Isolation and characterization of *Flexilinea flocculi* gen. nov., sp. nov., a filamentous, anaerobic bacterium belonging to the class *Anaerolineae* in the phylum *Chloroflexi*

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A novel obligately anaerobic bacterium, designated strain TC1T, was isolated from methanogenic granular sludge in a full-scale mesophilic upflow anaerobic sludge blanket reactor treating high-strength starch-based wastewater. Cells had a multicellular filamentous morphology, stained Gram-negative and were non-motile. The filaments were flexible, generally >100 μm long and 0.3–0.4 μm wide. Growth of the isolate was observed at 25–43 °C (optimum 37 °C) and pH 6.0–8.5 (optimum pH 7.0). Strain TC1T grew chemo-organotrophically on a range of carbohydrates under anaerobic conditions. Yeast extract was required for growth. The major fermentative end products of glucose, supplemented with yeast extract, were acetate, lactate, succinate, propionate, formate and hydrogen. Co-cultivation with the hydrogenotrophic methanogen *Methanospirillum hungatei* DSM 864T enhanced growth of the isolate. The DNA G+C content was determined experimentally to be 42.1 mol%. The major cellular fatty acids were anteiso-C15:0, iso-C15:0 and iso-C17:0 3-OH. Based on 16S rRNA gene sequence analysis, strain TC1T belonged to the class *Anaerolineae* in the phylum *Chloroflexi*, in which *Ornatilinea apprima* was its closest phylogenetic relative (88.3 % nucleotide identity). Phylogenomic analyses using 38 and 83 single-copy marker genes also supported the novelty of strain TC1T at least at the genus level. Based on phylogenetic, genomic and phenotypic characteristics, we propose that strain TC1T represents a novel species of a new genus, for which we suggest the name *Flexilinea flocculi* gen. nov., sp. nov. The type strain of *Flexilinea flocculi* is strain TC1T (=JCM 30897T =CGMCC 1.5202T).

Cultivation-independent 16S rRNA gene and metagenome surveys have significantly broadened the spectrum of known phylogenetic diversity of the bacterial phylum *Chloroflexi* (Campbell et al., 2014; Speirs et al., 2015; Yamada & Sekiguchi, 2009). More than 20 putative subphylum-level lineages can be discerned in the GreenGenes database (release 05_13; McDonald et al., 2012). Among them, the class *Anaerolineae* contains a large number of environmental 16S rRNA gene sequences (Yamada et al., 2006) from a diverse set of environments, including marine subsurface sediments, aquifers and anoxic waste/wastewater treatment sludges (Yamada & Sekiguchi, 2009). Their ubiquity suggests that members of the *Anaerolineae* are involved in key processes in these ecosystems. The first cultured representative...
(strain UNI-1T) of the class *Anaerolineae* was isolated from thermophilic methanogenic granular sludge (Sekiguchi et al., 2001) and described as *Anaerolinea thermophila* (Sekiguchi et al., 2003). Since then, nine more species of the class *Anaerolineae* have been isolated and described (Grégoire et al., 2011; Imachi et al., 2014; Nunoura et al., 2013; Podosokorskaya et al., 2013; Sekiguchi et al., 2003; Yamada et al., 2006, 2007). However, the majority of the lineages found previously in the *Anaerolineae* remain to be cultivated and described in detail.

To expand the coverage of the diversity of cultured members of the *Anaerolineae* further, we attempted to cultivate novel members of the *Anaerolineae* from a range of ecosystems. This resulted in the successful isolation of a novel bacterium belonging to the *Anaerolineae*, designated strain TC1T, from granular sludge in a mesophilic upflow anaerobic sludge blanket (UASB) reactor treating high-strength organic wastewater. Here, we describe the isolation and characterization of strain TC1T. Phenotypically and morphologically, the strain is similar to previously characterized members of the *Anaerolineae*. However, genome-scale comparisons underscored the conclusion that strain TC1T represents a distinct lineage in the class *Anaerolineae*. Its morphological, physiological, chemotaxonomic and genomic features suggested that strain TC1T represents a novel species of a new genus in the class *Anaerolineae*.

