Bacteroides paurosaccharolyticus sp. nov., isolated from a methanogenic reactor treating waste from cattle farms

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A strictly anaerobic bacterial strain (WK042^T) was isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms in Japan. Cells were Gram-staining-negative, non-motile, non-spore-forming rods. Growth was stimulated well by haemin, and was enhanced by cobalamin (vitamin B₁₂). Strain WK042^T utilized arabinose, xylose, glucose, mannose and aesculin as preferred substrates. Maltose, dextrin, glycogen, starch and pectin were also utilized, although growth on these substrates was much slower. The strain produced acetate, propionate and succinate from these saccharides. The strain was slightly alkaliphilic, with optimum growth at pH 7.7. The temperature range for growth was 10–40 °C, the optimum being 35 °C. The strain was sensitive to bile. The major cellular fatty acids were anteiso-C_{15:0}, iso-C_{17:0} 3-OH and C_{15:0}. Menaquinone 11 (MK-11) was the major respiratory quinone and the genomic DNA G + C content was 41.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequences placed the strain in the phylum *Bacteroidetes*. Strain WK042^T was related distantly to the type strains of species in the cluster including *Bacteroides massiliensis*, *Bacteroides vulgatus* and *Bacteroides dorei* (91–92% 16S rRNA gene sequence similarity). Based on data from the present phylogenetic, physiological and chemotaxonomic analyses, strain WK042^T is considered to represent a novel species of the genus *Bacteroides*, for which the name *Bacteroides paurosaccharolyticus* sp. nov. is proposed. The type strain is WK042^T (=JCM 15092^T =DSM 21004^T).

Bacteroides species are strictly anaerobic, Gram-staining-negative, non-spore-forming rods and are major members of the bacterial flora in the human colon and in human faeces (Holdeman *et al.*, 1984; Paster *et al.*, 1994). Although bacterial clones belonging to the genus *Bacteroides* have often been detected as dominant members of the bacterial flora in methanogenic reactors (Chouari *et al.*, 2005; Godon *et al.*, 1997; Levén *et al.*, 2007), only a few strains affiliated to *Bacteroides* have been isolated from this or similar habitats (Nishiyama *et al.*, 2009b; Ueki *et al.*, 2008; Whitehead *et al.*, 2005). In this study, we describe a novel species in the genus *Bacteroides* based on the phylogenetic, physiological and chemotaxonomic characteristics of strain WK042^T isolated from a methanogenic reactor.

Strain WK042^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, Hokkaido, Japan. The reactor was a vertically cylindrical type (1500 m³) operated at 35 °C. Rice straw used as matting at the cattle farms, containing cattle faeces and urine, was thrown into the reactor and treated as waste (Nishiyama *et al.*, 2009a, b; Ueki *et al.*, 2008).

Strain WK042^T was isolated by the anaerobic roll-tube method for enumeration of anaerobic fermentative bacteria (Hungate, 1966; Holdeman *et al.*, 1977). The rice-straw samples obtained from the reactor were washed several times with sterile anoxic diluent and homogenized in a Waring blender (10 000 r.p.m. for 10 min) under a N₂ atmosphere. The homogenized samples were successively diluted (10-fold) anaerobically and used as inocula to the anaerobic roll-tube agar (PY4S medium) for isolation of anaerobic bacteria (Akasaka *et al.*, 2003a). Colonies that formed on the agar were picked at random after incubation for 2 weeks at 30 °C and about 50 isolates were obtained from a sample. Strain WK042^T was selected as a

Abbreviation: CFA, cellular fatty acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WK042^T is AB298727.

representative of four strains that showed similar phenotypic properties and similar denaturing gradient gel electrophoresis profiles based on the V3 region of the 16S rRNA gene sequence (Ueki *et al.*, 2008). Strain WK042^T was picked from a roll-tube inoculated with a sample diluted 10⁻⁴.

