

Isolation of bacteria from mouse caecal samples and description of *Bacteroides sartorii* sp. nov

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Abstract Caecal samples from wild-type and TNF^{deltaARE} mice were cultured on selective media containing bile salts, amino acids or casein macro-peptides. Twenty-two strains were isolated and identified by 16S rRNA gene sequencing. Twenty-one strains showed >98% similarity to known bacteria (*Blautia* spp., *Clostridium innocuum*, *Enterococcus* spp., *Escherichia coli*, *Lactobacillus murinus*, *Parabacteroides goldsteinii* and *Shigella dysenteriae*). One additional isolate, strain A-C2-0, was a new bacterium. The closest relatives were *Bacteroides massiliensis*, *Bacteroides dorei* and *Bacteroides vulgatus* ($\leq 94\%$ similarity). Strain A-C2-0 is a Gram-negative rod that does not form spores and has a G + C content of DNA of 41.5%. Its major cellular fatty acid is C_{15:0} ANTEISO, and its major respiratory quinone is MK-9. Cells are aerotolerant but grow only under strict anoxic conditions. They are resistant to cefotaxime and tobramycin. When compared with related *Bacteroides* spp., the new bacterium was positive for α -arabinosidase, negative for glutamyl glutamic acid arylamidase and did not metabolise galactose, glucose, fructose, mannose, raffinose and sucrose. Strain A-C2-0 therefore merits recognition as a member of a novel species within the genus *Bacteroides*,

for which the name *Bacteroides sartorii* is proposed. The type strain is A-C2-0^T (= DSM 21941^T = CCUG 57211^T).

Keywords Mouse caecum · Bacterial diversity · *Bacteroidetes* · *Bacteroides sartorii* · TNF^{deltaARE} mice · Chronic intestinal inflammation

Introduction

Intestinal microbes are crucial for human health. They play important roles in barrier functions (Lievin-Le Moal and Servin 2006), maturation of the immune system (Rothkott and Pabst 1989), energy balance (Backhed et al. 2004) and production of bioactive metabolites (Clavel et al. 2005). However, under certain circumstances, intestinal microbes contribute to the development of diseases.

The involvement of bacteria in the onset and maintenance of inflammatory bowel diseases (IBD) has been demonstrated using germ-free animals and faecal stream diversion in IBD patients (Onderdonk et al. 1981; Rutgeerts et al. 1991). Although the occurrence of *Mycobacterium* spp. and *Chlamydia pneumoniae* may be associated with inflammation, a causative role in IBD remains to be proven (Muller et al. 2006; Mendoza et al. 2009). Molecular techniques have allowed in-depth assessment of intestinal microbiota in IBD (Manichanh et al. 2006; Sokol et al. 2006). Under chronic inflammation, intestinal microbiota seem to be characterised by global changes that affect commensal bacterial communities, including loss of functional groups and changes in spatial distribution (Clavel and Haller 2007). According to early studies comparing fluorescence and viable counts, not yet isolated and characterised bacteria may account for >60% of total intestinal bacteria (Langendijk et al. 1995). Thus, the main advantage of

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The GenBank accession number for the 16S rRNA and gyrase B gene sequences of strain A-C2-0 is GQ456204 and GQ409831, respectively.

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molecular-based techniques relies on the possibility to assess both cultured and uncultured bacterial taxonomic units. However, uncultured bacteria can also be considered as a pool of functions that remain to be characterised *in vitro*, and the isolation of novel commensal bacteria may give the opportunity to study in detail some of these functions, including modulation of host cell stress responses. The challenge is to gain access to novel bacteria out of highly diverse consortia of microorganisms.

