Carboxydothermus siderophilus sp. nov., a thermophilic, hydrogenogenic, carboxydotrophic, dissimilatory Fe(III)-reducing bacterium from a Kamchatka hot spring

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A novel anaerobic, thermophilic, Fe(III)-reducing, CO-utilizing bacterium, strain 1315T, was isolated from a hot spring of Geyser Valley on the Kamchatka Peninsula. Cells of the new isolate were Gram-positive, short rods. Growth was observed at 52–70 °C, with an optimum at 65 °C, and at pH 5.5–8.5, with an optimum at pH 6.5–7.2. In the presence of Fe(III) or 9,10-anthraquinone 2,6-disulfonate (AQDS), the bacterium was capable of growth with CO and yeast extract (0.2 g l−1); during growth under these conditions, strain 1315T produced H2 and CO2 and Fe(II) or AQDSH2, respectively. Strain 1315T also grew by oxidation of yeast extract, glucose, xylose or lactate under a N2 atmosphere, reducing Fe(III) or AQDS. Yeast extract (0.2 g l−1) was required for growth. Isolate 1315T grew exclusively with Fe(III) or AQDS as an electron acceptor. The generation time under optimal conditions with CO as growth substrate was 9.3 h. The G+C content of the DNA was 41.5 ± 0.5 mol%. 16S rRNA gene sequence analysis placed the organism in the genus Carboxydothermus (97.8 % similarity with the closest relative). On the basis of physiological features and phylogenetic analysis, it is proposed that strain 1315T should be assigned to a novel species, Carboxydothermus siderophilus sp. nov., with the type strain 1315T (=VKPM 9905B1 =VKM B-2474T =DSM 21278T).

Hydrogenogenic CO-oxidizing anaerobes represent a physiological group of thermophilic prokaryotes able to grow on CO, producing hydrogen and CO2 according to the reaction CO + H2O → CO2 + H2 (ΔG°θ = −20 kJ mol−1). They have been found in various hydrothermal environments, both terrestrial and submarine (Sokolova et al., 2007). Representatives of another physiological group of prokaryotes, Fe(III) reducers, are also widespread in thermal habitats (Slobodkin, 2005). An assumption has been made that these two types of chemolithotrophic growth (hydrogenogenic carboxydotrophy and ferric iron reduction) often co-exist in hydrothermal environments (Sokolova et al., 2007). The genera Carboxydothermus, Thermosinus, Thermincola and Thermolithobacter consist of hydrogenogenic carboxydotrophic and Fe(III)-reducing species (Svetlichny et al., 1991; Slobodkin et al., 2006; Sokolova et al., 2004, 2005, 2007; Zavarzina et al., 2007). One of these organisms, Thermosinus carboxydivorans, grows on CO, producing molecular hydrogen, and simultaneously reduces Fe(III) to Fe(II) (Sokolova et al., 2004). Here, we report the isolation of a novel thermophilic, hydrogenogenic, carboxydotrophic, dissimilatory Fe(III)-reducing bacterium from a Geyser Valley hot spring (Kamchatka Peninsula).

Strain 1315T was isolated from a sample of pink filaments from a hot spring with a temperature of 72 °C and a pH of 8.4. For enrichment and isolation of anaerobic carboxydothermous bacteria, the following basal medium was used (per litre): 0.66 g NH4Cl, 0.16 g MgCl2.6 H2O, 0.1 g CaCl2.6H2O, 0.33 g KCl, 0.5 g KH2PO4, 1 ml trace element solution (Kevbrin & Zavarzin, 1992) and 1 ml vitamin solution (Wolin et al., 1963). After boiling, the medium was flushed with N2 and cooled, NaHCO3 (0.5 g l−1) and yeast extract (0.2 g l−1) were added and the pH was adjusted to 6.8–7.0 with 6 M HCl or to 8.3 with 6 M NaOH. The medium was supplemented with amorphous ferric iron oxide (90 mM), which was prepared as described previously (Sokolova et al., 2004). Portions of medium (10 ml) were placed into 50 ml bottles and the
headspace was filled with 100% CO at atmospheric pressure. Bottles were inoculated with approximately 1 g sample and incubated at 70 °C. After 3 days of incubation, the pressure in the bottles had increased from 140 to 160–170 kPa at both pH 6.8 and pH 8.3. In addition, non-magnetic, brown, amorphous Fe(III) oxide was converted to a black, solid material of less volume that was strongly attracted to a magnet. Pure culture was obtained through serial dilutions on medium supplemented with amorphous Fe(III) oxide at pH 6.8 under 100% CO in the gas phase.

