Extremophiles
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ORIGINAL PAPER

# *Thermodesulfobacterium geofontis* sp. nov., a hyperthermophilic, sulfate-reducing bacterium isolated from Obsidian Pool, Yellowstone National Park

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**Abstract** A novel sulfate-reducing bacterium designated OPF15$^T$ was isolated from Obsidian Pool, Yellowstone National Park, Wyoming. The phylogeny of 16S rRNA and functional genes (*dsrAB*) placed the organism within the family *Thermodesulfobacteriaceae*. The organism displayed hyperthermophilic temperature requirements for growth with a range of 70–90 °C and an optimum of 83 °C. Optimal pH was around 6.5–7.0 and the organism required the presence of $H_2$ or formate as an electron donor and $CO_2$ as a carbon source. Electron acceptors supporting growth included sulfate, thiosulfate, and elemental sulfur. Lactate, acetate, pyruvate, benzoate, oleic acid, and ethanol did not serve as electron donors. Membrane lipid analysis revealed diacyl glycerols and acyl/ether glycerols which ranged from $C_{14:0}$ to $C_{20:0}$. Alkyl chains present in acyl/ether and diether glycerol lipids ranged from $C_{16:0}$ to $C_{18:0}$. Straight, iso- and anteiso-configurations were found for all lipid types. The presence of OPF15$^T$ was also shown to increase cellulose consumption during co-cultivation with *Caldicellulosiruptor obsidiansis*, a fermentative, cellulolytic extreme thermophile isolated from the same environment. On the basis of phylogenetic, phenotypic, and structural analyses, *Thermodesulfobacterium geofontis* sp. nov. is proposed as a new species with OPF15$^T$ representing the type strain.

**Keywords** Dissimilatory sulfate reduction · Hyperthermophile · Thermal environments · Thermodesulfobacterium · Membrane lipids

**Abbreviations**

| | |
|---|---|
| DSR | Dissimilatory sulfite reductase |
| SRM | Sulfate-reducing microorganisms |
| YNP | Yellowstone National Park |
| OP | Obsidian Pool |
| IPLs | Intact polar lipids |
| PE | Phosphoethanolamine |
| APT | Phosphoaminopentanetetrol |
| Phex | Phosphohexose |
| DAG | Diacyl glycerol |
| AEG | Acyl/ether glycerol |
| DEG | Diether glycerol |

## Introduction

The oxidation of inorganic (bi)sulfite to sulfide is an energy conserving process mediated by dissimilatory sulfite

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reductase (DSR). Phylogenetic analyses of archaeal and bacterial sulfate-reducing microorganisms (SRM) indicate that the key genes encoding DSR, *dsrA* and *dsrB*, were present early in the evolution of life and also horizontally transferred between domains (Wagner et al. 1998; Zverlov et al. 2005). The early emergence of microbial sulfate reduction is also evident in the geological record. Isotopic data suggest widespread sulfate reduction in the Precambrian oceans (Cameron 1982) or possibly as early as the Archaean age ~3.5 Ga (Shen et al. 2001). Terrestrial and marine hydrothermal systems mimic some putative early conditions on Earth and support the growth of a wide diversity of dissimilatory SRM (Widdel and Pfennig 1981; Stetter et al. 1987; Henry et al. 1994; Jeanthon et al. 2002). Within the bacteria, thermophilic sulfate reduction appears to be constrained within the phyla Nitrospirae (*Thermodesulfovibrio* spp.), Thermodesulfobacteria, and *Thermodesulfobiaceae* (Muyzer and Stams 2008; Mori and Hanada 2009). Archaea that respire sulfate include *Archaeoglobus* spp. (Achenbach-Richter et al. 1987; Stetter 1988; Zellner et al. 1989; Burggraf et al. 1990; Stetter et al. 1993; Steinsbu et al. 2010), *Caldirvirga maquilingensis* (Itoh et al. 1999), and *Thermocladium modestius* (Itoh et al. 1998). The temperature range for optimal growth of SRM varies greatly from 10 to 83 °C with the majority of cultured sulfate-reducing bacteria having temperature optima in the range of 30–70 °C. Hyperthermophilic, dissimilatory sulfate reduction has only been previously observed within the domain Archaea. *A. fulgidus*, for instance, grows optimally at 83 °C (Stetter 1988). The upper temperature limit has been shown to be much higher in situ with microbial sulfate reduction occurring at temperatures as high as 110 °C in hot sediments surrounding deep-sea hydrothermal vents (Jørgensen et al. 1992).

Thermal springs within Yellowstone National Park (YNP) harbor extremely thermophilic, chemoorganotrophic sulfate reducers including *Thermodesulfobacterium commune* and *Thermodesulfovibrio yellowstonii* (Zeikus et al. 1983; Henry et al. 1994). Through molecular surveys of 16S ribosomal RNA and dissimilatory sulfite reductase genes, novel SRM including deep-branching members of the genus *Thermodesulfobacterium*, have been detected in Obsidian Pool (OP), YNP, Wyoming (Hugenholtz et al. 1998; Fishbain et al. 2003). Rates of sulfate reduction in OP were reported to be approximately 104 nmol $SO_4^{2-}$ $cm^{-3}$ $day^{-1}$ (Fishbain et al. 2003). Despite this evidence indicating the presence of active SRM in this well-studied hydrothermal environment, cultivation attempts have previously been unsuccessful. In this report, we describe a novel sulfate-reducing bacterial isolate from OP that displays a hyperthermophilic temperature profile and chemolithoautotrophic requirements for growth. The name *Thermodesulfobacterium geofontis* OPF15$^T$ sp. nov. is proposed.

## Materials and methods

### Microorganisms

*Thermodesulfobacterium commune* YSRA-1$^T$ (DSMZ 2178), *Thermodesulfobacterium hveragerdense* JSP$^T$ (DSMZ 12571), and *Thermodesulfobacterium hydrogeniphilum* SL6$^T$ (DSMZ 14290) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. *Desulfovibrio vulgaris* (Hildenborough) was provided by Dwayne Elias. *Caldicellulosiruptor obsidiansis* ATCC BAA-2073 was obtained from our laboratory (Hamilton-Brehm et al. 2010).

### Enrichment and isolation

The original samples were collected by Brian P. Hedlund from OP, YNP, on 21 July 2001, and included sediment and spring water taken near the source vent and ranged from 78 to 92 °C, pH ~ 6.0. A primary enrichment culture was inoculated with fresh sediment and water from the sampling site and continuously fed with a complex growth medium while maintained at 85 °C under anaerobic conditions as described previously (Elkins et al. 2008). Secondary enrichments for this study were established from archived samples collected from the original primary enrichment and stored under anaerobic conditions in the dark at room temperature. The secondary enrichments were performed in 125 ml serum bottles containing 20 ml of the following medium: 4-(2-hydroxyethyl)-L-piperazineethanesulfonic acid (HEPES), 2.38 g; $Na_2SO_4$, 3.0 g; $Na_2HPO_4$, 0.5 g; $NH_4Cl$, 1.0 g; $MgCl_2 \times 6H_2O$, 0.4 g; KCl, 0.5 g; $CaCl_2 \times 2H_2O$, 0.07 g; $FeCl_3 \times 6H_2O$, 2 mg; sodium acetate, 2 mM; yeast extract (Difco), 0.2 g; resazurin, 0.5 mg; $Na_2S \times 6H_2O$, 0.5 g; Wolfe's vitamin and mineral solution, 10 ml each (Wolin et al. 1963); distilled $H_2O$, 980 ml. The medium was prepared anaerobically using a modified Hungate technique (Miller and Wolin 1974) and adjusted to pH 7.0 with 1 M NaOH. The serum bottle headspace contained a gas mixture of 80 % $H_2$ and 20 % $CO_2$ at 1 bar (15 psi) overpressure. For final isolation, serial dilutions were performed in roll tubes using the above medium solidified with 1 % (w/v) Gelzan CM (Sigma, USA) and 0.1 % (w/v) $MgCl_2 \times 6H_2O$. The tubes were charged with 80 % $H_2$ and 20 % $CO_2$ at 1 bar (15 psi) overpressure and incubated at 80 °C. After approx. 14 days of incubation, single colonies were selected and transferred anaerobically to serum bottles containing fresh growth medium. Another round of purification was performed using the same medium solidified with Gelzan CM. Isolates were identified by extracting genomic DNA using a method described by Barns et al. (1994) followed by 16S rRNA gene amplification with Eb246F (AGCTAGTTGGTGGGGT) and UA1406R (ACGGGCGGTGWGTRCAA) as the forward

