NOTE

***Methanobacterium congolense* sp. nov., from a methanogenic fermentation of cassava peel**

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Strain C^T, a non-motile, mesophilic, hydrogenotrophic, methanogenic bacterium, was isolated from an anaerobic digester used for the treatment of raw cassava-peel waste in Congo. The cells were rods, 0.4–0.5 × 2–10 µm in size, and stained Gram-positive. Hydrogen and carbon dioxide were the only substrates that supported growth and methane production. Methane production, but not growth, occurred with CO₂ in the presence of either 2-propanol, 2-butanol or cyclopentanol as hydrogen donors. The temperature range for growth was 25–50 °C, the optimum being between 37 and 42 °C. The optimum pH for growth was 7.2; consistent growth and methane production were not observed below pH 5.9 or above pH 8.2. The doubling time under optimal growth conditions was 7.5 h. The DNA base composition was 39.5 mol% G+C. On the basis of 16S rRNA gene sequence analysis and phenotypic characteristics, the isolate is proposed as a new species of the genus *Methanobacterium*, namely *Methanobacterium congolense* sp. nov. The type strain is strain C^T (= DSM 7095^T = OCM 779^T).

Keywords: methanoarchaea, *Methanobacterium congolense*, phylogeny, digester, cassava peel

Large quantities of solid waste (peel) are produced in the Congo (Central Africa) during the processing of cassava roots to produce cassava meal (fufu). This waste could be used to produce biogas and to generate part of the energy needed for the mechanical processing of cassava. However, the presence of cyanogenic glucosides in cassava peel can result in the release of cyanides, which are highly toxic to methanogenic bacteria (Cuzin & Labat, 1992). During the study of the anaerobic digestion of raw cassava peel in a 120 l plug-flow digester (Cuzin *et al.*, 1992), methanogens were enumerated from different parts of the digester and several methanogenic strains were isolated. This work characterized a hydrogenotrophic, mesophilic, methanogenic rod.

The techniques for cultivating strict anaerobes described by Hungate (1969), Macy *et al.* (1972) and Miller & Wolin (1974) were used throughout this study. Strain C^T was isolated and grown on a basal medium containing (l⁻¹ distilled water) 0.25 g K₂HPO₄, 0.30 g NH₄Cl, 1 g NaCl, 0.50 g KCl,

0.40 g MgCl₂ · 6H₂O, 0.16 g CaCl₂ · 2H₂O, 0.25 g CH₃COONa, 0.001 g resazurin and 1 ml trace-element solution (Imhoff-Stuckle & Pfennig, 1983). The medium was boiled under a stream of O₂-free N₂, cooled to room temperature and then dispensed into Hungate tubes or serum bottles. After the medium had been autoclaved at 110 °C for 40 min, NaHCO₃, sodium dithionite and Na₂S were added from heat-sterilized stock solutions to final concentrations of 0.25, 0.01 and 0.025 % (w/v), respectively. Just before inoculation, substrates and the vitamin solution of Balch *et al.* (1979) (1 ml l⁻¹ culture medium) were supplied from filter-sterilized stock solutions. Roll-tube medium was prepared from the above-mentioned basal medium supplemented with 1.8 % (w/v) Noble agar (Difco). Unless indicated otherwise, all growth experiments were carried out at 35 °C and at pH 7.0 under an atmosphere of H₂/CO₂ (80 % : 20 %, v/v). Growth was monitored directly in culture tubes by measuring optical density at 580 nm with a Bausch & Lomb Spectronic 21 spectrophotometer. The pH range for growth was estimated after adjustment of the pH with sterile KOH or HCl solutions. The temperature range for growth was determined as being between 20 and 50 °C. Nutritional tests were carried out in the basal

The GenBank accession number for the 16S rRNA sequence of strain C^T is AF233586.

medium with and without added 0.1% yeast extract (Difco) and 0.1% bio-Tryptase (bioMérieux). Prototrophic growth was tested by omitting the vitamin solution from the basal medium.

