Coprobacter fastidiosus gen. nov., sp. nov., a novel member of the family *Porphyromonadaceae* isolated from infant faeces

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A novel obligately anaerobic, non-spore-forming, rod-shaped, non-motile Gram-reaction-negative bacterium was isolated from infant faeces. The strain, designated NSB1^T, was able to grow on rich media at 30–37 °C, in the presence of up to 2 % (w/v) Oxgall and 2 % (w/v) NaCl. Cells of strain NSB1^T produced catalase, but not urease and indole. Aesculin was not hydrolysed. The strain was able to utilize D-glucose, lactose, maltose, mannose and raffinose as electron donors. When grown on D-glucose, the main metabolic end products were propionic and acetic acids, with a minor product being succinic acid. The major cellular fatty acids, iso-$C_{15:0}$ and anteiso-$C_{15:0}$, were present at a 1 : 1 molar ratio. The major menaquinone was MK-11. The DNA G+C content was found to be 38.5 mol%. According to 16S rRNA gene sequence analysis strain NSB1^T is a member of the family *Porphyromonadaceae*, phylum *Bacteroidetes*. The closest relatives of the strain were *Barnesiella viscericola* (88.2 % identity) and *Barnesiella intestinihominis* (87.4 % identity). On the basis of phenotypic and genotypic properties of strain NSB1^T we conclude that this strain represent a novel species in a new genus within the family of *Porphyromonadaceae* for which the name *Coprobacter fastidiosus* gen. nov., sp. nov. is proposed. The type strain of the species is NSB1^T (=DSM 26242^T,=VKM B-2743^T).

Human gastrointestinal tract is populated by a dense and diverse community of micro-organisms. The bacterial populations reach 10^{11}–10^{12} c.f.u. per g of intestinal contents in the distal colon and make up to 60 % of the wet faecal mass (O'Hara & Shanahan, 2006). Recent culture-independent studies of the intestinal microbiota identified more than 1000 prevalent bacterial species belonging to seven different phyla of the domain *Bacteria* (Eckburg *et al.*, 2005) with at least 160 prevalent species per individual (Costello *et al.*, 2009). At least 20 % of bacterial species in healthy adults belong to the order *Bacteroidales* of the phylum *Bacteroidetes* (Arumugam *et al.*, 2011). This taxon includes obligate anaerobic, non-sporulating, non-motile, Gram-negative bacteria with fermentative metabolism. In the last decade, advances in molecular phylogeny forced significant taxonomic rearrangements of the order *Bacteroidales*. Numerous species of the former genus *Bacteroides* were reclassified to new genera and families (Rautio *et al.*, 2003; Sakamoto & Benno, 2006; Hardham *et al.*, 2008). Also a number of new genera and novel species, including members of the family *Porphyromonadaceae* (genera *Petrimonas*, *Barnesiella* and *Macellibacteroides*), were discovered (Grabowski *et al.*, 2005; Sakamoto *et al.*, 2007; Jabari *et al.*, 2012). Rigorous analysis of metagenomic sequence data, generated from human stool samples, has detected the presence of several low-abundance, novel bacterial taxa, which span three major phyla: *Bacteroidetes*, *Proteobacteria* and *Firmicutes* (Wylie *et al.*, 2012). A significant proportion of these taxa were related to the recently discovered genus *Barnesiella*,

The GenBank/EMBL/DDBJ accession numbers for the partial sequences of the 16S rRNA gene and 60 kDa chaperonin (*cpn60*) genes of strain NSB1^T are JN703378 and JQ340477, respectively.

Two supplementary tables are available with the online version of this paper.

family *Porphyromonadaceae*, further encouraging efforts to characterize the members of this family and to study their functions in the human gut microbiota.

