Isolation and characterization of a thermophilic, chemolithotrophic nitrate-reducing bacterium from deep-sea hydrothermal vents

Susan Ellor*, James Voordeckers, and Costantino Vetriani

Department of Biochemistry and Microbiology, and Institute of Marine and Coastal Sciences, Rutgers University, New Brunswick, New Jersey, 08901

* Rutgers Undergraduate Research Fellow

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Abstract

The nitrogen cycle at deep-sea hydrothermal vents has yet to be thoroughly studied. Novel thermophilic microorganisms that couple autotrophic CO_2 fixation with the reduction of nitrate to ammonia have been suggested in recent studies to be important for primary production and nitrogen cycling in marine geothermal environments (Blochl, *et al.* 1997, Alain, *et al.* 2002b, Huber, *et al.* 2002, Miroshnichenko, *et al.* 2004, Vetriani, *et al.* 2004). In the summer of 2001, hydrothermal samples were collected from the Rainbow deep-sea vent field on the Mid-Atlantic Ridge (MAR), and several novel thermophilic organisms were obtained in pure culture. Among these novel isolates, cells of strain TB-1 were found to be Gram-negative rods with optimal growth occurring at 55°C, slightly acidic pH, and a salt concentration lower than that of seawater. This bacterium was capable of chemolithoautotrophic growth by coupling H_2-oxidation to NO_3^- reduction, which was reduced to ammonium. Under these conditions the generation time of TB-1 was about 50 minutes.

Isolate TB-1 is phylogenetically related to the epsilon-proteobacteria (genus *Caminibacter*). The ecological significance of such deep-sea hydrothermal vent bacteria is twofold: 1) these organisms contribute to the primary productivity by fixing CO_2, and 2) their nitrate respiratory metabolism (namely, the reduction of NO_3^- to NH_4^+) implicates that nitrogen is conserved and recycled within the vent system.

Introduction

The epsilon-proteobacteria comprise a complex subclass of Gram-negative, microaerophilic and/or anaerobic, chemoorganoheterotrophic or chemolithotrophic bacteria that are found to survive in an assortment of habitats. In microbial ecology studies, it is important to assess the composition and structure of the microbial communities inhabiting a given niche. Molecular surveys of 16S rRNA genes (amplified from a natural community by PCR and then sequenced) are used to examine the diversity and composition of microbial communities as they occur in their natural environment. For example, using a molecular ecological approach, the epsilon-proteobacteria have been found to represent a relevant fraction of the microbial communities at deep-sea vents located on both the Atlantic and the Pacific oceans (Longnecker and Reysenbach 2001, Alain, *et al.* 2002a, Lopez-Garcia, *et al.* 2003). Results from these studies revealed that this bacterial group accounted for between 40% to 80% of the 16S rRNA clones sequenced.

Overall, these molecular studies revealed an unsuspected genetic diversity that lacked a counterpart in organisms that could be brought into culture in the laboratory. This discrepancy between the diversity observed in 16S rRNA clones and in cultured organisms is worthy of investigation, because it questions whether each organism is really well represented at the site of isolation, or if chance selection of a rare organism occurred, despite its relative insignificance in the population as a whole. In an effort to bring into culture some of these "unculturable" organisms, this study sought to bridge the existing gap in the epsilon-proteobacteria, whose sequence diversity has not matched the diversity of the organisms grown in pure cultures in the laboratory.

A thermophilic epsilon-proteobacterium was isolated from a deep-sea hydrothermal vent on Mid-Atlantic Ridge (MAR), and was designated as TB-1. This organism was selected as the subject of this research project because it represented a novel, cultured member of the frequently detected epsilon proteobacteria in 16S rRNA based diversity surveys of vent microbial communities.

This paper contributes to the morphological and physiological characterization of TB-1, a novel thermophilic member of the epsilon-proteobacteria.

