Thermoflexus hugenholtzii gen. nov., sp. nov., a thermophilic, microaerophilic, filamentous bacterium representing a novel class in the *Chloroflexi*, *Thermoflexia* classis nov., and description of *Thermoflexaceae* fam. nov. and *Thermoflexales* ord. nov.

Jeremy A. Dodsworth,[1] Jonathan Gevorkian,[1] Fairuz Despujos,[1] Jessica K. Cole,[1]* Senthil K. Murugapiran,[1] Hong Ming,[2,3] Wen-Jun Li,[3,4] Gengxin Zhang,[5] Alice Dohnalkova[6] and Brian P. Hedlund[1]

Correspondence
Jeremy A. Dodsworth
jeremy.dodsworth@unlv.edu

[1]School of Life Sciences, University of Nevada, Las Vegas, NV 89154, USA

[2]Department of Life Sciences and Technology, Xinxiang Medical University, Xinxiang 453003, PR China

[3]Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, PR China

[4]Key Laboratory of Biogeography and Bioresource in Arid Land, CAS, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Ürümqi 830011, PR China

[5]Laboratory of Alpine Ecology and Biodiversity, Institute of Tibetan Plateau Research, Chinese Academy of Sciences, Beijing 10085, PR China

[6]Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, PO Box 999, Mail Stop K8-93, Richland, WA 99352, USA

A thermophilic, filamentous, heterotrophic bacterium, designated strain JAD2^T, a member of an as-yet uncultivated lineage that is present and sometimes abundant in some hot springs worldwide, was isolated from sediment of Great Boiling Spring in Nevada, USA. Cells had a mean diameter of 0.3 μm and length of 4.0 μm, and formed filaments that typically ranged in length from 20 to 200 μm. Filaments were negative for the Gram stain reaction, spores were not formed and motility was not observed. The optimum temperature for growth was 72.5–75 °C, with a range of 67.5–75 °C, and the optimum pH for growth was 6.75, with a range of pH 6.5–7.75. Peptone, tryptone or yeast extract were able to support growth when supplemented with vitamins, but no growth was observed using a variety of defined organic substrates. Strain JAD2^T was microaerophilic and facultatively anaerobic, with optimal growth at 1 % (v/v) O_2 and an upper limit of 8 % O_2. The major cellular fatty acids (>5 %) were $C_{16:0}$, $C_{19:0}$, $C_{18:0}$, $C_{20:0}$ and $C_{19:1}$. The genomic DNA G+C content was 69.3 mol%. Phylogenetic and phylogenomic analyses using sequences of the 16S rRNA gene and other conserved genes placed JAD2^T within the phylum *Chloroflexi*, but not within any existing class in this phylum. These results indicate that strain JAD2^T is the first cultivated representative of a novel lineage within the phylum *Chloroflexi*, for which we propose the name *Thermoflexus hugenholtzii* gen. nov., sp. nov., within *Thermoflexia* classis nov., *Thermoflexales* ord. nov. and *Thermoflexaceae* fam. nov. The type strain of *Thermoflexus hugenholtzii* is JAD2^T (=JCM 19131^T=CCTCC AB-2014030^T).

The GenBank/EMBL/DDBJ accession numbers for the sequences of the 16S rRNA gene and the *dnaG*, *frr*, *infC*, *nusA*, *pgk*, *pyrG*, *rplA*, *rplK*, *rplL*, *rplS*, *rplT*, *rpmA*, *rpoB*, *rpsB*, *rpsI*, *smpB* and *galE* genes of strain JAD2^T are KC526151, KF146916–KF146931 and KF981640, respectively.

Two supplementary figures, a supplementary table and other supplementary material are available with the online version of this paper.

*Pacific Northwest National Laboratories, Richland, Washington, 99352, USA.

The phylum *Chloroflexi*, formerly called the 'green non-sulfur bacteria', is a remarkably diverse, deeply branching lineage in the domain *Bacteria*. Originally consisting of a single class, taxonomic revisions and successful cultivation efforts have significantly expanded the taxonomic breadth of the phylum in the last 15 years. Currently, the phylum *Chloroflexi* contains seven recognized classes: *Chloroflexia* (Garrity & Holt, 2001; Gupta *et al.*, 2013), *Thermomicrobia* (Hugenholtz & Stackebrandt, 2004), *Ktedonobacteria* (Cavaletti *et al.*, 2006; Yabe *et al.*, 2010), *Dehalococcoidia* (Löffler *et al.*, 2013; Moe *et al.*, 2009), *Ardenticatenia* (Kawaichi *et al.*, 2013), *Anaerolineae* and *Caldilineae* (Yamada *et al.*, 2006). These lineages represent both mesophilic and thermophilic micro-organisms that collectively display a range of physiological capacities. In addition to obligately aerobic and facultatively or obligately fermentative chemo-organotrophic metabolisms, various members of the *Chloroflexi* are capable of anoxygenic phototrophy (Hanada & Pierson, 2006), chemolithotrophic nitrite oxidation (Sorokin *et al.*, 2012), anaerobic respiration coupled to reductive dechlorination (Taş *et al.*, 2010) or nitrate and ferric iron reduction (Kawaichi *et al.*, 2013) and diazotrophy (Lee *et al.*, 2009). Concomitant with the expansion of the phylum *Chloroflexi* by cultivation, studies utilizing cultivation-independent techniques have revealed a remarkable diversity of as-yet uncultivated micro-organisms affiliated with the phylum *Chloroflexi* (Hugenholtz *et al.*, 1998; Rappé & Giovannoni, 2003) that are apparently ubiquitous and often abundant in sediments, soils and wastewater treatment systems, as well as in extreme environments such as hypersaline lakes and geothermal springs (Yamada & Sekiguchi, 2009). In addition to its prominence in modern environments, the deeply branching nature of this lineage has embroiled the phylum *Chloroflexi* and characteristics of its members' physiology and cell structure in recent discussions regarding the nature and evolution of early life on Earth (Cavalier-Smith, 2010; Sutcliffe, 2011). This paper describes the isolation and characterization of strain JAD2^T, the first cultivated representative of a novel, divergent lineage affiliated with the phylum *Chloroflexi*.

