



Micropepsis pineolensis gen. nov., sp. nov., A Mildly Acidophilic Alphaproteobacterium Isolated From A Poor Fen, And Proposal Of Micropepsaceae fam. nov. Within Micropepsales ord. nov.

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An obligately anaerobic, mildly acidophilic, fermentative alphaproteobacterium, designated strain CS4T, was isolated from an acidic, oligotrophic (nutrient poor) poor fen located near Pineola, NC, USA. Cultures contained Gram-negative, slightly curved, non-motile, non-spore-forming, non-prosthecate rods (0.1–0.4 µm in diameter and 0.4–4 µm long, depending, in part, on the growth substrate). Growth optima were 35 °C (range 15–35 °C), pH 5.6 (range of 5.0–6.8), and with 0–50 mM added NaCl (range, 0–100 mM added NaCl). The culture fermented cellobiose, d-glucose, d-mannose, fructose, galactose, glycerol, lactose, maltose, peptone, sucrose, trehalose and xylose. Respiratory growth was not detected. Major fatty acids were C18:1ω7c, C19:0 cyclo ω8c, C14:0 and C16:0. The G+C content of the DNA was 61.9±0.3 mol%. The two most closely related species phylogenetically, Rhizomicrobium palustre A48T (AB081581) and Rhizomicrobium electricum Mfc52T (AB365487) shared 94 and 93 % SSU rRNA gene sequence identity, respectively, to that of strain CS4T. Lower SSU rRNA gene sequence identities resulted from pairwise comparisons with members of the order ‘Rhizobiales’(85–88%) or Sphingomonadales(85–86%). These findings all support the classification of strain CS4T as representative of a novel genus, family and order of Alphaproteobacteria. The type strain of the species pineolensis within the genus Micropepsis, family Micropepsaceae and order Micropepsales is CS4T (=JCM 30711T=ATCC BAA-2724T).

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Austin B. Harbison,¹ Laiken E. Price,¹ Michael D. Flythe² and Suzanna L. Bräuer^{1,*}

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An obligately anaerobic, mildly acidophilic, fermentative alphaproteobacterium, designated strain CS4^T, was isolated from an acidic, oligotrophic (nutrient poor) poor fen located near Pineola, NC, USA. Cultures contained Gram-negative, slightly curved, non-motile, non-spore-forming, non-prosthecate rods (0.1–0.4 µm in diameter and 0.4–4 µm long, depending, in part, on the growth substrate). Growth optima were 35 °C (range 15–35 °C), pH 5.6 (range of 5.0–6.8), and with 0–50 mM added NaCl (range, 0–100 mM added NaCl). The culture fermented cellobiose, D-glucose, D-mannose, fructose, galactose, glycerol, lactose, maltose, peptone, sucrose, trehalose and xylose. Respiratory growth was not detected. Major fatty acids were C_{18:1} ω7c, C_{19:0} cyclo ω8c, C_{14:0} and C_{16:0}. The G+C content of the DNA was 61.9±0.3 mol%. The two most closely related species phylogenetically, *Rhizomicrobium palustre* A48^T (AB081581) and *Rhizomicrobium electricum* Mfc52^T (AB365487) shared 94 and 93 % SSU rRNA gene sequence identity, respectively, to that of strain CS4^T. Lower SSU rRNA gene sequence identities resulted from pairwise comparisons with members of the order 'Rhizobiales' (85–88 %) or *Sphingomonadales* (85–86 %). These findings all support the classification of strain CS4^T as representative of a novel genus, family and order of *Alphaproteobacteria*. The type strain of the species *pineolensis* within the genus *Micropepsis*, family *Micropepsaceae* and order *Micropepsales* is CS4^T (=JCM 30711^T=ATCC BAA-2724^T).

Peatlands are unique environments that contain acidic soils, low redox potentials and associated low decomposition rates [1], and they serve as the largest natural source of atmospheric methane, releasing approximately 0.4 Gt CH₄ annually [2]. Numerous studies have shown that *Proteobacteria*, specifically *Alphaproteobacteria*, are prevalent in peatlands and thought to be involved in methane oxidation, as well as sugar fermentation and anaerobic respiration [3–5]. Herein, we describe the characteristics and phylogenetic placement of a novel alphaproteobacterium strain CS4^T isolated from a poor fen (more minerotrophic and less acidic than a true bog), Pineola Bog, in the southern Appalachian mountains, Pineola, NC, USA. Cultured and uncultured strains related to strain CS4^T form a novel family and order within *Alphaproteobacteria*.

