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

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A new anaerobic, thermophilic, facultatively carboxydotrophic bacterium, strain Nor1^T, was isolated from a hot spring at Norris Basin, Yellowstone National Park. Cells of strain Nor1^T 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 Nor1^T 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 H_2 and CO_2 according to the equation $CO+H_2O \rightarrow CO_2+H_2$. During growth on CO in the presence of ferric citrate or amorphous ferric iron oxide, strain Nor1^T reduced ferric iron but produced H_2 and CO_2 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 Nor1^T on CO and none of these chemicals was reduced. Strain Nor1^T 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, H_2 and CO_2 were produced. Thiosulfate was found to enhance the growth rate and cell yield of strain Nor1^T when it was grown on glucose, sucrose or lactose; in this case, acetate, H_2S and CO_2 were produced. In the presence of thiosulfate or ferric iron, strain Nor1^T was also able to grow on yeast extract. Lactate, acetate, formate and H_2 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 Nor1^T 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 Nor1^T be assigned to a new genus, *Thermosinus* gen. nov. The type species is *Thermosinus carboxydivorans* sp. nov. (type strain, Nor1^T=DSM 14886^T=VKM B-2281^T).

INTRODUCTION

Several phylogenetically diverse thermophilic prokaryotes perform the metabolic reaction $CO+H_2O \rightarrow CO_2+H_2$ ($\Delta G^0=-20$ kJ). These are representatives of the bacterial genera *Carboxydothermus* (Svetlichny *et al.*, 1991, 1994), *Caldanaerobacter* (formerly *Carboxydibrachium pacificum*)

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Nor1^T is AY519200.

(Sokolova *et al.*, 2001; Fardeau *et al.*, 2004) and *Carboxydocella* (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 (Svetlitchny *et al.*, 2001; Dobbek *et al.*, 2001). This physiological group of prokaryotes has been proposed to be named 'hydrogenogens' (Svetlitchny *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* subsp. *pacificus*, isolated from a deep-sea hydrothermal vent in the Okinawa Trough, besides having the capacity for anaerobic CO oxidation/H_2 formation, is capable of growing on several fermentable substrates (Sokolova *et al.*, 2001). *Thermococcus* strain AM4 grows on CO producing H_2, or chemo-organotrophically with elemental sulfur (Sokolova *et al.*, 2004). Herein, we report the isolation of a novel anaerobic, CO-utilizing, H_2-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 50 °C).

Culture conditions and strains. Enrichment and isolation of anaerobic carboxydotrophic 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}): NH_4Cl, 1; $MgCl_2.2H_2O$, 0·33; $CaCl_2.6H_2O$, 0·1; KCl, 0·33; KH_2PO_4, 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 N_2 atmosphere. Yeast extract (0·2 g l^{-1}) and $NaHCO_3$ (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 $FeCl_3$ 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 $Na_2S.9H_2O$ (0·5 g l^{-1}).

The reference strains used in this study were *Carboxydothermus hydrogenoformans* Z-2901^T (DSM 6008^T) (Svetlichny *et al.*, 1991), *Carboxydocella thermautotrophica* 41^T (DSM 12326^T) (Sokolova *et al.*, 2002) and *Caldanaerobacter subterraneus* subsp. *pacificus* JM^T (DSM 12653^T) (Sokolova *et al.*, 2001; Fardeau *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 % OsO_4 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 % N_2 in the gas phase was used, supplemented with the following substrates (2 g l^{-1}): peptone, yeast extract, sucrose, 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). H_2S 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 CCG CA-3′). The amplified DNA fragment was purified using the Qiagen PCR purification kit (Qiagen) and sequenced by the dideoxynucleotide 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 (5′-CCC CGT CAA TTC ATT TGA GTT T-3′) 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/DDBJ accession numbers of the 16S rRNA sequences used in this study are given in Fig. 4.

RESULTS AND DISCUSION

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 % H_2 and 30 % CO_2. 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 CO_2 and production of H_2 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).

