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Satomi Nishiyama, Atsuko Ueki, Nobuo Kaku, Kazuya Watanabe, Katsuji Ueki (2009) *Bacteroides graminisolvens* sp. nov., a novel, xylanolytic anaerobic rods isolated from a methanogenic reactor of cattle waste. *International Journal of Systematic and Evolutionary Microbiology* 59(8): pp. 1901-1907.

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***Bacteroides graminisolvens* sp. nov., a novel, xylanolytic anaerobic rods isolated from a methanogenic reactor of cattle waste**

Satomi Nishiyama¹, Atsuko Ueki¹, Nobuo Kaku¹, Kazuya Watanabe² and Katsuji Ueki¹

1. Faculty of Agriculture, Yamagata University, 1-23 Wakaba-machi, Tsuruoka 997-8555, Japan

2. Exploratory Research for Advanced Technology (ERATO), JST, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Author for correspondence: Atsuko Ueki. Tel: +81 235 28 2866. Fax: +81 235 28 2846.

E-mail: uatsuko@tds1.tr.yamagata-u.ac.jp

Abbreviations: CMC, carboxymethylcellulose; CFA, whole-cell fatty acid.

Key words: *Bacteroides graminisolvens*, methanogenic reactor, xylan, hemicellulose, propionate

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strains XDT-1^T is AB363973.

ABSTRACT

A strictly anaerobic bacterial strain XDT-1^T was isolated from plant residue in a methanogenic reactor treating waste from cattle farms. Cells of the strain were Gram-negative, non-motile, non-spore-forming rods. Haemin was required for growth. The strain utilized xylan as well as various sugars including arabinose, xylose, glucose, mannose, cellobiose, raffinose, starch and pectin. The strain produced acetate, propionate and succinate from saccharides in the presence of haemin. The optimum pH for growth was about 7.2 and the optimum growth temperature was 30-35°C. The strain was sensitive to bile. The major cellular fatty acids of the strain were anteiso-C_{15:0} and iso-C_{17:0} 3-OH. Menaquinones MK-10(H₀) was the major respiratory quinone, and the genomic DNA G + C content was 38.0%. Phylogenetic analysis based on

16S rRNA gene sequences placed the strain in the phylum *Bacteroidetes*. The most closely related species to the strain was *Bacteroides ovatus* with 16S rRNA gene sequence similarity of 94.2%. Based on the phylogenetic, physiological and chemotaxonomic analyses of the novel strain, *Bacteroides graminisolvens* sp. nov. is proposed to accommodate the strain. The type strain of the novel species is XDT-1^T (= JCM 15093^T = DSM 19988^T).

MAIN TEXT

The *Bacteroides-Prevotella* group mainly consists of species derived from human faecal and oral sources as well as other samples from mammalian organs such as the rumen (Holdeman *et al.*, 1984; Paster *et al.*, 1994; Shah & Collins, 1989). Although bacterial clones affiliated with the group have often been detected as dominant components from methanogenic reactors (Chouari *et al.*, 2005; Godon *et al.*, 1997; Levén *et al.*, 2007), the function of the group in the anaerobic degradation of organic matter in the methanogenic process remains to be clarified. Some *Bacteroides* species isolated from human faecal samples have been explored as important decomposers of hemicellulose or xylan (Chassard *et al.*, 2007, 2008; Hayashi *et al.*, 2005; Hespell & Whitehead, 1990; Hopkins *et al.*, 2003). Since hemicellulose is one of major components (30-40%) of plant cell wall as well as cellulose, concomitant decomposition of hemicellulose and cellulose is a key reaction for effective methanogenic fermentation of waste containing plant material (Collins *et al.*, 2005). In this study, we described a novel xylanolytic species affiliated with the genus *Bacteroides*, which was isolated from a plant residue sample (mainly rice-straw) in a methanogenic reactor treating waste from cattle farms.

