures of thermophilic heterotrophic anaerobes. X-ray diffraction analysis indicated that this sample was mainly composed of pyrite (FeS_2) and sphalerite (ZnS).

One subsample was used to inoculate (1/10, w/v) a sterile liquid medium called FRPFO, which was prepared anaerobically and kept under an atmosphere of highly purified 100 % nitrogen. FRPFO medium contained (g l^{-1}, unless stated otherwise): peptone (10), sea salts (30; Sigma), PIPES (6.05), cysteine hydrochloride (0.5), resazurin (1 mg) and amorphous Fe(III) oxyhydroxide (50 mM, pH 7.0) as an electron acceptor. Enrichment cultures were incubated at 60 °C. Between 3 to 5 days of incubation, the colour of the precipitates changed from brown to black, indicating Fe(III) reduction. The enriched microbial community was composed of motile long and small rods. One strain, designated DY22613^T, was unable to form colonies in solidified medium containing 1.5 % (w/v) agar or 0.2 % (w/v) Gelrite, therefore, strain DY22613^T was purified by three repeated dilution-to-extinction series. The purity of this isolate was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers (Lane, 1991) (Bac8F, Bac27F, 1100R, U1492R). Stock cultures were stored at −80 °C in FRPFO medium supplemented with 5 % (v/v) DMSO.

The morphological characteristics of cells of the novel isolate were determined by using light microscopy (CX21; Olympus) and transmission electron microscopy (JEM-1230; JEOL). For ultrathin section examination of the cell wall, bacterial cells were fixed with osmic acid and embedded in araldite; the samples were then sliced and stained with lead citrate (Reynolds, 1963). Cells of strain DY22613^T were regular to long rods (10 to 20 μm in length and 0.5 μm in width), motile, bearing flagella (Fig. S1a, available in the online Supplementary Material). Cells occurred mainly singly or formed short chains. The cells stained Gram-negative (Hangzhou Tianhe Micro-organism Reagent), and electron microscopy of ultrathin sections of cells revealed the presence of two layers characteristic of Gram-stain-negative bacteria (Fig. S1b). Moreover, the KOH reaction was positive, confirming the Gram-stain-negative type of the cells. Spores were not observed.

Physiological characterization of the novel isolate was carried out in FRPFO medium dispensed anaerobically in 50 ml vials sealed with butyl-rubber stoppers, reduced with 0.05 % (w/v) cysteine hydrochloride sterile solution, just before inoculation. Unless stated otherwise, experiments were carried out anaerobically under an atmosphere of N_2 (100 %, 1 bar) and incubations were performed in the dark at 60 °C and pH 7.0. Growth was routinely monitored by direct cell counting using a modified Thomas chamber (depth 10 μm). Growth rates were calculated using linear regression analysis of eight points along the linear portions of the growth curves that were exponentially transformed. The determination of the temperature range for growth was tested over the range 40–74 °C at 2 °C intervals. Growth was observed from 44 to 72 °C, with an optimum growth rate at 60–62 °C. Growth was also observed under high hydrostatic pressure, from 0.1 to 55 MPa (optimum 20 MPa; Fig. S2). The pH range for growth was tested from initial pH 4.0 to initial pH 10.0 at 60 °C in basal medium buffered and adjusted to the required pH (initial pH at 20 °C) with MES buffer (pH 4.0–6.0), PIPES buffer (pH 7.0–8.0), HEPES buffer (pH 8.0–9.0), AMPSO buffer (pH 9.0–10.0). Growth was observed from pH 5.0 to 9.0 and the optimum pH for growth was pH 7.0. Salt tolerance was tested at 60 °C in FRPFO medium prepared with various concentrations of NaCl (0–10 %, w/v, at 0.5 % intervals) or various concentrations of sea salts (0–100 g l^{-1} at 5 g l^{-1} intervals). Strain DY22613^T required salt and

grew at concentrations ranging from 0.65–5.20 % (w/v) NaCl (optimum 2.3 %, w/v, NaCl). Growth of strain DY22613^T was observed at sea salt concentration of 10–80 g l^{-1}, with an optimum sea salt concentration of 35 g l^{-1}. Under optimal growth conditions, the generation time was around 54 min at atmospheric pressure and around 27 min under 20 MPa.

