

# Isolation and characterization of a new CO-utilizing strain, *Thermoanaerobacter thermohydrosulfuricus* subsp. *carboxydovorans*, isolated from a geothermal spring in Turkey

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**Abstract** A novel anaerobic, thermophilic, Gram-positive, spore-forming, and sugar-fermenting bacterium (strain TLO) was isolated from a geothermal spring in Ayaş, Turkey. The cells were straight to curved rods, 0.4–0.6 µm in diameter and 3.5–10 µm in length. Spores were terminal and round. The temperature range for growth was 40–80°C, with an optimum at 70°C. The pH optimum was between 6.3 and 6.8. Strain TLO has the capability to ferment a wide variety of mono-, di-, and polysaccharides and proteinaceous substrates, producing mainly lactate, next to acetate, ethanol, alanine, H<sub>2</sub>, and CO<sub>2</sub>. Remarkably, the bacterium was able to grow in an atmosphere of up to 25% of CO as sole electron donor. CO oxidation was coupled to H<sub>2</sub> and CO<sub>2</sub> formation. The G + C content of the genomic DNA was 35.1 mol%. Based on 16S rRNA gene sequence analysis and the DNA–DNA hybridization data, this bacterium

is most closely related to *Thermoanaerobacter thermohydrosulfuricus* and *Thermoanaerobacter siderophilus* (99% similarity for both). However, strain TLO differs from *Thermoanaerobacter thermohydrosulfuricus* in important aspects, such as CO-utilization and lipid composition. These differences led us to propose that strain TLO represents a subspecies of *Thermoanaerobacter thermohydrosulfuricus*, and we therefore name it *Thermoanaerobacter thermohydrosulfuricus* subsp. *carboxydovorans*.

**Keywords** *Thermoanaerobacter* sp. · Geothermal springs · Thermophiles · Bacteria · *Thermoanaerobacter*

## Introduction

Diverse thermophilic heterotrophic anaerobes have been isolated from a variety of habitats. Members of the genus *Thermoanaerobacter*, in the order *Thermoanaerobacteriales*, are widely distributed in hydrothermal and oil-producing vents, volcanic hot springs, non-volcanic geothermally heated subsurface aquifers, soil, sugar beet, and sugar cane extraction juices (Klaushofer and Parkkinen 1965; Wiegel and Ljungdahl 1981; Schmid et al. 1986; Cayol et al. 1995; Cook et al. 1996; Kozianowski et al. 1997; Larsen et al. 1997; Slobodkin et al. 1999; Fardeau et al. 2000; Kim et al. 2001; Onyenwoke et al. 2007; Wagner et al. 2008). *Thermoanaerobacter* species are strictly anaerobic, thermophilic, rod-shaped bacteria, growing between 55 and 75°C, and most of them form round to oval terminal spores. *Thermoanaerobacter* species have been investigated for their sensitivity to different antibiotics but no differences have been found. A wide range of carbohydrates can be utilized by this group of organisms. Although the end products are mainly acetate,

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lactate, ethanol, H<sub>2</sub>, and CO<sub>2</sub>, the most abundant end product depends on the species and the growth conditions. Generally, thiosulfate can be used as electron acceptor in anaerobic respiration. Here we describe a new anaerobic thermophilic bacterium which belongs to the genus *Thermoanaerobacter*, and which differs from its closest relatives with respect to CO-utilization, lipid composition and fermentation pattern.

## Materials and methods

### Collection of the sample

Strain TLO was enriched from a mud sample obtained from a geothermal spring in Ayaş in Turkey. The geographical coordinates of the sampling site were 40.2° latitude, 32.4° longitude. The temperature and the pH of the sampling site was 75°C and 7.5 (at 75°C), respectively.

### Isolation

The mud sample (5 ml) was directly transferred to 50-ml sterilized bicarbonate-buffered medium in a 117-ml serum vial sealed with a butyl rubber stopper under a gas phase of N<sub>2</sub>/CO<sub>2</sub> (80/20, v/v). Cellobiose (20 mM) was used as electron donor. The enrichment culture was grown at 68°C and after growth, contained predominantly spore-forming rods. Isolation of the dominant bacterium was achieved by the soft-agar dilution method. The colonies, visible after three days of incubation, were uniformly round, 0.5–1.0 mm in diameter and white. They were picked from the highest dilution (10<sup>-8</sup>) with a sterile needle and subcultured in liquid medium containing 20 mM of cellobiose as a substrate. Serial agar and liquid dilutions in cellobiose-containing media were repeated until a pure culture was obtained. The pure culture was designated strain TLO (=DSM15750, =ATCC BAA-892).

