Bacteroides graminisolvens sp. nov., a xylanolytic anaerobe isolated from a methanogenic reactor treating cattle waste

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A strictly anaerobic bacterial strain, designated XDT-1^T, was isolated from plant residue from 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 approximately 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, MK-10(H₀) was the major respiratory quinone and the genomic DNA G + C content was 38.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequences placed the strain in the phylum *Bacteroidetes*. The closest phylogenetic neighbour of strain XDT-1^T was *Bacteroides ovatus* NCTC 11153^T, with a 16S rRNA gene sequence similarity of 94.2%. On the basis of data from the phylogenetic, physiological and chemotaxonomic analyses, strain XDT-1^T represents a novel species of the genus *Bacteroides*, for which the name *Bacteroides graminisolvens* sp. nov. is proposed. The type strain is XDT-1^T (=JCM 15093^T =DSM 19988^T).

The *Bacteroides*–*Prevotella* group consists mainly 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 investigated as important decomposers of hemicellulose or xylan (Chassard *et al.*, 2007, 2008; Hayashi *et al.*, 2005; Hespell & Whitehead, 1990; Hopkins *et al.*, 2003). As hemicellulose and cellulose are the main components (30–40 %) of the plant cell wall, their concomitant decomposition is a key reaction for the effective methanogenic fermentation of waste that contains plant material (Collins *et al.*, 2005). In this study, we describe a novel xylanolytic bacterial strain, affiliated with the genus *Bacteroides*, isolated from a plant-residue sample (mainly rice straw)

from a methanogenic reactor treating waste from cattle farms.

Strain XDT-1^T was isolated from a sample of rice-straw residue obtained from a methanogenic reactor treating waste collected from cattle farms (up to 1000 cattle in total) in Betsukai-machi in Hokkaido, Japan. The reactor was of the vertical cylindrical type (1500 m³) and was operated at 35 °C. Rice straw used as matting at the cattle farms, containing cattle faeces and urine, was thrown into the reactors and treated as waste (Ueki *et al.*, 2008).

Strain XDT-1^T was cultivated anaerobically at 30 °C (unless indicated otherwise) by using peptone/yeast extract (PY) medium (10 g peptone and 5 g yeast extract l⁻¹, respectively) as a basal medium, with O₂-free mixed gas (N₂/CO₂, 95 : 5) as the headspace, as described by Ueki *et al.* (2006a). PY medium supplemented with 0.25 g each of glucose, cellobiose, maltose and soluble starch l⁻¹ as well as 15 g agar l⁻¹ (Difco) was designated PY4S agar and used for maintenance of the strain in agar slants. For enrichment culture and the 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.*, 2009). PY

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

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, a B-vitamin mixture (10 ml l⁻¹) was added to PYH medium (PYHV medium) for cultivation, as described below. The composition of the B-vitamin mixture used was as follows (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 1 M NaOH. Growth in liquid medium was monitored by recording changes in OD₆₆₀.

Anaerobic sludge samples obtained from the reactor were filtered through a mesh (2 mm pore size) and plant residue remaining on the mesh was collected. The plant-residue samples obtained were washed several times with sterile, anoxic diluent and homogenized in a Waring blender (10 000 r.p.m. for 10 min) under N₂ (Kaku *et al.*, 2000). The homogenized samples were successively diluted anaerobically and the enrichment culture was started by inoculating 1 ml from 10-fold diluted samples (10⁻⁴–10⁻⁶) into 9 ml 1/10PY-x liquid medium (1/10PY medium containing birch-wood xylan at 5 g l⁻¹). After incubation for approximately 1 week, 1 ml culture was transferred to 9 ml fresh 1/10PY-x medium. Growth of xylanolytic bacteria in the enrichment culture was confirmed by a decrease in the pH of the medium relative to culture without xylan (1/10PY medium). After several subcultures of the xylanolytic enrichment, the diluted enrichment cultures were inoculated to anaerobic roll tubes using 1/10PY-x agar medium, and some colonies were picked after incubation for 1 week. Strain XDT-1^T was finally obtained, from among isolates that included non-xylanolytic, Gram-positive bacteria, after purification using 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 strain XDT-1^T under air was investigated as described previously (Ueki *et al.*, 2008). Spore formation was assessed by observing cells after Gram-staining as well as by means of phase-contrast microscopy. Catalase activity was determined as described previously (Ueki *et al.*, 2008). Oxidase and nitrate-reducing activities were determined according to the methods described by Akasaka *et al.* (2003b). The utilization of various carbon sources was tested in PYH liquid medium supplemented with each substrate at either 10 g l⁻¹ (for sugars and sugar alcohols) or 30 mM (for organic acids). The utilization of each substrate was determined by measuring growth (as OD₆₆₀) as well as by determining changes in the pH of the medium

