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REVIEW

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Thermincola ferriacetica sp. nov., a new anaerobic, thermophilic, facultatively chemolithoautotrophic bacterium capable of dissimilatory Fe(III) reduction

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Abstract A moderately thermophilic, sporeforming bacterium able to reduce amorphous Fe(III)-hydroxide was isolated from ferric deposits of a terrestrial hydrothermal spring, Kunashir Island (Kurils), and designated as strain Z-0001. Cells of strain Z-0001 were straight, Gram-positive rods, slowly motile. Strain Z-0001 was found to be an obligate anaerobe. It grew in the temperature range from 45 to 70°C with an optimum at 57–60°C, in a pH range from 5.9 to 8.0 with an optimum at 7.0–7.2, and in NaCl concentration range 0–3.5% with an optimum at 0%. Molecular hydrogen, acetate, peptone, yeast and beef extracts, glycogen, glycolate, pyruvate, betaine, choline, *N*-acetyl-D-glucosamine and casamino acids were used as energy substrates for growth in presence of Fe(III) as an electron acceptor. Sugars did not support growth. Magnetite, Mn(IV) and anthraquinone-2,6-disulfonate served as the alternative electron acceptors, supporting the growth of isolate Z-0001 with acetate as electron donor. Formation of magnetite was observed when amorphous Fe(III) hydroxide was used as electron acceptor. Yeast extract, if added, stimulated growth, but was not required. Isolate Z-0001 was able to grow chemolithoautotrophicaly with molecular hydrogen as the only energy substrate, Fe(III) as electron acceptor and CO_2 as the carbon source. Isolate Z-0001 was able to grow with 100% CO as the sole energy source, producing H_2 and CO_2, requiring the presence of 0.2 g l^{-1} of acetate as the carbon source. The G + C content of strain Z-0001^T DNA G + C was 47.8 mol%. Based on 16S rRNA sequence analyses strain Z-0001 fell into the cluster of family *Peptococcaceae*, within the low G + C content Gram-Positive bacteria, clustering with *Thermincola carboxydophila* (98% similarity). DNA–DNA hybridization with *T. carboxydophila* was 27%. On the basis of physiological and phylogenetic data it is proposed that strain Z-0001^T (= DSMZ 14005, VKM B-2307) should be placed in the genus *Thermincola* as a new species *Thermincola ferriacetica* sp. nov.

Keywords Fe(III)-reduction · Acetate-oxidation · CO-oxidation · Thermophile · Magnetite formation

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Introduction

Sediments containing amorphous Fe(III)-oxides are common in hydrothermal environments, in sites where acidic hydrothermal fluid bearing dissolved ferric iron is mixing with neutral water. Up to 1995 only mesophilic microorganisms capable of Fe(III)-reduction were known. The ability of thermophiles to reduce Fe(III) was first shown for the enrichment culture growing with acetate as the energy substrate (Slobodkin et al. 1995). At the same time the first thermophilic Fe(III)- and Mn(IV)-reducing bacterium *Bacillus infernus* was described (Boone et al. 1995). Since then it has been found that many thermophilic bacteria and archaea are capable of performing organotrophic with fermentable substrates or chemolithoautotrophic growth with molecular hydrogen reducing Fe(III) to Fe(II) (Slobodkin et al. 1997; Vargas et al. 1998; Zavarzina et al. 2002; Kashefi et al. 2002; Gavrilov et al. 2003). Acetate is an important intermediate of anaerobic organic matter mineralization and the pathways of its oxidations are of major importance for the understanding of anaerobic

