Carboxydocella thermautotrophica gen. nov., sp. nov., a novel anaerobic, CO-utilizing thermophile from a Kamchatkan hot spring

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INTRODUCTION

Anaerobic oxidation of CO coupled to equimolar hydrogen and CO₂ formation was first found in the purple non-sulfur, mesophilic, phototrophic bacteria Rubrivivax gelatinosus (Willems et al., 1991) (previously Rhodocyclus gelatinosus; Uffen, 1976, 1983) and Rhodospirillum rubrum (Bonam et al., 1989). Later, it was shown that the reaction \( \text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2 \) (\( \Delta G^\circ = -20 \text{ kJ} \)) might be performed by several phylogenetically diverse thermophilic bacteria. Among them, Carboxydothermus hydrogenoformans, isolated from terrestrial hot springs of the Kuril Islands, was the first taxonomically described representative (Svetlichny et al., 1991). Except for CO, the only substrate utilized by this organism was pyruvate. Another organism, ‘Carboxydothermus restrictus’, was isolated from a terrestrial hot vent on Raoul Island (Kermadek Archipelago, New Zealand) (Svetlichny et al., 1994). Carboxydobrachium pacificum was the first isolate found to originate from the deep-sea hot vents of the Okinawa Trough (Sokolova et al., 2001). In addition to being capable of anaerobic CO oxidation/\( \text{H}_2 \) formation, it could also ferment diverse organic substrates. All of these organisms are obligate or facultative lithotrophs, but have a requirement for yeast extract for growth. Here, we report the isolation of a novel CO-utilizing, \( \text{H}_2 \)-producing, thermophilic bacterium (from a thermal spring of the Geyser Valley, Kamchatka Peninsula) that is an obligate lithoautotroph.

METHODS

Sampling. Samples of hot water, mud and cyanobacterial mat from a hot spring (60 °C, pH 8.6) near the Giant Geyser (Geyser Valley, Kamchatka Peninsula) were taken anaerobically in tightly stoppered bottles and transported (at ambient temperature) to the laboratory.

Enrichment and isolation. For enrichment and isolation of anaerobic carboxydotrophic bacteria, the following medium (medium 1) was used (g l\(^{-1}\)): \( \text{NH}_4\text{Cl} \) (1); \( \text{MgCl}_2 \cdot 6\text{H}_2\text{O} \)
(0.33), CaCl₂, 6H₂O (0.1), KCl (0.33), KH₂PO₄ (0.5), resazurin (0.001), 1 ml trace-mineral solution (Kevbrin & Zavarzin, 1992) 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) and Na₂S₂O₃ (1.0 g l⁻¹) were then added and the pH was adjusted to 6.8 with 0.5 M HCl. Aliquots (10 ml each) of the medium were placed into 50 ml bottles and the heads were filled with 100% CO at atmospheric pressure. The incubation temperatures were 55 and 70 °C. Growth was determined using light microscopy and GLC detection of CO utilization and the formation of gaseous growth products. Positive enrichments were used for further purification. After a number of serial dilution transfers, colonies were obtained in roll-tubes prepared in 15 ml Hungate tubes on the same medium solidified with 5% agar, with CO in the gas phase. Well-separated colonies were transferred into the same liquid medium as that used for enrichments.

Light and electron microscopy. Light microscopy was carried out using a phase-contrast microscope with an oil-immersion objective (90/1.25). For electron microscopy, negative staining of whole cells was done 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. Embedded in Epon-812 resin, thin-sectioned and stained with uranyl acetate and lead citrate. Electron micrographs were taken with a JEM-100C electron microscope.

Physiology studies. Growth of the novel isolate was tested on different substrates in the same liquid mineral medium, with N₂ in the gas phase. Possible substrates were added individually at a concentration of 2 g l⁻¹ (final concentration). Possible electron acceptors were added at 2 g l⁻¹, and elemental sulfur at 10 g l⁻¹.

CO, CO₂, and H₂ were analysed using a Chrom-5 (CSFR) gas chromatograph equipped with a thermal conductivity detector and a 12 m glass column filled with activated carbon AG-3. The carrier gas was argon and the temperature in the incubator was 25 °C. 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.

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, except that the phosphate component of the medium was added in the form of 0.01 M phosphate buffer with the required pH.

Temperature and pH optima were determined from the growth rates. The cell density was measured by direct cell counting.

Sensitivity of the novel isolate to 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) was tested in the same medium under a CO atmosphere.