Strain WK042^T was cultivated anaerobically at 30 °C unless stated otherwise by using peptone/yeast extract (PY) as the basal medium with oxygen-free mixed gas (N₂/CO₂, 95:5) as the headspace, as described by Ueki *et al.* (2006a). PY medium supplemented with (per litre) 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 on agar slants. PY broth supplemented with haemin (at a final concentration of 5 mg l⁻¹) (Holdeman *et al.*, 1977) (PYH) and a B-vitamin mixture (10 ml l⁻¹) (PYHV) (Ueki *et al.*, 2006b, 2008) as well as 10 g glucose l⁻¹ (PYHVG) was used for cultivation of strain WK042^T for various physiological tests and chemotaxonomic analyses unless stated otherwise. When cyanocobalamin (cobalamin or vitamin B₁₂) was used as a sole vitamin added to the medium, it was used at the same concentration as that in the B-vitamin mixture. The pH of all media was adjusted to 7.0–7.3 except for determination of the pH range for growth of strain WK042^T. *Bacteroides massiliensis* JCM 13223^T and *Bacteroides vulgatus* JCM 5826^T were cultivated in the same media and under the same conditions as strain WK042^T and their phenotypic characteristics were determined by using the same methods described below.

Growth of strain WK042^T under aerobic conditions was examined as described previously (Ueki *et al.*, 2006a). Spore formation was assessed by observing cells after Gram staining, and production of thermotolerant cells was examined by cultivating heat-treated (80 °C for 10 min) cells in PYHVG broth. The motility of cells (from both slant and broth cultures) was examined via phase-contrast microscopy. Oxidase and nitrate-reducing activities were determined according to the methods described by Akasaka *et al.* (2003b). Catalase activity was examined by using cells cultivated in liquid medium and collected by centrifugation. A small amount of H₂O₂ solution (3 %, v/v) was mixed with the cell pellet (Wilkins *et al.*, 1978). The optimum growth conditions were tested at 5–45 °C (at 5 °C intervals with an exception at 37 °C), in the presence of 0, 0.5, 1, 2 and 3 % (w/v) NaCl in PHHVG broth and at pH 4.1, 4.8, 5.7, 6.8, 7.7, 8.2, 9.0 and 10.3 (pH verified after autoclaving). Bicine (Good's buffer; Dotite) (20 mM) was used to adjust the medium to pH values greater than 8.0 in modified PYHVG broth (Ueki *et al.*, 2008). Growth in liquid medium was monitored by changes in OD₆₆₀. Utilization of carbon sources was tested in PYHV broth, each substrate being added at 10 g l⁻¹ (for sugars and sugar alcohols) or 30 mM (for organic acids). Utilization of each substrate was determined from growth measurements (OD₆₆₀) as well as by determining fermentation products in the medium after cultivation. Fermentation

products were analysed by GC or HPLC as described previously (Ueki *et al.*, 1986; Akasaka *et al.*, 2003a). Bile sensitivity was determined in PYHVG broth supplemented with 0.1–2 % (w/v) oxgall (Difco). Production of urease, H₂S and indole as well as hydrolysis of aesculin and gelatin were tested according to the methods described by Holdeman *et al.* (1977).

Cells of strain WK042^T were Gram-staining-negative, non-motile rods. The strain grew as very thin, translucent colonies with a smooth surface on PY4S agar. Strain WK042^T did not grow in air, on either PY4S or nutrient agar. Spore formation was not observed and thermotolerant cells were not produced.

Strain WK042^T grew very slowly in PYG broth (without haemin and the B-vitamin mixture) at a growth rate (μ) of 0.075 h⁻¹, but the addition of haemin (PYHG) stimulated growth dramatically (μ =0.208 h⁻¹). Addition of the vitamin mixture or cobalamin to the medium further enhanced growth (μ =0.244 h⁻¹). The vitamin mixture and cobalamin each showed similar effects on growth and thus cobalamin appeared to stimulate growth of the strain. Addition of the vitamin mixture or cobalamin alone (without haemin) did not enhance growth. Further supplementation of the medium with vitamin K did not appear to affect the growth rate. Thus, physiological characteristics of the strain were usually determined in the presence of haemin and the vitamin mixture (PYHV broth). When cells rapidly grown in PYHVG broth to the stationary phase were used as inocula to the same fresh medium, growth was usually delayed significantly, suggesting that most of the cells had lost the ability to grow. Thus, the strain was usually maintained on PY4S agar slants (without haemin) with rather short transfer intervals for safe preservation.