Methanogenic granular sludge in a full-scale, high-rate, mesophilic (37 °C) UASB reactor treating high-strength starch-based organic wastewater discharged from a sweet-corn canning plant (operated at a volumetric loading rate around 11 kg chemical oxygen demand m⁻³ day⁻¹) was used for isolation of strain TC1T. All cultivations were performed using the basal medium and experimental procedures detailed by Sekiguchi et al. (2001). Unless otherwise stated, all incubations were performed at 37 °C under an atmosphere of N₂/CO₂ (80 : 20, v/v), in the dark and under static conditions. A primary enrichment culture was established by inoculating dispersed cells from the granular sludge in basal medium supplemented with starch (5 g l⁻¹) and yeast extract (0.1 %). This led to the growth of filamentous cells after 14 days of incubation. Following repeated transfers to fresh medium, a stable culture dominated by the filamentous cells was obtained and maintained. The growth yield of the filamentous cells was always low; a small number of cells were observed as flocs in liquid cultures after 1–2 weeks of anaerobic cultivation (Fig. 1a). Fluorescence *in situ* hybridization (FISH) with the *Chloroflexi*-specific probe GNSB941, performed as described previously (Sekiguchi et al., 1999; Yamada et al., 2005), identified the cells as members of the *Chloroflexi*. A pure culture of the filamentous cells, designated strain TC1T, was then established by >20 rounds of dilution/transfer of the enrichment culture, since repeated attempts at isolation using the roll-tube technique with starch-yeast extract medium supplemented with purified agar (20 g agar noble l⁻¹; Difco) failed to

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**Fig. 1.** (a) Photograph showing growth of strain TC1T in liquid basal medium supplemented with 20 mM glucose and 0.1 % yeast extract. The arrow indicates a floc-like aggregate of cells cultivated anaerobically for 4 weeks under optimum conditions. Bar, 5 mm. (b) Phase-contrast micrograph of cells of strain TC1T cultivated under the same conditions as those in (a). Bar, 10 μm. (c) Thin-section electron micrograph of cells of strain TC1T cultivated anaerobically in basal medium supplemented with 20 mM glucose and 0.1 % yeast extract. Bar, 100 nm.
produce visible colonies after 2 months of incubation. Purity of the strain was verified by FISH with probe GNSB941 for cultures grown on media supplemented with a mixture of carbohydrates, including glucose, sucrose, and mannose, at different concentrations (ranging from 1 to 20 mM). Purity of the culture was further confirmed by evaluating DNA shotgun sequencing data of genomic DNA extracted from the culture (Matsuura et al., 2015b). We found no putative contaminant sequences that were phylogenomically distantly related to strain TC1T.

Cell morphology of strain TC1T was observed for exponential-phase cultures with a phase-contrast microscope (Olympus AX80T). Agar-coated slides were prepared and cells of strain TC1T were immobilized on the slides (Pfenning & Wagener, 1986). Cells of strain TC1T showed a flexible filamentous morphology, generally longer than 100 μm and 0.3–0.4 μm wide (Fig. 1b). Gram staining performed by Hucker’s method (Doetsch, 1981) was negative. For ultrastructure observation, thin-section electron microscopy was performed as described previously (Sekiguchi et al., 2003). Briefly, thin sections (80 nm) of cells were prepared with an ultramicrotome (Leica EM UC7), stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (Hitachi H-7600). This demonstrated that cells of strain TC1T possessed a Gram-negative-type cell-wall structure, showing multicellular forms of filaments (Fig. 1c). Single cells of strain TC1T were 8–10 μm long. A sheath-like structure was not detected by light or electron microscopy (Fig. 1b, c). Gliding motility was evaluated as described previously (Sekiguchi et al., 2015), indicating that the cells were non-motile under the conditions tested.