and reverse primers, respectively. PCR products were cloned, sequenced, and analyzed as described previously (Hamilton-Brehm et al. 2010).

## Growth experiments

Serum bottles (100 ml) with a gas phase of $H_2/CO_2$ (80 %/20 %) at 1 bar overpressure were used in experiments to determine optimal pH and temperature. Cultures were incubated at 60.0, 65.0, 70.0, 75.0, 77.5, 80.0, 82.5, 85.0, 87.5, 90.0, and 92.5 °C without shaking. Buffers were added to 10 mM and included 2-(*N*-morpholino)ethanesulfonic acid (MES) for pH 6.0–6.5, 4-(2-hydroxyethyl)-L-piperazineethanesulfonic acid (HEPES) for pH 7.0, and *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) for pH 7.5–8.0. Salt tolerance was tested by adding NaCl to duplicate bottles from 0 to 200 mM while keeping other conditions constant. For substrate utilization experiments, serum bottles were prepared (as above) without sulfate or electron donors, which were added from sterile, anoxic stock solutions after autoclaving. *T. commune* was cultivated in DSM Medium 206 and *T. hydrogeniphilum* was grown in DSM Medium 829. Electron acceptors were tested at a concentration of 20 mM except for elemental sulfur, which was added at 0.1 % (w/v). Soluble electron donors were added at a concentration of 20 mM with oleic acid added at 0.5 % (w/v). High performance liquid chromatography (HPLC) analysis was used to determine the concentration of acetate, lactate, and ethanol using a refractive index detector as described (Hamilton-Brehm et al. 2010). Gas chromatography to determine $H_2$ and $CO_2$ gas phase concentrations was performed as previously described (Miller et al. 2010). Cell numbers were determined using a Thoma cell counting chamber (Blaubrand, Wertheim, Germany) with an Axioskop2 Plus microscope (Zeiss, Thornwood, NY, USA) with phase contrast illumination. Sulfide concentrations were determined using a procedure found in Cline (1969) modified by taking a 500 µl culture sample at each time point during growth, and transferred to 3.5 ml of sterile, anaerobic water in a $N_2$ flushed Balch tube. Then, 300 µl of 250–1000 µmol $l^{-1}$ diamine solution was added and allowed to sit for 20 min at 25 °C. A 1:40 dilution was made with aerobic water and then assayed using 670 nm on a spectrophotometer (UV-1800 Shimadzu, Japan). Sample absorbance values were compared to a standard curve generated with known concentrations of $Na_2S$. All samples were assayed in triplicate.

## Desulfoviridin assay

Cell suspensions were lysed with NaOH and exposed to UV light to determine the presence of desulfoviridin as described by Postgate (1959). *D. vulgaris* (Hildenborough) was used as a positive control.

## Co-cultivation experiments

*Caldicellulosiruptor obsidiansis* and *T. geofontis* OPF15$^T$ were grown together using the medium described above modified with 0.5 g $L^{-1}$ yeast extract and 4 g $L^{-1}$ cellulose (Avicel, Sigma). Serum bottles were inoculated with approx. $5 \times 10^6$ cells $ml^{-1}$ and co-cultures were established at a 1:1 ratio of *C. obsidiansis* and *T. geofontis* cells. Incubation temperature was maintained at 80 °C without shaking. After 168 h of incubation, the cultures were centrifuged at 6,000×*g* for 15 min in pre-weighed 50 ml conical centrifuge tubes. The supernatant was removed and the pellet was treated with a 0.2 M NaOH/1 % (w/v) SDS solution and sonicated with a Branson Sonifer 450 for 60 s (Danbury, CT). The cellulose pellet was washed two times with water and centrifuged at 6,000×*g* for 15 min. The remaining cellulose was dried overnight at 85 °C and then weighed.

## Electron microscopy

Cells were centrifuged at 3000 rpm for 1 min for each step of fixation. Growth medium was replaced with sodium cacodylate buffer (0.1 M, pH 7.0) for 5 min. Primary fixation was with 2 % glutaraldehyde/0.1 M sodium cacodylate buffer for 1 h at room temperature (RT). The cells were then washed three times with buffer and fixed in 1 % $OsO_4$/0.1 M sodium cacodylate buffer for 30 min at RT. Cells were washed twice for 10 min with distilled water and then dehydrated through an increasing concentration of ethanol in 25 % steps, 10 min each step, from 25 to 100 %. Scanning electron microscopy was performed as previously described (Hamilton-Brehm et al. 2010). For transmission electron microscopy, ethanol was replaced with two changes of propylene oxide (PO) as a transition step to infiltration with EmBed 812 (Electron Microscopy Sciences, Hatfield, PA). Infiltration of resin was in 1 h incremental steps of 25 % PO/EmBed 812–100 % resin and left overnight. After two changes of fresh 100 % resin (1 h each step), the cells were pelleted in 0.5 ml microcentrifuge tubes and placed in a 70 °C oven overnight. Then, 50–70 nm thick sections were cut on a RMC MT/X ultramicrotome (Boeckeler Instruments, Inc. Tucson AZ) and picked up on 300 mesh Cu grids. Sections were post-stained with 4 % aqueous UA and Reynold's lead citrate. Digital images were taken on a FEI Tecnai20 transmission electron microscope.

## Phylogenetic analysis

Full-length nucleotide sequences for the *dsrAB* genes were recovered from the annotated genome sequence of

*T. geofontis* (CP002829), and imported into the phylogenetic software package ARB (Ludwig et al. 2004). The genes from *T. geofontis* were aligned against a *dsrAB* reference database (Zverlov et al. 2005) using the ARB automated aligner, followed by visual inspection and correction. The aligned sequence data were exported from ARB using a positional filter that removed the 3rd position in each codon from the alignment. These data (1370 positions) were imported into the phylogenetic software package MEGA (Tamura et al. 2007) and into the software package MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) for phylogenetic tree construction.

Small subunit ribosomal RNA (SSU rRNA or 16S rRNA) gene sequences were recovered from the National Center for Biotechnology Information (NCBI) website, and aligned using the software package Greengenes (DeSantis et al. 2006). These sequences were imported into ARB, and exported from ARB using a three domain 50 % conservation filter which removes positions for which at least 50 % of the sequences are not the same. This alignment (1193 positions) was imported into the software packages MEGA and MrBayes for tree construction.

