Kallotenue papyrolyticum gen. nov., sp. nov., a cellulolytic and filamentous thermophile that represents a novel lineage (Kallotenuales ord. nov., Kallotenuaceae fam. nov.) within the class Chloroflexia

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Several closely related, thermophilic and cellulolytic bacterial strains, designated JKG1T, JKG2, JKG3, JKG4 and JKG5, were isolated from a cellulolytic enrichment (corn stover) incubated in the water column of Great Boiling Spring, NV. Strain JKG1T had cells of diameter 0.7–0.9 μm and length ~2.0 μm that formed non-branched, multicellular filaments reaching ~300 μm. Spores were not formed and dense liquid cultures were red. The temperature range for growth was 45–65 °C, with an optimum of 55 °C. The pH range for growth was pH 5.6–9.0, with an optimum of pH 7.5. JKG1T grew as an aerobic heterotroph, utilizing glucose, sucrose, xylose, arabinose, cellobiose, CM-cellulose, filter paper, microcrystalline cellulose, xylan, starch, Casamino acids, tryptone, peptone, yeast extract, acetate, citrate, lactate, pyruvate and glycerol as sole carbon sources, and was not observed to photosynthesize. The cells stained Gram-negative. Phylogenetic analysis using 16S rRNA gene sequences placed the new isolates in the class Chloroflexia, but distant from other cultivated members, with the highest sequence identity of 82.5 % to Roseiflexus castenholzii.

The major quinone was menaquinone-9; no ubiquinones were detected. The major cellular fatty acids (>5 %) were C18 : 0, anteiso-C17 : 0, iso-C18 : 0, iso-C17 : 0, C16 : 0, iso-C16 : 0 and C17 : 0. The peptidoglycan amino acids were alanine, ornithine, glutamic acid, serine and asparagine. Whole-cell sugars included mannose, rhamnose, glucose, galactose, ribose, arabinose and xylose. Morphological, phylogenetic and chemotaxonomic results suggest that JKG1T is representative of a new lineage within the class Chloroflexia, which we propose to designate Kallotenue papyrolyticum gen. nov., sp. nov., Kallotenuaceae fam. nov., Kallotenuales ord. nov. The type strain of Kallotenue papyrolyticum gen. nov., sp. nov. is JKG1T (=DSM 26889T=JCM 19132T).

The phylum Chloroflexi is a deeply branching lineage of bacteria composed of a limited number of cultivated representatives that display diverse metabolic strategies and phenotypes. The class Chloroflexia contains gliding filamentous bacteria divided into two orders, Chloroflexales and Herpetosiphonales. The order Chloroflexales comprises anoxygenic phototrophs that possess bacteriochlorophyll and may contain chlorosomes (Dubinina & Gorlenko, 1975; Hanada et al., 2002; Keppen et al., 1994; Pierson & Castenholz, 1974; Pierson et al., 1985). The order Herpetosiphonales includes only one genus, Herpetosiphon, the two described species of which, Herpetosiphon geysericola

Abbreviations: GBS, Great Boiling Spring; MK, menaquinone; SEM, scanning electron microscopy; TEM, transmission electron microscope.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JKG1T, JKG2, JKG3, JKG4 and JKG5 are JX848544, JX848545, JX848546, JX848547 and JX848548, respectively.
and Herpetosiphon aurantiacus, are mesophilic and aerobic heterotrophs. Herpetosiphon aurantiacus utilizes carbohydrates as carbon sources, with 6.5% of its genome devoted to carbohydrate transport and metabolism (Kiss et al., 2011). However, Herpetosiphon geysericola, reported to degrade cigarette paper, is the only member of the class Chloroflexia known to hydrolyse either soluble or insoluble cellulose (Lewin 1970).

