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ORIGINAL PAPER

Thermogladius shockii gen. nov., sp. nov., a hyperthermophilic crenarchaeote from Yellowstone National Park, USA

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Abstract A hyperthermophilic heterotrophic archaeon (strain WB1) was isolated from a thermal pool in the Washburn hot spring group of Yellowstone National Park, USA. WB1 is a coccus, 0.6–1.2 μm in diameter, with a tetragonal S-layer, vacuoles, and occasional stalk-like protrusions. Growth is optimal at 84°C (range 64–93°C), pH 5–6 (range 3.5–8.5), and <1 g/l NaCl (range 0–4.6 g/l NaCl). Tests of metabolic properties show the isolate to be a strict anaerobe that ferments complex organic substrates. Phylogenetic analysis of the 16S rRNA gene sequence places WB1 in a clade of previously uncultured Desulfurococcaceae and shows it to have ≤96% 16S rRNA sequence identity to *Desulfurococcus mobilis, Staphylothermus marinus, Staphylothermus hellenicus*, and *Sulfophobococcus zilligii*. The 16S rRNA gene contains a large insertion similar to homing endonuclease introns reported in *Thermoproteus* and *Pyrobaculum* species. Growth is unaffected by the presence of S^0 or SO_4^{2-}, thereby differentiating the isolate from its closest relatives. Based on phylogenetic and physiological differences, it is proposed that isolate WB1 represents the type strain of a novel genus and species within the Desulfurococcaceae, *Thermogladius shockii* gen. nov., sp. nov. (RIKEN = JCM-16579, ATCC = BAA-1607, Genbank 16S rRNA gene = EU183120).

Keywords Yellowstone national park · Desulfurococcaceae · Novel species · Thermophile

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Introduction

Yellowstone National Park (YNP) is the largest area of terrestrial hydrothermal activity on Earth, featuring geochemically and microbiologically diverse hot springs. Culturing efforts have yielded numerous thermophilic isolates, including strains of *Thermus, Thermosphaera*, *Thermocrinis*, *Hydrogenophilus*, *Roseiflexus*, *Sulfurihydrogenibium, and Metallosphaera* (Brock and Freeze 1969; Huber et al. 1998a, b; Stohr et al. 2001; Hanada et al. 2002; Nakagawa et al. 2005; Kozubal et al. 2008). Culture-independent investigations demonstrated significant archaeal and bacterial diversity in several YNP thermal environments encompassing a broad chemical diversity (Barns et al. 1994; Ward et al. 1997; Hugenholtz et al. 1998; Meyer-Dombard et al. 2005). A hot spring group on the flanks of Mt. Washburn (YNP) also features extreme geochemical conditions, with acid-sulfate waters rich in ammonium, sulfur species, organic carbon, and reduced gases (Spear et al. 2005; Windman et al. 2007). A first taxonomic survey of 16S rRNA genes suggested dominant hydrogen-driven primary production in the bacteria but provided very limited information on archaea (Spear et al. 2005). Here, we describe the first novel isolate (strain

WB1) from the Washburn hot spring group (YNP), which belongs phylogenetically to the Desulfurococcaceae but differs markedly in several key physiologic traits from established taxa. The name *Thermogladius shockii* is proposed.

Materials and methods

Isolation and culture conditions

Heated sediment and fluid were collected from "Boomerang Pool" (lat. 44°45′0.2″, long. 110°25′47.7″) and used to inoculate tubes containing anaerobic heterotrophic growth media designed from prior detailed geochemical analyses (Shock, pers. comm.). Tubes were incubated in situ for 5 h at 86.1°C and in the laboratory for two more weeks at 85°C. WB1 was isolated from one enrichment culture by seven rounds of serial dilution to extinction at 85°C. Filamentous and rod-shaped cells, present in the original enrichment, were no longer visible; only small cocci remained. These cocci were the only morphology observed during two subsequent rounds of serial dilution and were designated strain WB1. Purity was confirmed by gel electrophoresis where the 16S rRNA gene PCR product eluted as a single band of distinctive size and by sequencing in our laboratory and the RIKEN culture collection.

