

Thermincola carboxydiphila gen. nov., sp. nov., a novel anaerobic, carboxydotrophic, hydrogenogenic bacterium from a hot spring of the Lake Baikal area

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A novel anaerobic, thermophilic, alkalitolerant bacterium, strain 2204^T, was isolated from a hot spring of the Baikal Lake region. The cells of strain 2204^T were straight rods of variable length, Gram-positive with an S-layer, motile with one to two lateral flagella, and often formed aggregates of 3–15 cells. The isolate was shown to be an obligate anaerobe oxidizing CO and producing equimolar quantities of H₂ and CO₂ according to the equation CO + H₂O → CO₂ + H₂. No organic substrates were used as energy sources. For lithotrophic growth on CO, 0.2 g acetate or yeast extract l⁻¹ was required but did not support growth in the absence of CO. Growth was observed in the temperature range 37–68 °C, the optimum being 55 °C. The pH range for growth was 6.7–9.5, the optimum pH being 8.0. The generation time under optimal conditions was 1.3 h. The DNA G + C content was 45 mol%. Penicillin, erythromycin, streptomycin, rifampicin, vancomycin and tetracycline completely inhibited both growth and CO utilization by strain 2204^T. Thus, isolate 2204^T was found to be the first known moderately thermophilic and alkalitolerant H₂-producing anaerobic carboxydotroph. The novel bacterium fell within the cluster of the family *Peptococcaceae* within the low-G + C-content Gram-positive bacteria, where it formed a separate branch. On the basis of morphological, physiological and phylogenetic features, strain 2204^T should be assigned to a novel genus and species, for which the name *Thermincola carboxydiphila* gen. nov., sp. nov. is proposed. The type strain is strain 2204^T (= DSM 17129^T = VKM B-2283^T = JCM 13258^T).

Anaerobic, thermophilic, carboxydotrophic, hydrogenogenic prokaryotes are represented by several phylogenetically diverse prokaryotes which grow lithotrophically on CO, performing the metabolic reaction CO + H₂O → CO₂ + H₂ (ΔG⁰ = -20 kJ): *Carboxydotherrmus hydrogenofmans* (Svetlichny *et al.*, 1991), *Caldanaerobacter subterraneus* subsp. *pacificus* (Sokolova *et al.*, 2001; Fardeau *et al.*, 2004), *Carboxydocella thermautotrophica* (Sokolova *et al.*, 2002) and *Thermosinus carboxydivorans* (Sokolova *et al.*, 2004a). Recently, a hyperthermophilic archaeon of the genus *Thermococcus* able to grow by the same reaction was isolated from deep-sea hot vents (Sokolova *et al.*, 2004b). All these organisms are neutrophiles, growing in the pH range 6.5–7.8. To date, alkalitolerant, CO-utilizing,

H₂-producing, thermophilic anaerobes have not been reported. Here we describe a novel anaerobic, moderately thermophilic, moderately alkaliphilic, CO-oxidizing, H₂-producing bacterium isolated from an alkaline hot spring of the Baikal Lake area.

Samples of hot water, mud and cyanobacterial mats were taken from freshwater thermal springs of the Baikal Lake area; the pH of the water varied from 6.8 to 9.5, and the original temperatures were in the range 51–72 °C. The samples were taken anaerobically in tightly stoppered bottles and transported to the laboratory at ambient temperature.

Unless otherwise mentioned, the medium used for enrichments and cultures contained the following (g l⁻¹): NH₄Cl (1), MgCl₂·2H₂O (0.33), CaCl₂·6H₂O (0.1), KCl (0.33), KH₂PO₄ (0.5), 1 ml trace mineral solution (Kevbrin & Zavarzin, 1992), 1 ml vitamin solution (Wolin *et al.*, 1963) and resazurin (0.001). NaHCO₃ (0.5 g l⁻¹), Na₂CO₃

