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

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Running title: *Bacteroides paurosaccharolyticus* sp. nov.

Abbreviations: CFA, cellular fatty acid.

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

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 remarkably stimulated by haemin, and cobalamin (vitamin B_{12}) enhanced the growth. The strain utilized arabinose, xylose, glucose, mannose and aesculin as preferable substrates. Maltose, dextrin, glycogen, starch and pectin were also utilized, however, the growth on these substrates was much slower. The strain produced acetate, propionate and succinate from these saccharides. The strain was slightly alkaliphilic, having a pH optimum at 7.7. The temperature range for growth was 10-40^{o}C, the optimum being 35^{o}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 the 16S rRNA gene sequence placed the strain in the phylum *Bacteroidetes*. The strain was remotely related to the species in the cluster including *Bacteroides massiliensis*, *Bacteroides vulgatus and Bacteroides dorei* (91-92% 16S rRNA gene sequence similarity). Based on the phylogenetic, physiological and chemotaxonomic analyses, the new species *Bacteroides paurosaccharolyticus* sp. nov. is proposed to accommodate the strain. 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 colon or faeces of human (Holdeman *et al*., 1984; Paster *et al*., 1994). Although bacterial clones belonging to the genus *Bacteroides* have often been detected as one of the 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* species 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 in Japan.

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 m^3) operated at 35^{o}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 (10000 r.p.m. for 10 min.) under N_2 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 two weeks at 30ºC and about 50 isolates were obtained from a sample. Strain WK042^T was selected as a representative out of four strains, which showed similar phenotypic properties and DGGE 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 10^{-4} diluted sample.

Strain WK042^T was cultivated anaerobically at 30ºC unless otherwise stated using peptone/yeast extract (PY) as the basal medium with oxygen-free mixed gas (N_2/CO_2, 95/5) 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 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 otherwise stated. When cyanocobalamin (cobalamin or vitamin B_{12}) 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 pH 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 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 (80ºC for 10 min) cells in PYHVG broth. The motility of cells (from both slant and broth cultures) was examined using phase-contrast microscopy. Oxidase and nitrate-reducing activities were determined according to the methods described by Akasaka *et al*. (2003b). Catalase activity was examined using cells cultivated in liquid media and collected by centrifugation. A small amount of H_2O_2 solution (3%, v/v) was mixed with the cell pellet (Wilkins *et al*., 1978). The optimum growth conditions were tested on temperatures at 5-45ºC (at 5ºC intervals with an exception at 37ºC), 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 (as values verified after autoclaving). Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] (Good's buffer; Dotite) (20mM) was used to adjusted the pH higher than 8.0 with some modifications of PHHVG broth (Ueki *et al*., 2008). Growth in liquid medium was monitored by changes in OD_{660}. 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 measurement (OD_{660}) as well as by determining fermentation products in the medium after cultivation. Fermentation products were analyzed 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, H_2S 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 very thinly as translucent colonies with smooth surface on PY4S agar. The strain 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}, and the addition of haemin (PYHG) remarkably stimulated the growth (μ = 0.208 h^{-1}). Addition of the vitamin mixture or cobalamin to the medium further enhanced the growth (μ = 0.244 h^{-1}). The vitamin mixture and cobalamin each showed similar effects on growth and thus cobalamin appeared to stimulate the 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, the growth was usually significantly delayed, suggesting that most of the cells had lost ability to grow. Thus, the strain was usually maintained on PY4S agar slants (without haemin) with rather short intervals of the transfer for safe preservation.

Catalase activity was not detected in cells grown in PY4S agar slants or in liquid media (PYG, PYHG or

PYHVG). The strain utilized only a restricted range of substrates to support rapid growth, that is, 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 as compared with that on the substrates mentioned above. Rhamnose and pyruvate were weakly used. Substrates tested but not used by strain WK042^T are shown 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 produced as the dominant product (8.2 mmol l^{-1}) as well as acetate (4.2 mmol l^{-1}), while succinate was detected as only a minor product (1.0 mmol l^{-1}). The pH range for growth was pH 5.7-9.0, and the strain was slightly alkaliphilic with the pH optimum at about 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^{o}C, the optimum being at 35^{o}C (μ = 0.239, 0.348 and 0.302 h^{-1}at 30, 35 and 37^{o}C, respectively). The strain did not grow at 5 and 45^{o}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; even 0.1% (w/v) Oxgall completely inhibited the growth. Other physiological characteristics are shown in the species description.