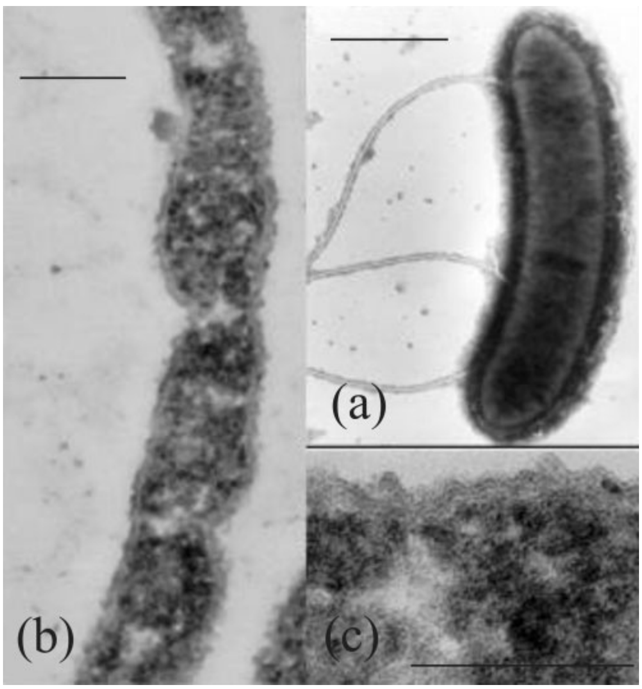

Fig. 1. Electron micrographs of cells of strain Nor1^T. Thin sections (b, c) and negative staining of whole cells (a). Bars, 0·5 μm.

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 H_2 and CO_2 formation in equimolar quantities according to the equation $CO + H_2O \rightarrow CO_2 + H_2$ (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 H_2 and CO_2 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 H_2 and CO_2 in nearly equimolar quantities. The isolate did not grow under an H_2/CO_2 (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,

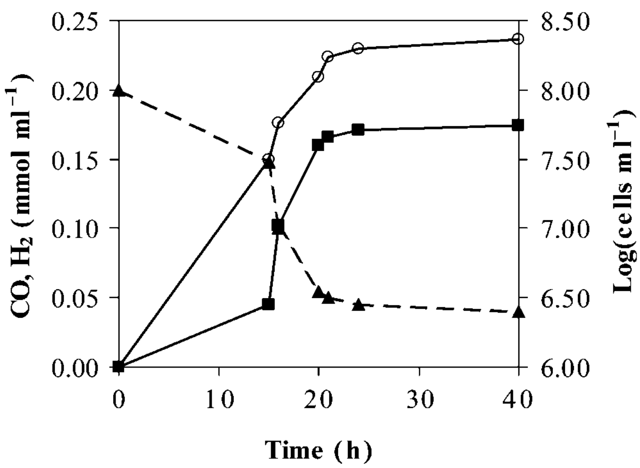

Fig. 2. Growth of strain Nor1^T at 60 °C in Medium 2 supplemented with 200 mg yeast extract l^{-1} under an atmosphere of CO: ○, cell number; ▲, CO consumption; ■, H_2 production. CO and H_2 are shown as their quantities in the gas phase per 1 ml of liquid culture.

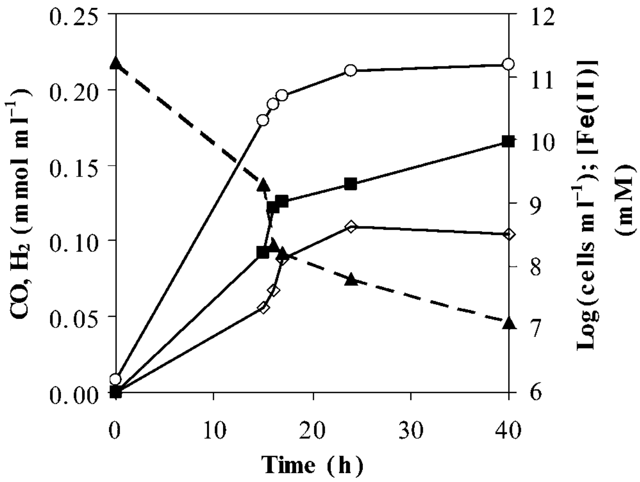

Fig. 3. Growth of strain Nor1^T at 60 °C in Medium 1 supplemented with 20 mM ferric citrate and 200 mg yeast extract l^{-1} under an atmosphere of CO: ○, cell number; ▲, CO consumption; ■, H_2 production; ◇, ferrous iron production. CO and H_2 are shown as their quantities in the gas phase per 1 ml of liquid culture.

arabinose, maltose, fructose, 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 mannitol. During growth on glucose, strain Nor1^T produced acetate, H_2 and CO_2. On Medium 1 (without reduction with sodium sulfide), strain Nor1^T 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 H_2S, respectively. Thiosulfate was found to stimulate the growth rate and cell yield of strain Nor1^T on glucose. During growth on glucose in the presence of thiosulfate, H_2 was not produced; the products were acetate, CO_2 and H_2S. No growth or H_2S 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 Nor1^T with CO or on organic substrates.