Materials and Methods

Sample Collection

In 2001, samples of black smokers (polymetallic sulfide structures venting hot hydrothermal fluids at the bottom of the ocean) were collected using the deep-submergence vehicle *Alvin* at the Rainbow vent field, located on the MAR (36°14'N, 33°54'W), at a depth of depth 2,300 meters. The in-situ temperature of the fluids emitted from the black smoker was 158°C. We can assume that a temperature gradient occurs between the inside of the chimney (158°C) and the surrounding seawater (2°C), creating several ecological niches that can be colonized by microorganisms. The samples were stored at 4°C until enrichment were initiated in the laboratory.

Culture Medium

The isolate was enriched and routinely cultivated in strictly anaerobic medium, which contained (per liter of solution): NaCl, 20.0 g; $MgSO_4 \cdot 7H_2O$, 3.5 g; $MgCl_2 \cdot 6H_2O$, 2.75 g; KCl, 0.325 g; KNO_3, 1.0 g; $CaCl_2 \cdot 2H_2O$, 0.750 g; KH_2PO_4, 0.5 g; NaBr, 0.05 g; H_3BO_3, 0.015 g; $SrCl_2 \cdot 6H_2O$, 7.5 mg; $(NH_4)_2SO_4$, 10 mg; KI, 0.05 mg in 0.1mL; $Na_2WO_4 \cdot 2H_2O$, 0.1 mg; $NiCl_2 \cdot 6H_2O$, 2 mg; trace element solution 141 (http://www.dsmz.de/media/med141.htm), 10 mL; Resazurin indicator, 1.0 mg. In order to reduce (i.e., scavenge oxygen) the medium, 10 mL of 50g/l Na_2S were added slowly, following boiling and subsequent cooling under a stream of H_2/CO_2. The pH of the medium was lowered to 5.5 using 10N H_2SO_4, and then 10 mL portions of the medium were aliquoted anaerobically in previously gassed (N_2) stoppered tubes.

Isolation of Pure Cultures

Enrichment cultures were initiated by inoculating a fragment (~ 1 g) of black smoker into the culture medium and were initially incubated at 65°C. After five subsequent transfers, growth was robust and rod shaped cells could be visualized under the microscope. Strain TB-1 was isolated by plating an aliquot of the liquid culture on solidified medium (obtained by supplementing the medium described above with 1 g/l of gelling agent). The inoculated Petri dishes were incubated at the desired temperature (65°C) in an anaerobic jar (Oxoid) under a H_2/CO_2 atmosphere, and it was then examined for the development of colonies. A colony, originating from a single cell, was transferred from solid to liquid medium, and the purity of the isolates was confirmed by microscopic observation and by sequencing of several independent 16S rRNA clones. In order to monitor the growth of strain TB-1, direct cell counts were carried out by visualization of acridine orange stained cells, using an Olympus BX-60 microscope equipped with an 100X oil immersion objective.

Sequencing and Phylogenetic Analysis of the 16S rRNA Gene

PCR amplification of the 16S rRNA gene from strain TB-1 was carried out, and the product was sequenced. In order to obtain the entire sequence of the 16S rRNA gene, 3.2 pmol of oligonucleotide primers 8F, 515F, 907R and 1517R, respectively (synthesized in the DNA Sequencing and Synthesis Core Facility of the Robert Wood Johnson Medical School; http://www2.umdnj.edu/dnalbweb/), were used in cycle sequencing reactions. The "Auto Assembler" computer program (Perkin Elmer) was used to assemble the sequencing products from the different primers. The assembled sequence was sent to a Sun Spark 10 workstation, and the Arb program (a database of several thousand organisms from the three Domains of life, Archaea, Bacteria, Eukarya; http://www.arb.de.vu/) was used to construct a phylogenetic tree.

Results

Characterization of *Caminibacter* strain TB-1

A thermophilic, chemolithoautotrophic, strictly anaerobic bacterium was isolated from the walls of a black smoker collected at the Rainbow hydrothermal vent site on the MAR. The organism grew by reduction of nitrate to ammonium using molecular hydrogen as the electron donor (H_2/CO_2 80%/20% v/v are supplied in the gas phase).