As a part of an ongoing longitudinal study of intestinal *Bacteroidales* populations in children (Kulagina *et al.*, 2012) a number of faecal samples from a healthy child were collected at different time points and stored frozen as 10^{-3} dilutions in Columbia broth/15 % (v/v) glycerol. One of the samples, collected at the age of 1 year, was serially diluted in saline, plated on Columbia agar supplemented with 5 % (v/v) horse blood and cultured anaerobically for 72 h at 37 °C. Grown colonies were assessed macroscopically and microscopically and replated aerobically. The isolates showing properties of the order *Bacteroidales* (Gram-reaction-negative, anaerobic, rod-shaped bacteria) were subjected to further identification using 16S rRNA gene sequencing. In addition to strains identified as *Alistipes putredinis* and *Alistipes onderdonkii* the studied sample also contained a strain, designated NSB1^T, which shares 88.2 % identity to *Barnesiella viscericola* JCM 13660^T in its 16S rRNA gene sequence, suggesting that it represents a distinct phylogenetic branch within the family *Porphyromonadaceae*. The goal of the current study was to determine the taxonomic position of this strain.

Strain NSB1^T was cultured anaerobically (in an atmosphere of 85 % N_2, 10 % H_2, 5 % CO_2) on Columbia blood agar (bioMérieux) and Eggerth–Gagnon (EG) agar (recipe available at http://www.jcm.riken.jp/cgi-bin/jcm/jcm_grmd?GRMD=14) supplemented with 5 % (v/v) defibrinated horse blood. The strain was also able to grow in EG broth without blood supplemented with 10 μM haemin. Susceptibility of the strain to bile and NaCl was tested in EG broth supplemented with 0–8 % (w/v) of either NaCl or Oxgall (Sigma–Aldrich). Media were inoculated from fresh plates and growth was examined visually after 48 h. Physiological properties and enzyme profiles were determined using API 20A and Rapid ID 32A identification systems (bioMérieux) according to the manufacturer's instructions. Aesculin hydrolysis and H_2S production were tested on Perfringens agar (Himedia) supplemented with 0.02 % (w/v) cystine, 0.05 % (w/v) cysteine–HCl, 0.4 % (w/v) Na_2HPO_4 and 0.5 % (w/v) aesculin. Growth and colony colour were monitored after 72 h incubation.

For transmission electron microscopy bacterial cell pellets were fixed in glutaraldehyde followed by osmium tetroxide (Zhao *et al.*, 2013). After fixation, samples were dehydrated and embedded in Epon 812 (Fluka). Embedded specimens were sliced into ultrathin sections (90 nm), stained with Reynolds lead citrate reagent (Reynolds, 1963) and uranyl acetate aqueous solution and examined with a Hitachi 700H microscope. For scanning electron microscopy, cell pellets were fixed as described above and dehydrated. Subsequently samples were critical-point-dried with liquid CO_2 in a Balzers apparatus, sputter-coated with gold–palladium and observed at 15 kV with a JSM-6380 scanning electron microscope (JEOL).

Metabolic end products were determined using GLC according to the method of Sakamoto *et al.* (2005) with broth cultures (48–72 h). Volatile fatty acids were analysed directly after extraction as described previously (Holdeman *et al.*, 1977). Non-volatile metabolites were converted to methyl esters in 6 % H_2SO_4/60 % (v/v) methanol solution at 60 °C (30 min) and then extracted using chloroform (Holdeman *et al.*, 1977). Products were separated using a model 5880A chromatograph (Agilent) equipped with 1.6 m glass column [2 mm, 10 % AT-1000/1 % (w/v) H_3PO_4 on Chromosorb W-AW, 100/120 mesh]. Cellular fatty acids and menaquinones were determined in late exponential phase cultures grown at the optimal temperature in EG broth. Long-chain fatty acids were analysed using GC–MS. Fatty acid methyl esters were prepared by acid methanolysis of dry biomass and extracted as described previously (Zhilina *et al.*, 2012) and processed on an AT-5850/5973 GC–MS system (Agilent Technologies) according to the method of Shcherbakova *et al.* (2005). Respiratory quinones were detected following the procedure of Collins (1985).