Strain DY22613^T was an obligate chemoorganoheterotroph, utilizing complex organic compounds including peptone, tryptone, beef extract and yeast extract. The ability of the isolate to use single carbon sources for growth was tested in triplicates at the optimal growth temperature using Biolog AN microplates in anaerobic jars as per the manufacturer's instructions. The Biolog AN plate results showed that strain DY22613^T was able to utilize amino acids (including L-alanine, L-alanyl-L-glutamine, L-glutamic acid, L-glutamine, L-methionine, L-phenylalanine, L-serine and L-threonine), sugars (including D-fructose, L-fucose, D-galactose, gentiobiose, D-glucose, D-glucose 6-phosphate, D-mannose, melibiose, 3-methyl D-glucose, palatinose, L-rhamnose and turanose) and organic acids (including pyruvate, D-lactic acid, methyl ester, erythritol, D-galacturonic acid and D-glucosaminic acid). Strain DY22613^T was not able to utilize D-gluconic acid, lactose, maltose, D-mannitol, melezitose, raffinose, salicin, D-sorbitol, stachyose, sucrose, trehalose, acetic acid, formic acid, fumaric acid, glyoxylic acid, malic acid, succinic acid, alaninamide, L-asparagine, L-valine, inosine, thymidine or uridine. The major fermentation products of glucose, determined by gas chromatography (QP2010; Shimadzu) were butyrate, acetate and hydrogen.

The ability of the novel isolate to use electron acceptors was tested by adding elemental sulfur (12 g l^{-1}), sulfite (1 mM), thiosulfate (20 mM), nitrate (10 mM), 9,10-anthraquinone-2,6-disulfonate (AQDS; 5 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), Fe(III) EDTA (20 mM) or oxygen (0.05–0.5 %, v/v) to the medium. Various forms of Fe(III) were synthesized by using modifications of previously described methods (Lovley & Phillips, 1986a). Strain DY22613^T was found to be strictly anaerobic. The strain grew only by fermentation with complex organic compounds and glucose, and also with Fe(III) as an electron acceptor. Strain DY22613^T was facultatively dependent on different forms of Fe(III) including Fe(III) oxyhydroxide (pH 7.0), amorphous iron(III) oxide (pH 9.0), goethite (α-FeOOH; pH 12.0), Fe(III) citrate or AQDS as an electron shuttle; but was unable to reduce Fe(III) chloride or Fe(III) EDTA. The cell yields of strain DY22613^T with peptone as an electron donor reached 10^8 cells ml^{-1}, both in the presence and in the absence of Fe(III). Reduced Fe(II) was measured by the accumulation of HCl-soluble Fe(II) over time with ferrozine (Lovley & Phillips, 1986b). The maximum concentration of reduced Fe(II) reached 16.82, 14.40, 14.29 and 4.51 mM (in the late stationary growth phase) when grown on amorphous iron(III) oxide (pH 9.0), Fe(III) citrate, Fe(III) oxyhydroxide (pH 7.0) and goethite (pH 12.0), respectively (experiments were performed in triplicate). The novel isolate reduced elemental sulfur (S°) to hydrogen sulfide (checked by colorimetry in the presence of $CuSO_4$/HCl), but did not reduce sulfite, sulfate, thiosulfate or nitrate.

The determination of the whole-cell fatty acid composition was performed on cultures grown on YTG medium at 60 °C. 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 Sherlock Microbial Identification System (MIDI). The fatty acids in strain DY22613^T comprised iso-$C_{15:0}$ (36.26 %), iso-$C_{14:0}$ 3-OH (20.61 %) and $C_{14:0}$ (7.36 %) and differed from the type strain *Halothermothrix orenii* H168^T in the proportion of several fatty acids including iso-$C_{15:0}$ (54.3 % in H168^T), $C_{16:0}$ (9.94 %), anteiso-$C_{15:0}$ (9.79 %) and $C_{14:0}$ (7.96 %) (Cayol *et al.*, 1994). The fatty acid profiles of both species are given in Table S1.

The genomic DNA G+C content of strain DY22613^T was 36.7 mol% as determined by genome sequencing using Illumina GAIIX (Meiji Company, Shanghai). An almost complete 16S rRNA gene sequence (1471 nt) was determined by double strand DNA sequencing. The identification of phylogenetic neighbours was initially carried out using BLAST (Altschul *et al.*, 1997) and MEGABLAST (Zhang *et al.*, 2000) against the database of type strains with validly published prokaryotic names (Chun *et al.*, 2007). A search of the most similar 16S rRNA gene sequences was also done against the web-based EzTaxon-e Server (Kim *et al.*, 2012). The 16S rRNA gene sequence of strain DY22613^T was found to be very distantly related to species in the orders *Halanaerobiales*, *Natranaerobiales*, *Thermoanaerobacterales* and *Clostridiales* in the phylum Firmicutes, with similarity below 87.0 %. The closest relative was *H. orenii* H168^T (Cayol *et al.*, 1994), with 86.92 % 16S rRNA gene sequence similarity, followed by *Natranaerobius trueperi* JW/NM-WN-LH1^T (85.73 %) (Mesbah & Wiegel, 2009) and *Moorella humiferrea* 64-FGQ^T (85.63 %) (Nepomnyashchaya *et al.*, 2012).