The almost-complete 16S rRNA gene sequence was PCR amplified using the primer pair (8f and 1546r) and a DNA sample 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 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 neighbor-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 the 16S rRNA gene sequence placed strain WK042^T in the phylum *Bacteroidetes* (Garrity & Holt, 2001). The strain had at least two different 16S rRNA gene sequences with C or T at position 183 (*Escheichia coli* numbering). The most closely related species was *Bacteroides massiliensis* JCM 13223^T (Fenner *et al*., 2005) isolated from blood culture of a healthy baby with a 16S rRNA gene sequence similarity of 92.2%, and *Bacteroides vulgatus* ATCC 8482^T (= JCM 5826^T) from human faeces (Holdeman *et al*., 1984) was the next closest species with almost the same similarity (92.1%). *Bacteroides dorei* JCM 13471^T, also isolated from human faeces (Bakir *et al*., 2006), was the third closest species (91.3%). The similarity values were calculated using the sequence giving the highest similarity, i.e., with T at position 183, for comparison with *B. massiliensis* and with C for comparison with *B. vulgatus*. In *B. dorei*, the base at this position was G, different from the three other strains. 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 (data not shown).

For cellular fatty acids (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 30ºC. 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 analyzed by GC (Hp6890; Hewlett-Packard or G-3000; Hitachi) equipped with a HP Ultra 2 column. Each peak was identified from their equivalent chain-lengths (ECL) (Miyagawa *et al*., 1979) according to the protocol of TechnoSuruga Co., Ltd (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 CFAs compositions of strain WK042^T and the two reference strains were rather similar (Table 1), sharing common features with all *Bacteroides* species having anteiso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and $C_{15:0}$ as major CFAs (Miyagawa *et al*., 1979; Moore *et al*., 1994). The profile of strain WK042^T, however, differed from those of related species by the respective proportions of some CFAs such as $C_{15:0}$ and iso-$C_{15:0}$.

Genomic DNAs of the three strains were extracted according to the method described by Akasaka *et al*. (2003b) and digested with P1 nuclease by using a YAMASA GC kit (Yamasa Shoyu) and their G + C contents were measured by HPLC (HITACHI L-7400) equipped with a μBondapak C18 column (3.9 × 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 analyzed 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 (cell morphology, haemin requirement, products from

glucose, respiratory quinone composition and so on) with the many recognized *Bacteroides* species (Holdeman *et al*., 1977, 1984; Shah & Collins, 1980), supporting its assignment to the genus *Bacteroides* based on the 16S rRNA gene sequence, however, strain WK042^T displayed a number of distinct 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 strongly stimulated by the presence of Oxgall (2%), while 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 rather restricted as shown above and the strain did not use the variety of saccharides utilized by the related species. The optimum temperature (35^{o}C) and the slightly alkaliphilic character of strain WK042^T were also at variance with the related species. The genomic DNA G + C content of strain WK042^T was slightly lower than those of both closest relatives.

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 mainly lives on monomers derived from plant biomass degradation by other hemicellulose or cellulose decomposers. The fact corresponds with the habitat (rice-straw residue in a methanogenic reactor) of the strain. The significance of the ability to decompose hemicellulose or xylan of the members of the *Bacteroides-Prevotella* group has been recognized (Chassard *et al*., 2008; Hespell & Whitehead, 1990; Nishiyama *et al*., 2009b; Ueki *et al*., 2006b, 2007).

Based on the phylogenetic, physiological and chemotaxonomic data, the new species *Bacteroides*

paurosaccharolyticus sp. nov. is proposed to accommodate the novel strain. The type strain is WK042^T (= JCM 15092^T = DSM 21004^T).