The human distal intestinal tract harbours very dense and complex communities of mostly anaerobic bacteria. At the phylum level, the *Firmicutes* and *Bacteroidetes* account for more than 95% of total intestinal bacterial diversity (Tap et al. 2009). However, at the species and strain level, bacterial populations are highly diverse, resulting in inter-individual differences in gut bacterial composition. Despite this high diversity, the notion of a core microbiome has emerged, referring to bacteria and underlying functions found in most healthy individuals. Members of the order *Bacteroidales*, including bacteria related to the species *Bacteroides fragilis*, *Bacteroides massiliensis*, *Bacteroides ovatus*, *Bacteroides thetaiotaomicron*, *Bacteroides vulgatus* and *Parabacteroides distasonis*, are dominant and prevalent bacteria in the intestine (Finegold et al. 1983; Rigottier-Gois et al. 2003; Tap et al. 2009). They carry functions of importance for gut homeostasis, including conversion of oligosaccharides and regulation of immune responses (Hooper et al. 1999; Mazmanian et al. 2005; Martens et al. 2009; Reichardt et al. 2009). However, they are also involved in bacterial infections (Brook 1989). In IBD patients, increased counts of *Bacteroides* in mucosal samples have been observed (Lucke et al. 2006). In a gnotobiotic HLA-B27 rat model of intestinal inflammation, *B. ovatus* led to more severe inflammation than that induced by *Escherichia coli* (Rath et al. 1999). To date, there is still little data available on bacterial diversity in the mouse intestine when compared with data in human subjects and most of the recent studies used molecular-based approaches (Salzman et al. 2002; Lupp et al. 2007; Ley et al. 2008).

Our aim was to isolate caecal bacteria from wild-type (WT) and TNF^{deltaARE} mice. Adult TNF^{deltaARE} mice develop chronic inflammation in the distal ileum (Kontoyiannis et al. 1999; Hormannsperger et al. 2009). Additionally, we focused on the genotypic and phenotypic description of strain A-C2-0, a new member of the genus *Bacteroides*.

Materials and methods

Sample collection

Male WT and heterozygous TNF^{deltaARE} C57BL/6 mice (n = 3 each) fed a standard diet (Ssniff, Soest, Germany,

cat. no. V1534-000 R/M-H) were killed by neck dislocation at the age of 5 weeks. Animal use was approved by the local institution in charge (Regierung von Oberbayern, approval no. 55.2-1-54-2531-74-06). Caecal contents were collected into 2-ml tubes and kept on ice for a maximum of 40 min prior to isolation. Wet weights were determined by weighing tubes before and after collection using a TB-215D precision balance (Denver Instrument). For histological analysis, 5-mm-long distal ileal segments adjacent to the caecum were fixed in formalin and embedded in paraffin. Sections were stained with haematoxylin and eosin. Histological scores, from 0 (not inflamed) to 12 (highly inflamed), were determined by assigning points to pathological criteria, as described previously (Katakura et al. 2005). There was no sign of inflammation in the 5-week-old TNF^{deltaARE} mice (mean histological score: 1.0 ± 0.3 and 0.7 ± 0.4 for TNF^{deltaARE} and WT mice, respectively).

Media

The basal solid medium (BM) contained: 4.2 g/l NaHCO₃, 720 mg/l Na₂HPO₄·H₂O, 415 mg/l PdCl₂ (Sigma, cat. no. 520657), 400 mg/l KH₂PO₄, 300 mg/l NaCl, 124 mg/l MgSO₄·7H₂O, 2.5 mg/l phenosafranine, 1.5% (w/v) agar and 1% (v/v) rumen fluid. After autoclaving (121°C, 15 min), BM was allowed to cool down (55°C) in a water bath and was supplemented with filter-sterilised solutions of L-cysteine and DTT to a final concentration of 0.025% (w/v) and 0.02%, respectively. The following compounds were added to BM to obtain the selective medium containing amino acids (A), bile salts (BS) or casein macro-peptides (CMP): 0.25% (w/v) each L-arginine (Sigma, cat. no. A8094) and glycine (AppliChem, cat. no. A1067), 0.25% bile salts (Fluka, cat. no. 48305) or 0.2% casein macro-peptides (LACRODAN[®] CGMP-10, Arla Foods Ingredients amba). The pH of the medium prior to autoclaving was set to: 6.5 (BS), 7.2 (BM and CMP) or 8.5 (A).

