Description of *Christensenella minuta* gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order *Clostridiales*, and proposal of *Christensenellaceae* fam. nov.

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A novel, strictly anaerobic, non-motile, non-spore-forming, Gram-negative, short, straight rod with tapered ends, designated YIT 12065^T, was isolated from human faeces. Strain YIT 12065^T was saccharolytic and negative for catalase, oxidase and urease, hydrolysis of aesculin and gelatin, nitrate reduction and indole production. The end products of glucose fermentation were acetic acid and a small amount of butyric acid. The DNA G + C content was 51.3 mol%. The predominant fatty acids were iso-C_{15:0}, C_{16:0} and C_{14:0}. Respiratory quinones were not detected. The cell wall contained glutamic acid, serine, alanine and LL-diaminopimelic acid. The whole-cell sugars were ribose, rhamnose, galactose and glucose. Phylogenetic analyses based on 16S rRNA gene sequences using three treeing algorithms revealed that the strain formed a novel family-level lineage within the phylum *Firmicutes*, class *Clostridia*, order *Clostridiales*. *Caldicoprobacter oshimai* JW/ HY-331^T was shown to be the closest named relative on the basis of 16S rRNA gene sequence similarity (86.9%), followed by *Tindallia californiensis* DSM 14871^T (86.3%) and *Clostridium ganghwense* JCM 13193^T (86.1%). Similar 16S rRNA gene sequences (98.6–96.7%) were found amongst faecal uncultured clones of human and dugong (*Dugong dugon*). They clustered with strain YIT 12065^T in a distinct and deep evolutionary lineage of descent in the order *Clostridiales*. The distinct phylogenetic position supports the proposal of *Christensenella* gen. nov., with the type species *Christensenella minuta* sp. nov. (type strain YIT 12065^T = DSM 22607^T = JCM 16072^T). A new family *Christensenellaceae* fam. nov. is also proposed.

The use of culture-independent molecular methods to analyse the gastrointestinal (GI) microbiota has allowed a more complete and accurate assessment of the biodiversity of this ecosystem (Eckburg *et al.*, 2005; Ley *et al.* 2008; Turnbaugh *et al.*, 2009; Tap *et al.*, 2009; Qin *et al.*, 2010). Such molecular methods using small-subunit rRNA gene sequence-based technologies are considered fruitful for finding potential links between microbes and a certain health and/or disease status. However, as pointed out by Zoetendal *et al.* (2008), it has to be realized that the results obtained with such approaches cannot necessarily be extrapolated beyond the description of the microbial diversity, since potential functions of these microbes are not always extracted from small-subunit rRNA gene sequence data. At the moment, although links have been

elucidated between the intestinal microbiota and GI tract disorders, causal effects have been determined only for cultured bacterial species such as *Helicobacter pylori* and *Clostridium difficile*. This observation, and the fact that the recent study of Eckburg *et al.* (2005) reported that the proportion of reported cultivable bacteria was only 20% for the human intestinal microbiota, indicated that cultivation of novel GI-tract inhabitants is indispensable for future studies of links between microbes and GI tract diseases such as inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS).

To understand better the physiological characteristics and function of the majority of the human GI microbiota, we have performed several intensive cultivation trials aimed at isolating so-called ‘unculturable’ or ‘as-yet-uncultured’ bacteria from the human GI tract (Sakon *et al.*, 2008; Morotomi *et al.*, 2008, 2009, 2010, 2011; Nagai *et al.*, 2009, 2010a, b; Watanabe *et al.*, 2010). Among these bacteria, 15 strains of 15 novel species including three new genera were adopted as reference genomes by the Human Microbiome Project (http://www.hmpdacc.org/reference_genomes/reference_genomes).

Abbreviations: A₂pm, diaminopimelic acid; GI, gastrointestinal; ML, maximum-likelihood; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIT 12065^T is AB490809.

A supplementary table is available with the online version of this paper.

php) and the assembled and annotated genomic sequences of these bacteria will be submitted to GenBank. In this article, we report the characterization and classification of strain YIT 12065^T, proposing its affiliation to a new family, genus and species.

