Caloranaerobacter ferrireducens sp. nov., an anaerobic, thermophilic, iron (III)-reducing bacterium isolated from deep-sea hydrothermal sulfide deposits

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A thermophilic, anaerobic, iron-reducing bacterium (strain DY22619T) was isolated from a sulfide sample collected from an East Pacific Ocean hydrothermal field at a depth of 2901 m. Cells were Gram-stain-negative, motile rods (2–10 μm in length, 0.5 μm in width) with multiple peritrichous flagella. The strain grew at 40–70 °C inclusive (optimum 60 °C), at pH 4.5–8.5 inclusive (optimum pH 7.0) and with sea salts concentrations of 1–10 % (w/v) (optimum 3 % sea salts) and NaCl concentrations of 1.5–5.0 % (w/v) (optimum 2.5 % NaCl). Under optimal growth conditions, the generation time was around 55 min. The isolate was an obligate chemoorganoheterotroph, utilizing complex organic compounds, amino acids, carbohydrates and organic acids including peptone, tryptone, beef extract, yeast extract, alanine, glutamate, methionine, threonine, fructose, mannose, galactose, glucose, palatinose, rhamnose, turanose, gentiobiose, xylose, sorbose, pyruvate, tartaric acid, α-ketobutyric acid, α-ketovaleric acid, galacturonic acid and glucosaminic acid. Strain DY22619T was strictly anaerobic and facultatively dependent on various forms of Fe(III) as an electron acceptor: insoluble forms and soluble forms. It did not reduce sulfite, sulfate, thiosulfate or nitrate. The genomic DNA G+C content was 29.0 mol%. Phylogenetic 16S rRNA gene sequence analyses revealed that the closest relative of strain DY22619T was Caloranaerobacter azorensis MV1087T, sharing 97.41 % 16S rRNA gene sequence similarity. On the basis of physiological distinctness and phylogenetic distance, the isolate is considered to represent a novel species of the genus Caloranaerobacter, for which the name Caloranaerobacter ferrireducens sp. nov. is proposed. The type strain is DY22619T (=JCM 19467T=DSM 27799T=MCCC1A06455T).

Iron minerals are abundant at deep-sea hydrothermal vents. The surfaces of active chimneys are frequently covered with deposits of iron oxides in different oxidative states. Thus, deep-sea hydrothermal vents can provide an ecological niche for Fe(III)-reducing micro-organisms (Slobodkin et al., 2001). Dissimilatory Fe(III)-reducing bacteria (DIRB)
conserve energy to support growth by coupling the oxidation of organic compounds and/or H₂ to the reduction of ferric iron (Lovley, 1995). The process of dissimilatory Fe(III)-reducing influences several biogeochemical elements cycles, causes the release of soluble Fe(II), phosphate and trace metals, and affects sediment properties (Lovley, 1995).

At the time of writing, only a few Fe(III)-reducing bacteria have been isolated and characterized from deep-sea hydrothermal areas, including potassium, silver and other metals, and affects sediment properties (Lovley, 1995). At the time of writing, only a few Fe(III)-reducing bacteria have been isolated and characterized from deep-sea hydrothermal areas, including Deferrispora abyssii (Miroshnichenko et al., 2003) and Deferrispora autotrophicus (Slobodkina et al., 2009) within the order Deferrisporales, and Geothermobacter ehrlichii (Kashefi et al., 2003) and Deferrisoma camini (Slobodkina et al., 2012) in the class Deltaproteobacteria. So far, only one species, Tepidimicrobium ferrilirum (Slobodkin et al., 2006), isolated from a hot spring, was described as an iron-reducing thermophilic prokaryote within the order Clostridiales. The genus Caloranaerobacter falls into the cluster XII of the Clostridium subphylum. At the time of writing, this genus comprises only one species of hydrothermal origin, Caloranaerobacter azorensis, which was isolated from the Mid-Atlantic Ridge (Wery et al., 2001b). In this paper, we describe an anaerobic, thermophilic, Fe(III)-reducing micro-organism, strain DY22619T, isolated from hydrothermal sulfide deposits, and characterized as representing a novel species of the genus Caloranaerobacter.