Isolation

All steps were carried out in a VA500 workstation (Don Whitley Scientific) containing 85% (v/v) N₂, 10% CO₂ and 5% H₂. The atmosphere was kept at 37°C and 75% humidity. It was tested for anaerobic conditions using Anaerostat[®] (Merck, cat. no. 1.15112.0001). All materials, including agar media, were brought into the workstation 24 h prior to isolation. According to caecal weight measurement, tenfold dilutions (w/v) were prepared by adding appropriate volumes of filter-sterilised phosphate-buffered saline solution (per litre dH₂O: 8.60 g NaCl, 0.87 g Na₂HPO₄, 0.40 g KH₂PO₄, pH 7.2) supplemented with 0.02% (w/v) peptone from meat and 0.05% L-cysteine. Samples were homogenised by vortexing using sterile

glass beads and left to stand for 3 min to sediment debris. Undiluted, ten- and hundredfold diluted cell suspensions (100 µl each) were spread onto the agar media using sterile glass beads. All colony morphology types observed after 6 days of growth were streaked onto blood agar plates (Biomérieux) to support better growth and ensure purity. After 4 days of growth, cells were transferred into GYBHlc broth (Clavel et al. 2009) prepared using anaerobic culture techniques (N₂ gas phase) (Attebery and Finegold 1969). Culture purity was examined by observing cell morphology after Gram-staining and colony morphology. Cryo-stocks (100 µl) were prepared by mixing bacterial suspensions with equal volumes of Tris-buffered aqueous solution (60 mM) containing 40% glycerol. Cryo-stocks were stored at –80°C after snap-freezing in liquid nitrogen.

Phylogenetic analysis and DNA base composition

Washed bacterial cell pellets were boiled in 0.02% (w/v) SDS (95°C, 5 min) and used as template for PCR reactions. If necessary, DNA was extracted from bacterial cell pellets using the innuPREP Bacteria DNA Kit (Analytik Jena). The 16S rRNA genes were amplified using primer 27F 5'-AGA GTT TGA TCC TGG CTC AG and 1492R 5'-GGT TAC CTT GTT ACG ACT T (Kageyama et al. 1999). The annealing temperature was 56°C. Amplicons were purified using agarose gel electrophoresis and the Wizard SV Gel and PCR Clean-Up System (Promega) and sequenced with primer 27F using the Qiagen Genomic Services. The 16S rRNA gene of strain A-C2-0 was further sequenced using primers 338F 5'-ACT CCT ACG GGA GGC AGC and 1492R. The gyrase B genes of strain A-C2-0, *Bacteroides uniformis* and *B. vulgatus* were amplified as described previously using primer gyrBBNDN1 (5' cgcgccacgtcggcrtengycat) and gyrBBAUP2 (5' gcggaagcggccngsnatgta) (Santos and Ochman 2004). Amplicons (approximately 1,500 bp) were purified as described above and sequenced using the aforementioned primers. Sequences of organisms closely related to the isolated strains were obtained using the BLAST function of the NCBI server (Altschul et al. 1990), the Ribosomal Database Project (Cole et al. 2003) and The All-Species Living Tree Project (Yarza et al. 2008). Ribosomal sequences were checked for anomalies using the program Pintail (Ashelford et al. 2005). All sequences were aligned using the Bioedit software, version 7.0.5.3 (Hall 1999). Percentages of similarity were calculated after unambiguous alignment of each isolated sequence with those of the most closely related species, using the DNA Distance Matrix function. The G + C content of DNA of strain A-C2-0 was determined at the German Collection of Microorganisms and Cell Cultures (DSMZ) according to standard methods (Cashion et al.

1977; Tamaoka and Komagata 1984; Mesbah et al. 1989; Visuvanathan et al. 1989).