### Media and cultivation

The composition of the BM medium used for routine growth and substrate utilization experiments were based on medium 120 of the DSMZ (<http://www.dsmz.de>), with the following modifications: casitone, methanol and cysteine-HCl were omitted and the amount of yeast extract was either lowered to 0.25 g l<sup>-1</sup> or not added for growth experiments. Routinely, cellobiose (20 mM) was used as the carbon and energy source. The pH of the medium (standard pH was 6.7) was adjusted using temperature corrected pH standards and by injecting calculated amounts of sterile Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> or HCl from the sterile, anaerobic stock solutions. The medium was boiled and

cooled to room temperature under a stream of O<sub>2</sub>-free N<sub>2</sub> gas. The medium was anaerobically dispensed into serum bottles under a N<sub>2</sub>/CO<sub>2</sub> (80/20, v/v) gas atmosphere. The bottles were closed with butyl rubber stoppers sealed with crimp seals. The medium was autoclaved for 20 min at 121°C. Prior to inoculation, the medium was reduced with sterile stock solutions of Na<sub>2</sub>S, 7–9 H<sub>2</sub>O and NaHCO<sub>3</sub> to obtain final concentrations of 0.04 and 0.2%, respectively.

The pure culture of strain TLO was either maintained by weekly transfer of a 1% (v/v) inoculum to fresh medium or inoculated from frozen glycerol stocks. Bottles were incubated in the dark without shaking. To investigate and optimize lactate production, some experiments were performed with phosphate-buffered medium 640 of the DSMZ (PB medium), which enabled a lower pH between 4.0 and 6.7. The reference strains used in this study were *Thermoanaerobacter siderophilus* (DSM 12299) (Slobodkin et al. 1999) and *T. thermohydrosulfuricus* (DSM 567) (Klaushofer and Parkkinen 1965).

Growth on different substrates was measured as the optical density at 600 nm (OD<sub>600</sub>).

Uninoculated medium served as a reference. The results represent duplicate experiments. The optimal growth temperature was determined by following growth at various temperatures between 35° and 85°C, with 5-degree intervals.

### Preparation of cell-free extract and enzyme measurement

Cell extracts used for enzyme assays were obtained from cells grown in the PB medium supplemented with 25% of CO as electron donor. The preparation of cell extracts was performed under anoxic conditions in an anaerobic glove box. Cells were collected by centrifugation at 10,000g for 10 min at 4°C. The cell pellet was suspended (1:2 [w/v]) in 15 mM potassium/sodium phosphate buffer, pH 7.2. The cells were disrupted by ultrasonic disintegration (Sonics & Materials Inc., Danbury, Conn.) at 40 Kc/s for 30 s followed by cooling for 30 s on ice. The cycle was repeated four times. Cell debris and whole cells were removed by centrifugation at 13,000g for 10 min at 4°C. The supernatant was used for enzyme assays. Carbon monoxide dehydrogenase (CODH) activity was assayed at the temperatures between 60 and 80°C by following the CO-dependent reduction of oxidized methyl viologen (MV) as described by Svetlitchnyi et al. (2001). One unit of CO oxidation activity was defined as the amount of enzyme that catalyzes the reduction of 2 μmol of MV min<sup>-1</sup>, which is equivalent to 1 μmol of CO oxidized min<sup>-1</sup>. The protein content of the cell extracts was determined according to the method of Bradford (1976) with bovine serum albumin as a standard.

## Substrates and electron acceptors utilization tests

The ability of strain TLO to metabolize substrates was tested in the bicarbonate-buffered medium (BM). Substrates were added from sterile, anoxic concentrated stock solutions to final concentrations of 20 mM, unless otherwise indicated. To test the use of potential electron acceptors with glucose as electron donor, thiosulfate (20 mM), elemental sulfur (2%, w/v), sodium sulfite (5 mM), FeCl<sub>3</sub> (10 mM), Fe(III)-NTA (10 mM), Fe(III)-citrate (10 mM), MnO<sub>2</sub> (5 mM), anthraquinone-2,6-disulfonate (AQDS) (20 mM) and arsenate (10 mM), sulfate (20 mM), nitrate (20 mM) or selenate (10 mM) was added to the medium at the indicated concentrations. The use of the electron acceptors was examined by following the optical density (600 nm) of the culture, detection of sulfide production (for sulfate, thiosulfate, sulfite and elemental sulfur), change of visible color (for AQDS) and measurements of the reduction of Fe(III) or formation of a precipitate (for MnO<sub>2</sub>) in the medium.

