Carboxydocella manganica sp. nov., a thermophilic, dissimilatory Mn(IV)- and Fe(III)-reducing bacterium from a Kamchatka hot spring

G. B. Slobodkina,¹ A. N. Panteleeva,² T. G. Sokolova,¹
E. A. Bonch-Osmolovskaya¹ and A. I. Slobodkin¹

Correspondence

G. B. Slobodkina
gslobodkina@mail.ru

¹Winogradsky Institute of Microbiology, Russian Academy of Sciences,
Prospect 60-letiya Oktyabrya 7/2, 117312 Moscow, Russia

²Bioengineering Center, Russian Academy of Sciences, Prospect 60-letiya Oktyabrya 7/1,
117312 Moscow, Russia

A thermophilic, anaerobic, dissimilatory Mn(IV)- and Fe(III)-reducing bacterium (strain SLM 61^T) was isolated from a terrestrial hot spring on the Kamchatka peninsula. The cells were straight rods, 0.5–0.6 µm in diameter and 1.0–6.0 µm long, and exhibited tumbling motility by means of peritrichous flagellation. The strain grew at 26–70 °C, with an optimum at 58–60 °C, and at pH 5.5–8.0, with an optimum at pH 6.5. Growth of SLM 61^T was observed at 0–2.0% (w/v) NaCl, with an optimum at 0.5% (w/v). The generation time under optimal growth conditions was 40 min. Strain SLM 61^T grew and reduced Mn(IV), Fe(III) or nitrate with a number of organic acids and complex proteinaceous compounds as electron donors. It was capable of chemolithoautotrophic growth using molecular hydrogen as an electron donor, Fe(III) but not Mn(IV) or nitrate as an electron acceptor and CO₂ as a carbon source. It also was able to ferment pyruvate, yeast extract, glucose, fructose, sucrose and maltose. The G + C content of DNA of strain SLM 61^T was 50.9 mol%. 16S rRNA gene sequence analysis revealed that the closest relative of the isolated organism was *Carboxydocella thermotrophica* 41^T (96.9% similarity). On the basis of its physiological properties and phylogenetic analyses, the isolate is considered to represent a novel species, for which the name *Carboxydocella manganica* sp. nov. is proposed. The type strain is SLM 61^T (=DSM 23132^T =VKM B-2609^T). *C. manganica* is the first described representative of the genus *Carboxydocella* that possesses the ability to reduce metals and does not utilize CO.

Metal-reducing micro-organisms play important roles in the cycling of carbon and metals in various anaerobic ecosystems, including thermal environments. Dissimilatory Fe(III)- and Mn(IV)-reducing micro-organisms have been found in a variety of thermal biotopes, including continental hot springs, a deep terrestrial subsurface, submarine petroleum reservoirs, deep-sea hydrothermal vents and anthropogenic systems (Lovley *et al.*, 2004; Slobodkin, 2005). Dissimilatory manganese reduction by thermophilic prokaryotes is much less well studied than iron reduction. Of 43 described species of Fe(III)-reducing thermophiles belonging to different phylogenetic groups of the *Bacteria* and *Archaea*, the capacity for Mn(IV) reduction has been reported for six species

(Slobodkin, 2005). Only two micro-organisms, *Bacillus infernus* (Boone *et al.*, 1995) and *Deferribacter thermophilus* (Greene *et al.*, 1997), were first isolated using Mn(IV) as an electron acceptor. In this article, we report the isolation and characterization of a novel anaerobic, thermophilic, Mn(IV)- and Fe(III)-reducing micro-organism from a hydrothermal spring on the Kamchatka peninsula.