Morphology. As shown in Figure 1, strain TB-1 is a short rod, approximately 1-2 µm in length, and 0.5 µm in width. Figure 1 shows the cytoplasmic membrane and a periplasmic space. Seen just interior to this are stacks of membranes whose function is unknown, although, in some cases, such membrane stacks have been found to be important in organizing internal enzymatic complexes. Further analysis may demonstrate an analogous function in TB-1.

Optimal temperature for growth. TB-1 was isolated at 65°C. In order to establish the isolate's optimal growth temperature, as well as its upper and lower limits of thermotolerance, growth of TB-1 was monitored at a temperature range varying between 45 and 65°C, in 5°C increments. Figure 2 shows that the optimal temperature for growth of isolate TB-1 is 50°C. At the optimal growth temperature, TB-1 exhibited the shortest generation (i.e., doubling) time (1 hour, 30 minutes minutes).

Oxygen Tolerance. TB-1 was tested in a medium with small oxygen content (supplied in the gas phase at 0.5% v/v). TB-1 did not grow in the presence of 0.5% oxygen, and it was only able to grow in oxygen depleted medium. Therefore we conclude that TB-1 is: i) not capable of using molecular oxygen as a terminal electron acceptor; and ii) a strictly anaerobic bacterium.

Energy Metabolism. When isolate TB-1 was grown with molecular hydrogen and nitrate as the electron donor and acceptor, respectively, nitrate was reduced to ammonium, which accumulated in the culture medium. Nitrate, nitrite, and ammonium were quantitatively measured on an ion analyzer. When nitrate was replaced with elemental sulfur as the terminal electron acceptor, a long lag phase (up to 2 days) was observed before exponential growth. This indicates that sulfur is a sub-optimal substrate for the energy-yielding metabolism of this organism.

Phylogenetic Analysis. Figure 3 shows a phylogenetic tree based on 16S rRNA sequences of strain TB-1 and related bacteria. Phylogenetic analysis revealed that isolate TB-1 belongs to the genus *Caminibacter*, within the epsilon subgroup of the proteobacteria. Isolate TB-1 is closely related to another bacterium isolated in our laboratory (TB-2; 99.6% sequence similarity), and to both *Caminibacter hydrogeniphilus* and *C. profundus* (sequence similarity ~ 96%). A comparison of the 16S rRNA sequence similarity of our organisms with that of *C. hydrogenophilus and C. profundus* is illustrated in Table 1, which shows noticeable differences between these organisms. Two organisms with a sequence similarity lower than 98% are considered different species, although further evidence, both at the physiological and genetic level, is usually required for microbial classification, according to internationally accepted conventions (Boone, *et al.* 2001).

Discussion and Conclusions

The mineralogy of deep-sea hydrothermal vent chimneys is determined mainly by the temperature, chemical composition and flux rate of the end member fluid (Tivey 1995). The chimney mineral composition determines its porosity, which in turn affects the steep thermal and chemical (oxygen, nutrients) gradients within the walls. The availability of redox couples for microbial processes depends largely on these gradients, which therefore influence the distribution of microorganisms within the chimney walls. *Caminibacter* strain TB-1 seems to be well adapted to live in the active chimney walls: the vent fluids enriched with molecular hydrogen provide its energy source, and it uses nitrate from the bottom seawater as an electron acceptor. Its optimal growth conditions, with a temperature of 55°C, slightly acidic pH, and a salt concentration lower than that of seawater, reflect conditions that may be found within the chimney walls where the hot, reduced hydrothermal fluids mix with the frigid, oxygenated seawater. The energy metabolism of isolate TB-1, namely the reduction of nitrate to ammonium, suggests that nitrogen is conserved in the vent ecosystem and it is recycled within the vent microbial communities (e.g., by reoxidation of ammonium to nitrite by ammonia oxidizing organisms). The relative abundance of the *Caminibacter* species in geothemal environments awaits more thorough assessment. However, available studies suggest that these organisms could play a critical role in vent ecology. In principle, the ammonium produced by these organisms through nitrate respiration could supply a nitrogen source to other vent inhabitants, as well as an electron donor to chemolithoautotrophic ammonia-oxidizing bacteria. In either case, nitrate-ammonifying organisms may play a pivotal role in the cycling of nitrogen at deep-sea hydrothermal vents.