Optimal growth conditions were determined by cultivating the isolate in basal medium supplemented with 20 mM glucose and 0.1 % yeast extract, at varying temperatures, pH and NaCl concentrations. All cultivations were set up in duplicate with 2 % (v/v) inoculum. Growth was evaluated based on the formation of cell flocs and generation of hydrogen and acetate after 4 weeks of incubation. All chemical analyses were performed as described previously (Imachi et al., 2002; Sekiguchi et al., 2000), including measurement of hydrogen and methane by gas chromatography and fermentative end products, such as acetate and lactate, by HPLC. Growth was evaluated at 20, 25, 30, 37, 43, 50 and 60 °C and observed in the range 25–43 °C, with an optimum at 37 °C. The medium was adjusted at room temperature to pH 5.5–9.0 using HCl or NaOH under a 100 % N2 atmosphere. Growth was detected at pH 6.0–8.5, with optimal growth at pH 7.0. Best growth was observed in the absence of added NaCl. Concentrations of 5–10 g NaCl l⁻¹ inhibited growth moderately, while the cells failed to grow at a concentration of 15 g NaCl l⁻¹.

To examine the growth and substrate utilization of strain TC1T, cells were cultivated in basal medium supplemented individually with autoclaved or filter-sterilized compounds such as proteins, sugars, alcohols, aromatic compounds and volatile fatty acids. All substrates and electron acceptors, including ferric nitroltriacetate [Fe(III)-NTA] (Roden & Lovley, 1993), were prepared as described previously (Sekiguchi et al., 2000). Each substrate was added at a final concentration of 20 mM unless otherwise specified. Cultivations were set up in duplicate under optimal growth conditions (pH 7.0, 37 °C and no added NaCl) with 2 % (v/v) inoculum. Growth and substrate utilization were evaluated after 4 weeks of incubation by inspecting the formation of cell flocs and generation of hydrogen and acetate. Substrate utilization was tested in both the presence and absence of 0.1 % yeast extract.

Strain TC1T is a strictly anaerobic micro-organism; no growth was observed in the presence of oxygen in the gas phase (1–20 %, v/v). It was not photosynthetic, because no changes were seen in growth rate, growth yield or the colour of cells of anaerobic cultures grown with incandescent light, which would be indicative of photosynthetic pigment formation. Yeast extract (0.1 %) was required for growth of strain TC1T. In the presence of yeast extract, the following compounds supported growth and acetate/hydrogen formation: glucose, fructose, ribose, xylose, arabinose, starch (5 g l⁻¹) and pyruvate. Yeast extract alone also supported growth, but to a much lesser extent than yeast extract supplemented with the above substrates. None of the following compounds supported growth or fermentative product formation in the absence of yeast extract: glucose, fructose, ribose, xylose, sucrose, raffinose, arabinose, starch (5 g l⁻¹), xylan, betaine, peptone, Casamino acids, pyruvate, hydrogen (1 atm) plus acetate, formate plus acetate, glycerol, crotonate, lactate, fumarate, acetate, propionate, n-butyrate, malate, succinate, ethanol, methanol, 1-propanol, ethylene glycol, hydroquinone and benzoate. Major fermentative end products in the medium containing glucose (20 mM) and yeast extract (0.1 %) were acetate, lactate, succinate, propionate, formate and hydrogen. Even after prolonged cultivation (4 weeks) under optimum conditions, only a limited fraction of the added glucose was degraded, and the maximum concentrations of these fermentation products were lower than 0.2 mM. Strain TC1T was not able to utilize sulfate (20 mM), sulfite (5 mM), thiosulfate (20 mM), nitrate (20 mM), fumarate (20 mM), elemental sulfur or Fe(III)-NTA (5 mM) as electron acceptors in medium supplemented with glucose (20 mM) and yeast extract (0.1 %). Syntrophic growth was assessed through co-cultivation with the hydrogen-/formate-consuming methanogen Methanospirillum hungatei DSM 864T. This experiment was performed with 20 mM glucose and 0.1 % yeast extract; a pre-grown culture of M. hungatei DSM 864T was added to the vials (2 % inoculum by volume) prior to cultivation. Increases (approx. twofold) in growth rate, yield and acetate formation of cells of strain TC1T were observed upon co-cultivation, and methane formation occurred in the co-culture. The addition of hydrogen (0.1 atm) to the gas phase inhibited the growth of cells of strain TC1T in glucose and yeast.
extract medium. Considering these results, we concluded that strain TC1\textsuperscript{T} is probably a semi-syntrophic carbohydrate fermenter benefitting from methanogenic hydrogen/formate consumers in the culture.