Five bacterial strains (JKG1T, JKG2, JKG3, JKG4 and JKG5) were isolated from a lignocellulosic enrichment incubated in Great Boiling Spring (GBS). GBS is a circumneutral geothermal spring, located in north-western Nevada, USA, at 40°39′41″N 119°21′58″W (Costa et al., 2009). Twenty grams of ammonia fibre explosion (AFEX)-treated corn stover were enclosed within a nylon filter bag of 100 μm pore size (Pentair Industrial) sewn shut with nylon thread. The bag was suspended in the spring water column of GBS ~10 cm below the air–water interface at Site 85 (85 °C) for 9 weeks (Peacock et al., 2013). A modified version of Castenholz Medium D (Castenholz, 1969), designated Castenholz medium D VN (VN medium), was prepared with the addition of 1 × Wolfe’s vitamins (Balch et al., 1979) and 0.027 g NH₄Cl l⁻¹. Ten millilitres of VN medium was prepared in 30 ml glass screw-top culture tubes with 1 % (w/v) filter paper (Whatman filter paper grade 1; GE Healthcare) as the sole carbon and energy source (VNF medium) and air as headspace. Material from the enrichment was obtained using sterile forceps and used to inoculate three screw-top tubes, each of which was incubated in the laboratory at 60, 70 or 80 °C. After 8 weeks of incubation a biofilm was visible in the 60 °C tube. The biofilm culture was maintained on VNF medium until pure cultures could be obtained. Traditional approaches of plating or dilution to extinction yielded weakly or non-cellulolytic isolates of the genera Thermus, Geobacillus and Rhodothermus; however, pure cultures were not obtained of a filamentous morphotype commonly observed at the medium/filter paper interface. Therefore, short filaments, representing one or a few cells, were separated from the mixed culture by using optical tweezers and a sterile microfluidic device (Dodsworth et al., 2013; Youssef et al., 2011) and recovered from the chip by flushing with VN medium. Five strains (JKG1, JKG2, JKG3, JKG4 and JKG5) of similar morphology were cultivated from single filaments. All five strains were checked for purity based on the recovery of a single colony type following plating on solid VN medium with 0.2 % (w/v) glucose and buffered at pH 7.5 with 15 mM HEPES (VNG medium). VNG medium was solidified by the addition of 1.5 % (w/v) agar (granulated; BD Difco). In addition, direct sequencing of 16S rRNA genes PCR-amplified from DNA prepared from each strain, described below, yielded a single, high quality sequence with no ambiguous nucleotides diagnostic of a mixed culture. Strain JKG1T was characterized in detail.

The colony morphology of strain JKG1T was observed after 7 days of incubation at 55 °C on VNG. Filament morphology was observed using phase-contrast microscopy with an Olympus BX51 phase-contrast microscope and Olympus V-TV1 × −2 camera. Detailed morphology was visualized using a Tecnai T-12 TEM (FEI) transmission electron microscope (TEM) with LaB6 filament operating at 120 kV. Images were collected digitally using a 2 × 2K Ultrscan 1000 charge-coupled device with a ‘U’ scintillator (Gatan) calibrated to the TEM camera length to enable direct measurements correlated with the magnification of the acquired images. DigitalMicrograph (Gatan) software was used for imaging and analyses of cellular features. Scanning electron microscopy (SEM) samples were examined using a Helios 600 Nanolab Dual Beam microscope (FEI).

For TEM, cells were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer at 4 °C overnight and washed three times in 0.1 M cacodylate buffer. Cells were then osmilicated with 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature and washed three times in 0.1 M cacodylate buffer. Cells were dehydrated in 30, 50 and 75 % ethanol for 30 min each, and three times in 100 % ethanol for 60 min. Cells were washed with a 1 : 1 mixture of LR white acrylic resin (Electron Microscopy Sciences) and ethanol for 30 min and then infiltrated in 100 % resin, three washes, 4 h each. Samples were cured at 60 °C for 24 h. Polymerized blocks were sectioned to 70 nm thin sections with a Leica Ultracut UCT ultramicrotome, sections were mounted on Formvar-coated 100 mesh Cu TEM grids, sputtered with carbon and post-stained for 7 min with aqueous 2 % uranyl acetate prior to TEM imaging.