Genomic DNA was extracted using a ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research). DNA G+C content was determined using HPLC analysis of free nucleosides released after nuclease P1/alkaline phosphatase treatment. DNA hydrolysis was performed by the method of Tamaoka & Komagata (1984). Nucleosides were separated as described by Sonoki *et al.* (1993) on a model 1200 HPLC system (Agilent) equipped with DAD-detector and YMC J'sphere ODS-M80 column (250 × 4.6 mm) using 0.2 M phosphate buffer (pH 5.2) : acetonitrile (95 : 5, v/v) as eluent with a flow rate of 0.8 ml min^{-1}.

Fragments of 16S rRNA gene (corresponding to nucleotide positions 8–1492 in *Escherichia coli*) and *hsp60* gene (corresponding to nucleotide positions 274–839 of *E. coli groL* gene) were amplified using PCR with primer pairs Bact8F/Bact1492R (Turner *et al.*, 1999) and H729/H730 (Sakamoto & Ohkuma, 2010), respectively. Reactions were performed in 50 μl volumes and contained 2 U TaqSE polymerase (Sibenzyme) along with the appropriate buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer and 50 ng DNA. PCR was carried out in an MJ Mini thermal cycler (Bio-Rad) using the following program: 95 °C for 2 min; followed by 30 cycles consisting of 94 °C for 20 s, 50 °C for 20 s and 72 °C for 60 s. PCR products were purified and cloned into pAL-TA vector (Evrogen) according to standard procedures (Sambrook & Russell, 2001). Plasmids were sequenced using universal primers (M13 forward and reverse) at the PYNNY sequencing centre (Moscow, Russia). Nucleotide sequences were deposited in GenBank/EMBL/DDBJ under accession numbers JN703378 and JQ340477. GenBank queries were performed using the Megablast algorithm. Phylogenetic analysis based on 16S rRNA and *hsp60* gene sequences was performed with the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony algorithms using the software MEGA5 (Tamura *et al.*, 2011) after multiple

alignment of data by CLUSTALW2 (Larkin *et al.*, 2007). Tree topologies were evaluated by a bootstrap analysis using 1000 resamplings of the sequences (Felsenstein, 1985). *Bacteroides thetaiotaomicron* JCM 5827^T was used as an outgroup. All positions containing gaps and missing data were eliminated. The evolutionary distances in the neighbour-joining algorithm were computed using the Kimura two-parameter substitution model (Kimura, 1980).

Strain NSB1^T comprised obligately anaerobic, non-spore-forming, non-motile, Gram-reaction-negative rods. When grown on EG agar supplemented with 5 % (v/v) horse blood cells were 0.2–0.3 × 0.5–2.0 μm in size (Fig. 1a) and occurred singly and in aggregates. Transmission electron microscopy confirmed the presence of outer membrane and periplasmic space typical of Gram-negative bacteria (Fig. 1b). No signs of additional envelopes, such as capsule or S-layer, were visible, and no surface appendages (pili, fimbriae or flagella) were observed.