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(0.5 g l⁻¹), Na₂S.9H₂O (1 g l⁻¹) and sodium acetate (2 g l⁻¹) were added after boiling and cooling of the medium under the flow of nitrogen, and the pH was adjusted to 8.5–9.0 with 5 M HCl. Samples (10 ml) of the medium were placed into 50 ml bottles and the headspaces were filled with 100% CO at atmospheric pressure. The incubation temperature was 55 °C. Growth was determined using light microscopy (MBI-3 microscope; LOMO) and GLC detection (GLC-Chrom 5; Laboratorni Přístroje Praha) of CO utilization and gaseous growth-product formation (Sokolova *et al.*, 2001). After incubation at 55 °C on the medium with CO, several samples produced significant microbial growth. In most cases, short oval rods were predominant. From the sample of mud and cyanobacterial mat from a hot spring on the bank of the Bolshaya River (Barguzin Reserve), an enrichment culture was obtained that grew at pH 9.0 by the utilization of CO and the production of equimolar quantities of H₂ and CO₂. After 7 days incubation, in the stationary growth phase, the gas pressure increased 1.5–2-fold and the pH of the medium changed to neutral. The enrichment was 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 by 5% agar, with CO in the gas phase. Round, white, semi-transparent colonies (up to 1 mm in diameter) developed after 5 days incubation at 55 °C. Well-separated colonies were transferred to the same liquid medium as that used for the enrichment. A pure culture was obtained and designated strain 2204^T.

Electron microscopy (JEM-100C apparatus; JEOL) and light microscopy were carried out as described previously (Sokolova *et al.*, 2002). The ability of the novel isolate to use diverse energy substrates was tested with the same liquid mineral medium (both in the presence of 0.5 g yeast extract l⁻¹ and in its absence) containing possible substrates at 2 g l⁻¹ (final concentration), with 100% N₂ as the gas phase. Possible electron acceptors were added at a concentration of 2 g l⁻¹ (or 10 g l⁻¹ in the case of elemental sulfur). Ferric iron hydromorphic oxide (90 mM) was added to the same medium devoid of Na₂S.9H₂O. The cell density was determined by direct cell counting using a phase-contrast microscope at a magnification of × 675.

The influence of the following antibiotics (50 µg ml⁻¹) on growth was tested on the medium with CO as the gas phase: penicillin, erythromycin, streptomycin, rifampicin, vancomycin and tetracycline.

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

16S rRNA gene amplification, sequencing and sequence analyses were done as described previously (Sokolova *et al.*, 2002).

The cells of isolate 2204^T were straight, thick rods with rounded ends, about 0.5 µm wide and 0.6–3.0 µm long. The cells were motile and single or in short chains of three to five cells; sometimes cells formed aggregates of up to 15 cells. Spores were never observed in cultures. Cultures were not transferable after 5 min heat treatment at 100 °C. Electron microscopy of whole cells showed the presence of one or two lateral flagella (Fig. 1a). Ultrathin sections revealed Gram-positive cell-wall structure and a globular S-layer (Fig. 1b). The cytoplasmic membrane was often invaginated (Fig. 1b).

Growth of isolate 2204^T occurred within the temperature range 37–68 °C, the optimum being at 55 °C; no growth was observed at 30 or 70 °C. Strain 2204^T grew at pH values in the range 6.7–9.5; the pH optimum for growth was 8.0.

Isolate 2204^T grew in an atmosphere of 100% CO on medium containing 0.2 g yeast extract l⁻¹ or 0.2 g sodium

