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The cellulolytic microflora of the human colon: evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects

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Abstract

In humans, plant cell wall polysaccharides (mainly cellulose and hemicelluloses) represent an important source of dietary fibres that are digested by the gut microflora. However, the fibrolytic micro-organisms involved in the breakdown of these substrates remain largely unknown. Our objective was to quantify the microcrystalline-cellulose-degrading and methanogenic microbial communities in faecal samples ($n = 34$) from both methane- and non-methane-excreting individuals and to identify the predominant cellulolytic organisms in these two categories of subjects. Microcrystalline cellulose degraders could only be enumerated in faecal samples from methane excretors while this community remained undetectable in non-methane-excretors. The cellulolytic isolates corresponded to new *Ruminococcus* species and to *Enterococcus* sp. closely related to *Enterococcus faecalis*. The presence of such fibrolytic species seems to be linked to that of methanogenic archaea in the gut, the relationships between these two microbial communities needing further investigation. Our findings suggest that the structure and activity of the cellulolytic communities differ in methane- and non-methane-excreting individuals.

Keywords: Human colon; Fibre hydrolysis; Cellulolytic bacterium; Methanogenic archaea

1. Introduction

In humans, dietary fibres, i.e. molecules escaping digestion by the endogenous enzymes of the upper digestive tract, represent an important source of substrates for the resident anaerobic microbial communities of the colon. Furthermore, fermentation of these dietary fibres by gut microbial communities is now well recognised to provide health benefits to the host (regulation of digestive transit, prevention of obesity and diabetes through control of glucose absorption and insulin response, and reduction of cardiovascular diseases and cancer through plant polymer-associated bioactive compounds with anti-oxidant activities) [1]. These fibres comprise many types of macromolecules from plant sources, including insoluble plant cell wall polysaccharides (mainly cellulose and hemicelluloses) that represent 20–30% of the dietary fibres ingested daily. The degradation and fermentation of these substrates in the colon require the contribution of different groups of micro-organisms linked in a trophic chain. The fibrolytic community acts at the beginning of this chain and plays a major role by hydrolysing polysaccharides into smaller fragments that are further fermented into short-chain fatty acids (mainly acetate, propionate and butyrate) and gases (H_2, CO_2). Despite the high percentage of digestibility of cellulose and hemicellulose (50–90%) in the human gut [2], the fibrolytic community involved in degradation of these plant cell wall polysaccharides has not been extensively explored. An important hemicellulolytic activity has been found in several bacterial species including the predominant *Bacteroides* sp. [3]. The presence of this activity in the numerically most important taxon in the human colon may explain the extensive degradation of hemicelluloses in the ecosystem. In contrast, attempts to identify the cellulolytic bacteria from human faeces have not been so successful. The few studies dealing with this microbial community have been performed on a small number of subjects (from two to six individuals),

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using different sources of cellulose [4–6]. The cellulolytic strains isolated phenotypically resembled *Ruminococcus* sp., *Clostridium* sp., *Eubacterium* sp. [5,6] and *Bacteroides* sp. [4].

Plant polysaccharide degradation in the colon was previously shown to be affected by hydrogenotrophic micro-organisms through interspecies H_2 transfer [7,8]. H_2 is a central microbial metabolite produced from sugar fermentation that must be eliminated from the ecosystem in order to favour the complete oxidation of organic substrates. H_2 utilisation by hydrogenotrophic micro-organisms remains the major route of H_2 disposal in the gut [9], part of this gas (15–20%) being also excreted in breath or flatus. The major H_2-utilising micro-organisms existing in the human gut are methanogenic archaea, sulfate-reducing and acetogenic bacteria [10,11]. Only 30–40% of the occidental adult population harbour a methanogenic community higher than a threshold level of 10^7–10^8 methanogens g^{-1} dry weight faeces, allowing detection of CH_4 in breath [12]. Therefore, the human population is commonly separated into methane excretors (CH_4^+) and non-methane-excretors (CH_4^-), although factors determining the methane production status remain unknown. H_2 transfer involving either methanogenic archaea or acetogenic bacteria was shown to affect differently plant polysaccharide degradation and fermentation by fibrolytic species [8]. This suggests that polysaccharide breakdown in the colon should be further influenced by the nature of the predominant H_2-utilising micro-organisms, in particular through modification of the structure and/or activities of the fibrolytic community.