Peat samples were collected on 12 September 2013, from Pineola Bog, at three locations within the bog, at a depth of 25–30 cm, in airtight jars and taken directly to an anaerobic (N₂ and H₂) glove box. Inoculum was prepared by homogenization with deionized water using an immersion blender and then pressed through a series of filters, with a final filter

size of 0.45 µm. Enrichment was performed in Balch tubes, with 5 ml of a basal medium, peat medium 1 (PM1), prepared anaerobically as described by Bräuer *et al.* [6]. Prior to inoculation, the tubes were flushed with ultra-high-purity N₂ gas and sterilized. Post-sterilization, the following additions were made (final concentrations): ca. 0.5 mM Ti(III) nitrilotriacetate (TiNTA) (preparation described by Bräuer *et al.* [6]), 20 mM MES (pH 5.7), vitamin solution (0.2, 1.0, 0.5 or 0.01 µg l⁻¹ of each vitamin as described by Bräuer *et al.* [6] and Balch *et al.* [7]), 40 mg yeast extract l⁻¹, 1 g glycerol l⁻¹ and 10 mg rifampicin l⁻¹. Tubes inoculated with 0.5 ml peat filtrate were incubated in the dark at 28 °C without shaking, and growth was monitored by visual inspection and by use of a spectrophotometer at OD₆₀₀.

Colonies from anaerobic enrichment cultures were isolated on 1.5 % agar plates (PM1 plates) supplemented with the PM1 additions, excluding TiNTA, streaked aerobically and incubated anaerobically in a mason jar with an N₂ headspace. After approximately 1 week of growth, single colonies were transferred to new plates. Colonies were re-streaked three times to ensure purity, and axenic colonies were

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Abbreviation: TiNTA, Ti(III) nitrilotriacetate.

The GenBank/EMBL/DDBJ accession number for the SSU rRNA gene sequence of strain CS4^T is KU738893.

transferred back to liquid medium in the anaerobic glovebox. Purity was visually inspected using fluorescence microscopy on an Olympus BX51 using acridine orange with a FITC filter. The SSU rRNA gene was PCR amplified from extracted DNA and sent to Beckman Coulter Genomics for Sanger sequencing. Once isolated, strain CS4^T was also grown in PM1 medium containing the addition of 500 mg l⁻¹ peptone and/or with 1 g l⁻¹ of either sucrose or dextrose substituted for glycerol.

Effects of pH, salinity and temperature on growth were examined in PM1 supplemented with glucose (1 g l⁻¹). For physiological studies, the isolate was grown in PM1 in at least duplicate or triplicate trials. Aerobic growth was tested by addition of 25, 50 or 100 % air in the N₂ headspace in Balch tubes with and without the reductant, TiNTA, or by incubating plates streaked with bacteria aerobically at 28 and 35 °C. Gram-staining, oxidase and catalase tests were completed according to previously described procedures [8]. Presence or absence of cell motility was examined visually under a phase-contrast microscope and by culturing anaerobically in swarm plates (0.8 % nutrient broth with 0.3 % agar), supplemented with 1 g l⁻¹ sucrose. Heat tolerance was examined by submerging inoculated PM1 in 80 °C (10 min) and determining subsequent bacterial viability. Cell morphology was observed by transmission electron microscopy of negatively stained [1 % phosphotungstic acid] cells fixed on a 100 square formvar coated grid. Cells were imaged using a JEM-1400 transmission electron microscope.