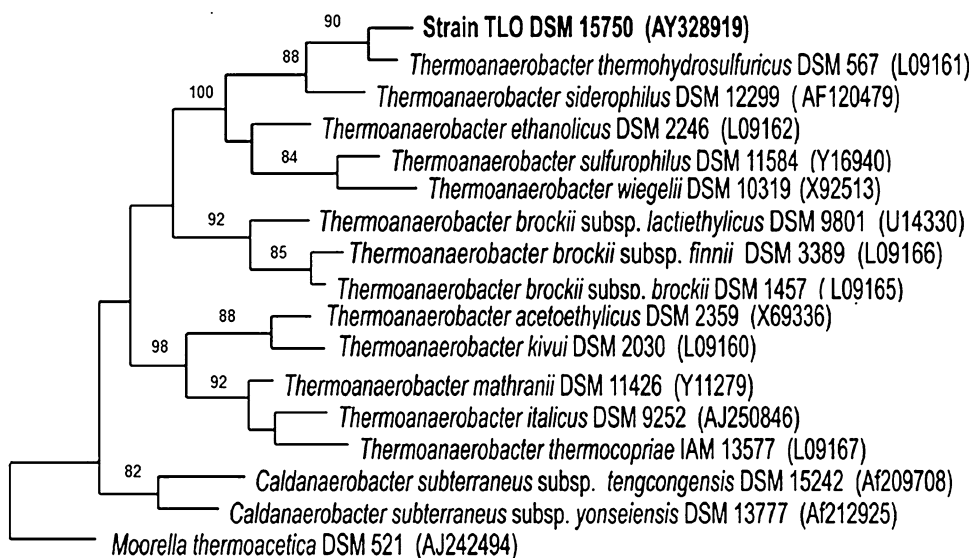
To test the utilization of CO, the bacterium was grown in 117-ml serum vials that contained 50 ml of the PB medium and that were sealed with butyl rubber stoppers and aluminum caps. These vials with 50 ml of the PB medium were flushed with N<sub>2</sub>. The PB medium was supplemented with 0.2 g yeast extract l<sup>-1</sup> and trypticase was omitted. Before addition of CO, an underpressure (0.2 bar) was created in the vials and CO was added to give a volume percentage (vol%) in the gas phase of 0 to 60 vol%. Then, N<sub>2</sub> was added to a pressure of 120 kPa (100 kPa = 1 bar). The culture was incubated at 65°C and shaken at 100 rpm. The measurements represent four replicates for each duplicate culture grown at the indicated temperature.

## Phylogeny, DNA base composition and DNA reassociation

DNA was extracted and purified using the UltraClean Soil DNA kit (MoBio). PCR was performed with the bacterial primers 7f and 1510r (Lane 1991) by using the *Taq* DNA polymerase kit (Life Technologies) to amplify the bacterial 16S rRNA gene. The PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing of the complete 16S rRNA gene was performed at Westburg Genomics (<http://genomics.westburg.nl>). A total of 1,676 nucleotides of the 16S rRNA gene were sequenced. The sequences were checked with the alignment programs of the ARB package (Ludwig et al. 2004), and a rooted neighbor-joining tree (*Escherichia coli* 8, D83536 positions 38-1449) was constructed using *Moorella thermoacetica* as an outgroup species (Fig. 1). On-line similarity analysis of the 16S rRNA gene sequences was performed with the BLAST program at NCBI and EMBL databases.

The G + C content determination and the DNA–DNA hybridizations were performed at the DSMZ (Braunschweig, Germany). DNA was isolated and purified by chromatography on hydroxyapatite (Cashion et al. 1977) and the G + C content of strain TLO was determined using the HPLC method described by Mesbah et al. (1989); unmethylated lambda DNA (Sigma) was used as the standard. DNA–DNA hybridization was carried out as described by De Ley et al. (1970), with the modifications described by Huss et al. (1983) and Escara and Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument

**Fig. 1** Dendrogram showing the position of strain TLO among the members of the order *Thermoanaerobacteriales* and related bacteria. Phylogenetic analysis based on 16S rRNA gene sequences available from GenBank data bases (accession numbers are given in parentheses). The neighbor-joining tree was reconstructed from distance matrices; bootstrap values (1000 replication) greater than 50% are listed at branching points. Bar evolutionary distance of 0.10



0.10

Laboratories). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke 1992).