Fatty acid methyl ester analysis was performed by converting cellular fatty acids to methyl esters by HCl/methanol treatment followed by GC-MS analysis (Hanada et al., 2002). Cells were harvested from cultures grown on basal medium supplemented with 20 mM glucose and 0.1 % yeast extract. The major fatty acids of the cells were anteiso-C\textsubscript{15}:0 (35 %), iso-C\textsubscript{15}:0 (25 %) and iso-C\textsubscript{17}:0 3-OH (21 %). The minor fatty acids were anteiso-C\textsubscript{17}:0 3-OH (5 %), iso-C\textsubscript{16}:0 (4 %), C\textsubscript{15}:0 (4 %), C\textsubscript{16}:0 (1 %), iso-C\textsubscript{16}:0 3-OH (1 %), iso-C\textsubscript{15}:0 3-OH (1 %), C\textsubscript{16}:0 3-OH (1 %), C\textsubscript{17}:0 3-OH (1 %), anteiso-C\textsubscript{17}:0 (1 %) and C\textsubscript{18}:0 (1 %). No unsaturated fatty acids were detected.

For 16S rRNA gene sequencing, DNA was extracted from the culture according to the method of Hiraishi (1992). For 16S rRNA gene sequencing, DNA was extracted from the culture according to the method of Hiraishi (1992). For 16S rRNA gene sequencing, DNA was extracted from the culture according to the method of Hiraishi (1992).

Phylogenetic relationships among members of the phylum Chloroflexi and strain TC1\textsuperscript{T} were further assessed using a draft genome of strain TC1\textsuperscript{T} (Matsuura et al., 2013b) along with other finished and draft genomes (e.g. Matsuura et al., 2015a) from members of the phylum Chloroflexi. From these genomes, 38 (Darling et al., 2014) or 85 (Soo et al., 2014) single-copy marker gene products were extracted using HMM searches as described previously (Sekiguchi et al., 2015). Homologous protein sequences were aligned using HMMALIGN in HMMER3, and subsequently concatenated. A mask was generated for the concatenated protein alignment using gblocks (Talavera & Castresana, 2007) with only conserved positions found in more than half of the sequences being considered. All tree topologies were tested for robustness using the maximum-likelihood methods from FASTTREE version 2 (with default parameters, JTT model, CAT approximation) (Price et al., 2010) and RAxML version 7 (JTT and Gamma models with rapid 100-time bootstrapping) (Stamatakis, 2006). The PHYLIP SEQBOOT module (Felsenstein, 1989) was used to generate 100 resampled alignments and FASTTREE was used to analyse the resampled alignments (n 100). A script (COMPAREBOOTSTRAP.PL) included in the FASTTREE package was used to compare the original tree to the resampled trees and generate bootstrap values. Generated trees were imported into ARB. The resulting phylogeny inferred based on concatenated protein sequences predicted from the two sets of marker genes (Fig. 2b, c) was in good agreement with the phylogeny based on the 16S rRNA gene. Again, strain TC1\textsuperscript{T} belonged to the class Anaerolineae, and formed an independent lineage within the class. It is interesting to note that subphylum-level associations among the classes Anaerolineae, Caldilineae and Thermoflexia could be inferred based on the genome-based trees (Fig. 2b, c).

