Bacteroides clarus sp. nov., *Bacteroides fluxus* sp. nov. and *Bacteroides oleiciplenus* sp. nov., isolated from human faeces

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Three Gram-stain-negative, obligately anaerobic, non-spore-forming, rod-shaped bacteria (strains YIT 12056^T, YIT 12057^T and YIT 12058^T) were isolated from human faeces. These strains were characterized by phylogenetic analyses based on 16S rRNA gene sequence and phenotypic tests. 16S rRNA gene sequence analyses revealed that strains YIT 12056^T, YIT 12057^T and YIT 12058^T were most closely related to the type strains of *Bacteroides gallinarum*, *Bacteroides uniformis* and *Bacteroides intestinalis* with approximate similarity values of 96.6, 95.0 and 96.7 %, respectively. The DNA G + C contents of the novel strains were 45.3 (YIT 12056^T), 45.2 (YIT 12057^T) and 43.6 mol% (YIT 12058^T) and the major respiratory quinones of all three isolates were menaquinones MK-10 and MK-11. These properties were typical for members of the genus *Bacteroides*. The results of the other phenotypic analyses also supported the affiliation of these strains to the genus *Bacteroides*. The 16S rRNA gene sequence analysis, analysis of the major cellular fatty acids and other biochemical tests enabled the genotypic and phenotypic differentiation of the three new strains. Based on these data, three novel species, *Bacteroides clarus* sp. nov., *Bacteroides fluxus* sp. nov. and *Bacteroides oleiciplenus* sp. nov. are proposed. The type strains of *B. clarus*, *B. fluxus* and *B. oleiciplenus* are YIT 12056^T (=JCM 16067^T=DSM 22519^T), YIT 12057^T (=JCM 16101^T=DSM 22534^T) and YIT 12058^T (=JCM 16102^T=DSM 22535^T), respectively.

The human gastrointestinal (GI) tract harbours a diverse and abundant microbiota and recent advances in culture-independent molecular techniques, such as 16S rRNA gene sequencing or metagenome shotgun sequencing, have contributed to increased understanding of this bacterial community (Eckburg *et al.*, 2005; Kurokawa *et al.*, 2007). Most of these organisms are members of the phyla *Bacteroidetes* and *Firmicutes* (Eckburg *et al.*, 2005; Hayashi *et al.*, 2002; Wang *et al.*, 2003). Among the phylum *Bacteroidetes*, species belonging to the genus *Bacteroides* in the family *Bacteroidaceae*, such as *Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, *Bacteroides caccae* and *Bacteroides fragilis*, are predominant members of the human GI microbiota (Eckburg *et al.*, 2005). At the time of writing, the genus *Bacteroides* comprised more than 37 recognized species (List of Prokaryotic names with Standing in Nomenclature; <http://www.bacterio.cict.fr>).

Although the use of culture-independent molecular techniques to analyse the GI microbiota has allowed more complete and accurate assessment of the biodiversity in this ecosystem, the application of molecular techniques can only examine the function or ecological role of specific micro-organisms indirectly. Therefore, a comprehensive understanding of the physiology of the GI microbiota requires their cultivation. For example, Hooper *et al.* (2001) revealed that *B. thetaiotaomicron*, one of the culturable and common members of the adult mouse and human GI tract microbiota, modulates expression of host genes that participate in diverse and fundamental physiological functions, indicating that host physiology is influenced by the indigenous microbiota. In view of this finding, we have attempted to facilitate the isolation of as yet uncultured bacteria from the human GI tract (Sakon *et al.*, 2008; Morotomi *et al.*, 2008, 2009, 2010; Nagai *et al.*, 2009, 2010). In this paper, we report the isolation of three novel species of the genus *Bacteroides*. Although novel taxonomic units (species) based on a single faecal isolate of a Japanese healthy adult are proposed, each isolate displayed >98.7 % 16S rRNA gene sequence similarity to some of the human and rat faecal uncultured clones

Abbreviations: FAMES, fatty acid methyl esters; GI, gastrointestinal; ML, maximum-likelihood; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains YIT 12056^T, YIT 12057^T and YIT 12058^T are AB490801, AB490802 and AB490803, respectively.

reported by several groups, indicating that these bacteria are common members of the intestinal microbiota of man and some other mammals.

