**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.

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A thermophilic, filamentous, heterotrophic bacterium, designated strain JAD2T, 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 JAD2T was microaerophilic and facultatively anaerobic, with optimal growth at 1 % (v/v) O2 and an upper limit of 8 % O2. The major cellular fatty acids (>5 %) were C16:0, C19:0, C18:0, C20:0 and C19:1. 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 JAD2T within the phylum Chloroflexi, but not within any existing class in this phylum. These results indicate that strain JAD2T 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 JAD2T (≡JCM 19131T≡CCTCC AB-2014030T).


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

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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 (Cavalloti 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 microorganisms 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; Rappe & 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\textsuperscript{T}, the first cultivated representative of a novel, divergent lineage affiliated with the phylum Chloroflexi.

Strain JAD2\textsuperscript{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\textsuperscript{−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-quad pressure cups (C. A. Technologies) modified for use as anaerobic incubation vessels (Balch et al., 1979), and 100 kPa N\textsubscript{2}/CO\textsubscript{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 \times 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\textsuperscript{T}.

A synthetic medium was designed to facilitate characterization of strain JAD2\textsuperscript{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 MQ-cm deionized water) 3 g NaCl, 0.15 g KCl, 0.3 g Na\textsubscript{2}SO\textsubscript{4}, 0.123 g MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.015 g CaCl\textsubscript{2}·2H\textsubscript{2}O, 0.107 g NH\textsubscript{4}Cl 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 Fe\textsubscript{2}O\textsubscript{3}·7H\textsubscript{2}O, 0.0984 g Mn\textsubscript{2}O\textsubscript{4}·2H\textsubscript{2}O, 0.0288 g ZnSO\textsubscript{4}·7H\textsubscript{2}O, 0.0238 g CoCl\textsubscript{2}·6H\textsubscript{2}O, 0.0172 g CuCl\textsubscript{2}·2H\textsubscript{2}O, 0.0242 g Na\textsubscript{2}MoO\textsubscript{4}·2H\textsubscript{2}O and 0.31 g H\textsubscript{3}BO\textsubscript{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\textsubscript{2}·7H\textsubscript{2}O were added. The medium was sparged with 100 % N\textsubscript{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\textsubscript{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\textsubscript{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-Hauser counting chamber (†3900; Hauser 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, 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 MgSO₄·7H₂O instead of Na₂SO₄ 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 O₂. H₂ 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: 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-TVIx-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 2000g 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% O₂. 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, frx, infC, nusA, pgk, pyrG, rplA, rplK, rplL, rplS, rplT, rpmA, rpoB, rpsB, rpsL, snpB) identified in a draft genome sequence of strain JAD2T using AMPHORA (Vu & Eisen, 2008); the genome sequence of JAD2T 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 phylogenograms 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 JAD2T 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 H₂ production was observed by GC under these conditions, suggesting that H₂ was a product of fermentative growth. Oxygen levels from 0.5 to 8% stimulated growth, with optimum cell yields obtained at 1% O₂, whereas no growth was observed at 12% O₂ or greater. The doubling time of strain JAD2T grown in the presence of 0.05% peptone and 1% O₂ at 75°C was approximately 1 day, with final culture densities ranging between 0.5 and 1.5 x 10⁶ filaments ml⁻¹, in comparison with 1–2 x 10⁵ 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 H2/CO2 (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, succrose, 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, while 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 NH4Cl, indicating that these could serve as sole nitrogen sources.

Cells of strain JAD2T 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, 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, JAD2T 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 JAD2T indicated that the following fatty acids were present at >1% abundance: C16 : 0 (42.0 %), C19 : 0 (16.1 %), C18 : 0 (7.7 %), C20 : 0 (7.2 %), C19 : 1 (6.6 %), C18 : 1ω7t (3.9 %), iso-C16 : 0 (3.6 %), C18 : 1ω9t (3.1 %), 10-methyl C16 : 0 (2.6 %), cyclopropane C19 : 0 (2.3 %), anteiso-C16 : 0 (1.8 %), C18 : 1ω7t (1.3 %) and C17 : 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 JAD2T 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 JAD2T had similar levels of identity to representatives of several classes in the Chloroflexi, including Ardenticatenia maritima 1105T (class Ardenticatenia; 83.6 % identity), Caldilinea aerophila DSM 14535T (Caldilineae; 83.5 %), Thermomicrobiurn roseum ATCC 27502T (Thermomicrobia; 82.6 %), 110C16 Anaerolinea thermophila UNI-1T (Anaerolineae; 82.0 %) and Dehalococcoides sp. BHI80-15 (Dehalococcoidia; 82.4 %). In previous versions