For *dsrAB* and 16S rRNA genes, neighbor-joining phylogenetic trees were constructed with aligned sequences using the maximum composite likelihood substitution model with complete deletion of gapped positions. The robustness of inferred tree topologies was evaluated by 1000 bootstrap resamplings of the data. For *dsrAB* gene alignments, the total number of positions compared was 1021, while 894 positions were compared for 16S rRNA genes. For maximum likelihood trees, the Tamura-Nei substitution model was employed, with complete deletion of gapped positions, and 1000 bootstrap resamplings of the data. The phylogenetic trees were compared, and nodes that were not supported by bootstrap values of 70 % or higher were treated as polytomies. In addition, Bayesian analyses were performed on the aligned sequence data by running five simultaneous chains (four heated, one cold) for one million generations, sampling every 1000 generations. The selected model was the general time reversible (GTR) using empirical base frequencies and estimating the shape of the gamma distribution and proportion of invariant sites from the data. A resulting 50 % majority-rule consensus tree (after discarding the burn-in of 25 % of the generations) was determined to calculate the posterior probabilities for each node. In all cases, the split-differential at 1 million generations was below 0.01.

## Lipid extraction

Intact polar lipids (IPLs) were extracted from lyophilized biomass from strains grown under parallel conditions as described above using a modified Bligh and Dyer extraction (Bligh and Dyer 1959).

## LC–$MS^n$ analysis of intact polar lipids

IPLs were analyzed according to a modified Sturt et al. (2004) method as described previously (Sinninghe Damsté et al. 2011). An aliquot of the Bligh and Dyer extract (BDE) was dissolved in hexane:isopropanol:water (72:27:1; v/v/v) and filtered through a 0.45 μm regenerated cellulose filter (Alltech Associates, Inc., Deerfield, IL, USA) prior to injection. Separation was achieved on an Agilent 1200 series LC (Agilent, San Jose, CA, USA) equipped with a Lichrosphere diol column (250 × 2.1 μm, 5 μm particles; Alltech Associates, Inc., Deerfield, IL, USA), maintained at 30 °C. The following elution program was used with a flow rate of 0.2 ml $min^{-1}$: 100 % A for 1 min, followed by a linear gradient to 66 % A:34 % B in 17 min, maintained for 12 min, followed by a linear gradient to 35 % A:65 % B in 15 min, where A = hexane/2-propanol/formic acid/14.8 M $NH_{3aq}$ (79:20:0.12:0.04; v/v/v/v) and B = 2-propanol/water/formic acid/14.8 M $NH_{3aq}$ (88:10:0.12:0.04; v/v/v/v). Detection was achieved with positive/negative ion switching $MS^2$ using a Thermo Scientific LTQ XL linear ion trap with Ion Max source with electrospray ionization (ESI) probe (Thermo Scientific, Waltham, MA). ESI settings were as follows: capillary temperature 275 °C, sheath gas ($N_2$) pressure 25 (arbitrary units), auxiliary gas ($N_2$) pressure 15 (arbitrary units), sweep gas ($N_2$) pressure 20 (arbitrary units), spray voltage 4.5 kV (positive ion ESI) or −5.5 kV (negative ion ESI). Each positive ion full scan (*m/z* 400–2000) was followed by data-dependent fragmentation of the four most abundant ions (normalized collision energy (NCE) 25, isolation width (IW) 5.0, activation Q 0.175). This was followed by a negative ion scan (*m/z* 400–2000) and subsequent fragmentation of the four most abundant ions (NCE 35, IW 5.0, Q 0.175). Our identification of head group composition is based upon characteristic fragmentations observed in the positive ion $MS^2$ spectra, which are based upon comparison with standard IPLs and previously published spectra (Sturt et al. 2004).

## Analysis of glycerol ester- and ether-linked alkyl chains

An aliquot of the BDE (∼1/3 by volume) was acid hydrolyzed to cleave polar head group moieties and release ester bound fatty acids using conditions previously described (Sinninghe Damsté et al. 2011). The resulting extract was reacted with diazomethane to convert any free acids to methyl esters and then applied to a short $Al_2O_3$ column and eluted with hexane/dichloromethane (DCM; 9:1; v/v) and DCM/methanol (1:1; v/v) to obtain the non-polar and polar fractions, respectively. The DCM/methanol fraction was silylated with BSTFA prior to analysis by GC and GC–MS. The analytical procedure for GC and

GC–MS analysis has been described by Sinninghe Damsté et al. (2011). Fatty acids, monoalkyl glycerols and dialkylglycerol diethers were identified by mass spectrometry and relative retention time.

## Results

### Enrichment and isolation

We had previously established a continuously fed microcosm-containing sediment from OP, which supported the growth of several hyperthermophilic archaea and bacteria (Elkins et al. 2008). Small-subunit rRNA gene sequencing confirmed the presence of an organism with >99.9 % sequence identity to the uncultivated OPB45 clone described previously (Hugenholtz et al. 1998). Secondary enrichments using the microcosm community as the inoculum were used to determine cultivation conditions to stimulate growth. Organic acids and alcohols including acetate, lactate, malate, and ethanol did not stimulate growth; however, addition of molecular hydrogen and carbon dioxide (80:20) resulted in increased sulfide production and strong enrichment of short, rod-shaped cells. Serial dilutions of the secondary enrichment in roll tubes containing media solidified with Gelzan CM and 1 bar overpressure of $H_2/CO_2$ produced 1–2 mm opaque colonies after approx. 2 weeks of incubation at 80 °C. After subsequent rounds of colony purification, multiple colonies were picked and verified by 16S rRNA gene sequence analysis to be the target organism detected within the primary enrichment (GenBank accession no. JX262247).

### Phenotypic characteristics

*Thermodesulfobacterium geofontis* OPF15$^T$ is a rod-shaped organism, approximately 2 µm long by 0.3 µm wide (Fig. 1a). Cells occur singly or in pairs, do not appear to be motile and spore formation was not observed following prolonged incubation. The cell envelope is Gram-negative in structure with a clearly defined periplasm with an inner and outer membrane (Fig. 1b). The temperature range for growth occurred between 60 and 90 °C, with optimal growth rate at approx. 83 °C (Fig. 2a). The organism grows optimally at pH 7.0, with a range of 6.0–8.5 (Fig. 2b). Sodium chloride had little effect on growth up to 75 mM; however, the specific growth rate decreased at higher salt concentrations with no growth occurring above 175 mM NaCl (Supplemental Fig. 1). Strict anaerobic conditions were required for growth. Molecular hydrogen and formate served as electron donors and $CO_2$ was consumed as a carbon source. Sulfate, thiosulfate, and elemental sulfur were reduced to sulfide when provided as dissimilatory electron acceptors. Growth was not observed on lactate, acetate, pyruvate, benzoate, oleic acid, or ethanol in the presence of sulfate (Table 1). Under optimal conditions, the doubling time was 5 h, producing up to 6 mM sulfide while decreasing the $CO_2$ concentration from 8.5 to 4.7 mM (Fig. 2c) and $H_2$ from 59.0 to 42.2 mM in the headspace (Fig. 2d). OPF15$^T$ did not fluoresce under UV light upon exposure to NaOH suggesting that desulfoviridin is absent. Cultivation of *T. geofontis* was not successful without the addition of yeast extract (0.02 %); however, acetate (2 mM) was not required for growth but increased overall cell densities by an order of magnitude when added.