In this context, the objective of our study was to quantify the cellulolytic and methanogenic microbial communities in faecal samples from CH_4-excreting and non-CH_4-excreting human subjects, to determine the diversity and activity of the fibrolytic microflora in the two categories of individuals and to characterise genetically and physiologically the predominant cellulolytic bacterial isolates. The cellulolytic microflora was investigated using purified microcrystalline cellulose as specific substrate for enumeration and isolation of these organisms.

2. Materials and methods

2.1. Human subjects

Thirty-four healthy subjects, comprising 13 males and 21 females, aged 19–53 years, took part in the present study. All consumed standard western diet. None had received antibiotics during the 3 months preceding the study and had any previously diagnosed gastrointestinal disease. All participants gave informed consent to the protocol, which was approved by the local ethical committee.

Breath methane concentration was determined with a Microlyser-SC gas chromatograph (Quintron Instrument, Biotech, France). Seventeen volunteers, who did not expire any CH_4, were selected as non-methane-excretors (CH_4^-). The other 17 subjects, selected as methane excretors (CH_4^+), excreted CH_4 at a concentration that was in excess of 1 ppm that of the ambient atmosphere [13].

2.2. Media and enumeration procedures

Freshly voided faeces were obtained from all subjects, stored at 4°C under anaerobic conditions and processed within 12 h. One gram of collected faecal sample was diluted 10-fold (wet weight/volume) in an anaerobic mineral solution. Serial 10-fold dilutions down to 10^{-11} were then carried out.

All liquid and solid media were prepared, dispensed and inoculated using strictly anaerobic techniques [14], with 100% O_2-free CO_2 gas. Total anaerobes, H_2-utilising methanogens and cellulolytic bacteria were enumerated in triplicate by the most probable number estimation (MPN) [15].

Total anaerobes were enumerated in a complex medium containing clarified rumen fluid [16] and bacterial growth was recorded in culture tubes of each dilution inoculated (10^{-7}–10^{-11}) after incubation for 48 h at 37°C.

H_2-utilising methanogens were enumerated as described by Doré et al. [10]. Faecal sample dilutions from 10^{-2} to 10^{-9} were inoculated into broth culture medium. After inoculation, culture tubes were pressurised with H_2/CO_2 (80/20 v/v, 202 kPa). Vials with CH_4 levels above 0.1% after 15 days incubation at 37°C were counted positive.

Cellulolytic bacteria were enumerated in the basal cellulolytic (BC) medium containing, per litre: 200 ml clarified rumen fluid, 75 ml mineral solution I, 75 ml mineral solution II, 10 ml trace element solution [17], 10 ml vitamin solution [18], 10 ml volatile fatty acid solution [18], 0.5 g yeast extract, 1.0 g tryptone, 1 ml resazurin (0.1%), 1 ml hemin (0.1%), 20 ml cysteine sulfide-reducing agent, 2.5 g $NaHCO_3$. Mineral solution I was composed of 6.0 g l^{-1} K_2HPO_4 and mineral solution II contained, per litre, 6.0 g KH_2PO_4, 12 g $(NH_4)_2SO_4$, 12.0 g NaCl, 1.2 g $MgSO_4$, 1.2 g $CaCl_2$. The cysteine sulfide-reducing agent contained 1.25% (w/v) each cysteine-HCl·H_2O and Na_2S·$9H_2O$. A piece of Whatman No. 1 filter paper cellulose (50 mg) was added to each tube before the addition of the pre-reduced BC medium (10 ml per tube). After incubation at 37°C for 21 days, cellulose utilisation in the culture tubes of each dilution inoculated (10^{-3}–10^{-9}) was detected visually by noting degradation of the filter paper.

2.3. Assay of cellulolytic and xylanolytic activities of faecal microflora

Cellulolytic and xylanolytic activities of the faecal microflora from 10 volunteers (five CH_4^+ and five CH_4^-) were determined by enzymatic assays in which reducing sugars released from carboxymethyl cellulose (CMC) and oat spelt xylan (Sigma Chemicals, St. Louis, MO, USA)