carbon cycle in microbial communities of thermal environments. Several thermophilic prokaryotes have been shown to use acetate as growth substrate, simultaneously reducing ferric iron to ferrous. Among them there are Gram-negative bacteria of genus *Deferribacter* (Greene et al. 1997; Takai et al. 2003; Miroshnichenko et al. 2003) and a novel member of genus *Geobacteraceae*, *Geothermobacter ehrlichii* (Kashefi et al. 2003). Recently it was found that acetate can be oxidized via ferrous iron reduction by hyperthermophilic archaea of *Euryarachaeota* kingdom such as *Ferroglobus placidus* and *Geoglobus ahangari* (Tor et al. 2001; Kashefi et al. 2002). All these organisms, with the only exception of *Deferribacter thermophilus*, obtained from the deep-subsurface thermal habitats, were isolated from the deep-sea hydrothermal habitats. The ability of Gram-positive thermophilic bacteria to oxidize acetate via iron reduction has been only reported for several strains of *Thermoanaerobacter ethanolicus* (Roh et al. 2002), although no growth and acetate utilization curves were presented. Here we report the isolation and characterization of a new thermophilic Gram-positive anaerobic Fe(III)-reducing, acetate-oxidizing bacterium *Thermincola ferriacetica* from a terrestrial hot spring of Kunashir Island, Kurils, Russia.

Materials and methods

Sampling

Samples of ochre deposits and water from the hydrothermal spring of Stolbovskiye group, Kunashir Island, Kurils were collected in August 1998. The pH of water at the sampling site was 6.8–7.0 and the temperature was 65°C. Samples were taken into glass vials with screw caps, without free volume and transported to the laboratory at ambient temperature, where they were stored at 4°C.

Enrichment

The initial enrichment was obtained by the inoculation of the anaerobically prepared medium with the slurry obtained by mixing of sediments and water from the hot spring of Stolobvskiye group. The composition of the basal medium used was (g l^{-1}): NH_4Cl, 0.33; KH_2PO_4, 0.33; $MgCl_2{\cdot}6H_2O$, 0.33; $CaCl_2{\cdot}6H_2O$, 0.33; KCl, 0.33; yeast extract (Difco), 0.05; $NaHCO_3$, 0.7; sodium acetate, 20 mM; trace element solution (Kevbrin and Zavarzin 1992), 1 ml l^{-1}; vitamin solution (Wolin et al. 1963), 1 ml l^{-1}. No reducing agent was added to the medium. The amorphous Fe(III) oxide was synthesized by titrating solution of $FeCl_3$ with 10% (w/v) NaOH (pH 9), with the final concentration of Fe(III) 90 mmol l^{-1}. The pH 7.0 was maintained with a CO_2–sodium bicarbonate buffer. The medium was dispensed into 15 ml Hungate tubes with screw caps, the head space (5 ml) filled with a N_2/CO_2 (8:2, v/v) gas mixture at atmospheric pressure. Inoculated tubes were incubated at 60°C.

Morphological studies

Light and electron microscopy were carried out as described previously (Zavarzina et al. 2002).

Growth characteristics

Organic substrates (peptides, alcohols and organic acids) at a concentration of 0.3% w/v and molecular hydrogen (100% gas phase) were added to the basal medium instead of acetate. The ability to ferment sugars and peptides was tested with medium without amorphous Fe(III) oxide and acetate and medium was pre-reduced with $Na_2S{\cdot}9H_2O$. When growth occured, three subsequent transfers on the same medium were performed. Utilization of alternative electron acceptors—sulfate, thiosulfate, sulfite, dithionite nitrate, fumarate, anthraquinone-2,6-disulfonate (AQDS), MnO_2, Fe(III) citrate and elemental sulfur was tested as described previously (Zavarzina et al. 2002). Furthermore synthetic magnetite (1% w/v) was added to the basal medium as possible electron acceptor. Magnetite was prepared by mixing of 0.5 M solutions of $FeSO_4$ and $Fe_2(SO_4)_3$ and mixture was titrated by 10% NaOH up to pH 9.0.

For testing growth on CO, 10 ml portions of basal medium with sodium acetate (0.2 g l^{-1}), yeast extract (0.2 g l^{-1}) and $Na_2S{\cdot}9H_2O$ (0.2 g l^{-1}) were placed into 60 ml bottles, and head space was filled with 100% CO at the atmospheric pressure. Chemolithoautotrophic growth with molecular hydrogen was tested on medium with the same mineral composition in the absence of yeast extract and amorphous Fe(III)-oxide as an electron acceptor. Cultivation was performed in 60 ml flasks with screw caps containing 10 ml of the medium. Head space (50 ml) was filled with 100% hydrogen.