Faecal samples were collected from three healthy Japanese males (subjects H, S and O, 57, 37 and 40 years old, respectively) and immediately transferred into an anaerobic glove box (Coy Laboratory Products, Inc.) which contained 88 % N₂, 7 % H₂ and 5 % CO₂. Each sample was weighed and diluted with pre-reduced 0.1 M PBS (0.145 M NaCl, 0.15 M sodium phosphate; pH 7) and spread on modified Gifu anaerobic medium (GAM; Nissui Pharmaceutical) agar plates supplemented with Bacto-Oxgall (Difco), NaCl or antibiotics to isolate subdominant groups of intestinal microbiota. The composition of the modified GAM agar has been described in a previous report (Sakon *et al.*, 2008). Plates were incubated at 37 °C for 3 days in an anaerobic glove box as described above. Strain YIT 12056^T was isolated from a modified GAM agar plate supplemented with 4 % Bacto-Oxgall and inoculated with a 10⁻⁵ serially diluted faecal sample from subject H. Strain YIT 12057^T was isolated from a modified GAM agar plate supplemented with 2 % Bacto-Oxgall and inoculated with a 10⁻⁵ serially diluted faecal sample from subject S. Strain YIT 12058^T was isolated from a modified GAM agar plate supplemented with 15.6 µg oleandomycin ml⁻¹ (Sigma) and inoculated with a 10⁻⁶ serially diluted faecal sample from subject O. Single colonies were picked and grown on modified GAM agar plates. This step was repeated until pure cultures were obtained.

The end products of bacterial metabolism in pre-reduced peptone-yeast extract (PY) medium and PY medium supplemented with glucose (PYG medium) (Holdeman *et al.*, 1977) were analysed by HPLC according to a previously reported procedure (Chonan *et al.*, 1995). Growth was tested in anaerobic and microaerophilic atmospheres which were created by using Anaeropack (Mitsubishi Gas Chemical) and CampyPak Plus microaerophilic system envelopes with palladium catalyst (BBL), respectively. Cellular morphology was observed after Gram-staining of 3-day plate cultures. Cell dimensions were determined by phase contrast microscopy (BX-50; Olympus) using an ocular micrometer (×10) and an objective (×100/1.25 numerical aperture) lens with 3-day plate cultures. Biochemical characteristics were determined using the API Rapid ID 32A and API 20A systems (bioMérieux) according to the manufacturer's instructions and performed in duplicate. Oxidase activity was examined using oxidase test strips (Eiken). The DNA G+C contents were determined by hydrolysing the DNA enzymically and quantifying the nucleosides by HPLC according to a previously described method (Ezaki *et al.*, 1990). Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and were analysed by an HPLC-atmospheric pressure chemical ionization (APCI)-MS/MS system (API3200, Applied Biosystems) using an L-column ODS (2.1 × 150 mm, Chemicals Evaluation and Research Institute) by a modified method of Katsuta *et al.*

(2005). Cellular fatty acid methyl esters (FAMES) were obtained from lyophilized cells by saponification, methylation and extraction using minor modifications (Kuykendall *et al.*, 1988) of the method described by Miller (1982). FAMES were determined by using the MIDI microbial identification system (Microbial ID). DNA extraction, PCR and sequencing of the 16S rRNA genes were performed as described previously (Sakon *et al.*, 2008). Closely related sequences were retrieved from the DNA Data Bank of Japan (DDBJ) using the FASTA program (Pearson & Lipman, 1985). Sequences were aligned and used to produce the unrooted phylogenetic tree by the neighbour-joining method (Saitou & Nei, 1987) using CLUSTAL_X software (version 2.0) (Thompson *et al.*, 1997). The topology of the tree was evaluated by a bootstrap analysis with 1000 replicates using CLUSTAL_X. The tree was visualized by 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 MEGA4 software package (Tamura *et al.*, 2007). The ML tree was calculated with the PhyML program (Guindon & Gascuel, 2003) using the K2P nucleotide substitution model (Kimura, 1980). The input file was prepared by the SEQBOOT program in the PHYLIP package (Felsenstein, 2005).

Cells of strains YIT 12056^T, YIT 12057^T and YIT 12058^T were Gram-stain-negative, non-motile, non-spore-forming and rod-shaped. None of the three strains grew under microaerobic or aerobic conditions. The cell sizes of strain YIT 12056^T were 0.5–1.0 µm wide and 0.7–2.9 µm long. Cells of strain YIT 12057^T were usually 1.0–1.4 µm wide and 1.0–2.5 µm long and extraordinarily long cells (7.5–52.4 µm long) occurred rarely. According to Shah & Collins (1989), the cell sizes of members of the genus *Bacteroides* are approximately 0.5–1.3 µm wide and 1.6–11 µm long, which were consistent with those of these two novel strains. However, the cell size of strain YIT 12058^T was 1.0–1.9 µm wide and 1.4–55.0 µm long, indicating that strain YIT 12058^T was quite different from other species of the genus *Bacteroides* due to its large cell size.