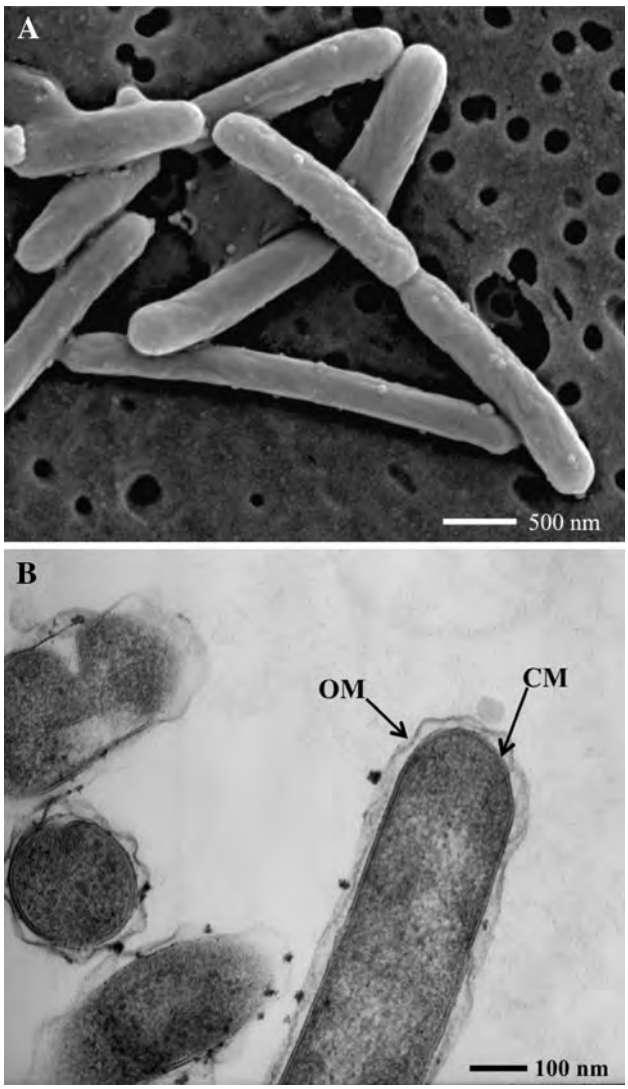


**Fig. 1** **a** Scanning electron micrograph of *T. geofontis* sp. nov. at 13,000×. **b** Transmission electron micrograph showing the outer membrane (OM) and cytoplasmic membrane (CM)

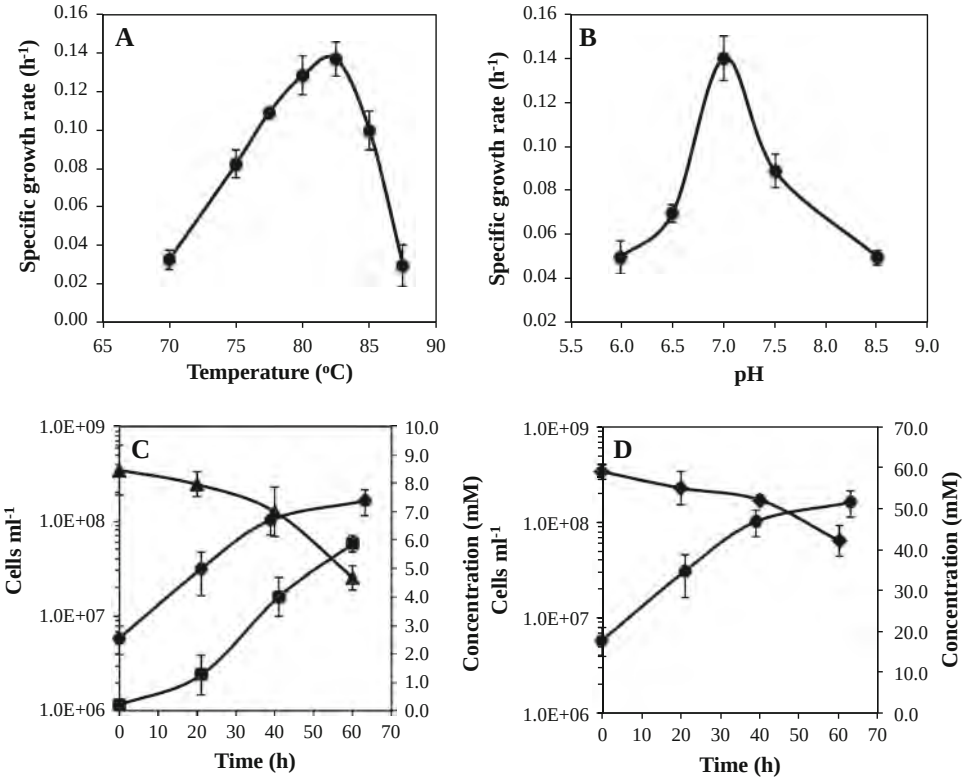


**Fig. 2** Specific growth rates for *T. geofontis* under **a** varying temperatures and **b** pH. Plot **c** cell growth at 83 °C (*circles*) with $H_2$ as the electron donor and $CO_2$ (*triangles*) as the carbon source with production of sulfide (*squares*). **d** cell growth (*circles*) with $H_2$ consumption (*diamonds*). Each data point represents the mean ± standard deviation calculated from 3 replicate cultures

## Phylogenetic analysis

The 16S rRNA gene sequence for OPF15$^T$ was most similar to sequences derived from uncultivated organisms within the genus *Thermodesulfobacterium* with 99.9 and 99.3 % identity to environmental clone OPB45 (AF027096; Hugenholtz et al. 1998) and clone WB3B011 (AY862050; Spear et al. 2005), respectively. Phylogenetic analysis comparing the 16S rRNA gene sequence with those from other cultivated representatives of SRM also confirmed placement of our isolate within the genus *Thermodesulfobacterium* (Fig. 3a); however, non-sulfate-reducing isolates *Caldimicrobium rimae* strain DS$^T$ (Miroshnichenko et al. 2009) and *Geothermobacterium ferrireducens* strain FW-1 (Kashefi et al. 2002) were the closest cultured relatives with 96.5 and 95.9 % identity, respectively. In comparison, *T. hydrogeniphilum* strain SL6$^T$, *T. commune* DSM 2178$^T$, and *T. thermophilum* DSM 1276$^T$ showed 95.5, 94.2, and 93.7 % identity, respectively. Inferred amino acid sequences produced from full-length dissimilatory sulfite reductase genes (subunits A and B; *dsrAB*) grouped with homologs from *T. commune* and *T. thermophilum* with high bootstrap support (Fig. 3b).