In July 2011, during the DY125-22 oceanographic cruise onboard the R/V Da Yang Yi Hao, fragments of hydrothermal sulfide deposits were collected at a depth of 2901 m on the East Pacific Rise (102.6°W 3.1°S). Samples were collected using a benthic seabed grab, stored hermetically in sealed sterile vials, and transported at 4 °C to the laboratory. X-ray diffraction analysis indicated that these samples were mainly composed of pyrite (FeS₂) and sphalerite (ZnS). One subsample was used to inoculate (at 1/10th w/v) a sterile liquid medium referenced as FRPFO, prepared anaerobically, under an atmosphere of highly purified 100 % N₂, 2001a). Unless stated otherwise, experiments were carried out anaerobically, under an atmosphere of N₂ (100 %, 1 bar), and incubation was performed in the dark at 60 °C and pH 7.0. Growth was routinely monitored by direct cell counting using a modified Thoma chamber (length 0.02 mm), or by counting after fixation with 1 % (v/v) glutaraldehyde and storage at –20 °C. All conditions were tested in triplicate. Growth rates were calculated using linear regression analysis of eight to ten points along the linear portions of the log-transformed growth curves. Determination of the temperature range for growth was investigated over the range 30–75 °C (at 30, 37, 40, 45, 50, 55, 60, 65, 70 and 75 °C). Growth was observed from 40 to 70 °C and the optimum temperature for growth was 60 °C.

Physiological characterization of strain DY22619T was carried out in YTG medium dispensed anaerobically in 50 ml vials sealed with butyl-rubber stoppers, reduced with 0.1 ml of a 10 % (w/v) Na₂SO₄ sterile solution, just before inoculation, as described previously (Wery et al., 2001a). Unless stated otherwise, experiments were carried out anaerobically, under an atmosphere of N₂ (100 %, 1 bar), and incubation was performed in the dark at 60 °C and pH 7.0. Growth was observed from 40 to 70 °C and the optimum temperature for growth was 60 °C. The pH range for growth was tested from initial pH 4.0 to initial pH 10.0, at 60 °C, in 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) or AMPSO buffer (pH 9.0–10.0). Strain DY22619T grew from pH 4.5 to pH 8.5 and the optimum pH for growth was 7.0. Salt tolerance was tested at 60 °C in YTG medium prepared with various concentrations of NaCl (0–10 % w/v, 0.5 % intervals) or various concentrations of sea salts (0–15 % w/v, 0.5 % intervals). Growth was observed with 1–10 % (w/v) sea salts (Sigma) and with 1.5–5.0 % (w/v) NaCl. The optimum salt concentration was 3.0 % for sea salts and 2.5 % for NaCl. Strain DY22619T was unable to grow without salt, but is not strictly halophilic. Under optimal growth conditions, the shortest generation time was 55 min.

Among the dissimilatory Fe(III)-reducing bacteria, strain DY22619T can be classified in the 'fermentative' group, which use Fe(III) reduction as a minor pathway for electron flow while fermenting sugars or amino acids to a mixture of volatile fatty acids (acetate, butyrate) and hydrogen. Strain DY22619T was obligately chemoorganoheterotrophic, 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 triplicate, under optimal growth conditions, by using Biolog
AN plates in the anaerobic jar according to the manufacturer’s instructions. Strain DY22619T was able to utilize amino acids (including alanine, glutamate, methionine and threonine), carbohydrates (including fructose, mannose, galactose, glucose, palatinose, rhamnose, turanose, gentiobiose, xylose and sorbose) and organic acids (including pyruvate, tartaric acid, 2-ketobutyric acid, 2-ketovaleric acid, galacturonic acid and glucosaminic acid). Methanol, ethanol, mannitol, formic acid, acetic acid, maltose, cellobiose and sucrose were not used. Strain DY22619T was not capable of chemoautotrophic growth in a H₂/CO₂ gas atmosphere.

