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ORIGINAL PAPER

Desulfomicrobium thermophilum sp. nov., a novel thermophilic sulphate-reducing bacterium isolated from a terrestrial hot spring in Colombia

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Abstract A moderately thermophilic, sulphate-reducing bacterium, designated strain P6-2^T, was isolated from a terrestrial hot spring located at a height of 2,500 m in the Andean region, Colombia (5°43′69″N, 73°6′10″W). Cells of strain P6-2^T were rod-shaped, stained Gram-negative and were motile by means of a single polar flagellum. The strain grew lithotrophically with H_2 as the electron donor and organotrophically on lactate, pyruvate, ethanol, malate, fumarate, *n*-propanol and succinate in the presence of sulphate as the terminal electron acceptor. Fumarate and pyruvate was fermented. Strain P6-2^T grew optimally at 55°C (range 37–60°C), pH 6.6 (range 5.8–8.8) in the presence of 0.5% NaCl (range 0–4.5%) with lactate and sulphate and produced acetate, CO_2 and H_2S as the major end-products. Sulphate, sulphite and thiosulphate could be used as electron acceptors but not elemental sulphur or nitrate. The G + C content of the genomic DNA was 58.7 mol%. The 16S rRNA sequence analysis indicated that strain P6-2^T was a member of the class *Deltaproteobacteria*, domain *Bacteria* with *Desulfomicrobium baculatum* being the closest relative (similarity value of 94%). Phylogeny of genes encoding α- and β-subunits of the dissimilatory sulphite reductase (*dsr*AB genes) supported its affiliation to members of the genus *Desulfomicrobium*. On the basis of this evidence, we propose to assign strain P6-2^T as new species of the genus *Desulfomicrobium*, *D. thermophilum* sp. nov., with strain P6-2^T as the type strain (= DSM 16697^T = CCUG 49732^T).

Keywords Anaerobe · Thermophile · Hot spring · Sulphate-reducing bacterium · Taxonomy

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Introduction

Sulphate-reducing bacteria (SRB) are considered to be ecologically important anaerobes in the numerous planetary ecosystems including the most extreme environments (e.g. saline, alkaline and thermal habitats) that they inhabit. SRB were once thought to grow on an extremely limited range of substrates, but it is now known that they are more nutritionally versatile and can degrade an extensive range of organic acids and sugars, as well as aromatic and xenobiotic compounds (Stahl et al. 2002; Fauque and Ollivier 2004). Therefore the SRB guild group of bacteria contribute to a variety of essential functions in anaerobic environments (Castro et al. 2000) and are thought to be involved, for example, in the biomineralization of more than 50% of organic matter in marine sediments (Fauque and Ollivier 2004). The 40 genera of SRB reported to date are members of four phylogenetically distinct clusters within domain

Bacteria with the majority being members of the *Deltaproteobacteria* (Castro et al. 2000). Members of the genera *Archaeoglobus*, *Caldivirga* and *Thermocladium* (Castro et al. 2000; Fauque and Ollivier 2004; Itoh et al. 1998, 1999) are the only SRB representative included in domain *Archaea*. Different studies of SRB have been undertaken on thermal environments such oil reservoirs, deep-sea hydrothermal vent systems and geothermal hot springs (Chang et al. 2001; Mori et al. 2003; Fauque and Ollivier 2004). The latter studies have resulted in the isolation and characterization of thermophilic SRB species represented in the genera *Desulfotomaculum*, *Thermodesulfobacterium*, *Thermodesulfovibrio*, *Thermodesulfobium* and *Caldivirga*. Recent culture- and culture-independent studies have suggested that an enormous SRB diversity still exists in thermal ecosystems (Blank et al. 2002; Fauque and Ollivier 2004; Ferris et al. 2003; Fishbain et al. 2003; Hugenholtz et al. 1998; Meyer-Dombard et al. 2005; Skirnisdottir et al. 2000).

Several geothermal systems are distributed in the Andean region of Colombia. Of these, the geothermal system of Paipa, located on the East slope of the Andean region, consists of 22 thermal springs, with maximal temperature of around 80°C, pH ranging from 3.6 to 7.4 and NaCl concentration ranging from 0.05 to 56 g l^{-1}. We have chosen to study these springs as part of our research program into the ecology and diversity of thermophilic anaerobic microorganisms and we report here on the isolation and characterization of strain P6-2^T, a thermophilic SRB member of the class *Deltaproteobacteria*.