Strain XDT-1^T (= JCM 15093^T = DSM 19988^T) was isolated from a rice-straw residue sample obtained from

a methanogenic reactor treating waste collected from cattle farms (up to 1000 cattle as a total) in Betsukai-machi in Hokkaido, Japan. The reactor was a vertically-cylindrical type (1500 m³) operated at 35°C. Rice-straw used as matting of the cattle farms was thrown into the reactor together with faeces and urine of animals and treated as waste (Ueki *et al.*, 2008).

The strain was cultivated anaerobically at 30°C unless otherwise stated by using peptone (10 g l⁻¹) /yeast extract (5 g l⁻¹) (PY) medium as basal medium with oxygen-free mixed gas (95% N₂/ 5% CO₂) as the headspace as described by Ueki *et al.* (2006a). PY medium supplemented with (l⁻¹) 0.25 g each of glucose, cellobiose, maltose and soluble starch as well as 15 g agar (Difco) was designated PY4S agar and used for maintenance of the strain in agar slants. For enrichment culture and isolation of xylanolytic bacteria, the concentrations of both peptone and yeast extract in the basal medium were decreased to one-tenth of those in PY medium (1/10PY medium) (Nishiyama *et al.*, in press). PY liquid medium supplemented with haemin (at a final concentration of 5 mg l⁻¹) (Holdeman *et al.*, 1977) (PYH medium) and 10 g glucose l⁻¹ (PYHG medium) was used for cultivation of the strain for various physiological tests and chemotaxonomic analyses of the cells unless otherwise stated (Ueki *et al.*, 2006b). In addition to haemin, B-vitamin mixture (10 ml l⁻¹) was added to PYH medium (PYHV medium) for the cultivation as described below. The composition of the B-vitamin mixture used was (100 ml⁻¹) 0.1 mg biotin, 0.1 mg cyanocobalamin (cobalamin or vitamin B₁₂), 0.3 mg *p*-aminobenzoic acid, 0.5 mg folic acid, 0.5 mg thiamine hydrochloride, 0.5 mg riboflavin and 1.5 mg pyridoxine hydrochloride (Akasaka *et al.*, 2004). The same concentration of cobalamin was used, when it was added to the medium as the sole vitamin. Media were usually adjusted to pH 7.2 with 1M NaOH. Growth in liquid medium was monitored by changes in OD₆₆₀.

Anaerobic sludge samples obtained from the reactor were filtrated through a mesh (2 mm of pore size) and plant residue remained on the mesh was collected. The plant residue samples obtained were washed several times with sterile anoxic diluent and homogenized by a Waring blender (10000 rpm, 10 min.) under N₂ gas (Kaku *et al.*, 2000). The homogenized samples were successively diluted anaerobically and the enrichment culture was started by inoculating 1 ml of 10-fold diluted samples (10⁻⁴ - 10⁻⁶) into 9 ml of 1/10PY-x liquid medium (1/10PY medium containing 5 g l⁻¹ of birchwood xylan). After incubation for about a week, 1 ml of the culture was transferred to fresh 9 ml of 1/10PY-x medium. Growth of xylanolytic bacteria in the enrichment culture was confirmed by the decrease in pH value of the medium as compared with the culture without xylan (1/10PY medium). After several subcultures of the xylanolytic enrichment, the diluted enrichment cultures were inoculated to the anaerobic roll tubes using 1/10PY-x agar medium, and some colonies were picked up after incubation for a week. Out of the isolates including non-xylanolytic, Gram-positive bacteria, strain XDT-1^T was finally obtained after purification by the anaerobic roll tube method (Hungate, 1966). Strain XDT-1^T was isolated from the enrichment culture initially inoculated with a 10⁻⁵ diluted sample of homogenized plant residue.