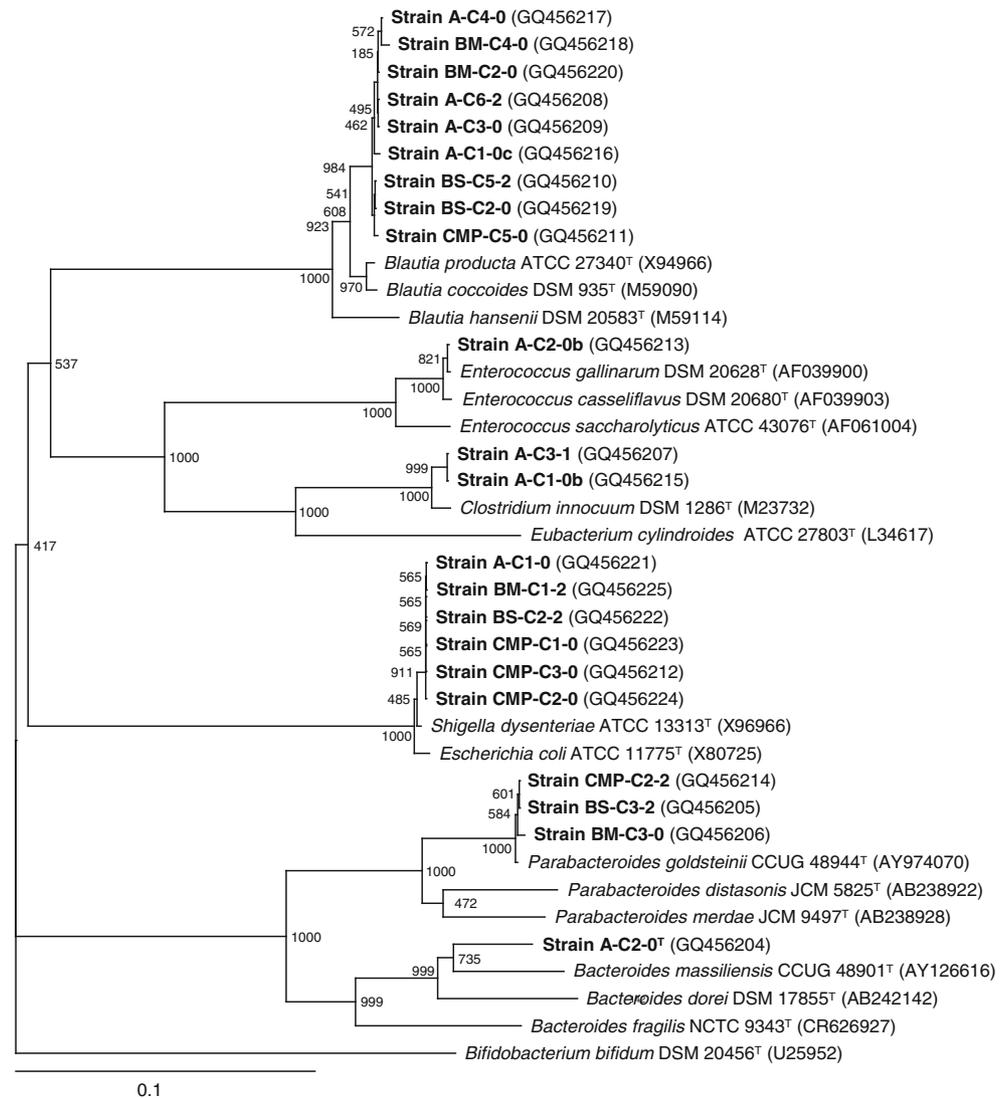
Phenotypic characterisation

All tests were performed as described previously (Clavel et al. 2009). Bacteria were grown in anoxic WCA broth (Oxoid). Quinone, peptidoglycan, polar lipid, whole cell sugar and API 50 CHL analyses were done at the Identification Service of the DSMZ (Braunschweig, Germany) according to standard procedures (Rhuland et al. 1955; Staneck and Roberts 1974; Whiton et al. 1985). Cellular fatty acids were analysed at the Culture Collection University of Göteborg (CCUG, Sweden). Bacteria were grown anaerobically at 37°C on Chocolate-agar (medium no. C376). Conditions for preparation of cell extracts and gas chromatography analysis are detailed in MIDI Technical Note #101 (Microbial ID Inc., Newark, Delaware, USA). Detailed experimental information is available online (<http://www.ccug.se>). Comparison analysis with reference strains was performed as described previously (Eerola and Lehtonen 1988).

Results and discussion

Twenty-two bacterial strains were isolated on four selective agar media from caecal contents obtained from six 5-week-old male WT and heterozygous TNF^{deltaARE} C57BL/6 mice. Figure 1 shows the phylogenetic position and the origin of the isolated strains. The fact that several strains of the same species were obtained from different mice, in spite of the relatively low number of colonies analysed, implies that the identified species belong to common bacterial communities in the mouse caecum. This is in agreement with previously published work, for instance with respect to the occurrence of *Blautia* spp. and *Clostridium innocum* (Lee et al. 1991; Wang et al. 1996). Limited data are available on the isolation and quantification of members of the genus *Parabacteroides* from mouse intestinal samples (Wang et al. 1996; Dewhirst et al. 1999). However, recent investigations using molecular tools have provided a multitude of cloned sequences similar to the sequence of the *Parabacteroides goldsteinii* strains isolated in the present study, suggesting that this taxon, originally recovered from clinical infections of human intestinal origin, is indeed common in the mouse intestine (Salzman et al. 2002; Stecher et al. 2007; Garner et al. 2009). Finally, although cultivation methods have been used for long to enumerate enterococci and *Enterobacteriaceae* from mouse intestinal samples (Tannock and Savage 1974) and the latter taxonomic group seems to be associated with inflammatory conditions (Lupp et al. 2007; Wohlgemuth et al. 2009), only a few mouse intestinal