## Lipid analysis of *T. geofontis*

HPLC/MS analysis of *T. geofontis* biomass showed the presence of lipids containing phosphoethanolamine (PE), phosphoaminopentanetetrol (APT), and phosphohexose (Phex) head groups (Table 2). Phex was the major head group present (~55 % of total peak area), while PE and APT were present in lower amounts (~14 and ~26 %, respectively). Lipids with a currently unknown headgroup of mass 256 Da were also present in minor amounts (~5 %). Diacyl glycerol phospholipids (DAG) were found to be the dominant lipid type in *T. geofontis* and comprised ~39 % of the total lipid composition. Based on GC and GC–MS analysis of the hydrolyzed IPLs, DAG-IPLs were found to contain saturated $C_{14:0}$ to $C_{20:0}$ acyl chains present as straight chain and branched (*iso* and *anteiso*; *i* and *ai*) isomers (Table 3). Acyl/ether glycerol phospholipids (AEG) comprise ~31 % of the total peak area based on HPLC–ESI–MS$^n$ analysis. During acid hydrolysis AEG-IPLs are converted into monoalkylglycerols and analysis by GC–MS indicated the presence of 1-*O*-monoalkylglycerols with a mixture of *n*- and *i*-$C_{16}$ alkanes (Table 3). In addition, 2-*O*-monoalkyl glycerols with *i*- and *n*-$C_{16}$, *ai*- and *i*-$C_{17}$, and, *i*- and *n*-$C_{18}$ alkyl chains were also identified (Table 3). Overall, monoalkylglycerols were present as trace amounts only, in contrast to AEG-IPLs analyzed using HPLC–ESI–MS$^n$. This discrepancy could be caused by differences in ionization efficiencies of respective DAG, AEG, and diether glycerol (DEG) phospholipid components during ESI–MS$^n$ analysis. The presence of monoalkylglycerols and the range of fatty acids identified during GC analysis are consistent with HPLC–ESI–MS$^n$ analysis

**Table 1** General characteristics of *T. geofontis* OPF15$^T$ sp. nov. and related isolates

| Characteristic | OPF15$^T$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|
| Domain | B | B | B | B | B | B | B | A |
| Morphology | Rod | Oval rod | Rod | Curved rod | Rod | Rod | Rod | Cocci |
| Dimensions (μm) | 0.7 × 2.0 | 0.5 × 1.0 | 0.5 × 1.2 | 0.3 × 1.5 | 0.3 × 0.9 | 0.5 × 0.8 | 0.5 × 2.8 | 0.5 |
| Motility | − | + | + | + | − | + | − | + |
| Doubling time (min) | 300 | 240 | 900 | 1446 | 240 | 186 | 324 | 240 |
| Temp. range (°C) | 65–90 | 52–82 | 65–100 | 40–70 | 60–80 | 50–80 | 55–74 | 60–95 |
| Optimal temp. (°C) | 83 | 75 | 85 | 65 | 70 | 75 | 70–74 | 83 |
| G + C (mol%) | 30.5 | 35.2 | ND | 29.5 | 34 | 28 | 40.0 | 48.6 |
| Electron acceptors | | | | | | | | |
| Sulfate | + | − | − | + | + | + | + | + |
| Sulfite | − | − | − | + | + | − | + | + |
| Thiosulfate | + | + | − | + | + | − | + | + |
| Elemental sulfur | + | + | − | − | − | − | − | − |
| Nitrate | − | − | − | − | − | − | − | − |
| Fe(III) oxide | − | − | + | ND | − | − | − | ND |
| Electron donors[a] | | | | | | | | |
| $H_2$ | + | + | + | + | + | + | − | + |
| Formate | + | − | − | + | + | + | − | + |
| Lactate | − | − | − | + | + | − | + | + |
| Acetate | − | − | − | − | − | − | − | − |
| Pyruvate | − | − | − | + | + | − | + | + |
| Benzoate | − | ND | − | − | − | − | − | ND |
| Ethanol | − | + | − | − | − | − | − | − |

Reference species: 1, *Caldimicrobium rimae* DS$^T$ (Miroshnichenko et al. 2009); 2, *Geothermobacterium ferrireducens* (Kashefi et al. 2002); 3, *Thermodesulfovibrio yellowstonii* YP87$^T$ (Henry et al. 1994); 4, *Thermodesulfobacterium commune* YSRA-1$^T$ (Zeikus et al. 1983); 5, *Thermodesulfobacterium hydrogeniphilum* SL6$^T$ (Jeanthon et al. 2002); 6, *Thermodesulfobacterium hveragerdense* JSP$^T$ (Sonne-Hansen and Ahring 1999); 7, *Archaeoglobus fulgidus* DSM 4304 (Stetter 1988; Zellner et al. 1989). Direct comparisons were performed between *T. geofontis*, *T. commune*, *T. hydrogeniphilum*, and *T. hveragerdense* for electron donor and acceptor experiments

*B* bacteria, *A* archaea, *ND* not determined

[a] $CO_2$ was provided as the carbon source for electron donor experiments

of DAG and AEG-IPLs from *T. geofontis* and indicates that the AEG lipids identified during HPLC–ESI–MS$^n$ analysis are composed of one ether-linked branched or straight chain $C_{16}$, $C_{17}$ or $C_{18}$ alkyl chain and a range of branched and straight chain $C_{14}$–$C_{20}$ ester-linked acyl chains. HPLC–ESI–MS$^n$ analysis showed that DEG phospholipids were also present (~25 % of the total peak area) and were found to contain a total of 32–38 carbon atoms between the two ether-linked alkyl chains. Acid hydrolysis of DEG-IPLs releases dialkylglycerol diethers and, by comparison with previously published spectra (e.g. Pancost et al. 2001; Bradley et al. 2009) we were able to identify a suite of diethers containing $C_{16}/C_{16}$, $C_{17}/C_{16}$, $C_{18}/C_{16}$, $C_{18}/C_{17}$, and $C_{18}/C_{18}$ alkyl chains (Table 3). Isomeric forms of each compound were present and consist of combinations of straight chain and *i*- and *ai*-branched alkyl chains.

The polar lipid compositions of *T. hydrogeniphilum* and *T. commune* were analyzed to provide a comparison to that observed in *T. geofontis* (Table 2). Phex was also the dominant head group present in the analysis of the lipid composition of *T. hydrogeniphilum* (45 % of total peak area; Table 2) and PE- and APT-containing IPLs were present in lower amounts. DEG was the most abundant lipid type identified (63 %) in *T. hydrogeniphilum*. The IPL composition of *T. commune* was found to contain IPLs with PE and APT head groups with relative peak area abundances of 44 and 35 %, respectively, while Phex was present in lower amounts with 23 % relative abundance. DEG, AEG, and DAG lipid types were identified in relative abundances of 46, 34, and 20 %, respectively.

## Co-cultivation on cellulose

Cellulose consumption was measured after culturing *T. geofontis* and *C. obsidiansis* individually, and in co-culture. As expected from the non-cellulolytic sulfate reducer, 98 % of the cellulose remained post-incubation, while *C. obsidiansis* consumed 41 % of the cellulose during the