Faecal samples were collected from two healthy Japanese males (subjects H and K, aged 57 and 27 years, respectively) and transferred immediately to anaerobic conditions. Each sample was weighed and diluted with pre-reduced 0.1 M PBS (pH 7) in an anaerobic glovebox (Coy Laboratory Products), which contained 88 % nitrogen, 7 % hydrogen and 5 % carbon dioxide. Each dilution was then spread on modified Gifu anaerobic medium (GAM; Nissui Pharmaceutical) containing 1.5 % (w/v) agar that was supplemented with bile (2, 4, 6 and 8 % Bacto oxgall; Difco), NaCl (1, 3, 6 and 10 %, w/v) or antibiotics (one of 12 compounds, at three different concentrations) in an attempt to isolate subdominant groups of the intestinal microbiota. The composition of the modified GAM agar was described previously (Sakon *et al.*, 2008). The inoculated plates were incubated at 37 °C for 3 days in an anaerobic glovebox. Subsequently, colonies showing different morphologies were selected and subcultured on GAM agar to obtain pure cultures. Strain YIT 12065^T was isolated from a GAM agar plate (pH 6.8) supplemented with 8 % Bacto oxgall (equivalent to 80 % bile) inoculated with a 10⁻⁶ serially diluted faecal sample from subject H.

The end products of strain YIT 12065^T grown in pre-reduced peptone-yeast extract (PY) medium (Holdeman *et al.*, 1977) supplemented with 1 % glucose (PYG), lactate or succinate were analysed by HPLC as described previously (Chonan *et al.*, 1995). Carbohydrate fermentation tests and other biochemical tests, such as indole production, urease activity and hydrolysis of gelatin and aesculin, were performed by the methods described by Holdeman *et al.* (1977) and by using the API 20A anaerobic test kit (bioMérieux). Additional enzyme activities were determined in duplicate by using the API Rapid ID32A and API ZYM systems (bioMérieux) according to the manufacturer's instructions. Oxidase activity was determined with oxidase test strips (Eiken Chemical). Sensitivity to bile and NaCl was determined by comparing growth on modified GAM agar with and without 2 % Bacto oxgall (Difco) or 1, 2, 3, 5, 10 or 20 % NaCl after 5 days of incubation at 37 °C. Growth on modified GAM agar at different temperatures was assessed over time for 14 days at 15, 20, 25, 30, 34, 37, 40, 43 and 45 °C under anaerobic conditions by use of an AnaeroPak system (Mitsubishi Gas Chemical). The effects of pH on growth were determined over the range pH 4.0–10.0 at intervals of 0.5 pH units using HCl and NaOH to adjust the pH after cultures had been incubated at 37 °C for 3 days, under anaerobic conditions with nitrogen to limit undesirable changes in pH during incubation.

Cellular fatty acids, isoprenoid quinones, cell-wall amino acids and whole-cell sugars were prepared from cells grown on PYG agar at 37 °C for 3 days. Cellular fatty acid methyl esters were obtained from lyophilized cells by saponification,

methylation and extraction using the method of Miller (1982) with minor modifications (Kuykendall *et al.*, 1988). Fatty acid methyl esters were identified by using the MIDI system with MOORE5 from the MIS Standard Libraries. Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analysed by using an HPLC atmospheric pressure chemical ionization (APCI)-MS/MS system (API 3200; Applied Biosystems) with an L-column ODS (2.1 × 150 mm; Chemicals Evaluation and Research Institute) and an HPLC-APCI-MS system (Micro-mass ZQ equipped with 2996 photodiode array detector; Waters) with a Cadenza CD-C18 column (3.0 × 150 mm; Imtakt), following the modified method of Katsuta *et al.* (2005). Isolation of the cell-wall fraction and preparation of the cell-wall hydrolysate were carried out by using the method of Schleifer (1985). The amino acid composition of the cell-wall hydrolysate was determined by using an Aquity ultraperformance liquid chromatography system (Waters). Whole-cell sugars were determined by HPLC [Shimadzu LC-20A system; Symmetry 300 TM C18 column (4.6 × 250 mm)] according to the method of Honda *et al.* (1989). The DNA G+C content was determined by hydrolysing the DNA enzymically and quantifying nucleosides by HPLC according to the method of Ezaki *et al.* (1990).

Caldicoprobacter oshimai DSM 21659^T and *Tindallia californiensis* DSM 14871^T, purchased from the DSMZ (Braunschweig, Germany), and *Clostridium ganghwense* JCM 13193^T, purchased from the JCM (Wako, Japan), were used as reference strains.