Methods

Sample collection

Sediment and water samples were collected from a Colombian geothermal spring (Escuela La Playa well), situated in the Andean region (5°43′69″N, 73°6′10″W) at a height of 2,500 m, in sterile glass containers. The containers were filled to the brim, capped, transported to the laboratory at Javeriana University, Bogotá, and kept at ambient temperature until used to initiate enrichment cultures. The temperature at the sampling point was 53.5°C, the pH was 6.8 and the NaCl content was approximately 2.5%.

Enrichment and isolation

Unless indicated otherwise, the technique of Hungate was used and cultures were incubated at 55°C under normal atmospheric pressure. The basal medium contained (per litre): 0.3 g of KH_2PO_4; 0.3 g of K_2HPO_4; 1.0 g of NH_4Cl; 5 g of NaCl; 0.1 g KCl; 3.0 g of $MgCl_2{\cdot}6H_2O$; 0.1 g of $CaCl_2{\cdot}2H_2O$, 0.5 g of cystein; 10 ml of trace mineral element solution (Balch et al. 1979); and 1 ml of 0.1% resazurin. The pH was adjusted to 7.2 with 10 M KOH. The basal medium was boiled, cooled to room temperature and 5 ml aliquots distributed in Hungate tubes with all procedures performed under a stream of O_2-free N_2 gas. The O_2-free N_2 gas phase was replaced with N_2–CO_2 (80:20, v:v) and the tubes autoclaved. Prior to use, 0.05 ml of 2% $Na_2S{\cdot}9H_2O$ and 0.1 ml of 10% $NaHCO_3$ were injected into each tube. Enrichments were initiated by inoculating 1 ml of the spring samples into a growth medium. The growth medium was the basal medium supplemented with sodium lactate (20 mM), sodium sulphate anhydrous (20 mM), 1 g yeast extract l^{-1} and 3 g $MgCl_2$ l^{-1}. Pure cultures were obtained using the roll tube technique. For this, positive enrichment cultures were subcultured several times, the enrichment cultures serially diluted and inoculated into growth medium supplemented with 2% Noble agar (Sigma). Several single well-isolated colonies that developed were picked, transferred into fresh growth medium and the procedure repeated at least twice before the cultures were regarded as pure. A strain designated P6-2^T was selected and used for further characterization. Routine microscopic examination revealing the presence of short rod-shaped cells and an absence of growth in medium containing 1 g yeast extract l^{-1} and 20 mM glucose, were used as indicators of culture purity.

Light and electron microscopy

Cell morphology was determined using a phase-contrast microscope (Nikon Eclipse E600). The preparation of thin sections and examination of strain P6-2^T by electron microscopy was performed as described previously (Fardeau et al. 1997).

pH, temperature and NaCl studies

All experiments were conducted in duplicate in basal medium supplemented with 20 mM lactate, 20 mM sulphate and 0.1 g yeast extract l^{-1}. The strain was subcultured at least once under the same experimental condition. For pH studies, the medium was adjusted with anaerobic stock solutions of either $NaHCO_3$ (10%) or Na_2CO_3 (10%) to give the desired pH. Temperature range for growth was determined between 30 and 60°C. For studies on NaCl requirements, NaCl was weighed directly into Hungate the tubes

(concentrations between 0 and 5%) and the growth medium dispensed.

Substrate utilization

Substrate utilization studies were performed in basal medium containing 0.1 g yeast extract l^{-1}. Ethanol, *n*-propanol, butanol and benzoate were tested at a final concentration of 5 mM, esculin and choline at 10 mM, glucose, fructose, lactose, galactose, formate, acetate, propionate, butyrate, fumarate, succinate, malate, lactate and pyruvate at 20 mM, methanol at 80 mM, casein, casamino acids and biotrypcase at 1% and H_2:CO_2 (80:20, v:v) at 2 bars atmosphere. Thiosulphate, sulphate and nitrate at 20 mM, sulphite and nitrite at 2 mM and elemental sulphur at 0.2% were tested as electron acceptors in basal medium containing 20 mM lactate. Fermentation of 10 mM lactate, fumarate, pyruvate, ethanol, malate and succinate were tested in basal medium.

