Carboxydocella sporoproducens sp. nov., a novel anaerobic CO-utilizing/H₂-producing thermophilic bacterium from a Kamchatka hot spring

Tatiana V. Slepova,¹ Tatyana G. Sokolova,¹ Anatoly M. Lysenko,¹
Tatyana P. Tourova,¹ Tatyana V. Kolganova,¹ Olga V. Kamzolkina,²
Genady A. Karpov³ and Elizaveta A. Bonch-Osmolovskaya¹

Correspondence

Tatiana V. Slepova
slepysh@gmail.com

¹Winogradsky Institute of Microbiology, Russian Academy of Sciences, Prospect 60 Let Oktyabrya, 7/2, 117811 Moscow, Russia

²Lomonosov Moscow State University, Biology faculty, Vorob'evy gory, 119899 Moscow, Russia

³Institute of Volcanology and Seismology, Far-East Division Russian Academy of Sciences, Piip Boulevard, 9, 683006 Petropavlovsk-Kamchatsky, Russia

A novel anaerobic, thermophilic, CO-utilizing bacterium, strain Kar^T, was isolated from a hot spring of Karymskoe Lake, Kamchatka Peninsula. The cells of the novel isolate were Gram-positive, spore-forming, short rods. The bacterium grew chemolithoautotrophically on CO, producing equimolar quantities of H₂ and CO₂ (according to the equation CO + H₂O → CO₂ + H₂), and in the absence of CO, under N₂ in the gas phase, chemoorganoheterotrophically with yeast extract, sucrose or pyruvate. Growth was observed in the temperature range 50–70 °C, with an optimum at 60 °C, and in the pH range 6.2–8.0, with an optimum at pH 6.8. The micro-organism did not grow on solid media; it was able to grow only in semi-solid medium containing 0.5% agar. The generation time under optimal conditions for chemolithoautotrophic growth was 1 h. The G + C content of the DNA was 46.5 ± 1 mol%. Growth was completely inhibited by penicillin, novobiocin, streptomycin, kanamycin and neomycin. Analysis of the 16S rRNA gene sequence showed that the isolate should be assigned to the genus *Carboxydocella*. On the basis of the results of DNA–DNA hybridization and morphological and physiological analyses, strain Kar^T represents a novel species of the genus *Carboxydocella*, for which the name *Carboxydocella sporoproducens* sp. nov. is proposed. The type strain is Kar^T (= DSM 16521^T = VKM B-2358^T).

Utilization of CO as a sole source of energy and/or carbon, with the production of H₂, is peculiar to a group of phylogenetically and metabolically diverse representatives of the anaerobic, thermophilic prokaryotes. They have been described as representing novel bacterial genera, namely *Carboxydotherrmus* (Svetlichny *et al.*, 1991), *Carboxydobrachium* (now *Caldanaerobacter*) (Sokolova *et al.*, 2001; Fardeau *et al.*, 2004), *Carboxydocella* (Sokolova *et al.*, 2002), *Thermosinus* (Sokolova *et al.*, 2004a), *Thermincola* (Sokolova *et al.*, 2005) and the archaeal genus *Thermococcus* (Sokolova *et al.*, 2004b). Here we report the isolation of a novel CO-utilizing, H₂-producing thermophilic bacterium from a thermal spring of Karymskoe Lake (Kamchatka Peninsula).

At a depth of 4–6 m in the Tokarev Crater of the volcanic Lake Karymskoe (Kamchatka Peninsula), a fracture zone

was found to be producing multiple sites at which gas was bubbling at a temperature of 60 °C and a pH of 6.6 (the ambient temperature and pH were 6 °C and 4.5). Cyano-bacterial mats developed in the form of hemispheres covering the gas exits. Samples of water, mud and microbial mats from these hot sites were taken anaerobically in tightly stoppered bottles and were transported to the laboratory at ambient temperature.

For the enrichment and isolation of anaerobic carboxydo-trophic bacteria, the following basal medium (medium 1) was used (l⁻¹): NH₄Cl (0.66 g), MgCl₂·6H₂O (0.16 g), CaCl₂·6H₂O (0.1 g), KCl (0.33 g), KH₂PO₄ (0.5 g), resazurin (0.001 g), 1 ml trace-element solution (Pfennig & Lippert, 1966) and 1 ml vitamin solution (Wolin *et al.*, 1963). After being boiled, the medium was flushed with N₂ and cooled; NaHCO₃ (0.5 g l⁻¹) was added, and the pH was adjusted to 6.8–7.0 with 6 M HCl. Na₂S·9H₂O (1.0 g l⁻¹) was added to reduce the medium; 10 ml aliquots of the medium were placed into 50 ml bottles, and the head space was then filled with 100% CO at atmospheric pressure. For the enrichment

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of thermophilic, anaerobic, carboxydophilic bacteria, bottles containing liquid anaerobic medium and a CO gas phase were inoculated with about 1 g sample. After 2 days incubation at 60 °C, the bottles showed an increase in pressure from 100 to 160–170 kPa, and light microscopy (phase-contrast, oil immersion objective 90/1.25) revealed growth of rod-shaped bacteria.