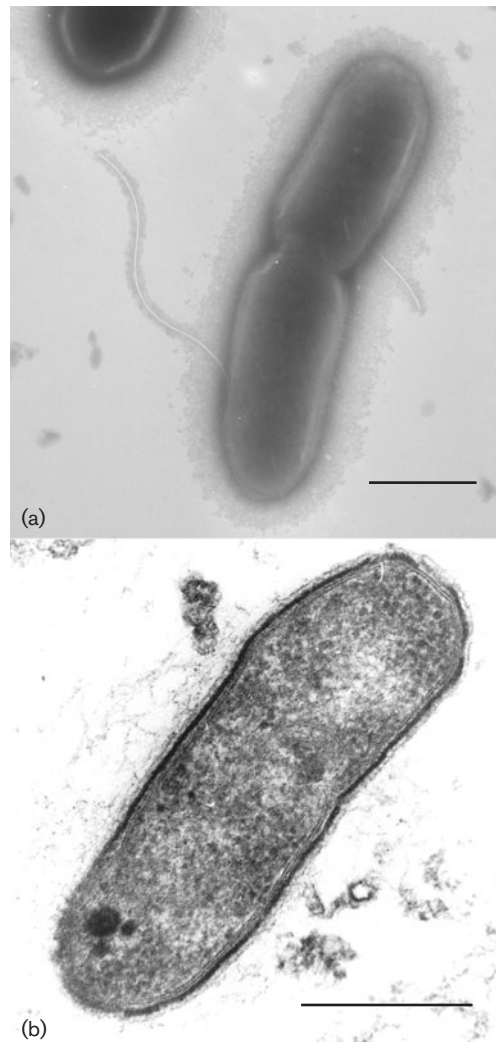


Fig. 1. Electron micrographs of cells of strain 2204^T. (a) Negative staining; (b) thin section. Bars, 0.5 µm.

acetate l^{-1} . CO oxidation was coupled with equimolar H_2 and CO_2 formation, like the CO oxidation of previously described anaerobic hydrogenogenic bacteria (Svetlichny *et al.*, 1991; Sokolova *et al.*, 2001, 2002). Neither methane formation nor acetate formation was detected during the growth of isolate 2204^T on CO. Either yeast extract or sodium acetate (0.2 g l^{-1}) was found to be required for growth. The generation time under optimal growth conditions (55°C , pH 8.0) was 1.3 h^{-1} . Strain 2204^T was unable to grow organotrophically on peptone or yeast extract or on starch, cellulose, cellobiose, sucrose, maltose, ribose, xylose, lactose, glucose, galactose, fructose, mannitol, sorbitol, pyruvate, acetate, formate, lactate, succinate, methanol, ethanol or glycerol. No growth was observed on an H_2/CO_2 gas mixture (80 : 20), on H_2 or CO with ferric iron or on H_2 or lactate with fumarate in the usual liquid mineral medium supplemented with 0.2 g sodium acetate l^{-1} and 0.5 g yeast extract l^{-1} . No growth was observed on peptone, yeast extract, sucrose, pyruvate, acetate, formate, lactate, succinate, methanol, ethanol or glycerol in the presence of elemental sulfur, sulfate, thiosulfate or ferric iron. Elemental sulfur, thiosulfate, sulfate and nitrate did not stimulate growth and were not reduced during growth on CO.

Penicillin, erythromycin, streptomycin, rifampicin, vancomycin and tetracycline completely inhibited both growth and CO utilization.

The DNA G + C content of strain 2204^T was $45.4 \pm 1 \text{ mol}\%$. The 16S rRNA gene sequence (1444 nt, corresponding to positions 38–1485 of *E. coli* numbering) was determined for strain 2204^T. Preliminary comparisons using BLAST (<http://www.ncbi.nih.gov/BLAST/>) performed with representatives of the domain *Bacteria* revealed that the novel isolate was a member of the *Bacillus*–*Clostridium* subphylum of Gram-positive bacteria but was not phylogenetically related to any named organism. The closest match (94% similarity) was with an uncultured clone, SHA-15, from an anaerobic 1,2-dichloropropane-dechlorinating mixed culture (Schlötterburg *et al.*, 2000). The closest organism of the species with validly published names (93% similarity) was *Pelotomaculum thermopropionicum* (Imachi *et al.*, 2000).