We further compared the draft genome of strain TC1\textsuperscript{T} with genomes of other members of the Anaerolineae based on average amino acid identity (AAI). Pairwise AAI values were computed for the following genomes: strain TC1\textsuperscript{T}, A. thermophila UNI-1\textsuperscript{T}, A. thermolimosa IMO-1\textsuperscript{T}, Bellilinea caldifiitulae GOMI-1\textsuperscript{T}, Leptolinea tardivitalis YMTK-2\textsuperscript{T}, Levilinea saccharolytica KIB-1\textsuperscript{T} and Longilinea arvoryzae KOME-1\textsuperscript{T}. Orthologous genes were identified as reciprocal best BLAST hits using the implementation in COMPAR E (version 0.0.7; https://github.com/dparks1134/CompareM) with default settings (e-value cut-off, 0.001; identity for defining homology, 30.0 %, alignment length, 70.0 %), except for the -BLASTP option. Pairwise AAI estimates for the genomes of members of the Anaerolineae, excluding strain TC1\textsuperscript{T}, varied between 56.2 and 60.9 % when comparing genomes from different genera, whereas the AAI within genera (A. thermophila UNI-1\textsuperscript{T} and A. thermolimosa IMO-1\textsuperscript{T}) was 67.4 %. By comparison, strain TC1\textsuperscript{T} shared only
Fig. 2. Phylogenetic structure of the class Anaerolineae based on comparative analysis of 16S rRNA gene sequences (a) and conserved protein sequences (b, c), indicating the position of strain TC1T. (a) Neighbour-joining phylogenetic tree of public data (accession numbers are shown) and the 16S RNA gene sequence of strain TC1T. Sequences from the bacterial phylum Chlorobi were used to root the tree (not shown). Reproducible nodes are marked based on bootstrap values from neighbour-joining, maximum-parsimony and maximum-likelihood inferences (closed circle, >95 % for all inferences; open circle, >85 %; open square, >70 %). Nodes that lack symbols were not reproducible among trees. Bar, 2 % estimated sequence divergence. The class Anaerolineae is bracketed to the right of the figure. (b) Maximum-likelihood phylogenetic inference of representative genomes of the class Anaerolineae. The tree was built using RAxML based on up to 38 universally conserved proteins. Reproducible associations (closed circle, >95 % bootstrap support; open circle, >85 % bootstrap support) are indicated at interior nodes. Accession numbers of genomes (IMGT or DDBJ/EMBL/GenBank databases) are shown in parentheses. Alignments of homologous proteins from genomes of members of the phylum Chlorobi were used to root the tree (not shown). Bar, 10 % estimated sequence divergence. (c) Maximum-likelihood phylogenetic inference of representative genomes of the class Anaerolineae. The tree was created as for (b), except that the tree was computed based on up to 83 proteins broadly conserved among bacteria. Bar, 10 % estimated sequence divergence.

50.4–53.5 % AAI with other members of the Anaerolineae, which represents mostly class- to order-level differences in the current bacterial taxonomy (Konstantinidis & Tiedje, 2005). The highest AAI value for strain TC1T was found with Levilinea saccharolytica KIBI-1T.

Based on the draft genome of strain TC1T, the DNA G+C content was estimated to be 41.35 % (Matsuura et al., 2015b). For the experimental determination of DNA base content, DNA of strain TC1T was extracted and purified as described previously (Kamagata & Mikami, 1991). The G+C content was determined by HPLC (Shimadzu LC-6A) with a UV detector as described elsewhere (triplicate determinations) (Shintani et al., 2000). The DNA G+C content was estimated to be 42.1 ± 0.1 mol%, which is consistent with the value estimated based on the genome sequence.

