Anaerolinea thermolimosa sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov. and *Caldilineae* classis nov. in the bacterial phylum *Chloroflexi*

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One thermophilic (strain IMO-1^T) and two mesophilic (strains KIBI-1^T and YMTK-2^T) non-spore-forming, non-motile, Gram-negative, multicellular filamentous micro-organisms, which were previously isolated as members of the tentatively named class '*Anaerolineae*' of the phylum *Chloroflexi*, were characterized. All isolates were strictly anaerobic micro-organisms. The length of the three filamentous isolates was greater than 100 µm and the width was 0.3–0.4 µm for strain IMO-1^T, 0.4–0.5 µm for strain KIBI-1^T and thinner than 0.2 µm for strain YMTK-2^T. Strain IMO-1^T could grow at pH 6.0–7.5 (optimum growth at pH 7.0). The optimal temperature for growth of strain IMO-1^T was around 50 °C (growth occurred between 42 and 55 °C). Growth of the mesophilic strains KIBI-1^T and YMTK-2^T occurred at pH 6.0–7.2 with optimal growth at pH 7.0. Both of the mesophilic strains were able to grow in a temperature range of 25–50 °C with optimal growth at around 37 °C. Yeast extract was required for growth of all three strains. All the strains could grow with a number of carbohydrates in the presence of yeast extract. The G + C contents of the DNA of strains IMO-1^T, KIBI-1^T and YMTK-2^T were respectively 53.3, 59.5 and 48.2 mol%. Major fatty acids for thermophilic strain IMO-1^T were anteiso-C_{17:0}, iso-C_{15:0}, C_{16:0} and anteiso-C_{15:0}, whereas those for mesophilic strains KIBI-1^T and YMTK-2^T were branched C_{14:0}, iso-C_{15:0}, C_{16:0} and branched C_{17:0}, and branched C_{17:0}, C_{16:0}, C_{14:0} and C_{17:0}, respectively. Detailed phylogenetic analyses based on their 16S rRNA gene sequences indicated that the isolates belong to the class-level taxon '*Anaerolineae*' of the bacterial phylum *Chloroflexi*, which for a long time had been considered as a typical uncultured clone cluster. Their morphological, physiological, chemotaxonomic and genetic traits strongly support the conclusion that these strains should be described as three novel independent taxa in the phylum *Chloroflexi*. Here, *Anaerolinea thermolimosa* sp. nov. (type strain IMO-1^T = JCM 12577^T = DSM 16554^T), *Levilinea saccharolytica* gen. nov., sp. nov. (type strain KIBI-1^T = JCM 12578^T = DSM 16555^T) and *Leptolinea tardivitalis* gen. nov., sp. nov. (type strain YMTK-2^T = JCM 12579^T = DSM 16556^T) are proposed. In addition, we formally propose to subdivide the tentative class-level taxon '*Anaerolineae*' into *Anaerolineae* classis nov. and *Caldilineae* classis nov. We also propose the subordinate taxa *Anaerolineales* ord. nov., *Caldilineales* ord. nov., *Anaerolineaceae* fam. nov. and *Caldilineaceae* fam. nov.

Abbreviations: FAME, fatty acid methyl ester; NTA, nitrilotriacetate; TEM, transmission electron microscopy; UASB, upflow anaerobic sludge blanket. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains IMO-1^T, YMTK-2^T and KIBI-1^T are AB109437–AB109439.

INTRODUCTION

In recent estimations of bacterial and archaeal diversity in nature on the basis of 16S rRNA gene sequences, the domains *Bacteria* and *Archaea* were both suggested to contain a number of clone clusters at the phylum and subphylum levels that are composed solely of environmental 16S rRNA gene clones (Hugenholtz, 2002; Hugenholtz *et al.*, 1998; Rappé & Giovannoni, 2003). The bacterial phylum *Chloroflexi* has also been recognized as a typical bacterial lineage that contains a number of environmental 16S rRNA gene sequences with a few cultured representatives. The phylum *Chloroflexi* is currently known to be divided phylogenetically into at least five major classes (subphyla), including the classes '*Chloroflexi*' and *Thermomicrobia*. The class '*Chloroflexi*' was proposed by Garrity & Holt (2001) as the best characterized class in the phylum, in which various photosynthetic bacteria (previously known as green non-sulphur bacteria) as well as non-photosynthetic microbes are classified. The class *Thermomicrobia*, with which two cultured species, *Thermomicrobium roseum* and *Sphaerobacter thermophilus*, are affiliated (Hugenholtz & Stackebrandt, 2004), was recently transferred to the phylum *Chloroflexi*. In addition to these formally proposed taxa, Hugenholtz & Stackebrandt (2004) informally proposed the remaining three subphylum-level taxonomic groups of the phylum *Chloroflexi*, the classes '*Anaerolineae*' and '*Dehalococcoidetes*' and a clone cluster (at subphylum level) called 'subphylum IV' (SAR202 cluster) (Morris *et al.*, 2004).