Closely related sequences were retrieved from GenBank/EMBL/DBJ by using the FASTA program (Lipman & Pearson, 1985). Sequences were aligned and used to produce an unrooted phylogenetic tree according to the neighbour-joining method (Saitou & Nei, 1987) using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). The stability of groupings was estimated by bootstrap analysis (1000 replications). Trees were visualized by using the TreeView program (version 1.6.6) (Page, 1996). Maximum-parsimony (MP) and maximum-likelihood (ML) methods were used to confirm the phylogenetic placement of the aligned sequences. MP analysis was performed using the software package MEGA4 (Tamura *et al.*, 2007). The ML tree was constructed via the PHYML program (Guindon & Gascuel, 2003) using Kimura's two-parameter nucleotide substitution model (Kimura, 1980). The input file was prepared via the SEQBOOT program in the PHYLIP software package (Felsenstein, 2004).

Cells of strain YIT 12065^T were Gram-reaction-negative, non-motile, short, straight rods with tapered ends (0.4 × 0.8–1.9 µm). Electron microscopy of ultrathin sections revealed a Gram-negative cell-wall structure (Fig. 1). Growth was observed under strict anaerobic conditions but not aerobic or microaerobic conditions created by use of the CampyPak Plus system (with 5–15 % O₂ and 5–12 % CO₂; BBL). After 4 days of anaerobic incubation at 37 °C on modified GAM agar, Wilkins–Chalgren anaerobe agar

Fig. 1. Morphology of strain YIT 12065^T. For transmission electron microscopy, cells were fixed in 2.5 % (w/v) glutaraldehyde, post-fixed in 1 % (w/v) OsO₄, dehydrated in a graded ethanol series and embedded in Epon 812 resin. Ultrathin sections were cut in an ultramicrotome (Reichert-Nissei), loaded onto nickel grids and stained with 2 % (w/v) uranyl acetate and Reynolds' lead citrate. Samples were examined using a JEOL JEM-1200EX operating at 80 kV accelerating voltage. (a) Phase-contrast micrograph. (b) TEM image of ultrathin-sectioned cells. (c) Enlargement of (b), showing the cell-wall structure. Bars, 10 µm (a), 250 nm (b) and 100 nm (c).

[Wilkins–Chalgren anaerobe broth (Oxoid) with 1.5 % agar] or Schaedler anaerobe agar [Schaedler anaerobe broth (Oxoid) with 1.5 % agar], colonies were punctiform, 0.1 mm in diameter, circular and beige. Strain YIT 12065^T was saccharolytic. Tests for aesculin hydrolysis, gelatin hydrolysis, indole production, nitrate reduction and

catalase, oxidase and urease activities were negative. Cells were resistant to 20 % bile. Under anaerobic conditions, growth was most rapid at 37–40 °C, slightly weak at 34 °C, weak at 25–30 °C and scant at 43 °C. No growth was observed below 20 °C or at 45 °C. The pH range for growth was pH 6.0–9.0, with optimal growth at pH 7.5. Numbers of colonies grown on medium containing 1, 2 and 3 % NaCl were 80, 27 and 6 %, respectively, compared with control medium without NaCl. No growth was observed on medium containing ≥5 % NaCl. Growth of strain YIT 12065^T in liquid media was very weak. Small amounts of acetic (3.6 mM) and butyric (0.3 mM) acids were produced as end products of glucose fermentation in PYG broth. Addition of lactate or succinate did not enhance growth or the production of short-chain fatty acids. In the API Rapid ID 32A and API ZYM test systems, strain YIT 12065^T was positive for α-arabinosidase, β-galactosidase, β-glucosidase, glutamic acid decarboxylase and naphthol-AS-BI-phosphohydrolase. Other biochemical characteristics obtained by using the API systems (API Rapid ID32A, API ZYM and API 20A) are included in the species description.

Cellular fatty acid profiles of strain YIT 12065^T and the type strains of phylogenetically related species are shown in Supplementary Table S1, available in IJSEM Online. Respiratory quinones were not detected. The cell wall contained glutamic acid, serine, alanine and LL-diaminopimelic acid (LL-A₂pm). Although LL-A₂pm is found mainly in species of Gram-positive bacteria, some Gram-negative bacteria such as *Syntrophomonas* species (Zhang *et al.*, 2004, 2005) and *Desulfosporosinus* species (Stackebrandt *et al.*, 2003; Ramamoorthy *et al.*, 2006; Vatsurina *et al.*, 2008) have been reported to possess LL-A₂pm as a diagnostic peptidoglycan diamino acid. All of these species are members of the order *Clostridiales*. The whole-cell sugars were ribose, rhamnose, galactose and glucose. The DNA G + C content of strain YIT 12065^T was 51.3 mol%.