Temperature and pH ranges were tested for cultures growing on basal medium with acetate as an electron donor and AQDS as an electron acceptor. The pH range for growth was determined at 60°C. The pH was adjusted with stock solutions of HCl (0.6 N) and NaOH (10%).

Analytical methods

Analytical methods used for description of new isolate were performed as described previously (after Zavarzina et al. 2000, 2002). The amounts of H_2 and CO were determined as described previously (Sokolova et al. 2002). Mössbauer studies of mineral phase were done as described previously (Chistyakova et al. 2002; Zavarzina 2004) DNA was isolated and purified as described by

Marmur (1961). The DNA G+C content was determined by the thermal denaturation method (Owen et al. 1969), *Escherichia coli* K-12 DNA used as a standard. For the DNA–DNA hybridization with the type species *Thermoincola carboxydophila*, DNA was obtained using a 'nick-translation' reaction based on [^{3}H]cytidine. The 16S rRNA gene amplification and sequencing were done as described previously (Zavarzina et al. 2000). The 16S rRNA gene sequence was aligned with a representative set of sequences obtained from GenBank by using CLUSTALW v 1.75 software. Positions that had not been sequenced in one or more reference organisms were omitted from analysis. Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (1969). The unrooted phylogenetic trees were constructed by neighbor-joining method by using the programs of the TREECON package (Van de Peer and De Wachter 1994).

Results and discussion

After 5 days of incubation of inoculated tubes with basal medium with ferric oxide, black magnetic sediment containing a significant amount of Fe(II) appeared. After several successive transfers, the enrichment was serially diluted in basal medium with 20 mM of AQDS substituting amorphous Fe(III)-oxide. Last dilution positive for AQDS reduction was serially diluted to extinction in roll-tubes with the same medium solidified with Bacto-agar, Difco (2.0 g l^{-1}). Single lens-shaped white-cream colonies, 0.2–0.4 mm in diameter, appeared after 3 days of incubation and were transferred to the liquid basal medium with amorphous Fe(III)-oxide. All isolates showed the same phenotypic characteristics: all of them reduced amorphous Fe(III)-oxide with acetate as an electron donors; were capable of growing with CO_2 as the only carbon source; had the same morphology. One of them designated as strain Z-0001^T was selected for the detailed characterization.

Morphology

Cells of strain Z-0001^T were straight to slightly curved rods, 0.4–0.5 μm in diameter and 1.0–3.0 μm in length. Single cells were observed in the medium with amorphous Fe(III)-oxide; when AQDS was used as an electron acceptor, cells occurred in large chains (4–50 cells in chain) (Fig. 1a). Cells exhibited a slight tumbling motility by means of one to four peritrichously located flagella (Fig. 1b). Strain Z-0001 ^{T} formed spores inside some of the cells in chains. Round spores of clostridial type were considerably larger in diameter than vegetative cells (from 2 to 3 μm) and were located in the middle of the cells. Spores were exceptionally thermoresistant and survived heating at 121°C for 30 min. Organism multiplied by binary fission (see *arrow* on Fig. 1a). Ultrathin sections showed a typical Gram-positive cell envelope profile with peptidoglycan layer in the cell wall (Fig. 1c).

Growth characteristics

Strain Z-0001^T was an obligate anaerobe and grew only in anaerobically prepared medium where O_2 was eliminated by boiling. In aerobically prepared medium and medium containing 1.5 or 2% O_2 no growth occurred. Growth of strain Z-0001^T occurred at temperatures ranging from 45 to 70°C, with an optimum between 57 and 60°C. The optimum pH for growth was 7.0–7.2. No growth occurred at pH lower than 5.9 or higher than 8.0. NaCl was not required for growth. Growth occurred at NaCl concentrations of up to 35 g l^{-1}.

