Parabacteroides chartae sp. nov., an obligately anaerobic species from wastewater of a paper mill

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A bacterial strain, designated NS31-3^T, was isolated from the wastewater of a paper mill. Cells of the isolate were obligately anaerobic, non-pigmented, non-motile, Gram-negative, short rods (0.7–1.0×1.4–2.5 μm). The isolate was able to grow on media containing 20 % bile salts. API 20A tests showed that acid was produced from glucose, lactose, sucrose, maltose, D-xylose, L-arabinose, cellobiose, D-mannose, D-melezitose, D-raffinose, D-trehalose, D-mannitol, salicin and D-sorbitol. The main fermentation products from PYG broth were lactic acid, propionic acid, formic acid and acetic acid. Chemotaxonomic analysis showed that the major fatty acids were anteiso-$C_{15:0}$, $C_{15:0}$ and iso-$C_{17:0}$ 3-OH and the predominant respiratory quinones were MK-9 and MK-10. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain NS31-3^T was related to members of genus *Parabacteroides* (91.2–93.2 % sequence similarity); the isolate had the closest affinity with *Parabacteroides merdae* JCM 9497^T. The G+C content of the genomic DNA was 37.2 mol%. On the basis of phenotypic, chemotaxonomic and phylogenetic analysis, strain NS31-3^T represents a novel species of the genus *Parabacteroides*, for which the name *Parabacteroides chartae* sp. nov. is proposed. The type strain is NS31-3^T (=JCM 17797^T =DSM 24967^T).

On the basis of phylogenetic distinction and menaquinone composition, it was proposed that three species of the genus *Bacteroides*, *Bacteroides distasonis* (Eggerth & Gagnon, 1933), *B. goldsteinii* (Song *et al.*, 2005) and *B. merdae* (Johnson *et al.*, 1986), did not belong to the genus *Bacteroides* and thus were reclassified as members of a new genus, *Parabacteroides*, by Sakamoto & Benno (2006). Subsequently, another two species, *Parabacteroides johnsonii* and *Parabacteroides gordonii*, were proposed by Sakamoto *et al.* (2007, 2009). Members of this genus are characteristically obligately anaerobic, Gram-negative, non-spore-forming, non-motile rods and can grow on media containing 20 % bile salts. Metabolically, members of the genus are saccharolytic and produce acetic acid and succinic acid as the major end-products of fermentation. In terms of chemotaxonomic properties, the major respiratory quinones are MK-9 and MK-10 and the major fatty acids are non-hydroxylated and 3-hydroxylated long-chain acids (Sakamoto & Benno, 2006). Most species of the genus *Parabacteroides* have been isolated from human faeces and clinical specimens (Song *et al.*, 2005; Simmon *et al.*, 2008).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NS31-3^T is JN029805.

A supplementary figure is available with the online version of this paper.

An obligately anaerobic bacterium was isolated from the wastewater of a paper mill in Lingqiao town (30° 15′ N 120° 10′ E), Zhejiang Province, China, in May 2010. The initial enrichment used medium Gs [containing l^{-1} distilled water: 10 g NaCl, 1.0 g $MgCl_2.6H_2O$, 0.5 g K_2HPO_4, 0.7 g KH_2PO_4, 0.025 g $FeSO_4.7H_2O$, 0.2 g $CaCl_2.2H_2O$, 1.0 g urea, 5 g yeast extract (Difco), 5 g tryptone, 1 ml trace element solution SL-10 (Widdel *et al.*, 1983), 0.4 g L-cysteine and 0.001 g resazurin]. The Hungate roll-tube technique (Hungate, 1969; Bryant, 1972) was used to isolate strains from the turbid enrichment cultures. After 2 days of incubation at 37 °C under a gas phase of O_2-free N_2, an off-white–grey colony, designated NS31-3^T, was picked from Gs agar. The isolate was purified by repeated subcultivation and was preserved in 25 % glycerol at −80 °C. Medium Gs supplemented with 1 % beef extract (medium Gss) was used to maintain the isolate and two reference strains, *Parabacteroides distasonis* JCM 5825^T and *Parabacteroides merdae* JCM 9497^T.