Analytical techniques

Growth was measured by inserting Hungate tubes directly into a Carry 50 Scan UV-visible spectrophotometer and measuring the optical density at 580 nm. Sulphide was determined photometrically (Cord-Ruwisch 1985). End-products were measured by high pressure liquid chromatography after 2 weeks incubation at 55°C (Fardeau et al. 2000). Cytochromes and desulphoviridin were determined as described by Postgate (1959).

Determination of G + C content

The G + C content was determined by the DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmb, Braunschweig, Germany) using the method of Mesbash et al. (1989).

Amplification and cloning of 16S rRNA and *dsr*AB genes

Genomic DNA of strain P6-2^T was extracted using the Wizard Genomic DNA Purification kit (Promega, Charbionnière, France). The 16S rRNA gene bacterial primers 8F (5′-CAGAGTTTGATCCTGGCTCAG-3′) and 1494R (5′-TACGGTTACCTTGTTACGAC-3′) were used to obtain a PCR product of approximately 1.5 kb corresponding to positions 8 to 1494 (*Escherichia coli* numbering). Approximately 1.9 kb of the *dsr*AB genes were amplified with the primers DSR1F (5′-ACSCACTGGAAGCACG-3′) and DSR4R (5′-GTGTAGCAGTTACCGCA-3′) (Wagner et al. 1998). PCR products were purified using the Nucleo Spin Extract kit (Macherey Nagel, Düren, Germany), and cloned using the pGEM-T-easy cloning kit (Promega, Charbionnière, France), according to the manufacturers protocols. Clone libraries were screened by direct PCR amplification from colonies using the vector specific primers SP6 (5′-ATTTAGGTGACACTATAG-3′) and T7 (5′-AATACGACTCACTATAGG-3′). Plasmids containing a length corresponding to the 16S rRNA gene were purified using the Wizard Plus SV Minipreps DNA Purification System (Promega, Charbionnière, France), according to the manufacturer's protocol and sequenced at Genome Express (Grenoble, France).

Phylogeny

The sequence alignment editor BioEdit (Hall 1999) was used to manually align the 16S rRNA gene sequences and the deduced amino-acid sequences of *dsr*AB genes with reference sequences of various members of the SRB. Reference sequences were extracted from the Ribosomal Database Project II (Maidak et al. 2001) and GenBank (Benson et al. 1999) databases. Positions of sequence and alignment uncertainties were omitted from the phylogenetic analyses. Pairwise evolutionary distances based on 1,342 unambiguous nucleotides (16S rRNA gene) and on 477 amino-acids (*dsr*AB gene) were computed by the methods of Jukes and Cantor (1969) and Kimura (1980), respectively. Dendrograms were constructed by the neighbour-joining method (Saitou and Nei 1987). Confidence of the tree topology was determined by bootstrap analysis using 100 resamplings of the sequences (Felsenstein 1993). All phylogenetic programs were implemented in the software package Treecon 1.3b (Van de Peer and De Wachter 1994).

Nucleotide sequence accession number

The 16S rRNA, *dsr*A and *dsr*B gene sequences of strain P6-2^T have been deposited in GenBank under accession numbers AY464939, DQ464346 and DQ464347, respectively.

Results

Enrichment and isolation

Growth of enrichment cultures in the basal medium containing lactate, sulphate and yeast extract was

observed after 3 days incubation at 55°C and microscopic examination revealed the presence of rod-shaped cells. Circular, smooth and white colonies developed in roll tubes after 3–4 days incubation at 55°C. Several morphologically similar cultures were isolated on the lactate and sulphate medium, and all strains were found to reduce sulphate into sulphide. Strain P6-2^T was characterized further.

Morphology

Cells of strain P6-2^T stained Gram-negative, were non-sporulating rods (0.7 μm × 2–3 μm) and were motile by means of a single polar flagellum (Fig. 1).