Strain Z-0001 ^{T} grew with amorphous Fe(III)-oxide as an electron acceptor and molecular hydrogen, acetate, peptone, yeast and beef extracts, glycogen, glycolate, pyruvate, betaine, choline, *N*-acetyl-D-glucosamine and casamino acids as growth substrates. No growth was observed with Fe(III) on: adonite, arginine, butyrate, citrate, formate, glutamate, lactate, malate, propionate, serine, succinate, tartrate, ethanol, mannitol, methanol, propanol, L-dulcitol, L-sorbitol, L-histidine, glycerol, DL-lysine, sarcosine, tryptone, cellulose, xylane, chitin and starch. Strain Z-0001^T was not able to grow by fermentation of such sugars as L-arabinose, cellobiose, fructose, galactose, glucose, D-cellobiose, D-lactose, maltose, mannose, melibiose, raffinose, D-ribose, saccharose, sorbose, trehalose, D-xylose, peptone, yeast and beef extracts.

Growth of strain Z-0001^T on the medium with acetate 20 mM and yeast extract (0.05 g l^{-1}) as potential electron donors and amorphous Fe(III)-oxide was studied (Fig. 2). The number of cells increased proportionally to Fe(II) accumulation. The maximal cell density did not exceed 3.0×10^7 cell m l^{-1}; the maximal production of Fe(II) ranged between 22 and 25 mmol l^{-1}. Mössbauer investigations showed that magnetite was the only solid magneto-ordering phase that was formed during the growth of strain Z−0001^T. During the stationary phase of bacterial growth the magnetite structural changes took place when Fe^{2+} cations were occupying vacancies in octahedral position of the crystal lattice (Chistyakova et al. 2002; Zavarzina 2004).

Strain Z-0001^T reduced amorphous Fe(III) oxide, magnetite, AQDS and MnO_2 and did not reduce citrate, fumarate, sulfate, sulfite, thiosulfate, dithionite, elemental sulfur and nitrate on the basal medium with acetate as the electron donor. Molecular hydrogen supported the growth of isolate on the same medium with Fe(III) oxide, AQDS, MnO_2, thiosulfate as the electron acceptors.

It was also found that strain Z-0001^T could grow lithoautotrophicaly with Fe(III) when molecular hydrogen was added as an electron donor and CO_2—as the only carbon source. Lithoautotrophic growth of