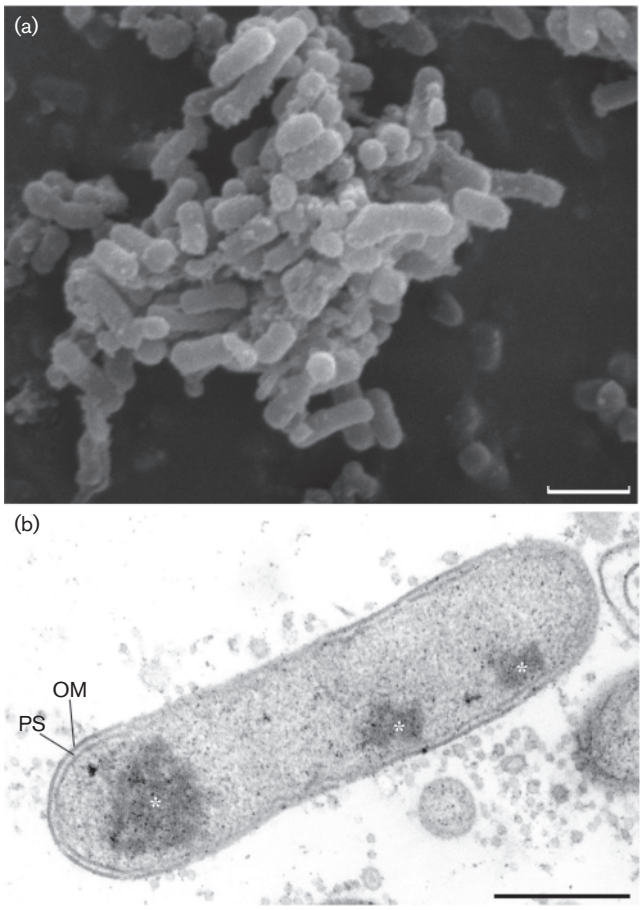

Fig. 1. Morphology of strain NSB1^T. Scanning electron micrograph of cells grown on EG agar (a) and transmission electron micrograph of longitudinal thin section. Bar, 1 μm (b) showing outer membrane (OM) and periplasmic space (PS) typical for Gram-negative-type cell envelope. Asterisks denote unrecognized electron-dense patches visible in some cells. Bar, 250 nm.

The strain appeared to be nutritionally demanding and yeast extract and haemin were required for growth. Colonies on EG blood agar appeared after 72 h incubation at 37 °C and were 0.5–2 mm in diameter, light brown, circular, entire, slightly convex and smooth. Weak haemolysis zones surrounding colonies were seen after prolonged incubation on EG blood agar plates. Growth on EG agar without blood was significantly retarded. On Columbia blood agar colonies were pigmented dark brown to black. In EG broth visible growth was obtained at 37 °C and 30 °C, but not at 42 °C after 48 h incubation. The strain was tolerant to Oxgall at concentrations up to 2 % (w/v). The addition of NaCl to final concentration of 2 % (w/v) inhibited growth partially while at 4 % (w/v) NaCl no growth was observed. The strain was unable to hydrolyse aesculin. Neither H_2S, nor cytochrome oxidase were produced. In aerobically prepared medium and medium containing trace amounts of O_2 no growth occurred. However, the cells were catalase-positive as determined by the formation of O_2 bubbles on a cell pellet treated with 3 % (v/v) H_2O_2 solution. Sodium azide added to 0.15 % (w/v) did not inhibit growth. According to API 20E tests NSB1^T was able to produce acid from D-glucose, lactose, maltose, mannose and raffinose (Table S1, available in IJSEM Online). The latter property separates this strain from *Barnesiella viscericola* JCM 13660^T and *Barnesiella intestinihominis* JCM 15079^T, which were unable to utilize raffinose. In addition, unlike the strains of the genus *Barnesiella*, NSB1^T did not ferment cellobiose, but produced catalase. The profile of enzymes assayed in Rapid ID 32A tests did not differ significantly in NSB1^T from those of type strains of species of the genus *Barnesiella* with the only difference being the absence of *β*-glucosidase in NSB1^T (Table S1).

The main metabolic end products produced by NSB1^T were propionic acid (4.25–4.38 mM in 48 h broth culture) and acetic acid (1.04–1.05 mM) in the approximate molar ratio of 4 : 1. Minor amounts of succinic acid were also detected. A similar composition of end products has been reported previously for *Paludibacter propionicigenes* DSM 17365^T (Ueki *et al.*, 2006), another member of the family *Porphyromonadaceae*. In contrast, the major metabolites of most closely related strains of species of the genus *Barnesiella* were acetic and succinic acids (Sakamoto *et al.*, 2007; Morotomi *et al.*, 2008).