For the whole mount preparation, 5 μl of a planktonic cell suspension was applied on a 100 mesh Cu grid with Formvar and carbon (Electron Microscopy Sciences). The cells were allowed to adhere to the grids for 30 s before blotting the liquid with a filter paper and then staining with 5 μl negative stain Nano-W (Nanoprobes) for 45 s, followed by air drying.

For SEM, cells were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer at 4 °C overnight, washed three times in buffer and dehydrated in an ethanol series (33 %, 50 %, 75 %), followed by three washes in 100 % ethanol. The samples were then critically point dried in a critical point dryer (CPD) instrument (Samdri-795; Tousimis) and processed according to an automated CPD scheme, with CO₂ as a transitional fluid. The CPD holder was lined with nanopore membrane to prevent cell loss during the processing. The cells were mounted on standard aluminium SEM stubs covered with double-sided carbon adhesive tape and sputter-coated with carbon.

Physiological tests carried out in VNG broth were done at 55 °C in the dark in glass 25 ml Balch tubes stopped with butyl rubber septa (Wheaton) with an air headspace and orbital shaking at an empirically determined optimum (100 r.p.m., 20° angle), except where noted. Growth at the following pH was tested with 15 mM of the indicated pH. 

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buffer: pH 5.5, 6.0 and 6.5, MES; pH 7.0, 7.5 and 8.0, HEPES; pH 7.5, 8.0, 8.5 and 9.0, Tris; and pH 8.5, 9.5 and 10.0 CHES. Growth temperature was tested in five-degree increments, from 40 to 75 °C. NaCl tolerance was tested at 0, 0.2, 0.4, 1.0, 1.5, 2.0, 2.5 and 3.0 % (w/v) NaCl.

VN medium buffered to pH 7.5 (at 55 °C) with 15 mM HEPES was used to test the utilization of a variety of potential growth substrates, tested at 0.2 % (w/v): D-glucose, sucrose, (+)-cellobiose, xylose, L-arabinose, pyruvate, Casamino acids (EMD), tryptone (BD Bacto), peptone (BD Bacto), yeast extract (EM Science, EMD), acetate, citrate, glycerol, lactate, CM-cellulose (Spectrum), filter paper (Whatman filter paper grade 1; GE Healthcare), microcrystalline cellulose (extra pure 90 μm; Acros Organics, Thermo Fisher Scientific), xylan (birch wood; Sigma-Aldrich) and starch (soluble potato starch powder; JT Baker, Avator Performance Materials). Three consecutive transfers were conducted for each substrate, with 72 h of growth allowed before each transfer, and the third transfer was performed in triplicate. Positive substrate utilization was determined by a compatible 1349-position Lane mask was applied (Lane, 1991). PHELYP v3.69 was used to produce neighbour-joining, maximum-parsimony and maximum-likelihood phylogenetic trees with 100 bootstraps per tree (Felsenstein, 2005). The following parameters in PHELYP were modified as indicated: input order of species was randomized, outgroup root was set to Bacillus subtilis, jumbles were 1, global rearrangements on, speedier but rougher analysis off, and more thorough search on. Trees were visualized and exported using Dendroscope v2 (Huson et al., 2007).

Strain JKG1T formed small, white, rhizoid colonies and gliding motility was observed. Dense liquid cultures were red and cells stained Gram-negative (Leboffe & Pierce, 2006). Phase-contrast microscopy revealed JKG1T to be a non-branched, multicellular and filamentous bacterium with a diameter of 0.7–0.9 μm (Fig. 1a). Filaments were flexible and composed of individual cells of ~2.0 μm in length. Spores were not observed. Transparent sections resembling the sleeves observed in species of the genus Herpetosiphon were sometimes observed between cells or at the ends of filaments (Lee & Reichenbach, 2006). Filaments in VNG medium typically ranged from 10–40 μm; in R2A medium filaments were longer, reaching >300 μm. SEM and TEM showed the filaments to be cylindrical with constrictions at cell junctions within the filaments (Fig. 1b–d). TEM of thin sections did not show the presence of chlorosomes or cellular inclusions (Fig. 1e, f). The cell envelope was similar to those of other members of the phylum Chloroflexi (Lee & Reichenbach, 2006; Yamada et al., 2006), composed of two electron-dense layers approximately 10 nm apart. The layers were present at the periphery of each cell, including the tip of the cells that terminated each filament (Fig. 1e), but neither layer was resolvable at the junctions of the cells within the filaments (Fig. 1f). The exact structure and composition of the cellular envelopes of members of the phylum Chloroflexi is