Among these classes, the '*Anaerolineae*' contained a vast number of environmental 16S rRNA gene sequences with only a few cultured strains. Since '*Anaerolineae*'-type clones have been found frequently within various ecosystems, the organisms within the group have been thought to be ubiquitous and to play important roles in these ecosystems (Hugenholtz *et al.*, 1998). Two thermophilic, multicellular filamentous organisms, *Anaerolinea thermophila* and *Calidilinea aerophila*, were recently isolated from an anaerobic granular sludge and a hot spring, respectively, and were characterized in detail (Sekiguchi *et al.*, 2003); these two strains are the only cultured representatives of the subphylum. In addition to the two thermophiles, we recently reported one thermophilic and two mesophilic filamentous strains belonging to the '*Anaerolineae*' newly isolated from mesophilic and thermophilic upflow anaerobic sludge blanket (UASB) sludge granules (Yamada *et al.*, 2005).

In this paper, we surveyed the characteristics of these three filamentous strains, IMO-1^T, KIBI-1^T and YMTK-2^T, in more detail to outline their taxonomic placements. Based on the morphological, physiological, chemotaxonomic and phylogenetic analysis, we propose novel species to include each strain, two of which are placed in new genera. In addition, we formally propose two classes, *Anaerolineae* classis nov. and *Calidilineae* classis nov., and also propose several subordinate taxa from order- to family-level lineages.

METHODS

Sources of micro-organisms. Strain IMO-1^T was originally isolated from granular sludge in a thermophilic UASB reactor that was fed with wastewater from a factory producing shochu (a Japanese distilled liquor made from sweet potato) (Yamada *et al.*, 2005). Strains KIBI-1^T and YMTK-2^T were isolated from sludge granules of mesophilic UASB reactors treating an actual wastewater discharged from a sugar-processing plant and an artificial wastewater mainly composed of sucrose and volatile fatty acids (Yamada *et al.*, 2005). Detailed isolation procedures for all the strains were described in our previous paper (Yamada *et al.*, 2005). *Methanospirillum hungatei* DSM 864^T and *Methanothermobacter thermautotrophicus* DSM 1053^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany).

Cultivation conditions. Cultivation of all filamentous microbes was performed with the basal medium used in previous studies (Sekiguchi *et al.*, 2000; Yamada *et al.*, 2005). Cultivation of strains KIBI-1^T and YMTK-2^T was carried out at 37 °C, whereas strain IMO-1^T was cultivated at 55 °C. Neutralized substrates were added to the vials from stock solutions prior to inoculation. *Methanospirillum hungatei* DSM 864^T and *Methanothermobacter thermautotrophicus* DSM 1053^T were incubated in the medium mentioned above, with an exception that hydrogen (approx. 1 Pa) was included in the gas phase (80 % N₂, 20 % CO₂, v/v) in the vials as an energy source. These cultures were incubated at 37 or 55 °C. 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.*, 1995) was used for phototrophic and aerobic growth tests. All the tests were performed under optimal pH and temperature conditions.

Effect of pH and temperature. To determine the optimal pH for growth, the pH of the basal medium was adjusted at room temperature to 5.0–9.0 by adding HCl or NaOH under a 100 % N₂ atmosphere. pH-adjusted media for strains IMO-1^T and KIBI-1^T were supplemented with sucrose (20 mM) and yeast extract (0.1 %), whereas media for strain YMTK-2^T were supplemented with yeast extract (0.1 %) as carbon and energy source. All cultivations were performed in triplicate (2 % inoculum per medium volume) and growth was evaluated by both the increase in optical density (OD₄₀₀) and production of hydrogen and acetate in the vials.

To judge the optimal temperature and temperature range for growth, we prepared the medium described above (i.e. gas phase of 80 % N₂ and 20 % CO₂, v/v; pH_{25 °C} 7.0). Temperature tests for the two mesophilic isolates were carried out at 15, 25, 30, 35, 37, 40, 45, 50, 55 and 60 °C, whereas those for the thermophilic strain were performed at 25, 37, 40, 42, 45, 50, 52, 55, 60, 65 and 70 °C. All tests were done in triplicate (2 % inoculum per medium volume). Growth was evaluated by measuring both OD₄₀₀ and the production of hydrogen and acetate in the vials.

Substrate utilization and syntrophic growth. To examine the substrate utilization of the isolates, they were cultivated in medium (containing 0.01 % yeast extract) supplemented individually with 36 autoclaved or filter-sterilized compounds such as proteins, sugars, alcohols, aromatic compounds and volatile fatty acids (see below for details). All substrates and electron acceptors, including ferric nitrotriacetate [Fe(III)-NTA] (Roden & Lovley, 1993), were prepared as described previously (Sekiguchi *et al.*, 2000). In all tests, cultivation was performed for 1 month under optimal growth conditions for each strain (2 % inoculum).