The growth medium (WX10) was designed to mimic Boomerang Pool thermal fluid and contained (per 1 of water) 2,814 mg Na_2SO_4, 530 mg NaCl, 360 mg $NaHCO_3$, 40 mg KCl, 3.25 mg $CaCl_2$, 1.67 mg $MgCl_2{\cdot}6H_2O$, 10 ml N/P solution (containing per 1 of water: 444.8 mg NH_4Cl and 83.0 mg K_2HPO_4), 3 g yeast extract, 3 g peptone, 3 g MES as pH buffer, and 0.5 ml 0.2% resazurin as an oxygen indicator. The medium was adjusted to pH 5 and autoclaved (30 min. at 121°C). Then, 2 ml Fe-EDTA solution (containing per 1 of water: 6.0 mg $FeSO_4{\cdot}7H_2O$ and 6.0 mg Na_2EDTA) and 10 ml trace element solution (containing per 1 of water: 689.2 mg $AlK(SO_4)_2{\cdot}12H_2O$, 36.2 mg RbCl, 15.5 mg $Na_2MoO_4{\cdot}2H_2O$, 11.8 mg H_2WO_4, 10.1 mg $ZnSO_4{\cdot}7H_2O$, 9 mg $MnCl_2{\cdot}4H_2O$, 6.53 mg $BaCl_2{\cdot}2H_2O$, 5.7 mg $SrCl_2{\cdot}6H_2O$, 0.4 mg $VOSO_4{\cdot}3.5H_2O$, 0.2 mg $PbCrO_4$, 0.1 mg $CdSO_4{\cdot}8H_2O$, and 0.1 mg $CuSO_4{\cdot}5H_2O$) were filter sterilized (0.22 μm) into the solution. The medium was boiled under N_2, and 10 ml aliquots transferred into sterile 25-ml Balch culture tubes containing ~0.3 g S^0 (sterilized by incubation at 95°C for 1 week, stirring under a stream of N_2). Tubes were capped, crimped (Al), and pressurized with N_2 (3 bar). Prior to inoculation, tubes were reduced with 0.3 ml of 2.5% Na_2S solution.

This medium was altered for specific tests of pH, metabolism, salinity, and gas toxicity as follows. For pH testing, buffers were changed from the original medium to K_2HPO_4 (pH 3–4.5), MES (pH 5–6), PIPES (pH 6.5–7.5), and HEPES (pH 8–9). Hydrogen toxicity was determined by replacing nitrogen in the headspace with pure hydrogen. Elemental sulfur was removed for half of the hydrogen toxicity experiments to determine its role in detoxification. Oxygen tolerance was tested by introducing known volumes of filtered (0.2 μm) laboratory air into the growth tubes upon inoculation. Sodium sulfide was not added to these trials, as it would reduce some or all of the added oxygen. The salinity requirements of WB1 were tested by varying the NaCl concentration of the medium from 0 to 15 g/l. Growth was also tested in tubes devoid of sulfate and elemental sulfur to evaluate possible metabolic requirements thereof. Experiments were conducted using yeast, peptone, casein, beef extract, starch, glucose, maltose, fructose, sucrose, ribose, cellulose, pectin, formic acid, lactic acid, propanoic acid, acetic acid, and citric acid, all at a concentration of 3 g/l to test potential carbon sources. The autotrophic capabilities of the isolate were evaluated using a pure base medium solution without organic carbon and with S^0 and an H_2/CO_2 (80:20) headspace. In trials of antibiotic sensitivity, the isolate was grown to early exponential phase before incubating with relevant antibiotics (100 μg/l) for an hour at room temperature and returning to growth temperature (Zillig et al. 1982; Fiala et al. 1986; Hensel et al. 1997).