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**Fig. 1.** Phase-contrast (a), transmission electron (b), scanning electron (c) and thin-section transmission electron (d) micrographs of strain JAD2T. 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\textsuperscript{2} 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 PHYL 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 $\geq$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\textsuperscript{2} (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. 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.
‘Candidatus Acetothermum autotrophicum’ (Takami et al., 2012). In the resulting phylogeny, JAD2\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{T} and Caldilinea aerophila (Fig. S2) do not support the recent proposal that only the classes Chloroflexi 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\textsuperscript{T} has the highest optimal growth temperature (72.5–75 °C), ravelled only by that of T. roseum ATCC 27502\textsuperscript{T} (70–75 °C; Jackson et al., 1973). JAD2\textsuperscript{T} can be distinguished from T. roseum ATCC 27502\textsuperscript{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\textsuperscript{T} from A. maritima 110\textsuperscript{ST}, the only described isolate in the class Ardenticatenia (Table 1). Phylogenetic analyses indicate that JAD2\textsuperscript{T} is a member of the phylum Chloroflexi (Figs 2b and S1); however, they do not support the affiliation of JAD2\textsuperscript{T} with any existing class within this phylum with high bootstrap support (Fig. 2b). Strain JAD2\textsuperscript{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\textsuperscript{T} represents a novel lineage within the phylum Chloroflexi, for which we propose the name Thermoflexus hugenholtzii gen. nov., sp. nov. JAD2\textsuperscript{T} with those of type strains representing other classes in the phylum Chloroflexi (Table 1). Classes are represented by the following type strains: Dehalobacterium autotrophicum 1-101\textsuperscript{T} (Pierson & Castenholz, 1974), Thermomonospora rosea ATCC 27502\textsuperscript{T} (Jackson et al., 1973), Ktedonobacter racemifer 21-64\textsuperscript{T} (Cavaletti et al., 2003), Thermoplasma acidophilum ATCC 27502\textsuperscript{T} (Piehler et al., 1995), Caldilinea aerophila ST-6-1-O (Seigfried et al., 2003), Caldarivibacterium thermophilum UNI-1\textsuperscript{T} (Sekiguchi et al., 2003), Caldilinea aerophila 110\textsuperscript{ST} (Pierson & Castenholz, 1974), Thermotoga maritima 110\textsuperscript{ST}, the only described isolate in the class Thermotogae, and Thermoflexus hugenholtzii JAD2\textsuperscript{T} (this study). No. No data available.

**Description of Thermoflexus gen. nov.**

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

**Table 1.** Comparison of characteristics of *Thermoflexus hugenholtzii* gen. nov., sp. nov. JAD2\textsuperscript{T} with those of type strains representing other classes in the phylum Chloroflexi (Table 1).

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<th>Characteristic</th>
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<th>Candidatus</th>
<th>Chloroflexi</th>
<th>Chloromicrobiota</th>
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<td><strong>DNA G+C content (mol%)</strong></td>
<td>48.9</td>
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* Determined at 70 °C (Bond & Langworthy, 1987).

**Description of Thermoflexus hugenholtzii gen. nov., sp. nov.**

JAD2\textsuperscript{T} has the highest optimal growth temperature (72.5–75 °C), ravalled only by that of T. roseum ATCC 27502\textsuperscript{T} (70–75 °C; Jackson et al., 1973). JAD2\textsuperscript{T} can be distinguished from T. roseum ATCC 27502\textsuperscript{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\textsuperscript{T} from A. maritima 110\textsuperscript{ST}, the only described isolate in the class Ardenticatenia (Table 1). Phylogenetic analyses indicate that JAD2\textsuperscript{T} is a member of the phylum Chloroflexi (Figs 2b and S1); however, they do not support the affiliation of JAD2\textsuperscript{T} with any existing class within this phylum with high bootstrap support (Fig. 2b). Strain JAD2\textsuperscript{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\textsuperscript{T} represents a novel lineage within the phylum Chloroflexi, for which we propose the name Thermoflexus hugenholtzii gen. nov., sp. nov. JAD2\textsuperscript{T} with those of type strains representing other classes in the phylum Chloroflexi (Table 1). Classes are represented by the following type strains: Dehalobacterium autotrophicum 1-101\textsuperscript{T} (Pierson & Castenholz, 1974), Thermomonospora rosea ATCC 27502\textsuperscript{T} (Jackson et al., 1973), Ktedonobacter racemifer 21-64\textsuperscript{T} (Cavaletti et al., 2003), Thermoplasma acidophilum ATCC 27502\textsuperscript{T} (Piehler et al., 1995), Caldarinea aerophila ST-6-1-O (Seigfried et al., 2003), Caldarivibacterium thermophilum UNI-1\textsuperscript{T} (Sekiguchi et al., 2003), Caldarinea aerophila 110\textsuperscript{ST} (Pierson & Castenholz, 1974), Thermotoga maritima 110\textsuperscript{ST}, the only described isolate in the class Thermotogae, and Thermoflexus hugenholtzii JAD2\textsuperscript{T} (this study). No. No data available.
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 Hugenholz, 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% O2 and optimal growth at 1%. In the absence of O2, fermentative growth and H2 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 C16:0, C19:0, C18:0, C20:0 and C19:1.

The type strain, JAD2T (=JCM 19131T=CCTCC AB2014030T), was isolated from Great Boiling Spring in Nevada, USA.

**Description of Thermoflexaceae fam. nov.**

*Thermoflexaceae* (Ther.mo.fle’xe.a.e. 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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