Strain JAD2^T was isolated from sediment collected ~20 cm below the water level from the north side of the main source pool of Great Boiling Spring (GBS) (samples collected at 40° 39.689′ N 119° 21.968′ W, corresponding to site A in Cole *et al.*, 2013a), located near the town of Gerlach, NV, USA. GBS is a circumneutral geothermal spring, with the temperature in the source pool typically ranging from ~74 to 87 °C, and has recently been the subject of several studies focusing on its microbiology and geochemistry (Cole *et al.*, 2013a; Costa *et al.*, 2009; Dodsworth *et al.*, 2011, 2012; Hedlund *et al.*, 2011; Rinke *et al.*, 2013). Cultivation-independent 16S rRNA gene surveys accompanying several of these studies have demonstrated that several as-yet uncultivated class- and phylum-level lineages are abundant in sediments of GBS, including novel sequences affiliated with the phylum *Chloroflexi* (Cole *et al.*, 2013a; Costa *et al.*, 2009; Dodsworth *et al.*, 2011). The enrichment medium, which is described in detail in the online Supplementary Material, was based on filtered water collected from GBS amended with (l^{-1}) 1 g sodium bicarbonate, 0.85 g sodium nitrate, 0.5 g peptone, 0.1 g yeast extract and trace element and vitamin supplements. For initial isolation, successive 10-fold dilutions of GBS sediment were made in an anaerobic chamber in liquid enrichment medium and streaked onto solid enrichment medium. Plates were sealed inside Bandit 2-quart pressure cups (C. A. Technologies) modified for use as anaerobic incubation vessels (Balch *et al.*, 1979), and 100 kPa N_2/CO_2 (4 : 1, v/v) was added to the headspace. After incubation for 12 days at 70 °C, isolated colonies developed that were predominantly 0.2–0.3 mm in diameter and clear to light cream in colour. Individual filaments and aggregates of filaments were observed in wet mounts made from several colonies of the predominant type at ×400 magnification using an Olympus BX51 phase-contrast microscope. Three of these colonies were used as inocula for four rounds of successive streak plating to obtain axenic cultures. Because of difficulty in confidently obtaining single colonies on these subsequent streak plates as a result of the small colony size, only one of the original selected colonies was successfully carried throughout this process, and was designated JAD2^T.

A synthetic medium was designed to facilitate characterization of strain JAD2^T. This medium, herein referred to as GBS salts medium, was formulated to approximate the concentrations of major salts present in GBS spring water (Costa *et al.*, 2009) and contained (per litre 18.2 MΩ-cm deionized water) 3 g NaCl, 0.15 g KCl, 0.3 g Na_2SO_4, 0.123 g $MgSO_4 . 7H_2O$, 0.015 g $CaCl_2 . 2H_2O$, 0.107 g NH_4Cl and 5 ml mineral solution. The mineral solution was based on that used for PE medium (Hanada *et al.*, 1995) and contained (per litre deionized water) 3.7 g EDTA, 1.1 g $FeSO_4 . 7H_2O$, 0.0984 g $MnCl_2 . 4H_2O$, 0.0288 g $ZnSO_4 . 7H_2O$, 0.0238 g $CoCl_2 . 6H_2O$, 0.0172 g $CuCl_2 . 2H_2O$, 0.0242 g $Na_2MoO_4 . 2H_2O$ and 0.031 g H_3BO_3, adjusted to pH 6 with KOH. For solid medium, 0.8 % (w/v) Gelrite gellan gum (#22168; Serva Electrophoresis) and 0.4 % (w/v) $MgCl_2 . 7H_2O$ were added. The medium was sparged with 100 % N_2 gas for 1 h and then transferred to an anaerobic chamber, and 5 ml aliquots were dispensed into serum tubes. Tubes were sealed with butyl rubber stoppers and aluminium crimps, the headspace was exchanged with N_2 gas and the medium was sterilized by autoclaving. Before each use, the stoppers used to seal tubes were boiled twice for 30 min each in 1 % (w/v) sodium sulfide nonahydrate, followed by boiling twice for 1 h each in water. Before inoculation, the following additions for routine cultivation were made from sterile, N_2-sparged stocks to the following final concentrations: sodium phosphate, pH 6.75 (10 mM), Bacto peptone (0.05 %, w/v; Becton Dickinson) and the vitamin solution used in the enrichment medium (1×; Balch *et al.*, 1979). Additionally, 1 ml air was added by syringe through a 0.2 μm filter. The typical inoculum was 0.05 ml (1/100 volume) of a 6–8 day old culture.