The metabolism of WB1 was determined from changes in aqueous and gas chemistry determined with ion and gas chromatography. Inorganic and organic ions were analyzed using a Dionex 600X Ion Chromatograph (IC). The analytes were separated on a Dionex AS11-HC column and detected by a Dionex ED50 Electrochemical Detector using suppressed conductivity with a multi-step gradient program ranging from 1.5 to 60 mM hydroxide as the eluent. The IC was calibrated from 1 to 100 ppm for sulfate and 0.5–10 ppm for all organic analytes. Changes in headspace gas content were monitored with an HP 6890 Plus Gas Chromatograph.

Cell cultures were observed using phase-contrast, epifluorescence, and electron microscopy. Phase-contrast light microscopy was used to observe approximate cell density and morphologic diversity. DAPI-stained cells were enumerated by epifluorescence microscopy, and growth rates computed from linear regression analysis of the exponential phase in triplicate experiments. Cell morphology was also determined using transmission electron microscopy (TEM) in late exponential growth by the Washington University Molecular Microbiology Imaging Facility. Cells were allowed to adsorb to freshly glow-discharged, 200-mesh, carbon-formvar-coated copper grids for 5 min and then rinsed twice with water and stained with 1% uranyl acetate (Ted Pella) for 1 min. Following three washes in phosphate

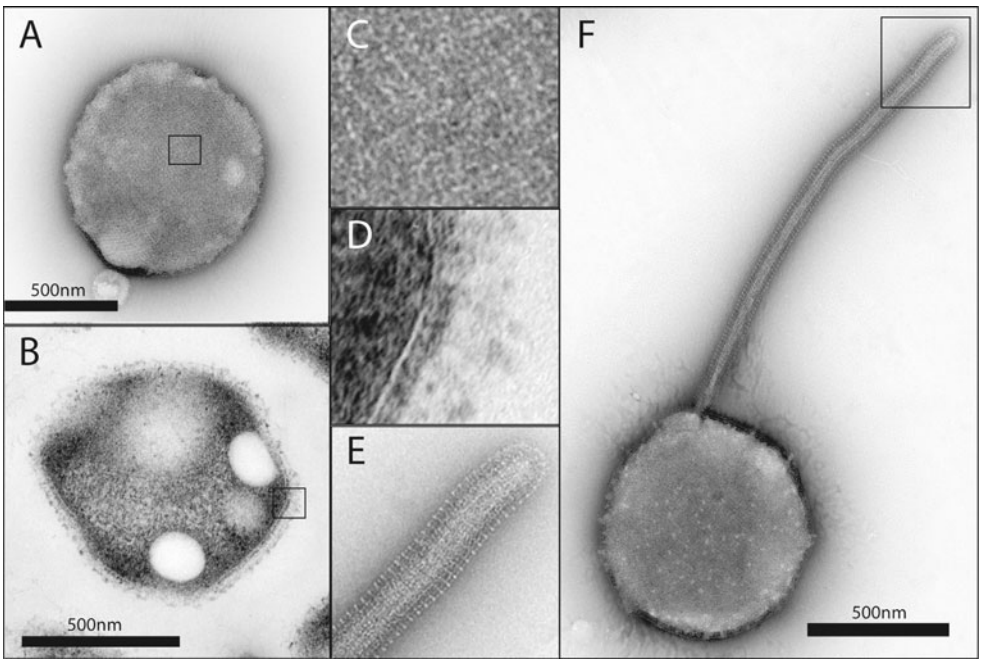

Fig. 1 TEM image of isolate WB1 **a** Negative staining method shows spherical morphology and a recently excreted vacuole; **b** Resin imbedded cell with vacuoles and cross section of cell membrane; **c** Magnified portion of '**a**' showing tetragonal pattern of outer surface proteins; **d** Magnification of '**b**' showing cross section of cell outer membrane and surface layer; **e** Subsection of '**f**' showing a cross section of the tip of a stalk; **f** Negative staining image of a cell with stalk-like protrusion

buffer, cells were postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 h at room temperature, rinsed extensively in dH_20, prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc.). Following several rinses in distilled water, cells were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 70–80 nm were cut and then stained with uranyl acetate and lead citrate. Image acquisition used a JEOL 1200 EX II Transmission Electron Microscope.