The ability of the novel isolate to use electron acceptors was tested by adding sulfate (1 mM), thiosulfate (20 mM), nitrate (10 mM), MnO₂(20 mM), Fe(III) oxyhydroxide (pH 7.0; 50 mM), apatiteous iron(III) oxide (pH 9.0; 50 mM), goethite (x-FeOOH, pH 12.0; 50 mM); Fe(III) citrate (20 mM), Fe(III) chlorite (20 mM), EDTA-Fe(III) (20 mM) or oxygen (0.05–0.5 % v/v) to the medium, without inoculation as a control. Various forms of Fe(III) were synthesized by using modifications of previously described methods (Lovley & Phillips, 1986a). Strain DY22619T was found to be strictly anaerobic and was able to grow only by fermentation. It was facultatively dependent on various forms of Fe(III), including insoluble forms such as amorphous Fe(III) oxyhydroxide (pH 7.0), amorphous iron(III) oxide (pH 9.0), goethite (x-FeOOH, pH 12.0), and soluble forms such as Fe(III) citrate, Fe(III) chlorite, EDTA-Fe(III). 9,10-anthraquinone-2,6-disulfonate (AQDS; 5 mM) could be used as an electron shuttle in Fe(III) respiration. Reduced Fe(II) was measured by recording the accumulation of HCl-soluble Fe(II) over time with ferrozine (Lovley & Phillips, 1986b). The maximum concentration of reduced Fe(II) could reach 12.32 mM in the medium when strain DY22619T reached the stationary phase, in medium supplemented with amorphous iron(III) oxide (pH 9.0) as a terminal electron acceptor. The Fe(III) reduction capability of strain DY22619T seems higher than that of Caloranaerobacter azorensis (4.12 mM). Various forms of Fe(III) did not stimulate the growth of strain DY22619T, which may be reduced as a minor pathway for electron flow. Strain DY22619T also could reduce MnO₂ to Mn(II). Strain DY22619T did not reduce sulfate, thiosulfate or nitrate.

Determination of the whole-cell fatty acid composition was performed on cultures of strain DY22619T and Caloranaerobacter azorensis MV1087T grown at 60 °C on YTg medium. YTg medium contained (g l⁻¹ unless otherwise stated): yeast extract, 1; peptone, 1; glucose, 2.5; artificial sea salts, 30; PIPES, 6.05; Wolf’s vitamin solution, 0.5 ml; Wolf’s trace elements solution, 5 ml; cysteine-HCl, 0.5; and resazurin, 1 mg. Cells were harvested at the end of the exponential phase of growth (36 h of incubation). Fatty acids were extracted and analysed following the instructions of the Microbial Identification System operating manual (MIDI). Fatty acids in strain DY22619T comprised three main species: iso-C₁₅:₀ (40.05 %), C₁₄:₀ (12.44 %) and iso-C₁₄:₀ 3-OH (11.66 %). This differed from the fatty acid profile of the closest relative Caloranaerobacter azorensis MV1087T, which included principally iso-C₁₅:₀ (44.67 %) and iso-C₁₄:₀ 3-OH (21.38 %). The detailed fatty acid profiles of strain DY22619T and Caloranaerobacter azorensis MV1087T are given in Table S1.

The G+C content of the chromosomal DNA was determined according to the methods described using reverse-phase HPLC (Mesbah & Whitman, 1989). The DNA G+C content of the novel isolate DY22619T was 29.0 mol%, which was similar to the DNA G+C content of Caloranaerobacter azorensis MV1087T (27.0 %). An almost-complete 16S rRNA gene sequence (1471 nt) of strain DY22619T was determined by double-strand sequencing and was deposited in the public database. The identification of phylogenetic neighbours was initially carried out using BLAST (Altschul et al., 1997) and megadBlast (Zhang et al., 2000) against the database of type strains with validly published prokaryotic names (Chun et al., 2007). A search of 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 DY22619T was most similar to that of Caloranaerobacter azorensis MV1087T, with 97.41 % sequence similarity. Comparisons of 16S rRNA gene sequences with those of other members of the ‘Clostridia’, showed that strain DY22619T shared only 92.70 % similarity with Brassinibacter mesophilus BM1, 92.39 % with Clostridium purinilyticum DSM 1384T, 92.16 % with Sporosalogibacteriaceae famiuarene SOL357T and 91.97 % with Thermohalobacter berrensis CTT3T. A phylogenetic tree of the representative members of cluster XII of the Clostridium subphylum was reconstructed from 16S rRNA gene sequences using 1374 conserved gene sequence positions (Fig. 1). Alignment of all sequences was performed using the software CLUSTAL X (version 2.3) and the phylogenetic trees were 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 tree topologies. These results indicate that strain DY22619T robustly branches with Caloranaerobacter azorensis MV1087T. Based on the above analyses, strain DY22619T could be assigned to the genus Caloranaerobacter, a member of cluster XII of the Clostridium subphylum, within the Gram-positive bacteria (Fig. 1). The 16S rRNA gene sequence similarity value between strain DY22619T and the type strain of Caloranaerobacter azorensis was well below the threshold value (98.7–99 %) currently recommended as the need for DNA–DNA hybridization to test for the genomic uniqueness of a novel species (Stackebrandt & Ebers, 2006). Consequently, the novel isolate displays sufficient molecular differences for delineation at the species level.

