

Note

Victivallis vadensis gen. nov., sp. nov.,
a sugar-fermenting anaerobe from human faecesErwin G. Zoetendal,^{1,2} Caroline M. Plugge,¹ Antoon D. L. Akkermans¹
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A novel strictly anaerobic, cellobiose-degrading bacterium, strain Cello^T, was isolated from a human faecal sample by combining enrichments in liquid and soft-agar basal media. A noteworthy characteristic was its inability to grow on normal agar plates and in roll tubes. The cells were coccus shaped and non-motile, with an extracellular slime layer. Growth of strain Cello^T occurred between 20 and 40 °C, with optimal growth at 37 °C. The pH range for growth was 5–7.5 with an optimum at 6.5. In pure culture, strain Cello^T could only grow on a variety of sugars. Glucose was converted to acetate, ethanol and H₂. The doubling time on glucose was 0.5 h. In a syntrophic co-culture with *Methanospirillum hungatei* strain JF-1^T, strain Cello^T converted glucose to acetate and H₂. The G+C content was 59.2 mol%. 16S rDNA analysis revealed that the closest relatives of strain Cello^T were two uncultured bacteria from anaerobic digesters, both with 94% 16S rDNA sequence similarity. The closest cultured representatives belong to genera of the bacterial division 'Verrucomicrobia'. The name *Victivallis vadensis* gen. nov., sp. nov. is proposed for strain Cello^T (=DSM 14823^T =ATCC BAA-548^T).

The human gastrointestinal (GI) tract is a very complex ecosystem in which bacteria are in close contact with each other and with the host's cells. The majority of these bacteria are strictly anaerobic and their presence in the GI tract seems to be influenced by the host (Bry *et al.*, 1996; Hooper *et al.*, 1999, 2000; Zoetendal *et al.*, 2001). Despite many intensive cultivation trials aimed at isolating bacteria from the human GI tract, it is estimated that at the moment only 10–50% of the bacteria in the human GI tract can be obtained in culture (McFarlane & Gibson, 1994; Langendijk *et al.*, 1995; Suau *et al.*, 1999; Wilson & Blitchington, 1996). Reasons for this unculturability include the unknown growth requirements of the bacteria, the selectivity of the very rich media that are used, the stress imposed by the cultivation procedures, the necessity of strictly anoxic conditions and the interactions of the bacteria with other microbes and host cells. Many novel bacteria have been detected in faeces using culture-independent approaches based on the variability of the 16S rRNA gene (Suau *et al.*, 1999; Wilson & Blitchington, 1996; Zoetendal *et al.*, 1998). This implies that the physiological characteristics and function of the majority of the human GI tract community

are unknown. In this study, we describe the isolation of a novel species belonging to a new genus, strain Cello^T, from the GI tract by using an alternative cultivation approach. Exposure of the faecal sample to oxygen was minimized and a combination of liquid and soft-agar basal media with cellobiose as the carbon source was used for isolation and cultivation.

A faecal sample (~0.5 g) from a healthy Dutch man (25 years old) was transferred within 1 min after defecation without homogenization into 10 ml sterile anoxic Ringers solution with a syringe. This faecal suspension was vortexed and diluted 10 000-fold in anaerobic Ringers solution. From this dilution, 0.3 ml was used to inoculate 30 ml of a bicarbonate-buffered anaerobic medium, as described previously by Stams *et al.* (1993), supplemented with 10 mM cellobiose and 0.7% (v/v) clarified sterile rumen fluid. This medium was subsequently used to isolate and cultivate strain Cello^T and to prepare plates and roll tubes by supplementation of 2% noble agar (Difco) and soft agar (0.75% noble agar). For the plates, the concentration of phosphates was increased 5-fold to replace the bicarbonate and to provide buffering capacity. Other media used to cultivate the isolated strain Cello^T included: Wilkens–Chalgren (WC) broth (Oxoid; 16 g l⁻¹), KA medium, as described by Kamlage *et al.* (1999) with minor modifications [no haemin, bacteriological peptone (Oxoid)

Abbreviations: DGGE, denaturing gradient gel electrophoresis; GI, gastrointestinal.