Metabolic properties

Strain P6-2^T was a strict anaerobe, which grew optimally at 55°C (temperature range 37–60°C) (Fig. 2a) and at a pH of 6.6 (pH growth range 5.8–8.8) (Fig. 2b). Strain P6-2^T was halotolerant and grew in the presence of NaCl concentrations ranging from 0 to 4.5% with an optimum at 0.5% NaCl (Fig. 2c). The cells contained c_3-type cytochromes, but not desulphovidirin. The isolate did not require peptides or vitamins although 0.1% biotrypcase enhanced growth.

Sulphate, thiosulphate and sulphite were utilized as electron acceptors, but not elemental sulphur, nitrate or nitrite. Strain P6-2^T grew lithotrophically with H_2 as the electron donor and organotrophically on lactate, pyruvate, ethanol, malate, fumarate, *n*-propanol and succinate in the presence of sulphate as the terminal electron acceptor. The main end-products resulting from lactate oxidation were acetate, CO_2 and H_2S. Strain P6-2^T fermented fumarate and pyruvate in the absence of sulphate. The products of fumarate fermentation were succinate and acetate. The following compounds did not support growth in the presence or absence of sulphate: butanol, benzoate, glucose, fructose, lactose, galactose, acetate, propionate, butyrate, methanol, casein, casamino acids, biotrypcase, choline and esculin.

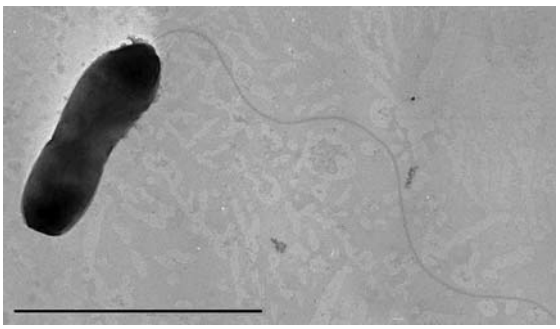

Fig. 1 Transmission electron micrograph of a negatively stained cell of strain P6-2^T showing a polar flagellum. *Bar* = 2.6 μm

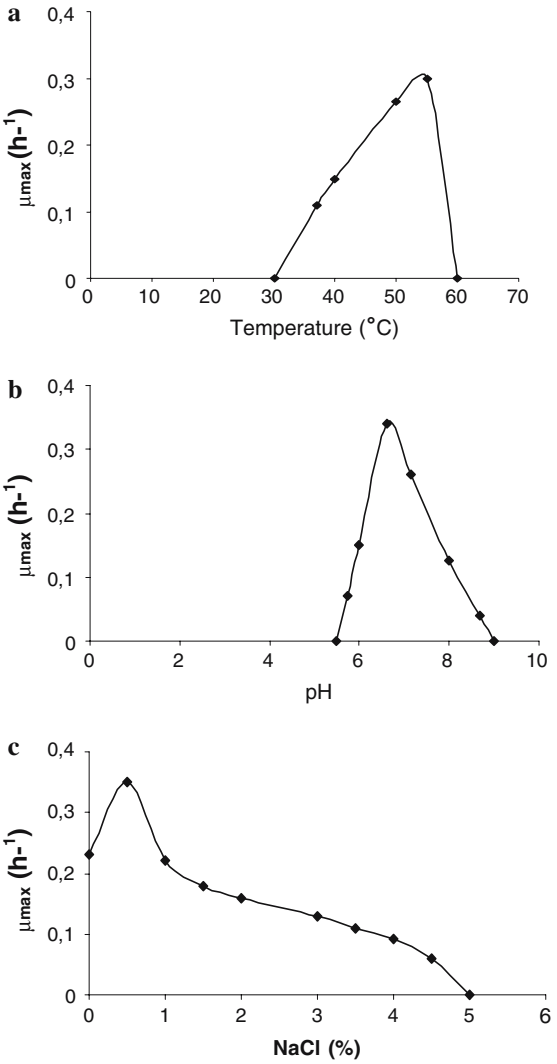

Fig. 2 Effect of **a** temperature, **b** pH and **c** NaCl concentration on the growth of strain P6-2^T cultivated in basal medium supplemented with lactate and sulphate. μ_{max} represents the growth rate measured during the exponential phase

