Anaerolinea thermophila gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov., novel filamentous thermophiles that represent a previously uncultured lineage of the domain *Bacteria* at the subphylum level

Yuji Sekiguchi,[1] Takeshi Yamada,[2] Satoshi Hanada,[1] Akiyoshi Ohashi,[2] Hideki Harada[2] and Yoichi Kamagata[1]

[1]Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan

[2]Department of Environmental Systems Engineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan

Correspondence
Yuji Sekiguchi
y.sekiguchi@aist.go.jp

Two thermophilic, Gram-negative, non-spore-forming, multicellular filamentous micro-organisms were isolated from thermophilic granular sludge in an upflow anaerobic sludge blanket reactor treating fried soybean-curd manufacturing waste water (strain UNI-1^T) and from a hot spring sulfur-turf in Japan (strain STL-6-O1^T). The filaments were longer than 100 μm and of 0·2–0·3 μm (strain UNI-1^T) or 0·7–0·8 μm (strain STL-6-O1^T) in width. Strain UNI-1^T was a strictly anaerobic organism. The optimum temperature for growth was around 55 °C; growth occurred in the range 50–60 °C. The optimum pH for growth was around 7·0; growth occurred in the range pH 6·0–8·0. Strain STL-6-O1^T was a facultatively aerobic bacterium. The optimum temperature for growth was around 55 °C; growth occurred in the range 37–65 °C. The optimum pH for growth was around 7·5–8·0; growth occurred in the range pH 7·0–9·0. The two organisms grew chemo-organotrophically on a number of carbohydrates and amino acids in the presence of yeast extract. The G+C content of the DNA of strains UNI-1^T and STL-6-O1^T was 54·5 and 59·0 mol%, respectively. Major cellular fatty acids for strain UNI-1^T were $C_{16:0}$, $C_{15:0}$, $C_{14:0}$ and $C_{18:0}$, whereas those for strain STL-6-O1^T were $C_{18:0}$, $C_{16:0}$, $C_{17:0}$ and iso-$C_{17:0}$. MK-10 was the major quinone from aerobically grown STL-6-O1^T cells. Phylogenetic analyses based on 16S rDNA sequences revealed that both strains belong to an uncultured, previously recognized clone lineage of the phylum *Chloroflexi* (formerly known as green non-sulfur bacteria). These phenotypic and genetic properties suggested that each strain should be classified into a new independent genus; hence, the names *Anaerolinea thermophila* and *Caldilinea aerophila* are proposed for strains UNI-1^T (=JCM 11387^T=DSM 14523^T) and STL-6-O1^T(=JCM 11388^T=DSM 14525^T), respectively. These strains represent the type and sole species of the genera *Anaerolinea* and *Caldilinea*, respectively.

INTRODUCTION

Recent cultivation-independent small subunit (SSU) rDNA-based analysis has been uncovering a vast diversity of micro-organisms in natural ecosystems. To date, a number of clone clusters which are distant from any other known phyla (divisions) in the domain *Bacteria* but contain no cultured micro-organisms are recognized (Hugenholtz, 2002; Hugenholtz *et al.*, 1998), while extensive efforts are also being made to isolate these yet-to-be-cultured micro-organisms by investigators (e.g. Zhang *et al.*, 2003). Among

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Abbreviations: NTA, nitrilotriacetate; PCE, tetrachloroethene; TCE, trichloroethene.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of *Anaerolinea thermophila* UNI-1^T and *Caldilinea aerophila* STL-6-O1^T are AB046413 and AB067647, respectively.

A phylogenetic tree (constructed using a large dataset) showing the relationships of *A. thermophila* UNI-1^T and *C. aerophila* STL-6-O1^T with members of the phylum *Chloroflexi* is available in IJSEM Online.

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these uncultured clades, the bacterial phylum *Chloroflexi* (formerly known as green non-sulfur bacteria) (Garrity & Holt, 2001) has been recognized as a typical bacterial cluster containing a number of diverse environmental clones with only a few cultured representatives (Hugenholtz *et al.*, 1998).

The phylum *Chloroflexi* has been divided into four major subphyla (subdivisions) on the basis of 16S rDNA/RNA sequences, i.e. subphyla I, II, III and IV (Hugenholtz *et al.*, 1998). The phylum *Chloroflexi* contains cultured microbes belonging to the genera *Chloroflexus*, *Oscillochloris*, *Herpetosiphon* and *Roseiflexus*, but they account for only a small portion of this group; all of the cultured microbes mentioned above are affiliated with subphylum III. The other three subphyla (I, II and IV) are composed of a wide variety of environmental clones, except for a purified organism, '*Dehalococcoides ethenogenes*', which is akin to subphylum II (Maymo-Gatell *et al.*, 1997). Subphylum I contains the most diverse environmental clones among the four subphyla of *Chloroflexi*; for example, it contains clones from hot springs, subsurfaces, aerobic and anaerobic waste water treatment sludges, and contaminated aquifers, which hint at its ecological and physiological breadth (Björnsson *et al.*, 2002; Hugenholtz *et al.*, 1998; Juretschko *et al.*, 2002; Sekiguchi *et al.*, 1999). However, there have been no descriptions of cultivable microbes belonging to this subphylum, although they are very likely to play significant roles in such environments.

