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*Kallotenuce papyrophylicum* gen. nov., sp. nov., a cellulolytic and filamentous thermophile isolated from Great Boiling Spring that represents a novel lineage (*Kallotenucales* ord. nov., *Kallotenuaceae* fam. nov.) within the class *Chloroflexia*

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Running Title: Novel cellulolytic and thermophilic *Chloroflexia*

Subject category: New Taxa (Other bacteria)

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Abbreviations: MK, menaquinone.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JKG¹⁷, JKG2, JKG3, JKG4, and JKG5 are JX848544, JX848545, JX848546, JX848547, and JX848548, respectively.
Summary

Several closely-related, thermophilic, and cellulolytic bacterial strains, designated JKG1\textsuperscript{T}, JKG2, JKG3, JKG4, and JKG5, were isolated from a cellulolytic enrichment (corn stover) incubated in the water column of Great Boiling Spring, NV. Strain JKG1\textsuperscript{T} had cells of a diameter of 0.7 - 0.9 μm and length of ~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 5.6-9.0, with an optimum of 7.5. JKG1\textsuperscript{T} grew as an aerobic heterotroph, utilizing glucose, sucrose, xylose, arabinose, cellobiose, carboxymethylcellulose, 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 C\textsubscript{18:0}, anteiso-C\textsubscript{17:0}, iso-C\textsubscript{18:0}, and iso-C\textsubscript{17:0}. C16:0, iso-C\textsubscript{16:0}, and C\textsubscript{17:0}. 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 JKG1\textsuperscript{T} is representative of a new lineage within the class Chloroflexia, which we propose to designate Kallothenue papyrolyticum gen. nov., sp. nov., Kallothenaceae fam. nov., Kallothenales ord. nov.

Keywords: Cellulolytic/Chloroflexi/hot spring/thermophile/Great Basin/Kallothenue papyrolyticum