Sequencing and taxonomy

Genomic DNA was extracted from isolate WB1 (Qiagen QIAamp DNA Mini Kit following the given protocol) in late exponential phase, and the 16S rRNA gene was amplified by PCR, once with primers 21F and 1391R, and a second time with Arc8F and Arc1492R (DeLong 1992; Barns et al. 1994; Sorensen et al. 2004). PCR products from isolate DNA were directly sequenced using standard reaction mixes and primers 21F, 780F, 956F, and 1391R (DeLong 1992; Barns et al. 1994). Sequence fragments were assembled, edited, and manually aligned with numerous other known archaeal 16S rRNA sequences. Phylogenetic trees were constructed using PAUP 4.8 beta software, and the evolutionary position of WB1 was assessed. The alignment was determined using the expected loop-stem secondary structure of *E. coli* as a guide (BioEdit). Using only highly conserved regions of the genes, we constructed neighbor joining and maximum parsimony trees from the alignment with PAUP 4.8 beta software. Maximum parsimony inferences used 1,000 random additions of taxa. Trees were bootstrapped using 50 replicates of 1,000 random taxa additions per replicate.

Results and discussion

Enrichment, isolation, morphology, and growth parameters

"Boomerang Pool" is a small crescent-shaped feature at the eastern edge of a group of springs, fumaroles, and mud pots that share the National Park Service Thermal Inventory ID WHSNN02. During the course of our long-term study (2000–2006), this pool averaged 83.8°C and pH 5.14.

Strain WB1 was isolated on medium WX10 at 85°C from an enrichment of a morphologically mixed community dominated by small coccoid-shaped cells and lesser numbers of rods and filaments. After seven serial dilutions to extinction, the coccoid cells in the tube at highest dilution of the final round were determined to be pure and subsequently named strain WB1.

Under normal growth conditions, the cells of WB1 are regular cocci, 0.6–1.2 μm in diameter, occurring singularly

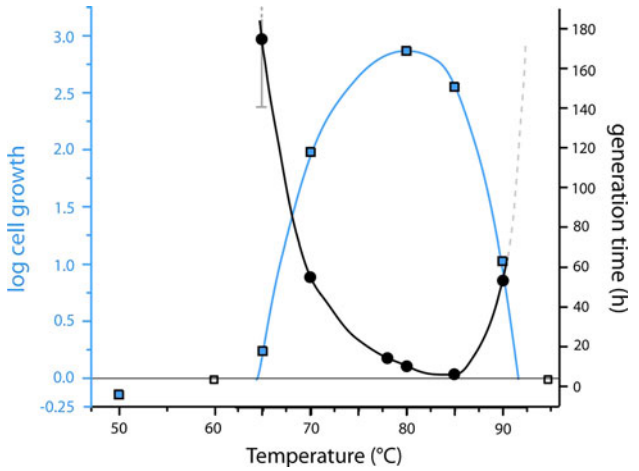

Fig. 2 Temperature growth experiments for strain WB1. Y1 shows the increase in cell density over a 2 week period for cultures grown at 50, 65, 70, 80, 85, and 90°C, along with growth curves that failed to increase in density at 60°C and 95°C. Y2 shows the generation time of isolate WB1 as a function of temperature. Points were calculated from exponential growth phases at each temperature (data not shown). The *dashed curve* shows the estimated extension of generation time above 90°C to the observed maximum (92°C) temperature

or in pairs (Fig. 1a). TEM images show that cells commonly contain vacuoles (Fig. 1a, b) and that the outer cellular membrane is surrounded by a thick, regularly patterned, protein surface layer (Fig. 1b–d). A small percentage of cells (~2%) exhibit long, straight extensions of the cellular membrane that resemble stalks described in *Caulobacter* species (Fig. 1e–f) (Gonin et al. 2000). The protrusions do not appear to vary in response to different nutrient conditions, as is the case in *Caulobacter*. Instead, they may be remnants of cell division as described in *Hyperthermus butylicus* (Zillig et al. 1990). The patterned S-layer of isolate WB1 appears tetragonal (Fig. 1c), consistent with that of *Desulfurococcus mobilis*, but divergent from other Desulfurococcales (Wildhaber et al. 1987; Zillig et al. 1990).