Growth of the strain under the air was examined as described previously (Ueki *et al.*, 2008). Spore formation was assessed by observation of cells after Gram-staining as well as by phase-contrast microscopy. Catalase activity was examined as described previously (Ueki *et al.*, 2008). Oxidase and nitrate-reducing activities were determined according to the methods described by Akasaka *et al.* (2003b). Utilization of carbon sources was tested in PYH liquid medium with each substrate added at 10 g l⁻¹ (for sugars and sugar

alcohols) or 30 mM (organic acids). Utilization of each substrate was determined by growth measured by OD₆₆₀ as well as by determining changes in the pH values of the medium after cultivation. Bile sensitivity was determined by the addition of Oxgall (Difco) (0.1-2%, w/v) or bile salts (Oxioid) (0.1-0.5%, w/v) to PYHG medium. Fermentation products were analyzed by GC or HPLC as described previously (Ueki *et al.*, 1986; Akasaka *et al.*, 2003a). Other characterizations were performed according to the methods as described by Holdeman *et al.* (1977).

Whole-cell fatty acids (CFAs) were converted to methyl esters according to the method of Miller (1982) and analyzed by GC (Hp6890; Hewlett-Packard or G-3000; Hitachi) equipped with a HP Ultra 2 column. CFAs were identified by equivalent chain-length (ECL) (Miyagawa *et al.*, 1979) according to the protocol of TechnoSuruga Co., Ltd (Shimizu, Japan) (Moore *et al.*, 1994). Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analyzed by using a mass spectrometer (JMS-SX102A; JEOL). Genomic DNA extracted according to the method as described by Akasaka *et al.* (2003b) was digested with P1 nuclease by using a YAMASA GC kit (Yamasa shoyu) and its G + C content was measured by HPLC (HITACHI L-7400) equipped with a μ Bondapak C18 column (3.9 \times 300 mm; Waters).

DNA was extracted from cells as described previously (Akasaka *et al.*, 2003b). Almost full-length of 16S rRNA gene was PCR amplified using a primer set of 27f and 1546r. The PCR-amplified 16S rRNA gene was sequenced by using an ABI Prism BigDye Terminator Cycle Sequencing ready reaction kit and ABI Prism 3730 automatic DNA sequencer (Applied Biosystems). Multiple alignments of the sequences with reference sequences in GenBank were performed with the BLAST program (Altschul *et al.*, 1997). A

phylogenetic tree was constructed with the neighbor-joining method (Saitou & Nei, 1987) by using the CLUSTAL W program (Thompson *et al.*, 1994). All gaps and unidentified base positions in the alignments were excluded before sequence assembly.

Cells of strain XDT-1^T were Gram-negative rods, and usually 0.4-0.6 µm in width and 1.2-4.5 µm in length. Rather long cells often occurred (Fig. 1). Cells were non-motile as observed phase-contrast microscopy. The strain formed very thin colonies with a smooth surface on PY4S agar, and had a pearl-like iridescence under light. The strain could not grow under aerobic conditions. Spore formation was not observed.

Strain XDT-1^T grew slowly ($\mu = 0.14 \text{ h}^{-1}$) in PYG liquid medium (without haemin and the B-vitamin mixture) and the addition of haemin to the medium (PYHG) remarkably stimulated the growth ($\mu = 0.39 \text{ h}^{-1}$). Although addition of the vitamin mixture alone to PYG medium did not affect the growth rate ($\mu = 0.13 \text{ h}^{-1}$), adding a vitamin mixture or cobalamin to PYHG liquid medium (PYHVG medium) slightly enhanced the growth ($\mu = 0.40\text{-}0.41 \text{ h}^{-1}$).