were measured colorimetrically [19,20]. All handling procedures of faecal samples were performed under anaerobic conditions using 100% N_2 gas. Faecal samples (10 g) were diluted 50% in N_2-saturated sodium phosphate buffer (50 mM, pH 6.8). The suspension was mixed in a stomacher, three times 1 min, and then centrifuged at 15 500 × *g* for 30 min at 4°C in order to eliminate food particles and bacteria. After a second similar centrifugation, the resulting faecal supernatant was used for enzymatic measurements. One hundred μl of 10-fold diluted (sodium phosphate buffer) faecal supernatant was incubated at 39°C for 5, 10, 15, 30, 60 and 90 min with either 1.4 ml of 50 mM sodium phosphate buffer, pH 5.5, containing 0.5% (w/v) CMC low viscosity or 1.4 ml of 50 mM sodium phosphate buffer, pH 6.8, containing 0.5% (w/v) oat spelt xylan. After incubation, the reaction was stopped by boiling for 10 min. Reducing sugars released from polysaccharide substrates were determined by the method of Miller [21]. Protein concentration in faecal supernatants was measured by the method of Bradford [22], with bovine serum albumin as standard. Each enzymatic assay was performed in triplicate.

2.4. Isolation of cellulolytic bacteria

Enrichments of cellulolytic bacteria were obtained from the highest dilution tubes in MPN enumeration in BC medium, showing filter paper cellulose degradation. Two to three weekly subcultures were performed in filter paper–BC broth medium before transferring the enriched cultures into roll tubes containing the same medium with 2% Bacto agar and 0.7% of hydrated Sigmacell® 50 cellulose (Sigma Chemicals). Colonies that formed clear zones in cellulose agar were picked from roll tubes and transferred into filter paper–BC broth medium. After three to five successive subcultures on roll tubes and BC broth medium, isolates were examined for purity, morphology and Gram staining by phase contrast microscopy, in cellulose- and glucose- or cellobiose- (2 g l^{-1}) grown culture.

2.5. Degradation and fermentation of microcrystalline cellulose by cellulolytic isolates

Cellulolytic isolates were cultivated in the liquid BC medium (10 ml per tube) containing 100 mg of Whatman No. 1 filter paper cellulose as sole energy source. Inocula were composed of 0.3 ml of 5-day-old cultures on filter paper–BC medium. Three culture tubes were inoculated for each incubation time and each strain studied. Kinetics of cellulose degradation by the isolates was determined by measuring the dry weight of cellulose remaining in the culture tubes after 2, 4, 6, 8 and 10 days of incubation at 37°C. The cellulose pellet was treated with 1 ml 1 M NaOH at 100°C for 10 min to remove bacterial material before drying to a constant weight at 80°C. The end products of cellulose fermentation were determined in the 10-day-old cultures. Gases in headspace of cultures and short-chain fatty acids in culture supernatants were analysed by gas phase chromatography [23]. Formate, succinate, ethanol and lactate production were measured by enzymatic methods (Boehringer, Mannheim, Germany).

2.6. Sequencing of 16S rRNA genes of cellulolytic isolates

Cellulolytic isolates were grown for 24 h in 50 ml BC medium with glucose or cellobiose (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 using the Easy DNA™ Genomic DNA Isolation kit (Invitrogen BV, Groningen, The Netherlands). Nucleic acid concentration was measured photometrically at 260 nm and quality was checked in ethidium bromide-stained agarose gels (0.7%, w/v). The 16S rRNA gene (approximately 1500 bp) was then amplified using the universal primers F8 (5′-AGAGTTTGATCMTGGCTC-3′) and 1492R (5′-GNTACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) amplification was performed using a thermal cycler (Gene Amp PCR System 2400, Applied Biosystems). Each reaction mixture (50 μl) contained 5 μl of 10 × PCR buffer, 4 μl $MgCl_2$ (25 mM), 1 μl dNTPs (10 mM), 2.5 μl each primer (10 μM), 1.25 U *Taq* polymerase (Promega), 1 μg μl^{-1} of extracted DNA and H_2O. Cycling conditions included denaturation at 94°C for 2 min, following by 25 cycles of denaturation at 94°C for 30 s, primer annealing at 62°C for 15 s and extension at 72°C for 5 min. After completion, an additional extension step was performed at 72°C for 5 min and the samples were then kept at 4°C. PCR products were purified using the Strataprep™ PCR Purification kit (OZYME, St Quentin en Yvelines, France) according to the manufacturer's instructions. Concentration of DNA after PCR amplification was measured in ethidium bromide-stained agarose gels and approximately 50 ng of each product was included in a 20-μl sequencing reaction. Sequences were obtained in both orientations from at least two separate PCR products. Sequencing reactions were performed with the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) according to the manufacturer's specifications. All reactions were carried out with the ABI Prism cycle sequencing kit (Applied Biosystems) and gels were run on an ABI Prism® 310 automated sequencer.