Previously, we reported the isolation of a novel thin, filamentous anaerobe (strain UNI-1^T) from a thermophilic anaerobic waste water treatment process (Sekiguchi *et al.*, 2001). 16S-rDNA-based phylogenetic analyses suggested that the isolate belongs to subphylum I of the phylum *Chloroflexi*, representing the first cultured organism in this clone cluster (Sekiguchi *et al.*, 2001). In addition, we have recently isolated a thermophilic, facultatively aerobic, filamentous microbe (strain STL-6-O1^T) from a hot spring sulfur-turf in Japan: the strain was found to be distantly related to strain UNI-1^T. Here, we report the detailed morphological, physiological and chemotaxonomic characteristics of strains UNI-1^T and STL-6-O1^T, and propose new genera and species to accommodate them.

METHODS

Sources of micro-organisms. Strain UNI-1^T was isolated as a part of a previous study on granular sludge in a thermophilic upflow anaerobic sludge blanket reactor treating soybean-curd manufacturing waste water (Sekiguchi *et al.*, 2001). Strain STL-6-O1^T was isolated from a sulfur-turf sample in a hot spring in Japan (Nakao hot spring, Gifu prefecture). The biomat was composed of macroscopic bundles of white filaments which might consist of colourless sulfur bacteria and elemental sulfur particles similar to those described by Yamamoto *et al.* (1998). The temperature and pH of the hot spring were about 60 °C and neutral, respectively. *Methanothermobacter thermautotrophicus* (formerly *Methanobacterium thermautotrophicum*) strain DSM 1053^T was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Cultivation conditions. The basal medium used for enrichment, isolation and maintenance of strain UNI-1^T was prepared according to Widdel & Pfennig (1981); its composition has been described previously (Sekiguchi *et al.*, 2000). All cultivations for strain UNI-1^T were carried out at 55 °C in 50 ml serum vials containing 20 ml medium (pH 7·0 at 25 °C) under an atmosphere of N_2/CO_2 (80 : 20, v/v) unless mentioned otherwise. Neutralized substrates were added to the vials containing the basal medium from stock solutions prior to inoculation. Solid medium was prepared by adding purified agar (Agar noble; Difco) to the medium described above at a final concentration of 20 g l^{-1}.

PE medium (Hanada *et al.*, 1995b) was used for the isolation and cultivation of strain STL-6-O1^T. All cultivations for strain STL-6-O1^T were carried out at 55 °C in liquid medium (pH 7·5 at 25 °C) under aerobic conditions unless mentioned otherwise.

M. thermautotrophicus DSM 1053^T was cultivated at 55 °C using the same medium used for strain UNI-1^T, except that hydrogen (approx. 0·5–1 atm) was included in the gas phase [N_2/CO_2 (80 : 20, v/v)] in the vials for energy source.

Effect of pH and temperature. To determine the optimum pH for growth of strain UNI-1^T, the pH values of the medium containing 10 mM sucrose and 0·1 % yeast extract were adjusted at room temperature to 5·5–9·0 by adding HCl or NaOH under a 100 % N_2 atmosphere. For strain STL-6-O1^T, the isolate was cultivated aerobically at 55 °C in PE medium; the pH value of the medium was adjusted to 5·5–9·5 as described above.

To evaluate the optimum temperature for the growth of strain UNI-1^T, the isolate was cultivated anaerobically in sucrose plus yeast extract medium (pH 7·0 at 25 °C) at 25, 37, 40, 45, 50, 55, 60, 65 and 70 °C. For strain STL-6-O1^T, cells were cultivated aerobically on PE medium (pH 7·5 at 25 °C) at the same temperatures used for strain UNI-1^T. All cultivations were done in duplicate (1 % inoculum) and optical density (OD_{400}) was measured.

Growth and substrate utilization. To test growth and substrate utilization of strain UNI-1^T, autoclaved or filter-sterilized substrates were added to the medium. All cultures were incubated anaerobically at 55 °C, pH 7·0 at 25 °C, for over 4 weeks. All substrates, including Fe(III)-NTA (nitrilotriacetate) (Roden & Lovley, 1993), were prepared as described previously (Sekiguchi *et al.*, 2000). Growth and substrate utilization of the strain were determined by monitoring the increase in the OD_{400} value with several carbon and energy sources (32 compounds), and the production of acetate and hydrogen, respectively. In syntrophic growth/substrate utilization tests, *M. thermautotrophicus* cells were added to the medium (2 % inoculum); growth and substrate utilization were checked by measuring the OD_{400} value and methane production.

For testing substrate utilization by strain STL-6-O1^T, 5 ml of medium containing one of various compounds (25 substances) as the sole carbon and energy sources to a final concentration of 0·25 % (w/v) were used in a multi-well plate (12 wells) as described previously (Hanada *et al.*, 2002). The plate was incubated at 55 °C under aerobic conditions.

To test tetrachloroethene (PCE) and trichloroethene (TCE) utilization by the isolates under anaerobic conditions, the strains were grown in glass vials stopped with Teflon-coated rubber septa. The gas phase was N_2/CO_2 (80 : 20, v/v). PCE or TCE was added separately to the vials as a solution in hexadecane at a final concentration of 100 μM. For

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strain UNI-1^T, sucrose plus yeast extract medium was used for this trial, while PE medium was employed for strain STL-6-O1^T. Cell growth was monitored by measuring an increase in the OD_{400} value; reduction of PCE and TCE was determined by measuring the concentrations of chlorinated ethenes, ethene and ethane in the gas phase.