### Lipid analysis

Bacterial cultures of strain TLO, *T. thermohydrosulfuricus* and *T. siderophilus* grown on glucose in the BM medium were harvested by centrifugation (14,500g, 20 min, 4°C) and pellets were washed with distilled water. Lipids from lyophilized cells were extracted ultrasonically using a mixture of dichloromethane (DCM):methanol of 2:1 (v/v) and this procedure was repeated four times. The residue was saponified with 1 N KOH in 96% of methanol by refluxing for 1 h and subsequently neutralized and extracted with DCM. After addition of internal standards, the “free” and “bound” lipid extracts were methylated and silylated and subsequently analyzed by GC and GC-mass spectrometry (GC-MS).

### Analytical methods

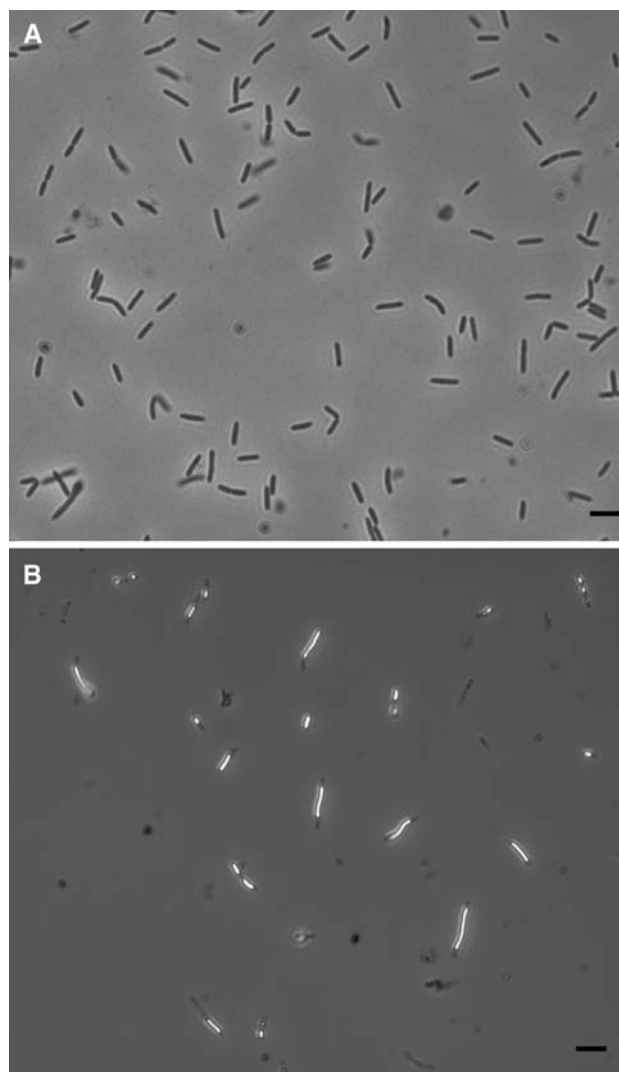
Most substrates were measured by HPLC as described previously by Stams et al. (1993). Gases and alcohols were measured by gas chromatography (Balk et al. 2003; Henstra and Stams 2004) and thiosulfate, nitrate and sulfate were analyzed by a HPLC system equipped with an Ionpac AS9-SC column and an ED 40 electrochemical detector (Dionex, Sunnyvale, CA) (Scholten and Stams 1995). Sulfide was analyzed by the method of Trüper and Schlegel (1964). Fe(III) reduction was determined by analyzing Fe(II) with the ferrozine method according to (Lovley and Phillips 1988).

To determine whether D- or L-lactic acid was produced, an enzymatic test kit (Enzytec, Scil Diagnostics GmbH) was used which involved D- or L-lactate dehydrogenase as the discriminating enzyme (From chicken heart, Sigma).

## Results

### Morphology

Cells of strain TLO were straight to slightly curved rods, 0.4–0.6 µm in diameter and 3.5–10 µm in length depending on the growth phase (Fig. 2a). The cells stained Gram-positive (Murray et al. 1994), occurred singly, in pairs or in long chains. Spores were terminal, round, heat resistant endospores. In the presence of thiosulfate and at acidic pH values, elemental sulfur was deposited inside the cells, which was visible as bright deposits under phase-contrast microscopy (Fig. 2b). Spores were usually found during the late exponential or early stationary growth phase in liquid cultures.



