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 DY22613T, 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 DY22613T 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 DY22613T 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 DY22613T 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 elemental sulfur. Products of fermentation were butyrate, acetate and hydrogen. Main cellular fatty acids were iso-C15 : 0, iso-C14 : 0 3-OH and C14 : 0. The genomic DNA G+C content of strain DY22613T 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 DY22613T (=JCM 19468T=DSM 28033T=MCCC 1A06456T).

Abbreviations: AODS, 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.

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Molecular inventories revealed a wide diversity of thermo-
philic 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
tepadiprofundus (Slobodkina et al., 2008). Recently, one
novel species, Vallitalea pronymosis, 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, H2 and
CO2. At the time of writing, the class Clostridia en-
compases four orders including Clostridiales, Halanaerobiales,
Natranaerobiales and Thermoanaerobacteriales and the sub-
order EuBacteriineae (Rainey, 2009). The orders Halonaero-
biales and Natranaerobiales were created to accommodate
dhalophilic anaerobes (Rainey et al., 1995; Mesbah et al.,
2007). The order Thermonaerobacteriales is polyphyletic,
comprising 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 characteri-
ization of a novel piezophilic, anaerobic, thermophilic,
fermentative bacterium (designated strain DY22613T) iso-
lated 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 DY22613T 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 trans-
ported 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 (FeS2) and sphalerite (ZnS).

One subsample was used to inoculate (1/10, w/v) a sterile
liquid medium called FRPFO, which was prepared anaero-
bically 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 DY22613T, was unable to form
colonies in solidified medium containing 1.5% (w/v) agar
or 0.2% (w/v) Gelrite, therefore, strain DY22613T was
purified by three repeated dilution-to-extinction series.
The purity of this isolate was confirmed routinely by
microscopic examination and by repeated partial sequen-
cing of the 16S rDNA 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
DY22613T 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
conducted 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 N2
(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 DY22613T 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<sup>T</sup> was observed at sea salt concentration of 10–80 g l<sup>−1</sup>, with an optimum sea salt concentration of 35 g l<sup>−1</sup>. Under optimal growth conditions, the generation time was around 54 min at atmospheric pressure and around 27 min under 20 MPa.

Strain DY22613<sup>T</sup> 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<sup>T</sup> 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-romanose and turanose) and organic acids (including pyruvate, D-lactic acid, methyl ester, erythritol, D-galacturonic acid and D-glucosaminic acid). Strain DY22613<sup>T</sup> grew at concentrations ranging from 0.65–5.20 % (w/v) NaCl (optimum 2.3 %, w/v, NaCl). Growth of strain DY22613<sup>T</sup> comprised iso-C<sub>15 : 0</sub> (36.26 %), iso-C<sub>14 : 0</sub> 3-OH (20.61 %) and C<sub>14 : 0</sub> (7.36 %) and differed from the type strain Halothermotheix oreii H168<sup>T</sup> in the proportion of several fatty acids including iso-C<sub>15 : 0</sub> (54.3 % in H168<sup>T</sup>), C<sub>16 : 0</sub> (9.94 %), anteiso-C<sub>15 : 0</sub> (9.79 %) and C<sub>14 : 0</sub> (7.96 %) (Cayol et al., 1994). The fatty acid profiles of both species are given in Table S1.

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<sup>T</sup> comprised iso-C<sub>15 : 0</sub> (36.26 %), iso-C<sub>14 : 0</sub> 3-OH (20.61 %) and C<sub>14 : 0</sub> (7.36 %) and differed from the type strain Halothermotheix oreii H168<sup>T</sup> in the proportion of several fatty acids including iso-C<sub>15 : 0</sub> (54.3 % in H168<sup>T</sup>), C<sub>16 : 0</sub> (9.94 %), anteiso-C<sub>15 : 0</sub> (9.79 %) and C<sub>14 : 0</sub> (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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> (Cayol et al., 1994), with 86.92 % 16S rRNA gene sequence similarity, followed by Natranaerobius trueperi JW/NM-WN-LH1<sup>T</sup> (85.73 %) (Mesbah & Wiegel, 2009) and Moorella humiferrea 64-FGQ<sup>T</sup> (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<sup>T</sup> 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<sup>T</sup> could be differentiated from its closest relatives H. orenii, N. trueperi and M. humiferrea.
Anoxybacter fermentans gen. nov., sp. nov.

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 DY22613T within the class Clostridia. Bar, expected number of changes per sequence position.

Table 1. Differential characteristics between strain DY22613T and its phylogenetically closest relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geographical origin</td>
<td>Sulfide of deep-sea hydrothermal vent, East Pacific rise</td>
<td>Sediment of salted lake, Tunisia</td>
<td>Sediment of alkaline, hypersaline lake, Egypt</td>
<td>Sediment of terrestrial hydrothermal spring, Russia</td>
</tr>
<tr>
<td>Cell size (μm)</td>
<td>10.0–20.0 × 0.5</td>
<td>10.0–20.0 × 0.4–0.6</td>
<td>4.0–20.0 (10)</td>
<td>9.9–21.6 (13.4)</td>
</tr>
<tr>
<td>NaCl range, %, w/v (optimum)</td>
<td>0.65–5.20 (2.3)</td>
<td>4.0–20.0 (10)</td>
<td>26.0–56.0 (51.0)</td>
<td>46.0–70.0 (65.0)</td>
</tr>
<tr>
<td>Temp. range, °C (optimum)</td>
<td>45.0–70.0 (60.0–62.0)</td>
<td>45.0–68.0 (60.0)</td>
<td>8.5–11.5 (9.9)</td>
<td>5.5–8.5 (7.0)</td>
</tr>
<tr>
<td>pH range (optimum)</td>
<td>5.0–9.0 (7.0)</td>
<td>5.5–8.2 (6.5–7.0)</td>
<td>41.7</td>
<td>51.0</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>36.7</td>
<td>39.6</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Major fatty acids (&gt;7 %)</td>
<td>iso-C_{15:0} (36.26 %); iso-C_{14:0} 3-OH (20.61 %); C_{14:0} (7.36 %)</td>
<td>iso-C_{15:0} (54.30 %); C_{16:0} (9.94 %); anteiso-C_{15:0} (9.79 %); C_{14:0} (7.96 %)</td>
<td>iso-C_{15:0} (80.40 %); anteiso-C_{15:0} (9.40 %)</td>
<td>ND</td>
</tr>
<tr>
<td>16S rRNA gene sequence similarity (%)*</td>
<td>100.0</td>
<td>86.92</td>
<td>85.73</td>
<td>85.63</td>
</tr>
</tbody>
</table>

*Calculated in reference to the 16S rRNA gene sequence of strain DY22613T.
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 DY22613T 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 oxygennium 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.**


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 (x-FeOOH; pH 12.0) and Fe(III) citrate can be reduced to Fe(II). Reduces S+ but does not reduce sulfite, sulfate, thiosulfate or sulfate.

The type strain, DY22613T (=JCM 19466T=DSM 28033T=MCCC 1A06456T), 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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**References**