Strain SLM 61^T was isolated from a sample of sediment collected from a hot spring at the 'Trostnikovyi' field, Uzon Caldera, Kamchatka, Russia. The conditions at the sampling site were 60 °C, pH 6.2, *E*_h –400 mV. Samples were taken anaerobically in tightly stoppered bottles and transported to the laboratory. Enrichment cultures were initiated by inoculation of 10% (w/v) of the sample into anaerobically prepared, bicarbonate-buffered, sterile (135 °C, 1 h) liquid medium with lactate (14 mM) as an electron donor and manganese(IV) oxide (25 mmol l^{–1}) as an electron acceptor. Medium composition and preparation techniques were described previously (Slobodkin *et al.*, 1999). MnO₂

Abbreviations: AQDS, 9,10-anthraquinone 2,6-disulfonate; CFA, cellular fatty acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SLM 61^T is GU584133.

A supplementary table is available with the online version of this paper.

(birnessite) was prepared by O₂ (air) oxidation of MnCl₂ in alkali medium (Feng *et al.*, 2004). A pure culture was obtained from an enrichment positive for Mn(IV) reduction by serial dilutions in the same medium followed by the selection of well-separated colonies that had developed in anaerobic agar blocks (1.5 % agar in growth medium). Since MnO₂ is a black, insoluble compound, it is inconvenient for colonies to be obtained in solidified medium, and it was replaced by KNO₃. Light-brown, irregular-shaped colonies, 0.5–1.0 mm in diameter, appeared after 3 days of incubation at 60 °C. Individual colonies were checked for manganese-reducing ability by transferring them to liquid medium with MnO₂ and, for positive cultures, the process of obtaining colonies was repeated two more times. Finally, a single colony that had developed in the highest agar block dilution (10^{−6}) and possessed Mn(IV)-reducing ability was designated strain SLM 61^T and used for further studies.

Physiological studies on substrate and electron acceptor utilization, temperature, pH and salinity ranges for growth, light and electron microscopy, analytical techniques, DNA extraction and determination of G + C content were performed as described previously (Slobodkin *et al.*, 1999). Manganese (Mn²⁺) contents were determined by the formaldoxime method (Goto *et al.*, 1962). Ammonium and nitrite were determined by HPLC with a conductivity detector (Aquilon C1P column, 4 mM HNO₃, for ammonium and Aquilon A1.2 column, 3.5 mM carbonate buffer, for nitrite). Cellular fatty acid (CFA) profiles were determined by GC-MS as methyl ester derivatives prepared from 5 mg dry cell material (Sasser, 1990). CFA content was determined as percentages of total ion current peak area. The 16S rRNA gene was selectively amplified from genomic DNA by PCR using primers 11F and 1492R (Lane, 1991). The PCR was carried out in 50 µl reaction mixture containing 50 ng DNA template, 5 pmol (each) primers, 12.5 nmol (each) dNTPs and 3 U *Taq* DNA polymerase (Fermentas) in *Taq* DNA polymerase reaction buffer (Fermentas). Temperature cycling was done by using the following program: first cycle of 9 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, followed by 30 amplification cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. A final extension step was carried out at 72 °C for 7 min. PCR products were purified using the Wizard PCR Preps kit (Promega) as recommended by the manufacturer. The 16S rRNA gene was sequenced in both directions with the use of Big Dye Terminator version 3.1 (Applied Biosystems), as described in the manufacturer's instructions, using an ABI PRISM 3730 sequencer (Applied Biosystems). Sequences were assembled and checked for accuracy manually using the alignment editor BioEdit version 5.0.9 (Hall, 1999). The full-length 16S rRNA gene sequence was compared with other sequences in GenBank (Benson *et al.*, 1999) using BLAST (Altschul *et al.*, 1997) to identify the closest relatives. Alignment with a representative set of other 16S rRNA gene sequences in GenBank was carried out using the CLUSTAL W program provided by the phylogenetic analysis package MEGA version 4 (Tamura *et al.*, 2007) and then adjusted

manually. Pairwise similarity values were calculated by means of EzTaxon (Chun *et al.*, 2007). A phylogenetic dendrogram was reconstructed by the neighbour-joining method (Saitou & Nei, 1987) with the maximum composite likelihood model (Tamura *et al.*, 2004) using MEGA version 4. *Carboxydocella thermautotrophica* DSM 12356^T and *C. sporoproducens* DSM 16521^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and used as reference strains.