G + C content of DNA and phylogeny

The G + C content of strain P6-2^T was 58.7 mol% (HPLC). The 16S rRNA analysis consistently placed strain P6-2^T in the class *Deltaproteobacteria* in the vicinity of the members of genus *Desulfomicrobium* (Fig. 3) with *Desulfomicrobium baculatum* being the closest relative (94% sequence similarity). The analysis derived from 477 amino-acids of the DsrAB is congruent with the 16S rRNA tree topology thereby supporting the phylogenetic affiliation of

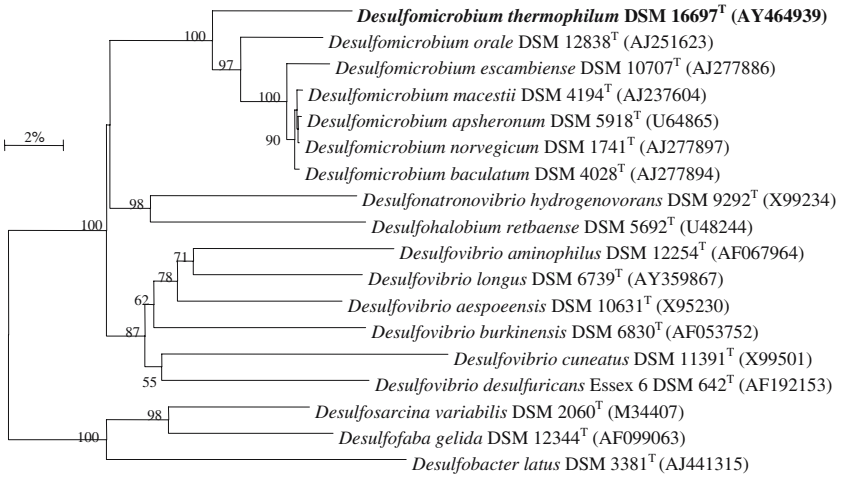

Fig. 3 Phylogenetic tree based on comparative analyses of SSU rRNA gene sequences (1,342 nucleotides) indicating the position of P6-2^T as a member of the *Desulfomicrobium* genus within the δ-subclass of the *Proteobacteria*. Sequence accession numbers are given in *parentheses*. *Numbers* at nodes represent bootstrap values expressed as percentages of 100 replications. *Bar*, two substitutions per 100 nucleotides

strain P6-2^T to the members of genus *Desulfomicrobium* (Fig. 4).

Discussion

Strain P6-2^T is the first report on a thermophilic, SRB isolated from a terrestrial hot spring in Colombia that is a member of the class *Deltaproteobacteria*, domain *Bacteria*. Past molecular studies carried out on terrestrial hot springs had provided evidence of the presence of *Deltaproteobacteria* SRB (Fishbain et al. 2003; Hugenholtz et al. 1998), but the representation of thermophilic SRB in *Deltaproteobacteria* is rare and so far, besides strain P6-2^T, only members of the genera *Desulfacinum* (Rees et al. 1995; Sievert and Kuever 2000), *Thermodesulforhabdus* (Beeder et al. 1995) and *Desulfonauticus* (Audiffrin et al. 2003) are reported as thermophiles. However, strain P6-2^T is phylogenetically distinct from the latter members. The G + C