Isolate WB1 grows over the temperature range 64–92°C, optimally at 80–85°C. Growth curves were constructed at 65, 70, 78, 80, 85, and 90°C; no growth was observed at 60 or 95°C (Fig. 2, y2). The temperature range was further constrained with a timed growth trial where initial and final cell concentrations were observed at 50, 65, 70, 80, 85, and 90°C (Fig. 2, y1). The two curves produced by independent methods are complementary and combine to constrain the temperature range. At 84°C, WB1 grows at pH 4–8.5 (optimally at 5–6), but not at pH 3.5 or 9. This strain grows only at low NaCl levels, up to 4.6 g/l, and best without any added NaCl. No growth was observed at 5 g/l NaCl. At optimal growth conditions, strain WB1 doubles in 4.9 h (Fig. 2) and reaches a final density of $\sim 10^9$ cells/ml. The temperature range and optimum of WB1 are similar to those of close relatives, but the pH range is considerably broader. Salinity requirements are highly variable between the organisms (Table 1). The large range of temperatures

Table 1 Comparison of isolate WB1 to closely related species of Desulfurococcaceae

Characteristic	1	2	3	4	5	6	7
Cell diameter (m)	0.6–1.2	0.5–1.0	0.5–1.0	3.0–5.0	0.8–1.0	1.2–3	0.2–0.8
Temperature range (opt) (°C)	64–93 (84)	NR (85)	65–98 (92)	70–95 (87)	70–100 (90–95)	70–98 (90)	65–90 (85)
pH range (opt)	3.5–8.5 (5–6)	4.5–7.0 (6)	4.5–8.5 (6.5)	6.5–8.5 (7.6)	5–9 (7.0)	3.8–6.5 (5.8)	5.0–7.0 (6.5)
NaCl range (w/v%)	0–0.46	NR	1–3.5	0–0.2	1.8–7.0 (3.5)	0.3–5.5 (2)	0–0.7
Optimum doubling time (h)	4.9	3	4.5	4.5	3.3	1.4	1.7
Maximum density (cells/ml)	10^9	3×10^8	3×10^8	1.5×10^8	1.5×10^9	4×10^7	4×10^8
Yeast extract	+	+	+	+	+	−	+
Peptone	+	+	+	−	+	−	−
Casein	+	+	−	−	−	−	+
Chemolithoautotrophic growth ($H_2/CO_2/S^0$)	−	−	−	−	−	+	−
Resistance to rifampicin	+	−	NR	+	NR	−	NR
Chloramphenicol	+	−	+	+	−	NR	NR
Streptomycin	+	+	+	+	NR	NR	NR
Kanamycin	+	+	+	NR	NR	NR	NR
Effect of S^0	No effect	Stimulating	Required	Inhibiting	No effect	Required	Strongly inhibiting
G + C (%)	51.3	50.8	34.9–35.3	54.7	67	41	46
Intron in 16S rRNA	+	−	−	−	+	−	−

1, strain WB1 (data from this study); 2, *Desulfurococcus mobilis* (Zillig et al. 1982); 3, *Staphylothermus marinus* (Fiala et al. 1986); 4, *Sulfophobococcus zilligii* (Hensel et al. 1997), 5, *Aeropyrum pernix* (Sako et al. 1996), 6, *Ignicoccus islandicus* (Huber et al. 2000), 7, *Thermosphaera aggregans* (Huber et al. 1998a); +, positive response; −, negative response; *NR*, not reported