A 1497 bp region of the 16S rRNA gene of strain YIT 12065^T was sequenced. Database searches revealed that strain YIT 12065^T was most closely related (≥86 % 16S rRNA gene sequence similarity) to *Caldicoprobacter oshimai* JW/HY-331^T (86.9 %), *Tindallia californiensis* DSM 14871^T (86.3 %) and *Clostridium ganghwense* JCM 13193^T (86.1 %). Both *Tindallia californiensis* and *Clostridium ganghwense* belong to the family *Clostridiaceae*, whereas *Caldicoprobacter oshimai* is proposed to belong to a novel family *Caldicoprobacteraceae*, consisting of a single genus and species, within the order *Clostridiales*. Although *Caldicoprobacter oshimai*, *Clostridium ganghwense* and *Tindallia californiensis* are phylogenetically most closely related to strain YIT 12065^T, they are significantly different from strain YIT 12065^T in their physiological characteristics (Table 1). Strain YIT 12065^T showed even lower similarities (79.2–85.0 %) to members of all other type genera of the families within the order *Clostridiales* (Fig. 2).

The 16S rRNA gene sequence of strain YIT 12065^T shared highest sequence similarities with uncultured faecal

Table 1. Major characteristics of strain YIT 12065^T and type strains of related species

Strains: 1, YIT 12065^T; 2, *Caldicoprobacter oshimai* DSM 21659^T; 3, *Tindallia californiensis* DSM 14871^T; 4, *Clostridium ganghwense* JCM 13193^T. Data are from the present study unless indicated. All strains are strictly anaerobic and negative for catalase, oxidase and urease activities. In API ZYM tests, all strains are negative for esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, trypsin, chymotrypsin, α -galactosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase.

Characteristic	1	2	3	4
Isolation source	Human faeces	Sheep faeces	Alkaline lake sediment	Tidal flat sediment
Morphology	Rods	Straight to curved rods	Slightly curved rods	Rod-shaped
Gram stain	Negative	Positive	Positive	Negative
Motility	—	—	+	+
Spore formation	—	+	+	+
Temperature range (optimum) (°C)	25–43 (37)	44–77 (70) ^{a*}	10–48 (37) ^b	15–40 (35) ^c
pH range (optimum) for growth	6.0–9.0 (7.5)	5.9–8.6 (7.2) ^a	8.0–10.5 (9.5) ^b	5.5–10.0 (7.5) ^c
NaCl or sea salt requirement	—	—	1–20 % NaCl ^b	1–9 % sea salts ^c
DNA G + C content (mol%)	51.3	45.4 ^a	40.2 ± 0.1 ^d	31.6
Indole production	—	—	+	+
Gelatin hydrolysis	—	—	—	+
Aesculin hydrolysis	—	—	—	+
API ZYM tests				
Alkaline phosphatase	—	+	+	+
Acid phosphatase	—	+	—	+
Naphthol-AS-BI-phosphohydrolase	+	—	—	—
β -Galactosidase	+	+	—	—
β -Glucosidase	+	+	—	—

*Data taken from: a, Yokoyama *et al.* (2010); b, Pikuta *et al.* (2003); c, Kim *et al.* (2006); d, Whitman (2006).

bacteria derived from studies of human (uncultured clone RL385_aao81d07, collected in the USA, GenBank accession no. DQ804839, 98.6 % similarity; Ley *et al.*, 2006) and dugong (uncultured clone dgD-165 from *Dugong dugon*,

96.7 % similarity, GenBank accession no. AB264080; Tsukinowa *et al.*, 2008) (Fig. 2). Based on their similar sources of isolation and 16S rRNA gene sequences, strain YIT 12065^T and unidentified clone RL385_aao81d07

Fig. 2. Phylogenetic tree showing relationships between strain YIT 12065^T and related taxa in the order *Clostridiales* based on 16S rRNA gene sequence analysis. The tree was rooted with *Bacillus subtilis* DSM 10^T and was reconstructed by using the neighbour-joining method and based on a comparison of 1170 nucleotide positions, with bootstrap values (>50 %) expressed as percentages of 1000 replications. Similar tree topologies were obtained by using the MP and ML methods (not shown). Filled circles indicate that the corresponding nodes were also recovered in trees generated with the MP and ML methods. GenBank/EMBL/DBJ accession numbers are shown in parentheses. Bar, 0.1 substitutions per nucleotide position.

belong to the same species, which presumably prevails in the human intestine. Phylogenetic analysis of these and other related sequences was performed and confirmed that strain YIT 12065^T was phylogenetically most closely associated with members of the families of the order *Clostridiales*, but formed a separate cluster (Fig. 2).