Syntrophic growth was tested for strains IMO-1^T and KIBI-1^T using medium supplemented with sucrose and yeast extract, whereas strain YMTK-2^T was evaluated using medium with yeast extract. For the two mesophilic strains (strains KIBI-1^T and YMTK-2^T), pre-grown cells of

Methanospirillum hungatei DSM 864^T (2 % inoculum) were added as the hydrogen-/formate-consuming organism in the vials prior to cultivation. For the thermophilic strain (IMO-1^T), cells of *Methanothermobacter thermautotrophicus* DSM 1053^T (2 % inoculum) were added. Substrate utilization and syntrophic growth were examined with production of methane and acetate, as well as increase in OD₄₀₀ in the vials.

Analytical methods. Short-chain fatty acids, methane, hydrogen and carbon dioxide were determined by GC as described previously (Imachi *et al.*, 2000, 2002). Alcohols and other compounds were determined by HPLC as described previously (Imachi *et al.*, 2000, 2002). Carbohydrates such as glucose and sucrose were measured by HPLC using an SCR-101-H column (Sekiguchi *et al.*, 2001).

Microscopy. Cell morphology of the strains was observed with a phase-contrast microscope (Olympus AX80T). For microscopy, agar-coated slides were prepared and microbial cells were immobilized on the slides (Pfennig & Wägener, 1986). Gram-staining was done by Hucker's method (Doetsch, 1981). Observation of the strains by transmission electron microscopy (TEM) was performed according to Sekiguchi *et al.* (2003).

Determination of DNA base content. DNA was extracted and purified as described previously (Kamagata & Mikami, 1991). The G+C content of all isolates was determined by HPLC (Shimadzu LC-6A) with a UV detector (Shintani *et al.*, 2000).

Determination of quinones and fatty acid methyl ester (FAME) analysis. Quinones were determined as described previously (Zhang *et al.*, 2003). For FAME analysis, cells of strains KIBI-1^T and IMO-1^T were harvested from cultures grown on medium containing sucrose (20 mM) plus yeast extract (0.1 %), whereas FAMES of strain YMTK-2^T were prepared from cultures grown on medium with yeast extract (0.1 %). All of the isolates were cultivated under optimal growth conditions. Fatty acids were converted to methyl esters by using HCl/methanol and analysed by GC with a mass spectrometer (Hitachi M7200A FC/3DQMS system) (Hanada *et al.*, 2002).

Sequencing and phylogenetic analyses. 16S rRNA gene sequences of all three strains were reported previously (Yamada *et al.*, 2005). The sequence data were aligned in an ARB dataset using the ARB program package, and the aligned data were corrected manually by using the editing tool in the package (Ludwig *et al.*, 2004). Phylogenetic trees based on the 16S rRNA gene dataset and various outgroups were constructed by three different algorithms, the neighbour-joining method (Saitou & Nei, 1987) within the ARB program package (Ludwig *et al.*, 2004), the maximum-parsimony method within the PAUP* 4.0 package (Swofford, 2002) and maximum-likelihood method within the TREEFINDER program package (Jobb, 2005). Bootstrap resampling analyses (Felsenstein, 1985) for 1000 replicates were performed for the neighbour-joining, maximum-likelihood and maximum-parsimony analyses to estimate the confidence of tree topologies. In addition, posterior probabilities of branching points were estimated by Bayesian inference using MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003) as mentioned previously (Zhang *et al.*, 2003).

RESULTS AND DISCUSSION

Morphology

Three filamentous strains (strains IMO-1^T, KIBI-1^T and YMTK-2^T) were isolated from UASB sludge granules treating high-strength organic wastewaters (Yamada *et al.*, 2005). In all cultures, small, colourless, lens-shaped colonies,

0.1–0.2 mm in diameter, were formed on anaerobic solid medium supplemented with sucrose (20 mM) and yeast extract (0.1 %) after 2–4 weeks of incubation (Yamada *et al.*, 2005).

All three strains formed flexible filaments of indefinite length (generally longer than 100 µm). The cell widths for strains IMO-1^T and KIBI-1^T were respectively 0.3–0.4 µm and 0.4–0.5 µm. Cells of strain YMTK-2^T were found to be much thinner (0.15–0.2 µm) than those of the other strains (Figs 1 and 2). The strains stained Gram-negative. TEM also indicated a Gram-negative-type cell-wall structure, showing

Fig. 1. Phase-contrast photomicrographs of strains IMO-1^T (a), KIBI-1^T (b) and YMTK-2^T (c) grown under their optimal conditions. Bars, 10 µm (a, b) and 20 µm (c).

Fig. 2. Transmission electron micrographs of ultrathin sections of cells of strains IMO-1^T (a), KIBI-1^T (b) and YMTK-2^T (c). Bars, 1 µm. The inset in (c) (bar, 0.2 µm) shows a cross section of filamentous cells of strain YMTK-2^T.

multicellular forms in filaments (Fig. 2). The electron micrographs also suggested that the strains did not possess a clearly visible sheath-like structure (Fig. 2). Individual cells of all three strains were found to be longer than 2 µm (Fig. 2). Based on observations of cell movement under a microscope and of colony morphology of all isolates formed on solid media, the strains were found to have no gliding motility. Spore formation was not observed under any culture conditions.