Using the GBS salts medium, various growth conditions were tested on triplicate cultures incubated horizontally without agitation in LabNet ProBlot hybridization ovens,

where incubation temperatures were tracked using Traceable Ultra thermometers (#4339; Control Company). Spectrophotometric methods failed to detect growth-induced changes in culture optical density because of low growth yields; therefore, growth was assessed by phase-contrast microscopy. Cultures were monitored for growth every 2 days for the first week after inoculation, and weekly thereafter to a maximum of 3 weeks. Cell counts were made using a Petroff-Hausser counting chamber (#3900; Hausser Scientific Partnership) using either direct culture samples or samples that had been concentrated 10-fold by centrifugation. Unless otherwise noted, conditions for routine cultivation listed above (pH 6.75, 1 ml air, 0.05 % peptone) were used with an incubation temperature of 75 °C. The pH range for growth was tested using 10 mM sodium phosphate buffer at pH 6.0–8.0 in increments of 0.25 pH units; the medium pH was measured both before and after growth experiments to confirm efficacy of the buffer. The temperature for growth was tested at 50, 55, 60, 65, 67.5, 70, 72.5, 75, 77.5 and 80 °C. Growth at different sodium levels was tested in the absence of added NaCl (~0.4 mM sodium due to the sodium content of the Bacto peptone estimated by the manufacturer) and at 0.5, 2, 5, 10, 20, 50, 100, 200 and 500 mM NaCl in modified GBS salts containing $MgSO_4 . 7H_2O$ instead of Na_2SO_4 and using a potassium phosphate buffer (pH 6.75) instead of sodium phosphate. The oxygen range for growth was tested by adding 0, 0.5, 1, 2, 4, 8, 12, 16 or 20 ml air to the culture headspace, where 1 ml air corresponds to approximately 1 % (v/v) or 1 kPa O_2. H_2 production during anaerobic growth was detected by GC using a thermal conductivity detector and argon as a carrier gas (Hedlund *et al.*, 2011). Potential growth substrates were tested in the presence of vitamins and the absence of peptone; any cultures that yielded growth were used to inoculate triplicate subcultures with the same substrate to confirm the phenotype. The following potential alternative electron acceptors were tested in medium with no air added (N_2 headspace) at a concentration of 2 mM unless otherwise noted: fumarate, nitrate, nitrite, sulfite, thiosulfate, ferric nitrilotriacetate (NTA) and elemental sulfur (0.1 and 3 %, w/v).

Microscopic morphological characterization by phase-contrast microscopy and Gram staining (Leboffe & Pierce, 2006) was performed on cultures grown in GBS medium under routine conditions for 6 days using an Olympus BX51 phase-contrast microscope equipped with a V-TV1x-2 camera (Olympus). For electron microscopy, larger volumes of cells were grown for 8 days to late exponential phase in 150 ml GBS salts medium in 500 ml glass bottles sealed with caps containing butyl rubber septa (#240680; Wheaton). Cells were pelleted by centrifugation at 2000 ***g*** for 10 min and fixed in 2.5 % glutaraldehyde buffered with 100 mM sodium cacodylate at 4 °C, and transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were performed as described previously (Cole *et al.*, 2013b). Colony morphology was observed on solid GBS salts medium incubated for 8 days at 75 °C in vessels containing 1 % O_2.

For chemotaxonomic and phylogenetic characterization, cell biomass was generated by cultivation in 500 ml bottles for 8 days (late exponential growth phase) under routine conditions as above for electron microscopy and collected on 0.2 μm filters (#60300; Pall Life Sciences). Extraction and identification of polar phospholipid fatty acids by GC-MS was performed as described by Zhang *et al.* (2003) using the MIDI Sherlock peak identification software version 6.2 (MIDI, Inc.), the TSBA6 peak library and the 1200-A calibration standards kit. DNA was isolated from biomass on filters using the FastDNA Spin kit for Soil (MP Biomedicals). The G+C content of genomic DNA was determined by HPLC using the method of Mesbah *et al.* (1989). Whole-cell sugars were determined by HPLC (Tang *et al.*, 2009). Sequencing and phylogenetic characterization of the nearly full-length (1466 nt) 16S rRNA gene were performed as described previously (Cole *et al.*, 2013b). Phylogenomic analyses were performed on predicted amino acid sequences of 16 conserved genes (*dnaG*, *frr*, *infC*, *nusA*, *pgk*, *pyrG*, *rplA*, *rplK*, *rplL*, *rplS*, *rplT*, *rpmA*, *rpoB*, *rpsB*, *rpsI*, *smpB*) identified in a draft genome sequence of strain JAD2^T using AMPHORA (Wu & Eisen, 2008); the genome sequence of JAD2^T will be described in more detail in a separate publication. Alignments were concatenated and phylogenies were inferred by maximum-likelihood using RAxML version 7.2.6 (Stamatakis, 2006). Resulting phylograms were visualized using iTOL version 2.2.1 (Letunic & Bork, 2011) and Dendroscope version 2.7.4 (Huson *et al.*, 2007). Alignments used for reconstruction of phylogenies presented in this paper are available in the online Supplementary Material.