Fermentative growth was examined in PM1 supplemented with sterile, anaerobic substrates (ca. 20 mM). Utilization of electron acceptors was tested in PM1 supplemented with 1 g l⁻¹ glucose as the sole carbon source and either sodium sulfate, ferric ammonium citrate, sodium fumarate, sodium malate (20 mM each) or O₂ (headspace replaced with O₂) as the sole electron acceptor. The Fe(III)-reducing capability of strain CS4^T was tested in PM1 supplemented with 1 g glucose l⁻¹ (with and without TiNTA) and with ferric ammonium citrate added to a final concentration of 2, 4 and 8 mM. FerroZine Iron Reagent Solution Pillows (Hach) were used at pH 7.0 to determine the presence of Fe(II) colorimetrically. The Fe(III)-reducing ability was additionally tested with the addition of acetate, ethanol, formate, glycerol, lactate, pyruvate or succinate (20 mM) as the sole electron donor. Nitrate or sulfate reduction was determined using previously described methods [8, 9]. The G+C content of the genomic DNA (in triplicate) was calculated as described by Shelobolina *et al.* [10] using linear regression analyses of a standard curve of melting temperatures (T_m) for standard DNA (in triplicate) from the following species: *Clostridium acetobutylicum*, *Flavobacterium capsulatum*, *Bacillus subtilis*, *Methanoregula boonei*, *Micrococcus luteus* and *Escherichia coli*. Phospholipid-derived fatty acids from freeze-dried cells harvested in stationary phase after growth in PM1 supplemented with 1 g glucose l⁻¹ were analysed via fatty acid methyl ester analysis at MIDI Labs. HPLC was utilized to determine the production of organic acids on a

Dionex platform operated isocratically (mobile phase, 0.085 M H₂SO₄; flow rate, 0.4 ml min⁻¹). Separation was completed with a Bio-Rad Aminex HPX-87H ion exchange column (50 °C); eluted compounds were detected with a refractive index detector and UV detector, in tandem.

Sequences similar to the partial SSU rRNA gene sequence for strain CS4^T (KU738893) as well as sequences for members of closely related alphaproteobacterial orders were collected from the GenBank database [11] using the BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and were aligned using the SILVA aligner of the ARB program (www.arb-silva.de/aligner/). Aligned sequences were trimmed utilizing BioEdit software (Ibis Biosciences). Phylogenetic trees were reconstructed with neighbour-joining [12] and maximum-likelihood methods using DNADIST, SEQBOOT with CONSENSE for bootstrapping, NEIGHBOR and DNAML programs within the PHYLIP package (<http://evolution.gs.washington.edu/phylip.html>). Tree output files were visualized in Tree Explorer and exported to Adobe Illustrator to allow the addition of full organism names.

Colonies of strain CS4^T grown anaerobically on PM1 plates, which had been poured and stored aerobically prior to inoculation, were smooth, translucent and ca. 2 mm in diameter. Strain CS4^T grew when streaked aerobically and incubated anaerobically, indicating a tolerance to brief oxygen exposure. However, bacterial growth was not observed with the addition of any percentage of air in the headspace or on the PM1 plates incubated aerobically; therefore, strain CS4^T is considered an obligate anaerobe. Cells stained Gram-negative. As observed thus far, cells averaged from 0.1 to 0.4 µm in diameter and were 0.4–4 µm long, as follows: when grown on glucose (1 g l⁻¹), cells were curved rods 0.2–0.3 µm in diameter and 1–3 µm long (Fig. 1); when grown on sucrose (1 g l⁻¹), cells were 0.2–0.4 µm in diameter and 2–4 µm long; and when grown on glycerol (1 g l⁻¹), cells were 0.1 µm in diameter and 0.4–0.5 µm long. Catalase and oxidase tests were negative. Division by binary fission was observed, but prosthecae and motility were not. Heat-tolerant cells were not produced. Strain CS4^T tolerated up to 100 mM NaCl and grew optimally with the addition of 0–50 mM NaCl to the medium. Optimal growth rates were observed at an initial pH of 5.6 (final pH 4.0; range, 5.0–6.8) and at 35 °C (range, 15–35 °C), unlike isolates of the phylogenetically closest related genus, *Rhizomicrobium*, members of which displayed a higher NaCl tolerance of up to 1% (171 mM) and a higher pH optimum of 6.7–6.8 (Table 1). Strain CS4^T grew fermentatively on numerous substrates including disaccharides derived from decomposing organic matter, such as cellobiose and maltose, as detailed below in the species description. Fermentation products from glucose (1 g l⁻¹) were ethanol (1.1±0.1 mM), formate (3.1±0.5 mM), acetate (1.6±0.1 mM), H₂ (2.1 mmole l⁻¹ culture), CO₂ (4.2 mmole l⁻¹ culture) and trace amounts of lactate. Low fermentation yields were likely due to cell growth and/or acidification of the medium because the cessation of cell growth occurred at pH 4.0. Unlike members of the *Rhizomicrobium*, strain

