Bacteroides cellulosilyticus sp. nov., a cellulolytic bacterium from the human gut microbial community

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A strictly anaerobic cellulolytic bacterium, strain CRE21^T, was isolated from a human faecal sample. Cells were Gram-negative non-motile rods that were about 1.7 µm in length and 0.9 µm in width. Strain CRE21^T degraded different types of cellulose and was able to grow on a variety of carbohydrates. Cellulose and sugars were mainly converted to acetate, propionate and succinate. The G+C content of the DNA was 41.1 mol%. 16S rRNA gene sequence analysis revealed that the isolate belonged to the genus *Bacteroides* with highest sequence similarity to the type strain of *Bacteroides intestinalis* (98%). DNA–DNA hybridization results revealed that strain CRE21^T was distinct from *B. intestinalis* (40% DNA–DNA relatedness). Strain CRE21^T also showed several characteristics distinct from *B. intestinalis*. In particular, it exhibited different capacity to degrade polysaccharides such as cellulose. On the basis of phylogenetic analysis and the morphological, physiological and biochemical data presented in this study, strain CRE21^T can be readily differentiated from recognized species of the genus *Bacteroides*. The name *Bacteroides cellulosilyticus* sp. nov. is proposed to accommodate this organism. The type strain is CRE21^T (= DSM 14838^T = CCUG 44979^T).

Most of the bacterial species making up the human gut microbiota are strict anaerobes, the predominant organisms being members of the genus *Bacteroides* (Harmsen *et al.*, 2002). Until recently, however, only a few *Bacteroides* species isolated from the human gut had been described (Salyers, 1984), most belonging to one of the ten species of the *Bacteroides fragilis* group. Recent investigations of this *Bacteroides* population have improved our knowledge of its structure, through isolation and description of novel species such as *Bacteroides plebeius*, *Bacteroides coprocola*, *Bacteroides helcogenes*, *Bacteroides intestinalis*, *Bacteroides finegoldii* and *Bacteroides dorei* (Kitahara *et al.*, 2005; Bakir *et al.*, 2006a, b, c). As *Bacteroides* species are numerically important, they are assumed to play key roles in the degradation and fermentation of organic matter present in the colon (Salyers, 1995). Some *Bacteroides* species were

indeed shown to be able to obtain carbon and energy from hydrolysis of soluble or hydrated polysaccharides (Bétian *et al.*, 1977; Salyers, 1995). In addition, degradation of maize bran, oat bran and wheat bran was evidenced in one strain of *Bacteroides ovatus* (Martin *et al.*, 1998) while one *Bacteroides* sp. isolate, but which was not further identified, was reportedly able to use pure cellulose (Bétian *et al.*, 1977). Although insoluble polysaccharides, mainly found in the plant cell wall (cereals, fruits, vegetables), constitute one of the main sources of carbohydrates that are extensively degraded by the human gut microbiota, the fibrolytic population involved in the breakdown of these substrates remains rather poorly explored. During the course of a study on the predominant cellulose-degrading community from the human colon, a Gram-negative bacterium that showed high 16S rRNA gene sequence similarity to members of the genus *Bacteroides* was isolated from a faecal sample. The present study describes this new isolate, designated strain CRE21^T, and further proposes that it represents a novel species of the genus *Bacteroides*.

Strain CRE21^T was isolated from faeces of a non-methane-excreting healthy volunteer (50 years old, male) by using a strictly anaerobic technique (Hungate, 1969). All liquid and solid media were prepared, dispensed and inoculated under 100% O₂-free CO₂ gas. Freshly voided faeces (1 g) were transferred into 10 ml sterile anoxic mineral solution and

Abbreviations: CMCase, carboxymethylcellulase; CWS, cell-wall spinach. The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain CRE21^T is AJ583243.