Strain JAD2^T grew consistently in the GBS salts medium supplemented with 0.05 % peptone at 75 °C provided that butyl rubber stoppers used to seal tubes were treated by boiling in sodium sulfide as described above. The temperature range for growth was 67.5–75 °C, with an optimum at 72.5–75 °C; no growth was observed at 65 or 77.5 °C. While the growth temperature range is unusually narrow, similarly narrow ranges have been observed among members of the phylum *Chloroflexi*, including *Anaerolinea thermophila* (50–60 °C; Sekiguchi *et al.*, 2003) and *Longilinea arvoryzae* (30–40 °C; Yamada *et al.*, 2007). The pH range for growth was 6.5–7.75, with an optimum at pH 6.75; no growth was observed at pH 6.25 or 8.0. Growth was observed at and below 200 mM NaCl (1.16 %, w/v), but not at 500 mM, with an optimum below 100 mM (0.58 %, w/v). Growth was observed in the absence of oxygen, and H_2 production was observed by GC under these conditions, suggesting that H_2 was a product of fermentative growth. Oxygen levels from 0.5 to 8 % stimulated growth, with optimum cell yields obtained at 1 % O_2, whereas no growth was observed at 12 % O_2 or greater. The doubling time of strain JAD2^T grown in the presence of 0.05 % peptone and 1 % O_2 at 75 °C was approximately 1 day, with final culture densities ranging between 0.5 and 1.5×10^6 filaments ml^{-1}, in comparison with $1–2 \times 10^5$ filaments ml^{-1} in the absence of oxygen. In

place of peptone, growth was supported by 0.05 % (w/v) tryptone (BD Bacto) or yeast extract (BD Bacto), and vitamins were required for consistent and efficient growth on peptone. No growth was observed on the following compounds (at 2 mM unless otherwise noted) as sole carbon and energy sources in the presence of vitamins: 25 kPa H_2/CO_2 (4 : 1, v/v) or methane, 0.1 % (w/v) microcrystalline cellulose (extra pure 90 mm; Acros Organics, Thermo Fisher Scientific), birch-wood xylan (Sigma-Aldrich), soluble potato starch (JT Baker; Avator Performance Materials), gelatin (EMD), CM-cellulose (Spectrum), pectin (TCI America), 0.05 % (w/v) Casamino acids (EMD), glutamate, L-histidine, L-arginine, L-lysine, L-methionine, L-aspartate, L-valine, L-isoleucine, L-leucine, glycine, sucrose, D-glucose, D-galactose, D-fructose, cellobiose (0.05 %, w/v), D-xylose (0.05 %, w/v), a mixture of D-arabinose, L-arabinose, D-xylose and D-mannose (each at 0.025 %, w/v), ethanol, methanol, D-mannitol, fumarate, pyruvate, lactate, propionate, succinate, isobutyrate, acetate or formate. In the absence of oxygen, fumarate increased total cell yield approximately 2- to 3-fold, and thus apparently served as an alternative electron acceptor, because this substrate did not support growth on its own in either the presence or absence of oxygen. Nitrate, nitrite, thiosulfate and ferric NTA had no effect on anaerobic growth yield, and sulfite and elemental sulfur inhibited anaerobic growth. After freezing on dry ice and storage at −80 °C, cultures preserved with 10 % (v/v) DMSO or 5–10 % (w/v) glycine betaine retained viability, while those preserved with 10–20 % (v/v) glycerol or with no preservative were not viable. Peptone, yeast extract and tryptone supported growth in the absence of NH_4Cl, indicating that these could serve as sole nitrogen sources.