For electron microscopy (negative staining), cultures were fixed as described previously (Sokolova et al., 2002) and examined under a JEM-100B microscope (JEOL). Cells of isolate 1315T were non-motile, straight, short rods, 0.7–1.5 μm long and 0.5 μm wide (Fig. 1). Cells divided by binary fission (not shown). Spores were not observed.

The effects of temperature and pH on growth were studied in medium supplemented with Fe(III) or AQDS, respectively, under a CO atmosphere. Since strain 1315T required amorphous Fe(III) oxide or AQDS, which are stable only at neutral and alkaline pH, it was impossible to study the growth of the strain under acidic conditions. Growth of strain 1315T occurred within a temperature range of 52–70 °C, with an optimum at 65 °C, and within a pH range of 5.5–8.5, with an optimum at 6.5–7.2. No growth was observed at 45 or 75 °C, or at pH 5.0 or 8.7. Cell density was determined by direct cell counting. Amorphous Fe(III) oxide was dissolved before cell counting by threefold dilution of 0.1 ml samples with an ammonium oxalate (28 g l⁻¹)/oxalic acid (15 g l⁻¹) solution (pH 3.5).

Growth of the new isolate on different substrates was tested in medium supplemented with amorphous Fe(III) oxide or with ferric citrate (20 mM), AQDS (20 mM) or Na2S·9H2O (0.5 g l⁻¹) under 100% N2 in the gas phase. Possible substrates were added to a final concentration of 2 g l⁻¹. Possible electron acceptors were added to a final concentration of 2 g l⁻¹ and elemental sulfur was added to 10 g l⁻¹ in medium reduced with Na2S·9H2O (0.5 g l⁻¹). CO, H₂ and CO₂ were determined by GLC as described previously (Sokolova et al., 2002). Strain 1315T grew chemolithotrophically on 100% CO only in medium supplemented with Fe(III) or AQDS. CO uptake was coupled to H₂ and CO₂ formation according to the equation CO + H₂O → CO₂ + H₂. Fe(III) reduction was monitored by measuring the accumulation of Fe(II) over time (Fig. 2) as described previously (Slobodkin et al., 1999). Ferric iron was reduced to ferrous iron, and this resulted in magnetite being formed. Yeast extract (0.2 g l⁻¹) was required for growth. The generation time of strain 1315T for growth on CO under optimal conditions was 9.3 h. No significant reduction of Fe(III) or AQDS in the presence or absence of CO in sterile medium was observed. No growth, CO consumption or H₂ production occurred in the absence of Fe(III) or AQDS.

Cell growth of the new isolate and reduction of amorphous Fe(III) oxide were observed on yeast extract (2.0 g l⁻¹), glucose, xylose and lactate. With reduction of AQDS, strain 1315T was capable of growing organotrophically with lactate only. Strain 1315T did not utilize peptone, sucrose, galactose, lactose, fructose, formate, acetate, pyruvate, succinate, oxalate, citrate, glycerol or ethanol under all conditions tested. The new isolate also did not grow under a H₂/CO₂ atmosphere (4:1, v/v) in either the presence or absence of Fe(III) or AQDS. Strain 1315T did not grow by fermentation of organic substrates in simple medium or in the same medium supplemented with Na₂S·9H₂O. Several attempts to grow strain 1315T in medium reduced with Na₂S·9H₂O and supplemented with different electron acceptors (sulfate, thiosulfate, sulfite, sulfur, nitrate or fumarate) and possible electron donors (CO, H₂ or lactate) were unsuccessful (Table 1).