A phylogenetic tree of representative members in the class *Clostridia* was reconstructed from 16S rRNA gene sequences using 1239 homologous gene sequence positions (Fig. 1). Alignment of all sequences was performed using the software CLUSTAL_X (version 2.3) and the phylogenetic tree was reconstructed using the neighbour-joining method with the software MEGA (version 5.1). Bootstrap analysis was performed with 1000 replications to provide confidence estimates for the tree topology. Based on this analysis, strain DY22613^T clearly belongs to the class *Clostridia*, but is not affiliated closely with any of the described lineages. Its closest neighbours belong to the order *Halanaerobiales* but are distantly related to the novel isolate (Fig. 1). Furthermore, strain DY22613^T could be differentiated from its closest relatives *H. orenii*, *N. trueperi* and *M. humiferrea*

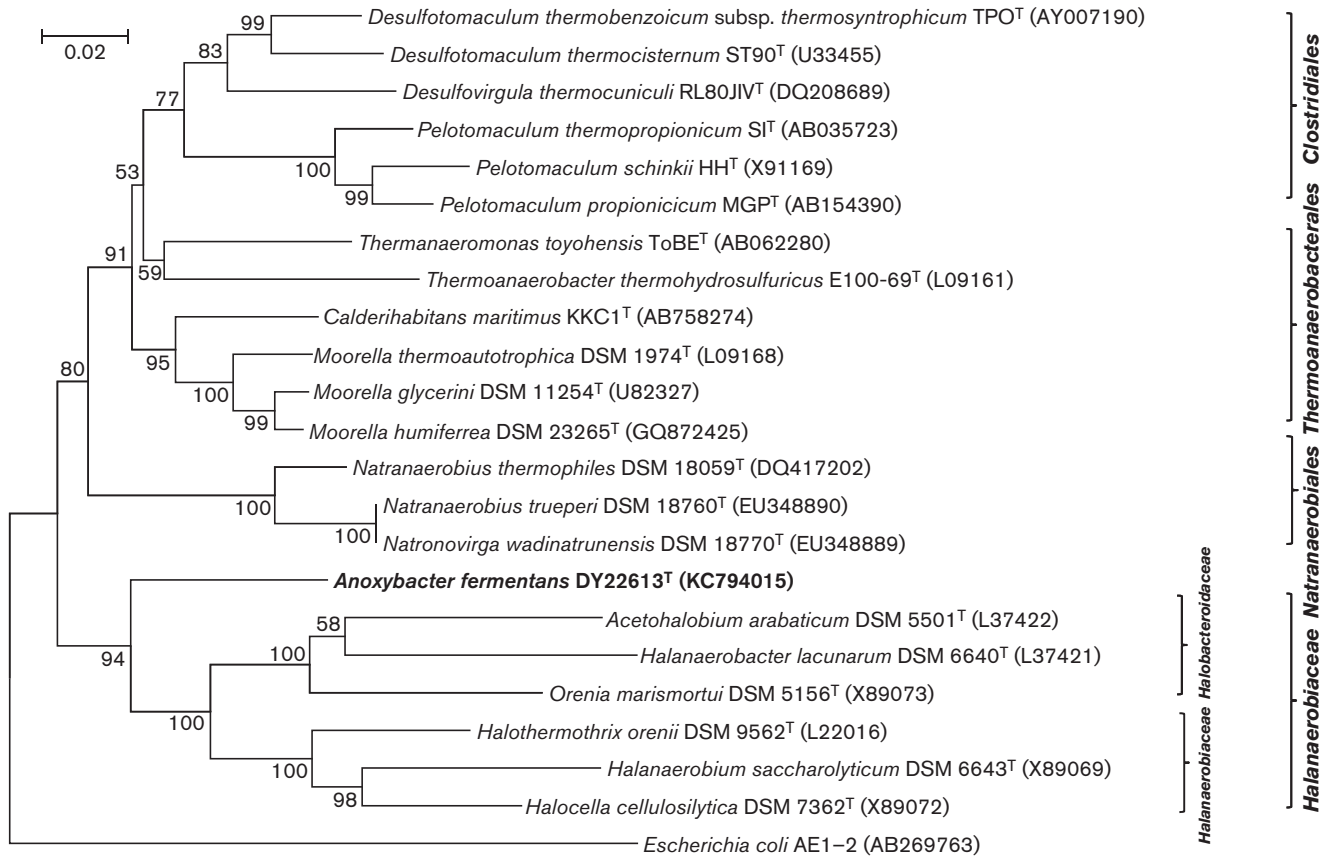

Fig. 1. Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences (1239 bp, omitting unaligned regions), showing the position of strain DY22613^T within the class *Clostridia*. Bar, expected number of changes per sequence position.

Table 1. Differential characteristics between strain DY22613^T and its phylogenetically closest relatives

Strains: 1, DY22613^T; 2. *H. orenii* H168^T; 3. *N. trueperi* JW/NM-WN-LH1^T; 4. *M. humiferrea* 64-FGQ^T. ND, No data.