Cells of strain JAD2^T displayed a filamentous morphology when grown both in liquid and on solid medium, and were negative for the Gram stain reaction. Filaments were typically between 20 and 200 μm long (Fig. 1a), although occasionally filaments of up to 500 μm and as short as 4 μm were observed. Mean filament length generally decreased after cultures reached their apparent maximum density (stationary phase). Although not readily apparent by light microscopy, structures consistent with septa between individual cells were observed by both TEM (Fig. 1b) and SEM (Fig. 1c). Based on measurements of cells in TEM images (n=16), the mean cell width and length were 0.27 ± 0.02 and 4.0 ± 1.3 μm (e.g. Fig. 1b). TEM of thin sections (Fig. 1d) revealed three distinct electron-dense layers in the cell envelope. The innermost layer probably represents the cytoplasmic membrane, while the two outer layers could represent either a cell wall and outer membrane or the inner and outer boundary of a cell wall-like structure. The difficulty to infer diderm vs monoderm cell structure based on EM images and Gram stain reaction in *Chloroflexi* has been noted, and firm conclusions await a more detailed analysis by EM and the presence or absence of specific genomic markers associated with an outer membrane (Sutcliffe, 2011). On solid GBS salts medium, JAD2^T formed translucent colonies that lacked obvious colour, approximately 0.2–0.3 mm in diameter, that were circular, convex and entire. Obvious swimming or gliding motility was not observed, and spores were not formed under any growth conditions tested. Analysis of phospholipid fatty acids in strain JAD2^T indicated that the following fatty acids were present at >1 % abundance: $C_{16:0}$ (42.0 %), $C_{19:0}$ (16.1 %), $C_{18:0}$ (7.7 %), $C_{20:0}$ (7.2 %), $C_{19:1}$ (6.6 %), $C_{18:1}\omega7t$ (3.9 %), iso-$C_{16:0}$ (3.6 %), $C_{18:1}\omega9t$ (3.1 %), 10-methyl $C_{16:0}$ (2.6 %), cyclopropane $C_{19:0}$ (2.3 %), anteiso-$C_{16:0}$ (1.8 %), $C_{18:1}$ (1.3 %) and $C_{17:0}$ (1.1 %). The genomic DNA G+C content was determined to be 69.3 mol%. Whole-cell sugars were mannose, rhamnose, glucose, galactose, ribose and xylose. The very low growth yields of this organism, typically <1 mg dry cell mass l^{-1}, rendered it impractical to perform additional chemotaxonomic analyses typically required for description of a new genus, including determination of major quinones, polar lipids and cell wall composition.

Phylogenetic analyses using the nearly complete 16S rRNA gene sequence indicated that JAD2^T formed a monophyletic group with other members of the phylum *Chloroflexi* (Fig. 2a), but was not affiliated with any existing class or class-level lineage within this phylum (Fig. 2b). The 16S rRNA gene sequence of strain JAD2^T had similar levels of identity to representatives of several classes in the *Chloroflexi*, including *Ardenticatena maritima* 110S^T (class *Ardenticatenia*; 83.6 % identity), *Caldilinea aerophila* DSM 14535^T (*Caldilineae*; 83.5 %), *Thermomicrobium roseum* ATCC 27502^T (*Thermomicrobia*; 82.6 %), *Anaerolinea thermophila* UNI-1^T (*Anaerolineae*; 82.0 %) and *Dehalococcoides* sp. BHI80-15 (*Dehalococcoidia*; 82.4 %). In previous versions

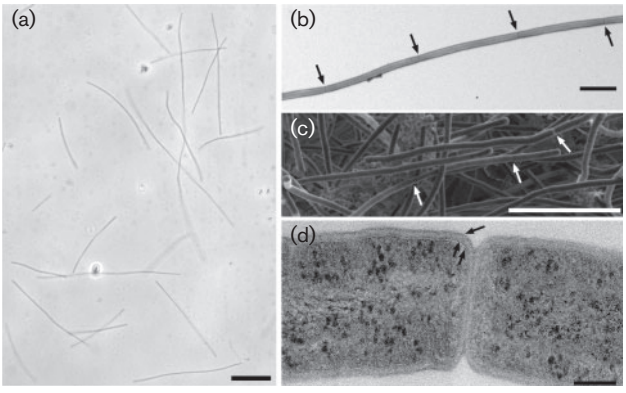


Fig. 1. Phase-contrast (a), transmission electron (b), scanning electron (c) and thin-section transmission electron (d) micrographs of strain JAD2^T. Negatively stained cells are shown in (b) and (d). Arrows in (b) and (c) indicate septa visible between individual cells in filaments. Arrows in (d) highlight three apparently distinct, electron-dense layers in the cell envelope. Bars, 20 μm (a), 2 μm (b), 4 μm (c) and 0.1 μm (d). Clumps of light-coloured particles in (c) represent precipitates formed during fixation and sample preparation.