CO utilization and the formation of gaseous growth products were studied using GLC as described previously (Sokolova *et al.*, 2002). The CO content in the gas phase decreased to 40 %, and about 30 % H₂ and 30 % CO₂ appeared. After several transfers in the same medium, the enrichments were serially diluted 10-fold and transferred to melted agar medium columns. No growth was observed in media solidified with 1, 2 or 3 % agar, under either CO or N₂, with or without the addition of organic compounds. After 3 days incubation at 60 °C, round white colonies were observed in semi-solid medium containing 0.5 % agar with the addition of 1 g pyruvate l⁻¹ under an atmosphere of N₂. Single colonies were transferred to liquid medium 1 under CO, and a pure culture, designated strain Kar^T, was obtained.

Cells of isolate Kar^T were straight, spore-forming rods varying in length from 1 to 6 µm and being about 0.5 µm in width (Fig. 1a). During spore formation, cells increased in width to 0.8 µm (Fig. 1b, c).

For electron microscopy, the cultures were fixed as described previously (Sokolova *et al.*, 2002) and examined under a JEM-100B microscope (JEOL). Electron microscopy of ultrathin sections revealed that the cell envelope was of the Gram-positive type, being composed of a cytoplasmic membrane and a double-layered cell wall consisting of an inner, electron-dense layer and an outer, lighter layer. Cells divided by means of binary transverse fission (Fig. 1a).

Fig. 1. Electron micrographs of cells of strain Kar^T. Bars, 0.5 µm.

Growth of the novel isolate was tested on different substrates in medium 1 with 100 % N₂ as the gas phase. Possible substrates were each added to a final concentration of 2 g l⁻¹. Possible electron acceptors were each added to a final concentration of 2 g l⁻¹ (except elemental sulfur, which was added to a final concentration of 10 g l⁻¹). A neutralized solution of ferric citrate or amorphous ferric iron oxide was added to medium 1 devoid of Na₂S.9H₂O to final concentrations of 20 or 90 mM, respectively. Amorphous ferric iron oxide was prepared by titrating a solution of FeCl₃ with 10 % (w/v) NaOH to pH 9.0.

Volatile fatty acids were determined on a Chrom-5 gas chromatograph with a flame-ionization detector; the column was filled with Chromosorb 10 (Sigma), the temperature was 170 °C and the carrier gas was argon at a flow rate of 40 ml min⁻¹. H₂S production was determined by the colorimetric method (Trüper & Schlegel, 1964) using a Beckman model 35 spectrophotometer at a wavelength of 670 nm.

Strain Kar^T grew only under strictly anaerobic conditions. It did not grow in medium lacking reducing agents or under a mixture of CO and air (4 : 1, v/v). It grew lithoautotrophically in an atmosphere of 100 % CO (0.23 mmol CO in the gas phase per 1 ml medium) on medium 1. CO oxidation was coupled to H₂ and CO₂ formation in equimolar quantities according to the equation CO + H₂O → CO₂ + H₂ (Fig. 2). Methane, acetate and other metabolic products were not detected. The generation time for strain Kar^T under optimal conditions was 1 h.

The effect of temperature on growth was studied in medium 1 under a CO atmosphere. The effect of pH on growth was studied under a CO atmosphere in the same medium, the pH being adjusted to different initial values with 6 M HCl.

Fig. 2. Growth of strain Kar^T at 60 °C in mineral medium under a CO atmosphere: ●, cell number; ▲, CO; ■, H₂. Quantities of CO and H₂ in the gas phase are given per ml liquid culture.

Temperature and pH optima were determined from the growth rates. The cell density was determined by direct cell counting. Growth of strain Kar^T occurred within the temperature range 50–70 °C, with an optimum at 60 °C. No growth was observed at 45 or 75 °C. Strain Kar^T grew at pH 6.2–8.0, with an optimum at pH 6.8; no growth was detected at pH 6.0 or 8.2.

The thermostability of spores was determined by boiling a spore-containing culture (10⁷ spores ml⁻¹) for 10–60 min. An aliquot of 0.1 ml boiled culture was then inoculated into medium 1 under an atmosphere of 100 % CO. Even after 60 min boiling, the spores produced growth in 2 days.

