Thermosinus carboxydivorans gen. nov., sp. nov., a new anaerobic, thermophilic, carbon-monoxide-oxidizing, hydrogenogenic bacterium from a hot pool of Yellowstone National Park

Tatyana G. Sokolova,1 Juan M. González,2† Nadezhda A. Kostrikina,1 Nikolai A. Chernyh,1 Tatiana V. Slepow,¹ Elizaveta A. Bonch-Osmolovskaya1 and Frank T. Robb2

1Institute of Microbiology, Russian Academy of Sciences, Prospect 60 Let Oktyabrya, 7/2, 117811 Moscow, Russia
2COMB, Columbus Center, 701 E. Pratt St, Baltimore, MD 21202, USA

A new anaerobic, thermophilic, facultatively carboxydotrophic bacterium, strain Nor1¹, was isolated from a hot spring at Norris Basin, Yellowstone National Park. Cells of strain Nor1¹ were curved motile rods with a length of 2.6–3 μm, a width of about 0.5 μm and lateral flagellation. The cell wall structure was of the Gram-negative type. Strain Nor1T was thermophilic (temperature range for growth was 40–68 °C, with an optimum at 60 °C) and neutrophilic (pH range for growth was 6.5–7.6, with an optimum at 6.8–7.0). It grew chemolithotrophically on CO (generation time, 1.15 h), producing equimolar quantities of H2 and CO2 according to the equation CO + H2O R CO2 + H2. During growth on CO in the presence of ferric citrate or amorphous ferric iron oxide, strain Nor1T reduced ferric iron but produced H2 and CO2 at a ratio close to 1 : 1, and growth stimulation was slight. Growth on CO in the presence of sodium selenite was accompanied by precipitation of elemental selenium. Elemental sulfur, thiosulfate, sulfate and nitrate did not stimulate growth of strain Nor1T on CO and none of these chemicals was reduced. Strain Nor1T was able to grow on glucose, sucrose, lactose, arabinose, maltose, fructose, xylose and pyruvate, but not on cellobiose, galactose, peptone, yeast extract, lactate, acetate, formate, ethanol, methanol or sodium citrate. During glucose fermentation, acetate, H2 and CO2 were produced. Thiosulfate was found to enhance the growth rate and cell yield of strain Nor1T when it was grown on glucose, sucrose or lactose; in this case, acetate, H2S and CO2 were produced. In the presence of thiosulfate or ferric iron, strain Nor1T was also able to grow on yeast extract. Lactate, acetate, formate and H2 were not utilized either in the absence or in the presence of ferric iron, thiosulfate, sulfate, sulfite, elemental sulfur or nitrate. Growth was completely inhibited by penicillin, ampicillin, streptomycin, kanamycin and neomycin. The DNA G+C content of the strain was 51.7 ± 1 mol%. Analysis of the 16S rRNA gene sequence revealed that strain Nor1¹ belongs to the Bacillus–Clostridium phylum of the Gram-positive bacteria. On the basis of the studied phenotypic and phylogenetic features, we propose that strain Nor1¹ be assigned to a new genus, Thermosinus gen. nov. The type species is Thermosinus carboxydivorans sp. nov. (type strain, Nor1¹ = DSM 14886¹ = VKM B-2281¹).

INTRODUCTION

Several phylogenetically diverse thermophilic prokaryotes perform the metabolic reaction CO + H2O R CO2 + H2 (ΔGº = −20 kJ). These are representatives of the bacterial genera Carboxydothermus (Svetlichny et al., 1991, 1994), Caldanaerobacter (formerly Carboxydibrachium pacificum)
(Sokolova et al., 2001; Fardeau et al., 2004) and Carboxydothermus (Sokolova et al., 2002). Recently, a hyperthermophilic archaeon of the genus *Thermococcus* capable of growth at the expense of the same reaction was isolated from deep-sea hydrothermal vents (Sokolova et al., 2004). The metabolism of *Carboxydothermus hydrogenoformans* has been studied at the enzymic level (Svetlichny et al., 2001; Dobbek et al., 2001). This physiological group of prokaryotes has been proposed to be named ‘hydrogenogens’ (Svetlichny et al., 2001). CO-oxidizing hydrogenogenic prokaryotes were shown to possess various metabolic capacities. Growth of *Carboxydocella thermautotrophica* was found to be obligately dependent on CO. Apart from growth on CO, *Carboxydothermus hydrogenoformans* is able to grow on pyruvate (Pusheva & Sokolova, 1995). *Caldanaerobacter subterraneus* is capable of growing on several fermentable substrates (Sokolova et al., 2001). *Thermococcus* strain AM4 grows on CO producing H2, or chemo-organotrophically with elemental sulfur (Sokolova et al., 2004). Herein, we report the isolation of a novel anaerobic, CO-utilizing, H2-producing, thermophilic bacterium capable of iron reduction during growth on CO.