Further efforts towards the characterization of strain TB-1 will continue to define the parameters of its survival, and the optima (with respect to temperature, salinity, pH, nutrient concentration) at which it thrives. Overall, the data presented in this paper suggest that isolate TB-1 represents a novel species within the newly described genus *Caminibacter*.

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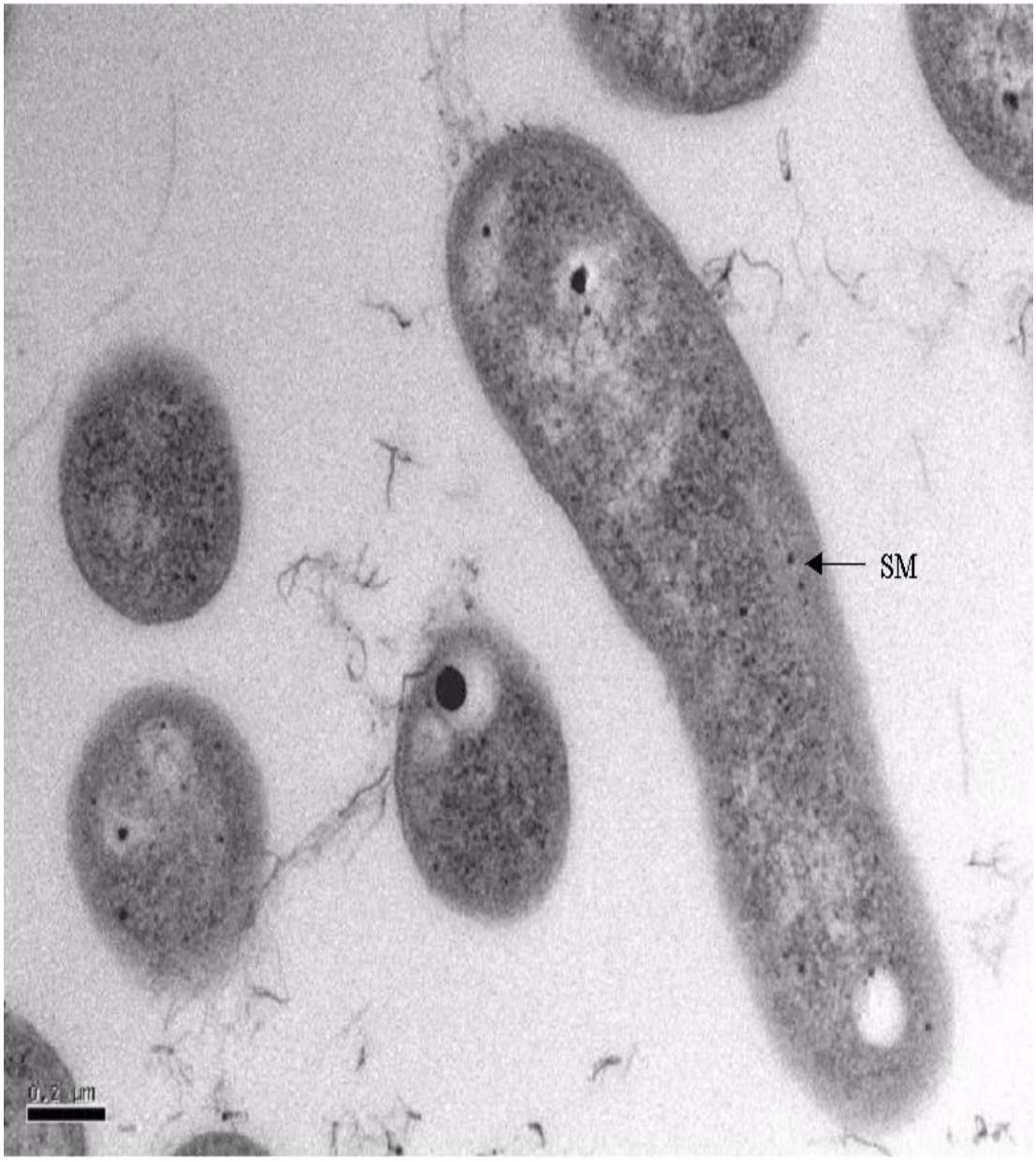