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

16S rDNA sequence determination and analysis. 16S rDNA was selectively amplified from genomic DNA by a PCR using 5'-AGAGTTTGTATCCTGCTAGCAG-3' as the forward primer and 5'-TACGGTTACCTGTTACGACTT-3' as the reverse primer (Lane, 1991). The PCR was carried out in 100 µl reaction mixture containing 1 µg DNA template, 200 µM (each) primers, 200 µM (each) dNTPs and 3 U Tet-z polymerase (BioMaster) in reaction buffer (100 mM Tris/HCl pH 8 ± 3, 500 mM KCl, 20 mM MgCl₂). The PCR was performed using the following programme: 30 amplification cycles of denaturation at 94 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 6 min. The PCR products were purified using a PCR-Prep kit (Promega) as recommended by the manufacturer. The 16S rDNA was sequenced in both directions by using a set of forward and reverse universal primers (Lane, 1991). DNA sequencing was performed by using Sequenase version 2 of the USB kit.

The 16S rDNA sequence was aligned with a representative set of 16S rRNA sequences obtained from the Ribosomal Database Project or from recent GenBank releases by using CLUSTALW. Positions that had not been sequenced in one or more reference organisms were omitted from the analysis. Pairwise evolutionary distances were computed by using the correction of Jukes & Cantor (1969). Phylogenetic trees were constructed by the neighbour-joining method using the programs of the TREECON package (Van de Peer & De Wachter, 1994), by the maximum-likelihood method using the PUZZLE program (Strimmer & von Haeseler, 1996) and by the maximum-parsimony method using program DNA pars of the PHYLIP package (Felsenstein, 1989) with bootstrap analysis of 100 trees.

RESULTS AND DISCUSSION

Enrichment and isolation

For the enrichment of anaerobic, carboxydotospheric, thermophilic bacteria, bottles with liquid anaerobic medium and a CO gas phase were inoculated with about 1 g sample. After 2 days incubation at 55 °C, two of the bottles showed an increase in pressure from 140 to 160–170 kPa. Growth of small rod-shaped bacteria was observed. The CO content in the gas phase had decreased to 40%, and about 30% H₂ and 30% CO₂ had appeared. After several transfers in the same medium, the enrichments were serially diluted 10-fold and transferred to solidified medium in roll-tubes. After 4 days incubation at 60 °C, round, white colonies, about 0.5 mm in diameter, were observed. Single colonies were isolated into liquid medium and two pure cultures were obtained. Strain 41₆ was selected for further characterization.

Morphology

Cells of isolate 41₆ were straight rods of various lengths from 1 to 3 µm and about 0.4–0.5 µm in width, arranged singly or in short chains of three to five cells. Cells were motile due to lateral flagella (Fig. 1a). 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-layer cell wall comprising an inner electron-dense layer and outer lighter layer. Cell membrane invaginations...
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were often observed (Fig. 1b, c). The cells divided by binary transverse fission (Fig. 1b).

Growth characteristics

Growth of strain 41\textsuperscript{T} occurred within the temperature range 40–68 °C, with an optimum at 58 °C. No growth was observed at 37 or 70 °C. Strain 41\textsuperscript{T} grew over a pH range of 6–7–6, with an optimum at 7–0, but no growth was detected at pH 6–2 or 7–8.

Strain 41\textsuperscript{T} grew only under strictly anaerobic conditions. It did not grow in medium without reducing agents, nor under a CO/air mixture (4:1, v/v). It grew in an atmosphere of 100% CO (0-23 mmol CO in the gas phase ml\textsuperscript{−1} medium) on the mineral medium without yeast extract or other organic compounds except vitamins. CO oxidation was coupled to H\textsubscript{2} and CO\textsubscript{2} formation in equimolar quantities according to the equation CO + H\textsubscript{2}O → CO\textsubscript{2} + H\textsubscript{2} (Fig. 2). Neither methane, acetate nor any other metabolic product was detected. The generation time of strain 41\textsuperscript{T} under optimal conditions was 1-1 h.

Strain 41\textsuperscript{T} did not grow on peptone, yeast extract, starch, cellobiose, sucrose, lactose, glucose, maltose, galactose, arabinose, fructose, acetate, formate, pyruvate, ethanol or methanol. A H\textsubscript{2}/CO\textsubscript{2} gas mixture (4:1, v/v) did not support growth. No growth was observed on acetate, ethanol or lactate in the presence or absence of elemental sulfur or sulfate. Neither sulfur nor sulfate was reduced during growth with CO. No growth or H\textsubscript{2}S production occurred in the medium with elemental sulfur or sulfate and H\textsubscript{2} in the gas phase.

Penicillin, ampicillin, streptomycin, kanamycin and neomycin completely inhibited CO utilization and the growth of strain 41\textsuperscript{T}.

DNA base composition

The G+C content of the DNA of strain 41\textsuperscript{T} was 46 ± 1 mol%.