Several phylogenetic trees were constructed by changing the compositions of reference organisms belonging to the *Clostridium* group. Regions of alignment uncertainties due to the presence of long inserts in 16S rRNA gene sequences around positions 80, 1040, 1140 and 1440 (*E. coli* numbering) of some members of this group (Rainey *et al.*, 1993; Slobodkin *et al.*, 1999) were omitted from sequence analyses. According to the phylogenetic analysis, the novel strain 2204^T fell within the radiation of the family *Peptococcaceae*. A final comparison of 1327 nt of 16S rRNA gene sequences of strain 2204^T and 40 reference strains of the family *Peptococcaceae* was made and used for the reconstruction of a phylogenetic tree and the calculation of sequence similarity. In a phylogenetic tree constructed by using

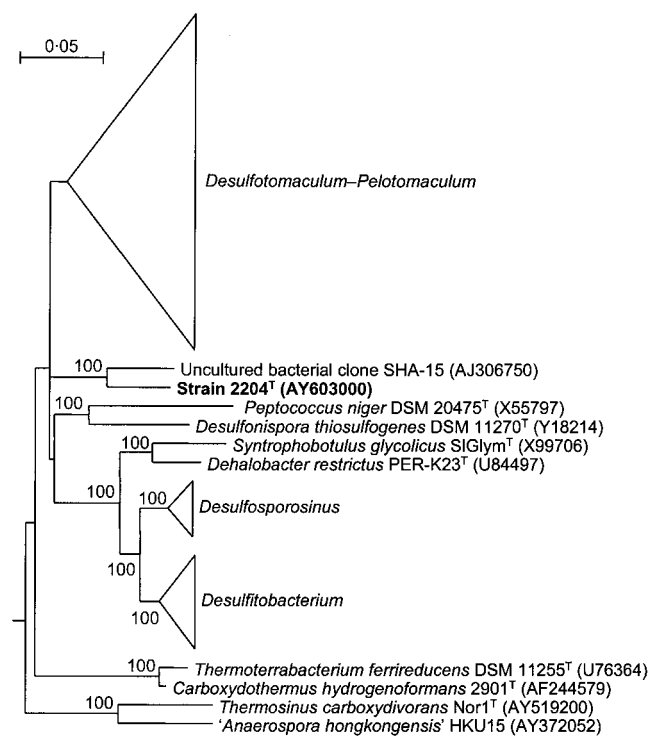


Fig. 2. Phylogenetic position of strain 2204^T in the tree of the family *Peptococcaceae*, constructed by the neighbour-joining method from a comparison of 16S rRNA gene sequences. *Clostridium butyricum* was used as the outgroup (not shown). Bootstrap values (from 100 replications) are shown at branch points; values greater than 95 were considered significant. Bar, 5 substitutions per 100 nt (Jukes & Cantor, 1969).

the neighbour-joining algorithm (Jukes & Cantor, 1969) (Fig. 2), strain 2204^T was not clustered exactly with any genus or species of the family *Peptococcaceae*, including the CO-utilizing hydrogenogens *Carboxydotherrmus hydrogenoformans* and *Thermosinus carboxydivorans*. The additional trees constructed by using other treeing algorithms, including option ‘transversion only’ for thermophilic organisms, had the same topology (data not shown). The level of sequence similarity of strain 2204^T was relatively low and almost equal for all reference strains (82.4–88.3%). Strain 2204^T formed a single cluster with maximal bootstrap support only with uncultured clone SHA-15 (Schlötterburg *et al.*, 2000); the level of similarity of complete 16S rRNA gene sequences was 92.9%, so this clone may represent a micro-organism phylogenetically related to strain 2204^T.

Strain 2204^T represents the physiological group of anaerobic carboxydophilic, hydrogenogenic thermophiles. CO was found to be the sole source of energy for this isolate. The same is true for *Carboxydocella thermautotrophica* (Sokolova *et al.*, 2002); however, strain 2204^T could not grow autotrophically, requiring acetate or yeast extract. All previously described carboxydophilic hydrogenogens are

Table 1. Characteristics of anaerobic, thermophilic, hydrogenogenic, CO-oxidizing prokaryotes

