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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 1315^T, 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 1315^T produced H_2 and CO_2 and Fe(II) or $AQDSH_2$, respectively. Strain 1315^T also grew by oxidation of yeast extract, glucose, xylose or lactate under a N_2 atmosphere, reducing Fe(III) or AQDS. Yeast extract (0.2 g l^{-1}) was required for growth. Isolate 1315^T 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 1315^T should be assigned to a novel species, *Carboxydothermus siderophilus* sp. nov., with the type strain 1315^T (=VKPM $9905B^T$ =VKM $B\text{-}2474^T$ =DSM 21278^T).

Hydrogenogenic CO-oxidizing anaerobes represent a physiological group of thermophilic prokaryotes able to grow on CO, producing hydrogen and CO_2 according to the reaction $CO+H_2O \rightarrow CO_2+H_2$ ($\Delta G'_0= -20$ 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 1315^T 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 carboxydotrophic bacteria, the following basal medium was used (per litre): 0.66 g NH_4Cl, 0.16 g $MgCl_2.6H_2O$, 0.1 g $CaCl_2.6H_2O$, 0.33 g KCl, 0.5 g KH_2PO_4, 1 ml trace element solution (Kevbrin & Zavarzin, 1992) and 1 ml vitamin solution (Wolin *et al.*, 1963). After boiling, the medium was flushed with N_2 and cooled, $NaHCO_3$ (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

Abbreviation: AQDS, 9,10-anthraquinone 2,6-disulfonate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 1315^T is EF542810.

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 1315^T 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 9,10-anthraquinone 2,6-disulfonate (AQDS), respectively, under a CO atmosphere. Since strain 1315^T 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 1315^T 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 $Na_2S.9H_2O$ (0.5 g l^{-1}) under 100 % N_2 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 $Na_2S.9H_2O$ (0.5 g l^{-1}). CO, H_2 and CO_2 were determined by GLC as described previously (Sokolova *et al.*, 2002). Strain 1315^T grew chemolithotrophically on 100 % CO only in medium supplemented with Fe(III) or AQDS. CO uptake was coupled to H_2 and CO_2 formation according to the equation $CO + H_2O \rightarrow CO_2 + H_2$. 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 1315^T 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 H_2 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 1315^T was capable of growing organotrophically with lactate only. Strain 1315^T 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 H_2/CO_2 atmosphere (4 : 1, v/v) in either the presence or absence of Fe(III) or AQDS. Strain 1315^T did not grow by fermentation of organic substrates in simple medium or in the same medium supplemented with $Na_2S.9H_2O$. Several attempts to grow strain 1315^T in medium reduced with $Na_2S.9H_2O$ and supplemented with different electron acceptors (sulfate, thiosulfate, sulfite, sulfur, nitrate or fumarate) and possible electron donors (CO, H_2 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.

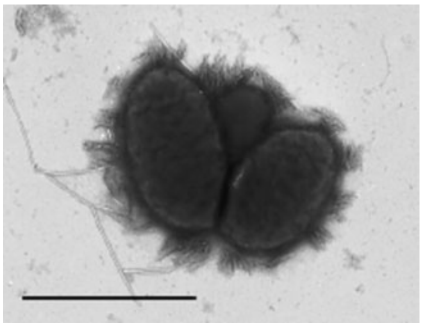

Fig. 1. Electron micrograph of cells of strain 1315^T. Bar, 1 μm.

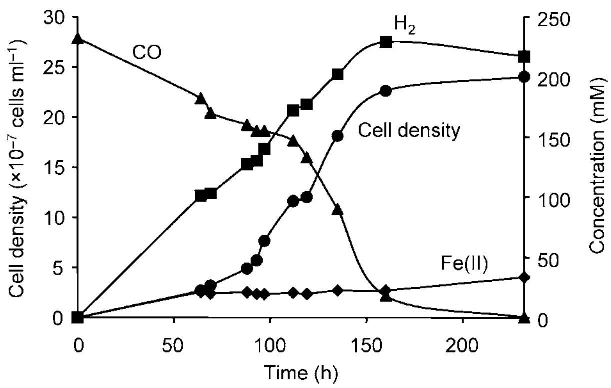

Fig. 2. Growth of strain 1315^T at 65 °C in medium supplemented with amorphous Fe(III) oxide under an atmosphere of 100 % CO. Concentrations of CO and H_2 are shown as amounts in the gas phase per litre liquid culture.