An unrooted neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain CRE21^T and some related members of the genus *Bacteroides*, a figure showing the kinetics of cellulose degradation by strain CRE21^T and a table detailing the end products of cellulose fermentation by strain CRE21^T are available as supplementary material with the online version of this paper.

serial 10-fold dilutions down to 10^{-11} were then carried out in mineral solution. These faecal dilutions were inoculated (0.3 ml) into liquid basal cellulolytic (BC) medium (10 ml per tube) (Robert & Bernalier-Donadille, 2003) with cell-wall spinach (CWS) fraction at a final concentration of 0.7 % as the sole energy source. CWS residue was obtained as described by Mosoni *et al.* (1993). After 15 days incubation at 37 °C, the presence of cellulolytic organisms in faecal dilution cultures was estimated by measuring carboxymethylcellulase (CMCase) activity. Detection of CMCase activity was performed by using an agar plate assay as described by Forano *et al.* (1994).

Strain CRE21^T was isolated from the highest dilution faecal cultures showing CMCase activity (10^{-8}). Isolation was carried out by using solid BC medium containing CWS as energy source and the roll-tube technique. After three to five successive subcultures on roll-tubes and broth BC medium, the isolate was examined for purity using phase-contrast microscopy, in CWS- and glucose- (2 g l^{-1}) grown cultures. The medium used for further routine cultivation of strain CRE21^T was BC medium while growth and nutrition studies of strain CRE21^T were carried out in semi-synthetic BC medium (Robert *et al.*, 2001). Cell morphology and motility were studied in 18-h glucose-grown cultures using phase-contrast microscopy and electron microscopy after negative staining of the whole-cell fraction with 2 % uranyl acetate. Gram staining was determined by using conventional methodology. Examination of cell morphology and cell-wall structure was also made on ultrathin sections by using transmission electron microscopy (Philips 400) after staining with uranyl acetate and lead citrate (Bernalier *et al.*, 1996). The presence of catalase and cytochrome oxidase was examined using standard methods. The optimal growth temperature of strain CRE21^T was determined in semi-synthetic BC medium containing glucose (2 g l^{-1}) at pH 6.8 over a temperature range of 25–45 °C (at 1 °C intervals). The influence of pH on growth of CRE21^T was evaluated in semi-synthetic BC medium containing glucose (2 g l^{-1}) at 37 °C. The pH range studied was 5.5–7.5 (at 0.1 pH unit intervals, adjusted with varying concentrations of NaHCO_3 in the medium).

Cells of strain CRE21^T were rods with rounded ends, with a mean length of 1.7 µm and mean width of 0.9 µm. Cells occurred singly or in pairs. Cells stained Gram-negative and thin sections examined via transmission electron microscopy showed a Gram-negative cell-wall structure. Negatively stained cells revealed the absence of flagella. Viable cells could not be recovered from cultures held at 100 °C for 10 min and no spores were observed in cellulose- or in glucose-grown cultures incubated at 37 °C for 30 days or more. Strain CRE21^T was strictly anaerobic and required an O_2 -free medium at a redox potential sufficient to decolorize resazurin ($E_0 = -50 \text{ mV}$). The cells did not possess catalase or cytochrome oxidase activity. With glucose as substrate, the optimal growth temperature was 37 °C with growth occurring from 30 to 39 °C. Strain CRE21^T grew from an

initial pH of 6.5 up to 7.2, with optimal growth at pH 6.8. These optimal conditions of growth are in line with those found in the human colon. Rumen fluid was not required for growth.

DNA extraction, PCR and sequencing of the 16S rRNA gene of strain CRE21^T were performed as reported by Bernalier *et al.* (1996). Strain CRE21^T was grown for 24 h in 50 ml BC medium with glucose (2 g l^{-1}) as carbon source. Cells were harvested by centrifugation for 15 min at 9000 g at 4 °C. The bacterial pellet was then subjected to DNA extraction (Easy DNA kit Genomic DNA Isolation; Invitrogen BV). The 16S rRNA gene was then amplified using the universal primers F8 (5'-AGAGTTTGATCMTGGCTC-3') and 1492R (5'-GNTACCTTGTTACGACTT-3'). Approximately 50 ng of purified PCR product was included in a 20-µl sequencing reaction. Sequencing reactions were performed using a Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) according to the manufacturer's specifications. All reactions were carried out with an ABI Prism cycle sequencing kit and gels were run on an ABI PRISM 310 automated sequencer. The closest known relatives of the new isolate were determined by performing database searches by using the program FASTA (Pearson & Lipman, 1985). These sequences and those of other related strains were retrieved from GenBank and aligned with the newly determined sequences according to the program SEQtools (<http://www.seqtools.dk>). The resulting multiple sequence alignment was corrected manually by using the program GeneDoc (Nicholas *et al.*, 1997) and a phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the programs SEQtools and TREEVIEW (Page, 1996). The G + C content of the genomic DNA was determined by HPLC by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany).