**METHODS**

**Collection of samples.** A sample of mud and water was taken from a small pool in the neutral (wooded) part of Norris Basin in the Yellowstone National Park. Organic matter (rotting wood, scum) was present in the pool. The geographical coordinates of the sampling site were 44° 43′ 797′′ N, 110° 42′ 506′′ W. The sample temperature was 50°C and its pH was 7.5 (at 30°C).

**Culture conditions and strains.** Enrichment and isolation of anaerobic carboxydothermic bacteria were carried out on Medium 1 supplemented with a neutralized solution of ferric citrate or amorphous ferric iron oxide. Medium 1 was of the following composition (g l−1): NH4Cl, 1; MgCl2·2H2O, 0.33; CaCl2·6H2O, 0.1; KCl, 0.33; KH2PO4, 0.5; 1 ml of trace element solution (Kevbrin & Zavarzin, 1992); 1 ml of vitamin solution (Wolin et al., 1963). After boiling, the medium was cooled under an N2 atmosphere. Yeast extract (0.2 g l−1) and NaHCO3 (0.5 g l−1) were added afterwards, and the pH was adjusted to 6.8–7.0 with 6 M HCl. A neutralized solution of ferric citrate or amorphous ferric iron oxide was added to a final concentration of 20 or 90 mM, respectively. Amorphous ferric iron oxide was prepared by titrating a solution of FeCl3 with 10% NaOH to pH 9. Aliquots (10 ml) of the medium were placed in 50 ml bottles, and the head-space was filled with CO (100 kPa). Pure cultures were obtained from colonies on the same medium solidified with 5% agar in roll-tubes under CO in the gas phase. Growth of pure cultures and physiological tests were performed using Medium 2. Medium 2 had the same composition as Medium 1 except it was supplemented with Na2S·9H2O (0.5 g l−1).

The reference strains used in this study were *Carboxydocella thermautotrophica* Z-2901T (DSM 6008T) (Svetlichny et al., 1991), *Carboxydothermus hydrogenoformans* Z-2901T (Svetlichny et al., 2001), *Caldanaerobacter subterraneus* subsp. *pacificus* Z-2901T (DSM 6008T) (Svetlichny et al., 2001), and *Caldanaerobacter pascuaicus* JM1 (DSM 12653T) (Sokolova et al., 2004).

**Light and electron microscopy.** Light microscopy was carried out using a phase-contrast microscope with a 90/1·25 oil immersion objective. Specimens of whole cells for electron microscopy were negatively stained with 2% phosphotungstic acid. For the preparation of thin sections, cells were fixed with 5% glutaraldehyde for 2 h and 1% OsO4 for 4 h at 4°C and then embedded in Epon-812. The thin sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken with a JEM-100C electron microscope.

**Physiological studies.** To test the growth of the novel isolate with various substrates and electron acceptors, Medium 2 with 100% N2 in the gas phase was used, supplemented with the following substrates (2 g l−1): peptone, yeast extract, succrose, lactose, glucose, galactose, ethanol, methanol, sodium salts of citrate, acetate, formate or pyruvate. Possible electron acceptors – elemental sulfur (10 g l−1) and sodium salts of nitrate, sulfate, thiosulfate (2 g l−1), sulfite (2 mM) or selenate (2 mM) – were tested on Medium 2 with various growth substrates. Growth with amorphous ferric iron oxide (90 mM) or selenite (2 mM) on various substrates was tested in Medium 1.

Bacterial growth was determined by direct cell count under a phase-contrast microscope.

Determinations of CO, gaseous products of metabolism, short-chain organic acids and alcohols were performed by GC as described previously (Sokolova et al., 2001). Fe(III) was determined by colorimetric reaction (Trüper & Schlegel, 1964). Fe(III) reduction was determined by measuring the accumulation of Fe(II) in the growth medium. For that, a 0.5 ml sample was added to 5 ml of 0·6 M HCl and, after a 24 h extraction, HCl-soluble Fe(II) was determined by the reaction with 2,2′-dipyridyl (Balashova & Zavarzin, 1980).

**DNA isolation and base composition.** DNA was prepared as described by Marmur (1961). The DNA G+C content was determined by melting-point analysis (Marmur & Doty, 1962) using *Escherichia coli* K-12 DNA as a reference.