Table 1. Characteristics of strain 1315^T, *Carboxydothermus hydrogenoformans* 2901^T and *C. ferrireducens* JW/AS-$Y7^T$

Data for reference strains were taken from Svetlichny *et al.* (1991) (*C. hydrogenoformans* 2901^T) and Henstra & Stams (2004) and Slobodkin *et al.* (2006) (data for both strains). None of the strains uses lactate as an electron donor with sulfate as an electron acceptor.

Characteristic	*C. hydrogenoformans* 2901^T	*C. ferrireducens* JW/AS-$Y7^T$	Strain 1315^T
Morphology	Slightly curved rods	Straight to slightly curved rods	Straight rods
Flagellation	Lateral flagella	Peritrichous flagella	Non-motile
Temperature for growth (°C)			
Range	40–78	50–74	52–70
Optimum	70–72	65	65
pH for growth			
Range	6.6–8.0	5.5–7.6	5.5–8.5
Optimum	7.0	6.0–6.2	6.5–7.2
G+C content of DNA (mol%)	39–41	41	41.5
Anaerobic respiration of selected electron donors and acceptors			
CO as electron donor with acceptor:			
Fe(III)	−	+*	+†
AQDS	+†	+*	+†
H_2 as electron donor with Fe(III) or AQDS as acceptor	+	+	−
Lactate as electron donor with acceptor:			
Sulfite	+	+	−
Thiosulfate	+	+	−
Sulfur	+	+	−
Nitrate	+	+	−
Fumarate	+	−	−

*Growth without H_2 production
†Growth with H_2 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 1315^T 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 µl 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′-TACGGYTACCTT-GTTACGACTT-3′) as described by Subbotina *et al.* (2003). Preliminary comparisons (using BLAST) with 16S rRNA gene sequences available in GenBank revealed that isolate 1315^T was a member of the phylum *Firmicutes*, order *Clostridiales*, family *Peptococcaceae*. A phylogenetic tree (Fig. 3) demonstrated that strain 1315^T 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 1315^T 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-$Y7^T$ and

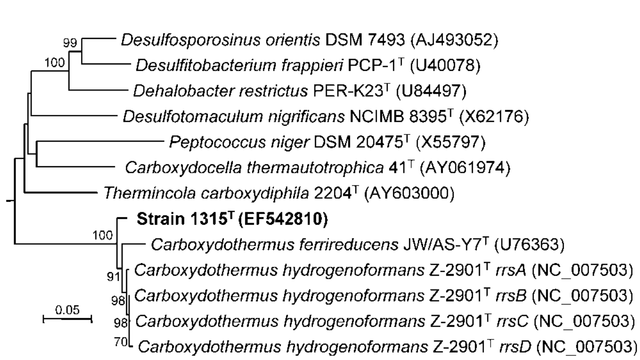

Fig. 3. Phylogenetic tree generated by the neighbour-joining method on the basis of 16S rRNA gene sequences, showing the position of strain 1315^T. Bar, 0.05 changes per sequence position. Bootstrap values from 100 replications are shown at branch points.

97.64–97.8 % with four different 16S rRNA genes from the total genome of *C. hydrogenoformans* Z-2901^T. 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 1315^T to a novel species is also supported by significant phenotypic differences between strain 1315^T and the two previously known species of the genus *Carboxydothermus* (Table 1). *C. hydrogenoformans* reduces Fe(III) with H_2 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 1315^T reduces Fe(III) and grows on CO with production of H_2. 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 1315^T also differed from the two species by several other phenotypic features. The new isolate could not reduce sulfite, 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 1315^T 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 N_2. Grows chemolithotrophically with CO, but not H_2. Yeast extract (0.2 g l^{-1}) is required for growth. During growth on CO in the presence of Fe(III) or AQDS, hydrogen, CO_2 and Fe(II) or $AQDSH_2$, 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, sulfite, 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, 1315^T (=VKPM 9905B^T =VKM B-2474^T =DSM 21278^T), was isolated from a terrestrial hot spring of Geyser Valley, Kamchatka Peninsula, Russia.

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