Catalase activity was not detected in cells grown in PY4S agar slants or in PYG liquid medium as well as the cells grown in PYHG medium. Oxidase activity was not detected. The strain utilized various sugars in addition to xylan and glucose as shown below in Table 1 and the species description. Substrates tested but not utilized are also shown in the description. The strain produced acetate (1.9 mmol l^{-1}), propionate (1.4 mmol l^{-1}) and succinate (4.3 mmol l^{-1}) from glucose (PYHG medium). Without haemin (PYG medium), a small amount of lactate was produced in addition to acetate, while propionate and succinate were not

detected. A small amount of pyruvate was sometimes formed in PYHG medium. When grown in the presence of the vitamin mixture as well as haemin (PYHVG medium), propionate production was enhanced with concomitant decrease of succinate production (acetate 1.5 mmol l⁻¹, propionate 3.6 mmol l⁻¹ and succinate 2.9 mmol l⁻¹). The final pH was 4.9. Almost the same amounts of products were formed from xylose in PYH medium. When grown on xylan (5 g l⁻¹) in PYH medium, propionate was produced as the most dominant product (5.8 mmol l⁻¹) with lower amounts of acetate (3.4 mmol l⁻¹) and succinate (2.0 mmol l⁻¹). In the presence of the vitamin mixture as well as haemin (PYHV medium), propionate production from xylan increased to 10.3 mmol l⁻¹ with acetate and succinate as minor products (3.1 and 1.3 mmol l⁻¹, respectively) after cultivation for two days. The final pH with xylan was 5.2-5.3. Almost the same amounts of products were detected when grown in the medium containing 10 g l⁻¹ of xylan even after seven days of incubation. The decrease in pH value of the medium should limit the xylan utilization. The vitamin mixture and cobalamin showed the same effect on the fermentation products, and thus it appeared that cobalamin in the vitamin mixture stimulated propionate production of the strain.

The optimum pH was pH 7.2 ($\mu = 0.39 \text{ h}^{-1}$) and the pH range for growth was 6.1-8.2. The strain did not grow at the initial pH values of 5.0 and 8.7. Growth temperature range was 5-40°C with the optimum at 30-35°C ($\mu = 0.39-0.41 \text{ h}^{-1}$). Although the growth rate at 40°C ($\mu = 0.45 \text{ h}^{-1}$) was higher than that at 35°C, the maximum OD₆₆₀ of the culture reached was lower than that at 35°C and the value of OD₆₆₀ declined soon, indicating cell lysis of the culture. The strain grew even at 5°C at a rather low growth rate ($\mu = 0.006 \text{ h}^{-1}$). The NaCl concentration range for growth was 0-4% (w/v) in PYHG medium with the optimum at 0% (w/v) NaCl. Aesculin and starch were hydrolyzed, but not gelatin. Nitrate-reducing and urease activities

were not detected. The strain did not grow in the presence of 2% (w/v) Oxgall or 0.1% (w/v) bile acids; the growth of the strain was strongly inhibited even in the presence of 0.1% (w/v) of Oxgall. The strain did not produce indole and sulfide.

The major CFAs of the strain were anteiso-C_{15:0} (33.4%), iso-C_{17:0} 3-OH (20.0%), iso-C_{15:0} (8.9%) and anteiso-C_{13:0} (7.0%). Minor acids such as iso-C_{13:0} (4.9%), C_{15:0} (4.0%), anteiso-C_{17:0} 3-OH (3.6%), C_{16:0} (3.0%), iso-C_{14:0} (2.0%), C_{14:0} (1.9%), iso-C_{16:0} (1.5%), C_{16:0} 3-OH (1.3%) and anteiso-C_{17:0} (1.2%) were detected. Menaquinone MK-10(H₀) was the major respiratory quinone, and the genomic DNA G + C content was 38.0%.