The GenBank accession number for the 16S rDNA sequence of strain Cello^T is AY049713.

instead of tryptic peptone from meat], both supplemented with 0.7% clarified sterile rumen fluid, and Biolog Universal Anaerobic (BUA) agar plates (Stag). Antibiotics streptomycin (100 mg l⁻¹) and polymyxin B (20 mg l⁻¹) were used to remove a faecal contaminant. Aerobic Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) was used to check the presence of the contaminant. All incubations were at 37 °C. Basal media containing several carbon sources were used to determine the utilization of different carbon sources by strain Cello^T. Carbon sources were added from sterile stock solutions to a final concentration of 10 mM, unless stated otherwise. The optimal growth temperature of strain Cello^T was determined in basal medium containing cellobiose at pH 7 over the temperature range 4 to 45 °C. The pH range was determined in WC broth at 37 °C over the range pH 5–8 (at 0.5 pH unit intervals, adjusted with NaOH or HCl). Incubations were done for at least 1 month to determine carbon source utilization, optimal growth and pH range of strain Cello^T. HPLC and GC analyses were performed as described previously (Stams *et al.*, 1993).

Methanospirillum hungatei JF-1^T (=DSM 864^T) was used as partner organism in syntrophic cultures. For the cultivation of *M. hungatei*, a gas phase of 182 kPa H₂/CO₂ (80:20, v/v) was used and after growth, the gas phase was changed to N₂/CO₂ and the bottles were inoculated with strain Cello^T (1%, v/v) and glucose (approx. 10 mM final concentration). Gram staining and light microscopy were performed as described elsewhere (Plugge *et al.*, 2000). For TEM, cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 0 °C for 2 h. After rinsing with the same cacodylate buffer, post-fixation was performed in a 2:1 mixture of 1% OsO₄ and 5% K₂Cr₂O₇ at room temperature for 2 h. After rinsing with water, the cells were post-stained with 1% uranyl acetate overnight, dehydrated in a graded ethanol series and embedded in Epon [Glycid ether (Serva)]. Ultrathin sections were post-stained with lead citrate (Cole & Popkin, 1981) and analysed using a Philips CM10 TEM.

DNA isolation, PCR and sequencing of the complete 16S rDNA was performed as reported recently (Plugge *et al.*, 2002). BLAST was used for homology searches (Altschul *et al.*, 1990) and the ARB and RDP programs were used for phylogenetic analysis (Maidak *et al.*, 2001; Strunk & Ludwig, 1995). A neighbour-joining tree was constructed as described previously (Plugge *et al.*, 2002) using *Escherichia coli* as the outgroup species. PCR and denaturing gradient gel electrophoresis (DGGE) analysis of the V6 to V8 regions of 16S rDNA of the enrichment cultures and faeces were performed as described before (Zoetendal *et al.*, 2001).

Anaerobic basal medium with cellobiose as sole carbon source was inoculated with a diluted faecal suspension in Ringers solution. Growth was observed after 2 days of incubation at 37 °C. Microscopic analysis revealed that a coccus-shaped bacterium became predominant. In addition,

a rod-shaped organism was observed, which grew very well under oxic conditions in LB medium at 37 °C. Repeated transfers in serial dilution of the enrichment to fresh medium showed a decrease in the number of rod-shaped bacteria. However, it was never outcompeted completely, since the rod-shaped bacteria could still be found in parallel incubations in LB medium. Therefore, the enrichment was transferred to medium containing streptomycin and polymyxin B to inhibit growth of the rod-shaped bacterium. After 1 week of incubation, growth of the coccus-shaped bacterium was observed while no growth of the enrichment culture was observed in LB medium. This could also be visualized when the V6–V8 regions of the 16S rDNA from the cultures were analysed by DGGE. Before the antibiotic treatment, a tiny band representing the rod-shaped bacterium was still present, while it was absent after the antibiotic treatment. This indicates that the rod-shaped bacterium was no longer present in the enrichment. Cultivation of the enrichment in two different very rich media for growing fermentative bacteria (KA medium and WC broth) resulted in the growth of only the coccus-shaped bacterium after 1 and 2 days, respectively, as indicated by microscopic and DGGE analysis. Despite several attempts to cultivate this isolate on solid medium containing 2% agar (plates and roll tubes) with the same components as the liquid medium, colonies were not obtained. In addition, colonies were not formed on BUA agar plates, which is regarded as a universal medium for anaerobic bacteria. Incubation in soft agar (0.75%) was more successful. Serial dilution of the enrichment in basal medium having cellobiose as carbon source resulted in the development of beige, shiny, lens-shaped colonies after 10 days. Single colonies were transferred to liquid medium followed by incubation in soft agar. This procedure was performed three times and the same colony, cell shape and DGGE band position of its 16S rRNA gene were always observed. Therefore, it was concluded that the strain was pure and this pure culture was designated strain Cello^T and characterized further. Incubation of strain Cello^T in basal medium without rumen fluid did not affect its growth and rumen fluid was therefore omitted from the following experiments.