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 smooth surface. Haemin remarkably stimulates the growth, and cobalamin (vitamin B_{12}) slightly enhances the growth. Utilizes arabinose, xylose, glucose, mannose and aesculin. Maltose, dextrin, glycogen, starch, and pectin are also used, although the growth is rather slow. Weakly uses rhamnose and pyruvate. Produces acetate, propionate and succinate from saccharides in the presence of haemin and cobalamin. Does not utilize ribose, fructose, galactose, sorbose, cellobiose, lactose, melibiose, saccharose, trehalose, melezitose, raffinose, carboxymethylcellulose (CMC), cellulose powder, xylan, inulin, amygdalin, salicin, glycerol, dulcitol, inositol, mannitol, sorbitol, ethanol, fumarate, lactate, malate and succinate. Slightly alkaliphilic, having a pH optimum at 7.7. The temperature range for growth is 10-40^{o}C, the optimum being 35^{o}C. NaCl concentration range is 0-2% (w/v), the optimum being 0%. Catalase, oxidase, nitrate-reducing and urease activities are absent. Does not produce indole and hydrogen sulfide. Aesculin is hydrolyzed, but not gelatin. Sensitive to bile. The major cellular fatty acids 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 is 41.0 mol%. The type strain is WK042^T (= JCM 15092^T = DSM 21004^T) isolated from a sample of rice-straw residue in a methanogenic reactor treating waste from cattle farms in Japan.

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339 **Figure legend**

340

341 **Fig. 1.** Neighbor-joining tree showing the phylogenetic relationship of strain WK042^T and other species in the

342 genus *Bacteroides* based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000

343 replications) above 70% are shown at branch nodes. The sequence of *Prevotella melaninogenica* ATCC

344 25845^T was used as the outgroup. The tree topology evaluated by using the maximum-likelihood method was

345 almost the same as that obtained with the neighbour-joining method. Bar, 2% estimated difference in

346 nucleotide sequence position.