Catalase activity was not detected in cells grown in PY4S agar slants or in liquid media (PYG, PYHG or PYHVG). Strain WK042^T utilized only a restricted range of substrates to support rapid growth, namely arabinose, xylose, glucose, mannose and aesculin. The final pH after growth was 4.9–5.1. Although maltose, dextrin, glycogen, starch and pectin were also utilized by the strain, growth on these substrates was much delayed compared with that on the substrates mentioned above. Rhamnose and pyruvate were utilized weakly. Substrates tested but not used by strain WK042^T are listed in the species description. The strain produced acetate (2.8 mmol l⁻¹), propionate (4.6 mmol l⁻¹) and succinate (6.8 mmol l⁻¹) from glucose in PYHV medium. Almost the same amounts of products were formed from arabinose and xylose. When grown on pyruvate, propionate was the dominant product (8.2 mmol l⁻¹) together with acetate (4.2 mmol l⁻¹), while succinate was detected as only a minor product (1.0 mmol l⁻¹). Strain WK042^T grew at pH 5.7–9.0; it was slightly alkaliphilic, with optimum growth at about pH 7.7 (μ =0.233, 0.271 and 0.132 h⁻¹ at pH 6.8, 7.7 and 8.2, respectively). The temperature range for growth was 10–40 °C, the optimum

being at 35 °C ($\mu=0.239, 0.348$ and 0.302 h^{-1} at 30, 35 and 37 °C, respectively). The strain did not grow at 5 or 45 °C. The NaCl concentration range for growth was 0–2 % (w/v, in PYHVG medium) and optimum growth occurred in the absence of added NaCl. The strain was sensitive to bile; oxgall inhibited growth completely, even at 0.1 % (w/v). Other physiological characteristics are detailed in the species description.

The almost-complete 16S rRNA gene sequence of strain WK042^T was PCR-amplified by using the primer pair 8f and 1546r and a DNA sample was extracted from cells as described previously (Akasaka *et al.*, 2003b). The PCR-amplified 16S rRNA gene was sequenced by 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 amplified sequence (1476 bp) 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) and with the maximum-likelihood program (DNAML) of the PHYLIP 3.66 package (Felsenstein, 2006). All gaps and unidentified base positions in the alignments were excluded before sequence assembly.

Phylogenetic analysis based on 16S rRNA gene sequences placed strain WK042^T in the phylum *Bacteroidetes* (Garrity & Holt, 2001). Strain WK042^T had at least two different 16S rRNA gene sequences, with C or T at position 183 (*Escherichia coli* numbering). Strain WK042^T was related most closely to *B. massiliensis* B84634^T (Fenner *et al.*, 2005)

isolated from blood culture of a healthy baby (92.2 % 16S rRNA gene sequence similarity), *B. vulgatus* ATCC 8482^T (Holdeman *et al.*, 1984) isolated from human faeces (92.1 %) and *Bacteroides dorei* JCM 13471^T (Bakir *et al.*, 2006), also isolated from human faeces (91.3 %). Levels of similarity were calculated by using the sequence giving the highest value, i.e. with T at position 183 for comparison with *B. massiliensis* B84634^T and with C for comparison with *B. vulgatus* ATCC 8482^T. In contrast to the three other strains, in *B. dorei* JCM 13471^T the base at this position was G. Strain WK042^T formed a distinct branch in the phylogenetic tree constructed by using the neighbour-joining method (Fig. 1). The tree topology evaluated by the maximum-likelihood method was essentially the same (not shown).

For cellular fatty acid (CFA) analysis, strain WK042^T, *B. massiliensis* JCM 13223^T and *B. vulgatus* JCM 5826^T were cultivated in PYHVG broth for 72 h at 30 °C. CFAs were converted to methyl esters by saponification and methylation by using the cell biomass according to the method of Miller (1982). CFAs were extracted from the reaction mixtures, and the compositions were analysed by GC (Hewlett Packard HP6890 or Hitachi G-3000), the chromatograph being equipped with an HP Ultra 2 column. Each peak was identified from its equivalent chain-length (Miyagawa *et al.*, 1979) according to the protocol of TechnoSuruga (Shimidu, Japan) (Moore *et al.*, 1994). The major CFAs of strain WK042^T were anteiso-C_{15:0} (24.5 %), iso-C_{17:0} 3-OH (15.5 %), C_{15:0} (12.9 %), C_{16:0} (7.2 %), anteiso-C_{13:0} (6.9 %), iso-C_{15:0} (6.7 %) and iso-C_{13:0} (6.6 %). The overall CFA profiles of strain

Fig. 1. Neighbour-joining tree showing the phylogenetic relationship between strain WK042^T and the type strains of recognized *Bacteroides* species based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) above 70 % are shown at branch nodes. The sequence of *Prevotella melaninogenica* ATCC 25845^T was used as the outgroup. The tree topology evaluated by using the maximum-likelihood method was almost the same as that obtained with the neighbour-joining method. Bar, 2 % estimated difference in nucleotide sequence position.