**rRNA gene sequence.** The 16S rRNA gene sequence was PCR-amplified by using the primer pair 519F(5′-GTT TCA GCM GCC GCG GTA ATW C-3′) and 1522R (5′-AAG GAG GTG ATC CAG CGG CA-3′). The amplified DNA fragment was purified using the Qiagen PCR purification kit (Qiagen) and sequenced by the dye-deoxy-nucleotide chain-termination method on an ABI 373A sequencer (Applied Biosystems). Sequencing was performed using the primers 519F and 341F (5′-CC TAC GGG AGG CAG CAG-3′), forward strand, and primers 907R, 1522F and 1522R, reverse strand. Sequence alignment was performed using the software suite ARB (Ludwig et al., 2004). The alignment was edited manually considering the expected sequence secondary structure. An unrooted phylogenetic tree was constructed by maximum-likelihood using the program FastDNAmL (Felsenstein, 1981) embedded in ARB. The obtained tree topology was reconstructed by quartet-puzzling using the program TreePuzzle (Strimmer & von Haeseler, 1996) also available in the ARB package. The quartet-puzzling tree represented a consensus tree showing well supported branching. It was based on 1000 puzzling trials. The reliability value of each internal branch indicates as a percentage how often the corresponding cluster was found. The GenBank/EMBL/DDJB accession numbers of the 16S rRNA sequences used in this study are given in Fig. 4.

**RESULTS AND DISCUSSION**

**Enrichment and isolation.** For the enrichment of anaerobic, thermophilic, CO-oxidizing bacteria, 100 ml serum bottles containing 20 ml Medium 1 with ferric citrate and CO as the gas phase were...
inoculated with about 1 g of sample. After 3 days incubation at 55 °C, the gas pressure in several bottles increased from 100 to 120–150 kPa. Growth of curved rod-shaped cells was observed. The CO content in the gas phase decreased; the resulting gas phase composition was about 25–30 % CO, 30 % H2 and 30 % CO2. The colour of the medium changed from yellow–brown to green, indicating the reduction of Fe(III) to Fe(II). When transferred to Medium 2, the culture retained the ability to grow by oxidation of CO to CO2 and production of H2 and had the same morphology. After seven passages performed by serial tenfold dilutions, the culture was transferred to solid medium in roll-tubes filled with CO. After 4 days incubation at 55–60 °C of tubes inoculated with aliquots from the 10^6 and 10^7 dilutions, round white colonies of about 0.5 mm in diameter developed. Several colonies were isolated and transferred to Medium 2 under 100 % CO. From all the colonies the growth of motile curved rods was obtained. One isolate, designated Nor1^T, was chosen for further characterization.

**Morphology**

Cells of strain Nor1^T were curved rods with a length of 2–6–3 μm and a width of about 0.5 μm, arranged singly or in pairs (Fig. 1a, b). Cells were motile due to lateral flagella (Fig. 1a). Electron microscopy of ultrathin sections revealed a Gram-negative cell wall structure (Fig. 1c). The outer membrane had a folded structure (Fig. 1b, c). Cells divided by binary transverse fission (Fig. 1b, c).

**Growth parameters**

Strain Nor1^T grew between 40 and 68 °C, with an optimum at 60 °C. No growth occurred at 37 or 70 °C. Growth was possible between pH 6.5 and 7.6; no growth was detected at pH 6.2 or 7.8. Optimum pH for growth was 6.8–7.0.

**Physiology of growth**

Strain Nor1^T was able to grow on Medium 1 with or without ferric iron with 100 % CO in the gas phase, as well as on Medium 2 with 100 % CO. CO oxidation was coupled with H2 and CO2 formation in equimolar quantities according to the equation CO + H2O \(\rightarrow\) CO2 + H2 (Fig. 2 and Fig. 3). No methane, acetate or any other metabolic products were produced. The generation time of strain Nor1^T grown on CO without ferric iron was 1.15 h. During the growth on CO in Medium 1 in the presence of ferric citrate or amorphous ferric iron oxide, strain Nor1^T reduced ferric iron (Fig. 3); however, the amount of ferric iron reduced was not large enough to shift significantly the ratio of H2 and CO2 produced. The generation time of strain Nor1^T during growth on CO in the presence of ferric citrate was 1.07 h. The isolate grew in Medium 1 on CO in the presence of selenite, reducing it to elemental selenium, visible as red precipitate, but producing H2 and CO2 in nearly equimolar quantities. The isolate did not grow under an H2/CO2 (4:1) mixture either in Medium 2 or in Medium 1 supplemented with ferric citrate or amorphous ferric iron oxide.