Physiological properties

Strains IMO-1^T, KIBI-1^T and YMTK-2^T were strictly anaerobic, filamentous micro-organisms. No growth of the strains was observed in PE medium under aerobic conditions (20 % oxygen, v/v, in the gas phase) and phototrophic metabolism was not observed in PE medium under anaerobic conditions. Yeast extract was required for growth of all three strains.

Utilization of various substrates (added at final concentrations of 20 mM unless specified) was tested in the presence of yeast extract (0.01 %). All three strains utilized glucose, fructose, ribose, xylose, sucrose, raffinose and tryptone (0.1 %). Strain IMO-1^T also utilized mannose, galactose, arabinose and pyruvate, strain KIBI-1^T utilized pyruvate and strain YMTK-2^T utilized mannose, xylan, pectin and betaine. Casamino acids (0.1 %) supported weak growth and acid formation for all three strains, as did the following compounds: starch (5 g l⁻¹), xylan, pectin and peptone (0.1 %) for strain IMO-1^T, xylan, pectin, peptone (0.1 %), betaine, mannose and galactose for strain KIBI-1^T and starch (5 g l⁻¹), peptone (0.1 %), galactose, arabinose and pyruvate for strain YMTK-2^T. None of the strains utilized crotonate, H₂/CO₂ (1 atm) plus acetate (10 mM), lactate, glycerol, fumarate, acetate, propionate, butyrate, malate, succinate, ethanol, methanol, 1-propanol, ethylene glycol, hydroquinone (1 mM), phenol (1 mM), benzoate (5 mM) or formate plus acetate (10 mM). Strain IMO-1^T did not utilize betaine.

Major fermentative products in medium containing glucose (20 mM) and yeast extract (0.01 %) were acetate, lactate and hydrogen for strain IMO-1^T, acetate, formate and hydrogen with a small amount of lactate for strain KIBI-1^T and acetate, lactate, pyruvate and hydrogen with small amounts of succinate and formate for strain YMTK-2^T. The three strains could not utilize any of the following compounds as electron acceptors within 4 weeks of incubation (glucose and yeast extract medium): 20 mM sulphate, 1 mM sulphite, 20 mM thiosulphate, 20 mM elemental sulphur, 20 mM nitrate, 20 mM fumarate and 5 mM Fe(III)-NTA.

The optimal temperatures for growth were 50 °C (range 42–55 °C) for strain IMO-1^T and around 37 °C (range 25–50 °C) for strains KIBI-1^T and YMTK-2^T. The optimal pH for growth was around pH 7.0 for all three strains; the strains grew at pH 6.0–7.5 (strain IMO-1^T) or pH 6.0–7.2 (strains KIBI-1^T and YMTK-2^T).

Growth of strain IMO-1^T was found to stagnate after an amount of hydrogen (approx. 10 kPa) accumulated in medium supplemented with glucose and yeast extract. No significant improvement of growth of strain KIBI-1^T or YMTK-2^T was observed in co-cultivation with the hydrogenotrophic methanogen *Methanospirillum hungatei* DSM 864^T (Yamada *et al.*, 2005). NaCl was slightly inhibitory for growth of all three strains at 2.5 g l⁻¹; growth of strains

IMO-1^T and YMTK-2^T was inhibited completely by 15 g NaCl l⁻¹, whereas growth of strain KIBI-1^T was inhibited only slightly by 30 g NaCl l⁻¹.

Chemotaxonomic analyses

The DNA G + C content of strain IMO-1^T was 53.3 mol%, and those of strains KIBI-1^T and YMTK-2^T were respectively 59.5 and 48.2 mol%. FAME analysis showed that cells of strain IMO-1^T contained anteiso-C_{17:0} (21%), iso-C_{15:0} (19%), C_{16:0} (16%) and anteiso-C_{15:0} (12%) as the major fatty acids, with C_{18:0} (6%), iso-C_{17:0} (5%), iso-C_{13:0} (5%), C_{14:0} (5%), anteiso-C_{13:0} (3%), C_{17:0} (3%), iso-C_{16:0} (2%), C_{15:0} (2%) and C_{12:0} (1%) as minor components. Cells of strain KIBI-1^T contained branched C_{14:0} (30%), iso-C_{15:0} (22%), C_{16:0} (14%) and branched C_{17:0} (13%) as the major fatty acids, with C_{16:1} (Δ 9) *cis* (8%), C_{12:0} (4%), branched C_{13:0} (4%), branched C_{14:0} (3%), C_{18:0} (1%) and branched C_{15:0} (1%) as minor fatty acids. Cells of strain YMTK-2^T contained branched C_{17:0} (41%), C_{16:0} (25%), C_{14:0} (11%) and C_{17:0} (9%) as the major fatty acids, with branched C_{15:0} (5%), C_{12:0} (2%), branched C_{13:0} (2%), C_{16:1} (Δ 9) *cis* (2%), anteiso-C_{15:0} (1%), branched C_{16:0} (1%) and branched-C_{19:0} (1%) as minor components. No quinones were detected from cells of any of the strains cultivated under anaerobic conditions.