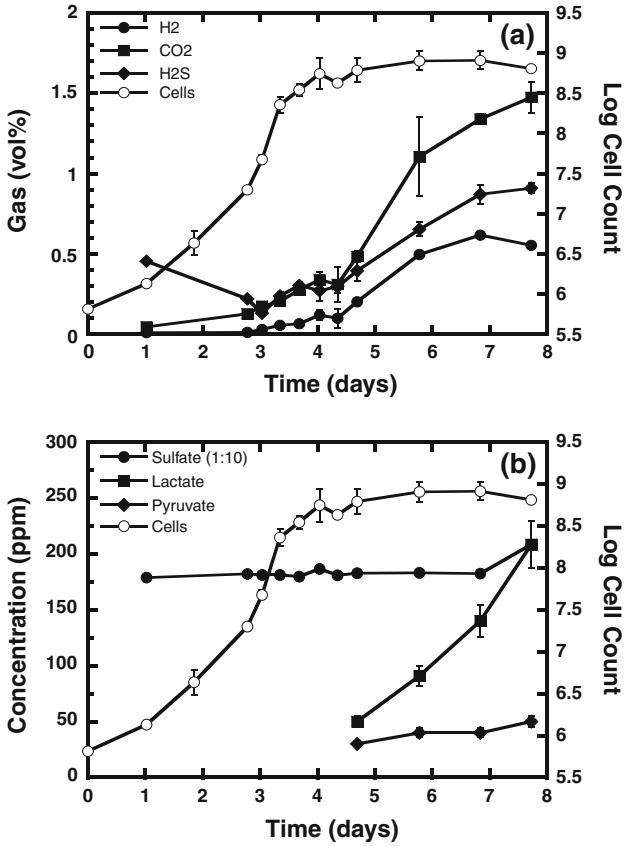

Fig. 3 Concentrations of gaseous CO_2, H_2, and H_2S (in vol%) in the headspace (Fig. 3a) and SO_4^{2-}, *lactate*, and *pyruvate* (in ppm) in the aqueous phase (Fig. 3b), together with cell densities (per mL) during growth of strain WB1. Sulfate concentrations are 10× the *plotted values*. Lactate and pyruvate concentrations for the first ~4.7 days were below detection limits

and pHs tolerated by WB1 may be of ecological advantage in a terrestrial environment such as "Boomerang Pool" that is subject to seasonal chemical variations.

Metabolic properties

Isolate WB1 grows only on anaerobic media containing yeast extract, peptone, tryptone, beef extract, or casein as carbon and energy sources. No growth was observed on glucose, sucrose, lactose, maltose, fructose, ribose, formic acid, acetic acid, propanoic acid, citric acid, pectin, cellulose, or starch (each at 3 g/l). Further, WB1 did not grow autotrophically when H_2 was provided as the sole energy source (under an H_2:CO_2 headspace 80:20 at 300 kPa). Growth density and growth rate were not affected by the absence of S^0 and/or SO_4^{2-} under normal conditions; however, both O_2 and H_2 in the medium appeared to inhibit growth of WB1. Trace amounts of O_2 (< 0.1%, 0.2 μm filter sterilized) completely inhibited WB1 growth and caused rapid cell death. In the absence of S^0, H_2 caused a considerable decrease in the growth rate, but it did not completely prevent growth or stationary phase survival even as 100% of the headspace. In the presence of S^0, growth was only minimally affected by the H_2 atmosphere.

To further investigate the metabolism of WB1, concentrations of potential key reactants and products, along with cell densities, were monitored during growth (Fig. 3). Note in Fig. 3a that the concentrations of CO_2, H_2, and H_2S (in the presence of S^0) increased moderately during the exponential growth phase (circa the first 4 days) and increased strongly at high cell concentration. In growth studies without S^0, no H_2S was generated, and final cell densities were lower (data not shown). Note in Fig. 3b that sulfate levels remained constant at ~1,800 ppm during the incubation period, but that pyruvate and lactate were detected at the end of the exponential growth phase, and then levels increased moderately (pyruvate) and strongly (lactate) during the stationary phase. Because the growth medium had to be diluted considerably to permit sulfate analysis by IC, the detection limits for the organic acids were unfortunately relatively high. This limited our ability to observe increases in organic acids during the exponential phase. Concentrations of other organic acids (e.g., acetate, propanoate) were below detection limit (data not shown). These data demonstrate fermentation as the primary catabolism of isolate WB1. The H_2 produced during fermentation then reacts with S^0 to generate H_2S. It does not appear that sulfur reduction can be used as a primary metabolism as WB1 could not grow autotrophically on H_2, CO_2, and S^0. The results further demonstrate that WB1 is neither a sulfate reducer, nor an obligate S^0 reducer, owing to WB1's ability to grow in the absence of S^0. The carbon metabolism and preferred substrates of WB1 are consistent with those from related genera *Desulfurococcus*, *Staphylothermus*, and *Thermosphaera*, but these organisms differ in their requirements for or against S^0.