Strain Nor1^T was found to be an obligate anaerobe. It did not grow under mixtures of CO and air, which contained 0.5, 1.0, 1.5, 2, 5 or 10 % molecular oxygen.

On Medium 2 (reduced with sodium sulfide), strain Nor1^T was capable of growth with glucose, sucrose, lactose,

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**Fig. 1.** Electron micrographs of cells of strain Nor1^T. Thin sections (b, c) and negative staining of whole cells (a). Bars, 0.5 μm.
carbohydrates, xylose and pyruvate, but not with cellobiose, galactose, peptone, yeast extract, lactate, acetate, formate, ethanol, methanol or sodium citrate. Weak growth was detected on the medium with mannanol. During growth on glucose, strain Nor1T produced acetate, H2 and CO2. On Medium 1 (without reduction with sodium sulfide), strain Nor1T was able to grow on organic substrates (glucose, sucrose and lactose) only in the presence of electron acceptors (ferric iron or thiosulfate), producing ferrous iron or H2S, respectively. Thiosulfate was found to stimulate the growth rate and cell yield of strain Nor1T on glucose. During growth on glucose in the presence of thiosulfate, H2 was not produced; the products were acetate, CO2 and H2S. No growth or H2S production was observed on the Medium 2 with peptone, lactate or acetate, in the presence of thiosulfate. The isolate showed poor growth and weak ferrous iron formation in Medium 1 with peptone or yeast extract and amorphous ferric iron oxide. The isolate did not reduce selenite or selenate during growth on peptone, sucrose or lactose.

Nitrate, sulfite and sulfate were not reduced during growth of strain Nor1T with CO or on organic substrates.

No growth was detected under an atmosphere of H2 + CO2 or H2 + air.

Our tests of other hydrogenogenic carboxydotrophs, Carboxythermus hydrogenoformans, Carboxydothermus hydrogenoformans and Caldanaerobacter subterraneous subsp. pacificus, for the capacity to reduce Fe(III) during growth on CO showed that none of these three organisms was able to reduce ferric citrate or amorphous oxide of ferric iron.

Sensitivity to antibiotics

Penicillin (100 µg ml−1), ampicillin (100 µg ml−1), streptomycin (100 µg ml−1), kanamycin (50 µg ml−1) and neomycin (50 µg ml−1) completely inhibited CO utilization and growth of strain Nor1T.

DNA base composition

The DNA G+C content of strain Nor1T was 51.7 ± 1 mol%.

16S rRNA gene sequence analysis

Sequencing of the 16S rRNA gene from strain Nor1T placed this isolate in the domain Bacteria; this is in agreement with the profile of antibiotic inhibition of growth. A BLAST search (Altschul et al., 1997) showed Dendrosporobacter quercicolum (formerly Clostridium quercicolum) (Strompl et al., 2000) as the closest relative (89.6% similarity). Other phyletic relatives of strain Nor1T, such as Acetronema (88.1%), Sporomusa (87.4%) and Selenomonas (86.2%) species, showed lower similarity values but phylogenetic analysis presented them clustered with strain Nor1T (Fig. 4).