Phylogenetic analysis

In our previous study, the most closely related, previously characterized micro-organism to all the strains was *A. thermophila* (16S rRNA gene sequence similarity of strains IMO-1^T, KIBI-1^T and YMTK-2^T with *A. thermophila* UNI-1^T of respectively 93, 88 and 88%; Yamada *et al.*, 2005). In addition, the 16S rRNA gene sequence similarity among the three strains was found previously to be lower than 89% (Yamada *et al.*, 2005), indicating at least species-level novelty of each strain.

To examine the monophyly of the tentatively named, subphylum-level group 'Anaerolineae' (Hugenholtz & Stackebrandt, 2004), we performed more detailed phylogenetic analyses including 16S rRNA gene sequences of members of the class *Thermomicrobia* (containing the genera *Thermomicrobium*, *Sphaerobacter* and *Thermobaculum*), with sequences of a variety of bacteria representing multiple phyla as outgroups (Fig. 3). In addition to posterior probabilities of branching points based on Bayesian inference, bootstrap values on the basis of maximum-parsimony and maximum-likelihood indicated that the node support of the group 'Anaerolineae' was lower than 90%, although the node support estimated with neighbour-joining analysis was 94% (Fig. 3). These facts suggested that the group 'Anaerolineae' might not be a monophyletic taxon as suggested by Hugenholtz & Stackebrandt (2004). In particular, the *Caldilineae* cluster often did not form a clade with other members of the group 'Anaerolineae' within four phylogenetic algorithms. In addition, the group 'Anaerolineae' clearly exhibited monophyletic taxon status in the same

estimations when members affiliated with the *Caldilineae* cluster were excluded (data not shown). Thus, it was concluded that the group 'Anaerolineae' should be subdivided phylogenetically into two class-level taxa (Fig. 3).

Taxonomic remarks on the strains

Physiological, chemotaxonomic and genetic analyses of the three strains indicated clear differences among the strains and other known organisms. *A. thermophila*, which is the most closely related species to the strains characterized in this study, is a strictly anaerobic, thermophilic and multicellular filamentous organism and can utilize various carbohydrates fermentatively such as glucose and fructose (Sekiguchi *et al.*, 2001, 2003). The phenotypic features of strain IMO-1^T were very similar to those of *A. thermophila*. However, comparative analysis of the 16S rRNA gene sequence of strain IMO-1^T with that of *A. thermophila* suggested that strain IMO-1^T should be differentiated from *A. thermophila* at the species level (Stackebrandt & Goebel, 1994). In addition, strain IMO-1^T can be differentiated from *A. thermophila* in several phenotypic respects (Table 1): (i) the presence of branched fatty acids as major cellular fatty acids; (ii) slightly thicker filaments; (iii) clearly faster growth. These genotypic and phenotypic differences justify the creation of a novel species of the genus *Anaerolinea* to accommodate strain IMO-1^T, for which we propose the name *Anaerolinea thermolimosa* sp. nov.

One of the striking differences that distinguishes strains KIBI-1^T and YMTK-2^T from species of the genus *Anaerolinea* is the temperature range for growth; the genus *Anaerolinea* was described as containing thermophilic anaerobes, whereas strains KIBI-1^T and YMTK-2^T grew under mesophilic conditions (Table 1). In addition, the mesophilic strains KIBI-1^T and YMTK-2^T also exhibited several distinct phenotypic features: (i) cells of strain KIBI-1^T were clearly thicker than those of strain YMTK-2^T; (ii) strain KIBI-1^T had a higher G + C content than strain YMTK-2^T; (iii) substrate utilization for the two strains was slightly different. Phylogenetic analyses with 16S rRNA gene sequences of strains KIBI-1^T and YMTK-2^T indicated that the two organisms formed a cluster independent from *A. thermophila* and strain IMO-1^T, and the sequence similarity of the strains with strains of the genus *Anaerolinea* was lower than 90%. Furthermore, the 16S rRNA gene sequence similarity between strain KIBI-1^T and YMTK-2^T (91%) was too low to classify both strains into a single genus. These findings justify the creation of the new genera and species *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov. for strains KIBI-1^T and YMTK-2^T, respectively. Both genera should be affiliated with the same family as the genus *Anaerolinea*.

Subdivision of the group 'Anaerolineae'

The putative class- (subphylum)-level group 'Anaerolineae' now contains four cultured genera, *Anaerolinea*, *Caldilinea*, *Levilinea* and *Leptolinea* (Sekiguchi *et al.*, 2003). Based on