after cultivation. Bile sensitivity was determined by adding Bacto oxgall (Difco) (0.1–2 %, w/v) or bile salts (Oxoid) (0.1–0.5 %, w/v) to PYHG medium. Fermentation products were analysed by means of GC or HPLC, as described previously (Ueki *et al.*, 1986; Akasaka *et al.*, 2003a). Other characterizations were performed according to the methods described by Holdeman *et al.* (1977).

Whole-cell fatty acids were converted to methyl esters according to the method of Miller (1982) and were analysed using a gas chromatograph (Hewlett Packard model Hp6890 or Hitachi model G-3000) equipped with an HP Ultra 2 column. Whole-cell fatty acids were identified from equivalent chain-lengths (Miyagawa *et al.*, 1979) according to the protocol of TechnoSuruga (Moore *et al.*, 1994). Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analysed using a mass spectrometer (JMS-SX102A; JEOL). Genomic DNA extracted according to the method described by Akasaka *et al.* (2003b) was digested with P1 nuclease by using a Yamasa GC kit (Yamasa Shoyu) and its DNA G + C content was measured by means of HPLC (L-7400; Hitachi) equipped with a µBondapak C18 column (3.9 × 300 mm; Waters).

DNA was extracted from cells as described previously (Akasaka *et al.*, 2003b). The almost-complete 16S rRNA gene was PCR-amplified using primers 27f and 1546r. The PCR-amplified 16S rRNA gene was sequenced using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit and an ABI Prism 3730 automatic DNA sequencer (Applied Biosystems). Multiple alignments of the sequence with reference sequences in GenBank were performed with the BLAST program (Altschul *et al.*, 1997). A phylogenetic tree was constructed with the neighbour-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, usually 0.4–0.6 µm wide and 1.2–4.5 µm long. Rather long cells often occurred (Fig. 1). Under phase-contrast microscopy, cells were observed to be non-motile. The strain formed very thin colonies with smooth surfaces 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 ($\mu=0.14$ h⁻¹) in PYG liquid medium (without haemin and the B-vitamin mixture); the addition of haemin to the medium (PYHG) stimulated growth considerably ($\mu=0.39$ h⁻¹). Although addition of the vitamin mixture alone to PYG medium did not affect the growth rate ($\mu=0.13$ h⁻¹), the addition of a vitamin mixture or cobalamin to PYHG liquid medium (PYHVG medium) slightly enhanced growth ($\mu=0.40$ – 0.41 h⁻¹).

Catalase activity was not detected in cells grown in PY4S agar slants or in PYG liquid medium as well as cells grown

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

in PYHG medium. Oxidase activity was not detected. The strain utilized various sugars in addition to xylan and glucose (see the species description and Table 1). Substrates that were not utilized are also listed in the species 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 and there was a concomitant decrease in succinate production (acetate, 1.5 mmol l⁻¹; propionate, 3.6 mmol l⁻¹; succinate, 2.9 mmol l⁻¹). The final pH was 4.9. Almost the same concentrations of products were formed from xylose in PYH medium. When growth was performed on PYH medium containing xylan (5 g l⁻¹), propionate was the predominant product (5.8 mmol l⁻¹), with smaller concentrations 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 2 days. The final pH with xylan was 5.2–5.3. Almost the same concentrations of products were detected when growth was performed in the medium containing 10 g xylan l⁻¹, even after 7 days incubation. The decrease in pH of the medium should limit xylan utilization. The vitamin mixture and cobalamin showed the same effect on the fermentation products; thus, cobalamin in the vitamin mixture appeared to stimulate propionate production.

The optimum pH was 7.2 ($\mu=0.39$ h⁻¹) and the pH range for growth was 6.1–8.2. The strain did not grow at an initial pH of 5.0 or 8.7. The growth temperature range was 5–40 °C, with an optimum at 30–35 °C ($\mu=0.39$ –0.41 h⁻¹). Although the growth rate at 40 °C ($\mu=0.45$ h⁻¹) was

Table 1. Characteristics that serve to differentiate strain XDT-1^T from related species of the genus *Bacteroides*

Strains/species: 1, strain XDT-1^T; 2, *B. ovatus*; 3, *B. thetaiotaomicron*; 4, *B. xylanisolvens* DSM 18836^T. Data for reference taxa were taken from Holdeman *et al.* (1984) and Chassard *et al.* (2008). +, Positive; –, negative; v, variable, depending on strain; ND, no data available.