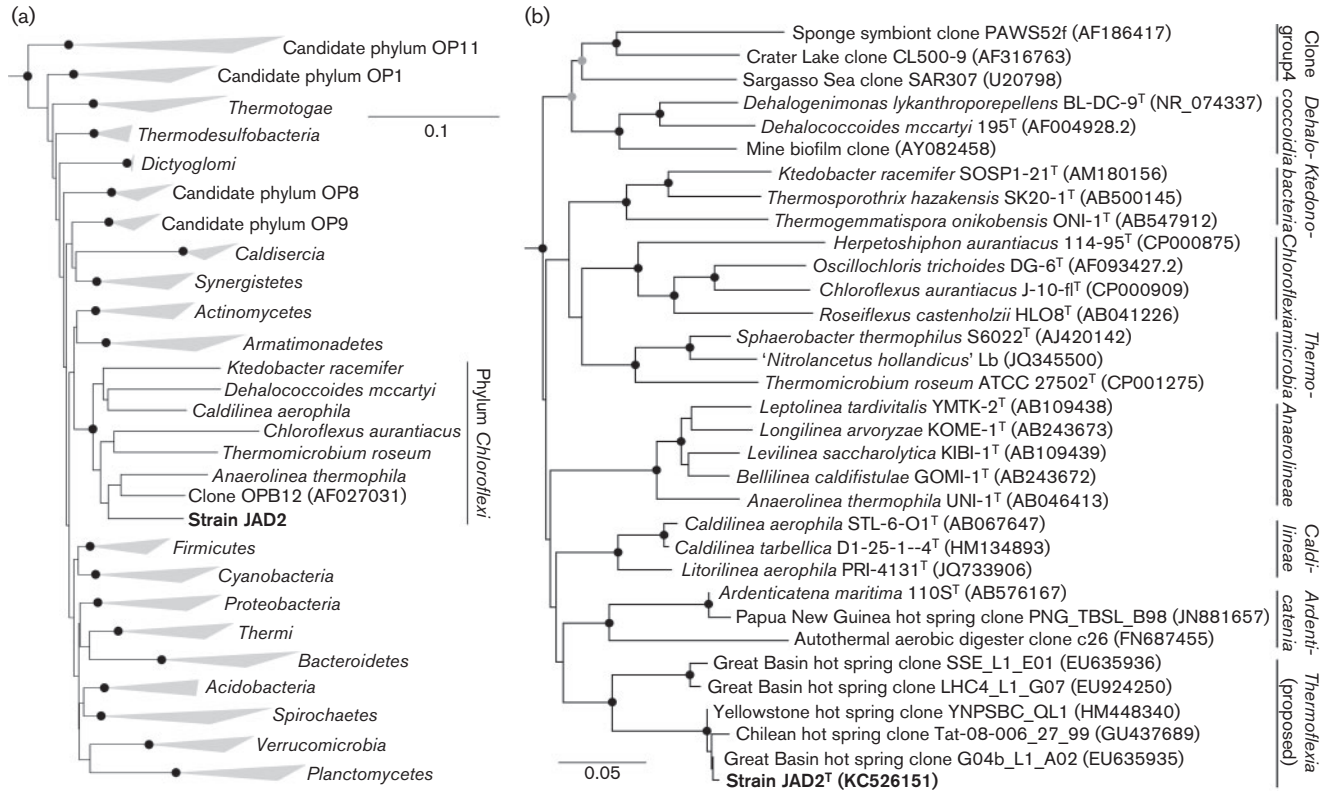


Fig. 2. Inferred phylogeny of strain JAD2^T based on 16S rRNA gene sequences within the context of recognized and candidate phyla within the domain *Bacteria* (a) and members of the phylum *Chloroflexi* (b). Bars, 0.1 (a) and 0.05 (b) substitutions per nucleotide position. Alignments used to generate the phylogenies are available in the online Supplementary Material. (a) Neighbour-joining phylogram based on a distance matrix reconstructed using PHYLIP using the F84 substitution model (Felsenstein, 2005) and excluding nucleotide positions with <50 % conservation. Individual sequences within the phylum *Chloroflexi* are shown, while sequences in other recognized and candidate phyla are represented by wedges. Within the phylum *Chloroflexi*, strains and accession numbers are the same as those shown in (b); a full list of the sequences used appears in Table S1. Members of the phylum *Aquificae* served as an outgroup (not shown). Major nodes supported in >80 % of 1000 bootstrap pseudoreplicates (filled circles) are indicated. (b) Neighbour-joining phylogram of cultivated members of the phylum *Chloroflexi* and selected environmental sequences, including those affiliated with strain JAD2^T (i.e. affiliated with the GAL35 group) and those present in a previous phylogeny of the phylum *Chloroflexi* (Hugenholtz & Stackebrandt, 2004), which identified the class-level lineage denoted as 'clone group 4'. Classes or class-level lineages are indicated by vertical bars. *Bacillus subtilis* 168 (GenBank accession no. D26185) and *Pseudomonas aeruginosa* ATCC 27853 (AY268175) served as an outgroup. Nodes supported in ⩾90 % (filled circles) or >80 % (shaded circles) of bootstrap pseudoreplicates for both maximum-likelihood (100 resamplings; tree not shown) and neighbour-joining (1000 resamplings) methods are indicated. Nodes that join the proposed *Thermoflexia* lineage with the class *Ardenticatenia* and nodes that join these lineages with the classes *Caldilineae* and *Anaerolineae* all had <50 % bootstrap support using either of the above phylogenetic inference methods.