Analytical methods. Short-chain fatty acids, methane, hydrogen and carbon dioxide were determined by GC (Sekiguchi *et al.*, 2000). Alcohols and other compounds were determined by HPLC as described previously (Imachi *et al.*, 2000). Carbohydrates such as sucrose and glucose were determined by HPLC using a SCR101-H column (Sekiguchi *et al.*, 2001). PCE, TCE, *cis*-1,2-dichloroethene and vinyl chloride were determined by GC (Shimadzu GC-14A; detector type, FID; packing material, 20 % tricresylphosphate on Chromosorb WAW; column temperature, 70 °C). Ethene and ethane were determined by GC (Shimadzu GC-14A; detector type, FID; packing material, Porapak type Q; column temperature, 60 °C).

Microscopy. The cell morphology of the strains was examined under a phase-contrast microscope (Olympus AX80T). Gram-staining was done by Hucker's method (Doetsch, 1981). Phase-contrast micrographs were taken by using wet mounts on agar-coated slides (Pfennig & Wagener, 1986) for exponential-phase cultures. Cells of strain UNI-1^T for thin-section electron microscopy were fixed with 2·5 % glutaraldehyde overnight, then post-fixed in 1 % osmium tetroxide at 4 °C for 3 h. The fixed cells were dehydrated and embedded in Spurr low-viscosity resin. Since thin-section electron microscopy for strain STL-6-O1^T using the above protocol seemed to be unsuccessful (the integrity of the cells seemed to be lost), cells of the strain were rapidly frozen at −185 °C and soaked in 100 % acetone containing 2 % osmium tetroxide at −80 °C for 2 days. The cells were then gradually warmed to room temperature, placed in a freezer at −20 °C for 2 h and then in a refrigerator at 4 °C for 1 h. The samples were subsequently rinsed with 100 % acetone followed by infiltration with propylene oxide at room temperature; they were then embedded in Spurr resin. Thin-sections (80 nm) of the cells of both strains were made with an ultramicrotome (Reichert ULTRACUT N) and stained with uranyl acetate and lead citrate; they were then examined using a transmission electron microscope (Hitachi H-7000).

Determination of DNA base content. DNA 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 (Shintani *et al.*, 2000).

Determination of quinone and fatty acid methyl ester (FAME) analysis. Quinones were determined as described previously (Zhang *et al.*, 2000). For FAME analysis, fatty acids of cells were converted to methyl esters by using HCl/methanol and determined by GC with MS (Hitachi M7200A FC/3DQMS system) (Hanada *et al.*, 2002). For quinone and FAME analyses, cells of strain UNI-1^T were harvested from cultures grown on the medium containing sucrose plus yeast extract, while cells of strain STL-6-O1^T were obtained from cultures grown aerobically on PE medium.

16S rDNA sequence analysis. The 16S rDNA sequence of strain UNI-1^T was reported as part of our previous study (Sekiguchi *et al.*, 2001). For strain STL-6-O1^T, DNA was recovered according to the method of Hiraishi (1992). The 16S rRNA gene (rDNA) of the strain was amplified by PCR with *Taq* polymerase (Perkin Elmer) as described previously (Sekiguchi *et al.*, 2000). The PCR primers used in the amplification were the bacterial domain universal primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′; positions 8–27, *Escherichia coli* numbering) and the prokaryote universal primer 1490R (5′-GGTTACCTTGTTACGACTT-3′; positions 1491–1509, *E. coli* numbering) (Weisburg *et al.*, 1991). The PCR product was directly sequenced on an ABI 377 DNA sequencer using a dRhodamine Dye Terminator Cycle Sequencing kit (Applied Biosystems). Sequence data were aligned in an ARB dataset using the ARB program package (http://www.arb-home.de/); the aligned data were corrected manually by using the editing tool in the package. Phylogenetic trees based on 16S rDNA sequences were constructed by the neighbour-joining method (Saito & Nei, 1987) within the MEGA2 package (Kumar *et al.*, 2001). Bootstrap resampling analysis (Felsenstein, 1985) was performed to estimate the confidence of tree topologies (1000 replicates).

RESULTS AND DISCUSSION

Isolation and morphology

Strain UNI-1^T was isolated as part of our previous study on granular sludge in a thermophilic upflow anaerobic sludge blanket reactor for waste water treatment; the detailed isolation procedure for this strain has been described previously (Sekiguchi *et al.*, 2001). On an anaerobic solid medium containing glucose and yeast extract, the strain formed very small colonies that were white, lens-shaped and 0·1–0·2 mm in diameter.

Strain STL-6-O1^T was obtained from a natural hot spring sample in Japan. Part of a biomat (sulfur-turf) collected from the hot spring was gently homogenized and used as inoculum for further cultivation and isolation of microorganisms. When the biomat in the hot spring was directly cultivated aerobically in organic medium (PE medium) containing glutamate, succinate, acetate, yeast extract and Casamino acids, only limited microbes, such as *Thermus* spp., were obtained in pure culture (data not shown). We also made a primary aerobic enrichment culture using thiosulfate/HCO_3^- as carbon and energy sources to focus on isolation of chemolithotrophic thermophiles. A number of successive transfers to fresh thiosulfate/HCO_3^- medium were conducted for the enrichment, in which we found that the microbial community that developed in the enrichment was stable, consisting of several morphotypes of microbes. To isolate thiosulfate/HCO_3^--utilizing microbes, a portion of the enrichment transferred successively over 6 months was inoculated onto thiosulfate/HCO_3^- agar medium, but no visible growth occurred on the plate. However, when we plated the enrichment on PE medium, orange to slightly pinkish, glossy, irregular colonies became visible within a week. A colony was picked and transferred to fresh agar medium, resulting in the isolation of strain STL-6-O1^T.