DNA was extracted using the FastDNA Spin kit for Soil (MP Biomedicals) using modifications specified previously (Dowdsworth et al., 2011). 16S rRNA genes were amplified by PCR using primers 9bF (Eder et al., 1999) and 1512uR (Eder et al., 2001) as described previously (Costa et al., 2009) and sequenced at Functional Biosciences (Madison, WI, USA) using the PCR primers 704bR (TCTAG YATTTCACYGCT) and 516uF (TGBCA GMGCGCGG– TAA) to obtain overlapping reads. Reads were trimmed to remove bases with quality scores of less than 20, aligned against the mother–provided SILVA alignment in the program mothur v1.20.2 (Schloss et al., 2009) and trimmed to the shortest sequence, resulting in an alignment of the near-full-length 16S rRNA gene sequences of all five strains 1355 nt in length.

The nucleotide sequence of the near-full-length 16S rRNA gene of strain JKG1T was aligned with reference sequences of members of the phylum Chloroflexi using the mother–provided SILVA alignment in the program mothur. The sequences of Heliotherix oregenensis and Chloronema giganteum were excluded due to poor sequence quality. BioEdit v7.0.5.3 was used to manually curate the alignment and calculate the pairwise 16S rRNA gene sequence identities (Hall, 1999). The mother–provided SILVA–compatible 1349-position Lane mask was applied (Lane, 1991). PHELYP v3.69 was used to produce neighbour-joining, maximum-parsimony and maximum-likelihood phylogenetic trees with 100 bootstraps per tree (Felsenstein, 2005). The following parameters in PHELYP were modified as indicated: input order of species was randomized, outgroup root was set to Bacillus subtilis, jumbles were 1, global rearrangements on, speedier but rougher analysis off, and more thorough search on. Trees were visualized and exported using Dendroscope v2 (Huson et al., 2007).
currently unknown, but genomic evidence has failed to reveal components necessary for synthesis of an outer membrane and protein secretion to or across an outer membrane, suggesting a monoderm cell structure (Sutcliffe, 2010, 2011).

Strain JKG1T was able to grow heterotrophically under aerobic conditions utilizing glucose, sucrose, xylose, arabinose, cellobiose, CM-cellulose, filter paper, microcrystalline cellulose, xylan, starch, Casamino acids, tryptone, peptone, yeast extract, acetate, citrate, lactate, pyruvate and glycerol as sole carbon sources. Optimal growth occurred at 55 °C and pH 7.5. Growth occurred from 45 to 65 °C and pH 6.5–9.5. Photoheterotrophic, photoautotrophic, fermentative and anaerobic respiration were not observed.

The major cellular fatty acids of strain JKG1T were C₁₈:0 (26.3 % of total fatty acids), anteiso-C₁₇:0 (15.0 %), iso-C₁₈:0 (12.7 %), iso-C₁₇:0 (11.5 %), C₁₆:0 (8.9 %), iso-C₁₆:0 (6.2 %) and C₁₇:0 (5.0 %). The minor fatty acids were anteiso-C₁₉:0 (3.9 %), anteiso-C₁₅:0 (1.9 %), iso-C₁₉:0 (1.4 %), C₁₈:1ω9c (1.4 %), C₁₉:0 (1.0 %), C₁₇:0 3-OH (0.9 %), C₁₈:1ω7c (0.8 %), iso-C₁₅:0 (0.7 %), iso-C₁₇:1ω9c (0.6 %), anteiso-C₁₇:1ω9c (0.5 %), anteiso-C₁₃:0 (0.5 %), C₁₅:0 (0.5 %) and C₁₄:0 (0.4 %).