No growth was detected under an atmosphere of H_2+CO_2 or H_2 + air.

Our tests of other hydrogenogenic carboxydotrophs, *Carboxydothermus hydrogenoformans*, *Carboxydocella thermautotrophica* 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 Nor1^T.

DNA base composition

The DNA G+C content of strain Nor1^T was 51·7 ± 1 mol%.

16S rRNA gene sequence analysis

Sequencing of the 16S rRNA gene from strain Nor1^T 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 quercicolus* (formerly *Clostridium quercicolum*) (Strömpl *et al.*, 2000) as the closest relative (89·6 % similarity). Other phyletic relatives of strain Nor1^T, such as *Acetonema* (88·1 %), *Sporomusa* (87·4 %) and *Selenomonas* (86·2 %) species, showed lower similarity values but phylogenetic analysis presented them clustered with strain Nor1^T (Fig. 4).

The ability to grow anaerobically on CO with the production of H_2 as the only reduced product was first observed in the mesophilic purple, non-sulfur bacteria *Rhodocyclus gelatinosus* and *Rhodospirillum rubrum* (Uffen, 1976; Bonam *et al.*, 1989). *Carboxydothermus 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 CO_2 and H_2 production were described (Svetlichny *et al.*, 1994; Sokolova *et al.*, 2001, 2004). Strain Nor1^T is similar to *Caldanaerobacter subterraneus* 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 Nor1^T differs from *Caldanaerobacter subterraneus* subsp. *pacificus* JM^T also in its cell morphology: Nor1^T cells are short, curved, motile rods, while *Caldanaerobacter subterraneus* 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 Nor1^T 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, H_2-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 Nor 1^T resembles *Thermanaerovibrio* species (Baena *et al.*, 1999; Zavarzina *et al.*, 2000), which are thermophilic, anaerobic organotrophs with vibrioid cells. As for strain Nor1^T, *Thermanaerovibrio*