Chloramphenicol (100 μg ml⁻¹), penicillin (100 μg ml⁻¹) and erythromycin (100 μg ml⁻¹) inhibited growth, CO oxidation and Fe(III) reduction completely. Ampicillin (100 μg ml⁻¹), streptomycin (100 μg ml⁻¹) and tetracycline (100 μg ml⁻¹) did not inhibit growth, CO oxidation or Fe(III) reduction.

Fig. 1. Electron micrograph of cells of strain 1315T. Bar, 1 μm.

Fig. 2. Growth of strain 1315T at 65 °C in medium supplemented with amorphous Fe(III) oxide under an atmosphere of 100% CO. Concentrations of CO and H₂ are shown as amounts in the gas phase per litre liquid culture.
The DNA G+C content was determined by melting-point analysis (Marmur & Doty, 1962) using *Escherichia coli* K-12 DNA as a reference. The DNA G+C content in strain 1315T was 41.5 ± 0.5 mol% (mean ± SD of three determinations).

The phylogenetic position of the new isolate was determined based on its partial 16S rRNA gene sequence. DNA was isolated from 50 ml cell pellet by a modified alkaline Birnboim–Doly method (Boulygina et al., 2002) and by Wizard technology (Wizard MaxiPreps DNA purification resin; Promega). Selective PCR amplification of the 16S rRNA gene and its sequencing were performed as described previously (Subbotina et al., 2003).

Amplification of the template DNA was performed with the modified bacterial forward primer Bact 8-27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal reverse primer Univ1492R (5′-TACGGYTACCTTGGCAGCG-3′) as described by Subbotina et al. (2003). Preliminary comparisons (using BLAST) with 16S rRNA gene sequences available in GenBank revealed that isolate 1315T was a member of the phylum *Firmicutes*, order *Clostridiales*, family *Peptococcaceae*. A phylogenetic tree (Fig. 3) demonstrated that strain 1315T was a member of the genus *Carboxydothermus*, which to date contains two species with validly published names, *Carboxydothermus hydrogenoformans* (Svetlichny et al., 1991) and *Carboxydothermus ferrireducens* (Slobodkin et al., 1997, 2006). A direct comparison of the 16S rRNA gene sequence of strain 1315T with reference sequences of these species was carried out and the level of sequence similarity was found to be 96.4% with *C. ferrireducens* JW/AS-Y7T.

### Table 1. Characteristics of strain 1315T, *Carboxydothermus hydrogenoformans* 2901T and *C. ferrireducens* JW/AS-Y7T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>C. hydrogenoformans</em> 2901T</th>
<th><em>C. ferrireducens</em> JW/AS-Y7T</th>
<th>Strain 1315T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Slightly curved rods</td>
<td>Straight to slightly curved rods</td>
<td>Straight rods</td>
</tr>
<tr>
<td>Flagellation</td>
<td>Lateral flagella</td>
<td>Peritrichous flagella</td>
<td>Non-motile</td>
</tr>
<tr>
<td>Temperature for growth (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>40–78</td>
<td>50–74</td>
<td>52–70</td>
</tr>
<tr>
<td>Optimum</td>
<td>70–72</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>pH for growth</td>
<td>6.6–8.0</td>
<td>5.5–7.6</td>
<td>5.5–8.5</td>
</tr>
<tr>
<td>Optimum</td>
<td>7.0</td>
<td>6.0–6.2</td>
<td>6.5–7.2</td>
</tr>
<tr>
<td>G+C content of DNA (mol%)</td>
<td>39–41</td>
<td>41</td>
<td>41.5</td>
</tr>
</tbody>
</table>

### Anaerobic respiration of selected electron donors and acceptors

<table>
<thead>
<tr>
<th>CO as electron donor with acceptor:</th>
<th>Fe(III)</th>
<th>AQDS</th>
<th>H2 as electron donor with Fe(III) or AQDS as acceptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H2 as electron donor with Fe(III) or AQDS as acceptor</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lactate as electron donor with acceptor:</th>
<th>Sulfite</th>
<th>Thiosulfate</th>
<th>Sulfur</th>
<th>Nitrate</th>
<th>Fumarate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</table>

*Growth without H2 production
†Growth with H2 production.