Cell motility and morphology were examined using light microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) during the late-exponential phase. Growth at pH 5.0–10.0 was determined by adjusting the medium with the following buffers (25 mM): MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) and CAPSO (pH 9.0–10.0). Conditions for growth were

also determined at 4, 10, 16, 22, 28, 32, 37, 40 and 45 °C and with 0–6 % (w/v) NaCl (in increments of 0.5 %). Bacteroides bile salts aesculin medium (Shah, 1992) was used to check whether bile salts inhibited growth of the isolate. Physiological and biochemical reactions were determined in duplicate using the API 20A anaerobe test kit and the API ZYM test kit (bioMérieux), according to the manufacturer's instructions. Catalase and urease, as well as hydrolysis of gelatin and aesculin, were examined using standard approaches (Gerhardt *et al.*, 1994). The end-products of fermentation were determined after growth in PYG broth (1 % peptone, 1 % yeast extract, 1 % glucose).

Fatty acid methyl esters of strain NS31-3^T, *P. distasonis* JCM 5825^T and *P. merdae* JCM 9497^T were obtained from cells grown to late-exponential phase in medium Gss at 37 °C for 24 h and then freeze dried, as described by Kuykendall *et al.* (1988). The identification and quantification of the fatty acid methyl esters as well as the numerical analysis of the fatty acids were examined as described by Zhang *et al.* (2010), matching the results with the MOORE 3.90 library. Respiratory quinones were extracted from freeze-dried cells and purified as described by Tindall (1989). The prepared quinones were analysed using LC-MS (Agilent HC-C_{18}, methanol/2-propanol 75 : 25 as the mobile phase at 1.0 ml min^{-1}, λ=270 nm, column temperature 40 °C; LCQ DECA XP 123 MAX; Thermo Finnigan).

Genomic DNA was extracted as described by Rainey *et al.* (1996). The DNA G+C content was determined by HPLC, as described by Mesbah *et al.* (1989), using salmon sperm

Table 1. Differential phenotypic, physiological and genotypic characteristics of strain NS31-3^T and two members of the genus *Parabacteroides*

Strains: 1, *Parabacteroides chartae* sp. nov. NS31-3^T; 2, *P. merdae* JCM 9497^T; 3, *P. distasonis* JCM 5825^T. Data were taken from this study. All strains were positive for growth on medium containing 20 % bile salts, aesculin hydrolysis, alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase and acid production from glucose, lactose, sucrose, maltose, xylose, mannose, raffinose and trehalose. All strains were negative for indole production, gelatin hydrolysis, urease, lipase, trypsin and acid production from glycerol. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3
Temperature for growth (°C)			
Optimum	35–37	32	37
Range	10–40	16–45	20–40
pH for growth			
Optimum	7.0–7.5	6.9	6.0
Range	5.5–8.5	5.5–8.0	5.5–8.0
NaCl for growth (%, w/v)			
Optimum	0	0.5–1.0	0.5
Range	0–2	0–4	0–2
Catalase	–	–	+
Acid production from:			
Arabinose	+	–	–
Cellobiose	+	–	+
Mannitol	+	–	–
Melezitose	+	+†	+
Salicin	+	+†	+
Sorbitol	+	–	–
Rhamnose	–	–	+
Enzymes			
Esterase	+	+	w
Esterase lipase	+	+	w
α-Chymotrypsin	–	–	+
β-Glucuronidase	–	–	+
β-Glucosidase	+	+	–
α-Mannosidase	+	–	–
α-Fucosidase	+	–	–
Main fermentation products*	LA, PA, FA, AA	PP, SA, AA, LA	LA, PA, AA, FA
DNA G+C content (mol%)	37.2	41.2	42.1

*From PYG broth: AA, acetic acid; FA, formic acid; LA, lactic acid; PA, propionic acid; SA, succinic acid.
†Reported as negative by Sakamoto & Benno (2006).