The 16S rRNA gene of strain CRE21^T was sequenced (1422 bases) and phylogenetic analysis revealed that this cellulolytic bacterium was most closely related to members of the phylum *Bacteroidetes*. Strain CRE21^T shared 97–98 % 16S rRNA gene sequence similarity with its most closely related species, *B. intestinalis*, and only 92 % with *B. helcogenes*. A phylogenetic tree showing the relationships of strain CRE21^T to other *Bacteroides* species is available as Supplementary Fig. S1 in IJSEM Online. The G + C content of the DNA of strain CRE21^T was 41.1 mol%; reported values for *B. intestinalis* and *B. helcogenes* are 44 and 45 mol%, respectively (Table 1).

Given that the 16S rRNA gene sequence similarity between strain CRE21^T and *B. intestinalis* was close to 98 %, DNA–DNA hybridization experiments between these two taxa were performed by the DSMZ service. DNA of strain CRE21^T and of *B. intestinalis* DSM 17393^T was isolated by using a French pressure cell from cells harvested from brain heart infusion broth medium and was further purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977). DNA–DNA hybridization was carried out as described by

Table 1. Differential characteristics between strain CRE21^T and related *Bacteroides* species

Taxa: 1, strain CRE21^T; 2, *B. intestinalis* DSM 17393^T (data from Bakir *et al.*, 2006a); 3, *B. helcogenes* DSM 20613^T (Kitahara *et al.*, 2005; Bakir *et al.*, 2006a). +, Positive; –, negative; W, weak.

Characteristic	1	2	3
Aesculin hydrolysis	W	+	+
Acid produced from:			
Salicin	W	+	+
D-Cellobiose	W	+	+
L-Arabinose	W	+	–
Trehalose	–	–	–
Enzymic reactions:			
α -Fucosidase	W	+	+
6-Phospho- β -galactosidase	–	–	+
α -Arabinosidase	+	+	–
β -Glucuronidase	–	–	+
Proline arylamidase	–	–	+
Phenylalanine arylamidase	W	–	+
Tyrosine arylamidase	W	–	–
Major cellular fatty acids	anteiso-C _{15:0} , C _{15:0} , iso-C _{17:0} 3-OH	anteiso-C _{15:0} , iso-C _{17:0} 3-OH, C _{18:1ω7c} , C _{16:0} 3-OH	anteiso-C _{15:0} , iso-C _{17:0} 3-OH, C _{18:1ω7c}
DNA G + C content (mol%)	41.1	44	45

De Ley *et al.* (1970) and modified by Huß *et al.* (1983) by using a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe. Strain CRE21^T showed DNA–DNA relatedness of 40 % with *B. intestinalis* DSM 17393^T. This low DNA–DNA relatedness value (<70 %) with its most closely related species indicated that strain CRE21^T represented a novel species.

Utilization of different substrates by strain CRE21^T was determined with semi-synthetic BC medium containing several carbon sources. Carbon sources were added from sterile stock solutions to a final concentration of 10 mM. Carbon source utilization by strain CRE21^T was determined after incubation of the cultures at 37 °C for at least 4 days and by examination of the ability of the strain to maintain growth after three successive transfers on the same substrate. Bacterial growth was monitored by determining the optical density of the culture at 600 nm (OD₆₀₀). Other biochemical and enzymic activity tests were performed by using the API Rapid ID 32AN and API ZYM test kit systems (bioMérieux) according to the manufacturer's instructions and anaerobic incubation at 37 °C. Furthermore, the cellular fatty acid profile of strain CRE21^T was determined using gas chromatography at the Culture Collection, University of Göteborg (Sweden). End products of cellulose and glucose fermentation were determined in 12-day and 24-h cultures, respectively. Gases in the headspace of cultures and short-chain fatty acids in culture supernatants were analysed by gas phase chromatography (Robert *et al.*, 2001). Formate, succinate, ethanol and lactate production were measured using enzymic methods (Roche).