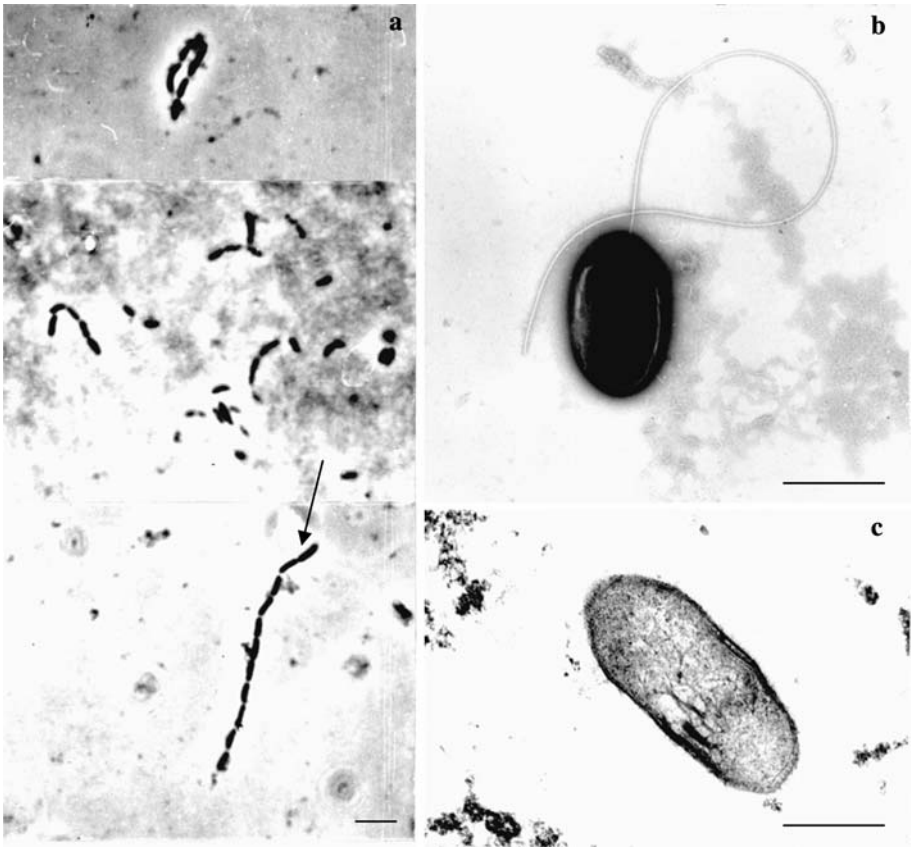

Fig. 1 a-c. Morphology of strain Z-0001^T. **a** Cells as viewed under phase-contrast microscope. *Bar* = 5 μm. **b** Negatively stained flagellated cell. *Bar* = 0.5 μm. **c** Longitudinal section shows that cell-wall structure is Gram-positive type. *Bar* = 0.5 μm

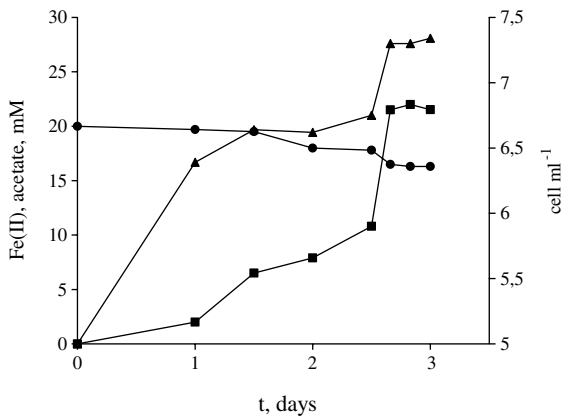

Fig. 2 Growth and Fe(II) production by strain Z-0001^T with acetate as an electron donor and amorphous Fe(III) oxide as an electron acceptor. Growth of strain Z-0001 (*filled triangle*), Fe(II) production (*filled square*) and acetate degradation (*filled circle*)

strain Z-0001^T was stable and did not decrease after three consequent transfers on the same medium.

Yeast extract when added stimulated growth of strain Z-0001^T

Strain Z-0001^T grew under 100% CO atmosphere on the medium containing 0.2 g l^{-1} of sodium acetate and 0.2 g l^{-1} of yeast extract. CO oxidation was coupled with equimolar H_2 and CO_2 production.

Chloramphenicol, neomycin, penicillin, kanamycin completely inhibited growth at a concentration of 100 μg l^{-1}. Polymyxin B and streptomycin at the same concentration did not inhibit growth.

DNA and phylogenetic analyses

The DNA G+C content of strain Z−0001^T was 47.8 ± 1 mol%. Almost complete sequence of the 16S rRNA (1444 nucleotides) of strain Z-0001^T covering the region between position 50 and 1498 (*E. coli* numbering) was determined. The preliminary analysis performed with representatives of the domain *Bacteria* revealed that the new isolate Z-0001^T was a member of the *Bacillus*/*Clostridium* subphylum of Gram-positive bacteria. Several phylogenetic trees were constructed by changing the spectrum of reference organisms. These trees demonstrated that strain Z-0001^T was a member of *Clostridium* group and fell into radiation of the family *Peptococcaceae*. A final comparison of the 16S rDNA sequences of strain Z-0001^T and 39 reference strains of the family *Peptococcaceae* up was carried out and used for reconstruction of the phylogenetic tree (Fig. 3) and calculation of sequence similarity. The tree showed that the closest relative of strain Z-0001^T was *T. carboxydophila* (Sokolova et al. 2005). A direct

comparison of 1444 nucleotides of the 16S rDNA sequence of strain Z-0001^T with *T. carboxydophila* was carried out and the sequence similarity was found to be 98.0%. DNA–DNA hybridization with *T. carboxydophila* DSM 17129 was 27 ± 1%.