**Fig. 2** Phase-contrast micrograph of strain TLO (a). Elemental sulfur deposits in thiosulfate supplemented medium (b). Bar 2.5 µm

pH-, temperature-, and sodium chloride concentration ranges for growth

The pH of the medium was adjusted using temperature corrected pH standards and by injecting calculated amounts of sterile Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> or HCl from the sterile, anaerobic stock solutions. The effects of the pH and NaCl concentration were determined at the optimal temperature for growth. Under these conditions, strain TLO grew between 40°C and 80°C with optimum growth around 70°C, while no growth was detected at 35°C and 85°C. Growth was observed between pH 4.5 and 9, with optimum growth around pH 6.5 (pH 6.3–6.5). Growth occurred at NaCl concentrations ranging from 0 to 20 g l<sup>-1</sup>, with optimum growth at 5 g NaCl l<sup>-1</sup>. No growth was observed at 30 g l<sup>-1</sup>. Under



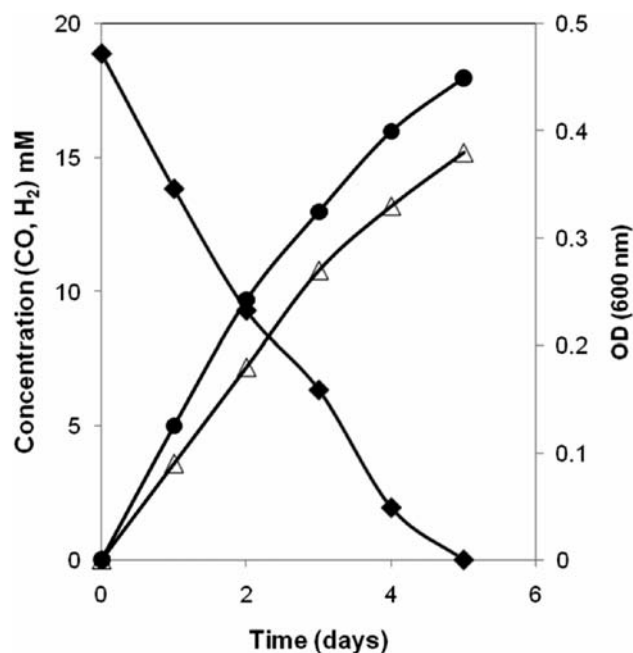
the optimal conditions of growth and in the presence of thiosulfate (20 mM), the doubling time of the isolate was about 70 min and the cell density ( $OD_{600}$ ) reached 0.8.

### Growth substrates and products

Strain TLO grew on the following substrates (at a concentration of 20 mM unless indicated otherwise) in the presence or absence of thiosulfate as the electron acceptor: yeast extract, peptone, beef extract, fructose, glucose, galactose, lactose, maltose, mannose, ribose, sucrose, xylose, xylan, starch, pectin, inulin, and cellobiose. Slow growth was also observed on mannitol and carboxymethylcellulose (CMC). No growth occurred with formate, acetate, lactate, methanol, ethanol, propanol, butanol, propionate, succinate, malate, glycine, alanine, arginine, cellulose, and lichenan.

Under standard conditions at pH 6.7, lactate was the major end product of sugar fermentation (in the absence of thiosulfate), but acetate, ethanol, alanine,  $H_2$ , and  $CO_2$  were formed as well, although in minor amounts. This metabolic profile was largely influenced by the growth conditions. At more acidic pH values, lactate production increased, and when the pH of the medium was between 4.5 and 5.0 around 1.7 mol lactate and 0.1 mol ethanol were produced per mol glucose fermented and acetate could not be detected. Increasing the pH up to 9.0 resulted in more acetate (1.6 mol/mol glucose) and less lactate (0.4 mol/mol glucose). Thiosulfate utilization also affected the metabolic patterns during sugar utilization. In the absence of thiosulfate, per mol of glucose 1.2 mol of lactate, 0.3 mol of acetate, 0.4 mol of ethanol, 0.3 mol of alanine, 0.2 mol of hydrogen, and 0.7 mol of  $CO_2$  were produced. In the presence of thiosulfate, the product formation from glucose of the strain changed with an increase in acetate production and a decrease in alanine production. Around 1.3 mol of acetate was produced per mol glucose fermented, and the other products were lactate (0.6 mol), ethanol (0.2 mol), alanine (0.02 mol), hydrogen (0.03 mol), and  $CO_2$  (1.5 mol). Under these conditions 1 mol of thiosulfate was reduced to 2 mol of sulfide. Remarkably, when the phosphate-buffered and  $N_2$ -flushed medium (PB medium; pH 5.8) was used, thiosulfate was converted to elemental sulfur instead of sulfide as in the BM medium.

Strain TLO was also found to grow in an atmosphere of up to 25% of CO as a sole electron donor. The bacterium either did not grow or did not completely oxidize CO at higher concentrations than 25% of CO in the gas phase. The complete conversion of CO was only observed in the bottles with CO up to 25%.