Colonies after anaerobic incubation on modified GAM agar plates for 4 days were 1–2 mm in diameter, translucent whitish, butyrous, entire, circular, convex, shiny, slightly opaque and similar to a pearl in appearance (strain YIT 12056^T); 1–2.5 mm in diameter, grey-white, butyrous, entire, circular, convex, shiny and opaque (strain YIT 12057^T); and 0.1–3 mm in diameter, grey-white, butyrous, entire, circular, convex, shiny and opaque (strain YIT 12058^T). Growth of strain YIT 12057^T persisted only for a short period, approximately 1–2 days, on modified GAM agar plates at 37 °C.

The major metabolic end products of strain YIT 12056^T from glucose in PYG broth were lactic, acetic and succinic acids (9.3, 2.2 and 1.9 mM, respectively) and minor amounts of formic and propionic acids were also

produced. Those of strain YIT 12057^T were succinic and acetic acids (19.2 and 12.8 mM, respectively) and minor amounts of formic and propionic acids were also produced. The major metabolic end products of strain YIT 12058^T were acetic, succinic, formic and lactic acids (9.8, 8.3, 3.3 and 1.7 mM, respectively). The results of the phenotypic analyses of the novel strains using the API systems are summarized in Table 1. Although many of the characteristics of the novel isolates were similar to those of related species of the genus *Bacteroides*, some differences were found.

The major cellular fatty acids of strain YIT 12056^T were anteiso-C_{15:0} (26.0%), iso-C_{15:0} (25.6%) and summed feature 11 (comprising iso-C_{17:0} 3-OH and/or C_{18:2} dimethylacetal; 20.3%). Those of strain YIT 12057^T were

anteiso-C_{15:0} (28.8%), summed feature 11 (27.8%), C_{18:1ω9c} (14.0%) and iso-C_{15:0} (9.3%). Those of strain YIT 12058^T were anteiso-C_{15:0} (26.1%), C_{18:1ω9c} (24.5%), iso-C_{15:0} (11.7%) and summed feature 11 (10.3%). These results indicate that the major cellular fatty acid compositions of these strains were basically consistent with those of the members of the genus *Bacteroides*, which contain major amounts of anteiso- and iso-methyl branched chain acids (Shah & Collins, 1983).

The major quinones of members of the genus *Bacteroides* are menaquinones MK-10 and MK-11 (Shah, 1992). The major quinones of strains YIT 12056^T and YIT 12057^T were MK-10 (53 and 81%, respectively) and MK-11 (47 and 19%, respectively). Strain YIT 12058^T also had a trace

Table 1. Phenotypic characteristics of strains YIT 12056^T, YIT 12057^T, YIT 12058^T and phylogenetically related members of the genus *Bacteroides*