The Gram type of strain Cello^T was determined using the Gram stain combined with light microscopy and TEM. Light microscopic analysis revealed that strain Cello^T stained Gram-negative and that the coccus-shaped cells were variable in size (Fig. 1a). Moreover, haloes surrounding the cells were often observed, indicating the presence of an extracellular slime layer. Electron microscopy confirmed that the cell wall of strain Cello^T was Gram-negative, although clear visualization of the cell membrane was hampered by the extracellular slime layer, which completely surrounded the coccus-shaped cells (Fig. 1b). In addition, electron microscopy revealed that the cells varied in diameter from 0.5 to 1.3 µm and contained many intracellular electron-dense structures. These structures are most likely intracellular protein precipitates or storage material.

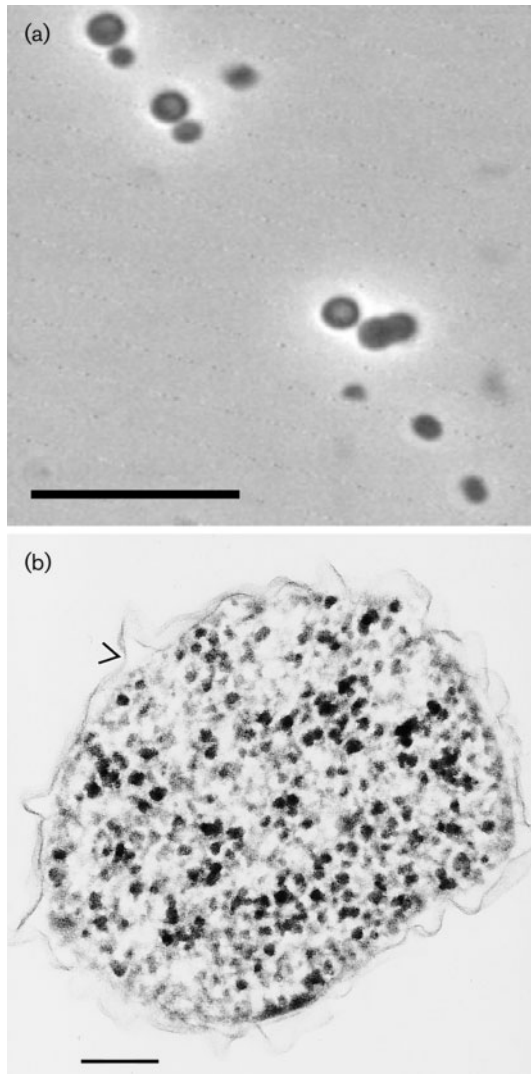


Fig. 1. Phase-contrast micrograph (a) and transmission electron micrograph (b) of cells of strain Cello^T. The arrowhead in (b) indicates the extracellular slime layer. Bars, 5 (a) and 0.25 (b) μm .