(e.g. April 2011) of the Greengenes phylogenetic tool (McDonald *et al.*, 2012), the 16S rRNA gene sequence of JAD2^T was placed within the 'GAL35 group', named after a sequence amplified from a hot spring in Yellowstone National Park (YNP) (GenBank accession no. DQ324886; 99.5 % identity to JAD2^T). Additional sequences affiliated with the GAL35 group have been recovered from geothermal environments in YNP, Chile (Engel *et al.*, 2013), Bulgaria, China (Hou *et al.*, 2013) and the US Great Basin, where they are sometimes abundant (Cole *et al.*, 2013a; Costa *et al.*, 2009; Dodsworth *et al.*, 2011). Depending on the sequences, phylogenetic inference method and position mask used to infer the domain-level phylogeny (Fig. 2a), JAD2^T was sometimes monophyletic with sequences in the candidate phylum OP1 (not shown). To examine the relationship between JAD2^T and the phylum *Chloroflexi*, candidate phylum OP1, and other bacterial lineages more rigorously, a phylogeny was inferred based on concatenated alignments of the predicted amino acid sequences of 16 conserved genes identified by AMPHORA (Wu & Eisen, 2008) in the draft genome sequence of strain JAD2^T and other bacterial genomes. These 16 markers were chosen because they were also identified in the only available partial genome sequence dataset for a member of the candidate phylum OP1,

'*Candidatus* Acetothermum autotrophicum' (Takami *et al.*, 2012). In the resulting phylogeny, JAD2^T formed a monophyletic lineage with all other members of the phylum *Chloroflexi* with high bootstrap support (100 % of 100 pseudoreplicates), and was not affiliated with '*Candidatus* Acetothermum autotrophicum' (Fig. S1, available in the online Supplementary Material); similar results were obtained when all 31 markers identified by AMPHORA were used (not shown). A comparison of signature indels in ribosomal protein L19 (RplS) and UDP-glucose 4-epimerase (GalE) of strain JAD2^T with those of other members of the phylum *Chloroflexi* (Fig. S2), based on the dataset presented by Gupta *et al.* (2013), also support an affiliation of JAD2^T with the phylum *Chloroflexi*. It should be noted that the robust monophyly of the phylum *Chloroflexi* in phylogenomic analyses (Fig. S1) and the presence of signature indels in strain JAD2^T and *Caldilinea aerophila* (Fig. S2) do not support the recent proposal that only the classes *Chloroflexia* and *Thermomicrobia* should be considered within the phylum *Chloroflexi sensu stricto* (Gupta *et al.*, 2013).

In comparison with other members of the phylum *Chloroflexi*, JAD2^T has the highest optimal growth temperature (72.5–75 °C), rivalled only by that of *T. roseum* ATCC 27502^T (70–75 °C; Jackson *et al.*, 1973). JAD2^T can be distinguished from *T. roseum* ATCC 27502^T by its filamentous (versus irregular rod) morphology and growth under anaerobic conditions. A higher genomic DNA G+C content and the lack of growth at or below 65 °C distinguish JAD2^T from *A. maritima* 110S^T, the only described isolate in the class *Ardenticatenia* (Table 1). Phylogenetic analyses indicate that JAD2^T is a member of the phylum *Chloroflexi* (Figs 2b and S1); however, they do not support the affiliation of JAD2^T with any existing class within this phylum with high bootstrap support (Fig. 2b). Strain JAD2^T has <84 % 16S rRNA gene sequence identity to any cultivated member of this lineage, which is within the range of the majority (~90 %) of pairwise 16S rRNA gene sequence identity comparisons for members of different classes in a given phylum (75–85 %; Lasher *et al.*, 2009). On the basis of these and other phenotypic, genotypic, morphological and chemotaxonomic features, we propose that strain JAD2^T represents a novel lineage within the phylum *Chloroflexi*, for which we propose the name *Thermoflexus hugenholtzii* gen. nov., sp. nov. We further propose the designation of *Thermoflexaceae* fam. nov., *Thermoflexales* ord. nov. and *Thermoflexia* classis nov. to accommodate this novel lineage and other as-yet uncultivated organisms whose 16S rRNA gene sequences were previous placed in the GAL35 group. A comparison of characteristics of *T. hugenholtzii* JAD2^T with type strains of members of other classes in the phylum *Chloroflexi* is shown in Table 1.

Table 1. Comparison of characteristics of *Thermoflexus hugenholtzii* gen. nov., sp. nov. JAD2^T with those of type strains representing other classes in the phylum *Chloroflexi*

Classes are represented by the following type strains: *Dehalococcoides mccartyi* 195^T (data from Löffler *et al.*, 2013), *Anaerolinea thermophila* UNI-1^T (Sekiguchi *et al.*, 2003), *Caldilinea aerophila* STL-6-O1^T (Sekiguchi *et al.*, 2003), *Chloroflexus aurantiacus* J-10-fl^T (Pierson & Castenholz, 1974), *Thermomicrobium roseum* ATCC 27502^T (Jackson *et al.*, 1973), *Ktedonobacter racemifer* SOSP1-21^T (Cavaletti *et al.*, 2006), *Ardenticatena maritima* 110S^T (Kawaichi *et al.*, 2013) and *Thermoflexus hugenholtzii* JAD2^T (this study). ND, No data available.