**Fig. 3** CO conversion by strain TLO performed at 65°C and shaken at 100 rpm with CO as sole substrate. The results represent the average of duplicate experiments. Carbon monoxide (filled diamond), hydrogen (filled circle) and OD (open triangle)

CO oxidation was coupled to  $H_2$  and  $CO_2$  formation (Fig. 3). In agreement with the observed CO-utilization, cell-free extracts were shown to exhibit CODH activity.

The maximum CODH activity in cell-free extracts at 60, 65, 70, 75, and 80°C was found to be  $66 \pm 4.7$ ,  $72 \pm 3.9$ ,  $46 \pm 2.9$ ,  $12 \pm 2.1$ , and  $2.2 \pm 0.17$  U mg of protein<sup>-1</sup>, respectively.

### Electron acceptors

In addition to thiosulfate (20 mM), elemental sulfur (2%, w/v), sodium sulfite (5 mM),  $FeCl_3$  (10 mM), Fe(III)-NTA (10 mM), Fe(III)-citrate (10 mM),  $MnO_2$  (5 mM), anthraquinone-2,6-disulfonate (AQDS) (20 mM), and arsenate (10 mM) could serve as electron acceptors. Sulfate (20 mM), nitrate (20 mM), and selenate (10 mM) could not be utilized.

### Yeast extract dependence

Yeast extract did affect product formation. When the yeast extract concentration was raised to 0.1% or higher, glucose fermentation was shifted from lactate and ethanol to more lactate and acetate formation.

Lactate produced from glucose, fructose, or maltose was mostly L-lactate with a content of more than 99% based on total lactic acid produced.

### Antibiotic susceptibility

Penicillin, ampicillin, streptomycin, and novobiocin at 100 µg ml<sup>-1</sup> did not inhibit growth. However, chloramphenicol, neomycin, and kanamycin completely inhibited growth at concentrations of 100 µg ml<sup>-1</sup> medium.

### DNA base composition

The G + C content of the DNA of strain TLO was found to be 35.1 mol%.

### Phylogeny and DNA reassociation

A total of 1,676 nucleotides of the 16S rRNA gene of strain TLO were sequenced from and compared with the members of the genus *Thermoanaerobacter* and related organisms. The closest phylogenetic relatives of strain TLO were *T. siderophilus* and *T. thermohydrosulfuricus* (formerly *Clostridium thermohydrosulfuricum*, Lee et al. 1993) (similarity of 99% for both) (Fig. 1).

DNA–DNA hybridization values (all in duplicate) between strain TLO and the two bacteria, *T. siderophilus* and *T. thermohydrosulfuricus*, were between 37% and 45%, and 72% and 76%, respectively.

### Lipid composition

The most abundant fatty acids of strain TLO, *T. thermohydrosulfuricus* and *T. siderophilus* were iso-C<sub>15:0</sub> and

iso-C<sub>17:0</sub>. Table 2 shows the lipid composition of strain TLO in comparison with phylogenetically closely related species.

### Discussion

Here we describe the characterization of a novel thermophilic, Gram-positive, anaerobic bacterium, which was isolated from a geothermal spring in Ayaş in Turkey. Based on the 16SrRNA sequence, strain TLO is phylogenetically closely related to *T. thermohydrosulfuricus* and *T. siderophilus* (Fig. 1), although its G + C content of 35.1 mol% is different from that of *T. hydrosulfuricus* (37.6%) and *T. siderophilus* (32.0%). Strain TLO shares several phenotypic features with its close relatives, like a broad substrate specificity, optimal pH- and T-range and the facultative use of thiosulfate.

However, several obvious differences were found as well (Table 1). First, in contrast to the type strain *T. thermohydrosulfuricus* and also *T. siderophilus*, strain TLO is able to use CO as electron donor, which is converted to H<sub>2</sub> and CO<sub>2</sub>. This feature is not new among Gram-positive anaerobes, and various recently isolated thermophiles have been shown to grow chemolithoautotrophically through the conversion of CO + H<sub>2</sub>O to H<sub>2</sub> + CO<sub>2</sub> (Svetlichnyi et al. 1994, 2001; Sokolova et al. 2001, 2002, 2004, 2005, 2007; Slepova et al. 2006). Utilization of CO has also been demonstrated for several representatives of a subdivision of the *Thermoanaerobacter* genus (Group 3) (Subbotina et al. 2003),