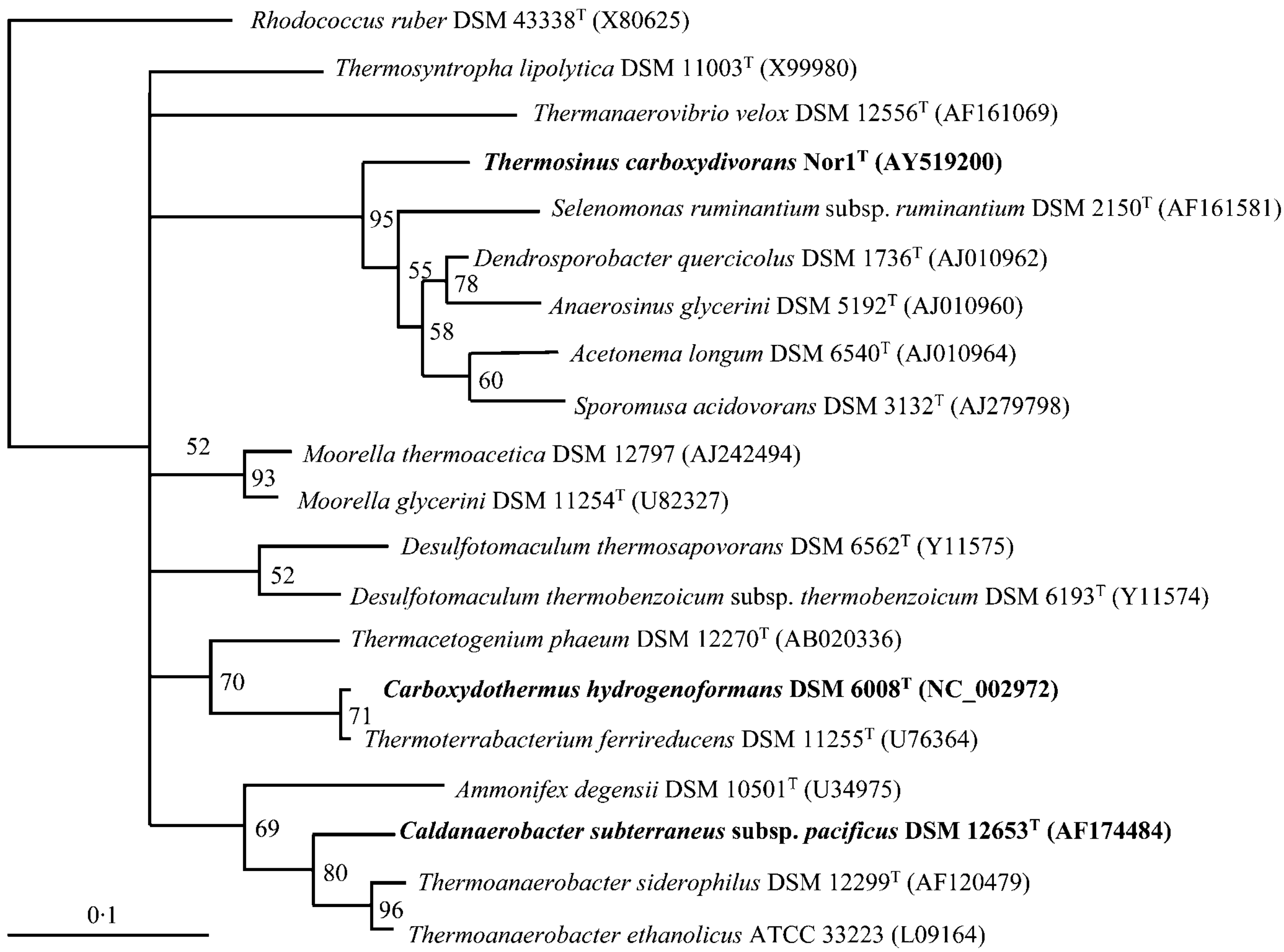

Fig. 4. Unrooted phylogenetic tree showing the position of strain Nor1^T. The scale bar represents the expected number of changes per sequence position. Reliability values of internal branches are expressed as percentages. Hydrogenogenic carboxydotrophs are shown in bold. GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are in parentheses.

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 H_2S (Zavarzina *et al.*, 2000). Strain Nor1^T differs from *Thermanaerovibrio* species by the ability to grow chemolithotrophically on CO, producing H_2 and CO_2, and the phylogenetic distance between Nor1^T strain and *Thermanaerovibrio* species. A phylogenetic analysis revealed that strain Nor1^T 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 *D. quercicolus* (Strömpl *et al.*, 2000). *D. quercicolus*, formerly *Clostridium quercicolum* (Stankewich *et al.*, 1971), was isolated from discoloured tissue of living oak trees, and its cells are spore-forming, peritrichously flagellated rods. *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, *Selenomonas ruminantium* was the closest species (86·2 % similarity) to strain Nor1^T (Fig. 4). *Selenomonas ruminantium* is an amino-acid-fermenting anaerobic bacterium, generally found in the digestive tract of mammals (Bryant, 1956). Neither *D. quercicolus* nor *Selenomonas ruminantium* was shown to grow on CO.

On the basis of its phenotypic and genotypic properties, we propose strain Nor1^T as representative of the type species of a new genus and species, *Thermosinus carboxydivorans* gen. nov., sp. nov.

Description of *Thermosinus* gen. nov.

Thermosinus (Ther.mo.sin′us. Gr. adj. *thermos* hot; L. masc. n. *sinus* bend; N.L. masc. n. *Thermosinus* thermophilic curved rod).

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.

Thermosinus carboxydivorans (car.bo.xy.di.vo′rans. N.L. neut. n. *carboxydum* carbon monoxide; L. part. adj. *vorans* devouring, digesting; N.L. part. adj. *carboxydivorans* digesting carbon monoxide).

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, H_2 and CO_2. Grows chemolithotrophically on CO. Utilizes CO as the sole energy source with equimolar formation of H_2 and CO_2 according to the equation $CO + H_2O \rightarrow CO_2 + H_2$. Reduces ferric iron during growth on CO, sucrose or lactose. Elemental sulfur, thiosulfate, sulfate and nitrate do not stimulate growth and these are not reduced during growth on CO. Thiosulfate enhances growth rate and cell yield during growth on glucose, sucrose or lactose; in this case, metabolic products are acetate, H_2S and CO_2. Does not utilize lactate, acetate, formate or H_2, neither in the absence nor in the presence of ferric iron, thiosulfate, sulfate, 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 Nor1^T (= DSM 14886^T = VKM B-2281^T); isolated from a hot pool at Norris Basin, Yellowstone National Park.

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