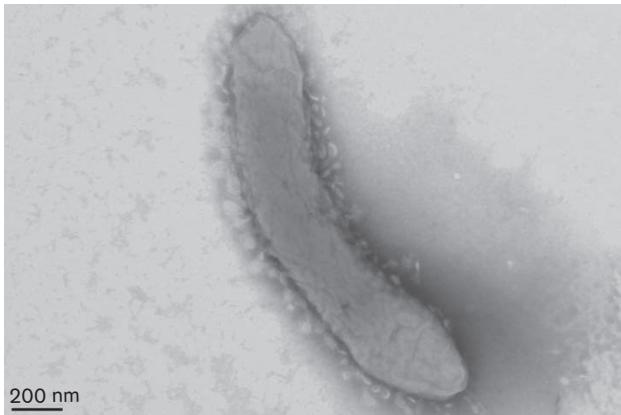


Fig. 1. Transmission electron micrograph of strain CS4^T grown in PM1 supplemented with glucose, negatively stained using phosphotungstic acid and showing curved rods. Bar, 200 nm.

CS4^T did not use sodium sulfate, ferric ammonium citrate, sodium fumarate, sodium malate or O₂ as electron acceptors (Table 1). Fe(III) was not reduced with either acetate, ethanol, formate, glucose, lactate, pyruvate or succinate as the sole electron donor.

The major fatty acids (>5 %) of strain CS4^T were C_{18:1}ω7c (36.7 %), C_{19:0} cyclo ω8c (22 %), C_{14:0} (17 %) and C_{16:0} (13.2 %). Other fatty acids detected (>0.1 %) included C_{16:0} 3-OH (4.6 %); C_{18:0} (2.2 %), C_{14:0} 3-OH and/or iso-C_{14:0} I (1.2 %), C_{18:1}ω7c 11-methyl (0.9 %), C_{16:1}ω7c and/or C_{16:1}ω6c (0.8 %), C_{20:2}ω6,9c (0.6 %), C_{12:0} (0.4 %), C_{19:1}ω6c and/or C_{19:1}ω7c and/or C_{19:0} cyclo (0.2 %), C_{15:0} (0.2 %) and C_{15:0} 3-OH (0.1 %). Notably, the phospholipid-derived fatty acid profile demonstrates that strain CS4^T can adapt to low pH environments by the significant presence of the saturated, cyclopropane-containing fatty acid C_{19:0} cyclo ω8c [13], indicating a stiffer and more stress-tolerant membrane structure. Members of both the *Rhizomicrobium* and strain CS4^T also contain C_{18:1}ω7c as their most abundant fatty acid. The genomic DNA G+C content of strain CS4^T was estimated at 61.9±0.3 mol% using melt curve analysis.

A database search of sequences closely related to strain CS4^T revealed that it was most closely related (99 %) to two environmental clones 3C003352 (EU801969) and MUP3F11 (HQ178872) (Fig. 2). Two sequences from cultured strains *Rhizomicrobium palustre* A48^T (ABO81581) and *Rhizomicrobium electricum* Mfc52^T (AB365487) shared 94 and 93 % identity, respectively, to that of strain CS4^T. *R. palustre* was isolated from the roots of rice plants in a flooded rice paddy and was originally phylogenetically placed in *Alphaproteobacteria* as a representative of a novel genus related to *Sphingomonadales* [14]. *R. electricum* was later isolated from a cellulose-fed microbial fuel cell and phylogenetically placed in the genus *Rhizomicrobium* as most closely related to the ‘Rhizobiales’, but not fully affiliated with any established order [15]. Strain CS4^T also shared relatively high