WK042^T and the two reference strains were rather similar (Table 1), sharing common features with all *Bacteroides* species in having anteiso-C_{15:0}, iso-C_{17:0} 3-OH and C_{15:0} as major components (Miyagawa *et al.*, 1979; Moore *et al.*, 1994). However, the profile of strain WK042^T differed from those of related species by the respective proportions of some CFAs, such as C_{15:0} and iso-C_{15:0}.

Genomic DNA of the three strains was extracted according to the method described by Akasaka *et al.* (2003b) and digested with P1 nuclease by using a Yamasa GC kit (Yamasa Shoyu). DNA G+C contents were measured by HPLC (L-7400; Hitachi) equipped with a μ Bondapak C18 column (3.9 $\times$ 300 mm; Waters). The genomic DNA G+C content of strain WK042^T was 41.0 mol%. Those of *B. massiliensis* JCM 13223^T and *B. vulgatus* JCM 5826^T determined in this study were 45.8 and 44.3 mol%, respectively. Isoprenoid quinones were extracted as described by Komagata & Suzuki (1987) and analysed by using a mass spectrometer (JMS-SX102A; JEOL). Menaquinone 11 (MK-11) was the major respiratory quinone of strain WK042^T.

Strain WK042^T shared common characteristics (e.g. cell morphology, haemin requirement, products from glucose and respiratory quinone composition) with recognized

Table 1. Cellular fatty acid compositions of strain WK042^T and the type strains of related *Bacteroides* species

Strains: 1, WK042^T; 2, *B. massiliensis* JCM 13223^T; 3, *B. vulgatus* JCM 5826^T. Data were obtained in this study. Values are percentages of total fatty acids; fatty acids amounting to <0.5% of the total in all three strains are not shown. tr, Trace (<0.5%); –, not detected.

Fatty acid	1	2	3
Saturated straight chain			
C _{13:0}	1.0	–	tr
C _{14:0}	2.0	1.3	0.7
C _{15:0}	12.9	2.9	14.7
C _{16:0}	7.2	5.4	1.5
C _{17:0}	1.3	–	–
C _{18:0}	–	1.6	–
Saturated branched chain			
iso-C _{13:0}	6.6	1.6	1.3
anteiso-C _{13:0}	6.9	4.1	2.8
iso-C _{14:0}	1.0	1.5	2.4
iso-C _{15:0}	6.7	14.0	7.7
anteiso-C _{15:0}	24.5	34.1	30.3
iso-C _{16:0}	tr	1.7	0.6
iso-C _{17:0}	1.4	4.1	tr
anteiso-C _{17:0}	1.4	2.6	tr
Hydroxy			
C _{15:0} 3-OH	tr	0.9	3.1
C _{16:0} 3-OH	3.0	3.1	2.9
iso-C _{17:0} 3-OH	15.5	16.5	21.8
anteiso-C _{17:0} 3-OH	2.0	–	2.4
C _{17:0} 3-OH	1.5	–	1.8

Bacteroides species (Holdeman *et al.*, 1977, 1984; Shah & Collins, 1980), supporting its assignment to the genus *Bacteroides* based on 16S rRNA gene sequence analysis. However, strain WK042^T displayed a number of characteristics that differentiated it from closely related species (*B. massiliensis* and *B. vulgatus*) (Table 2). The two closest relatives of strain WK042^T were derived from human faeces. Their growth was stimulated strongly in the presence of oxgall (2%), whereas strain WK042^T did not grow even in the presence of only a small amount (0.1%) of oxgall. The substrate utilization range of strain WK042^T was relatively restricted, and the strain did not use the variety of saccharides utilized by *B. massiliensis* and *B. vulgatus*. The optimum temperature for growth (35 °C) and the slightly alkaliphilic character of strain WK042^T were in contrast to its closest relatives. The genomic DNA G+C content of strain WK042^T was slightly lower than those of the type strains of *B. massiliensis* and *B. vulgatus*.