Strain CRE21^T was able to utilize a variety of substrates. Sugar utilization and enzymic activity patterns of strain CRE21^T were similar to those of *B. intestinalis*. However, strain CRE21^T could be differentiated from *B. intestinalis* and *B. helcogenes* based on several characteristics (Table 1). The major cellular fatty acid of strain CRE21^T was anteiso-C_{15:0} (37.8 %) in agreement with data for the genus *Bacteroides* (Miyagawa *et al.*, 1979) (Table 1). Other cellular fatty acids found in significant amounts in strain CRE21^T included iso-C_{17:0} 3-OH (12.7 %) and C_{15:0} (15 %). The hydroxy acid iso-C_{17:0} 3-OH was also found in *B. intestinalis* and *B. helcogenes* but at a higher proportion of the total (19.2 and 17.7 %, respectively). The other cellular fatty acids found in *B. intestinalis* and *B. helcogenes* were different from those found in strain CRE21^T (Table 1). The end products of cellulose and glucose fermentation by strain CRE21^T were mainly acetate, propionate and succinate, with formate and lactate also being produced in smaller quantities (results are given in Supplementary Table S1 in IJSEM Online). Other *Bacteroides* species isolated from the human gut such as *B. ovatus*, *Bacteroides thetaiotaomicron* and *B. fragilis* similarly produced acetate, propionate and succinate from sugar fermentation (Salysers *et al.*, 1981) but, in contrast to strain CRE21^T, they did not produce lactate.

The ability of strain CRE21^T and of *B. intestinalis* DSM 17393^T to degrade the main polysaccharides that make up dietary fibres was compared. Strain CRE21^T and *B. intestinalis* DSM 17393^T were cultivated in liquid BC medium (10 ml per tube) containing Avicel pH 101 cellulose (100 mg), Sigmacell type 101 cellulose (100 mg), oat spelt xylan (100 mg), pectin from citrus (20 mg), starch from

potatoes (20 mg) or CWS (100 mg) as sole energy source. Cultures were incubated at 37 °C for 2–4 days and three culture tubes were inoculated for each substrate. After incubation, substrate utilization was estimated for the two strains by measuring bacterial growth (OD₆₀₀). In cellulose-, xylan- and CWS-grown cultures, substrate disappearance was measured (disappearance of dry matter) and CMCase and xylanase activities were detected by using an agar plate test (Forano *et al.*, 1994). Cellulase activities of strain CRE21^T were further determined by using enzymic assays in which reducing sugars released from carboxymethylcellulose and Avicel pH 101 cellulose were measured colorimetrically with glucose as standard (Robert & Bernalier-Donadille, 2003). Each enzymic assay was performed in triplicate.

Strain CRE21^T and *B. intestinalis* DSM 17393^T showed different capacities to utilize dietary polysaccharides (Table 2). Strain CRE21^T was able to degrade different types of cellulose (results are shown in Supplementary Fig. S2 in IJSEM Online), including the CWS cellulose, and expressed CMCase [54.0 ± 0.2 µg glucose min⁻¹ (mg protein⁻¹)] and avicellase [24.0 ± 0.1 µg glucose min⁻¹ (mg protein⁻¹)] activities in cellulose-grown cultures. *B. intestinalis* DSM 17393^T was not able to grow on cellulosic substrates. Nevertheless, *B. intestinalis* showed the ability to degrade xylan whereas poor growth and weak xylanase activity were detected for strain CRE21^T. The two bacterial taxa were able to use pectin and starch but *B. intestinalis* showed poor ability to degrade starch compared with strain CRE21^T. Polysaccharide utilization profiles thus differentiated between strain CRE21^T and *B. intestinalis* DSM 17393^T. Strain CRE21^T remains the sole *Bacteroides* representative isolated from the human gut and described to date that is able to degrade cellulose.