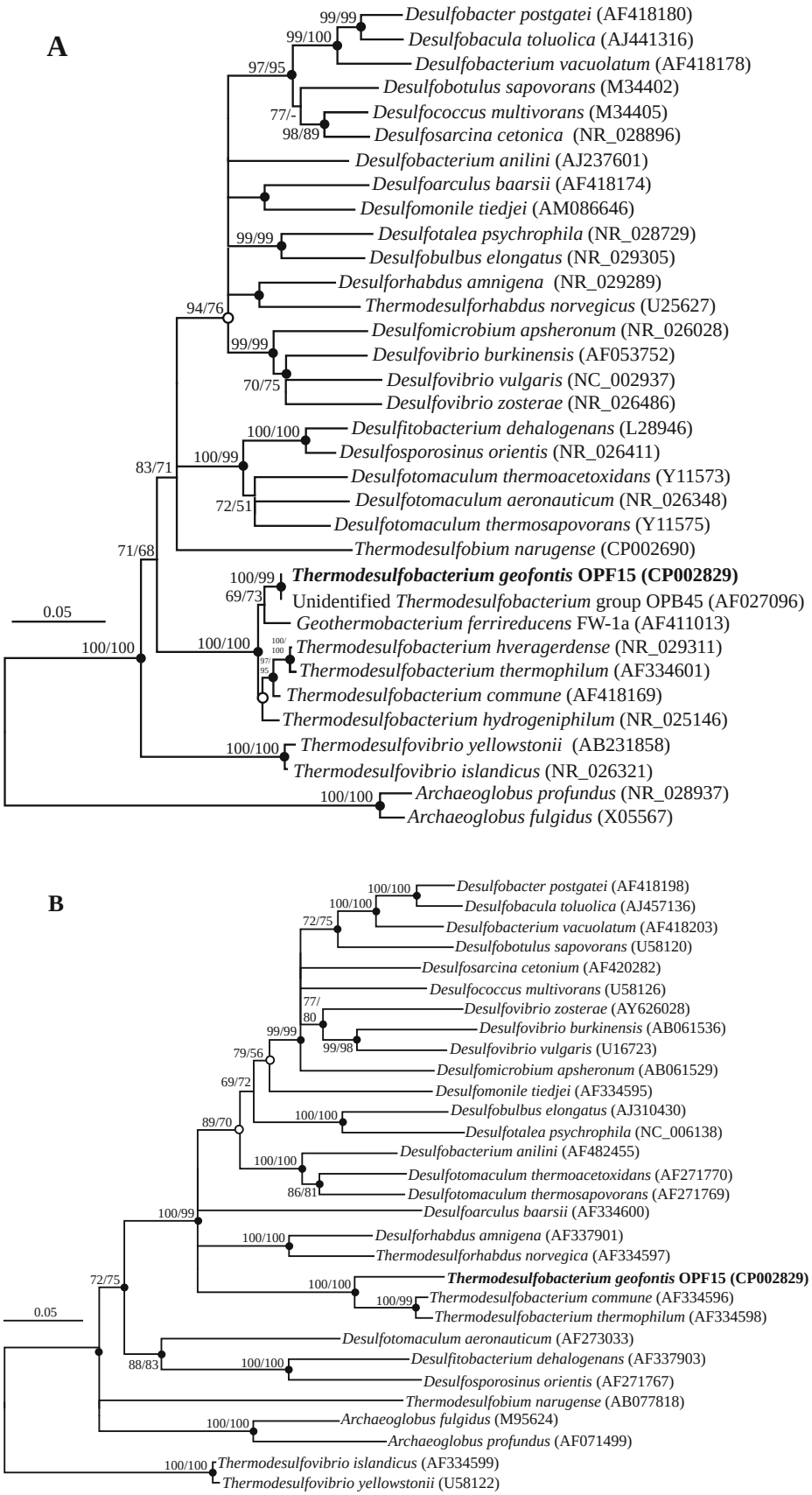


**Fig. 3** Phylogenetic tree reflecting the relationships of **a** filtered 16S rRNA gene sequences; and **b** filtered *dsrAB* gene sequences. The tree topology was obtained from a boot-strapped neighbor-joining analysis. Nodes for which bootstrap values equaled or exceed 70 % are indicated by a *numerical value*. The bootstrap value derived from maximum likelihood analysis is also indicated (NJ/ML). Polytomies indicate branching points that were not consistently supported by bootstrap analyses. Nodes supported by Bayesian analysis, with posterior probability values greater than 95 %, are indicated with *black circles*. Nodes with posterior probability values greater than 70 % are indicated with *white circles*. The *scale bars* indicate 0.05 substitutions per nucleotide position

**Table 2** Intact polar lipid composition identified in three *Thermodesulfobacterium* spp.

| IPL[a] | *T. geofontis* | *T. hydrogenophilum* | *T. commune* |
|---|---|---|---|
| DEG APT | **2** ($C_{34:0}$-$C_{37:0}$; $C_{35:0}$) | **9** ($C_{32:0}$–$C_{37:0}$; $C_{36:0}$) | **8** ($C_{33:0}$–$C_{37:0}$; $C_{35:0}$) |
| AEG APT | **14** ($C_{34:0}$–$C_{37:0}$; $C_{34:0}$) | **16** ($C_{33:0}$–$C_{37:0}$; $C_{34:0}$) | **17** ($C_{33:0}$–$C_{35:0}$; $C_{34:0}$) |
| DAG APT | **10** ($C_{34:0}$–$C_{36:0}$; $C_{34:0}$) | **5** ($C_{34:0}$–$C_{35:0}$; $C_{35:0}$) | **10** ($C_{32:0}$–$C_{35:0}$; $C_{34:0}$) |
| DEG PE | **10** ($C_{32:0}$–$C_{38:0}$; $C_{35:0}$) | **13** ($C_{32:0}$–$C_{38:0}$; $C_{34:0}$) | **20** ($C_{33:0}$–$C_{37:0}$; $C_{34:0}$) |
| AEG PE | **3** ($C_{32:0}$–$C_{36:0}$; $C_{34:0}$) | **11** ($C_{33:0}$–$C_{36:0}$; $C_{34:0}$) | **14** ($C_{32:0}$–$C_{36:0}$; $C_{34:0}$) |
| DAG PE | **1** ($C_{34:0}$–$C_{35:0}$; $C_{35:0}$) | – | **8** ($C_{33:0}$–$C_{35:0}$; $C_{33:0}$) |
| DEG Phex | **13** ($C_{36:0}$–$C_{38:0}$; $C_{36:0}$) | **41** ($C_{32:0}$–$C_{35:0}$; $C_{32:0}$) | **18** ($C_{33:0}$–$C_{35:0}$; $C_{35:0}$) |
| AEG Phex | **14** ($C_{35:0}$–$C_{37:0}$; $C_{36:0}$) | **4** ($C_{33:0}$–$C_{36:0}$; $C_{34:0}$) | **3** ($C_{35:0}$; $C_{35:0}$) |
| DAG Phex | **28** ($C_{35:0}$–$C_{38:0}$; $C_{36:0}$) | – | **2** ($C_{34:0}$–$C_{36:0}$; $C_{34:0}$) |

Figures reported as percent of total intact polar lipids by peak area of extracted ion plots. Range of total carbon number and double bond equivalents in the core lipids presented in brackets with most abundant in italics

*DEG* diethyl glycerol, *AEG* acyl/ether glycerol, *DAG* diacyl glycerol, *APT* aminopentanetetrol, *PE* phosphoethanolamine, *Phex* phosphohexose

[a] Intact Polar Lipid

**Table 3** Fatty acids, glycerol monoethers and glycerol diethers released by acid hydrolysis of the IPL fraction of *T. geofontis*

| Compound | Relative amount (%) |
|---|---|
| Fatty acids | |
| *n*-$C_{14:0}$ | 1 |
| *i*-$C_{15:0}$ | tr |
| *n*-$C_{15:0}$ | tr |
| *i*-$C_{16:0}$ | 5 |
| *n*-$C_{16:0}$ | 5 |
| *i*-$C_{17:0}$ | 3 |
| *ai*-$C_{17:0}$ | 8 |
| *n*-$C_{17:0}$ | 1 |
| *i*-$C_{18:0}$ | 6 |
| *n*-$C_{18:0}$ | 10 |
| *i*-$C_{19:0}$ | tr |
| *ai*-$C_{19:0}$ | tr |
| *n*-$C_{19:0}$ | tr |
| *i*-$C_{20:0}$ | tr |
| *n*-$C_{20:0}$ | tr |
| Monoalkyl glycerols | |
| *$C_{16:0}$ | tr |
| *$C_{17:0}$ | tr |
| *$C_{18:0}$ | tr |
| Dialkylglycerol diethers[a] | |
| *$C_{16}$/$C_{16}$ | 1 |
| *$C_{17}$/$C_{16}$ | 4 |
| *$C_{18}$/$C_{16}$ | 22 |
| *$C_{18}$/$C_{17}$ | 24 |
| *$C_{18}$/$C_{18}$ | 10 |