Micro-organism	Morphology	Cell-wall structure	Carboxydrotrophy	Optimum growth conditions		Minimum doubling time (h)	DNA G+C content (mol%)	Reference(s)
				Temperature (°C)	pH			
<i>Carboxydothermus hydrogenoformans</i>	Short, slightly curved rods with lateral flagella	Gram-positive, globular S-layer	Facultative	70–72	7.0	2	41	Svetlichny <i>et al.</i> (1991)
<i>Carboxydocella thermautotrophica</i>	Short, straight rods with peritrichous flagella	Gram-positive	Obligate	58	7.0	1.1	46	Sokolova <i>et al.</i> (2002)
<i>Thermosinus carboxydivorans</i>	Curved rods with lateral flagella	Gram-negative	Facultative	60	6.8–7.0	1.15	51.7	Sokolova <i>et al.</i> (2004a)
<i>Caldanaerobacter subterraneus</i>	Long thin rods, sometimes branched, non-motile	Gram-positive, globular S-layer	Facultative	70	6.8–7.1	7.1	33	Sokolova <i>et al.</i> (2001), Fardeau <i>et al.</i> (2004)
<i>Thermococcus</i> sp. AM4	Cocci	Layer of protein subunits*	Facultative	85	7.0*	3.1*	55	Sokolova <i>et al.</i> (2004b)
Strain 2204 ^T	Straight, thick rods of variable length with lateral flagella	Gram-positive, globular S-layer	Obligate	55	8.5	1.3	48	This work

*Unpublished results obtained by T. G. Sokolova.

neutrophiles; strain 2204^T, which grows at pH values in the range 6.7–9.5, is the first alkalitolerant representative of this group (Table 1). According to 16S rRNA gene analyses, strain 2204^T forms a new separate line of descent within the low-G + C-content Gram-positive subdivision of the *Bacteria* and could not be assigned to any genus with a validly published name. Thus, we propose a novel genus for it, namely *Thermincola*, with *Thermincola carboxydiphila* as the type species.

Description of *Thermincola* gen. nov.

Thermincola (Therm.in'co.la. Gr. adj. *thermos* hot; L. fem. n. *incola* inhabitant; N.L. fem. n. *Thermincola* inhabitant of a hot spring).

Cells are non-spore-forming rods. Cell wall is of the Gram-positive type. Anaerobic, thermophilic bacteria. Capable of growth by anaerobic CO oxidation, producing molecular hydrogen and CO₂. Does not grow by fermentation of organic substrates. The type species is *Thermincola carboxydiphila*. Habitat: terrestrial hot springs.

Description of *Thermincola carboxydiphila* sp. nov.

Thermincola carboxydiphila (car.bo.xy.di.phi'la. N.L. neut. n. *carboxydum* carbon monoxide; Gr. adj. *philos* loving; N.L. fem. adj. *carboxydiphila* loving carbon monoxide).

Cells are straight, thick rods, with rounded ends, about 0.5 µm wide and 0.6–3.0 µm long. Motile due to one or two lateral flagella. Cell wall is of the Gram-positive type. Obligately anaerobic. Grows in the temperature range 37–68 °C, with an optimum at 55 °C. Alkalitolerant: pH range from 6.7 to 9.5, with an optimum at pH 8.0. Grows chemolithotrophically on CO. Utilizes CO as sole energy source, with equimolar formation of H₂ and CO₂ according to the equation CO + H₂O → CO₂ + H₂. The presence of 200 mg yeast extract or acetate I⁻¹ is required for growth. Elemental sulfur, thiosulfate, sulfate and nitrate do not stimulate growth and are not reduced during growth on CO. Does not grow organotrophically on peptone, yeast extract, starch, cellulose, cellobiose, sucrose, maltose, ribose, xylose, lactose, glucose, galactose, fructose, mannitol, sorbitol, pyruvate, acetate, formate, lactate, succinate, methanol, ethanol or glycerol. H₂/CO₂ gas mixture (80:20) does not support growth. Does not grow on H₂ or CO with ferric iron. Does not grow on peptone, yeast extract, sucrose, pyruvate, acetate, formate, lactate, succinate, methanol, ethanol or glycerol, in the presence of elemental sulfur, sulfate, thiosulfate or ferric iron. Growth is completely inhibited by penicillin, erythromycin, streptomycin, rifampicin, vancomycin and tetracycline. The DNA G + C content of the type strain is 45.4 ± 1 mol%.

The type strain is strain 2204^T (= DSM 17129^T = VKM B-2283^T = JCM 13258^T), isolated from a hot spring of the Baikal Lake region.

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