Subject Category: New Taxa (Other Bacteria)
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*. *Chloroflexales* is comprised of anoxygenic phototrophs that possess bacteriochlorophyll and may contain chlorosomes (Dubinin & Gorlenko, 1975; Hanada et al., 2002; Keppen et al., 1994; Pierson & Castenholz, 1974; Pierson et al., 1985). *Herpetosiphonales* includes only one genus, *Herpetosiphon*, the two described species of which, *H. geysericola* and *H. aurantiacus*, are mesophilic and aerobic heterotrophs. *H. aurantiacus* utilizes carbohydrates as carbon sources, with 6.5% of its genome devoted to carbohydrate transport and metabolism (Kiss et al., 2011). However, *H. geysericola*, reported to degrade cigarette paper, is the only member of the class *Chloroflexia* known to hydrolyze 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 northwestern Nevada at N40°39′41″ W119°21′58″, USA (Costa et al., 2009). Twenty grams of ammonia fiber explosion (AFEX)-treated corn stover was enclosed within a nylon filter bag of 100 μm pore size (Pentair Industrial, Hanover Park, IL) 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 nine weeks (Peacock et al., 2013). A modified version of Castenholz Medium D (Castenholz, 1969), designated Castenholz medium D VN (abbreviated VN medium), was prepared with the addition of 1X Wolfe's Vitamins (Balch & Wolfe, 1976) and 0.027 g NH₄Cl per L. Ten mL 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, Piscataway, NJ) as the sole carbon and energy source (abbreviated 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 °C, 70 °C, or 80 °C. After eight 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 *Thermus*, *Geobacillus*, and *Rhodothermus* isolates; 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 (Youssef et al., 2011; Dodsworth et al., 2013) 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (abbreviated VNG medium). VNG medium was solidified by the addition of 1.5% w/v agar (BD Difco agar granulated). 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 seven 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-TV1X-2 camera. Detailed morphology was visualized using Tecnai T-12 TEM (FEI) transmission electron microscope (TEM) with LaB6 filament operating at 120 kV. Images were collected digitally using a 2x2K Ultrascan 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. DigitalMicrographTM (Gatan) software was used for imaging and analyses of cellular features. Scanning electron microscopy (SEM) samples were examined at 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 osmicated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at room temperature and washed three times in 0.1 M cacodylate buffer. Cells were dehydrated in 30%, 50%, and 75% ethanol for 30 minutes each, and three times in 100% ethanol for 60 minutes. Cells were washed with a 1:1 mixture of LR white acrylic resin (Electron Microscopy Sciences, Hatfield, PA [EMS]) and ethanol for 30 minutes, and then infiltrated in 100% resin, 3 washes, 4 hours 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
poststained 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 (EMS). The cells were allowed to adhere to the grids for 30
seconds before blotting the liquid with a filter paper, and staining then with 5 μL of negative stain
Nano-W™ (Nanoprobes, Yaphank NY) for 45 seconds, 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 ethanol series (33%, 50%, 75%), followed
by 3 washes in 100% ethanol. The samples were then critically point dried in a CPD instrument
(Tousimis Samdri-795), and processed according to an automated CPD scheme, with CO₂ as a
transitional fluid. The CPD holder was lined with nanopore membrane to prevent the cell loss during
the processing. The cells were mounted on standard aluminum 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 stoppered with butyl rubber septa (Wheaton, Millville, NJ) with an air headspace and
orbital shaking at an empirically determined optimum (100 RPM, 20° angle), except where noted. The
following pH values were tested with 15 mM of the indicated buffer: pH 5.5, 6.0, and 6.5, 2-(N-
Morpholino) ethanesulfonic acid (MES); pH 7.0, 7.5, and 8.0, 4-(2-hydroxyethyl)-1-
-piperazineethanesulfonic acid (HEPES); pH 7.5, 8.0, 8.5, 9.0, tris (hydroxymethyl) aminomethane
(Tris); and pH 8.5, 9.5, and 10.0 2-(N-cyclohexylamino)-ethanesulfonic acid (CHES). Temperature
was tested in five-degree increments, including 40 to 75 °C. NaCl concentration was tested at 0, 0.2,
0.4, 1.0, 1.5, 2.0, 2.5, and 3.0% w/v.

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, D(+)-cellulbiose,
xylose, L-arabinose, pyruvate, casamino acids (EMD, Billerica, MA), tryptone (BD Bacto, Franklin
Lakes, NJ), peptone (BD Bacto, Franklin Lakes, NJ), yeast extract (EM Science, EMD, Billerica,
MA), acetate, citrate, glycerol, lactate, carboxymethylcellulose (Spectrum, Gardena, CA), filter paper
(Whatman Filter Paper Grade 1, GE Healthcare, Piscataway, NJ), microcrystalline cellulose (extra
pure 90 μm, Acros Organics, Thermo Fisher Scientific, Fair Lawn, NJ), xylan (birch wood, Sigma-Aldrich, St. Louis, MO), and starch (soluble potato starch powder, JT Baker, Avator Performance Materials, Center Valley, PA). Three consecutive transfers were conducted for each substrate, with 72 hours of growth allowed before each transfer, and the third transfer was performed in triplicate. Positive substrate utilization was determined by a final average optical density (OD) exceeding that of a no carbon source control by 0.050 OD units (Spectronic 20D spectrophotometer, Milton Roy).

Growth under anaerobic conditions was tested using Balch tubes with N₂-gassed VN medium buffered to pH 7.5 with 15 mM HEPES and a headspace of N₂ using glucose, casamino acids, and yeast extract (0.2% w/v) as potential fermentation substrates. Anaerobic respiration was tested in VNG with the following possible terminal electron acceptors at 2 mM except where noted: nitrate, dimethylsulfoxide, fumarate, ferric iron (ferric-nitritoltriacetic acid or 18 mM hydrous ferric oxide (Schwertmann & Cornell, 2009)), thiosulfate, and sulfate.