The G+C content of genomic DNA in strain NSB1^T was 38.5 mol%, which is close to that of type strains of species of the genera *Dysgonomonas* (38.0–38.5 mol%, Hofstad *et al.*, 2000) and *Paludibacter* (39.3 mol%, Ueki *et al.*, 2006). In the most closely related bacteria of the genus *Barnesiella* the molar G+C content is higher and reaches 52 mol% in *Barnesiella viscericola* C46^T.

The major cellular fatty acids in strain NSB1^T were: iso-$C_{15:0}$ (26.1–27.4 %) and anteiso-$C_{15:0}$ (23.4–27.1 %). Significant amounts of $C_{16:0}$ 3-OH, iso-$C_{17:0}$ 3-OH and $C_{16:0}$. were also present (Table 1). The roughly 1 : 1 molar

ratio of anteiso-$C_{15:0}$ to iso-$C_{15:0}$ is a distinctive feature of strain NSB1^T. Similar proportion of major cellular fatty acids in members of the family *Porphyromonadaceae* have been reported only for *Porphyromonas catoniae* (Willems & Collins, 1995), while in *Barnesiella viscericola* the ratio was 2.3–2.5 : 1 (Sakamoto *et al.*, 2007).

The predominant menaquinone in strain NSB1^T was MK-11 (~95 %), and minor amounts of MK-10 and MK-12 were also present. In contrast, the only two species of the family *Porphyromonadaceae* possessing MK-11 as a major menaquinone, *Barnesiella viscericola* JCM13660^T and *Tannerella forsythensis* JCM 10827^T contain both MK-11 and MK-12 in the mass ratios of 66 : 21 and 48 : 33, respectively (Sakamoto *et al.*, 2007; Sakamoto *et al.*, 2002).

The search for nucleotide sequences similar to NSB1^T 16S rRNA gene fragment (1490 nt) in the GenBank 'nr' database using the Megablast algorithm revealed numerous almost identical (99 % identity) hits to 16S rRNA sequences of uncultured bacteria originating from several metagenomic studies of human intestinal microbiota (Ley *et al.*, 2006; Li *et al.*, 2012). In addition, search in the whole-genome shotgun ('wgs') database revealed the 99 % identity of 16S rRNA between the strain NSB1^T and an uncharacterized bacterial isolate designated *Tannerella* sp. 6_1_58FAA_CT1, for which the draft full-genome nucleotide sequence is available (GenBank accession NZ_ACWX01000000).

The phylogenetic analysis was performed using 1425 bp 16S rRNA sequence fragments (positions 47–1487; *E. coli* numbering system) of each species and showed that strain NSB1^T represented a novel subline within the family *Porphyromonadaceae* (Fig. 2). Among the characterized species the closest relatives to NSB1^T were *Barnesiella viscericola* JCM 13660^T (88.2 % identity, Sakamoto *et al.*, 2007) and *Barnesiella intestinihominis* JCM15079^T (87.4 % identity, Morotomi *et al.*, 2008). More distantly related taxa within the family *Porphyromonadaceae* included *Parabacteroides johnsonii* JCM 13406^T (85.3 %), *Macellibacteroides fermentans* LIND7H^T (85.2 %) and *Tannerella forsythia* JCM 10827^T (85.2 %). The use of an alternative phylogenetic marker, *cpn60* (*hsp60*) chaperonin gene (Sakamoto & Ohkuma, 2010), confirmed the separate lineage of NSB1^T. As judged by *hsp60* phylogeny, the closest relatives of NSB1^T were *Barnesiella intestinihominis* JCM15079^T (80.1 %), *Parabacteroides gordonii* JCM 15724^T (78.5 %) and *Parabacteroides johnsonii* JCM 13406^T (77.8 %).