**Table 1** Phenotypic characteristics of strain TLO in comparison with phylogenetically closely related species

Feature	Strain TLO	<i>T. thermohydrosulfuricus</i>	<i>T. siderophilus</i>
Source	Geothermal spring	Extraction juices of beet sugar factory	Hydrothermal vent
Gram reaction	+	V	+
Spores	+	+	+
Temperature range (°C)	40–80	40–78	39–78
Optimum temperature (°C)	68–70	65–68	69–71
pH range	4.5–9	5–9	4.8–8.2
Optimum pH	6.3–6.8	6.9–7.5	6.3–6.5
DNA G + C content (mol%)	35.1	37.6	32
Reduction of arsenate	+	+ <sup>a</sup>	+ <sup>a</sup>
Growth substrates			
Ribose	+	+	NR
Mannitol	W	W <sup>a</sup>	V
Starch	+	+	+
Pectin	+	+	NR
Xylan	+	+	–
Carboxymethylcellulose	W	+	–
CO (up to 25% v/v)	+	– <sup>a</sup>	– <sup>b</sup>

Data for reference species were obtained from Klaushofer and Parkkinen (1965) and Slobodkin et al. (1999)

NR not reported, V variable, W weak

<sup>a</sup> Tested in this report; <sup>b</sup> Data from Gavrilov et al. (2003)

**Table 2** Lipid composition (in % of total quantified lipids) of strain TLO in comparison with phylogenetically closely related species

Lipids	Strain TLO		<i>T. thermohydrosulfuricus</i>		<i>T. siderophilus</i>	
	FLF	BLF	FLF	BLF	FLF	BLF
Summed concentration (mg/g dry weight)	6.7	2.0	17.4	2.9	8.6	0.6
<i>n</i> -C <sub>14</sub> FA	4	1	n.d.	n.d.	7	2
Iso-C <sub>15</sub> FA	39	25	81	60	3	40
Anteiso-C <sub>15</sub> FA	2	2	2	2	0	8
<i>n</i> -C <sub>16</sub> FA	5	1	2	2	19	5
Iso-C <sub>17</sub> FA	16	5	12	22	66	16
Anteiso-C <sub>17</sub> FA	2	1	1	2	5	4
<i>n</i> -C <sub>18</sub> FA	n.d.	n.d.	n.d.	n.d.	n.d.	1
Iso-C <sub>15</sub> -OH	10	n.d.	1	n.d.	<1	3
<i>n</i> -C <sub>16</sub> -OH	7	1	n.d.	n.d.	n.d.	1
Iso-C <sub>17</sub> -OH	9	9	1	n.d.	n.d.	n.d.
Anteiso-C <sub>17</sub> -OH	4	<1	n.d.	n.d.	n.d.	n.d.
$\alpha,\omega$ -13,16-Dimethyl-octacosanedioic acid	2	50	n.d.	12	n.d.	20
$\alpha,\omega$ -13,16-Dimethyl-triacontanedioic acid	n.d.	2	n.d.	n.d.	n.d.	n.d.
30-Hydroxy-13,16-dimethyl-triacontanoic acid	n.d.	3	n.d.	n.d.	n.d.	n.d.

The strains were grown in glucose-containing bicarbonate-buffered (BM) medium at 65°C for 24 h

*n.d.* not detected, *FA* fatty acid, *OH* alcohol, *FLF* free lipid fraction, *BLF* bound lipid fraction

which was recently reassigned to the genus *Caldanaerobacter* (Fardeau et al. 2004). For example, *Caldanaerobacter subterraneus* subsp. *tengcongensis* and *C. subterraneus* subsp. *pacificus* are able to use CO. However, among the more distantly related, true *Thermoanaerobacter* strains, CO-dehydrogenase activity has not been demonstrated or determined yet, with the exception of strain TLO, as shown here (Table 1). It is important to realize that the bacterium either did not grow or did not completely oxidize CO at concentrations higher than 25% (v/v) in the gas phase. Often, growth is only tested in the presence of 100% CO, which may be toxic and then gives rise to false conclusions regarding the ability to grow on CO. Moreover, levels of CO in natural hot environments are probably much lower, and microorganisms growing on CO in such environments are likely to be able to use very low CO concentrations. For example, *Carboxydotherrmus hydrogenoformans* consumed CO to below detectable levels of 2 ppm, when the CO<sub>2</sub> concentration was kept low (A.M. Henstra et al., unpublished data).

Second, significant differences in the lipid composition of strain TLO and the phylogenetically closely related species, *T. thermohydrosulfuricus* and *T. siderophilus*, were detected as shown in Table 2. In *T. siderophilus* the iso-C17 FA was substantially more abundant than in the TLO strain and *T. thermohydrosulfuricus*. Interestingly, in all three strains the uncommon membrane-spanning  $\alpha,\omega$ -13,16-dimethyl-octacosanedioic acid, previously detected in the phylogenetically related *T. ethanolicus* (Jung et al. 1994; Lee et al. 2002) was present in substantial amounts,

especially in strain TLO. Since increasing ethanol concentrations were found to be associated with high levels of C<sub>30</sub> fatty acids in *T. ethanolicus* (Burdette et al. 2002), the high tolerance to ethanol further verifies also the potential of strain TLO.