The DNA G+C content was determined by melting-point analysis (Marmur & Doty, 1962) using *Escherichia coli* K-12 DNA as a reference. The DNA G+C content in strain 1315T was 41.5 ± 0.5 mol% (mean ± SD of three determinations).

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97.64–97.8% with four different 16S rRNA genes from the total genome of *C. hydrogenoformans* Z-2901T. 16S rRNA gene sequence similarity lower than 98.7% has been used as evidence that organisms belong to different species (Stackebrandt & Ebers, 2006).

The affiliation of strain 1315T to a novel species is also supported by significant phenotypic differences between strain 1315T and the two previously known species of the genus *Carboxydothermus* (Table 1). *C. hydrogenoformans* reduces Fe(III) with H2 but not CO and is hydrogenogenic (Svetlichny et al., 1991; Slobodkin et al., 2006), whereas *C. ferrireducens* reduces Fe(III) with CO but without hydrogen production (Slobodkin et al., 2006). Strain 1315T reduces Fe(III) and grows on CO with production of H2. Dependence of growth of the strain on the presence of Fe(III) indicated the dissimilatory nature of Fe(III) reduction. Ferric iron could be replaced only by AQDS. This physiological feature is common to many other known Fe(III) reducers (Lovley et al., 2004; Slobodkin, 2005). Natural analogues of AQDS (humic acids) are regarded as possible extracellular electron carriers to insoluble Fe(III) in natural environments (Lovley et al., 2004). Strain 1315T also differed from the two species by several other phenotypic features. The new isolate could not reduce sulfate, thiosulfate, sulfur, nitrate or fumarate, whereas *C. hydrogenoformans* and *C. ferrireducens* can reduce these substrates (Henstra & Stams, 2004). Differences in morphology, temperature and pH ranges, G+C content of DNA and substrates used by the three species in the course of anaerobic respiration are summarized in Table 1. Thus, based on phenotypic and 16S rRNA differences, we propose to assign strain 1315T to a novel species of the genus *Carboxydothermus*, *Carboxydothermus siderophilus* sp. nov.

**Description of Carboxydothermus siderophilus** sp. nov.

*Carboxydothermus siderophilus* (si.de.ro’ phi.lus. Gr. n. sideros iron; Gr. adj. philos loving. N.L. masc. adj. siderophilus iron-loving). Cells are short, non-motile, straight rods, 0.5 μm wide and 0.7–1.5 μm long. Gram-positive. Grows at 50–70 °C, with optimum growth at 65 °C, and at pH 5.5–8.5, with optimum growth at pH 6.5–7.2. Grows only in the presence of Fe(III) or AQDS. Grows chemoheterotrophically with glucose, xylose, lactate or yeast extract under N2. Grows chemolithotrophically with CO, but not H2. Yeast extract (0.2 g l–1) is required for growth. During growth on CO in the presence of Fe(III) or AQDS, hydrogen, CO2 and Fe(II) or AQDS, respectively, are produced. The product of amorphous Fe(III) oxide reduction is magnetite. No growth occurs with peptone, sucrose, galactose, lactose, fructose, maltose, formate, acetate, pyruvate, succinate, oxalate, citrate, malate, fumarate, glycerol, ethanol or methanol, either in the presence or absence of Fe(III) or AQDS. Does not reduce sulfate, thiosulfate, elemental sulfur, nitrate or fumarate. Growth is inhibited by chloramphenicol, penicillin and erythromycin but not by ampicillin, streptomycin or tetracycline. The DNA G+C content of the type strain is 41.5±0.5 mol%.

The type strain, 1315T (=VKPM 9905T = VKM B-2474T = DSM 21278T), was isolated from a terrestrial hot spring of Geyser Valley, Kamchatka Peninsula, Russia.

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**References**