Taxa: 1, YIT 12056^T; 2, YIT 12057^T; 3, YIT 12058^T; 4, *B. uniformis* JCM 5828^T (data from Bakir *et al.*, 2006; Lan *et al.*, 2006); 5, *B. intestinalis* JCM 13265^T (Bakir *et al.*, 2006); 6, *B. gallinarum* JCM 13658^T (Lan *et al.*, 2006). Data for strains YIT 12056^T, YIT 12057^T and YIT 12058^T are from this study. All strains were positive for hydrolysis of aesculin, indole production, acid production from cellobiose, lactose, maltose, D-mannose, raffinose, sucrose and D-xylose. All strains were also positive for alanine arylamidase, alkaline phosphatase, β-galactosidase, α-glucosidase, β-glucosidase and leucyl glycine arylamidase. All strains were negative for nitrate reduction, urease, arginine dihydrolase and histidine-, leucine-, phenylalanine-, proline-, pyroglutamic acid- and serine arylamidase. +, Positive; −, negative; w, weakly positive; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6
Isolation source	Human faeces	Human faeces	Human faeces	Human faeces	Human faeces	Chicken caecum
Catalase	−	−	+	−	ND	−
Gelatin hydrolysis	−	−	+	−	−	−
Acid production from:						
L-Arabinose	−	+	+	+	+	+
Glycerol	w	+	+	−	−	−
D-Mannitol	w	+	+	−	−	−
L-Rhamnose	+	+	+	−	+	w
Salicin	w	+	+	+	−	−
D-Sorbitol	w	+	+	−	−	−
Trehalose	w	+	+	−	−	−
Enzyme activities:						
N-Acetyl-β-glucosaminidase	w	+	+	+	+	+
α-Arabinosidase	−	−	+	+	+	+
Arginine arylamidase	−	w	−	−	−	−
α-Fucosidase	−	+	−	+	+	−
α-Galactosidase	+	+	+	+	+	−
6-Phospho-β-galactosidase	−	−	−	v	−	−
β-Glucuronidase	−	+	−	−	−	−
Glutamic acid decarboxylase	+	−	+	+	+	+
Glutamyl glutamic acid arylamidase	+	+	−	+	+	+
Glycine arylamidase	−	w	−	−	−	−
Valine arylamidase	−	−	w	−	−	−
DNA G + C content (mol%)	45.3	45.2	43.6	46.1–47.8	44	47
Distinctive major cellular fatty acids*	iso-C _{15:0} , summed feature 11	C _{18:1ω9c} , summed feature 11, iso-C _{15:0}	C _{18:1ω9c} , iso- C _{15:0} , summed feature 11	iso-C _{17:0} 3-OH, iso-C _{15:0}	iso-C _{17:0} 3-OH	iso-C _{17:0} 3-OH, iso-C _{15:0}

*Anteiso-C_{15:0} is major component in all strains. Summed features represent group of two or three fatty acids that could not be separated using GC with the MIDI system. Summed feature 11 contains iso-C_{17:0} 3-OH and/or C_{18:2} dimethylacetal.

amount of MK-10 and MK-11, with no other quinones detected. The DNA G + C contents of strains YIT 12056^T, YIT 12057^T and YIT 12058^T were 45.3, 45.2 and 43.6 mol%, respectively, which supported their affiliation to the genus *Bacteroides*, members of which have DNA G + C contents of between 40 and 48 mol% (Shah, 1992)

Almost complete 16S rRNA gene sequences of strains YIT 12056^T (1481 bp), YIT 12057^T (1477 bp) and YIT 12058^T (1476 bp) were determined. Database searches revealed that the most similar sequences to those of the novel isolates were *Bacteroides gallinarum* JCM 13658^T with approximately 96.6% similarity (YIT 12056^T), *Bacteroides uniformis* JCM 5828^T with approximately 95.0% similarity (YIT 12057^T) and *Bacteroides intestinalis* JCM 13265^T with approximately 96.7% similarity (YIT 12058^T). The 16S rRNA gene sequences of strains YIT 12056^T, YIT 12057^T, YIT 12058^T and related type species of the genus *Bacteroides* were aligned and a phylogenetic tree was constructed using *Escherichia coli* as an outgroup, with closely related uncultured clones obtained from human faeces described below (Fig. 1). The phylogenetic tree showed that all three new isolates belonged to the genus *Bacteroides*. The position of each strain was phylogenetically distinct and their specific lineages were robust, supported by high bootstrap values. These results were congruent with those obtained using the MP and ML methods (data not shown).

The database searches also revealed that several uncultured clones displayed >98.7% sequence similarity to strains YIT

12056^T, YIT 12057^T and YIT 12058^T. For example, strain YIT 12056^T was related to the faecal uncultured clones from human subjects reported by Li *et al.* (2008) (GenBank accession no. EF405059) and Suau *et al.* (1999) (AF132259). Strain YIT 12057^T was also related to the human faecal uncultured clones reported by Ley *et al.* (2006) (DQ793778 and another 9 clones). Strain YIT 12058^T was related to the faecal uncultured clones from humans (Dethlefsen *et al.*, 2008; EU763372 and 4 other clones) and rats (S. P. J. Brooks and others, unpublished, FJ880188 and 3 other clones). These data indicated that these three novel strains occur in faeces of human and some other mammals as common members of the intestinal microbiota. It is evident from the results of the taxonomic study that the Gram-negative-staining strains YIT 12056^T, YIT 12057^T and YIT 12058^T recovered from human faeces represent hitherto unknown species of the genus *Bacteroides*. On the basis of phylogenetic and phenotypic analyses, we propose that these strains represent three novel species of the genus *Bacteroides*: *Bacteroides clarus* sp. nov., *Bacteroides fluxus* sp. nov. and *Bacteroides oleiciplenus* sp. nov. Differential characteristics of the novel species and related species of the genus *Bacteroides* are shown in Table 1.