An acetate-utilizing Fe(III)-reducing enrichment culture obtained from the ochre deposits in hot hydrothermal spring Stolbovskiye, Kuril Islands, was found to contain dissimilatory Fe(III)-reducing bacterium capable to oxidize anaerobically acetate, H_2 and a number of organic compounds. Thus, this organism could be involved in therminal reactions of organic matter mineralization in anaerobic microbial communities of terrestrial hot springs. 16S rRNA analysis placed the new isolate into a novel genus *Thermincola* (Sokolova et al. 2005), *T. carboxydophila* being its only close relative with 98% similarity. These results were in agreement with phenotypic characteristics of both microorganisms, as they both are "hydrogenogenic CO-trophs" (Svetlitchnyi et al. 2001) able to grow on CO with concurrent production of molecular hydrogen. This capacity was found to be widely spread among phylogenetivally diverse thermophilic anaerobes: new Gram-positive bacteria (Sokolova et al. 2002, 2004a) and hyperthermophilic archaea of genus *Thermococcus* (Sokolova et al. 2004b). However, strain Z-0001^T turned to be the first thermophilic bacterium capable both of dissimilatory iron reduction and the anaerobic growth on CO, coupled with hydrogen formation. The level of DNA–DNA hybridization between strain Z-0001^T and *T. carboxydophila* was 27 ± 1%, indicating that they belong to different species. This evidence was supported by significant phenotypic differences between the two organisms (Table 1): contrary to *T. carboxydophila*, strain Z-0001^T formed spores and, apart of growth on CO, was capable of performing chemolithoautotrophic and organotrophic growth with Fe(III) and several other electron acceptors. On the basis of physiological properties and phylogenetic analysis, we propose to describe strain Z-0001^T as a type strain of a new species of the genus *Thermincola*, namely *T. ferriacetica*.

Description of *T. ferriacetica* sp. nov.

T. ferriacetica (fer.ri.ace.ti.ca Latin *n. ferrum* iron; Latin *n. aceticum* acetate; Latin *part. adj. ferriacetica* iron oxide and acetate-utilizing).