Characteristic	1	2	3	4
Isolation source	Methanogenic reactor	Human faeces	Human faeces	Human faeces
Optimum growth temperature (°C)	35	37	37	38
Genomic DNA G + C content (mol%)	38	39–43	40–43	42.8
Predominant quinone(s)	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	+	+	–	+

*A, Acetate; P, propionate; S, succinate. Minor products are given in parentheses.

higher than that at 35 °C, the maximum OD₆₆₀ of the culture was lower than that reached at 35 °C and the OD₆₆₀ soon declined, indicating cell lysis. The strain even grew at 5 °C, albeit 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 an optimum at 0 % (w/v) NaCl. Aesculin and starch were hydrolysed, but gelatin was not hydrolysed. Nitrate-reducing and urease activities were not detected. The strain did not grow in the presence of 2 % (w/v) Bacto oxgall or 0.1 % (w/v) bile acids; growth was strongly inhibited even in the presence of 0.1 % (w/v) Bacto oxgall. The strain did not produce indole or sulfide.

The major whole-cell fatty acids of strain XDT-1^T 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 fatty acids included 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 %). MK-10(H₀) was the major respiratory quinone and the genomic DNA G+C content was 38.0 mol%.

Phylogenetic analysis based on the 16S rRNA gene sequence placed the strain in the phylum *Bacteroidetes*. The closest relative of strain XDT-1^T was *Bacteroides ovatus* NCTC 11153^T, with 16S rRNA gene sequence similarity of 94.2 %. The next closest relatives were *Bacteroides thetaiotaomicron* ATCC 29148^T (similarity of 94.0 %) and *Bacteroides xylanisolvens* DSM 18836^T (93.6 %). *Bacteroides eggerthii* NCTC 11155^T (93.3 %), *Bacteroides acidifaciens* JCM 10556^T (93.3 %), *Bacteroides caccae* ATCC 43185^T (93.1 %), *Bacteroides finegoldii* JCM 13345^T (92.7 %) and *Bacteroides fragilis* ATCC 25285^T (92.5 %) were the next most closely related members of the genus *Bacteroides*.

Of the few xylan-decomposing species that belong to the genus *Bacteroides*, *B. ovatus* is one that has been investigated extensively with regard to its xylanase system (Cooper *et al.*, 1985; Hopkins *et al.*, 2003). *B. xylanisolvens*, isolated from human faeces, was described quite recently as a xylanolytic bacterium (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* was described as producing acids from xylan (Holdeman *et al.*, 1984). Furthermore, recent reports have indicated that *Bacteroides intestinalis* and *Bacteroides dorei* (Bakir *et al.*, 2006a, b), both of which were isolated from human faeces, have the ability to decompose hemicellulose (Chassard *et al.*, 2007). Thus, the phylogenetic placement of strain XDT-1^T is close to known xylanolytic species of the genus *Bacteroides* (Fig. 2).

Hemicellulose and xylan are the predominant components of plant biomass, and thus their decomposition is an important function of fermentative bacteria in anaerobic environments containing plant material. Recent reports have indicated the importance of species from the genus *Bacteroides* in the decomposition of hemicellulose in the

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

human intestine. Hopkins *et al.* (2003) reported that xylan breakdown by human intestinal microbes resulted in increased propionate formation relative to that formed from starch breakdown (which was associated with the production of acetate and butyrate). Strain XDT-1^T produced acetate and propionate, with smaller amounts of succinate, in the presence of the B-vitamin mixture or cobalamin from various saccharides. Furthermore, more propionate and less succinate were produced from xylan than were produced from glucose or xylose. The data are consistent with the results of Hopkins *et al.* (2003) and suggest that hemicellulose decomposition is an important function of the *Bacteroides* species in anaerobic environments and that this may cause increases in propionate production in such habitats.