Description of *Bacteroides clarus* sp. nov.

Bacteroides clarus (cla'rus. L. masc. adj. *clarus* shining, referring to the shiny appearance of the colonies).

Cells are obligately anaerobic, Gram-stain-negative, non-motile, non-spore-forming, rod-shaped and approximately

Fig. 1. Phylogenetic tree showing the positions of *Bacteroides clarus* sp. nov. YIT 12056^T, *Bacteroides fluxus* sp. nov. YIT 12057^T, *Bacteroides oleiciplenus* sp. nov. YIT 12058^T, other members of the genus *Bacteroides* and selected uncultured bacterial clones based on 16S rRNA gene sequence similarity data. The tree was rooted with *Escherichia coli* ATCC 11775^T and constructed by using the neighbour-joining method; bootstrap values from 1000 replications are given at the nodes. GenBank/EMBL/DBJ accession numbers are shown in parentheses. Bar, 0.1 substitutions per nucleotide position.

0.5–1.0 µm wide and 0.7–2.9 µm long. Colonies on modified GAM agar plates after 4 days anaerobic incubation are 1–2 mm in diameter, translucent whitish, butyrous, entire, circular, convex, shiny and slightly opaque. Major end products of glucose metabolism in PYG broth are lactic, acetic and succinic acids. The major cellular fatty acids are anteiso-C_{15:0}, iso-C_{15:0} and summed feature 11 (comprising iso-C_{17:0} 3-OH and/or C_{18:2} dimethylacetal). The main respiratory quinones are MK-10 and MK-11. The phenotypic characteristics of the type strain obtained with API systems are shown in Table 1.

The type strain, YIT 12056^T (=JCM 16067^T=DSM 22519^T), was isolated from human faeces. The DNA G + C content of the type strain is 45.3 mol%.

Description of *Bacteroides fluxus* sp. nov.

Bacteroides fluxus (flu'xus. L. masc. adj. *fluxus* transient or perishable, referring to the short-lived nature of this bacterium during cultivation).

Cells are obligately anaerobic, Gram-stain-negative, non-motile, non-spore-forming, rod-shaped and usually 1.0–1.4 µm wide and 1.0–2.5 µm long. Extraordinarily long cells (7.5–52.4 µm long) occur rarely. Colonies on modified GAM agar plates after 4 days anaerobic incubation are 1–2.5 mm in diameter, grey-white, butyrous, entire, circular, convex, shiny and opaque. Major end products of glucose metabolism in PYG broth are succinic and acetic acids. The major cellular fatty acids are anteiso-C_{15:0}, summed feature 11 (comprising iso-C_{17:0} 3-OH and/or C_{18:2} dimethylacetal), C_{18:1}ω9c and iso-C_{15:0}. The main respiratory quinones are MK-10 and MK-11. The phenotypic characteristics of the type strain obtained with API systems are shown in Table 1.

The type strain, YIT 12057^T (=JCM 16101^T=DSM 22534^T), was isolated from human faeces. The DNA G + C content of the type strain is 45.2 mol%.

Description of *Bacteroides oleiciplenus* sp. nov.

Bacteroides oleiciplenus (o.le.i.ci.ple'nus. N.L. n. *acidum oleicum* oleic acid; L. adj. *plenus* full of, abounding in; N.L. masc. adj. *oleiciplenus* abounding in oleic acid, referring to the cellular fatty acid composition).

Cells are obligately anaerobic, Gram-stain-negative, non-motile, non-spore-forming, rod-shaped and approximately 1.0–1.9 µm wide and 1.4–55.0 µm long. Colonies on modified GAM agar plates after 4 days anaerobic incubation are 0.1–3 mm in diameter, grey-white, butyrous, entire, circular, convex, shiny and opaque. Major end products of glucose metabolism in PYG broth are acetic, succinic, formic and lactic acids. The major cellular fatty acids are anteiso-C_{15:0}, C_{18:1}ω9c, iso-C_{15:0}, and summed feature 11 (comprising iso-C_{17:0} 3-OH and/or C_{18:2} dimethylacetal). The main respiratory quinones are MK-10 and MK-11.

Phenotypic characteristics of the type strain obtained with the API systems are shown in Table 1.

The type strain, YIT 12058^T (=JCM 16102^T=DSM 22535^T), was isolated from human faeces. The DNA G + C content of the type strain is 43.6 mol%.

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