Fig. 3. Evolutionary dendrogram of strains IMO-1^T, KIBI-1^T and YMTK-2^T in the bacterial phylum *Chloroflexi*, inferred on the basis of 16S rRNA gene sequences. The sequences were aligned and the phylogenetic tree was constructed by the neighbour-joining method. 16S rRNA gene sequences of *Escherichia coli* ATCC 11775^T (X80725), *Bacillus subtilis* 168 (D26185) and *Corynebacterium diphtheriae* NCTC 11397^T (X84248) were used as outgroups (not shown). Filled circles indicate bootstrap probabilities (based on neighbour-joining analyses with 1000 replicates) above 95 %, and open circles show bootstrap probabilities above 85 %. Nodes without symbols were not highly resolved (< 85 %) as specific groups in the analysis. Probability support for the node of 'Anaerolineae' was evaluated with neighbour-joining (NJ), maximum-parsimony (MP), maximum-likelihood (ML) and Bayesian (Bayes) analyses, and respective bootstrap/posterior probability values are given as percentages. Bar, 5 nucleotide substitutions per 100 nucleotides. Class-level lineages in the phylum *Chloroflexi* are indicated to the right, as suggested by Hugenholtz & Stackebrandt (2004). Subdivision of the 'Anaerolineae' (broken bracket in the figure) into classes *Anaerolineae* and *Caldilineae* proposed in this study is indicated. Numbers in brackets following these names refer to the class-level lineage numbering ([1]–[4]) of Hugenholtz *et al.* (1998) and include numbers for classes *Thermomicrobia* ([5]) and *Caldilineae* ([6]).

the phylogenetic placements of these organisms, we propose to subdivide 'Anaerolineae' into two new classes, *Anaerolineae* classis nov. and *Caldilineae* classis nov. (Fig. 3). The notable phenotypic property that clearly distinguishes members of *Anaerolineae* (the genera *Anaerolinea*, *Levilinea* and *Leptolinea*) from *Caldilineae* (the genus *Caldilinea*) is oxygen respiration: members of the class *Anaerolineae* are obligately anaerobic, while members of the class *Caldilineae* are facultatively aerobic. More strains belonging to the two classes (as well as comparative genomics of the two classes) might lend further support to the distinctiveness of these classes.

We also propose subordinate taxa from order (*Anaerolineales* ord. nov. and *Caldilineales* ord. nov.) to family (*Anaerolineaceae* fam. nov. and *Caldilineaceae* fam. nov.) for the classes *Anaerolineae* and *Caldilineae*.

Description of *Anaerolineae* classis nov.

Anaerolineae (An.ae.ro.li.ne'ae. N.L. fem. pl. n. *Anaerolineales* type order of the class; -ae ending to denote a class; N.L. fem. pl. n. *Anaerolineae* the *Anaerolineales* class).

Table 1. Phenotypic characteristics of species affiliated with the classes *Anaerolineae* and *Caldilineae*

Only differences found among the species shown are listed. Data for *Anaerolinea thermolimos*, *Levilinea saccharolytica* and *Leptolinea tardivitalis* were obtained in our previous study (Yamada *et al.*, 2005) and this study. Data for *Anaerolinea thermophila* and *Caldilinea aerophila* were taken from Sekiguchi *et al.* (2001, 2003). –, Negative; $\pm$, variable; +, positive; ND, not determined. All species are multicellular filamentous organisms that are capable of fermentative metabolism, require yeast extract for growth and utilize glucose, sucrose and raffinose and not ethanol for growth in the presence of yeast extract.

Characteristic	Class <i>Anaerolineae</i>				Class <i>Caldilineae</i>
	<i>Levilinea saccharolytica</i> KIBI-1 ^T	<i>Leptolinea tardivitalis</i> YMTK-2 ^T	<i>Anaerolinea thermolimos</i> IMO-1 ^T	<i>Anaerolinea thermophila</i>	<i>Caldilinea aerophila</i>
Cell diameter (μ m)	0.4–0.5	0.15–0.2	0.3–0.4	0.2–0.3	0.7–0.8
Temperature for growth (°C):					
Range	25–50	25–50	42–55	50–60	25–50
Optimum	37–40	37	50	55	55
pH for growth:					
Range	6.0–7.2	6.0–7.2	6.0–7.5	6.0–8.0	7.0–9.0
Optimum	7.0	7.0	7.0	7.0	7.5–8.0
Minimum doubling time (h)	56	50	48	72	5
O ₂ respiration	–	–	–	–	+
Major cellular fatty acids	C _{14:0} , iso-C _{15:0} , C _{16:0} , branched C _{17:0}	Branched C _{17:0} , C _{16:0} , C _{14:0} , C _{17:0}	Anteiso-C _{17:0} , iso-C _{15:0} , C _{16:0} , anteiso-C _{15:0}	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}
Major quinone	None	None	None	None	MK-10
DNA G+C content (mol%)	59.5	48.2	53.3	54.5	59.0
Utilization in the presence of yeast extract:					
Casamino acids	$\pm$	$\pm$	$\pm$	$\pm$	–
Tryptone	+	+	+	$\pm$	+
Pyruvate	+	$\pm$	+	$\pm$	+
Lactate	–	–	–	–	+
Fumarate	–	–	–	–	+
Acetate	–	–	–	–	+
Succinate	–	–	–	–	+
Mannose	$\pm$	+	+	+	ND
Galactose	$\pm$	$\pm$	+	+	+
Fructose	+	+	+	+	–
Arabinose	–	$\pm$	+	$\pm$	–
Xylose	+	+	+	$\pm$	–
Ribose	+	+	+	$\pm$	–
Pectin	$\pm$	+	$\pm$	$\pm$	ND
Starch	–	$\pm$	$\pm$	+	+
Isolation source	Mesophilic anaerobic sludge	Mesophilic anaerobic sludge	Thermophilic anaerobic sludge	Thermophilic anaerobic sludge	Hot spring

The class *Anaerolineae* is defined on the basis of a phylogenetic tree by comparative 16S rRNA gene sequence analysis from four representatives and a wide variety of environmental clones. Gram-negative. Cells are not coloured. Negative for gliding motility. Multicellular filamentous organisms. Spores are not formed. No photosynthetic growth is observed. Grow under strictly anaerobic conditions. Chemo-organotrophs. No quinone is detected. The type order is *Anaerolineales*.