identity (91–92 %) to a group of isolates obtained on aerobic plates supplemented with xylan: Ellin362, Ellin335, Ellin332, among others. The related Ellin isolates have been described as representatives of a novel order within the class *Alphaproteobacteria* [16], named Ellin329, in the Greengenes database [17]. In addition to the two *Rhizomicrobium* species, strain CS4^T also shares high identity (88 %) with several members of the ‘Rhizobiales’ (*Sinorhizobium morelense*, *Devasia submarina* strain S174 and *Ochrobactrum tritici*, among others). However, it shares equal identity (85–86 %) with other members of the ‘Rhizobiales’ (85 % identity to *Methylobacterium thiocyanatum* strain ALL/SCN-P^T and *Cucumibacter marinus* strain CL-GR60^T) and members of the order *Sphingomonadales* (85 % to *Sphingomonas paucimobilis* strain ATCC 29837 and 86 % identity to *Erythrobacter longus* strain DSM 6997). Furthermore, in several different phylogenetic trees, either built here or presented in the literature, organisms related to strain CS4^T can form a larger cluster with *Sphingomonadales* [14], *Caulobacterales* [16] or ‘Rhizobiales’ [15], in all cases with low bootstrap support, thus demonstrating a lack of strong affiliation with any of the identified orders. Similar to strain CS4^T, *R. electricum* shared 85 % identity to both *Sphingomonas paucimobilis* and *Erythrobacter longus* and 87 % identity to *Sinorhizobium morelense*, *D. submarina* and *O. tritici*. According to analyses by Yarza *et al.* [18], of 85 orders analysed, the median SSU rRNA gene sequence identity is 89.2 % (95 % confidence interval of 88.25–90.1 %). Although Yarza *et al.* [18] recommend a more stringent cutoff of 82 % or lower for identifying a novel order based on sequence information alone, the values of 85–88 % or lower identity between strain CS4^T and members of other orders are in line with those for distinct orders within the *Alphaproteobacteria*.

Several lines of evidence implicate members of the candidate order Ellin329, herein proposed as *Micropepsales*, in the utilization of both simple and more complex plant sugars such as cellobiose, maltose and/or xylan [14, 16, 19]. At least one study has previously demonstrated the abundance of sequences affiliating with the candidate order Ellin329 in the top layers of soil where plant litter and root exudates are abundant [20]. Additionally, *R. electricum* was isolated from a cellulose-fed microbial fuel cell, suggesting at least indirect involvement in polysaccharide degradation [15]. Finally, at least two known studies have classified members of Ellin329 as having the capability to hydrolyse not only monomers such as xylose and arabinose but also glycosidic bonds of complex carbohydrates including cellulose and xylan [16, 19].

Overall, strain CS4^T displays characteristics that clearly define it from previously characterized strains of the genus of closest relation, *Rhizomicrobium* (Table 1). The G+C content, growth optima (pH, salinity tolerance and O₂ requirement) and utilization of growth substrates (Table 1) support our consideration that strain CS4^T is a member of a novel genus, *Micropepsis*, for which the proposed name is

Table 1. Select characteristics of strain CS4^T, *Rhizomicrobium electricum* Mfc52^T and *Rhizomicrobium palustre* A48^T

+, Positive; -, negative.

Characteristic	CS4 ^T	<i>R. electricum</i> Mfc52 ^{T*}	<i>R. palustre</i> A48 ^{T†}
Prosthecae	-	+	+
Cell division	Binary fission	Budding, binary fission	Budding, binary fission
pH optimum (range)	5.6 (5.0–6.8)	6.7 (5.0–7.5)	6.8 (5.5–7.3)
NaCl range (%)	0–0.3 %	0–1 %	0–1 %
O ₂ requirement	Anaerobic	Facultatively anaerobic	Facultatively anaerobic
Anaerobic reduction of:			
Nitrate	-	+	-
Fumarate	-	+	-
Fe(III)	-	+	+
Fermentative growth on:			
Cellobiose	+	+	+
Glycerol	+	-	-
Trehalose	+	+	-
Mannose	+	+	-
Ribose	-	+	-
Arabinose	-	+	+
Xylan	-	-	+
DNA G+C content (mol%)	61.9	64.7	55.4
Major fatty acids (%)	C _{18:1} ω7c (36.7), C _{19:0} cyclo ω8c (22.0), C _{14:0} (17.0) and C _{16:0} (13.2)	C _{18:1} ω7c (47.3), anteiso-C _{15:0} (14.0), C _{16:0} (8.6)	C _{18:1} ω7c (29.4), anteiso-C _{15:0} (16.2), C _{16:0} (13.2)

*Data collected by Kodama and Watanabe [15].

†Data collected by Ueki *et al.* [14].

Micropepsis pineolensis. Additionally, based on phylogenetic analyses of the SSU rRNA gene, comparisons of metabolic capabilities and assertions from previous work [15, 16], we propose that the genera *Micropepsis* and *Rhizomicrobium* form a new family, *Micropepsaceae*, within the new order *Micropepsales*.