2.7. Phylogenetic placement

Phylogenetic analysis was performed using the ARB software package. Sequences were aligned and the tree was generated by the neighbour-joining analysis (*Escherichia coli* position 93–1432) and corrected with a mask that included only 50% of the conserved regions. Five hundred bootstrap analyses were performed.

Table 1
Distribution of total anaerobes, cellulolytic bacteria and H_2-utilising methanogenic archaea in faecal samples from CH_4^+ and CH_4^- individuals[a]

Subjects		Cellulolytic bacteria	Methanogenic archaea	Total anaerobes
CH_4^+ ($n=17$)	$n=13$	6.7 ± 0.5	7.7 ± 0.9	10.5 ± 0.4
		[5.7–7.7]	[6.5–9.4]	[9.9–11.3]
	$n=4$	< 3.0	7.2 ± 0.9	10.7 ± 0.3
			[6.4–8.2]	[10.5–11.2]
CH_4^- ($n=17$)	$n=2$	[4.3–7.2]	< 2.0	[10.4–10.9]
	$n=15$	< 3.0	2.9 ± 1.7	10.6 ± 0.4
			[2.0–6.0]	[9.7–11.6]

[a]Results are expressed as mean $\log_{10}$ micro-organisms g^{-1} faeces (wet weight) ± S.E.M., [range].

2.8. Data and statistical analysis

Bacterial counts are reported as $\log_{10}$ cells g^{-1} wet weight of faeces. Data are expressed as mean ± S.E.M. (n = number of faecal samples studied). Statistical analyses were performed with Instat 2.01 GraphPad software for Macintosh using Student's t-test. All tests were two-tailed and the level used to establish significance was $P < 0.05$.

3. Results

3.1. Evaluation of the cellulolytic population in faecal samples of CH_4^+ and CH_4^- subjects

Results of microbial counts are shown in Table 1. The cellulolytic population enumerated in the different faecal samples covered a very wide range, the level of this filter paper-degrading population varying from undetectable ($<$ log 3 g^{-1} faeces) to log 7.7 g^{-1} faeces, depending on the subject considered (Table 1). The fibrolytic microbial community could only be detected in 15 faecal samples of the 34 specimens analysed, no cellulolytic bacteria being cultivated from the other 19 faecal samples. The majority of individuals harbouring filter paper-degrading micro-organisms (13 of 15) were CH_4 excretors whereas only two non-CH_4-excreting subjects appeared to carry such fibrolytic bacteria. Among these two CH_4^- individuals, only one harboured a cellulolytic population to a high level (log 7.2 g^{-1} faeces), the second one showing a very small count of filter paper-degrading bacteria (log 4.3 g^{-1} faeces). By contrast, most of the individuals showing no detectable filter paper-degrading flora were non-CH_4-excretors (15 of 19), only four CH_4^+ individuals showing no detectable cellulolytic bacteria in their faeces. Therefore, it appears that the majority of CH_4^+ subjects harboured a filter paper-degrading microbial community, ranging between log 5.7 and log 7.7 g^{-1} faeces, while most of the CH_4^- subjects showed undetectable filter paper-degrading community ($<$ log 3 g^{-1} faeces).

The average counts of methanogenic archaea were significantly different ($P = 0.01$) in CH_4^+ and CH_4^- individuals (Table 1). In CH_4^+ subjects, the methanogenic population ranged between log 6.4 and log 9.4 g^{-1} faeces, whereas this methanogenic population remained undetectable ($<$ log 2 g^{-1} faeces) in most of the CH_4^- subjects examined. Only three CH_4^- subjects harboured an average count of methanogens equal to log 6.0 g^{-1} faeces. In contrast to methanogens, the numbers of total anaerobes were not significantly different between faeces of CH_4^+ and CH_4^- subjects (Table 1).

3.2. Cellulolytic and xylanolytic activities of faecal microflora from CH_4^+ and CH_4^- subjects

Among the 34 faecal samples, five specimens from CH_4^+ subjects harbouring cellulolytic bacteria and five specimens from CH_4^- subjects who did not have such cultivable cellulolytic organism were used to measure carboxymethyl cellulase (CMCase) and xylanase activities of the faecal

Table 2
CMCase and xylanase activities in faecal samples of CH_4^+ and CH_4^- subjects

Faecal microflora	CMCase activity		Xylanase activity	
	Specific activity[a]	Maximal activity[b]	Specific activity[a]	Maximal activity[b]
CH_4+ ($n=5$)	6.2 ± 3.5	92.8 ± 21.8	3.3 ± 2.1	86.3 ± 26.7
CH_4- ($n=5$)	7.1 ± 2.0	95.7 ± 29.1	3.2 ± 0.8	83.0 ± 15.2
P	NS[c]	NS	NS	NS

Results are expressed as mean activity ± S.E.M.
[a]μg reducing sugars min^{-1} (mg protein)$^{-1}$.
[b]μg reducing sugars (mg protein)$^{-1}$.
[c]NS: not statistically significant ($P > 0.05$).