On the basis of the phenotypic, genotypic and phylogenetic data presented here, we propose that strain CRE21^T represents a novel species of the genus *Bacteroides*. Strain CRE21^T showed properties that characterize it as typical for the genus *Bacteroides*: Gram-negative rods, non-spore-forming,

non-motile, obligately anaerobic with a G+C content ranging from 40 to 48 mol% (Shah, 1992), saccharolytic, producing acetate and succinate as the major metabolic end products (Holdeman & Moore, 1974) and anteiso-C_{15:0} as the major cellular fatty acid (Miyagawa *et al.*, 1979). Strain CRE21^T was most closely related to *B. intestinalis*, which was isolated from the human colon, but these two taxa showed a low level of DNA–DNA relatedness (<70 %). In addition, phenotypic differences could be shown between strain CRE21^T and *B. intestinalis*, in particular in their abilities to degrade polysaccharides. Strain CRE21^T is characterized by its ability to degrade cellulose compared with *B. intestinalis* as well as other *Bacteroides* species described from the human gut. Given this physiological characteristic, we propose the name *Bacteroides cellulosilyticus* sp. nov. to accommodate strain CRE21^T.

Description of *Bacteroides cellulosilyticus* sp. nov.

Bacteroides cellulosilyticus (cell.u'lo.si.ly'ti.cus. N.L. n. *cellulosum* cellulose; Gr. adj. *lutikos* loosening, dissolving; N.L. adj. *lyticus* -a -um dissolving; N.L. masc. adj. *cellulosilyticus* cellulose-dissolving).

Cells are non-motile rods, 1.7 µm in length and 0.9 µm in width. Gram-negative by staining and cell-wall ultrastructure. No heat-resistant endospores are formed. Strictly anaerobic. Colonies developed on glucose-BC agar medium are white to slightly brown, translucent, circular with entire margins and about 2–5 mm in diameter. Cytochrome oxidase- and catalase-negative. Metabolizes cellulose to acetate, propionate and succinate. Also ferments glucose, sucrose, fructose, maltose, xylose, galactose, ribose, melibiose, mannose, lactulose, galacturonic acids, pectin, starch and cellulose. Poor growth is exhibited with lactose, raffinose, arabinose, cellobiose, aesculin, xylan and salicin. No growth is observed on trehalose, mannitol, inositol, sorbitol or fucose. Positive reactions are obtained with the API Rapid ID 32AN and API ZYM systems for *N*-acetyl-β-glucosaminidase, alkaline phosphatase, alanine arylamidase, α-arabinosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, glutamic acid decarboxylase, glutamyl glutamic acid arylamidase, leucyl glycine arylamidase, mannose, raffinose, indole α-fucosidase (weak), phenylalanine arylamidase (weak), tyrosine arylamidase (weak), acid phosphatase, naphthol-AS-BI-phosphohydrolase and esterase lipase C8 (weak). Growth occurs between 30 and 39 °C, and pH 6.5 and 7.2. Optimal growth conditions are 37 °C and pH 6.8. Rumen fluid is not required for growth. Major fatty acids are anteiso-C_{15:0} (37.8 %), iso-C_{17:0} 3-OH (12.7 %) and C_{15:0} (15 %). The G+C content of the DNA of the type strain is 41.1 mol%.

The type strain, CRE21^T (= DSM 14838^T = CCUG 44979^T), was isolated from human faeces of a non-methane-excreting individual.

Table 2. Differential abilities of strain CRE21^T and *B. intestinalis* DSM 17393^T to degrade various polysaccharides

+, Positive; –, negative; w, weak. Both strains degraded pectin.

Polysaccharide degradation	Strain CRE21 ^T	<i>B. intestinalis</i> DSM 17393 ^T
Cellulose Avicel pH 101	+	–
Cellulose Sigmacell 101	+	–
Starch	+	w
Xylan	w	+
CWS	+	–

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