DESCRIPTION OF MICROPEPSIS GEN. NOV.

Micropepsis (*Mi.cro.pep'sis*. Gr. adj. *mikros* small; Gr. fem. *n. pepsis* digestion; N.L. fem. n. *Micropepsis* small digester).

Cells are Gram-negative, non-spore-forming, curved rods without prosthecae that reproduce by binary fission. Growth occurs between 15 and 35 °C, between pH 5.0 and 6.8 and with 0–100 mM NaCl. Anaerobic, mesophilic and mildly acidophilic. The major cellular fatty acids (>5 %) are C_{18:1}ω7c, C_{19:0} cyclo ω8c, C_{14:0} and C_{16:0}.

The type species is *Micropepsis pineolensis*.

DESCRIPTION OF MICROPEPSIS PINEOLENSIS SP. NOV.

Micropepsis pineolensis (*pi.ne.ol.en'sis* N.L. fem. adj. *pineolensis* pertaining to Pineola, NC, USA; N.L. fem. adj. *Micropepsis pineolensis* small digester from Pineola).

Cells are non-spore-forming and non-motile, slightly curved rods (0.1–0.4 µm in diameter and 0.4–4 µm long). The optimum growth temperature is 35 °C, and the optimum pH is 5.6. The optimum NaCl concentration for growth is 0–50 mM added NaCl (growth observed ≤100 mM NaCl). Grows fermentatively on cellobiose, D-glucose, D-mannose, fructose, galactose, glycerol, lactose, maltose, sucrose, trehalose, xylose and peptone. Does not utilize 2-butanol, acetate, alanine, arabinose, aspartic acid, cellulose, citrate, cysteine, aesculin, ethanol, fumarate, glycine, inulin, lactate, mannitol, methanol, propanol, proteose, pyruvate, rhamnose, ribose, serine, sodium acetate, sodium formate, soluble starch, sorbitol, sorbose, succinate or xylan. Major fermentation products on glucose (1 g l⁻¹) are as follows: E (ca. 1 mM), F (ca. 3 mM), A (ca. 1.5 mM), H₂ (ca. 2 mmoles l⁻¹ culture) and CO₂ (ca. 4 mmoles l⁻¹ culture). Does not utilize acetate, ethanol, formate, lactate, pyruvate or succinate as an electron donor. Does not utilize Fe(III), Na₂SO₄, sodium fumarate, sodium malate or O₂ as an electron acceptor.

The type strain CS4^T (=JCM 30711^T=ATCC BAA-2724^T) was isolated from anoxic peat soil in a southern Appalachian peatland known as Pineola Bog, Pineola, NC, USA. The genomic DNA G+C content of the type strain is 61.9 ± 0.3 mol%.