On the basis of these phylogenetic and physiological data, strain YIT 12065^T is proposed as the type strain of the novel taxon *Christensenella minuta* gen. nov., sp. nov. and *Christensenella* gen. nov. is proposed as the type genus of the novel family *Christensenellaceae* fam. nov. within the order *Clostridiales* of the phylum *Firmicutes*.

Description of *Christensenella* gen. nov.

Christensenella (Chris.ten.se.nel'la. N.L. fem. dim. n. *Christensenella* named after Professor Henrik Christensen, in honour of his many contributions to systematic bacteriology).

Cells are Gram-negative, strictly anaerobic, non-spore-forming, non-motile, short rods. Utilize various sugars and produce volatile fatty acids as fermentation end products. The type species is *Christensenella minuta*.

Description of *Christensenella minuta* sp. nov.

Christensenella minuta (mi.nu'ta. L. fem. adj. *minuta* little, small, minute, referring to the cell and colony size).

Displays the following properties in addition to those given for the genus. Cells are approximately 0.4×0.8 – $1.9 \mu\text{m}$ and occur singly or in pairs. Colonies after 4 days of growth at 37 °C on modified GAM agar under anaerobic conditions are 0.1 mm in diameter, circular and beige. Grows at 25–43 °C, with optimum growth at 37–40 °C. The pH range for growth at 37 °C is pH 6.0–9.0, with optimal growth at pH 7.5. Resistant to 20 % bile. No growth is observed on medium containing $\geq 5\%$ NaCl. Major end products of metabolism of glucose are acetic and butyric acids. Negative for aesculin hydrolysis, catalase, gelatin hydrolysis, indole production, nitrate reduction, oxidase and urease. Acid is produced from glucose, salicin, D-xylose, L-arabinose and L-rhamnose. Acid is produced weakly from D-mannose. Acid is not produced from cellobiose, D-mannitol, melezitose, raffinose, D-sorbitol, trehalose, glycerol, lactose, maltose or sucrose. By using API test systems (API Rapid ID 32A and API ZYM), positive reactions are obtained for α -arabinosidase, β -galactosidase, β -glucosidase, glutamic acid decarboxylase and naphthol-AS-BI-phosphohydrolase. Negative for N-acetyl- β -glucosaminidase, acid and alkaline phosphatases, alanine arylamidase, arginine arylamidase, arginine dihydrolase, chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), α -fucosidase, α -galactosidase, α -glucosidase, β -glucuronidase, glutamyl glutamic acid arylamidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, leucyl glycine arylamidase, lipase (C14), α -mannosidase, phenylalanine arylamidase, 6-phosphate- β -galactosidase, proline arylamidase, pyroglutamic acid

arylamidase, serine arylamidase, trypsin, tyrosine arylamidase and valine arylamidase. Dominant cellular fatty acids of cells grown on PYG agar at 37 °C are iso-C_{15:0}, C_{16:0} and C_{14:0}. Respiratory quinones are not detected. The cell wall contains glutamic acid, serine, alanine and LL-A₂pm. Whole-cell sugars are ribose, rhamnose, galactose and glucose.

The type strain, YIT 12065^T (=DSM 22607^T =JCM 16072^T), was isolated from human faeces. The DNA G + C content of the type strain is 51.3 mol%.

Description of *Christensenellaceae* fam. nov.

Christensenellaceae (Chris.ten.se.nel.la'ce.a.e. N.L. fem. n. *Christensenella* type genus of the family; suff. -aceae ending to denote a family; N.L. fem. pl. n. *Christensenellaceae* family of the genus *Christensenella*).

The family is described on the basis of phylogenetic analyses of 16S rRNA gene sequences. Cells are rod-shaped, Gram-negative and anaerobic. Belong to the order *Clostridiales* within the class *Clostridia* of the phylum *Firmicutes*. The type genus is *Christensenella*.

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