Figure 1. Transmission Electron Micrograph of cells of isolate TB-1. The arrow indicates the location of stacked membranes (SM) inside the cytoplasm.

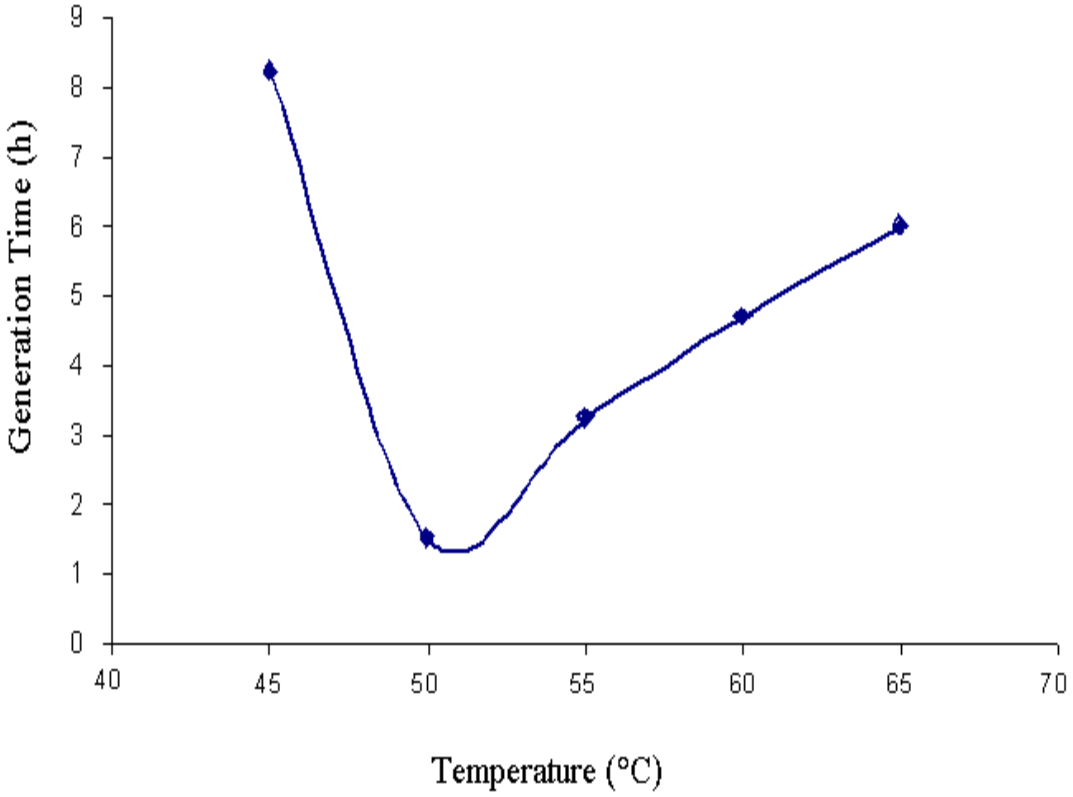

Figure 2. Generation time (in h) of strain TB-1 during growth at different temperatures