Phylogenetic analysis based on the 16S rRNA gene sequence placed the strain in the phylum *Bacteroidetes* (Garrity & Holt, 2001). The most closely related species to the strain was *Bacteroides ovatus* NCTC 11153^T (Holdemen *et al.*, 1984) with 16S rRNA gene sequence similarity of 94.2%. The next closely related species were *Bacteroides thetaiotaomicron* ATCC 29148^T (Holdemen *et al.*, 1984) (similarity of 94.0%) and *Bacteroides xylanisolvens* DSM 18836^T (Chassard *et al.*, 2008) (93.6%). *Bacteroides* species such as *Bacteroides eggerthii* NCTC11155^T (Holdemen *et al.*, 1984) (93.3%), *Bacteroides acidifaciens* JCM 10556^T (Miyamoto & Itoh, 2000) (93.3%), *Bacteroides caccae* ATCC 43185^T (Johnson *et al.*, 1986) (93.1%), *Bacteroides finegoldii* JCM 13345^T (Bakir *et al.*, 2006b) (92.7%) and *Bacteroides fragilis* ATCC 25285^T (Holdemen *et al.*, 1984) (92.5%) were the next closely related species with similar levels of the sequence similarities.

Out of rather restricted species of xylan-decomposers in the *Bacteroides* species, the xylanase system of *B. ovatus* has been investigated extensively (Cooper *et al.*, 1985; Hopkins *et al.*, 2003). *B. xylanisolvans* is described quite recently as a xylanolytic bacterium isolated from human faeces (Chassard *et al.*, 2008). In addition, a subspecies of *B. fragilis* from human faecal samples is also known to be xylanolytic (Hespell & Whitehead, 1990; Hopkins *et al.*, 2003), and *B. eggerthii* is described to produce acids from xylan (Holdeman *et al.*, 1984). Furthermore, recent reports indicated that *Bacteroides intestinalis* (Bakir *et al.*, 2006a) and *Bacteroides dorei* (Bakir *et al.*, 2006c), both recent isolates from human faeces, have the ability to decompose hemicellulose (Chassard *et al.*, 2007). Thus, strain XDT-1^T was placed closely related to the major xylanolytic species in the genus *Bacteroides* (Fig. 2).

Hemicellulose or xylan is one of dominant components of plant biomass, and thus the decomposition is an important function of fermentative bacteria in anaerobic environments containing plant biomass. Recent reports have indicated the importance of the *Bacteroides* species in decomposition of hemicellulose in the human intestine. Hopkins *et al.* (2003) reported that xylan breakdown by human intestinal microbes resulted in the increased propionate formation as compared with the starch breakdown, which was associated with production of acetate and butyrate. Strain XDT-1^T produced acetate and propionate with a lesser amount of succinate in the presence of the B-vitamin mixture or cobalamin from various saccharides. Furthermore, an increased amount of propionate and a lower amount of succinate were produced from xylan as compared with glucose or xylose. The results are consistent with the above-mentioned report and suggest that hemicellulose decomposition is an important function of the *Bacteroides* species in anaerobic environments and which may cause increase in the propionate production in the habitats.

Some characteristics of strain XDT-1^T and the most closely related species are compared in Table 1. Only a few species in the genus *Bacteroides* have been isolated from sources other than mammalian samples (Ueki *et al.* 2008; Whitehead *et al.*, 2005), and all the closest relatives of strain XDT-1^T were also derived from human faeces. Although strain XDT-1^T had the common characteristics to the *Bacteroides* species isolated from mammalian samples in morphology, physiology and chemotaxonomy, the strain had some distinct features different from the relatives. Although *B. ovatus* and *B. thetaiotaomicron* usually have catalase activity (Holdeman *et al.*, 1984), we did not detect it in strain XDT-1^T in spite of careful examination (Ueki *et al.*, 2008; Wilkins *et al.*, 1978). Bile resistance is one of specific characteristics of the *Bacteroides* species, however, our strain was highly sensitive to bile. The strain did not produce indole. *B. thetaiotaomicron* was reported to be clearly differentiated from *B. ovatus* in the incapability of xylan fermentation (Cooper *et al.*, 1985), and the range of substrate utilization of strain XDT-1^T was different from any of the closely related species. The CFA profile of strain XDT-1^T was basically consistent with those of *Bacteroides* species, although the percentages of some CFAs such as anteiso-C_{13:0} (7.0%), C_{16:0} (3.0%) and C_{16:0} 3-OH (1.3%) were rather different from those of the related species (Miyagawa *et al.*, 1979; Shah & Collins, 1980).