Strain Kar^T grew organotrophically with yeast extract, sucrose or pyruvate under N₂ in the gas phase. It did not grow on peptone, starch, cellobiose, glucose, arabinose, fructose, xylose, galactose, lactose, maltose, glycerol, acetate, citrate, succinate, formate, ethanol or methanol. A mixture of H₂ and CO₂ gas (4:1, v/v) did not support growth. No growth was observed on acetate, lactate, glycerol or xylose in the presence of sulfate, thiosulfate, sulfide, elemental sulfur or nitrate. Sulfate, thiosulfate, sulfur and nitrate were not reduced during growth with CO. Elemental sulfur, sulfide and nitrate inhibited growth. No growth or H₂S production occurred in medium with sulfate, thiosulfate, sulfide, elemental sulfur or nitrate with H₂ in the gas phase. Strain Kar^T showed growth and ferric iron reduction only after the first transfer to liquid mineral medium supplemented with amorphous ferric iron oxide under a CO atmosphere. In addition, the strain did not grow in the same medium in the presence of amorphous ferric iron oxide under an H₂ atmosphere.

The sensitivity of the novel isolate to penicillin (100 µg ml⁻¹), novobiocin (100 µg ml⁻¹), streptomycin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹) and neomycin (50 µg ml⁻¹) was tested in the same medium under a CO atmosphere. Penicillin, novobiocin, streptomycin, kanamycin and neomycin completely inhibited CO utilization and the growth of strain Kar^T.

DNA was isolated 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. The DNA G+C content of strain Kar^T was 49.5 ± 1 mol%.

16S rRNA gene sequence determination and analysis was carried out as for *Carboxydocella thermautotrophica* (Sokolova *et al.*, 2002). A partial 16S rRNA gene sequence (1430 nt) was determined for strain Kar^T, corresponding to positions 37–1467 in *E. coli* numbering. Preliminary comparisons (using BLAST) with 16S rRNA gene sequences available in GenBank revealed that the novel isolate, Kar^T, was a member of the *Bacillus/Clostridium* subphylum of Gram-positive bacteria. The highest similarity was found with the 16S rRNA gene sequence of *C. thermautotrophica* 41^T (99.5 % similarity). The level of DNA–DNA hybridization

was determined spectrophotometrically (De Ley *et al.*, 1970). There was 45 % DNA–DNA hybridization between strain Kar^T and *C. thermautotrophica* 41^T. Thus, we can conclude that strain Kar^T is a representative of a novel species of the genus *Carboxydocella*. This is confirmed by the physiological features of strain Kar^T. Strain Kar^T also differs from *C. thermautotrophica* in its capacity to form spores and its ability to grow organotrophically (Table 1) (*C. thermautotrophica* is non-spore-forming and obligately autotrophic). On the basis of its physiological and phylogenetic features, strain Kar^T represents a novel species of the genus *Carboxydocella*, for which we propose the name *Carboxydocella sporoproducens* sp. nov.

Description of *Carboxydocella sporoproducens* sp. nov.

Carboxydocella sporoproducens (spo.ro.pro.du'cens. Gr. n. *spora* a seed and, in biology, a spore; L. part. adj. *producens* producing; N.L. part. adj. *sporoproducens* spore-producing).

Has the characteristics of the genus. Cells are short, straight rods, about 0.5 µm in width and varying in length from 1 to 6 µm. Motile. Cell wall is of the Gram-positive type. Cells divide by binary transverse fission. Grows within the temperature range 50–70 °C, with an optimum at 60 °C. Growth pH ranges from 6.2 to 8.0, with an optimum at pH 6.8. Grows chemolithoautotrophically on CO. Grows organo-heterotrophically with yeast extract, sucrose or pyruvate under N₂ in the gas phase. Does not grow on peptone, starch, cellobiose, glucose, arabinose, fructose, xylose, galactose, lactose, maltose, glycerol, acetate, citrate, succinate, formate, ethanol or methanol or in an H₂/CO₂ gas mixture (4:1, v/v). Does not reduce elemental sulfur, sulfate or thiosulfate at the expense of H₂, acetate, lactate, glycerol or xylose or during growth with CO. Elemental sulfur, sulfide and nitrate inhibit growth. Growth is inhibited by penicillin, novobiocin, streptomycin, kanamycin and neomycin. The DNA G+C content of the type strain is 49.5 ± 1 mol%.

Table 1. Differential characteristics of strain Kar^T and *C. thermautotrophica* 41^T

Data for *C. thermautotrophica* 41^T were taken from Sokolova *et al.* (2002). Cells of both strains are short rods with a Gram-positive wall structure.

Characteristic	41 ^T	Kar ^T
Spore production	–	+
Carboxydotrophy	Obligate	Facultative
Optimum growth conditions:		
Temperature (°C)	58	60
pH	7.0	6.8
Capacity for growth on solid media	+	–
DNA G+C content (mol%)	46	49.5

The type strain, strain Kar^T (=DSM 16521^T=VKM B-2358^T), was isolated from a hot spring of Lake Karymskoe on the Kamchatka Peninsula.

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