In summary, the strain NSB1^T differs from the phylogenetically neighbouring strains of *Barnesiella viscericola* and *Barnesiella intestinihominis* in the composition of metabolic end products, fatty acid and menaquinone profile, enzymic activity, bile tolerance and nucleotide composition of genomic DNA. On the basis of phenotypic and genotypic properties of strain NSB1^T we conclude that this strain represent a novel species in a new genus within the family *Porphyromonadaceae* for which the name *Coprobacter fastidiosus* gen. nov., sp. nov. is proposed. The main chemotaxonomic properties of *Coprobacter fastidiosus* gen. nov., sp. nov. are given in Table 2 in comparison with other genera within family *Porphyromonadaceae*. The antibiotic susceptibility profile of strain NSB1^T is given in Table S2.

Table 1. Cellular fatty acid compositions of strain NSB1^T

Values shown are percentages of total fatty acids as determined in three independent experiments.

Fatty acid	Percentage
iso-$C_{14:0}$	0.2–0.3
$C_{14:0}$	1.5–1.6
iso-$C_{15:0}$	26.1–27.4
anteiso-$C_{15:0}$	23.4–27.1
$C_{15:0}$	3.9–6.0
$C_{16:1}$	0.2–0.8
$C_{16:0}$	6.2–7.2
$C_{14:0}$ 3-OH	0.4–0.5
iso-$C_{15:0}$ 3-OH	3.0–4.4
anteiso-$C_{15:0}$ 3-OH	0.3–0.4
$C_{15:0}$ 3-OH	1.9–2.0
iso-$C_{16:0}$ 3-OH	0.2–0.4
$C_{18:2}$	0.5–0.6
$C_{18:1}$	1.5–2.4
$C_{18:0}$	1.4–1.7
$C_{16:0}$ 3-OH	7.9–10.6
iso-$C_{17:0}$ 3-OH	12.1–12.4
anteiso-$C_{17:0}$ 3-OH	0.6–0.8
$C_{17:0}$ 3-OH	0.6–0.7

Description of *Coprobacter* gen. nov.

Coprobacter (Co.pro.bac' ter. Gr. n. *kopros* excrement; N.L. masc. n. *bacter* rod; N.L. masc. n. *Coprobacter* a rod isolated from excrements).

Cells are Gram-reaction-negative, rod-shaped, obligately anaerobic, non-spore-forming, non-motile, 0.2–0.3 × 0.5–2.0 μm in size and occur singly and in aggregates. The cells are mesophilic, saccharolytic and tolerant to Oxgall in concentrations of up to 2 % (w/v). The main metabolic end products are propionic and acetic acids. Succinic acid is produced to a lesser extent. The major cellular fatty acids, iso-$C_{15:0}$, anteiso-$C_{15:0}$, are present at 1 : 1 molar ratio. The major menaquinone is MK-11. A member of the family *Porphyromonadaceae*, phylum *Bacteroidetes*, according to 16S rRNA gene sequence analysis.

The type species is *Coprobacter fastidiosus*.

Description of *Coprobacter fastidiosus* gen. nov., sp. nov.

Coprobacter fastidiosus (fas.ti.di.o' sus. L. masc. adj. *fastidiosus* fastidious, referring to its fastidious character).