The ability to grow anaerobically on CO with the production of H2 as the only reduced product was first observed in the mesophilic purple, non-sulfur bacteria Rhodococcus gelatinosus and Rhodospirillum rubrum (Uffen, 1976; Bonam et al., 1989). Carboxythermus hydrogenoformans was the first anaerobic, thermophilic, non-photosynthetic bacterium found that performed this process (Svetlichny et al., 1991). Later, several other organisms performing the reaction of anaerobic CO oxidation with CO2 and H2 production were described (Svetlichny et al., 1994; Sokolova et al., 2001, 2004). Strain Nor1T is similar to Caldanaerobacter subterraneous subsp. pacificus (formerly Carboxydibrachium pacificum) (Sokolova et al., 2001; Fardeau et al., 2004) in its ability to ferment some carbohydrates. However, they differ in the ability to reduce ferric iron. Strain Nor1T differs from Caldanaerobacter subterraneous subsp. pacificus JM1 also in its cell morphology: Nor1T cells are short, curved, motile rods, while Caldanaerobacter subterraneous subsp. pacificus cells are non-motile, straight, long, thin rods, sometimes branching. The isolate described in this work differs from other previously described anaerobic CO-oxidizing hydrogenogens by its capacity for fermentative growth on several carbohydrates and for thiosulfate reduction. Unlike other anaerobic CO-oxidizing hydrogenogenic bacteria, strain Nor1T is able to reduce ferric iron during the growth on CO in the presence of ferric citrate or ferric iron amorphous oxide. All previously described CO-dependent, H2-generating bacteria show a cell wall structure typical of Gram-positive bacteria; they belong to the Bacillus–Clostridium phylum of Gram-positive bacteria and do not form a single phylogenetic cluster. Morphologically, strain Nor1T resembles Thermaanaerobacterium species (Baena et al., 1999; Zavarzina et al., 2000), which are thermophilic, anaerobic organotrophs with vibrioid cells. As for strain Nor1T, Thermaanaerobacterium...
species have curved cells, which are motile by means of a tuft of lateral flagella located on the concave side of the cell, Gram-negative, non-spore-forming and capable of growing chemo-organotrophically with fermentable substrates or lithoheterotrophically with molecular hydrogen and elemental sulfur, reducing sulfur to H₂S (Zavarzina et al., 2000). Strain Nor1ᵀ is different from \textit{Thermanaerovibrio} species by the ability to grow chemolithotrophically on CO, producing H₂ and CO₂, and the phylogenetic distance between Nor1ᵀ strain and \textit{Thermanaerovibrio} species. A phylogenetic analysis revealed that strain Nor1ᵀ adds to the list of bacterial genera that show Gram-negative-type cell walls but belong to the phylogenetic lineage of Gram-positive bacteria (Lee et al., 1978). By similarity percentage, the closest relative appears to be \textit{D. quercicolus} (Strömpel et al., 2000). \textit{D. quercicolus}, formerly \textit{Clostridium quercicolum} (Stankevich et al., 1971), was isolated from discoloured tissue of living oak trees, and its cells are spore-forming, peritrichously flagellated rods. \textit{D. quercicolus} is able to ferment fructose or glycerol, producing acetate and propionate. The G+C content of its genomic DNA is 52–54 mol%. Based on consensus tree topology, \textit{Selenomonas ruminantium} was the closest species (86–92% similarity) to strain Nor1ᵀ (Fig. 4). \textit{Selenomonas ruminantium} is an amino-acid-fermenting anaerobic bacterium, generally found in the digestive tract of mammals (Bryant, 1956). Neither \textit{D. quercicolus} nor \textit{Selenomonas ruminantium} was shown to grow on CO.

On the basis of its phenotypic and genotypic properties, we propose strain Nor1ᵀ as representative of the type species of a new genus and species, \textit{Thermosinus carboxydivorans} gen. nov., sp. nov.

**Description of \textit{Thermosinus} gen. nov.**

\textit{Thermosinus} (Ther.mo.sin’us. Gr. adj. thermos hot; L. masc. n. sinus bend; N.L. masc. n. \textit{Thermosinus} thermophilic curved rod).

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**Fig. 4.** Unrooted phylogenetic tree showing the position of strain Nor1ᵀ. The scale bar represents the expected number of changes per sequence position. Reliability values of internal branches are expressed as percentages. Hydrogenogenic carboxydrotrophs are shown in bold. GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are in parentheses.
Cells are motile, curved, non-spore-forming rods. Cell wall of Gram-negative type. Cells divide by binary transverse fission. Obligately anaerobic. Thermophilic. Neutrophilic. Ferment carbohydrates. DNA G+C content is 51.7 ± 1 mol%. The habitat is terrestrial hot spring.

The type species is Thermosinus carboxydivorans.

**Description of Thermosinus carboxydivorans sp. nov.**


Has the characteristics of the genus. Cells are curved rods with a length of 2.6–3 μm and a width of about 0.5 μm. Motile by means of lateral flagellation. Thermophile, grows in the temperature range 40–68 °C, with an optimum at 60 °C. Neutrophile, grows in the pH range 6.5–7.6, with an optimum at 6.8–7.0. Grows on glucose, sucrose, lactose, arabinose, maltose, fructose, xylose and pyruvate, but not on cellobiose, galactose, peptone, yeast extract, lactate, acetate, formate, ethanol, methanol or sodium citrate. During glucose fermentation produces acetate, H₂ and CO₂. Grows chemolithotrophically on CO. Utilizes CO as the sole energy source with equimolar formation of H₂ and acetate, formate, ethanol, methanol or sodium citrate. Does not utilize lactate, acetate, formate or H₂, neither in the absence nor in the presence of ferric iron, thiourea, sulfite, sulfite, elemental sulfur or nitrate. Growth is completely inhibited by penicillin, ampicillin, streptomycin, kanamycin and neomycin. The DNA G+C content is 51.7 ± 1 mol%.

The type strain is Nor1T (= DSM 14886T = VKM B-2281T); isolated from a hot pool at Norris Basin, Yellowstone National Park.

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anaerobic, CO-utilizing thermophile from Kamchatkan hot spring.