Both isolates formed flexible filaments, which were longer than 100 μm (Fig. 1). Strain UNI-1^T cells were 0·2–0·3 μm wide (Fig. 1A), while those of STL-6-O1^T were 0·7–0·8 μm wide (Fig. 1B). Spore formation was not observed, and Gram-staining was negative for both isolates. Gliding motility was not observed in either isolate. A sheath-like structure was not clearly visible in either strain by light or electron microscopy (Figs 1 and 2). Electron microscopy demonstrated that cells of both isolates possessed a Gram-negative-type cell wall structure, showing multicellular

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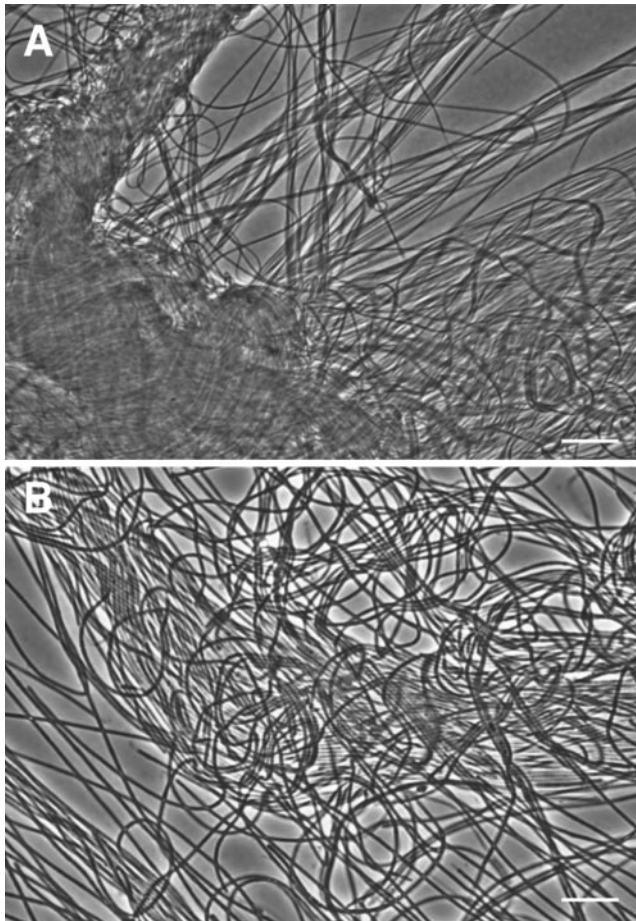

Fig. 1. Photomicrographs of strains UNI-1^T and STL-6-O1^T, which belong to subphylum I of *Chloroflexi*. (A) Strain UNI-1^T was isolated from a thermophilic anaerobic granular sludge. (B) Strain STL-6-O1^T was isolated from the biomat of a natural hot spring. Bars, 10 μm.

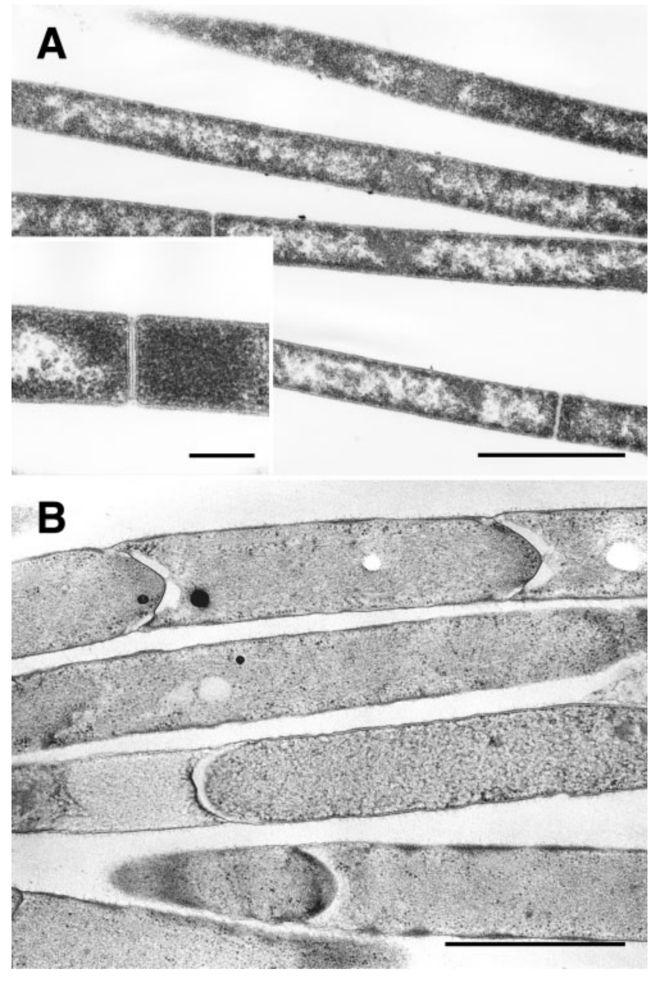

Fig. 2. Transmission electron micrographs of ultra-thin sections of (A) strain UNI-1^T (bar, 1 μm; bar in the inset, 0·1 μm) and (B) strain STL-6-O1^T (bar, 1 μm).

forms of filaments (Fig. 2A, B). The micrographs also suggest that cells of both strains are longer than 2 μm.