Phylogenetic analyses

We determined a partial 16S rDNA sequence (1456 nt) for strain 41\textsuperscript{T}, corresponding to positions 37–1467 of the E. coli numbering. Preliminary comparisons (using BLAST) with 16S rDNA sequences available in GenBank revealed that the novel isolate 41\textsuperscript{T} was a member of the Bacillus/Clostridium subphylum of the Gram-positive bacteria. The highest score was found with 16S rDNA sequences of the Thermoanaerobacter–Syntrophomonas group, but it was no
more than 89%, so there were no sequences that were closely related to that of strain 41\textsuperscript{T}.

Several phylogenetic trees were constructed by changing the composition of reference organisms belonging to the *Clostridium* group. Regions of alignment uncertainty resulting from the presence of long inserts in 16S rDNA sequences of some members of this group (Rainey et al., 1993; Slobodkin et al., 1999) were omitted from the sequence analysis. A final comparison was carried out of 1322 nt of 16S rDNA sequences of strain 41\textsuperscript{T} and 57 of the closest reference strains of the *Thermoanaerobacter—Syntrophomonas* group, combined members of clusters V–VIII of the *Clostridium* spectrum (Collins et al., 1994). This group, including species of the genera *Desulfovomaculum*, *Thermoanaerobacter*, *Thermoanaerobacterium*, *Moorella*, *Anaerobranca* and some others, was used for the reconstruction of a phylogenetic tree and the calculation of sequence similarity.

The level of sequence similarity of strain 41\textsuperscript{T} was relatively low and almost equal for all reference strains (85.3–90.7%). In the phylogenetic tree constructed by using the neighbour-joining algorithm (Fig. 3), strain 41\textsuperscript{T} was not clustered exactly with any genus or species of the *Clostridium* group. Trees constructed using other treeing algorithms had the same topology (data not shown).

A variety of diverse bacteria possess CO-oxidizing enzymes, termed CO dehydrogenases, which allow them to assimilate CO as a sole source of carbon and energy. Aerobic carboxydotrophic bacteria grow heterotrophically on various organic compounds or chemolithoautotrophically on CO; they are taxonomically diverse, consisting of more than 15 described species from eight genera (Zavarzin & Nozhevnikova, 1977; Meyer et al., 1986, 1993).

In anaerobes, CO dehydrogenases are key enzymes of the acetyl-CoA pathway found in the following phylogenetically distant micro-organisms: aceticogenic bacteria (Wood & Ljungdahl, 1991; Ljungdahl, 1994; Drake, 1994), sulfate-reducing bacteria (Schauer et al., 1987; Janssen & Schink, 1995; Oude Elferink et al., 1999; Fukui et al., 1999), photosynthetic bacteria (Uffen, 1983; Ensign, 1995), methanogenic archaea (Deppenmeier et al., 1996; Fry, 1999) and non-methanogenic archaea (Möller-Zinkhan & Thauer, 1990; Vorholt et al., 1995, 1997). Some of them can grow on CO as the only energy source. During growth on CO, homooacetogenic and methanogenic bacteria respectively produce acetate and methane. CO is a common component of volcanic gases in terrestrial and submarine hot springs. Thus, the ability to utilize CO might be assumed to be a widespread capacity of thermophilic micro-organisms. However, only a limited number of thermophilic species are capable of anaerobic CO utilization. Among the thermophilic prokaryotes, CO-utilizing methanogens are represented only by *Methanothermobacter thermautotrophicus* (Zeikus & Wolfe 1972; Wasserfallen et al., 1981) and 57 of the closest reference strains to the *Clostridium* species from eight genera (Zavarzin & Nozhevnikova, 1977; Meyer et al., 1986, 1993).

![Fig. 3. Phylogenetic tree showing the position of strain 41\textsuperscript{T}](https://example.com/fig3.jpg)
Table 1. Characteristics of anaerobic, thermophilic, carboxydrotrophic bacteria

Data for reference species were taken from Svetlichny et al. (1991) (*Carboxydothermus hydrogenoformans* 2901T), Svetlichny et al. (1994) (*Carboxydothermus restrictus* R1) and Sokolova et al. (2001) (*Carboxydococcus pacificum* JM1).
neomycin. Does not grow on peptone, yeast extract, starch, cellulose, sucrose, lactose, glucose, maltose, galactose, arabinose, fructose, acetate, formate, pyruvate, ethanol, methanol or a H₂/CO₂ gas mixture (4:1, v/v). Does not grow on H₂, acetate, ethanol or lactate in the presence or absence of elemental sulfur or sulfate. Does not reduce elemental sulfur or sulfate during growth with CO. The G+C content of the DNA of the type strain is 46 ± 1 mol %. The type strain is strain 41T (= DSM 12356T = VKM B-2282T), isolated from a terrestrial hot vent of the Geyzer Valley, Kamchatka Peninsula.

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