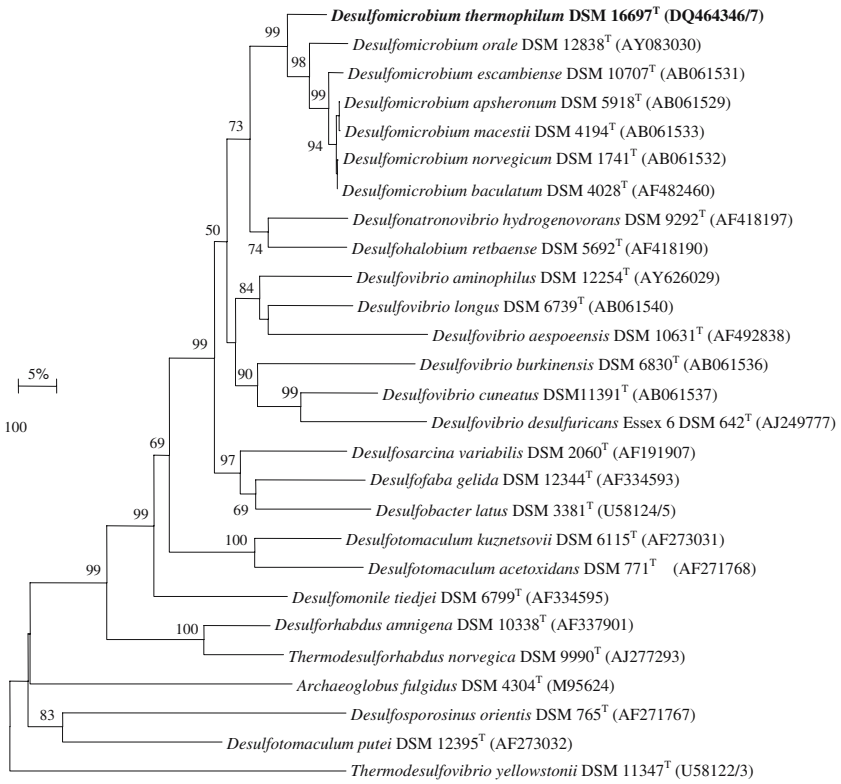

Fig. 4 Phylogenetic tree based on comparative analyses of 477 amino-acid sequences deduced from *dsr*AB genes of strain P6-2^T and its relatives. Nucleotidic sequence accession numbers are given in *parentheses*. *Numbers* at nodes represent bootstrap values expressed as percentages of 100 replications. *Bar*, five substitutions per 100 amino-acids

Table 1 Differential characteristics of the species of the genus *Desulfomicrobium* and strain P6-2^T

Characteristic	Strain P6-2^T	*D. baculatum*	*D. apsheronum*	*D. escambiense*	*D. norvergicum*	*D. macestii*	*D. orale*
Isolation source	Terrestrial hot spring	Water-saturated manganese carbonate ore	Stratal waters of oil-bearing deposits	Aquatic sediments	Harbour water	Water of a sulphide spring	Human subgingival plaque
Morphology and size	Rods 0.7 × 2–3 μm	Rods 0.5–0.7 × 0.9–1.9 μm	Rods 0.7–0.9 × 1.4–2.9 μm	Rods 0.5 × 1.7–12.2 μm	Rods 0.5 × 3.0–5.0 μm	Straight rods 0.7 × 1.9–2.0 μm	Rods 0.6–0.8 × 1.8–3.0 μm
Gram stain	Gram (–)	Gram (–)	Gram (–)	Gram (–)	Gram (–)	Gram (–)	Gram (–)
Mol% G + C content of DNA	58.7	56.8	52.5	59.6	56.3	58	59.7
Spore-forming ability	–	–	–	–	–	–	–
Motility	(+) Single polar flagellum	(+) Single polar flagellum	(+) Single polar flagellum	n.r.	(+) Peritrichous flagellation	(+) Single polar flagellum	(+) Single polar flagellum
Optimal temperature (°C)	55	28–37	25–30	25–30	25–30	35	37
Desulphoviridin	–	–	–	–	–	–	–
C_3-type cytochromes	+				+	+	+
NaCl or vitamins required for growth	(–) Halotolerant (0–4.5%)	(–) Halotolerant (0–6%)	(–) Halotolerant (0–6%)	–	–	1.3% NaCl, range 0–2.5%	n.r.
Terminal electron acceptors							
Sulphur	–	–	–	n.r.	n.r.	–	n.r.
Thiosulphate	+	+	+	+	+	+	n.r.
Sulphite	+	+	+	n.r.	–	+	n.r.
Nitrate	–	–	–	–		n.r.	n.r.
Electron donors							
Lactate	+	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+	+
Ethanol	+	+	+	+	+	+	+
Formate	n.d.	+	+	+	+	+	+
Hydrogen	+	+	+	+	+	+	+
Succinate	+	–	–	–	–	n.r.	n.r.
Acetate	–	–	–	–	–	–	–
Propionate	–	–	–	–	–	n.r.	–
Butyrate	–	–	–	–	–	–	n.r.
Methanol	–	–	–	–	–	–	n.r.
Propanol	+	–	–	–	–	+	n.r.
Butanol	–	–	–	–	–	+	n.r.
Choline	–	–	–	–	–	–	n.r.
References	This study	Rozanova et al. (1988)	Rozanova et al. (1988)	Sharak Genthner et al. (1994)	Sharak Genthner et al. (1997)	Hippe et al. (2003)	Langendijk et al. (2001)