Table 3
Origin and morphological characteristics of cellulolytic strains isolated from five human faecal samples

Subject	CH_4 status	Number of cellulolytic bacteria[a]	Strains	Isolated from faecal dilution	Cell form	Gram[b]
1	CH_4^+	7.5	18P13, 18H3, 18A3	−8	Coccobacillus	+
			18P16, 18A1	−8	Coccus	+
			18A2	−8	Rod	+
2	CH_4^+	7.0	27C61, 27C66	−7	Rod	+
			27C63, 27C65	−7	Coccus	+
7	CH_4^+	7.2	7L71, 7L74, 7L75	−7	Coccobacillus	+
			7L72, 7L76	−7	Coccus	+
			7L73	−7	Rod	+
15	CH_4^+	6.6	15A, 15C	−7	Coccobacillus	+
25	CH_4^-	7.2	25F2, 25F6, 25F7,	−7	Coccobacillus	+
			25F8, 25F9	−7	Coccobacillus	+

[a]Expressed as $\log_{10}$ cellulolytic bacteria g^{-1} wet weight faeces.
[b]Results of Gram staining. + = Gram-positive.

communities. The two fibrolytic activities were detected in faecal communities from both CH_4^+ and CH_4^- subjects (Table 2). The CMCase and xylanase activities of CH_4^- faecal communities compared with those of CH_4^+ ones. The specific and maximal activities of these two hydrolases were not significantly different in CH_4^+ and CH_4^- subjects (Table 2). These enzymatic activities were found to vary greatly according to the faecal communities considered, independently of the CH_4 status of the donor. This observation explained the large standard error of the mean found for both activities in CH_4^+ and CH_4^- faecal samples.

3.3. Enrichments and isolation of cellulolytic bacteria

Enrichments of cellulolytic bacteria were successfully obtained from faecal samples harbouring a high level of cellulolytic population. These faecal specimens came from four CH_4^+ subjects and from the only CH_4^- individual harbouring a level of cellulolytic bacteria similar to that of CH_4^+ individuals (Table 3). The highest dilution tubes from MPN enumeration exhibiting filter paper degradation (dilution 10^{-7}–10^{-8}) were used for enrichment of cellulolytic bacteria (Table 3). After two to three weekly transfers of the enrichment series, degradation of filter paper cellulose could be observed after an 8-day incubation period at 37°C. From these filter paper enrichments, a total of 23 cellulolytic strains were obtained after three to four successive isolations from agar roll tubes.

All the cellulolytic isolates exhibited similar positive Gram staining (Table 3). However, different morphological types could be identified. Four strains were rods while the 19 other strains corresponded to cocci (six strains) or coccobacilli (13 strains). Coccobacillus strains were found in nearly all faecal samples (Table 3) and were strict anaerobes. Rod and coccus isolates were present in three of the five faecal samples examined (Table 3), and were facultative anaerobes, as demonstrated by their ability to grow in the presence and absence of O_2 in the gas phase (data not shown).

3.4. Sequencing of 16S rRNA genes of cellulolytic isolates

The almost complete sequence of the 16S rRNA gene (around 1400 nucleotides) was determined for nine cellulolytic strains selected as representatives of the different morphological types. Five coccobacilli (strains 18P13, 7L75, 25F6, 25F7, 25F8), three cocci (18P16, 27C63 and 7L76), isolated from three different faecal samples (two CH_4^+ and one CH_4^- subjects), and one rod strain (27C61 from a CH_4^+ subject) were chosen. Sequence similarity analysis showed that all five coccobacillus isolates were *Ruminococcus*-like strains and were phylogenetically positioned with representatives of cluster IV of the Clostridiaceae as defined by Collins et al. [24]. Among these *Ruminococcus*-like strains, sequence similarity varied between 92.3% and 97.6%. The closest relatives of these isolates were *R. callidus* (95–96%) and *R. flavefaciens* (94–95%) with a mean sequence similarity of 95.1%. A bootstrapped phylogenetic tree of *Clostridium* clusters (III and IV) is shown in Fig. 1. The isolates did not show high sequence homology with any of the members of cluster IV and represent new *Ruminococcus* species. 16S rDNA sequences of these *Ruminococcus* isolates were deposited in EMBL database under the accession numbers AJ515913, AJ515914, AJ515915, AJ515916 and AJ515917.