Characteristic	1	2	3	4
Geographical origin	Sulfide of deep-sea hydrothermal vent, East Pacific rise	Sediment of salted lake, Tunisia	Sediment of alkaline, hypersaline lake, Egypt	Sediment of terrestrial hydrothermal spring, Russia
Cell size (μm)	10.0–20.0 × 0.5	10.0–20.0 × 0.4–0.6	4.0–5.0 × 0.3–0.4	2.0–5.0 × 0.3–0.5
NaCl range, %, w/v (optimum)	0.65–5.20 (2.3)	4.0–20.0 (10)	9.9–21.6 (13.4)	0–15.0 (0)
Temp. range, °C (optimum)	45.0–70.0 (60.0–62.0)	45.0–68.0 (60.0)	26.0–56.0 (51.0)	46.0–70.0 (65.0)
pH range (optimum)	5.0–9.0 (7.0)	5.5–8.2 (6.5–7.0)	8.5–11.5 (9.9)	5.5–8.5 (7.0)
DNA G+C content (mol%)	36.7	39.6	41.7	51.0
Major fatty acids (>7 %)	iso-$C_{15:0}$ (36.26 %); iso-$C_{14:0}$ 3-OH (20.61 %); $C_{14:0}$ (7.36 %)	iso-$C_{15:0}$ (54.30 %); $C_{16:0}$ (9.94 %); anteiso-$C_{15:0}$ (9.79 %); $C_{14:0}$ (7.96 %)	iso-$C_{15:0}$ (80.40 %); anteiso-$C_{15:0}$ (9.40 %)	ND
16S rRNA gene sequence similarity (%)*	100.00	86.92	85.73	85.63

*Calculated in reference to the 16S rRNA gene sequence of strain DY22613^T.

based on a number of physiological characteristics such as NaCl range for growth and optimal salinity for growth, genomic DNA G+C content (mol%) and fatty acid profile (Table 1).

In conclusion, on the basis of the wide phylogenetic distance from its closest relatives (far below the threshold level of 94.5 % identity for the delineation of a new genus and close to the threshold level of 86.5 % for a new family delineation) (Yarza *et al.*, 2014), and with phenotypic differences with the closest neighbours, we propose to place strain DY22613^T as the type strain of a novel species within a new genus, for which the name *Anoxybacter fermentans* gen. nov., sp. nov., is proposed. A novel family will have to be established in the future to encompass this genus and related genera yet to be described, when more isolates are available.

Description of *Anoxybacter* gen. nov.

Anoxybacter (An.o.xy.bac′ter. Gr. pref. *an* without; M.L. *oxy* shortened from *oxygenium* oxygen; N.L. *bacter* masc. equivalent of Gr. neut. n. *bakterion* rod or sta; N.L. masc. n. *Anoxybacter* rod growing without oxygen).

Cells are Gram-stain-negative. Endospores are not observed. Thermophilic. Strictly anaerobic. Chemoorganoheterotrophic. The genomic DNA G+C content is approximately 37 mol%.

Description of *Anoxybacter fermentans* sp. nov.

Anoxybacter fermentans (fer.men′tans. L. part. adj. *fermentans* fermenting).

Cells are motile, round-ended rods with flagella. Cells grow in the temperature range of 44 to 72 °C (optimum 60–62 °C), in the hydrostatic pressure ranging from 0.1 to 55 MPa (optimum 20 MPa), pH range of 5.0 to 9.0 (optimum pH 7.0) and with 10 to 85 g l^{-1} sea salts (optimum 35 g l^{-1}). The shortest doubling time is 27 min at 60 °C under 20 MPa. It can utilize complex organic compounds, amino acids, sugars and organic acids including peptone, tryptone, beef extract, yeast extract, alanine, glutamine, methionine, phenylalanine, serine, threonine, fructose, fucose, galactose, gentiobiose, mannose, melibiose, glucose, palatinose, rhamose, rhamnose, turanose, pyruvate, lactic acid, galacturonic acid, glucosaminic acid, methyl ester and erythritol. Insoluble Fe(III) compounds, including amorphous Fe(III) oxyhydroxide (pH 7.0), amorphous iron (III) oxide (pH 9.0), goethite (α-FeOOH; pH 12.0) and Fe(III) citrate can be reduced to Fe(II). Reduces S° but does not reduce sulfite, sulfate, thiosulfate or nitrate.

The type strain, DY22613^T (=JCM 19466^T=DSM 28033^T=MCCC 1A06456^T), was isolated from a hydrothermal sulfide sample collected from an East Pacific Ocean hydrothermal field (GPS position: 102.6° W 3.1° S) at a depth of 2891 m. The genomic DNA G+C content of the type strain is 36.70 mol%.

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