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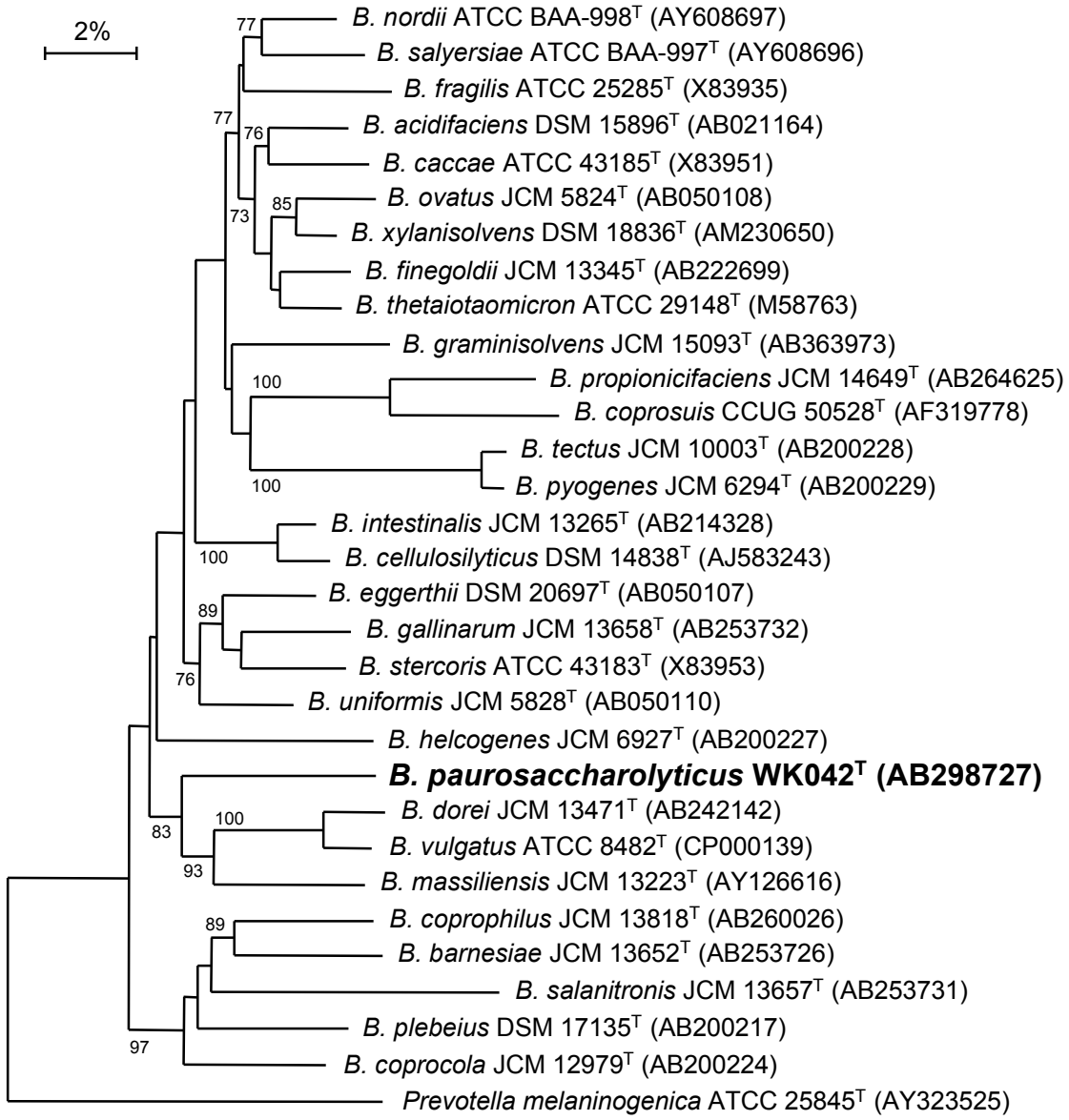
2%
B. nordii ATCC BAA-998T (AY608697)
B. salyersiae ATCC BAA-997T (AY608696)
B. fragilis ATCC 25285T (X83935)
B. acidifaciens DSM 15896T (AB021164)
B. caccae ATCC 43185T (X83951)
B. ovatus JCM 5824T (AB050108)
B. xylanisolvens DSM 18836T (AM230650)
B. finegoldii JCM 13345T (AB222699)
B. thetaiotaomicron ATCC 29148T (M58763)
B. graminisolvens JCM 15093T (AB363973)
B. propionicifaciens JCM 14649T (AB264625)
B. coprosuis CCUG 50528T (AF319778)
B. tectus JCM 10003T (AB200228)
B. pyogenes JCM 6294T (AB200229)
B. intestinalis JCM 13265T (AB214328)
B. cellulosilyticus DSM 14838T (AJ583243)
B. eggerthii DSM 20697T (AB050107)
B. gallinarum JCM 13658T (AB253732)
B. stercoris ATCC 43183T (X83953)
B. uniformis JCM 5828T (AB050110)
B. helcogenes JCM 6927T (AB200227)
B. paurosaccharolyticus WK042T (AB298727)
B. dorei JCM 13471T (AB242142)
B. vulgatus ATCC 8482T (CP000139)
B. massiliensis JCM 13223T (AY126616)
B. coprophilus JCM 13818T (AB260026)
B. barnesiae JCM 13652T (AB253726)
B. salanitronis JCM 13657T (AB253731)
B. plebeius DSM 17135T (AB200217)
B. coprocola JCM 12979T (AB200224)
Prevotella melaninogenica ATCC 25845T (AY323525)

Table 1. Cellular fatty acid composition (%) of strain WK042^T and other related *Bacteroides* species.
Strains: 1, WK042^T; 2, *Bacteroides massiliensis* JCM13223^T; 3, *Bacteroides vulgatus* JCM 5826^T. All data from this study.
CFAs amounting to <0.5% of the total fatty acids in the three strains listed are not shown. Tr, traces (<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 acids			
$C_{15:0}$ 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

Table 2. Characteristics that differentiate strain WK042^T from the related *Bacteroides* species. Strains: 1, Strain WK042^T; 2, *Bacteroides massiliensis* JCM 13223^T ; 3, *Bacteroides vulgatus* JCM 5826^T. +, Positive; -, negative; S, stimulatory; +w, weakly positive. All data from this study. The DNA G + C contents reported in the original species descriptions of *B. massiliensis* (Fenner *et al*., 2005) and *B. vulgatus* (Holdemen *et al*., 1984) are shown in parentheses. All other data coincide 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 temp. (ºC)	35	37	37
Growth in bile	-	S	S
Acid production from:			
Arabinose	+	-	+
Xylose	+	-	+
Fructose	-	+	+
Lactose	-	+	+
Melibiose	-	+	+w
Saccharose	-	+	+
Raffinose	-	+	+