Almost all substrates preferably utilized by strain WK042^T (arabinose, xylose, glucose and mannose) are major components of hemicellulose and cellulose in plant biomass (Collins *et al.*, 2005; Hopkins *et al.*, 2003). Thus, it seems likely that the bacterium lives mainly on monomers derived from the degradation of plant biomass by other hemicellulose or cellulose decomposers. This is in agreement with the source of isolation (rice-straw residue in a methanogenic reactor) of strain WK042^T. The significance of the ability of members of the *Bacteroides*–*Prevotella* group to decompose hemicellulose or xylan has been discussed previously (Chassard *et al.*, 2008; Hespell &

Table 2. Characteristics that differentiate strain WK042^T from type strains of related *Bacteroides* species

Strains: 1, WK042^T; 2, *B. massiliensis* JCM 13223^T; 3, *B. vulgatus* JCM 5826^T. +, Positive; –, negative; s, stimulatory; w, weakly positive. All data are from this study. The DNA G+C contents reported in the original species descriptions of *B. massiliensis* (Fenner *et al.*, 2005) and *B. vulgatus* (Holdeman *et al.*, 1984) are shown in parentheses. All other data are in agreement with the original descriptions.

Characteristic	1	2	3
Genomic DNA G+C content (mol%)	41.0	45.8 (49)	44.3 (40–42)
Optimum growth temperature (°C)	35	37	37
Growth in bile	–	s	s
Acid production from:			
Arabinose	+	–	+
Xylose	+	–	+
Fructose	–	+	+
Lactose	–	+	+
Melibiose	–	+	w
Sucrose	–	+	+
Raffinose	–	+	+

Whitehead, 1990; Nishiyama *et al.*, 2009b; Ueki *et al.*, 2006b, 2007).

Based on the phylogenetic, physiological and chemotaxonomic data presented, strain WK042^T is considered to represent a novel species of the genus *Bacteroides*, for which the name *Bacteroides paurosaccharolyticus* sp. nov. is proposed.

Description of *Bacteroides paurosaccharolyticus* sp. nov.

Bacteroides paurosaccharolyticus (pau.ro.sac.cha.ro.ly'ti.cus. Gr. adj. *pauros* little or a few; Gr. n. *sakchâr* sugar; Gr. adj. *lutikos* dissolving; N.L. masc. adj. *paurosaccharolyticus* dissolving a few kinds of sugars).

Cells are Gram-staining-negative, non-motile, non-spore-forming rods, 0.7–0.8 µm wide and 1.3–5.0 µm long. Strictly anaerobic. Colonies are thin and translucent with a smooth surface. Haemin stimulates growth considerably, and cobalamin (vitamin B₁₂) enhances growth slightly. Utilizes arabinose, xylose, glucose, mannose and aesculin. Maltose, dextrin, glycogen, starch and pectin are also used, although growth is quite slow. Utilizes rhamnose and pyruvate weakly. Produces acetate, propionate and succinate from saccharides in the presence of haemin and cobalamin. Does not utilize ribose, fructose, galactose, sorbose, cellobiose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, CM-cellulose, cellulose powder, xylan, inulin, amygdalin, salicin, glycerol, dulcitol, inositol, mannitol, sorbitol, ethanol, fumarate, lactate, malate or succinate. Slightly alkaliphilic; optimum growth at pH 7.7. The temperature range for growth is 10–40 °C (optimum 35 °C). Grows in the presence of 0–2 % (w/v) NaCl, with optimum growth in the absence of NaCl. Negative for catalase, oxidase, nitrate reduction and urease activity. Does not produce indole or hydrogen sulfide. Aesculin is hydrolysed but gelatin is not. Sensitive to bile. The major CFAs are anteiso-C_{15:0}, iso-C_{17:0} 3-OH and C_{15:0}. The major respiratory quinone is menaquinone 11 (MK-11). The genomic DNA G+C content of the type strain is 41.0 mol%.

The type strain, WK042^T (=JCM 15092^T =DSM 21004^T), was isolated from a sample of rice-straw residue in a methanogenic reactor treating waste from cattle farms in Japan.

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