Sequence analysis of the three coccus isolates (18P16, 27C63 and 7L76) demonstrated that these strains could be assigned to *Enterococcus* species, their sequences being 99% similar to *E. faecalis*. The rod isolate, strain 27C61, was found to belong to the *Propionibacterium* genus.

3.5. Degradation and fermentation of microcrystalline cellulose by some cellulolytic isolates

The cellulolytic isolates related to *Ruminococcus* and *Enterococcus* species efficiently degraded filter paper cellulose during the incubation period (Fig. 2). The total amount of cellulose degraded by *Ruminococcus* strains 18P13 and 25F7 and *Enterococcus* strain 7L76 was close

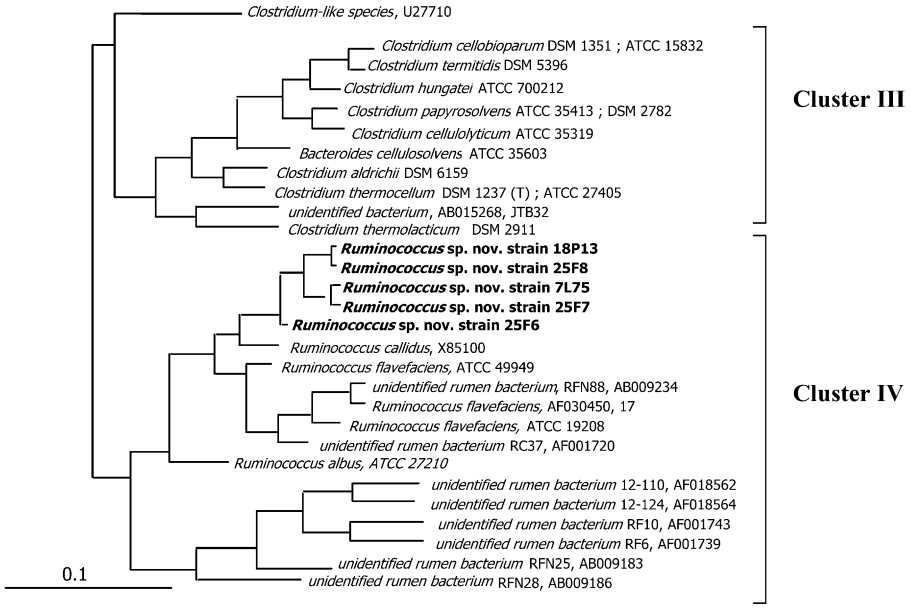

Fig. 1. Tree showing the phylogenetic relationships of *Ruminococcus* strains 18P13, 7L75, 25F6, 25F7 and 25F8 within cluster IV of the Clostridiaceae. The tree was constructed using the neighbour-joining method based on comparison of approximately 1350 nucleotides.

to 30 mg after incubation for 10 days. In contrast, the *Propionibacterium* isolate (27C61) appeared to lose its ability to degrade filter paper cellulose after two to three successive transfers on this substrate. The end products of cellulose fermentation by the *Ruminococcus* isolates 18P13 and 25F7 and *Enterococcus* strain 7L76 were mainly succinate, acetate and ethanol, the ratio of these different metabolites varying according to the species considered (Table 4). In addition, these cellulolytic isolates were shown to produce large amounts of H_2 during cellulose degradation (Table 4). The *Propionibacterium* isolate was shown to metabolise glucose into propionate, without production of H_2 (data not shown).