Description of *Anaerolineales* ord. nov.

Anaerolineales (An.ae.ro.li.ne.al'es. N.L. fem. n. *Anaerolinea* type genus of the order; -ales ending to denote an order; N.L. fem. pl. n. *Anaerolineales* order of the genus *Anaerolinea*).

The description is the same as for the class *Anaerolineae*. The type genus is *Anaerolinea*.

Description of *Anaerolineaceae* fam. nov.

Anaerolineaceae (An.ae.ro.li.ne.a'ce.ae. N.L. fem. n. *Anaerolinea* type genus of the family; -aceae ending to denote a family; N.L. fem. adj. *Anaerolineaceae* family of the genus *Anaerolinea*).

The description is the same for the class *Anaerolineae*. The type genus is *Anaerolinea*.

Emended description of the genus *Anaerolinea* Sekiguchi et al. 2003

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 organisms not living in air).

Gram-negative. Cells are non-motile and filamentous. Spores are not formed. Thermophilic. Grow under strictly anaerobic conditions. Neither photosynthetic nor aerobic growth is observed. The cellular fatty acids consist mainly of straight-chain saturated fatty acids (e.g. C_{16:0} and C_{18:0}) and branched-chain fatty acids (e.g. anteiso-C_{17:0}). The G+C content of genomic DNA is 53.3–54.5 mol%. Phylogenetic position is in the family *Anaerolineaceae*, order *Anaerolineales*, class *Anaerolineae* of the phylum *Chloroflexi*. The type species is *Anaerolinea thermophila*.

Description of *Anaerolinea thermolimosa* sp. nov.

Anaerolinea thermolimosa (ther.mo.li.mo'sa. Gr. adj. *thermos* hot; L. fem. adj. *limosa* muddy, pertaining to sludge; N.L. fem. adj. *thermolimosa* living in thermophilic sludge).

Has the following properties in addition to those described for the genus. The main fatty acids are anteiso-C_{17:0}, iso-C_{15:0}, C_{16:0} and anteiso-C_{15:0}. The G+C content of genomic DNA is 53.3 mol%. Multicellular filaments are longer than 100 µm in length and 0.3–0.4 µm in width. Growth occurs between 42 and 55 °C with optimum growth at 50 °C. Growth is observed at pH 6.0–7.5 with optimum

growth at pH 7.0. The doubling time for growth is 48 h under optimal growth conditions. Growth is stimulated significantly when the organism is co-cultivated with hydrogenotrophic methanogens. Yeast extract is required for growth. In the presence of 0.01 % yeast extract, growth and substrate utilization are observed with glucose, mannose, galactose, fructose, ribose, xylose, arabinose, sucrose, raffinose, tryptone and pyruvate. Weak growth occurs with the following substrates with medium supplemented with 0.01 % yeast extract: Casamino acids, starch, xylan, pectin and peptone. The following substrates are not utilized in the presence of 0.01 % yeast extract: crotonate, H₂/CO₂ plus acetate, betaine, lactate, glycerol, fumarate, acetate, propionate, butyrate, malate, succinate, ethanol, methanol, 1-propanol, ethylene glycol, hydroquinone, phenol, benzoate and formate plus acetate. Sulphate, sulphite, thiosulphate, elemental sulphur, nitrate, fumarate and Fe(III)-NTA are not utilized as electron acceptors.

The type strain is strain IMO-1^T (=JCM 12577^T=DSM 16554^T), isolated from sludge from a thermophilic UASB reactor in which wastewater from manufacture of a Japanese distilled alcohol (shochu) is treated.

Description of *Levilinea* gen. nov.

Levilinea (Le.vi.li'ne.a. L. adj. *levis* smooth; L. fem. n. *linea* line; N.L. fem. n. *Levilinea* smooth, line-shaped organism).

Gram-negative. Cells are non-motile. Multicellular and filamentous morphology with indefinite length. Mesophiles. Spores are not formed. Grow under strictly anaerobic conditions. No photosynthetic growth is observed. The main fatty acids are branched C_{14:0}, iso-C_{15:0}, C_{16:0} and branched C_{17:0}. The G+C content of genomic DNA is 59.5 mol%. The phylogenetic position is in the family *Anaerolineaceae*. The type species is *Levilinea saccharolytica*.

Description of *Levilinea saccharolytica* sp. nov.