The major quinone was MK-9(H₆) (84.1 % relative abundance), with a smaller proportion of MK-8(H₂) (15.9 %). The peptidoglycan amino acids were alanine, ornithine, glutamic acid, serine and asparagine. meso-Diaminopimelic acid was not present and peptide cross-links are likely formed between ornithine and D-alanine, as in other members of the phylum Chloroflexi (Cavaletti et al., 2006; Jürgens et al., 1987, 1989; Yabe et al., 2010, 2011). In particular, ornithine has been found to replace diaminopimelic acid as the diamino acid that forms the cross-peptide bonds (Garrity & Holt, 2001). Whole-cell sugars were mannose, rhamnose, glucose, galactose, ribose, arabinose, xylose, and two unknown compounds. The DNA G+C content was 72.4 mol% and was determined by HPLC (Mesbah et al., 1989).

The 16S rRNA gene sequences of all five strains were identical over the length of the alignment shared by all five sequences (1355 nt). Phylogenetic analysis of JKG1T using the 16S rRNA gene sequence placed the strain in the class Chloroflexia, with bootstrap values of at least 90 % at the node defining the class in all three phylogenetic methods utilized (Fig. 2). Members of the class Chloroflexia share an unbranched filamentous morphology, with the majority of the isolates capable of aerobic respiration (Table 1). However, strain JKG1T is distinguishable from these other members of the class as the only thermophilic chemo-organotroph not capable of photosynthesis. In addition, strain JKG1T harbours distinct profiles of fatty acids, quinones, whole-cell sugars and cell wall amino acids. Finally, the 16S rRNA gene sequence identity between strain JKG1T and other members of the class Chloroflexia was ≤82.5 %, which is well below the mean pairwise 16S rRNA gene sequence identity between taxa whose lowest shared rank is class, ~87 % (Lasher et al., 2009; Konstantinidis & Tiedje, 2005), and the 16S rRNA gene sequence identity commonly used to circumscribe microbial orders in cultivation-independent studies, 85 % (Cole et al., 2013; Peacock et al., 2013; Schmitt et al., 2012; Webster et al., 2010). Altogether, the unique phylogenetic, phenotypic and chemotaxonomic features of strain JKG1T suggest that it is representative of a novel genus, family and order within the class Chloroflexia. The designations Kallotenue gen. nov., Kallotenuaceae fam. nov. and Kallotenuales ord. nov., are proposed.