[a] Indicates the summed relative abundance of branched (*i*- and *ai*-) and straight-chain isomers

same period. Co-cultivation resulted in 66 % of the insoluble substrate being consumed under parallel incubation conditions (Fig. 4a). During growth on cellulose, *C. obsidiansis* produced 7.4 mM acetate, 3.3 mM lactate, and no ethanol, while the co-culture produced 10.5 mM acetate, 3.8 mM lactate, and 2.0 mM ethanol (Fig. 4b). Hydrogen production from *C. obsidiansis* growing on cellulose reached a concentration of 10.8 mM. During co-cultivation with *T. geofontis*, the concentration of headspace $H_2$ increased to 2.6 mM which was 1.6 mM above the uninoculated control.

## Discussion

SRM play a fundamental role in geochemical cycles in both terrestrial and aquatic environments. This includes hot biotopes where escaping geothermal energy generates conditions where only organisms adapted to high temperatures can survive (Stetter 1999). Cultured isolates from deep-sea hydrothermal vents have defined the known upper temperature for life and have demonstrated growth well above 100 °C (Blöchl et al. 1997; Kashefi and Lovley 2003). The maximum temperature limit for biological sulfate reduction from pure culture studies has been obtained from an *Archaeoglobus* sp. growing at temperatures between 90 and 95 °C (Stetter 1988; Burggraf et al. 1990). In situ reduction of $^{35}$S-labeled sulfate has been measured in sediment samples from Guaymas Basin at temperatures up to 110 °C, suggesting uncultivated groups exist that carry out sulfate reduction near the upper temperature limit for life (Jørgensen et al. 1992). For terrestrial thermal environments, many SRM have been described,

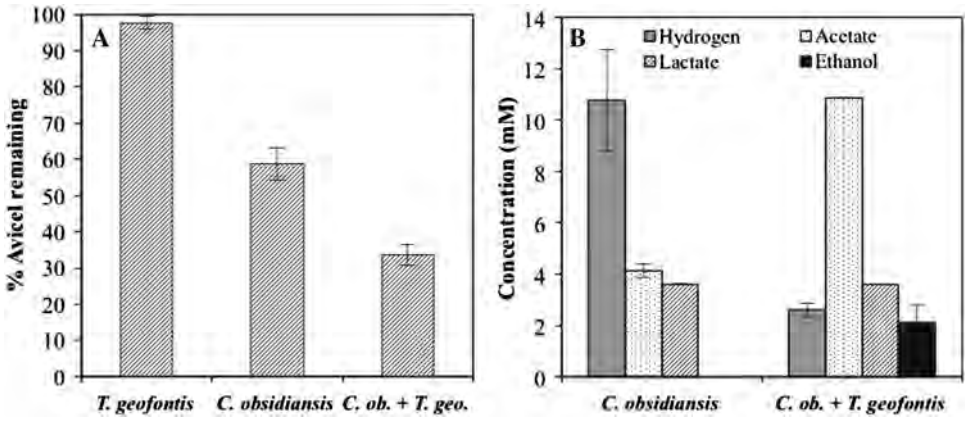


**Fig. 4** **a** Cellulose (Avicel) consumption by pure and co-cultures of *C. obsidiansis* and *T. geofontis*. **b** Comparison of fermentation end products from *C. obsidiansis* versus co-culture growing on cellulose. Each *bar* represents the mean ± standard deviation calculated from 3 replicate cultures incubated at 80 °C for 168 h

including isolates from YNP. These organisms, however, fall into the thermophilic to extreme thermophilic range with maximum temperatures for growth occurring between 65 and 80 °C (Hatchikian and Zeikus 1983; Henry et al. 1994). The temperature profile for *T. geofontis* extends the upper limit for bacterial sulfate reduction ($T_{opt}$ at 83 °C), and overlaps that known for hyperthermophilic archaeal isolates. Fresh water members of the Archaeoglobales have been detected in OP (Barns et al. 1994) and can be enriched in microcosms with the same temperature and pH profiles as those supporting growth of *T. geofontis* (Elkins et al. 2008).

The 16S rRNA gene sequence identity is below 97 % threshold compared to validly described species within the genus *Thermodesulfobacterium.* However, it is interesting to note that the closest relatives among cultured organisms, *C. rimae* and *G. ferrireducens*, do not utilize sulfate as an electron acceptor but are able to reduce elemental sulfur or ferric iron oxides, respectively (Kashefi et al. 2002; Miroshnichenko et al. 2009). The physiology of OPF15$^T$ is similar to the chemolithoautotroph *T. hydrogeniphilum* SL6$^T$, which was isolated from a deep-sea hydrothermal vent (Jeanthon et al. 2002); however, OPF15$^T$ is able to reduce a wider range of sulfur compounds including elemental sulfur and thiosulfate. SL6$^T$ is able to grow without any non-specific nutrient sources, while OPF15$^T$ requires traces of yeast extract suggesting a deficiency in vitamin or cofactor biosynthesis. OPF15$^T$ grows optimally at NaCl concentrations from 0 to 4 g l$^{-1}$, while SL6$^T$ prefers 30 g l$^{-1}$ which corresponds to the spring water and marine environments these organisms were isolated from, respectively.

Dissimilatory sulfate reduction in hyperthermophilic archaea and in thermophilic members of the bacterial lineages *Thermodesulfobacterium* and *Desulfotomaculum* was likely acquired by horizontal transfer of *dsrAB* functional genes from unidentified donors within the Deltaproteobacteria (Klein et al. 2001; Zverlov et al. 2005). This is indicated by incongruences in the phylogeny of 16S rRNA genes and *dsrAB* homologs, particularly in organisms from thermophilic clades (Klein et al. 2001; Zverlov et al. 2005). The *dsrAB* gene sequences from *T. geofontis* are most similar to sequences of *dsrAB* from other members of the genus *Thermodesulfobacterium*, and consistent with the hypothesis of lateral gene transfer, these genes are more similar to genes from Deltaproteobacteria than to sequences from other deep branching thermophiles (Fig. 3b). Ribosomal RNA phylogeny places the genus *Thermodesulfobacterium*, including *T. geofontis*, as a deep-branching lineage well outside the Proteobacteria (Fig. 3a).