Levilinea saccharolytica (sac.cha.ro.ly'ti.ca. Gr. n. *saccharon* sugar; N.L. fem. adj. *lytica* from Gr. adj. *lutikos* able to loosen; N.L. fem. adj. *saccharolytica* saccharolytic, using various sugars).

Multicellular filaments are longer than 100 µm and 0.4–0.5 µm wide. Growth occurs between 25 and 50 °C with optimum growth at 37 °C. Growth is observed at pH 6.0–7.2 with optimum growth at pH 7.0. Doubling time for growth is 56 h under optimal growth conditions. Yeast extract is required for growth. In the presence of 0.01 % yeast extract, growth is observed with glucose, fructose, xylose, ribose, sucrose, raffinose, tryptone, pyruvate and pectin. Weak growth occurs with the following substrates with medium supplemented with 0.01 % yeast extract: Casamino acids, peptone, betaine, mannose, galactose and xylan. The following substrates are not utilized in the presence of yeast extract: crotonate, H₂/CO₂ plus acetate, lactate, glycerol, fumarate, acetate, propionate, butyrate, malate, succinate, ethanol, methanol, 1-propanol, arabinose, starch,

ethylene glycol, hydroquinone, phenol, benzoate and formate plus acetate. Sulphate, sulphite, thiosulphate, elemental sulphur, nitrate, fumarate and Fe(III)-NTA are not utilized as electron acceptors.

The type strain is KIBI-1^T (=JCM 12578^T=DSM 16555^T), isolated from sludge in a mesophilic UASB reactor in which sugar-manufacturing wastewater is treated.

Description of *Leptolinea* gen. nov.

Leptolinea (Lep.to.li'ne.a. Gr. adj. *leptus* fine; L. fem. n. *linea* line; N.L. fem. n. *Leptolinea* fine, line-shaped organism).

Gram-negative. Cells are non-motile, forming very fine multicellular filaments (0.2 µm wide or less). Mesophiles. Spores are not formed. Grow under strictly anaerobic conditions. No photosynthetic growth is observed. The main fatty acids are branched C_{17:0}, C_{16:0}, C_{14:0} and C_{17:0}. The G+C content of genomic DNA is 48.2 mol%. The phylogenetic position is in the family *Anaerolineaceae*. The type species is *Leptolinea tardivitalis*.

Description of *Leptolinea tardivitalis* sp. nov.

Leptolinea tardivitalis (tar.di.vi.ta'lis. L. adj. *tardus* slow; L. fem. adj. *vitalis* vital, alive; N.L. fem. adj. *tardivitalis* having a slow lifestyle).

Multicellular filaments are longer than 100 µm and thinner than 0.2 µm. Growth occurs between 25 and 50 °C with optimum growth at 37 °C. Growth is observed at pH 6.0–7.2 with optimum growth at pH 7.0. Doubling time for growth is 50 h under optimal growth conditions. In the presence of 0.01 % yeast extract, growth is observed with glucose, mannose, fructose, xylose, ribose, sucrose, raffinose, xylan, pectin, tryptone and betaine. Weak growth occurs with the following substrates with medium supplemented with 0.01 % yeast extract: Casamino acids, peptone, galactose, arabinose, starch and pyruvate. The following substrate are not utilized in the presence of 0.01 % yeast extract: crotonate, H₂/CO₂ plus acetate, lactate, glycerol, fumarate, acetate, propionate, butyrate, malate, succinate, ethanol, methanol, 1-propanol, ethylene glycol, hydroquinone, phenol, benzoate and formate plus acetate. Sulphate, sulphite, thiosulphate, elemental sulphur, nitrate, fumarate and Fe(III)-NTA are not utilized as electron acceptors.

The type strain is strain YMTK-2^T (=JCM 12579^T=DSM 16556^T), isolated from sludge in a mesophilic UASB reactor in which an artificial wastewater composed mainly of peptone, sucrose, propionate and acetate is treated.

Description of *Caldilineae* classis nov.

Caldilineae (Cal.di.li'ne.ae. N.L. fem. pl. n. *Caldilineales* type order of the class; -ae ending to denote a class; N.L. fem. pl. n. *Caldilineae* the *Caldilineales* class).

The description is the same as that for the genus *Caldilinea* given by Sekiguchi *et al.* (2003). The type order is *Caldilineales*.

Description of *Caldilineales* ord. nov.

Caldilineae (Cal.di.li.ne.al'es. N.L. fem. n. *Caldilinea* type genus of the order; -ales ending to denote a order; N.L. fem. pl. n. *Caldilineales* order of the genus *Caldilinea*).

The description is the same as that for the genus *Caldilinea* given by Sekiguchi *et al.* (2003). The type genus is *Caldilinea*.

Description of *Caldilineaceae* fam. nov.

Caldilineaceae (Cal.di.li.ne.a'ce.ae. N.L. fem. n. *Caldilinea* type genus of the family; -aceae ending to denote a family; N.L. fem. n. *Caldilineaceae* family of the genus *Caldilinea*).

The description is the same as that for the genus *Caldilinea* given by Sekiguchi *et al.* (2003). The type genus is *Caldilinea*.

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