DNA G + C content and phylogenetic affiliation

The G + C content of the genomic DNA of isolate WB1 was 51.3 mol% (analysis by DSMZ by HPLC). The 16S rRNA gene (2,089 base pairs, including introns) of this strain was sequenced (GenBank EU183120), and exon regions were ≤96% identical to its closest cultured relatives, *D. mobilis, S. marinus, S. hellenicus,* and *S. zilligii.* Phylogenetic analysis (Fig. 4) placed WB1 within a clade of uncultured organisms found elsewhere in YNP, specifically the acid-sulfate thermal fluids at Sylvan Springs and Cistern Springs (Meyer-Dombard et al. 2005; Korf et al. 2006). This clade consistently branches within the Desulfurococcaceae but separate from all known genera. Another tree constructed using a neighbor joining algorithm instead of maximum parsimony shows nearly

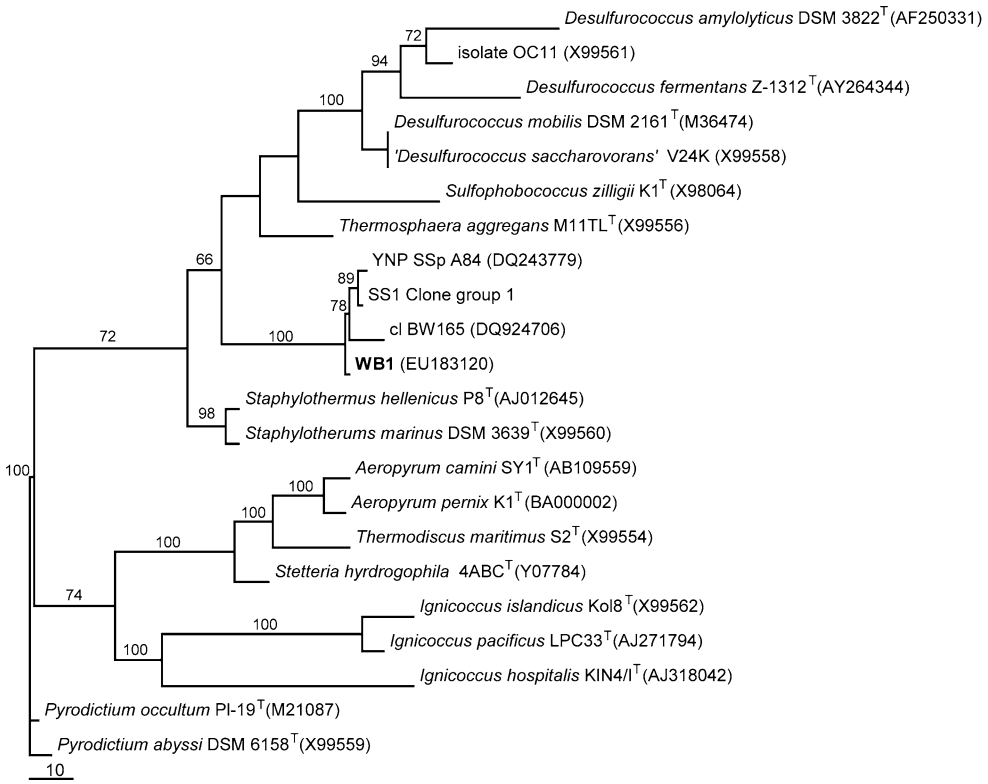

Fig. 4 Phylogenetic tree of the Desulfurococcales. Tree was prepared using maximum parsimony comparison of 1,000 replicates. Bootstrap values were produced using 50 iterations of 1,000 replicates. GenBank accession numbers are given in *parentheses* and superscript *T* indicates type strain

identical topology, further supporting the classification of WB1 as a new genus (Fig. S1).