Physiological properties of strain UNI-1^T

Strain UNI-1^T was a strictly anaerobic organism: no growth occurred in the presence of oxygen (20 %, v/v, in the gas phase). It was not photosynthetic (data not shown). Yeast extract was required for growth and it could not be replaced with vitamin mixtures. In the presence of yeast extract (0·01 %), growth and substrate utilization were observed with the following substrates (all at 20 mM): glucose, fructose, galactose, mannose, raffinose, sucrose and starch. Yeast extract itself (0·5 %) also allowed good growth. Weak growth occurred with the following substrates (all at 20 mM, unless shown otherwise) with medium supplemented with yeast extract (0·01 %): Casamino acids (0·1 %), tryptone (0·1 %), pyruvate, ribose, xylose, arabinose, xylan and pectin. Growth and acid formation were not observed in the following substrates (all at 20 mM, unless shown otherwise) in the presence of yeast extract (0·01 %): crotonate, H_2/CO_2 (1 atm) plus acetate, lactate, glycerol, acetate, propionate, butyrate, malate, succinate, ethanol, methanol, 1-propanol, benzoate, hydroquinone, phenol or formate plus acetate. In glucose medium supplemented with yeast extract (0·01 %), cells of the strain produced acetate and hydrogen as the main end products of fermentation, with small amounts of lactate, succinate and formate.

The following compounds were tested as electron acceptors with sucrose and yeast extract medium, but none of them was utilized: 20 mM sulfate, nitrate, sulfite, thiosulfate, fumarate, 5 mM Fe(III)-NTA. PCE and TCE (100 μM each) were also tested as electron acceptors, but neither of them was utilized within 4 weeks incubation.

Strain UNI-1^T grew between 50 and 60 °C with optimum growth at 55 °C, whereas no growth was observed below 45 °C or above 65 °C with 4 weeks incubation. The pH range for growth was between 6·0 and 8·0, with the optimum at

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around pH 7·0. Under the optimum conditions (pH 7·0, 55 °C), the approximate doubling time of the strain in pure culture on sucrose plus yeast extract medium was 3 days (Sekiguchi *et al.*, 2001). As described in our previous report, growth of strain UNI-1^T ceased after a certain amount of hydrogen had accumulated in the medium. However, growth improved significantly when the strain was cultivated in the presence of the hydrogenotrophic methanogen *M. thermautotrophicus* (the approximate doubling time of the co-culture, estimated based on methane-production rate, was 1·5 days). NaCl was slightly inhibitory at 5 g l^{-1}, and 10 g NaCl l^{-1} completely inhibited growth of the strain.

Physiological properties of strain STL-6-O1^T

Strain STL-6-O1^T was a facultatively aerobic microbe: it could grow under either aerobic or anaerobic (fermentative) conditions. It was not photosynthetic (data not shown). Strain STL-6-O1^T was able to use yeast extract as the best substrate for growth under aerobic and anaerobic conditions. Yeast extract (0·01 %) was required for growth when the strain grew aerobically on the following substrates: glucose, tryptone, sucrose, maltose, raffinose, starch, glycerol, acetate, pyruvate, lactate, succinate, fumarate and glutamate. The following substrates were not utilized by the strain: mannose, fructose, arabinose, xylose, ribose, ethanol, formate, malate, alanine, serine and Casamino acids. Growth was observed under anaerobic conditions with PE medium (containing 0·05 % yeast extract) as well as with the sucrose plus yeast extract medium used for strain UNI-1^T. Under anaerobic conditions, hydrogen was not formed by the strain at detectable levels in the cultures. In addition, the strain did not utilize thiosulfate/HCO_3^- as carbon and energy sources, the substrates for the primary enrichment. This indicates that the strain might survive on some remnants from the community that had constantly formed in the primary enrichment.

The following compounds were tested as electron acceptors with PE medium under anaerobic conditions, but none of them was utilized: 20 mM sulfate, nitrate, sulfite, thiosulfate, fumarate, 5 mM Fe(III)-NTA. PCE and TCE (100 μM each) were also tested, but neither was utilized within 4 weeks under anaerobic conditions.

Strain STL-6-O1^T grew between 37 and 65 °C, with optimum growth at 55 °C; no growth was observed below 30 °C or above 70 °C with 30 days incubation. The pH range for growth was between 7·0 and 9·0, with the optimum at pH 7·5–8·0. The approximate doubling time of growth was 5 h under optimum growth conditions in aerobic liquid PE medium (pH 7·5, 55 °C). NaCl was slightly inhibitory at 5 g l^{-1}, and 15–20 g NaCl l^{-1} completely inhibited growth of the strain.