Table 2. Cellular fatty acid compositions of strain NS31-3^T and two members of the genus *Parabacteroides*

Strains: 1, *Parabacteroides chartae* sp. nov. NS31-3^T; 2, *P. merdae* JCM 9497^T; 3, *P. distasonis* JCM 5825^T. Data were taken from this study.

Fatty acids (%)	1	2	3
Saturated straight-chain			
$C_{14:0}$	2.9	2.0	2.9
$C_{15:0}$	12.7	6.6	12.5
$C_{16:0}$	5.1	6.3	4.7
$C_{18:0}$	0.4	3.2	0.8
Unsaturated straight-chain			
$C_{16:1}\omega9c$	2.6	2.2	2.9
$C_{18:1}\omega9c$	2.5	4.7	1.8
$C_{18:2}\omega9,12c$	0.5	1.0	0.6
Hydroxy			
$C_{16:0}$ 3-OH	3.8	3.7	3.6
iso-$C_{16:0}$ 3-OH	2.0	0.7	0.4
$C_{17:0}$ 3-OH	0.7	1.0	0.5
iso-$C_{17:0}$ 3-OH	13.1	13.7	10.7
anteiso-$C_{17:0}$ 3-OH	8.2	1.0	0.6
iso-$C_{15:0}$ ALDE	1.0	1.4	1.1
Saturated branched-chain			
iso-$C_{13:0}$	1.2	1.3	1.9
anteiso-$C_{13:0}$	2.7	0.9	2.1
iso-$C_{15:0}$	4.7	11.0	10.5
anteiso-$C_{15:0}$	31.1	34.4	35.9
Summed feature 4*	0.5	0.4	1.6

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 4 consisted of equivalent chain-length 12.616 and/or $C_{15:1}\omega7c$.

DNA as the calibration standard. The 16S rRNA gene was amplified using the universal bacterial primers 27F and 1492R and the amplification products were cloned into the vector pMD19-T (TaKaRa) for sequencing (Xu *et al.*, 2007). The 16S rRNA gene sequence was compared with closely related sequences of reference organisms using FASTA and the EzTaxon service (Chun *et al.*, 2007). Multiple sequence alignment was performed using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Similarity values were calculated and converted to a distance matrix by the neighbour-joining method (Saitou & Nei, 1987) using MEGA4 (Tamura *et al.*, 2007). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980).

Strain NS31-3^T was an obligately anaerobic, non-pigmented, non-motile, Gram-negative, short rod (0.7–1.0 × 1.4–2.5 μm; Fig. S1, available in IJSEM Online). Growth was not inhibited on medium containing 20 % bile salts. Colonies were circular, entire, smooth and off-white–grey (0.5–1.0 mm in diameter after 24 h on medium Gs at 37 °C). Catalase was negative. The isolate grew at 10–40 °C (optimum 35–37 °C), at pH 5.5–8.5 (optimum pH 7.0–7.5) and with 0–2 % NaCl (optimum 0 %). Phenotypic characteristics are summarized in Table 1 and the species description. Strain NS31-3^T could be differentiated from reference strains by the ability to ferment mannitol, arabinose and sorbitol (API 20A) and the presence of α-mannosidase and α-fucosidase (API ZYM). In addition, the isolate differed from *P. distasonis* JCM 5825^T by the presence of β-glucosidase and the absence of α-chymotrypsin and β-glucuronidase.

The major cellular fatty acids of the isolate and the reference strains were anteiso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and $C_{15:0}$ (Table 2), although the proportion of anteiso-$C_{15:0}$ was slightly lower in strain NS31-3^T. In addition, strain NS31-3^T contained significantly higher proportions of iso-$C_{16:0}$ 3-OH and anteiso-$C_{17:0}$ 3-OH and significantly lower proportions of iso-$C_{15:0}$. The major menaquinones of the isolate were MK-9 (49.7 %) and MK-10 (44.3 %), and small amounts of MK-7 (0.3 %) and MK-8 (5.7 %) were also present. These major and minor menaquinones were in line with those of *P. merdae* JCM 9497^T and *P. distasonis* JCM 5825^T, although there were some differences in the proportions: 66.8 and 24.3 % for MK-9, 16.2 and