Although some differences in phenotype may be suggested, such as the type of fermentation end product (Table 1), strain TC1T and all of the previously cultivated and described members of the class Anaerolineae showed relatively uniform phenotypic characteristics in terms of morphology (multicellular filamentous morphology) and physiology (strictly anaerobic, chemo-organotrophic and neutrophilic growth) (Table 1). In addition, strain TC1T grew semi-syntrophically with hydrogenotrophic methanogens, which is one of the characteristic traits of the Anaerolineae (Sekiguchi et al., 2003; Yamada et al., 2006). Genome-scale comparison, however, clearly distinguished strain TC1T from other known members of the Anaerolineae. Strain TC1T had the lowest genome DNA G+C content among the known members of the Anaerolineae; this was confirmed by measuring the DNA base composition both using HPLC (in this study) and by determining its draft genome sequence (Matsuura et al., 2015b). 16S rRNA gene-based phylogeny and phylogenomic analyses as well as pairwise AAI also supported the novelty of strain TC1T within the Anaerolineae. Taken together, these findings underscore the conclusion that traditional methods for characterizing bacterial phenotypes may have significant limitations in identifying phenotypic differences among members of the Anaerolineae, or that genome-level dissimilarities do not necessarily correlate with phenotypic differences. Comparative genome analysis and additional physiological evaluation of strain TC1T based on its genomic content will shed further light on the tangible features of its novelty. Following the taxonomic legacy of the Anaerolineae, based upon the phenotypic and genomic characteristics, we propose a novel species of a new genus, Flexilinea flocculi gen. nov., sp. nov., for strain TC1T.

Description of Flexilinea gen. nov.

Flexilinea (Flex.i.li’ne.a. L. masc. n. flexus bending; L. fem. n. linea a line; N.L. fem. n. Flexilinea a flexible line-shaped organism).

Strictly anaerobic organisms. Cells are non-motile. Filamentous. Gram-staining reaction is negative. Phylogenetic position is in the class Anaerolineae within the bacterial phylum Chloroflexi. The type species is Flexilinea flocculi.

Description of Flexilinea flocculi sp. nov.

Flexilinea flocculi (floc’cu.li. N.L. dim. gen. n. flocculi of a small floc).

Cells are longer than 100 μm and 0.3–0.4 μm wide. Yeast extract is required for growth. In the presence of 0.1 % yeast extract, growth occurs with glucose, fructose, ribose, xylose, arabinose, starch (5 g l⁻¹) and pyruvate. Yeast extract (0.1 %) itself also supports growth. The following substrates are not utilized: sucrose, raffinose, xylan, betaine, peptone, Casamino acids, hydrogen (1 atm) plus acetate, formate plus acetate, glycerol, crotonate, lactate, fumarate, acetate, propionate, n-butylate, malate, succinate, ethanol, methanol, 1-propanol, ethylene glycol, hydroquinone and benzoate. The following compounds are not utilized as electron acceptors: sulfate, sulfite, thiosulfate, nitrate, fumarate, elemental sulfur and
Table 1. Characteristics of strain TC1<sup>T</sup> and related members of the class Anaerolineae

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<td>Cell diameter (μm)</td>
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<td>DNA G+C content (mol%)</td>
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<td>52.4</td>
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<td>Major cellular fatty acids*</td>
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<td>Growth enhancement in syntrophic growth with methanogens</td>
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<td>Marine subsurface sediment</td>
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<td>Thermophilic anaerobic sludge</td>
<td>Thermophilic anaerobic sludge</td>
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<td>Shallow sea hydrothermal vent</td>
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</table>

*ai, Anteiso-branched; br, branched; i, iso-branched.
†A, Acetate; E, ethanol; F, formate; L, lactate; Pr, propionate; Py, pyruvate; S, succinate.
Fe(III)-NTA. Growth is observed at 25–43 °C with optimum growth at 37 °C. Growth is observed at pH 6.0–8.5 with an optimum at pH 7.0. Growth is enhanced in co-cultivation with hydrogenotrophic methanogens. The major fatty acids are anteiso-C₁₅:₀, iso-C₁₅:₀ and iso-C₁₇:₀ 3-OH.

The type strain is TC₁₁ᵀ (=ICM 30897ᵀ=CGMCC 1.5202ᵀ), which was isolated from methanogenic granular sludge in a full-scale mesophilic UASB reactor treating high-strength starch-based organic wastewater. The DNA G+C content of the type strain is 42.1 mol% (HPLC).

Acknowledgements

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References