**Description of Kallotenue gen. nov.**

*Kallotenue* (Kal.ō.te′nu.e. Gr. neut. n. kallos beauty, grace; L. adj. tenuis -is -e slender, fine, thin; N.L. neut. n. Kallotenue a thin beauty).
Filamentous, non-branching and non-spore-forming bacteria. Gram-stain negative. Thermophilic, aerobic chemoorganotrophs that grow at 45–65 °C, pH 5.6–9.0 and with 0–2.0 % (w/v) NaCl. Peptidoglycan amino acids include alanine, ornithine, glutamine, serine, and asparagine. Major whole-cell sugars comprise mannose, rhamnose...
Table 1. Phenotypic characteristics of strain JKG1^T and related members of the class Chloroflexia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Aerobic chemotrophs</th>
<th>Chlorosome-less phototrophs</th>
<th>Chlorosome-containing phototrophs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JKG1^T</td>
<td>Herpetosiphon aurantiacus DSM 785^T</td>
<td>Herpetosiphon geysericola ATCC 23076^T</td>
</tr>
<tr>
<td>Cell diameter (μm)</td>
<td>0.7–0.9</td>
<td>1.0–1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Sleeves</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gas vesicles</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Gram stain results</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Optimum growth temperature (°C)</td>
<td>55</td>
<td>30–37</td>
<td>30–37</td>
</tr>
<tr>
<td>Metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photoheterotrophy</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Photoautotrophy</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Aerobic respiration</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fermentation</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cellulolytic ability</td>
<td>+</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>−</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Insoluble cellulose</td>
<td>+</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>Major cellular fatty acids (&gt;5%)</td>
<td>C18 : 0, anteiso-C17 : 0, iso-C18 : 0, iso-C17 : 0, C16 : 0, iso-C16 : 0, C17 : 0</td>
<td>C16 : 1, C16 : 0, C18 : 1, C16 : 1, C16 : 0, C18 : 1, C16 : 1, C16 : 0, C18 : 1</td>
<td>C16 : 0, C14 : 0, C15 : 0, C18 : 0, C16 : 0, C18 : 1, C17 : 0, C18 : 1, C16 : 0, C16 : 1</td>
</tr>
<tr>
<td>Predominant menaquinone</td>
<td>MK-9</td>
<td>MK-6</td>
<td>ND</td>
</tr>
<tr>
<td>DNA G+C content</td>
<td>72.4</td>
<td>50.9</td>
<td>51.8</td>
</tr>
<tr>
<td>Whole-cell or [cell wall] sugars</td>
<td>Man, Rham, Glc, Ara, Xyl, Unk (× 2), Gal, Rib</td>
<td>Man, Gal, Rham, Ara, Xyl</td>
<td>ND</td>
</tr>
<tr>
<td>Cell wall amino acids</td>
<td>Ala, Orn, Glu, Ser, Asn</td>
<td>Glu, Ala, Orn, Gly, His</td>
<td>ND</td>
</tr>
<tr>
<td>Isolation environment</td>
<td>Lignocellulosic enrichment in terrestrial hot spring</td>
<td>Slimy coating of Chara sp. in freshwater lake</td>
<td>Rock surface beside hot spring</td>
</tr>
</tbody>
</table>
and glucose. The major quinone is menaquinone-9. The type species is *Kallotenue papyrolyticum*.

**Description of Kallotenue papyrolyticum sp. nov.**

*Kallotenue papyrolyticum* [pa.py.roy.li’ti.cum. Gr. n. papyros papyrus, paper; N.L. neut. adj. *lyticum* (from Gr. neut. adj. *lytikon*) able to loose, able to dissolve; N.L. neut. adj. *papyrolyticum* paper-dissolving].

The following properties are displayed, in addition to those specified for the genus. Growth occurs at an optimum temperature of 55 °C and optimum pH of 7.5. Colonies are small, 1–2 mm diameter, white and rhizoid. The following substrates support growth: glucose, sucrose, xylose, arabinose, cellobiose, CM-cellulose, filter paper, microcrystalline cellulose, xylan, starch, Casamino acids, tryptone, peptone, yeast extract, acetate, citrate, lactate, pyruvate and glycerol. The major cellular fatty acids are C₁₈:₀, anteiso-C₁₇:₀, iso-C₁₈:₀, iso-C₁₇:₀, C₁₆:₀, iso-C₁₆:₀, C₁₇:₀ and anteiso-C₁₉:₀.

The type strain is JKG1T (=DSM 26889®=JCM 19132ᵀ) isolated from a lignocellulosic enrichment incubated in a hot spring. The G+C content of the type strain is 72.4 mol%.

**Description of Kallotenaceae fam. nov.**

*Kallotenaceae* (Kal.lo.te.nu.ea.e. N.L. n. *Kallotenue* type genus of the family; suff. -aceae ending denoting a family; N.L. fem. pl. n. *Kallotenaceae* the family of the genus *Kallotenue*).

The description is the same as for the genus *Kallotenue*. The family is a member of the order *Kallotenuales*. The type genus is *Kallotenue*.

**Description of Kallotenuales ord. nov.**

*Kallotenuales* (Kal.lo.te.nu.a’les. N.L. n. *Kallotenue* type genus of the order; suff. -ales ending denoting an order; N.L. fem. pl. n. *Kallotenuales* the order of the genus *Kallotenue*).

The description is the same as for the genus *Kallotenue*. The order is a member of the class *Chloroflexia*. The type genus is *Kallotenue*.

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