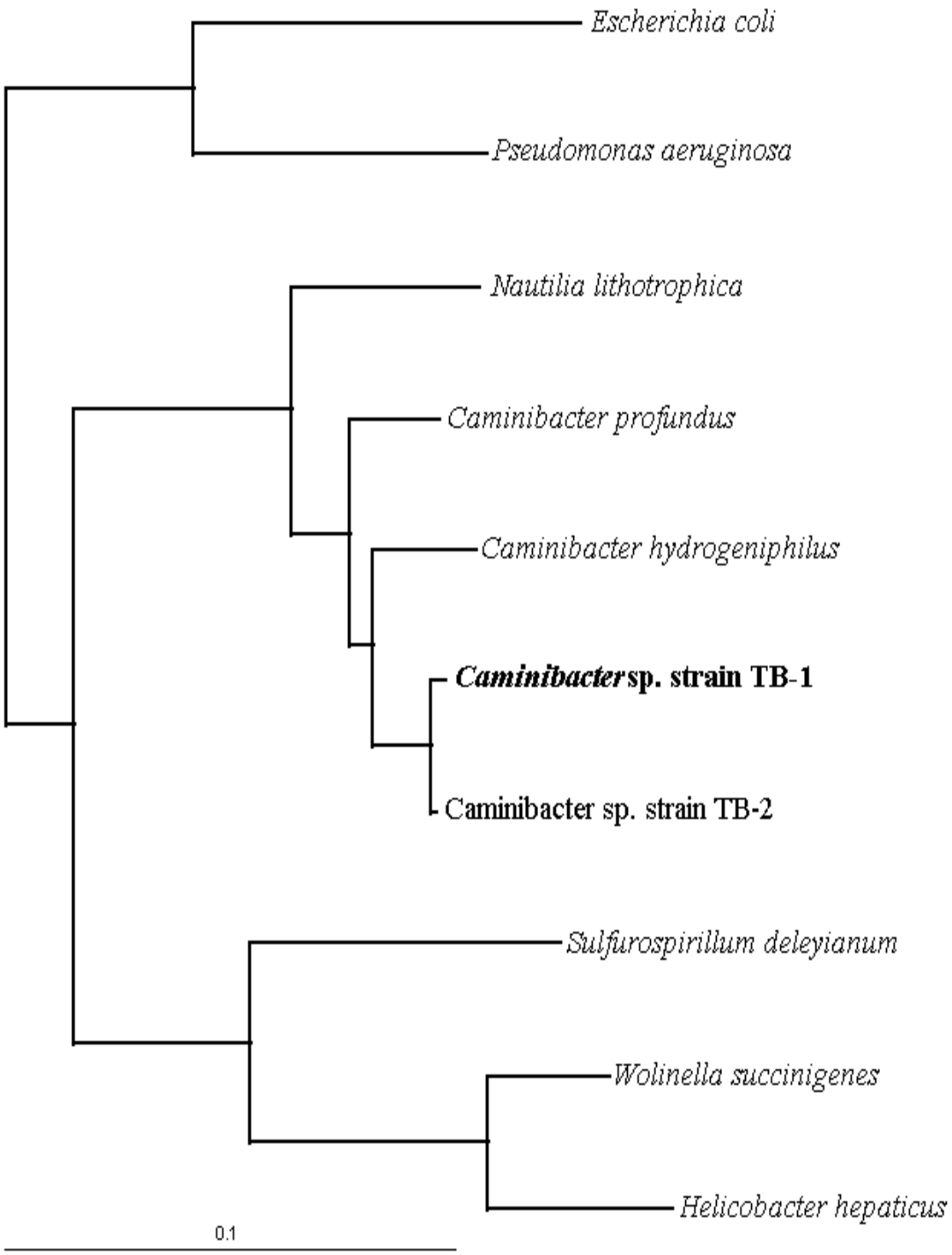

Figure 3. Phylogenetic position of strain TB-1. The neighbor joining tree includes members of the epsilon and gamma proteobacteria. Bar, 10 substitutions per 100 nucleotides

Table 1. Comparison of sequence similarity among all known members of the genus *Caminibacter*

	Isolate TB-1	**Isolate TB-2**	***C. hydrogeniphilus***
Isolate TB-1	100%	99.6%	95.9%
C. hydrogeniphilus	96%	95.9%	100%
C. profundus	96%	96.3%	94.9%