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

Tatiana V. Slepova, Tatiana G. Sokolova, Anatoly M. Lysenko, Tatiana P. Tourova, Tatyana V. Kolganova, Olga V. Kamzolkina, Genady A. Karpov and Elizaveta A. Bonch-Osmolovskaya

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, Pip Boulevard, 9, 683006 Petropavlovsk-Kamchatsky, Russia

A novel anaerobic, thermophilic, CO-utilizing bacterium, strain KarT, 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, chemooorganoheterotrophically 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 KarT represents a novel species of the genus Carboxydocella, for which the name Carboxydocella sporoproducens sp. nov. is proposed. The type strain is KarT (= DSM 16521T = VKM B-2358T).

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 Carboxythermus (Svetlichny et al., 1991), Carboxydocella (now Caldanaerobacter) (Sokolova et al., 2001; Fardeau et al., 2004), Carboxydocella (Sokolova et al., 2002), Thermosinus (Sokolova et al., 2004a), Thermocina (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). Cyanobacterial 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 carboxydocellar 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. Na₂SO₄·H₂O (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...
of thermophilic, anaerobic, carboxydrotrophic 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 KarT, was obtained.

Cells of isolate KarT 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).

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 KarT 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 KarT 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. 1. Electron micrographs of cells of strain KarT. Bars, 0.5 μm.

Fig. 2. Growth of strain KarT 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 KarT occurred within the temperature range 50–70 °C, with an optimum at 60 °C. No growth was observed at 45 or 75 °C. Strain KarT 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 (10⁵ 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 KarT 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 KarT 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 KarT.

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 KarT 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 KarT, 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, KarT, 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 41T (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 KarT and C. thermautotrophica 41T. Thus, we can conclude that strain KarT is a representative of a novel species of the genus Carboxydocella. This is confirmed by the physiological features of strain KarT. Strain KarT 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 KarT 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 organoheterotrophically 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 KarT and C. thermautotrophica 41T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>41T</th>
<th>KarT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore production</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Carboxydotrophy</td>
<td>Obligate</td>
<td>Facultative</td>
</tr>
<tr>
<td>Optimum growth conditions:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
<td>6.8</td>
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<tr>
<td>Capacity for growth on solid media</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>46</td>
<td>49.5</td>
</tr>
</tbody>
</table>

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