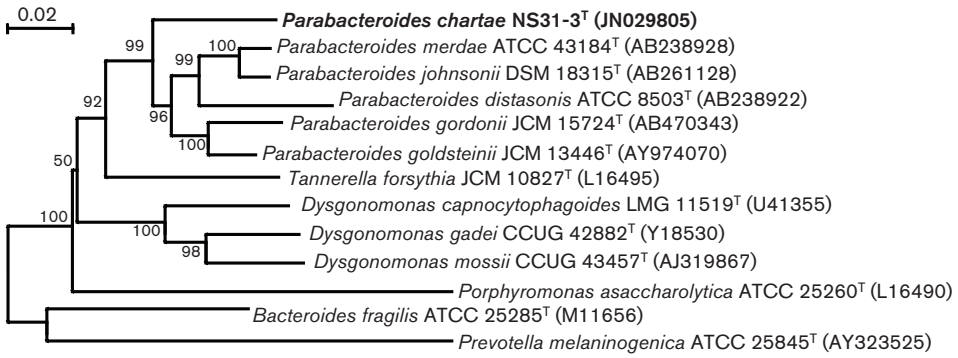

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain NS31-3^T and related taxa. Bootstrap values (⩾50 %) based on 1000 replicates are shown at branch nodes. Bar, 0.02 substitutions per nucleotide position.

72.7 % for MK-10, 0.3 and 8.9 % for MK-7 and 8.6 and 2.2 % for MK-8, respectively. In general, the menaquinone composition of strain NS31-3^T was consistent with those of members of the genus *Parabacteroides* (Sakamoto & Benno, 2006). The genomic DNA G+C content of strain NS31-3^T was 37.2 mol%, which is slightly lower than for *P. merdae* JCM 9497^T and *P. distasonis* JCM 5825^T (41.2 and 42.1 mol%, respectively) (Table 1).

An almost-complete 16S rRNA gene sequence (1481 nt) was obtained from strain NS31-3^T. Phylogenetic analysis indicated that the isolate belonged to a distinct lineage within the cluster containing members of the genus *Parabacteroides* (Fig. 1). Strain NS31-3^T exhibited 91.2–93.2 % 16S rRNA gene sequence similarity with the genus *Parabacteroides*. The isolate's closest neighbour was *P. merdae* JCM 9497^T.

On the basis of the phenotypic, chemotaxonomic, genotypic and phylogenetic characterization, strain NS31-3^T represents a novel species of the genus *Parabacteroides*, for which the name *Parabacteroides chartae* sp. nov. is proposed.

Description of *Parabacteroides chartae* sp. nov.

Parabacteroides chartae (char′tae. L. gen. n. *chartae* of/from paper, pertaining to paper milling).

Cells are obligately anaerobic, non-spore-forming, non-pigmented, non-motile, Gram-negative, short rods (0.7–1.0 × 1.4–2.5 μm). On medium Gs, colonies are circular, entire, smooth, off-white–grey and 0.5–1.0 mm in diameter. Grows at 10–40 °C (optimum 35–37 °C), at pH 5.5–8.5 (optimum pH 7.0–7.5) and with 0–2 % NaCl (optimum 0 %). Grows on medium containing 20 % bile salts. Negative for catalase. Indole and urease are not produced. Gelatin and aesculin are hydrolysed. Acid is produced from glucose, lactose, sucrose, maltose, D-xylose, L-arabinose, D-cellobiose, D-mannose, D-melezitose, D-raffinose, D-trehalose, D-mannitol, salicin and D-sorbitol, but not from glycerol or L-rhamnose. With API ZYM, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, but negative for lipase (C14), trypsin, α-chymotrypsin and β-glucuronidase. Mannose and raffinose are fermented. The major end-products from fermentation of PYG broth are lactic acid, propionic acid, formic acid and acetic acid. The major cellular fatty acids are anteiso-$C_{15:0}$, $C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The predominant respiratory quinones are MK-9 and MK-10.

The type strain, NS31-3^T (=JCM 17797^T =DSM 24967^T), was isolated from wastewater of a paper mill. The DNA G+C content of the type strain is 37.2 mol%.

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