Haemin requirement is commonly found in the *Bacteroides* species. We recently described three novel species related to the *Bacteroides-Prevotella* group, which were isolated from plant residue collected from irrigated rice field soil as well as a methanogenic reactor. All the three species also required haemin for growth (Ueki, *et al.*, 2006b, 2007, 2008). These results suggest that major bacterial species related to the *Bacteroides-Prevotella* group living in the habitats other than mammalian bodies also need haemin for growth.

Cobalamin requirement was also commonly recognized for propionate-producing strains in the above-mentioned novel species.

Xylanolytic *Bacteroides* species such as *B. ovatus* and *B. fragilis* are known to be rarely isolated from human faecal samples when xylan is used as a selective substrate (Wedekind *et al.*, 1988, Chassard *et al.*, 2007). In this study to enrich the xylanolytic bacteria, concentrations of pepton and yeast extract in the medium were reduced to one-tenth of those in the normal PY medium. According to our experiences, higher concentrations of these components in the medium for enrichment of xylan- or cellulose-decomposing bacteria usually enhanced the growth of fast-growing proteolytic or aminolytic bacteria and these bacteria usually outcompeted the polysaccharide-decomposing bacteria (Nishiyama *et al.*, in press). By using the reduced concentrations of pepton and yeast extract, the growth of the non-xylanolytic bacteria should be suppressed as compared with that in the normal PY medium, and thus the xylan-decomposing *Bacteroides* species should be enriched successfully. Furthermore, we used homogenized plant residue as an inoculum for the enrichment. We have already isolated the three novel species belonging to the *Bacteroides-Prevotella* group from plant residue samples obtained from rice field soil and the methanogenic reactor as described above (Ueki *et al.*, 2006b, 2007, 2008). Thus, it seems likely that the bacterial species related to the *Bacteroides-Prevotella* group often live on plant residue as decomposers of hemicellulose or other plant material (e.g., pectin) in various anaerobic environments.

Based on the phylogenetic, physiological and chemotaxonomic analyses of the novel strains, *Bacteroides graminisolvens* sp. nov. is proposed to accommodate the strains. The type strain of the novel species is

XDT-1^T (= JCM 15093^T = DSM 19988^T).

Description of *Bacteroides graminisolvens* sp. nov.

Bacteroides graminisolvens (gra.mi.ni.sol'vens. L. neut. n. *gramen* grass; L. v. *solvere* dissolve; N.L. part. adj. *graminisolvens* grass-dissolving).

Cells are Gram-negative, non-motile, non-spore-forming rods (0.4-0.6 µm in width and 1.2-4.5 µm in length) with some longer cells. Produces thin and smooth surface colonies with pearl-like, iridescent luster under the light. Growth is strongly stimulated by the addition of haemin, and cobalamin (vitamin B₁₂) slightly enhances the growth. Uses arabinose, xylose, fructose, galactose, glucose, mannose, cellobiose, lactose, maltose, melibiose, sucrose, raffinose, xylooligosacchride, dextrin, glycogen, starch, pectin, xylan (birchwood), amygdalin and salicin. Ribose, rhamnose, inulin, aesculin and pyruvate are only weakly used. Produces acetate and propionate with a small amount of succinate in the presence of haemin and cobalamin from glucose. Propionate is a most abundant product from xylan in the presence of haemin and cobalamin. Final pH values in the presence of glucose and xylan are pH 4.9 and 5.2-5.3, respectively. Does not use sorbose, trehalose, melezitose, carboxymethylcellulose (CMC), cellulose (filter paper fragments and ball-milled powder), glycerol, dulcitol, inositol, mannitol, sorbitol, ethanol, fumarate, lactate, malate and succinate. The optimum pH is 7.2. Growth temperature range is 5-40°C; optimum at 30-35°C. NaCl concentration range for growth is 0-4.0% (w/v) in PYHG medium; optimum at 0% (w/v). Does not have catalase, oxidase, nitrate-reducing and urease activities. Does not produce hydrogen sulfide and indole. Hydrolyzes aesculin and starch, but not gelatin. Sensitive to bile. The major cellular fatty acids are