Chemotaxonomic analyses

The DNA G+C content of strains UNI-1^T and STL-6-O1^T was calculated to be 54·5 and 59·0 mol%, respectively. Fatty acid methyl ester analysis showed that strain UNI-1^T contained $C_{16:0}$ (35 %), $C_{15:0}$ (14 %), $C_{14:0}$ (12 %) and $C_{18:0}$ (12 %) as the major fatty acids, with $C_{17:0}$ (7 %), branched $C_{17:0}$ (6 %), branched $C_{19:0}$ (6 %), anteiso-$C_{17:0}$ (6 %), $C_{12:0}$ (1 %) and branched $C_{14:0}$ (1 %) as the minor fatty acids. Strain STL-6-O1^T contained $C_{18:0}$ (33 %), $C_{16:0}$ (28 %), $C_{17:0}$ (24 %) and iso-$C_{17:0}$ (11 %) as the major fatty acids, with branched $C_{19:0}$ (2 %), $C_{12:0}$ (1 %) and $C_{19:0}$ (1 %) as the minor fatty acids. No unsaturated fatty acids were detected in either strain. Quinone analysis revealed that strain STL-6-O1^T contained MK-10 as the major quinone when grown aerobically. No quinone was detected from cells of strain UNI-1^T.

Phylogenetic analysis and taxonomic conclusions

On the basis of the phylogenetic analysis reported previously and that shown in Fig. 3, strain UNI-1^T belongs to subphylum I of the phylum *Chloroflexi* (formerly known as green non-sulfur bacteria) (Garrity & Holt, 2001) in the domain *Bacteria* (Sekiguchi *et al.*, 2001). For strain STL-6-O1^T, a total of 1427 nt of its 16S rDNA were sequenced and used for phylogenetic analyses to determine its phylogenetic position in the domain *Bacteria*. According to neighbour-joining analysis, strain STL-6-O1^T also fell into subphylum I of *Chloroflexi* (Fig. 3). Strains UNI-1^T and STL-6-O1^T displayed 81·1 % 16S rDNA sequence similarity, indicating that they belonged to different genera. Sequence similarity values for the 16S rDNA of the two isolates compared to those of related species, such as *Chloroflexus* species, were all less than 80 %; these values were low enough for the creation of two new genera in subphylum I of *Chloroflexi* (Stackebrandt & Goebel, 1994).

At present, the two isolates reported here are the only cultivated representatives of subphylum I of *Chloroflexi*, which had long been considered a typical uncultured bacterial lineage consisting solely of environmental clones (Hugenholtz *et al.*, 1998). Members of subphylum I have been known to be widespread in diverse environments: clones have been retrieved in great numbers from mainly anoxic environments such as sediments, subsurfaces, anaerobic dechlorinating consortia, hot springs and anaerobic waste water sludges, but also from virtually aerobic environments such as freshwater and activated sludge systems (Hugenholtz *et al.*, 1998). This evidence strongly suggests that members of subphylum I are very likely to be ubiquitous organisms playing certain roles in such environments. In fact, it has been shown that they are numerically abundant in anaerobic, thermophilic granular sludge for waste water treatment and that they are important constituents in sludge granules for maintaining the granule structure as well as for triggering bulking (Sekiguchi *et al.*, 1998, 1999, 2001). Recently, molecular studies on activated sludge systems have also shown a remarkable abundance and distribution of microbes belonging to subphylum I in these systems (Björnsson *et al.*, 2002; Juretschko *et al.*, 2002). Interestingly, studies employing

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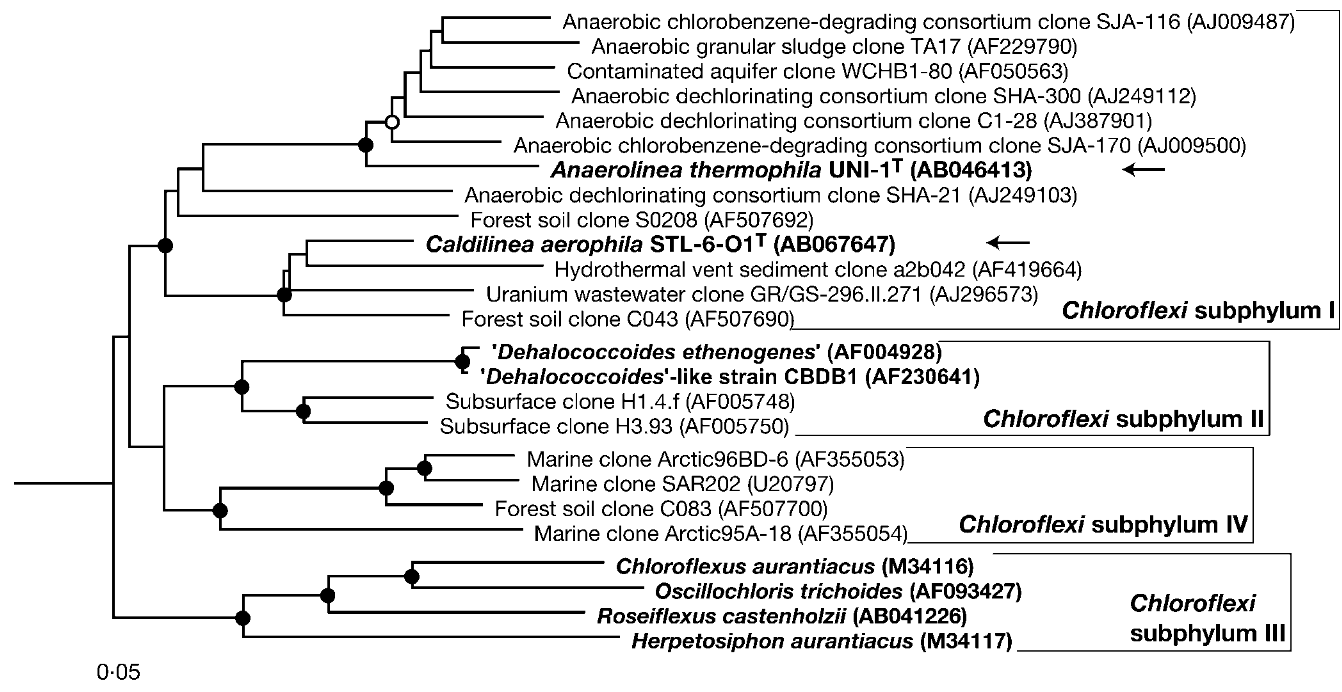