DNA was isolated from strain Cello^T and the complete 16S rRNA gene was amplified. The nucleotide sequence (1456 bp) of the amplified 16S rRNA gene was determined in duplicate and analysed phylogenetically. It revealed that strain Cello^T is most closely related to a group of uncultured bacteria (AA08, vadinHB65 and vadinBE97) that were retrieved from an anaerobic digester by a PCR and cloning approach (Fig. 2). This group is related to members of the division 'Verrucomicrobia' (Hedlund *et al.*, 1997), a division that is defined mainly on 16S rRNA sequences of uncultured bacteria and consists of five proposed subgroups (Hugenholtz *et al.*, 1998). The group to which strain Cello^T belongs is related most closely to subgroup 5 of the division 'Verrucomicrobia', but forms a separate cluster (Fig. 2). Some cultured members of the division 'Verrucomicrobia' have been described, including the genera *Prostheco* (Hedlund *et al.*, 1996) and *Opirit* (Janssen

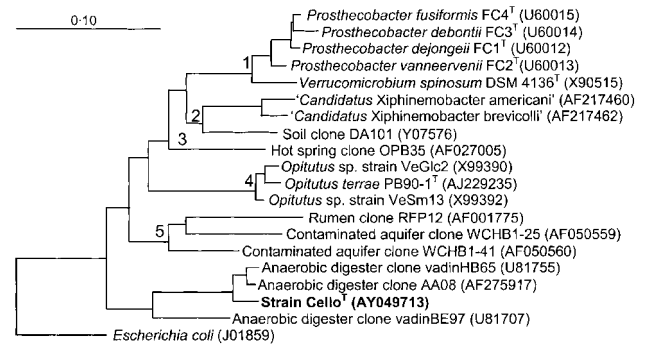


Fig. 2. Neighbour-joining tree showing the phylogenetic relationship of 16S rDNA sequences (*E. coli* positions 64–1491) of strain Cello^T (bold) and its closest relatives in the DNA databases. Habitat sources are indicated before the names of cloned 16S rDNA sequences of uncultured bacteria. The numbers before interior branch-points indicate the five major lineages of the division 'Verrucomicrobia' as proposed by Hugenholtz *et al.* (1998). *E. coli* was used as outgroup species to root the tree. Bar, 10% sequence divergence.

et al., 1997; Chin *et al.*, 2001). However, these cultured members respectively belong to subdivisions 1 and 4 and are therefore not closely related to strain Cello^T (Fig. 2). The closest relatives of strain Cello^T are clone AA08 and clone vadinHB65, with only 94% sequence similarity. Bacteria having 16S rRNA sequence similarity values below 97% are considered to represent different species (Stackebrandt & Goebel, 1994). No described cultured bacterium is even closely related to strain Cello^T, indicating that this newly isolated strain belongs to an as yet undescribed genus. The G+C content of strain Cello^T was 59.2 mol%.

To characterize strain Cello^T, it was cultured in basal liquid medium supplemented with several carbon and energy sources. A variety of carbohydrates and other carbon sources were tested. It appeared that strain Cello^T grows well on a variety of sugars, including cellobiose, fructose, galactose, glucose, lactose, lactulose, maltose, maltotriose, mannitol, melibiose, *myo*-inositol, rafterose, rhamnose, ribose, sucrose and xylose. No growth was observed on cellulose, fucose, gelatin, mucus (extracted from Ht-29 cultures), pectin, raffinose, starch, fumarate, Casamino acids, oleate, pyruvate, yeast extract or H₂/CO₂. In addition, no alcohols (methanol, ethanol, 1-propanol, 2-propanol and 2-butanol were tested) or proteins (casein, peptone and tryptone were tested at 0.1%, w/v) were utilized by strain Cello^T. Growth of strain Cello^T on cellobiose was observed between 20 and 40 °C, with optimum growth at 37 °C. Growth on WC broth was observed between pH 5 and 7.5 with an optimum at pH 6.5. These optimal conditions are in line with those found in the human colon. Strain Cello^T did not grow under micro-aerophilic conditions, nor did it use sulphate or nitrate as electron acceptor.

In pure culture, the stoichiometry of glucose degradation by strain Cello^T was: glucose (13·1)→acetate⁻ (10·4)+ ethanol (14·0)+H₂ (19·1)+HCO₃⁻ (24·4). The amount of bicarbonate was calculated assuming that, with the formation of 1 mol acetate and/or ethanol from glucose, 1 mol bicarbonate was formed. The yield on glucose was 26–30 g (mol glucose)⁻¹. The carbon recovery was 91–93 % (excluding the biomass formed). The doubling time was approximately 0·5 h when grown on glucose plus 0·02 % yeast extract. In syntrophic co-culture with *M. hungatei* JF-1^T, strain Cello^T converted glucose (12·1 mM) exclusively to acetate (20·6 mM) and H₂ (44·3 mM).