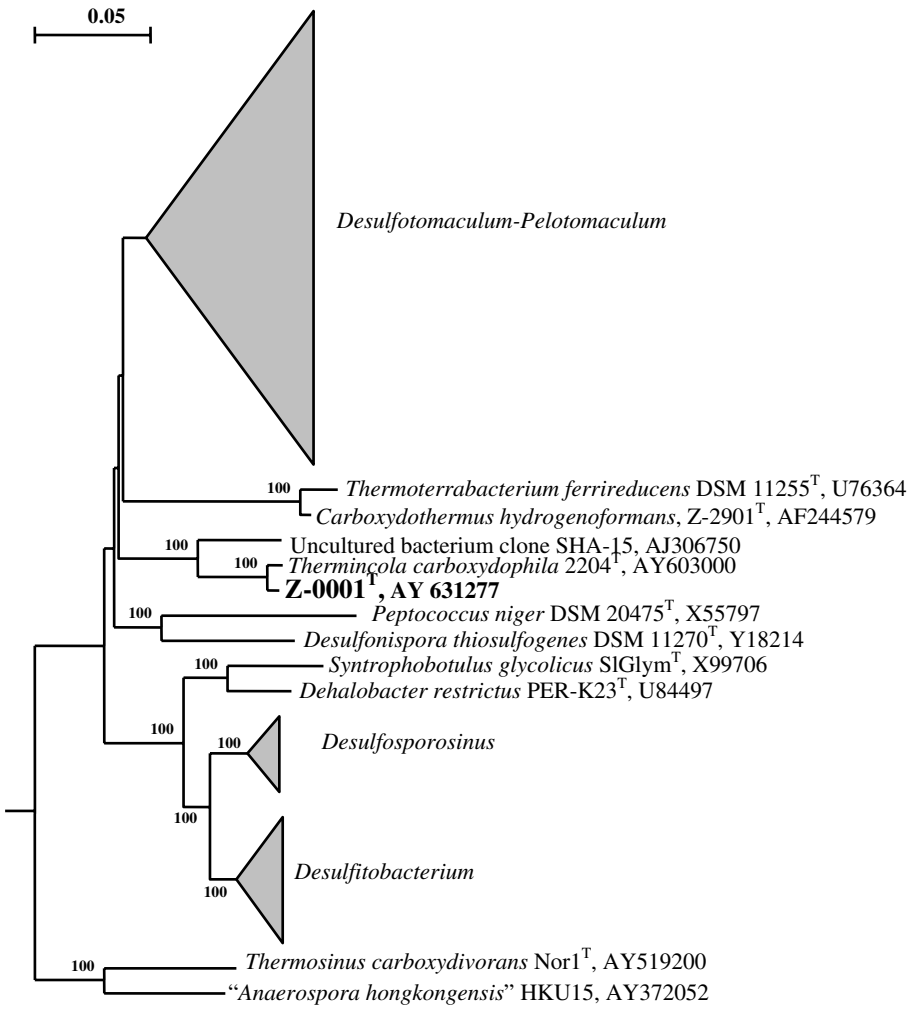

Fig. 3 Phylogenetic position of strain Z-0001^T in the tree of the family *Peptococcaceae*. Tree was constructed by the comparison of the 16S rRNA gene nucleotide sequences. *Clostridium butyricum* was used as the outgroup. Tree topography and evolutionary distances are given by the neighbor-joining method with Jukes and Cantor distances. Bootstrap values (from100 replicates) are shown as branch points; values greater than 70 were considered significant. Bar, 5 substitutions per 100 nt

Table 1 Characteristics that differentiate strain Z-0001^T from *Thermincola carboxydophila*

Characteristic	*Thermincola carboxydophila* (Sokolova et al. 2005)	Strain Z-0001^T
Morphology	Straight thick rods with rounded ends	Straight to slightly curved rods
Cell size (μm)	0.5 × 0.6–3.0	0.4–0.5 × 1.0–3.0
Spore formation	−	+
Temperature		
range (°C)	37–68	45–67
Optimum	55	57–60
pH		
Range	6.7–9.5	5.9–8.0
Optimum	8.0	7.0–7.1
NaCl range (%)	ND	0–3.5
G + C content of DNA	45.4 ± 1%	47.8 ± 1%
Oxidation with Fe(III) of		
Acetate	−	+
H_2	−	+

ND not determined

Cells are straight to slightly curved rods, 0.4–0.5 μm in diameter and 1.0–3.0 μm in length. Cells occur singly or in large chains. Cells exhibit a slight tumbling motility by means of one to four peritrichously located flagella. Form round spores of clostridial type in the middle of the rod-shaped cell. The cell wall has Gram-positive structure. Anaerobe. Growth occurs from 45 to 70°C, with an optimum between 57 and 60°C, and in a pH range from 5.9 to 8.0 with an optimum at pH 7.0–7.1. Growth occurs in NaCl concentration range of 0–3.5% with an optimum at 0%. Reduces amorphous Fe(III)-oxide by oxidation of molecular hydrogen, acetate, peptone, yeast and beef extracts, glycogen, glycolate, pyruvate, betaine, choline, *N*-acetyl-D-glucosamine and casamino acids. No growth occurs on: adonite, arginine, butyrate, citrate, formate, glutamate, lactate, malate, propionate, serine, succinate, tartrate, ethanol, mannitol, methanol, propanol, L-sorbitol, L-histidine, glycerol, DL-lysine, sarcosine, tryptone, cellulose, chitin and starch. Does not grow by fermentation of sugars, peptone, yeast and beef extracts. With acetate, apart from amorphous Fe(III) oxide, uses magnetite, AQDS and MnO_2 as the electron acceptors, but not citrate, fumarate, sulfate, sulfite, thiosulfate, dithionite, elemental sulfur and nitrate. With molecular hydrogen and yeast extract (0.2 g l^{-1}) it reduces Fe(III) oxide, AQDS, MnO_2, and thiosulfate. Grows chemolithoauthotrophicaly with hydrogen as the energy source, Fe(III) as the electron acceptor and CO_2 as carbon source. Yeast extract stimulates growth. Capable of growing on CO, producing H_2 and CO_2, in the presence of 0.2 g l^{-1} of yeast extract and acetate. Chloramphenicol, neomycin, penicillin, kanamycin, but not polymyxin B and streptomycin completely inhibit the growth.

The type strain is Z-0001^T (DSMZ 14005, VKM B-2307). The DNA G + C content is 47.8 ± 1 mol%. Source of isolation: ochre deposits in hot spring Stolbovskiye, Kuril Islands, Russia.

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