In summary, strain DY22619T shared many physiological and chemotaxonomic characteristics with its closest phylogenetic relative, Caloranaerobacter azorensis. Nevertheless, it
can be distinguished from *Caloranaerobacter azorensis* by its clear phylogenetic distance, cell morphology, fatty acid profile, doubling time under optimal growth conditions and different use of soluble iron compounds as electron acceptors (Table 1).

Therefore, based on the data presented and far phylogenetic distance with closest relatives (98.65 % 16S rRNA gene sequence similarity can be used as the threshold for differentiating two species; Kim *et al.*, 2014), we suggest that strain DY22619<sup>T</sup> represents a novel species of the genus *Caloranaerobacter*, for which the name *Caloranaerobacter ferrireducens* sp. nov. is proposed.

**Description of Caloranaerobacter ferrireducens** sp. nov.

*Caloranaerobacter ferrireducens* [fer.ri.re.du’cens. L. n. *ferrum* iron; L. part. adj. *reducens* converting to a different state; N.L. part. adj. *ferrireducens* reducing iron (III)].

**Table 1.** Characteristics that differentiate strain DY22619<sup>T</sup> from its closest relative *Caloranaerobacter azorensis* MV1087<sup>T</sup>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Hydrothermal vent from the East Pacific Rise</td>
<td>Hydrothermal vent from the Mid-Atlantic ridge</td>
</tr>
<tr>
<td>Cell diameter (µm)</td>
<td>2.0–10.0 × 0.5</td>
<td>0.5–2.0 × 0.3–0.5</td>
</tr>
<tr>
<td>Gram stain</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NaCl range (optimum) (g l&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>15.0–50.0 (25.0)</td>
<td>6.5–65.0 (20.0)</td>
</tr>
<tr>
<td>Temperature range (optimum) (°C)</td>
<td>40.0–70.0 (60.0)</td>
<td>45.0–65.0 (65.0)</td>
</tr>
<tr>
<td>pH range (optimum)</td>
<td>4.5–8.5 (7.0)</td>
<td>5.5–9.0 (7.0)</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>29.0</td>
<td>27.0</td>
</tr>
<tr>
<td>Doubling time (min)</td>
<td>55</td>
<td>15</td>
</tr>
<tr>
<td>Electron acceptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfur</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Fe(III) chlorite</td>
<td>+</td>
<td>–*</td>
</tr>
<tr>
<td>Fe(III) citrate</td>
<td>+</td>
<td>–*</td>
</tr>
<tr>
<td>EDTA-Fe(III)</td>
<td>+</td>
<td>–*</td>
</tr>
</tbody>
</table>

*Data from this study.
Cells are motile, round-ended rods (2–10 μm in length, 0.5 μm in width) with flagellum. Endospores are never observed. Cells grow at 37–70 °C (optimum 60 °C), at pH 4.5–8.5 (optimum pH 7.0) and with 10–100 g l⁻¹ sea salts (optimum 30 g l⁻¹). Doubling time is 55 min under optimal growth conditions. Strictly anaerobic and obligately chemoorganoheterotrophic. Can utilize complex organic compounds, amino acids, sugars, and organic acids including peptone, tryptone, beef extract, yeast extract, alanine, glutamate, methionine, threonine, fructose, mannose, galactose, glucose, palatinose, rhamnose, turanose, gentiobiose, xylose, sorbose, pyruvate, tartaric acid, α-ketobutyric acid, α-ketovaleric acid, galacturonac acid and glucosaminic acid. Insoluble and soluble formed Fe(III) chemicals, including amorphous Fe(III) oxyhydroxide (pH 7.0), amorphous iron(III) oxide (pH 9.0), goethite (α-FeOOH, pH 12.0), Fe(III) citrate, Fe(III) chloride and EDTA-Fe(III) can be reduced. Does not reduce sulfate, sulfite, thiosulfate or nitrate. The type strain, DY22619^T (=JCM 19467^T=DSM 27799^T=MCCC1A064555^T), was isolated from a hydrothermal sulfide sample collected from an East Pacific Ocean hydrothermal field (102.6°W 3.1°S) at a depth of 2901 m. The DNA G+C content of the type strain is 29.0 mol%.

Acknowledgements

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