n.d. not determined, *n.r.* not reported

content of genomic DNA of strain P6-2^T is 58.7% and therefore differentiates it from the low G + C DNA containing thermophilic members of the genus *Desulfotomaculum* species, such as *D. luciae*, *D. solfataricum* and *D. australicum* (Goorissen et al. 2003; Liu et al. 1997; Love et al. 1993). Members of the genera *Thermodesulfobacterium* and *Thermodesulfovibrio* are also thermophilic SRB isolated from thermal habitats (Henry et al. 1994; Sonne-Hansen and Ahring 1999; Zeikus et al. 1983) with the former placed in a deep-branch of the phylogenetic tree and the latter closely related to the phylum *Nitrospira*. Recently, a novel SRB, *Thermodesulfobium narugense*, was isolated from a Japanese hot spring but is phylogenetically located at the periphery of the *Nitrospira* phylum (Mori et al. 2003). The phylogenetic placement of strain P6-2^T as a member of *Deltaproteobacteria* clearly sets it apart from the members of these three genera.

Desulfomicrobium baculatum isolated from a water-saturated manganese carbonate ore (Rozanova and Nazina 1976; Rozanova et al. 1994) and strain P6-2^T are both members of *δ-Proteobacteria* are the most closely related (94% sequence similarity). Both *D. baculatum* and strain P6-2^T oxidized lactate incompletely, and do not possess desulphoviridin. However, in contrast to *D. baculatum*, strain P6-2^T uses fumarate, malate and succinate as electron donors. *Desulfomicrobium* genus contains six validly described species (Table 1), found in different ecological habitats from freshwater to brackish, in anaerobic stratal or overlying water, marine anaerobic sediments, human subgingival plaque and sulphide springs: *D. baculatum*, *D. apsheronum* (Rozanova et al. 1994, 1988), *D. escambiense* (Sharak Genthner et al. 1994, 1996), *D. norvergicum* (Sharak Genthner et al. 1997), *D. orale* (Langendijk et al. 2001) and *D. macestii* (Hippe et al. 2003).

Desulfomicrobium baculatum and other members of the genus *Desulfomicrobium* are mesophiles, but strain P6-2^T is a moderate thermophile. The Colombian hot spring from where strain P6-2^T was isolated is associated with volcanism and subduction zones of the Andean region. In this ecosystem, sulphate reduction (measured sulphate concentration within the pool was 136 mM) is expected to be an important metabolic activity to which strain P6-2^T could contribute. In addition, with the isolation of strain P6-2^T, we extend our knowledge of the biodiversity of culturable thermophilic SRB inhabiting terrestrial hot springs.

On the basis of phylogenetic and phenotypic characteristics we propose to assign strain P6-2^T as a new species of the genus *Desulfomicrobium* for which we propose the name *D. thermophilum* sp. nov.

Description of *Desulfomicrobium thermophilum* sp. nov. (Gr. n. *therme* heat; Gr. adj. *philus* loving; M. L. adj. *thermophilum* heat-loving)

Anaerobic strains Gram-negative, moderately thermophilic rods (0.7 μm × 2–3 μm). Spores are not observed. Motile by a single polar flagellum. Growth occurs between 37 and 60°C (optimum 55°C). The pH range for growth is 5.8–8.8 (optimum 6.6). Growth does not occur at NaCl concentrations above 4.5% (w:v). Sulphate, thiosulphate and sulphite are used as electron acceptors, but not elemental sulphur, nitrate or nitrite. Electron donors utilized in the presence of sulphate are lactate, pyruvate, ethanol, *n*-propanol, malate, fumarate, succinate and H_2. Grows autotrophically on hydrogen. Ferments fumarate and pyruvate (Table 1).

The G + C content of genomic DNA is 58.7%. The type strain is P6-2^T (DSM 16697, CCUG 49732). It has been isolated from a terrestrial hot spring (Paipa, Colombia).

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