Gel electrophoresis showed the 16S rRNA gene of WB1 to be significantly larger than that of the archaeon *Palaeococcus helgesonii,* our laboratory control archaeon. This was confirmed by sequencing, where a 684-bp insertion was identified within the 16S rRNA gene. This apparent intron has a 44 mol% G + C value, compared to 66 mol% in the surrounding sequence. An intron of this size was found in the 16S rRNA gene of *Pyrobaculum aerophilum* and was part of the argument for assigning it as a separate species from *Pyrobaculum islandicum* (Burggraf et al. 1993). Comparison of this sequence to protein databases showed it to be related to a family of homing-endonucleases previously reported in the *Thermoproteus* and *Pyrobaculum* genera (Burggraf et al. 1993; Lykke-Anderson et al. 1994; Itoh et al. 2003).

Phylotype and phenotype comparison to closest relatives

Strain WB1 differs in several key physiological, metabolic, and molecular characteristics from its closest relatives (Table 1). For example, WB1 tolerates a larger and more acidic pH range, uses a wider variety of organic substrates for growth, and features a very large intron in the 16S rRNA gene. Perhaps most notably, its growth is not tightly associated with the presence of S^0 or SO_4^{2-}, as opposed to *D. mobilis, S. marinus,* and *S. zilligii*. Of the cultured relatives, WB1 shares most physiological similarity with *D. mobilis*, but phylogenetically groups outside of the cluster of *Desulfurococcus* species. Based on the aforementioned characteristics, a novel species within a novel genus for which the name *Thermogladius shockii* gen. nov., sp. nov. is hereby proposed.

Description of *Thermogladius* gen. nov

Thermogladius (Ther.mo.gla.di.us. Gr. Adj. *thermos* hot; L. n. *gladius* sword; L. m. n. *Thermogladius* the hot sword).

Regular to slightly irregular cocci growing singularly or in pairs with vacuoles and tetragonal patterned S-layer. Heterotrophic metabolism using fermentation of a variety of complex organic substrates to produce organic acids. Cells are not capable of autotrophic growth and are strictly anaerobic. Cells can thrive in high temperatures and in a broad pH range but require low NaCl concentrations. The presence of elemental sulfur has no effect on growth rate or final density except for detoxification of elevated H_2. Found primarily in terrestrial acid-sulfate type hot springs. Type species is *Thermogladius shockii*.

Description of *Thermogladius shockii* sp. nov

T. shockii (shock'i.i. N.L. gen. masc. n. *shockii* of Shock, in honor of Everett L. Shock who pioneered the investigation into Yellowstone hot springs with coupled culturing, phylogenetic, geochemical, and thermodynamic approaches).

Organism possesses the properties characteristic of the genus along with the following traits. Cells are 0.6–1.2 μm in diameter with rigid, stalk-like protrusions on a subset (<2%) of the population. Species is capable of fermenting yeast, peptone, tryptone, beef extract, and casein. Grows optimally at 84°C (range 64–93°C), pH 5–6 (range 3.5–8.5), with little or no added NaCl (range 0–4.6 g/l). At optimal conditions, cells grow with a doubling time of 4.9 h to a maximum density of 10^9 cells/ml. Species does not show sensitivity to the antibiotics ampicillin, kanamycin, rifampicin, chloramphenicol, or streptomycin. The G + C content of genomic DNA is 51.3 mol%. Organism was isolated from the Washburn hot spring group in Yellowstone National Park, USA. The type strain is *Thermogladius shockii* WB1, which has been deposited at RIKEN (JCM-16579) and ATCC (BAA-1607).

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