Cells of strain SLM 61^T were straight rods, 0.5–0.6 µm in diameter and 1.0–6.0 µm long. The cells occurred singly or in pairs and were motile by means of peritrichous flagellation. Spores were not observed. Ultrathin sectioning of strain SLM 61^T revealed the presence of a distinct electron-dense material typical of the peptidoglycan layer of a Gram-positive-type cell wall.

The temperature range for growth of strain SLM 61^T was 26–70 °C, with an optimum at 58–60 °C. No growth was detected at or above 71 °C or at 25 °C or below after incubation for 3 weeks. The pH range for growth was 5.5–8.0, with an optimum at pH 6.5. No growth was noted at or below pH 5.0 or at or above pH 8.5. Growth of strain SLM 61^T was observed at 0–2.0 % (w/v) NaCl with an optimum at 0.5 % (w/v); no growth was evident at or above 2.5 % (w/v) NaCl. Yeast extract was not required for growth, but addition of 0.20 g yeast extract l^{−1} increased final cell yield 10- to 15-fold. Potential electron acceptors were tested with sodium lactate (14 mM) as an energy source in the presence of 0.20 g yeast extract l^{−1}. Manganese(IV) oxide (25 mmol MnO₂ l^{−1}), poorly crystalline iron(III) oxide (ferrihydrite) [90 mmol Fe(III) l^{−1}], iron(III) citrate (10 mM) and potassium nitrate (10 mM) were used as electron acceptors for growth of strain SLM 61^T. Sulfate (14 mM), elemental sulfur (10 g l^{−1}), thiosulfate, 9,10-anthraquinone 2,6-disulfonate (AQDS), fumarate (20 mM each) and oxygen (3 or 20 %, v/v, in the gas phase) were not reduced and did not support growth. During Mn(IV) reduction, black insoluble MnO₂ turned to a light-brown precipitate containing 5–7 mM Mn(II). Ferrihydrite was reduced to a black magnetic precipitate with an Fe(II) content of 25–30 mM. No changes in colour or volume of precipitate were observed in uninoculated controls with Mn(IV) and Fe(III) incubated under the same conditions. Nitrate was reduced to ammonium. Lactate was oxidized incompletely to acetate. Strain SLM 61^T was able to grow and reduce Fe(III), Mn(IV) and nitrate with lactate, pyruvate (14 mM each), formate, butyrate, succinate (20 mM each), yeast extract, peptone, tryptone (2.5 g l^{−1} each) and H₂/CO₂ (80/20 v/v) as electron donors. Strain SLM 61^T could grow chemolithoautotrophically in the absence of yeast extract, using molecular hydrogen as an electron donor and ferrihydrite [but not Mn(IV) or nitrate] as an electron acceptor and bicarbonate/CO₂ as a carbon source. It also was able to grow without electron acceptors by fermentation of pyruvate, glucose, fructose, sucrose, maltose (15 mM each) and yeast extract (2.5 g l^{−1}). The main product of glucose fermentation (15 mM initial concentration) was acetate (28–30 mM),