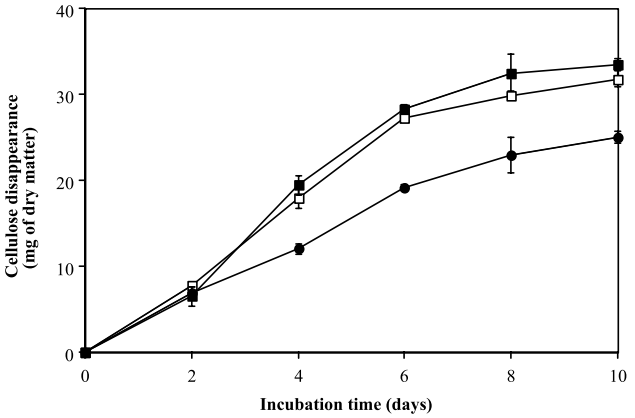

Fig. 2. Kinetics of filter paper cellulose degradation by *Ruminococcus* and *Enterococcus* related isolates. ■, *Enterococcus* sp. strain 7L76; ●, *Ruminococcus* sp. strain 25F7; □, *Ruminococcus* sp. strain 18P13.

4. Discussion

As previously demonstrated [10,12], the status of the CH_4 excretor was always correlated with the presence of a high level of methanogens in the colon whereas non-CH_4-excreting individuals have less than 10^6–10^7 methanogenic archaea g^{-1} wet weight faeces.

Only 40% of the volunteers included in our study were shown to harbour filter paper microcrystalline cellulose-degrading micro-organisms in the gut. This result is consistent with previous observations from Bétian et al. [4], Montgomery [5] and Wedekind et al. [6] who reported that only a restricted number of individuals (one or two subjects of the six studied) harboured such cellulolytic micro-organisms. These authors explained the failure to detect

Table 4
End products of cellulose fermentation by three cellulolytic strains related to *Ruminococcus* and *Enterococcus* species

	End products of fermentation (mM/100 mM glucose equivalent)		
	Ruminococcus sp.		*Enterococcus* sp.
	18P13	25F7	7L76
Formate	0.0	0.2	0.8
Acetate	41.2	43.6	90.0
Succinate	62.6	64.1	79.3
Lactate	0.3	1.4	1.3
Ethanol	54.3	35.7	24.9
H_2	36.1	43.7	32.0
C recovery[a] (%)	73.7	69.9	91.9

Cellulolytic isolates were incubated for 10 days at 37°C with 100 mg of Whatman No. 1 filter paper cellulose as sole energy source.
[a]Carbon recovery did not take into account CO_2 production and cell carbon.

cellulolytic organisms in human faecal samples by the nature of the cellulose used to enumerate this population, whereas the presence of such cellulose degraders was tentatively justified by the diet of the volunteers. We showed here that the presence or the absence of a detectable crystalline cellulose-degrading community appears to be mainly related to the CH_4 status of the volunteers. Indeed, the presence of microcrystalline cellulose-degrading flora was evidenced in faecal samples from most of the CH_4^+ subjects studied while this cellulolytic population could not be detected in specimens from the majority of CH_4^- individuals. The size of the cellulolytic community was close to 10^7 g^{-1} wet weight faeces, which is consistent with previous findings [6]. The colonic microbial community of CH_4^+ subjects is thus able to degrade microcrystalline cellulose efficiently in contrast to CH_4^- subjects. The absence of a detectable microcrystalline cellulose-degrading flora in CH_4^- faecal samples probably does not reflect the absence of cellulolytic micro-organisms in these subjects. Indeed, comparable CMCase activity was found in faecal communities of CH_4^+ and CH_4^- volunteers, which suggests the presence of micro-organisms able to cleave β1-4 linkages such as those found in plant polymers like cellulose, in all subjects. Similarly, xylanase activities detected in all faecal samples demonstrated the presence of hemicellulolytic micro-organisms in both individuals. The absence of detectable cellulolytic microflora in CH_4^- subjects using our culture conditions suggests that the structure and activity of this fibrolytic community differ from that of CH_4^+ individuals. The cellulolytic microflora from CH_4^- individuals should thus be investigated using celluloses from different natures and different botanic origins.

Only a few CH_4^+ subjects (four of the 17 studied) did not harbour a microcrystalline cellulose-degrading flora whereas this bacterial population was found at high levels in only one CH_4^- volunteer. In these cases, it could be hypothesised that the level of fibres in the diet of these volunteers has influenced the structure of the intestinal cellulolytic community. Indeed, the amount of dietary fibres ingested daily by the volunteers was not recorded in our study. It is also possible that this bacterial community might have been influenced by host factors (genetic or physiological). In this context, investigation of the cellulolytic microflora in a cohort of individuals receiving a dietary fibre-controlled diet would be necessary.