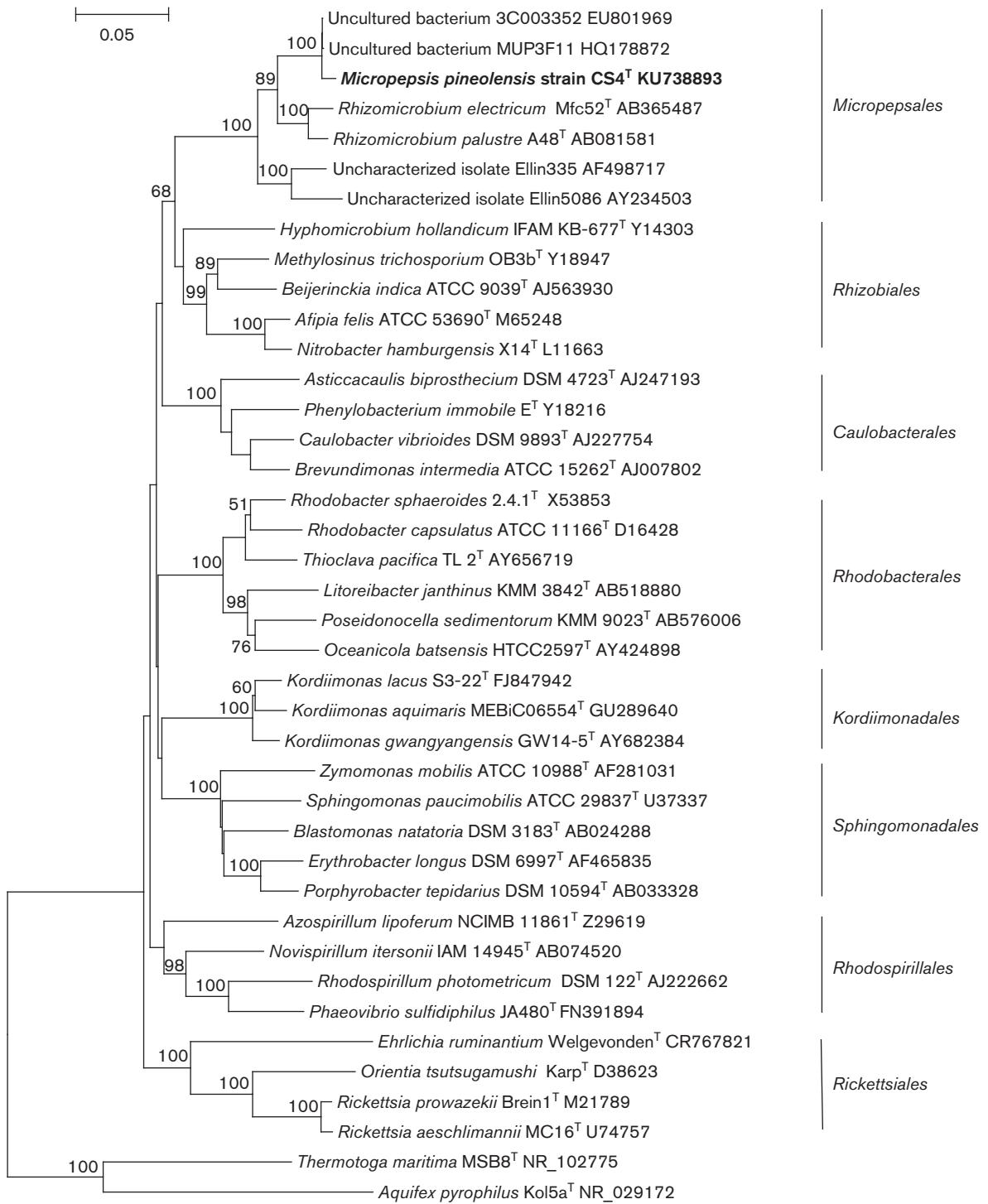


Fig. 2. Neighbour-joining dendrogram, based on SSU rRNA gene sequences, showing the phylogenetic position of strain CS4^T (KU738893) in relation to the genus *Rhizomicrobium*. Members of *Micopepsales* ord. nov. are shown in comparison to other alphaproteobacterial orders including 'Rhizobiales' and *Sphingomonadales*. Nodal support was determined using bootstrapping values of 100 replicates in neighbour joining, and values are displayed only for those nodes with support values >50 % that were also supported by maximum-likelihood analyses. Bar, 0.05 substitutions per nucleotide position.

DESCRIPTION OF *MICROPEPSACEAE* FAM. NOV.

Micropepsaceae (Mi.cro.pep.sa.ce'ae. N.L. fem. n. *Micropepsis* the type genus of the family; -aceae to denote a family; N. L. fem. pl. n. *Micropepsaceae* the *Micropepsis* family).

Gram-negative, non-spore-forming rod-shaped bacteria that reproduce by binary fission or budding from prosthecate mother cells. Obligately anaerobic, facultative or aerobic. Neutrophilic or mildly acidophilic. Grow fermentatively on mono-, di- and polysaccharides such as sucrose, dextrose, xylose, maltose, cellobiose and/or xylan. Ferric iron, nitrate, oxygen and/or fumarate may serve as electron acceptors. DNA G+C content is 55–64 mol%.

The nomenclatural type is the genus *Micropepsis*.

DESCRIPTION OF *MICROPEPSALES* ORD. NOV.

Micropepsales (Mi.cro.pep.sa'les. N.L. fem. n. *Micropepsis* the type genus of the order; -ales ending denoting an order; N.L. fem. pl. n. *Micropepsales* the order of *Micropepsis*).

Description of the order is the same as that for the family.

The nomenclatural type is the genus *Micropepsis*.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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