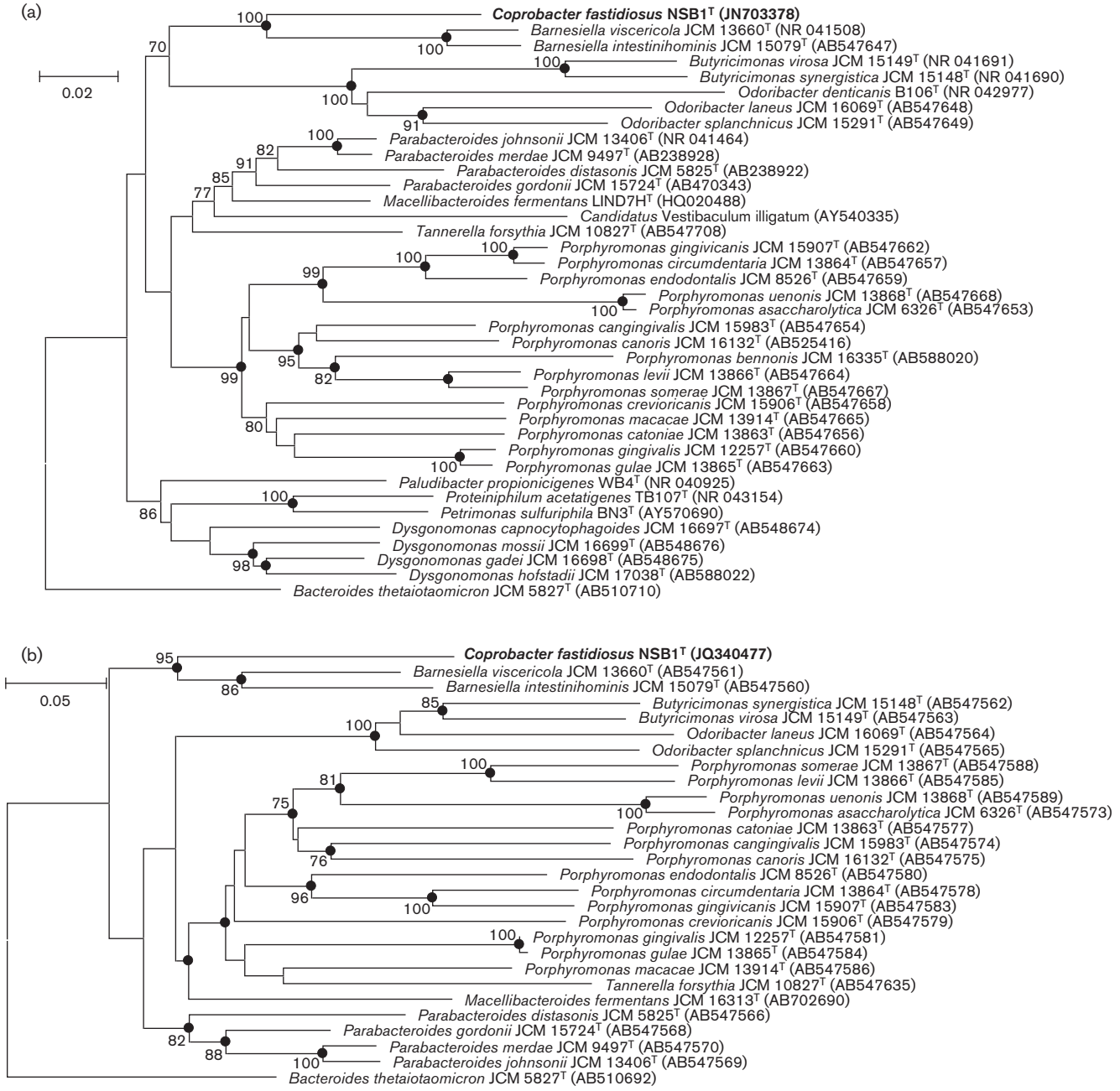

Fig. 2. Neighbour-joining phylogenetic trees based on 16S rRNA (a) and *hsp60* (b) gene sequences showing the relationships between strain NSB1^T and related representatives of the family *Porphyromonadaceae*. Bootstrap values of 70 % or higher are shown at branch nodes. Filled circles indicate that the corresponding nodes also received bootstrap values greater than 70 % in the tree generated with the maximum-parsimony algorithm. The scale bars represent substitutions per nucleotide position.