with trace amounts (<1.0 mM) of ethanol, formate, lactate and succinate; no molecular hydrogen was produced. The generation time under optimal growth conditions on pyruvate was 40 min. Isolate SLM 61^T did not utilize acetate (10 mM), propionate, fumarate, maleinate, methanol, ethanol or glycerol (20 mM each) with iron(III) citrate (5 mM), manganese(IV) oxide (25 mmol l⁻¹) or potassium nitrate (10 mM) as electron acceptor. No growth was observed on peptone or tryptone (2.5 g l⁻¹ each) without an electron acceptor. Xylose, L-arabinose, lactose, galactose, cellobiose (15 mM each), starch (2.5 g l⁻¹) and citrate (10 mM) were not utilized with or without an electron acceptor. No growth or electron acceptor reduction was observed during cultivation on carbon monoxide (5, 15, 45 or 100 % CO in gas phase) with ferrihydrite [90 mmol Fe(III) l⁻¹], manganese(IV) oxide (25 mmol l⁻¹) or KNO₃ (10 mM) or without an acceptor added. Penicillin, ampicillin, streptomycin, novobiocin, kanamycin and neomycin (each at 100 µg ml⁻¹) inhibited growth of strain SLM 61^T. The fatty acids comprised a mixture of straight-chain and iso- and anteiso-branched fatty acids (Table S1, available in IJSEM Online). C_{16:0} predominated among the fatty acids (31.38 %); iso-C_{15:0}, C_{16:1ω7c}, iso-C_{17:0}, iso-C_{17:1ω8}, C_{18:0}, C_{18:1ω7} (5.23–9.95 %) and C_{14:0}, C_{15:0}, iso-C_{16:0}, C_{16:1ω9}, iso-C_{17:1ω6c}, anteiso-C_{17:0}, C_{17:0} and C_{18:1ω9} (1.10–2.75 %) were also present.

The G + C content of the genomic DNA of strain SLM 61^T was 50.9 mol% (T_m). A comparison of 1522 nt of the 16S rRNA gene sequence of strain SLM 61^T with those available in the GenBank database showed that strain SLM 61^T belonged to the genus *Carboxydocella* (Fig. 1). The 16S rRNA gene sequence of the new isolate had the highest pairwise similarity to that of *C. thermautotrophica* 41^T (96.9 %). The level of 16S rRNA gene sequence similarity to *C. sporoproducens* Kar^T was 96.7 %. Trees reconstructed by using the maximum-likelihood and maximum-parsimony algorithms displayed the same topology (not shown). Transversion analysis (Woese *et al.*, 1991) did not affect the phylogenetic position of the novel strain.

The new isolate described in this report represents a thermophilic micro-organism capable of reduction of Mn(IV) and Fe(III) and, phylogenetically, it is closest to members of the genus *Carboxydocella*. At the time of writing, the genus *Carboxydocella* consists of two species with validly published names, *C. thermautotrophica* Sokolova *et al.* 2002 (type species) and *C. sporoproducens* Slepova *et al.* 2006, and the novel isolate shares many phenotypic features with them. All members of the genus *Carboxydocella* display motile, rod-shaped cells and grow under anaerobic conditions, have very similar ranges of growth temperature and pH and were isolated from hot springs in Kamchatka. However, strain SLM 61^T displays a number of significant differences (Table 1). The most significant distinction of strain SLM 61^T from the other representatives of the genus *Carboxydocella* is its inability to grow on carbon monoxide, not only at 100 % CO in the gas phase but also at lower concentrations. It was reported

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences indicating the position of strain SLM 61^T. The tree was reconstructed using the neighbour-joining algorithm. GenBank accession numbers are given in parentheses. Bar, 2 substitutions per 100 nucleotide positions. Only bootstrap values greater 70 % are indicated.

previously that nitrate inhibits growth of *C. sporoproducens*. In our study, we found that *C. thermautotrophica* DSM 12326^T could also not grow and reduce nitrate with lactate or CO as electron donor. Unlike other members of the genus *Carboxydocella*, strain SLM 61^T is able to use nitrate as an electron acceptor. It utilizes a wider spectrum of substrates, including hydrogen, mono- and disaccharides, organic acids and complex proteinaceous compounds. We performed additional studies on sugar utilization by *C. thermautotrophica* DSM 12326^T in the presence of yeast extract in the culture medium used for cultivation of strain SLM 61^T and confirmed its inability to consume carbohydrates. Strain SLM 61^T was enriched on Mn(IV) as electron acceptor and possesses the capacity for Mn(IV) and Fe(III) reduction. This ability was not shown previously for micro-organisms belonging to the genus *Carboxydocella*. In our study, we found that *C. thermautotrophica* DSM 12326^T could not reduce Mn(IV) or Fe(III) with lactate, molecular hydrogen or CO as electron donor. In contrast, *C. sporoproducens* DSM 16521^T could grow and reduce Mn(IV) and Fe(III) with lactate as electron donor, at least in three sequential transfers. So, metal-reducing ability is not unique to the new isolate, but is inherent to the genus *Carboxydocella*. The strains also had some differences in G + C content of DNA and CFA profiles. The major fatty acid for all strains was C_{16:0}; the second most abundant fatty acid for *C. sporoproducens* DSM 16521^T and *C. thermautotrophica* DSM 12326^T was the saturated straight-chain acid C_{18:0}, whereas for strain SLM 61^T, the next most abundant fatty acids were C_{16:1ω7c} and iso-C_{17:0} (Table 1). On the basis of the phylogenetic position and phenotypic and physiological properties, we propose that strain SLM 61^T represents a novel species, *Carboxydocella manganica* sp. nov.