Fig. 1 Phylogenetic position of the isolated strains among closely related species. The GenBank accession numbers of the 16S rRNA gene sequences (5'-end, 548 bp) used to construct the tree are indicated in brackets. The strains isolated in the present study are written in *bold letters*. Strain numbers indicate the medium used for isolation (A, BM, BS, or CMP), the mouse caecum number (C1 to 6; mouse 1, 2 and 4 were TNF^{delta}ARE mice) and the dilution plated (10^{-x}). Sequences were aligned using the Bioedit Software, and the tree was constructed with Clustal X 1.8 using the neighbour-joining method with bootstrap values calculated from 1,000 trees. Major groupings were confirmed using the maximum parsimony method. *Bifidobacterium bifidum*, a member of the family *Actinobacteria*, was used as outgroup to root the tree. The bar represents 10 nucleotide changes per 100 nucleotides



isolates are available (Berg 1980; Kim et al. 2005; Clavel et al. 2009; Wohlgemuth et al. 2009).

From the data obtained, it was determined that one of the twenty-two isolates, strain A-C2-0, was a novel bacterium isolated from the caecum of a TNF^{delta}ARE mouse on the selective medium containing amino acids. It was identified as a new member of the phylum *Bacteroidetes*. The partial 16S rRNA gene sequence of strain A-C2-0 (1,327 bp) (GQ456204) was: (1) 99% similar to the as yet not described isolate *Bacteroides* sp. TP-5 (AB499846), originating from the intestine of TCR-beta and p53 double-knockout mice, and to cloned sequences from monkey, mouse and rat intestinal samples (Turnbaugh et al. 2006; Ley et al. 2008); (2) \leq 94% similar to sequences of described species: *B. massiliensis* (94.0%), isolated from blood culture of a newborn (Fenner et al. 2005), *Bacteroides dorei* (93.7%) (Bakir et al. 2006) and *B. vulgatus*

(93.0%) (Eggerth and Gagnon 1933), which are both members of human gut microbiota. Detailed phylogenetic analysis, including all representative members of the order *Bacteroidales*, for which 16S rRNA gene sequences are available, showed that strain A-C2-0 belongs to the genus *Bacteroides* (Fig. 2). Since strain A-C2-0 clustered together with numerous cloned sequences obtained in the course of several studies on mammalian gut bacteria, we hypothesise that strain A-C2-0 is a dominant taxon in the intestine of mammals. Additional work on quantitative analysis of strain A-C2-0 and related strains is needed to test this hypothesis. Based on partial sequence analysis of gyrase B genes (1,337 bp), strain A-C2-0 (GQ409831) was 70.4% similar to *B. vulgatus* (GQ409833), 69.2% to *B. fragilis* (CR626927), the type species of the genus, and 69.1% to *B. uniformis* (GQ409832). These data confirm that strain A-C2-0 differs from previously described species of the