Fig. 3. Phylogenetic positions of strains UNI-1^T and STL-6-O1^T among the phylum *Chloroflexi*. The 16S rDNA sequences obtained and reference sequences were aligned by using the ARB program. The phylogenetic tree was constructed by the neighbour-joining method. 16S rDNA sequences of members of the order *Thermotogales* were used to root the tree. ●, Bootstrap probabilities (based on 1000 replicates) above 95 %; ○, bootstrap probabilities above 85 %. Branch points without circles were not highly resolved (<85 %) as specific groups in the analyses. Bar, number of changes per sequence position.

whole cell *in situ* hybridization analyses for these microbes suggested that members of subphylum I all have the filamentous morphotype with a wide range of filament thickness (Björnsson *et al.*, 2002; Juretschko *et al.*, 2002; Sekiguchi *et al.*, 1999, 2001). These observations are consistent with our findings, in that strains UNI-1^T and STL-6-O1^T are both filamentous but are significantly different to each other in their thickness.

In addition, relatively high 16S rDNA sequence divergence of environmental clones belonging to this subphylum implies that they can be physiologically diverse as well. In fact, the two strains characterized in this study showed obvious differences in their physiological properties such as aerobic growth, pH and temperature ranges for growth and syntrophic growth, although they share, to some extent, several traits such as substrate range and morphology. Also, there are significant differences in the physiological and chemotaxonomic properties of the present isolates and their phylogenetic relatives such as members in the family '*Chloroflexaceae*' (Table 1) (Hanada & Pierson, 2002). These phenotypic properties of the two isolates also supported the creation of new taxa.

The reasons why such diverse members of the subphylum are abundantly present in diverse environments still remain to be clarified. To better understand the typical features of the subphylum, much more tangible microbes belonging to this subphylum need to be characterized further.

In conclusion, the two strains described here belong to subphylum I of the phylum *Chloroflexi* but are distantly related to each other. In addition, several significant differences in their physiological and chemotaxonomic properties seem to justify the creation of two individual genera for the two isolates. Therefore, in conclusion, we propose the creation of *Anaerolinea thermophil*a gen. nov., sp. nov. and *Caldilinea aerophila* gen. nov., sp. nov. for strains UNI-1^T and STL-6-O1^T, respectively.

Description of *Anaerolinea* gen. nov.

Anaerolinea (An.ae.ro.li.ne′a. Gr. pref. *an* not; Gr. masc. n. *aer* air; L. fem. n. *linea* line; N.L. fem. n. *Anaerolinea* line-shaped not living in air).

Gram-negative. Cells are non-motile and filamentous. Spores are not formed. Thermophilic. Grows under strictly anaerobic conditions. Neither photosynthetic nor aerobic growth is observed. The main fatty acids are $C_{16:0}$, $C_{15:0}$, $C_{14:0}$ and $C_{18:0}$. The G+C content of genomic DNA is 54·5 mol%. Phylogenetic position is in subphylum I of the phylum *Chloroflexi*.

The type species is *Anaerolinea thermophila*.

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Table 1. Phenotypic characteristics of valid genera affiliated with the phylum *Chloroflexi*

The table was created based on a review by Hanada & Pierson (2002). Data for *Anaerolinea* and *Caldilinea* are from this study. Species of the genus *Chloroflexus* (Pierson & Castenholz, 1974; Gorlenko, 1976; Hanada *et al.*, 1995a): *Cfl. aurantiacus* (type species), *Cfl. aurantiacus* var. *mesophilus*, *Cfl. aggregans*. Species of the genus *Oscillochloris* (Keppen *et al.*, 1993, 1994, 2000; Gorlenko & Pivovarova, 1977): *Osc. chrysea* (type species), *Osc. trichoides*. Species of the genus *Herpetosiphon* (Holt & Lewin, 1968; Lewin, 1970): *Hph. aurantiacus* (type species), *Hph. geysericola*. *Sphaerobacter thermophilus* (type species; Demharter *et al.*, 1989). *Roseiflexus castenholzii* (type species; Hanada *et al.*, 2002). –, Negative; ±, variable; +, positive; ND, not determined.