Since no cultivable bacterium is closely related to strain Cello^T, a detailed physiological comparison with related bacteria is not possible. The recently discovered *Opiritatus terrae* (Janssen *et al.*, 1997; Chin *et al.*, 2001) is able to ferment some sugars. However, these bacteria have a different stoichiometry of glucose fermentation to acetate and propionate. Furthermore, *O. terrae* can reduce nitrate to nitrite, whereas strain Cello^T is not able to reduce any external electron acceptor.

To determine whether strain Cello^T is a predominant member of the faecal flora, DGGE analysis of 16S rDNA amplicons of strain Cello^T and faeces was performed. This analysis demonstrated that the band position of strain Cello^T did not correspond to any of the band positions in the faecal DGGE profile from which strain Cello^T was isolated. This indicates that strain Cello^T is not a predominant bacterium in this faecal sample.

Despite many cultivation trials in the past decade, it is estimated that only a small minority of the bacteria in natural environments can be obtained in culture. One of the reasons for the difficulty of cultivating GI tract bacteria could be a combination of strict anaerobic conditions and specific growth requirements in order to allow them to compete successfully for substrates in the media used. In this study, we used an alternative approach to enrich and isolate faecal bacteria, which resulted in the isolation of strain Cello^T. Instead of plating the bacteria on rich media, we enriched the bacteria in basal liquid medium containing cellobiose as the carbon source. Only rumen fluid was added as a supplement of unknown growth factors, although it seemed to be unnecessary for growth in the latter enrichment stages. Strain Cello^T belongs to a new genus and its 16S rDNA sequence clusters in a group that only included sequences from uncultured bacteria. Therefore, we propose a novel genus and species, *Victivallis vadensis* gen. nov., sp. nov.

Since most cultivation trials of bacteria from ecosystems including the human GI tract are performed on agar media, and since strain Cello^T does not grow on normal agar plates, we speculate that this is the main reason why Cello^T-like bacteria have never been isolated before. The limited carbon source utilization of strain Cello^T could also be a reason why such strains have not been obtained

in pure culture. Therefore, we suggest that alternative cultivation approaches, such as the approach described here, may result in the isolation of novel species. Remarkably, the closest uncultured relatives of strain Cello^T are derived from anaerobic digesters fed with wastewater containing plant material. Since cellulose is a major component of plant material, consisting of cellobiose units, on which strain Cello^T was isolated, cellobiose could be used as carbon source in cultivation trials of similar strains from such reactors.

Description of *Victivallis* gen. nov.

Victivallis (Vic.ti.val'lis. L. masc. n. *victus* food; L. fem. n. *vallis* valley; N.L. fem. n. *victivallis* food valley, referring to the Wageningen 'Food Valley', which includes Wageningen and surroundings, an area of The Netherlands in which Food Science is a major research topic).

Non-motile, Gram-negative cocci. Non-spore-forming. Strictly anaerobic. Cells produce extracellular material. Growth on sugars. The type species is *Victivallis vadensis*.

Description of *Victivallis vadensis* sp. nov.

Victivallis vadensis (va.den'sis. M.L. fem. adj. *vadensis* of Vada, referring to Wageningen).

Gram-negative, non-motile cocci. Single cells, diameters vary between 0·5 and 1·3 µm. In pure culture, the cells can grow on a variety of sugars, only under strictly anaerobic conditions. Glucose is converted to acetate, ethanol, H₂ and bicarbonate. Growth on solid agar media is possible below 0·75 % (w/v) agar. Cells grow optimally on bicarbonate-buffered mineral salts medium with cellobiose at 37 °C and pH 6·5. The DNA G+C base composition is 59·2 mol%. The type strain is strain Cello^T (=DSM 14823^T =ATCC BAA-548^T).

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