Description of *Carboxydocella manganica* sp. nov.

Carboxydocella manganica (man.ga'ni.ca. N.L. fem. adj. *manganica* pertaining to manganese).

Cells are straight, motile rods, 0.5–0.6 µm in diameter and 1.0–6.0 µm long. Spores are not observed. The cell wall is of the Gram-positive type. Cells form light-brown,

Table 1. Characteristics that differentiate strain SLM 61^T from the type strains of *Carboxydocella* species

Strains: 1, *C. manganica* sp. nov. SLM 61^T; 2, *C. sporoproducens* DSM 16521^T; 3, *C. thermautotrophica* DSM 12326^T. Data were obtained in this study.

Characteristic	1	2	3
CO utilization	—	+	+
Electron acceptors			
Mn(IV)	+	+	—
Fe(III)	+	+	—
KNO ₃	+	—	—
Electron donors			
Yeast extract	+	+	—
Peptone, tryptone	+	—	—
Molecular hydrogen	+	—	—
Formate	+	—	—
Lactate	+	+	—
Pyruvate	+	+	—
Glucose, fructose, maltose	+	—	—
Sucrose	+	+	—
Main CFAs (% of total CFA content)	C _{16:0} (31.38), C _{16:1ω7c} (9.95), iso-C _{17:0} (9.90)	C _{16:0} (35.84), C _{18:0} (16.35)	C _{16:0} (38.58), C _{18:0} (15.47)
DNA G + C content (mol%)	50.9	49.5	46.0

irregular-shaped colonies (0.5–1.0 mm in diameter) in anaerobic agar blocks. The temperature range for growth is 26–70 °C, with an optimum at 58–60 °C. The pH range for growth is 5.5–8.0, with an optimum at pH 6.5. Growth occurs at 0–2.0 % (w/v) NaCl, with an optimum at 0.5 % (w/v). Anaerobic. Reduces insoluble oxides of Mn(IV) and Fe(III). Utilizes peptone, tryptone, yeast extract, molecular hydrogen, formate, butyrate, lactate, pyruvate and succinate with Mn(IV), Fe(III) or nitrate as an electron acceptor. Lactate is oxidized incompletely to acetate. Capable of chemolithoautotrophic growth using molecular hydrogen as an electron donor, Fe(III) as an electron acceptor and CO₂ as a carbon source. Ferments pyruvate, yeast extract, glucose, fructose, sucrose and maltose. The main product of glucose fermentation is acetate. Does not utilize acetate, propionate, fumarate, maleinate, methanol, ethanol, glycerol, xylose, L-arabinose, lactose, galactose, cellobiose, starch or citrate, with or without electron acceptor. Sulfate, elemental sulfur, thiosulfate, AQDS, fumarate and oxygen are not reduced and do not support growth. No growth on carbon monoxide with or without electron acceptor. Penicillin, ampicillin, streptomycin, novobiocin, kanamycin and neomycin inhibit growth at 100 µl ml⁻¹. The major fatty acid is C_{16:0}. The G + C content of DNA of the type strain is 50.9 mol% (*T_m*).

The type strain, SLM 61^T (=DSM 23132^T =VKM B-2609^T), was isolated from a terrestrial hot spring at Kamchatka, Russia.

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