Characteristic	*Dehalococcoidia*	*Anaerolineae*	*Caldilineae*	*Chloroflexia*	*Thermomicrobia*	*Ktedonobacteria*	*Ardenticatenia*	*Thermoflexia*
Temperature for growth (°C)								
Range	20–34	50–60	37–65	35–65	45–80	17–40	30–75	67.5–75
Optimum	28–34	55	55	52–60	70–75	28–33	55–70	75
pH for growth								
Range	6.0–8.0	6.0–8.0	7.0–9.0	ND	7.5–8.7	4.2–7.2	5.5–8.0	6.5–7.75
Optimum	7.0–7.5	7	7.5–8.5	7.6–8.4	8.2–8.5	6	7	6.75
Morphology	Disc-shaped cocci	Filamentous	Filamentous	Filamentous	Irregular rods	Filamentous	Filamentous	Filamentous
O_2 respiration	Anaerobic	Anaerobic	Facultative	Facultative	Aerobic	Aerobic	Facultative	Facultative (microaerophilic)
Major cellular fatty acids	10-Methyl 16:0, 16:0, 14:0, 18:0	16:0, 15:0, 14:0, 18:0	18:0, 16:0, 17:0, iso-17:0	18:1, 16:0, 18:0	12-Methyl 18:0, 18:0, 20:0*	16:1 2-OH, iso-17:0, iso-16:0,	22:0, 19:0, 23:0, 21:0	16:0, 19:0, 18:0, 20:0
DNA G+C content (mol%)†	48.9	54.5	59	56.9	64	54	51.1	69.3

*Determined at 70 °C (Pond & Langworthy, 1987).
†Determined by HPLC except for *Dehalococcoides mccartyi* 195^T (genome sequence) and *Thermomicrobium roseum* ATCC 27502^T (melting temperature).

Description of *Thermoflexus* gen. nov.

Thermoflexus (Ther.mo.fle′xus. Gr. fem. n. *therme* heat; L. adj. *flexus* curved from L. v. *flecto* to bend, to curve; N.L. masc. n. *Thermoflexus* the hot, curved one).

Thermophilic. Facultatively microaerophilic, chemoheterotrophic growth. Negative reaction for Gram stain. Filamentous morphology. Spores are not formed. Cells are not motile. The DNA G+C content of the type strain of the type species is 69.3 mol%. The genus represents a distinct phylogenetic lineage in the family *Thermoflexaceae*, the order *Thermoflexales* and the class *Thermoflexia* of the phylum *Chloroflexi*. The type species is *Thermoflexus hugenholtzii*.

Description of *Thermoflexus hugenholtzii* sp. nov.

Thermoflexus hugenholtzii (hu.gen.holt′zi.i. N.L. gen. n. *hugenholtzii* of Hugenholtz, referring to Australian microbiologist Dr Philip Hugenholtz, who contributed much to our understanding of bacterial diversity in geothermal environments).

Has the following properties in addition to those given in the genus description. Individual cells (0.3 μm wide and 4.0 μm long) form multicellular filaments typically ranging from 20 to 200 μm in length; septa between cells in filaments are not readily visible by light microscopy. Only complex carbon and energy sources including peptone, tryptone or yeast extract support growth, and vitamins are required for efficient growth. Temperature range for growth is 67.5–75 °C, with an optimum at 72.5–75 °C. pH range for growth is 6.5–7.75, with an optimum at pH 6.75. Growth is observed at up to 200 mM NaCl, with optimal growth below 100 mM. Microaerophilic, with growth observed up to 8 % O_2 and optimal growth at 1 %. In the absence of O_2, fermentative growth and H_2 production are observed. Anaerobic growth is enhanced by fumarate, inhibited by sulfite or elemental sulfur and not affected by nitrate, nitrite, thiosulfate or ferric NTA. The major cellular fatty acids (>5 % abundance) are $C_{16:0}$, $C_{19:0}$, $C_{18:0}$, $C_{20:0}$ and $C_{19:1}$.

The type strain, JAD2^T (=JCM 19131^T=CCTCC AB-2014030^T), was isolated from Great Boiling Spring in Nevada, USA.

Description of *Thermoflexaceae* fam. nov.

Thermoflexaceae (Ther.mo.fle.xa′ce.ae. N.L. n. *Thermoflexus* type genus of the family; L. suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Thermoflexaceae* the family of the genus *Thermoflexus*).

The description is the same as for the genus *Thermoflexus*. The type genus is *Thermoflexus*.

Description of *Thermoflexales* ord. nov.

Thermoflexales (Ther.mo.fle.xa′les. N.L. n. *Thermoflexus*, type genus of the order; L. suff. *-ales* ending to denote an order; N.L. fem. pl. n. *Thermoflexales* the order of the genus *Thermoflexus*).

The description is the same as for the genus *Thermoflexus*. The type family is *Thermoflexaceae*.

Description of *Thermoflexia* classis nov.

Thermoflexia (Ther.mo.fle′xi.a. N.L. n. *Thermoflexus* type genus of the class; L. suff. *-ia* ending to denote a class; N.L. neut. pl. n *Thermoflexia* the class of the order *Thermoflexales*).

The description is the same as for the genus *Thermoflexus*. The class is a member of the phylum *Chloroflexi*. The type order is *Thermoflexales*.

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