Strain TLO showed some additional features that are noteworthy. The use of thiosulfate as terminal electron acceptor is a common property of many thermophilic fermentative bacteria. Both hydrogen sulfide and elemental sulfur have been reported as reduced end product. The formation of elemental sulfur deposits was observed for *Thermoanaerobacter italicus* (Kozianowski et al. 1997) and *T. uzonensis* (Wagner et al. 2008), whereas *Thermoanaerobacter sulfurignens* (Lee et al. 2007) only produces elemental sulfur, similar to most species of *Thermoanaerobacterium* (Schink and Zeikus 1983; Lee et al. 1993). The ability to produce either sulfide or sulfur from thiosulfate has been proposed as a differentiating feature between the genera *Thermoanaerobacter* and *Thermoanaerobacterium* (Lee et al. 1993). However, for strain TLO we could show that the formation of hydrogen sulfide or sulfur is very much dependent on the type of medium and the pH. At pH 6.7 (BM-medium), sulfide was exclusively formed, whereas at pH 5.8 (PB-medium) substantial sulfur deposition was observed. The reason for this drastic change in thiosulfate reduction is not known, but it indicates that the ability to produce either hydrogen sulfide or sulfur is not as group-specific as previously thought.

The pH of the growth medium severely influenced the fermentation pattern. Whereas at basic and neutral conditions significant amounts of acetate, ethanol, and alanine were formed in addition to the major product lactate, at pH values between 4.5 and 5, an almost homolactic fermentation was observed. This latter feature combined with the ability to convert a wide array of substrates, and the growth on non-complex media, make this organism an interesting candidate for industrial lactic acid production.

In the absence of thiosulfate, strain TLO also produced substantial amounts of alanine (0.3 mol/mol glucose). Alanine has been reported before as fermentation end product in sugar-fermenting thermophiles, and it is regarded as a sink for reducing equivalents (Kengen and Stams 1994; Balk et al. 2002). Accordingly, in the presence of the electron acceptor thiosulfate, alanine formation was almost negligible (0.02 mol/mol glucose).

In the last two decades, intensive research on anaerobic, thermophilic, carbohydrate-fermenting microorganisms from marine and terrestrial volcanic hot springs has led to the isolation of several new genera and species in the domains Bacteria and Archaea. The major aim for this research stems from the biotechnological potential and the basic evolutionary traits of these microbes. CO-utilizing thermophilic microorganisms are able to grow by converting CO with water to H<sub>2</sub> and CO<sub>2</sub>. This feature makes these microorganisms interesting for cost effective hydrogen production. Hydrogen gas attracts great interest as a potential clean future fuel. Besides its potential as a future energy carrier, H<sub>2</sub> is a potent electron donor in various reductive processes, both in chemical and biotechnological applications. The use of thermophilic microorganisms for these processes could offer some advantages; although to date, few thermophiles are known that grow well on CO. The identification of new isolates that would broaden the product range of synthesis gas fermentations is desirable. Strain TLO can be one of the possible candidates for further research in this area.

In conclusion, a new thermophilic anaerobic bacterium is described that differs from its closest phylogenetic relatives, *T. thermohydrosulfuricus* and *T. siderophilus*, in the ability to use carbon monoxide, its G + C content, and fatty acids composition. The lipid profile of strain TLO is different from *T. thermohydrosulfuricus* and *T. siderophilus* and characterized by the predominance of membrane-spanning lipids. Moreover, in the absence of thiosulfate strain TLO produced almost entirely lactate instead of ethanol or acetate. These differences led us to propose that strain TLO represents a subspecies of *Thermoanaerobacter thermohydrosulfuricus*, and we therefore name it *Thermoanaerobacter thermohydrosulfuricus* subsp. *carboxydovorans*.

The description of *T. thermohydrosulfuricus* is as given by Klaushofer and Parkkinen (1965) with the following

modifications. Arsenate is used as electron acceptor. The most abundant fatty acids are iso-C<sub>15:0</sub> and iso-C<sub>17:0</sub> and the membrane-spanning lipids,  $\alpha,\omega$ -13,16-dimethyl-octacosanedioic acid, was also found. One of the subspecies of *T. thermohydrosulfuricus*, strain TLO, is able to grow on CO (<25% v/v).

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