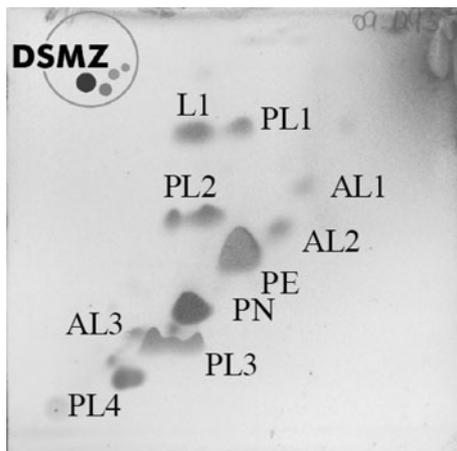


Fig. 3 Two-dimensional thin-layer chromatogram of the polar lipids of strain A-C2-0. Batch cultures (1.5 l) of strain A-C2-0 grown under anoxic conditions for 48 h in WCA broth supplemented with 0.05% (w/v) cysteine were centrifuged ($5,525 \times g$, 10 min, RT) in 500-ml-containers using a 4K15C centrifuge (Sigma). Pellets were re-suspended in sterile PBS, pooled in 50-ml-FALCON tubes and bacterial suspensions were centrifuged ($5,525 \times g$, 15 min, RT). Supernatants were discarded, cell pellets were frozen at -80°C and dried by lyophilisation for 24 h (Alpha 1-4 LDplus, Christ) prior to shipping. Polar lipid analysis was done by the Identification Service of the DSMZ and Dr. B.J. Tindall (Braunschweig, Germany). AL, unidentified aminolipid (AL1 stains orange); L, lipid; PE, phosphatidylethanolamine; PL, phospholipid; PN, aminophospholipid

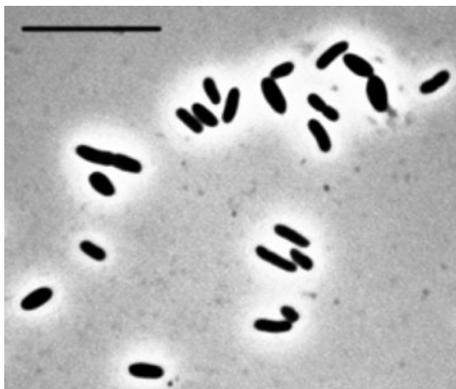


Fig. 4 Phase contrast microscopic picture showing the cell morphology of strain A-C2-0. The bar represents 10 μm

and negative control, respectively. Enzymatic tests using rapid ID 32 A and API 50 CHL strips (Biomérieux) showed that strain A-C2-0 was positive for α - and β -galactosidase, α -glucosidase, α -arabinosidase, N-acetyl- β -glucosaminidase, mannose and raffinose fermentation, glutamic acid decarboxylase, α -fucosidase, alkaline phosphatase, arginine, leucyl glycine, leucine and alanine arylamidase as well as lactose, maltose and melibiose

metabolism. Parameters that distinguish the isolate from phylogenetically closely related species are given in Table 1. The sensitivity of strain A-C2-0 was tested towards ten antimicrobial agents as described previously (Clavel et al. 2009). Each antibiotic was tested in duplicate in three independent experiments and the MIC breakpoint was expressed as the average of those six replicates. MIC breakpoints were ($\mu\text{g/ml}$): cefotaxime (3rd generation cephalosporin), >32 ; ciprofloxacin, $3,000 \pm 0.967$; clarithromycin, 0.006 ± 0.010 ; clindamycin, <0.016 ; erythromycin, 0.151 ± 0.032 ; metronidazole, 0.110 ± 0.036 ; oxacillin (class penicillin), $8,500 \pm 1.708$; tetracycline, 0.089 ± 0.014 ; tobramycin, >256 ; vancomycin, 4.667 ± 0.615 . Thus, strain A-C2-0 is resistant to the broad spectrum β -lactam antibiotic cefotaxime and to the aminoglycoside tobramycin. Most of the other antimicrobial agents tested, with the exception of oxacillin, a narrow-spectrum β -lactam antibiotic, and vancomycin demonstrated high susceptibility of strain A-C2-0. With respect to cefotetan (2nd generation cephalosporin), clindamycin, metronidazole and vancomycin, *B. massiliensis* showed a similar pattern of antibiotic resistance (Fenner et al. 2005).

In conclusion, the work has led to the isolation and identification of six cultivable species from the mouse caecum, including one novel bacterium. Ongoing investigations aim at characterising the prevalence and functions of the new species with respect to host cellular responses in the gut.

Description of *Bacteroides sartorii* sp. nov

Bacteroides sartorii (sar.to'ri.i. N.L. gen. n. *sartorii*, in honour of Balfour Sartor, Professor of Medicine, Microbiology and Immunology at the University of North Carolina in Chapel Hill, for his outstanding contribution to the understanding of microbial ecology in IBD).