Phototrophic growth was tested using N₂-gassed VN medium, N₂ as headspace, and 0.3 mM Na₂S or 3 mM thiosulfate as possible electron donors. Tubes were incubated under the illumination of a full-spectrum LED bulb (Grow Lite, Stimulus Brands, Burnsville, MN) or incandescent bulb.

Photoheterotrophic growth was tested using glucose, citrate, and yeast extract (0.2% w/v) as potential substrates. Photoautotrophic growth was tested by the addition of 50 mM NaHCO₃.

Chemotaxonomic analyses were conducted on lyophilized cells that were grown aerobically in R2A medium (Reasoner & Geldreich, 1985). Glucose was prepared separately as a sterile solution and added after autoclaving. Fatty acid methyl ester analysis was performed by MIDI Labs (Newark, DE) (Sasser, 2006). Preparation of purified cell-wall and peptidoglycan analysis were performed by TLC and HPLC as described by Schleifer (Schleifer, 1985) and Tang et al. (Tang et al., 2009). The whole-cell sugars were prepared and analyzed according to the method as described by Tang et al. (Tang et al., 2009). Menaquinones were extracted as described by Collins et al. (Collins et al., 1977) and Minnikin et al. (Minnikin et al., 1984) and then analyzed by HPLC (Tamaoka et al., 1983).

DNA was extracted using the FastDNA Spin Kit for Soil (MP Biometrics, Solon, OH) using modifications specified previously (Dodsworth 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 (Costa et al., 2009).
and sequenced at Functional Biosciences (Madison, WI) using the PCR primers, 704bR (TCTACG YATTTCACYGCT) and 516uF (TGBCAGCMGCCGCGGTAA) to obtain overlapping reads. Reads were trimmed to remove bases with quality scores of less than 20, aligned against the mothur-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 1,355 nt in length.

The nucleotide sequence of the near-full length 16S rRNA gene of strain JKG1T was aligned along with reference sequences of members of the phylum Chloroflexi using the mothur-provided SILVA alignment in the program mothur. The sequences of Heliothrix oregonensis 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 mothur-provided SILVA-compatible 1,349-position Lane mask was applied (Lane, D. J., 1991). PHYLIP v3.69 was used to produce neighbor-joining, maximum parsimony, and maximum likelihood phylogenetic trees with 100 bootstraps per tree (Felsenstein, 2005). The following parameters in PHYLIP were modified as indicated: input order of species was randomized, outgroup root was set to B. 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 Herpetosiphon sp. 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 a 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 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 JKGlT was able to grow heterotrophically under aerobic conditions utilizing glucose, sucrose, xylose, arabinose, cellobiose, carboxymethylcellulose, 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 45-65 °C and pH 6.5-9.5. Photoheterotrophic, photoautotrophic, fermentative, and anaerobic respiration were not observed.

The major cellular fatty acids of strain JKGlT 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 and anteiso-C₁₉:₀ (3.9%), anteiso-C₁₅:₀ (1.9%), iso-C₁₉:₀ (1.4%), ø9c-C₁₈:₁ (1.4%), C₁₉:₀ (1.0%), 3OH-C₁₇:₀ (0.9%), ø7c-C₁₈:₁ (0.8%), iso-C₁₅:₀ (0.7%), iso-ø9c-C₁₇:₁ (0.6%), anteiso-ø9c-C₁₇:₁ (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 lower concentration 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 DAP 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 G + C content was 72.4 mol% and was determined by high-performance liquid chromatography (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 (1,355 nt). Phylogenetic analysis of JKGI\textsuperscript{T} 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 JKGI\textsuperscript{T} is distinguishable from these other members of the class as the only thermophilic chemoorganotroph not capable of photosynthesis. In addition, strain JKGI\textsuperscript{T} harbors distinct profiles of fatty acids, quinones, whole-cell sugars, and cell wall amino acids. Finally, the 16S rRNA gene identity between strain JKGI\textsuperscript{T} and other members of the class Chloroflexia was ≤ 82.5%, which is well below the mean pair-wise 16S rRNA gene identity between taxa whose lowest shared rank is class, ~87% (Lasher et al., 2009; Konstantinos & Tiedje, 2005), and the 16S rRNA gene 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 JKGI\textsuperscript{T} suggest that it is representative of a novel genus, family, and order within the class Chloroflexia. The designations Kallotenue gen. nov., Kallotenuceae fam. nov., and Kallotenuales ord. nov., are proposed.