Some characteristics of strain XDT-1^T and its closest relatives 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 of the closest relatives of strain XDT-1^T were also derived from human faeces. Although XDT-1^T shares common morphological, physiological and chemotaxonomic characteristics with *Bacteroides* species isolated

from mammalian samples, the strain has some features that serve to differentiate it from its 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 despite careful examination (Ueki *et al.*, 2008; Wilkins *et al.*, 1978). Bile resistance is one of the characteristics specific to *Bacteroides* species, yet our strain was highly sensitive to bile. The strain did not produce indole. *B. thetaiotaomicron* has been clearly differentiated from *B. ovatus* on the basis of its inability to ferment xylan (Cooper *et al.*, 1985) and the range of substrates utilized by strain XDT-1^T differed from those of any of the closely related species. The whole-cell fatty acid profile of XDT-1^T was basically consistent with those of *Bacteroides* species, although the percentages of some whole-cell fatty acids, such as anteiso-C_{13:0} (7.0%), C_{16:0} (3.0%) and C_{16:0} 3-OH (1.3%), were rather different in the novel strain (Miyagawa *et al.*, 1979; Shah & Collins, 1980).

A haemin requirement is commonly found in *Bacteroides* species. We recently described three novel species (isolated from plant residue from irrigated rice-field soil as well as a methanogenic reactor) that were related to those in the *Bacteroides-Prevotella* group. All three species also required haemin for growth (Ueki *et al.*, 2006b, 2007, 2008). These results suggest that bacterial species related to those of the *Bacteroides-Prevotella* group living in habitats other than the mammalian body also require haemin for growth. A cobalamin requirement was also commonly found in propionate-producing strains of the above-mentioned novel species.

Xylanolytic *Bacteroides* species such as *B. ovatus* and *B. fragilis* are known to be isolated only rarely 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 for xylanolytic bacteria, the peptone and yeast extract concentrations in the medium were reduced to one-tenth of those in the normal PY medium. In our experience, higher concentrations of these components in medium used for the enrichment of xylan- or cellulose-decomposing bacteria usually enhanced the growth of fast-growing proteolytic or aminolytic bacteria, and these bacteria usually outcompeted polysaccharide-decomposing bacteria (Nishiyama *et al.*, 2009). The use of reduced concentrations of peptone and yeast extract should suppress the growth of non-xylanolytic bacteria compared with that produced in the normal PY medium, and thus xylan-decomposing *Bacteroides* species should be enriched successfully. Furthermore, we used homogenized plant residue as an inoculum for the enrichment. We have previously isolated three novel species belonging to the *Bacteroides-Prevotella* group from plant residue samples obtained from rice-field soil and a methanogenic reactor, as described above (Ueki *et al.*, 2006b, 2007, 2008). Thus, it seems likely that bacterial species related to those of the *Bacteroides-Prevotella* group often live on plant residue as decomposers of hemicellulose or other plant material (e.g. pectin) in various anaerobic environments.

On the basis of the data from these phylogenetic, physiological and chemotaxonomic analyses, strain XDT-1^T represents a novel species of the genus *Bacteroides*, for which the name *Bacteroides graminisolvens* sp. nov. is proposed.

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 wide and 1.2–4.5 µm long); some longer cells occur. Produces thin, smooth-surfaced colonies with a pearl-like, iridescent lustre under light. Growth is strongly stimulated by the addition of haemin; cobalamin (vitamin B₁₂) slightly enhances growth. Utilizes arabinose, xylose, fructose, galactose, glucose, mannose, cellobiose, lactose, maltose, melibiose, sucrose, raffinose, xylo-oligosaccharides, dextrin, glycogen, starch, pectin, xylan (birch wood), amygdalin and salicin. Ribose, rhamnose, inulin, aesculin and pyruvate are utilized only weakly. In the presence of haemin and cobalamin, acetate and propionate are produced from glucose, along with small amounts of succinate. Propionate is the most abundant product from xylan in the presence of haemin and cobalamin. In the presence of glucose and xylan, the final pH is 4.9 and 5.2–5.3, respectively. Does not use sorbose, trehalose, melezitose, CM-cellulose, cellulose (filter-paper fragments and ball-milled powder), glycerol, dulcitol, inositol, mannitol, sorbitol, ethanol, fumarate, lactate, malate or succinate. The optimum pH is 7.2. The growth temperature range is 5–40 °C; the optimum is 30–35 °C. The NaCl concentration range for growth is 0–4.0% (w/v) in PYHG medium; optimum growth in the absence of NaCl. Does not have catalase, oxidase, nitrate-reducing or urease activities. Does not produce hydrogen sulfide or indole. Hydrolyses 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 and the major respiratory quinone is MK-10(H₀). The genomic DNA G+C content of the type strain is 38.0 mol%.

The type strain, XDT-1^T (=JCM 15093^T =DSM 19988^T), was isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms.

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