The species has the features of the genus. It is phylogenetically related to *Bacteroides massiliensis*. Cells are approximately $1.0\text{--}1.5 \times 2.0\text{--}4.0 \mu\text{m}$, are aerotolerant but grow only under strict anoxic conditions. They grow well at pH values between 6.0 and 9.0, in the temperature range from 25 to 40°C and in the presence of 0.5% (v/v) bile salts. Slight turbidity is observed at pH values between 4.5 and 6.0. After 48 h of growth at 37°C on blood agar, colonies are circular, entire, raised, grey and non-haemolytic. The species is positive for α -arabinosidase, negative for glutamyl glutamic acid arylamidase and does not metabolise galactose, glucose, fructose, mannose, raffinose and sucrose. Its major menaquinone is MK-9 and its major fatty acid is $\text{C}_{15:0}$ ANTEISO. The G + C content of DNA is 41.5 mol%. Resistance to cefotaxime and tobramycin has been shown.

Table 1 Characteristics of strain A-C2-0 and of phylogenetically closely related species of the genus *Bacteroides* (Symbols: +, positive reaction; –, negative reaction; ±, ambiguous reaction (Fenner et al. 2005; Bakir et al. 2006); ND not determined)

	1	2	3	4
Origin	Mouse caecum	Newborn blood	Human faeces	Human faeces
G + C content of DNA (mol%)	41.5	49	40–42	43
α -Arabinosidase [†]	+	–	+	+
β -glucuronidase [†]	–	–	+	+
Glutamic acid decarboxylase [†]	+	±	+	+
Glutamyl glutamic acid arylamidase [†]	–	±	+	+
Leucine arylamidase [†]	+	+	–	+
Galactose*	–	+	ND	ND
Glucose*	–	+	+	+
Fructose*	–	+	+	ND
Lactose*	+	+	+	+
Maltose*	+	+	+	+
Mannose*	–	+	+	+
Melibiose*	+	+	ND	ND
Raffinose*	–	+	+	+
Sucrose*	–	+	+	+
Respiratory quinones	MK-9	ND	MK-10, -11	ND
Cellular fatty acids [§]				
13:0	–	–	0.9	–
13:0 ISO	0.5	0.7	0.6	–
13:0 ANTEISO	1.0	1.2	1.7	0.7
14:0	0.6	0.3	1.1	0.4
14:0 ISO	–	–	3.2	1.2
14:0 aldehyde	–	–	–	0.5
15:0	12.9	7.7	26.2	12.5
15:0 ISO	7.3	9.6	6.3	7.0
15:0 ANTEISO	43.6	31.1	28.4	35.2
15:0 ISO aldehyde	3.9	9.7	1.5	7.8
15:0 3OH	–	–	4.1	2.0
16:0	4.3	2.9	3.3	3.4
16:0 ISO	0.5	0.4	–	–
16:0 3OH	1.9	0.0	4.2	2.3
16:0 ISO 3OH	–	–	1.1	0.6
16:1 w7c	0.0	0.4	0.3	–
17:0 ISO	0.5	0.7	–	–
17:0 ANTEISO	0.8	0.8	–	0.4
17:0 2OH	–	–	1.2	1.9
17:0 3OH	–	–	2.9	2.0
17:0 ISO 3OH	11.6	21.3	5.2	11.8
17:1 w9c ANTEISO	0.8	1.2	–	–
18:0	1.0	1.1	0.6	1.2
18:0 ANTE	4.7	6.1	3.3	4.4
18:1 w9c	3.1	3.7	2.5	2.7
Unidentified	–	1.1	1.3	2.1

Taxa: 1, *Bacteroides sartorii* sp. nov. (strain A-C2-0^T); 2, *B. massiliensis* (Fenner et al. 2005); 3, *B. vulgatus* (Holdeman et al. 1984); 4, *B. dorei* (Bakir et al. 2006)

[†], * Analysed using the rapid ID 32 A and the API 20 A identification system, respectively

[§] Strains were *B. sartorii* CCUG 57211^T, *B. massiliensis* CCUG 48901^T, *B. vulgatus* CCUG 12546 and *B. dorei* CCUG 53892^T

The type strain, A-C2-0^T (= DSM 21941^T = CCUG 57211^T), was isolated from the caecum of a 5-week-old male heterozygous TNF^{deltaARE} C57BL/6 mouse.

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