Exhibits the following characteristics in addition to those given in the description of the genus. Growth on EG blood agar is visible after 72 h incubation at 37 °C. Colonies are 0.5–2 mm in diameter, light brown, circular, entire, slightly convex and smooth. Aesculin is not hydrolysed. Indole and urease are not produced. Catalase is produced. Gelatin is digested. Acid is produced from D-glucose, lactose, maltose, mannose and raffinose, but not from D-mannitol, sucrose, salicin, D-xylose, L-arabinose, glycerol, cellobiose, melezitose, D-sorbitol, L-rhamnose and trehalose. Positive reactions using Rapid ID32A are obtained for α-galactosidase, β-galactosidase, α-glucosidase, β-*N*-acetylglucosaminidase, glutamic acid decarboxylase, alkaline phosphatase, leucyl glycine arylamidase and alanine arylamidase. Raffinose and mannose are fermented. The rest of the reactions give negative results.

Table 2. Differential characteristics of strain NSB1^T

Taxa: 1, NSB1^T; 2, genus *Macellibacteroides*; 3, genus *Butyricimonas*; 4, genus *Barnesiella*; 5, genus *Dysgonomonas*; 6, genus *Odoribacter*; 7, genus *Paludibacter*; 8, genus *Parabacteroides*; 9, genus *Porphyromonas*; 10, genus *Proteiniphilum*; 11, genus *Tannerella*. Data for the type strains of species of these genera are taken from Jabari *et al.* (2012) and Sakamoto *et al.* (2009). +, Positive; −, negative; NT, not tested; F, fermentative; NF, non-fermentative.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Growth with bile	+	nt	−	−	+	+	−	+	−	−	−
Aerobic growth	−	−	−	−	+	−	−	−	−	−	−
Pigment produced	−	−	−	−	−	−	−	−	+ (except for *Porphyromonas catoniae*)	−	−
Metabolism	F	F	F	F	F	F	F	F	Mostly NF	NF	NF
Major end products (organic acids)	Propionic, acetic, succinic	Lactic, acetic, butyric, isobutyric	Butyric, isobutyric	Acetic, succinic	Propionic, lactic, succinic	Acetic, succinic	Acetic, propionic	Acetic, succinic	Acetic, butyric, isovaleric, propionic, phenylacetic, succinic	Acetic, propionic	Acetic, butyric, isovaleric, propionic, phenylacetic
Major cellular fatty acids	Iso-$C_{15:0}$ anteiso-$C_{15:0}$	Anteiso-$C_{15:0}$, $C_{15:0}$, $C_{17:0}$ 2-OH	Iso-$C_{15:0}$	Anteiso-$C_{15:0}$	Iso-$C_{14:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH	Iso-$C_{15:0}$	Anteiso-$C_{15:0}$, $C_{15:0}$, anteiso-$C_{17:0}$ 3-OH	Anteiso-$C_{15:0}$	Iso-$C_{15:0}$	Anteiso-$C_{15:0}$	Anteiso-$C_{15:0}$
Ratio of anteiso-$C_{15:0}$ to iso-$C_{15:0}$	0.9–1.0	5.6	0.028–0.033	2.3–3.5	6.0–8.8	0.34	28	3.1–10.3	0.025–0.9	12.3	22.8–95.2
Predominant menaquinone	MK-11	MK-9, MK-10	MK-10	MK-11, MK-12	NT	MK-9	MK-8	MK-9, MK-10	MK-9, MK-10	NT	MK-10, MK-11
Growth at 37 °C	+	+	+	+	+	+	−	+	+	+	+
G+C content (mol%)	38.5	41.4	46	45–52	38.0–38.5	46	39.3	43–46	40–55	46.6	44–48
Isolation source	Faeces	Abattoir waste waters	Faeces	Faeces	Human clinical specimen	Faeces	Irrigated rice-field soil	Faeces	Oral cavities	Sludge from UASB reactor	Periodontal pockets

The type strain of the species, isolated from infant faeces, is NSB1^T (=DSM 26242^T =VKM B-2743^T). The DNA G+C content is 38.5 mol%.

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