Our study of the structure of the microcrystalline cellulose-degrading population, examined through isolation and identification of the predominant organisms forming a clear zone on cellulose solid medium, provides new information on the diversity of the cellulolytic flora from the human colon. It is likely that most of the subjects studied harbour more than one cellulolytic species. The most common cellulolytic strains isolated correspond to *Ruminococcus* and *Enterococcus* species. Using pebble-milled filter paper cellulose as substrate, Montgomery [5] has also reported isolation of two cellulolytic strains from a faecal specimen that phenotypically resemble *Ruminococcus* sp. Similarly, the cellular and phenotypic characteristics of our coccobacillus strains were consistent with the genus *Ruminococcus* [25]. Phylogenetic analysis of these isolates demonstrated that they represent unknown groups within rRNA cluster IV of *Clostridium* that includes *R. flavefaciens*, the type species of the *Ruminococcus* genus [24]. Our findings constitute the first demonstration of the existence of cellulolytic ruminococci from the human gut, the strains being related to *R. flavefaciens*, one of the main cellulolytic organisms from the gastrointestinal tract of herbivores [26]. In addition, *Ruminococcus* was recently identified as a predominant bacterial group from the human gut community [27]. Further characterisation of our new *Ruminococcus* species is now in progress. These cellulolytic *Ruminococcus* species were found in three CH_4^+ subjects of the four studied, and in the sole CH_4^- individual harbouring such a microcrystalline cellulose-degrading flora. Other cellulolytic isolates from CH_4^+ individuals were identified as *Enterococcus* species. This represents a new finding since no facultative anaerobes were previously described within the cellulolytic microflora of gastrointestinal ecosystems. Based on 16S rDNA gene sequence comparison, the closest relative of our isolates was shown to be *E. faecalis*. Similarly, analyses of metabolic profiles of our strains using the API system showed that they could be identified as *E. faecalis* species. However, no cellulolytic activity was shown in *E. faecalis* [28], in contrast to our *Enterococcus* strains. Therefore, DNA/DNA hybridisation between *E. faecalis* and our strains as well as more metabolic characterisation are needed for a complete identification of the *Enterococcus* isolates. Phylogenetic identification of our rod isolate indicated that it is closely related to *Propionibacterium* sp. In contrast, cellulolytic rods previously isolated using purified crystalline cellulose were tentatively assigned to *Eubacterium* sp. and one to *Bacteroides* sp. [4,6].

Ruminococcus and *Enterococcus* strains showed similar efficiency to degrade, in vitro, microcrystalline cellulose. The end products of cellulose fermentation were also comparable, acetate and succinate being the main metabolites formed by all species. In addition, both *Ruminococcus* and *Enterococcus* species were shown to produce large amounts of hydrogen from cellulose fermentation. Interspecies H_2 transfer between this H_2-producing fibrolytic species and methanogenic archaea could thus explain the relationships that were observed between the presence of microcrystalline cellulose-degrading flora and the CH_4-excreting status of individuals. Similarly, a correlation between numbers of methanogens and those of microcrystalline cellulose-degrading bacteria was demonstrated in the intestinal tract of different herbivorous mammals [29]. While factors involved in determining CH_4 status in humans remain unknown, our finding suggests that microcrystalline cellulose-degrading bacteria could play a role in the establishment and the development of methanogens in

the human gut. As metabolism of the methanogenic archaea from the human colon is restricted to utilisation of few energetic substrates (H_2/CO_2, formate and, in some species, methanol), their presence in the ecosystem is strongly dependent on other bacterial species able to produce H_2 and/or formate on fermentation of organic substrates. In this context, the H_2-producing fibrolytic bacteria such as *Ruminococcus* and *Enterococcus* isolates could be essential for the development of methanogens in the colon. This interspecies H_2 transfer would also undoubtedly affect cellulose breakdown and fermentation products by fibrolytic species, as previously demonstrated [7,8]. Furthermore, it can also be hypothesised that non-H_2-producing fibrolytic species predominate in the colon of CH_4^- individuals, which could thus explain the absence of methanogens in this colonic ecosystem.

In conclusion, our results suggest that the structure and activity of the colonic cellulolytic microbial community differ greatly in CH_4^+ and CH_4^- individuals. H_2-producing microcrystalline cellulose-degrading bacteria were evidenced in CH_4^+ subjects. The presence of such species seems to be linked with that of methanogens. The relationships existing between these two microbial communities and their possible roles in determining CH_4 status in humans need to be further investigated. Furthermore, the structure of the cellulolytic community from CH_4^- individuals needs to be evaluated using different sources of cellulose. This is in progress in our laboratory.

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