In addition to rRNA and *dsrAB* gene sequences, we also compared the composition of polar lipids, including head group composition and glycerol ester and ether moieties, of *T. geofontis* with other thermophilic sulfate reducers (Table 2). Dialkylglycerol diethers comprised a significant fraction (i.e., 25 % total peak area during HPLC–ESI–MS$^n$) of the total lipid composition of *T. geofontis*, similar to other thermophilic bacteria including *T. commune* (Langworthy et al. 1983) and *Aquifex pyrophilus* (Huber et al. 1992). From our investigations using HPLC–ESI–MS$^n$, the lipid content of *T. geofontis* appears to be similar to both *T. hydrogeniphilum* and *T. commune*. For instance, all three cultures were found to contain phospholipids with the same three head groups, PE, APT, and Phex, albeit in different relative amounts. Similar to *T. hydrogeniphilum*, Phex was found to be the major head group in *T. geofontis*. However, glycerol ester and glycerol ether lipid types were more similar to *T. commune*, as in both cultures DEG, AEG, and DAG lipids were present in similar relative amounts, whereas *T. hydrogeniphilum* contained predominantly DEG lipids (~62 % of total peak area), with a smaller contribution of AEG lipids (~31 %) and a minor contribution of DAG lipids. Although the distribution of ester or ether lipid types may be similar between *T. geofontis* and *T. commune*, the length of the ester and ether-linked alkyl chains in the lipids appears to be slightly greater than that observed for *T. commune* or *T. hydrogeniphilum*, i.e., the total number of carbon atoms in alkyl

chains of Phex-containing lipids ranges from $C_{35}$ to $C_{38}$, whereas a total number of carbon atoms in core lipid moieties of Phex-containing lipids of *T. commune* and *T. hydrogeniphilum* range from $C_{33}$ to $C_{36}$. This would be consistent with the higher growth temperature of *T. geofontis*. A series of phospholipids with an unknown head group of 256 Da were also identified as a minor component (~5 %) of the IPL composition of *T. geofontis*. This particular type of IPL has also been detected during analysis of the IPL distribution of *Aquifex aeolicus* which grows at 85 °C (R. Gibson, unpublished data). IPLs containing a head group of 256 Da were not observed in *T. commune* or *T. hydrogeniphilum*. The composition of the acyl and alkyl chains of the IPLs in *T. geofontis* appears to be similar to that reported for *T. commune* (Langworthy et al. 1983) in that they are composed of *n*-, *i*-, and *ai*-isomeric forms. Langworthy et al. (1983) reported that branched $C_{17}$ was the predominant fatty acid species released from the phospholipids of *T. commune*, however, the *n*-$C_{18}$ fatty acid was the most abundant in *T. geofontis* (Table 3). We were also able to identify minor amounts of *i*-$C_{19}$, *ai*-$C_{19}$, *n*-$C_{19}$, *n*-$C_{20}$, and *i*-$C_{20}$ fatty acids that were not reported in *T. commune*. Previously, $C_{16}/C_{16}$, $C_{17}/C_{16}$, $C_{17}/C_{17}$, $C_{17}/C_{18}$, and $C_{18}/C_{18}$ diethers have been reported from *T. commune*, of which diethers containing $C_{17}/C_{17}$ alkyl chains were the most abundant. Analysis of the glycerol diethers in *T. geofontis* revealed that a $C_{18}/C_{17}$ diether was the most abundant (24 %; Table 3), and the alkyl chains were composed of combinations containing *n*-, *i*-, and *ai* isomers.

Molecular hydrogen is an important energy source in many terrestrial hot springs which drives geochemical cycles and supports primary production of biomass from $CO_2$ (Huber et al. 2000; Spear et al. 2005). Hydrogen-oxidizing bacteria, belonging primarily the order *Aquificales*, dominate OP where they thrive at high temperatures under microaerobic conditions (Reysenbach et al. 1994, 2000; Hugenholtz et al. 1998; Spear et al. 2005). Reduced oxygen solubility at elevated temperatures combined with oxygen removal from venting reducing gasses and $H_2$ oxidizing microbes generates low redox conditions necessary for sulfate reduction to occur. Environmental sequence evidence from both 16S rRNA gene (Hugenholtz et al. 1998; Spear et al. 2005) and *dsrAB* gene surveys suggests that SRM comprise an active component of the OP community, with sulfate reduction rates at OP reported to be up to 104 nmol of $SO_4^{2-}$ $cm^{-3}$ $day^{-1}$ (Fishbain et al. 2003). These data indicate that *T. geofontis* and its close relatives at the strain level form a bacterial sulfate-reducing community at hyperthermophilic temperatures (above 80 °C) in OP.

With an interest in microbial cellulose utilization in thermal environments, we established co-cultures between OPF15$^T$ and *C. obsidiansis*, an extreme thermophile isolated from OP that grows on crystalline cellulose (Hamilton-Brehm et al. 2010). Co-cultivation of the two organisms resulted in markedly different patterns of overall cellulose consumption and end product formation relative to pure cultures (Fig. 4). *C. obsidiansis* primarily produces acetate, lactate, $H_2$, $CO_2$, and traces of ethanol from polysaccharides (Hamilton-Brehm et al. 2010). Higher titers of acetate and ethanol accumulated in co-cultures with a concomitant drop in headspace hydrogen. Molecular hydrogen accumulating to 5–10 mM has been shown to cause metabolic shifts toward lactate production for *Caldicellulosiruptor saccharolyticus*; while removing, hydrogen increased acetate production (van Niel et al. 2003). In *C. saccharolyticus*, pyruvate:ferredoxin oxidoreductase oxidizes pyruvate to produce acetyl-CoA and reduced ferredoxin which is then oxidized by hydrogenases to produce $H_2$ (Verhaart et al. 2010). Hydrogenotrophic organisms including methanogens and sulfate reducers cooperate metabolically with fermentative microbes by removing inhibitory molecular hydrogen from the immediate environment (Schink 2002). Decreasing the partial pressure of $H_2$ would increase flux of acetyl-CoA through phosphotransacetylase and acetate kinase to yield ATP and acetate. Ethanol could also be produced from acetyl-CoA or acetaldehyde although specific pathways for ethanol are not well characterized in *Caldicellulosiruptor* sp. Sasaki et al. (2012) recently combined a hydrogenotrophic methanogen with *Clostridium clariflavum*, a cellulolytic microbe, to increase cellulose digestion efficiency by 3-fold with a shift to higher acetate concentrations. Carbon and sulfur cycles are tightly coupled in anaerobic environments and more studies are needed to further investigate specific consortial interactions at high temperatures (>80 °C) particularly as they pertain to the hydrolysis of biomass.

## Description of *Thermodesulfobacterium geofontis* OPF15$^T$ sp. nov.

*Thermodesulfobacterium geofontis* (ge.o.fon'tis. Gr. n. gê, earth; L. masc. n. fons, *fontis*, a spring or fountain; N.L. gen. n. *geofontis*, from a terrestrial hot spring). Cells are short, straight rods approximately 2 μm long by 0.7 μm wide occurring singly or in chains. Cell wall is Gram-negative type. Cells are not motile and endospore formation is not observed. Intact polar lipid composition includes diacyl, acyl/ether, and diether glycerols with phosphohexose comprising the most abundant head group. Acyl and alkyl chains in straight, iso-, and anteiso-configurations range from $C_{14:0}$ to $C_{20:0}$ with straight-chain $C_{18:0}$ fatty acids being the most common. Optimal growth temperature is 83 °C, with a $T_{max}$ of 90 °C and minimum at 60 °C. Optimal pH is 7.0 with a range of 5.5–8.5. Molecular hydrogen or formate serve as electron donors and $CO_2$ is

required as the carbon source. Sulfate, thiosulfate, and elemental sulfur are reduced to sulfide when provided as dissimilatory electron acceptors. Growth is not observed on lactate, acetate, pyruvate, benzoate, oleic acid, or ethanol in the presence of sulfate. Traces of yeast extract are required for growth. When grown in the presence of 80:20 $H_2/CO_2$ and 20 mM sulfate, the doubling time is 5 h. The G + C content of the gDNA from *T. geofontis* is 30.5 mol%. The type strain of *Thermodesulfobacterium geofontis* is OPF15$^T$ (ATCC BAA 2454, JCM 18567).

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