anteiso-C_{15:0} and iso-C_{17:0} 3-OH. Menaquinone MK-10(H₀) is the major respiratory quinone, and the genomic DNA G + C content is 38.0%. The type strain of the novel species, XDT-1^T (= JCM 15093^T = DSM 19988^T), was isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms.

ACKNOWLEDGEMENTS

This work was partly supported by a Grant-in-Aid from the Institute for Fermentation, Osaka, and also by the Project for Development of Technology for Analysing and Controlling the Mechanism of Biodegrading and Processing supported by the New Energy and Industrial Technology Development of Organization (NEDO). We are grateful to Dr. T. Hoaki for sampling of sludge and Dr. K. Takahashi for quinone analysis.

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Figure legends

Fig. 1. Phase-contrast photomicrograph of cells of strain XDT-1^T grown anaerobically on agar slant of PY4S medium. Bar, 10 μm.

Fig. 2. Neighbor-joining tree showing the phylogenetic relationship of strain XDT-1^T based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) above 50% are shown at branch nodes. Bar, 2% estimated difference in nucleotide sequence position. The sequence of *Prevotella melaninogenica* ATCC 25845^T was used as the outgroup.

Fig. 1

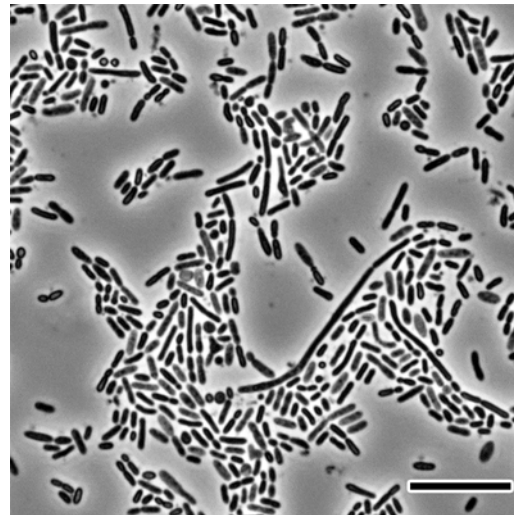


Fig. 2

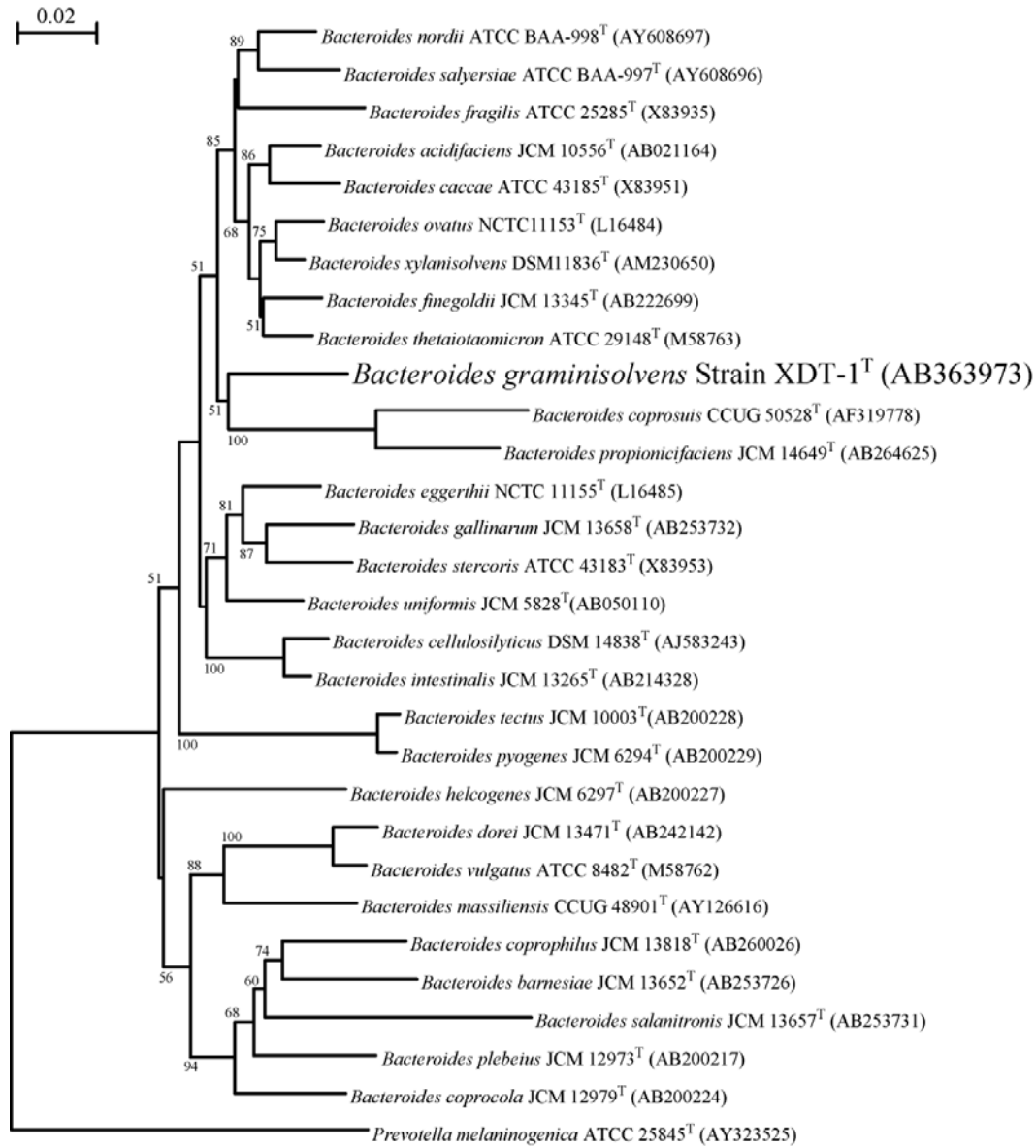


Table 1. Characteristics that differentiate strain XDT-1^T from other related *Bacteroides* species. Strains: 1, Strain XDT-1^T; 2, *Bacteroides ovatus* ATCC 8483^T (Holdeman *et al.*, 1984); 3, *Bacteroides thetaiotaomicron* ATCC 29148^T (Holdeman *et al.*, 1984); 4, *Bacteroides xyloxylicus* DSM 18836^T (Chassard *et al.*, 2008). +, Positive; -, negative; v, variable depending on strains; +w, weak reaction; ND, no data available. A, acetate; P/p, propionate; S/s, succinate; py, pyruvate. Lower-case letters indicate minor products.

Characteristic	1	2	3	4
Isolation source	Methanogenic reactor	Human faeces	Human faeces	Human faeces
Optimum growth temp. (°C)	35	37	37	38
Genomic DNA G+C content (%)	38	39-43	40-43	42.8
Predominant quinones	MK-10	MK-10, MK-11	MK-10, MK-11	ND
Catalase activity	-	v	v	-
Growth in bile	-	+	+	ND
Indole production	-	+	+	-
Products from glucose	A, P, s	A, S, p	A, S, p	A, P, S
Acid production from:				
Trehalose	-	+	+	+
Melezitose	-	-	-	+
Starch	+	+	+	-
Xylan	+	+	-	+
Mannitol	-	-	-	+
Sorbitol	-	-	-	+
Glycerol	-	-	-	+
Salicin	+	+	-	+