Description of Kallotenue gen. nov.

Kallotenue. Kal.lo.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, nonbranching, and non-spore-forming bacteria. Gram-stain negative. Thermophilic, aerobic chemoorganotrophs that grow at 45-65 °C, pH 5.6-9.0, and 0-2.0% w/v NaCl. Peptidoglycan amino acids include alanine, ornithine, glutamine, serine, and asparagine. Major whole-cell sugars comprise mannose, rhamnose, and glycogen. The major quinone is menaquinone-9. The G+C content of the type species is 72.4 mol%. The type species is Kallotenue papyrolyticum.
Description of Kallo tenue papyrolyticum sp. nov.


The following properties are displayed, in addition to those specified for the genus. Growth occurs at 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, carboxymethylcellulose, filter paper, microcrystalline cellulose, xylan, starch, casamino acids, tryptone, peptone, yeast extract, acetate, citrate, lactate, pyruvate, and glycerol. The major cellular fatty acids are C<sub>18:0</sub>, anteiso-C<sub>17:0</sub>, iso-C<sub>18:0</sub>, iso-C<sub>17:0</sub>, C<sub>16:0</sub>, iso-C<sub>16:0</sub>, C<sub>17:0</sub>, and anteiso-C<sub>19:0</sub>. The type strain is JKG1<sup>T</sup> (DSM 26889<sup>T</sup> and JCM 19132<sup>T</sup>), isolated from a lignocellulosic enrichment incubated in a hot spring.

Description of Kallo tenaceae fam. nov.


The description is the same as for the genus *Kallo tenue*. The family is a member of the order *Kallo tenuales*. The type genus is *Kallo tenue*. a.e. N.L. n. *Kallo tenue*, type genus of the family; suff. -aceae, ending denoting a family; N.L. fem. pl. n. *Kallo tenaceae*, the family of the genus *Kallo tenue*.

The description is the same as for the genus *Kallo tenue*. The family is a member of the order *Kallo tenuales*. The type genus is *Kallo tenue*.

Description of Kallo tenuales ord. nov.
Kallotenuales: Kal.lo.te.nu.a’les. N.L. n. Kallotenu, type genus of the order; suff. -ales, ending
denoting an order; N.L. fem. pl. n. Kallotenuales, the order of the genus Kallotenu.

The description is the same as for the genus Kallotenu. The order is a member of the class
Chloroflexia.

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Figure 1. Micrographs of strain JKG1T. (a) Phase-contrast micrograph of whole filaments. (b) SEM image of whole filaments. (c) TEM image of a whole mount focused on a single cell and (d) the junction between cells in a filament. (e) TEM image of a single cell thin section and (f) the junction between cells in a filament.

Figure 2. Maximum-likelihood phylogenetic tree of the phylum Chloroflexi, with strain JKG1T indicated by an arrow. The tree was constructed based upon 1,183 aligned positions that remained after the application of the Lanè mask to the 16S rRNA gene sequences. Bootstrap values are out of 100 replicates for each tree-building method represented (maximum likelihood (ML), maximum parsimony (MP), and neighbor-joining (NJ)) and are indicated at each node using black (≥90% recovery), grey (89-80% recovery), or no shading (<80% recovery). Bootstrap support for nodes supported by <80% recovery from all three methods are not shown. Scale bar indicates 0.02 changes per nucleotide. Accession numbers are included in parentheses and strains are included for cultivated representatives. Outgroups were Bacillus subtilis 168T (D26185.1), Escherichia coli ATCC 11775T (X80725.1), and Corynebacterium diphtheriae NCTC 11397T (X84248.1).


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