Characteristic	Genus of subphylum I of *Chloroflexi*		Genus of subphylum III of *Chloroflexi*				
	Anaerolinea	***Caldilinea***	***Chloroflexus***	***Oscillochloris***	***Herpetosiphon***	***Sphaerobacter***	***Roseiflexus***
Morphology	Multicellular filaments	Multicellular filaments	Multicellular filaments	Multicellular filaments	Multicellular filaments	Coccoid rod	Multicellular filaments
Cell diameter (µm)	0·2–0·3	0·7–0·8	0·7–1·5	1·0–5·5	0·5–1·5	1·0–3·0	0·8–1·0
Sheath	−	−	±	±	+	−	−
Gram stain	−	−	−	±	−	+	−
Optimum growth temp. (°C)	55	55	20–25, 55	28–30	25–30	55	50
Metabolism:							
Photoheterotroph	−	−	+	+	−	ND	+
Photoautotroph	−	−	±	+	−	ND	−
O_2 respiration	−	+	+	±	+	+	+
Fermentation	+	+	±	ND	−	−	−
Major cellular fatty acids	$C_{16:0}$, $C_{15:0}$, $C_{14:0}$, $C_{18:0}$	$C_{18:0}$, $C_{16:0}$, $C_{17:0}$, iso-$C_{17:0}$	$C_{18:0}$, $C_{16:0}$, $C_{18:1}$	$C_{18:1}$, $C_{16:0}$, $C_{16:1}$	ND	ND	$C_{16:0}$, $C_{14:0}$, $C_{15:0}$
Major quinone	–	MK-10	MK-10 (and MK-4)	MK-10	MK-6	MK-8	MK-11
DNA G+C content (mol%)	54·5	59·0	56·9–57·1	59·2	48·1–48·5	66·3	62·0

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Description of *Anaerolinea thermophila* sp. nov.

Anaerolinea thermophila (ther.mo.phi′la. Gr. adj. *thermos* hot; Gr. adj. *philos* loving; N.L. fem. adj. *thermophila* heat-loving).

Cells are filament-shaped (longer than 100 μm and 0·2–0·3 μm wide). Growth occurs between 55 and 60 °C; optimum growth at 55 °C. The pH range for growth is 6·0–8·0; optimum growth at pH 7·0. The doubling time of growth is 3 days under optimum growth conditions. Growth is significantly stimulated when the organism is co-cultivated with hydrogenotrophic methanogens. Growth is observed with yeast extract as substrate. Yeast extract is required for growth. In the presence of yeast extract, growth and substrate utilization could be observed with the following substrates: glucose, fructose, galactose, mannose, raffinose, sucrose and starch. Weak growth occurs with the following substrates with medium supplemented with yeast extract: Casamino acids, tryptone, pyruvate, ribose, xylose, arabinose, xylan and pectin. The following substrates are not utilized in the presence of yeast extract: crotonate, H_2/CO_2 plus acetate, lactate, glycerol, acetate, propionate, butyrate, malate, succinate, ethanol, methanol, 1-propanol, benzoate, hydroquinone, phenol and formate plus acetate. None of the following compounds is utilized as electron acceptor: sulfate, nitrate, sulfite, thiosulfate, fumarate, Fe(III)-NTA, PCE and TCE.

The type strain is UNI-1^T (=JCM 11387^T=DSM 14523^T). Isolated from a thermophilic upflow anaerobic sludge blanket reactor treating soybean-curd manufacturing waste water.

Description of *Caldilinea* gen. nov.

Caldilinea (Cal.di.li.ne′a. L. adj. *caldus* hot; L. fem. n. *linea* line; N.L. fem. n. *Caldilinea* line-shaped living in a hot environment).

Gram-negative. Cells are non-motile and filamentous. Spores are not formed. Thermophilic. Cells grow under aerobic and anaerobic conditions. No photosynthetic growth is observed. The main fatty acids are $C_{18:0}$, $C_{16:0}$, $C_{17:0}$ and iso-$C_{17:0}$. The major quinone is menaquinone MK-10. The G+C content of genomic DNA is 59·0 mol%. Phylogenetic position is in subphylum I of the phylum *Chloroflexi*.

The type species is *Caldilinea aerophila*.

Description of *Caldilinea aerophila* sp. nov.

Caldilinea aerophila (ae.ro.phi′la. Gr. masc. n. *aer* air; Gr. adj. *philos* loving; N.L. fem. adj. *aerophila* air-loving).

Cells are filament-shaped (longer than 100 μm and 0·7–0·8 μm wide). Growth occurs between 37 and 65 °C; optimum growth at 55 °C. The pH range for growth is 7·0–9·0; optimum growth at pH 7·5–8·5. The doubling time of growth is 5 h under optimum growth conditions. Utilizes yeast extract as the best substrate for growth under aerobic and anaerobic conditions. Yeast extract is required for growth. In the presence of yeast extract, growth and substrate utilization could be observed with the following substrates: glucose, tryptone, sucrose, maltose, raffinose, starch, glycerol, acetate, pyruvate, lactate, succinate, fumarate and glutamate. The following substrates are not utilized in the presence of yeast extract: mannose, fructose, arabinose, xylose, ribose, ethanol, formate, malate, alanine, serine and Casamino acids. Growth is observed under anaerobic conditions. None of the following substances is utilized as electron acceptor under anoxic conditions: sulfate, nitrate, sulfite, thiosulfate, fumarate, Fe(III)-NTA, PCE and TCE.

The type strain is STL-6-O1^T (=JCM 11388^T=DSM 14525^T). Isolated from a hot spring sulfur-turf in Japan.

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