A sample of digester juice was serially diluted into Hungate tubes with basal medium; the tubes with the highest dilution showing methane production after 2 weeks of incubation at 35 °C were used to inoculate roll tubes. After incubation at 35 °C for 2 weeks, the roll tubes were transferred into an anaerobic glove box and individual colonies were then picked with a sterile Pasteur pipette and transferred to sterile basal medium for serial dilution into roll-tube agar medium. This procedure was repeated several times until a single colony type was obtained. Strain purity was checked by inoculating individual colonies into a basal medium supplemented with 0.25% (w/v) each of yeast extract, peptone, bio-Tryptase and glucose. The cultures were examined microscopically after 3 weeks of incubation at 35 °C under aerobic and anaerobic conditions. Fluorescence was determined microscopically by using a 420 nm filter set (Zeiss). Phase-contrast micrographs were obtained by using a Nikon microscope. Electron micrographs of negatively stained (1%, w/v, sodium phosphotungstate) cells were obtained by using a JEOL model JEM-1200 EX transmission electron microscope. The Gram reaction was performed with a staining kit (Sigma). Methane was measured by GC as described previously (Labat & Garcia, 1986). The DNA base composition was determined at the DMSZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cells from the mid-exponential growth phase were harvested, washed with 10 mM saline-EDTA (pH 7.0), resuspended in 30 ml MUP buffer (8 M urea, 0.24 M sodium phosphate, pH 6.8) and disrupted by two passages (16000 p.s.i., 110400 kPa) through a French pressure cell. The DNA was purified on hydroxyapatite according to the procedure of Cashion *et al.* (1977). The DNA was hydrolysed with P₁ nuclease and the nucleotides were dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The resulting deoxyribonucleosides were analysed by HPLC (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989). Non-methylated λ DNA (Sigma) was used as the standard.

Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA and purification of the PCR products were performed at the DSMZ as described previously (Rainey *et al.*, 1996). Purified PCR products were sequenced using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A DNA sequencer. The resulting sequence data were put into the alignment editor ae2 (Maidak *et al.*, 1996), aligned manually and then compared with representative 16S rRNA gene sequences of organisms belonging to the *Euryarchaeota* (Maidak *et al.*, 1996). For comparison, 16S rRNA sequences were obtained from the EMBL

Fig. 1. Electron micrograph of (a) a negatively stained culture of strain C¹ and (b) an ultrathin section of strain C¹ showing the cytoplasmic membrane (cm) and the pseudomurein cell wall (cw). Bars, 1 μ m (a) and 0.1 μ m (b).

database or the Ribosomal Database Project (Maidak *et al.*, 1996). 16S rRNA gene-similarity values were calculated by pairwise comparison of the sequences within the alignment. For construction of the phylogenetic dendrogram, operations of the PHYLIP package (Felsenstein, 1993) were used. Pairwise evolutionary distances were computed from percentage similarities by the correction of Jukes & Cantor (1969). On the basis of evolutionary distance values, a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The root of the tree was determined by including the 16S rRNA gene sequence of *Methanospaera stadtmanae* in the analysis.

After 7–14 d of incubation at 35 °C in roll-tubes, circular surface colonies appeared. They were white, opaque, slightly raised at the centre, with a rough surface and entire margins, and up to 1 mm in diameter. Subsurface colonies were similar, but had a lenticular shape. Cells were rod-shaped and 0.4–0.5 μ m wide by 2–10 μ m long (Fig. 1a). They appeared singly or in pairs, rarely in small aggregates or long chains. The cells stained Gram-positive and motility was not observed by phase-contrast microscopy. Some features

Fig. 2. Phylogenetic dendrogram showing the position of strain C^T amongst the representative members of the genus *Methanobacterium*. Sequences were obtained from GenBank under the following accession numbers: *Methanobacterium bryantii* M.o.H.^T (= DSM 863^T), M59124; *Methanobacterium espanolae* GP9^T (= NRC 5912^T), AF095260; *Methanobacterium formicicum* MF^T (= DSM 1535^T), Z29436; *Methanobacterium ivanovii* Ivanov^T (= DSM 2611^T), AF095261; *Methanobacterium oryzae* FPI^T (= DSM 11106^T), AF028690; *Methanobacterium palustre* F^T (= DSM 3108^T), AF093061; *Methanobacterium subterraneum* A8p^T (= DSM 11074^T), X99044; *Methanobacterium uliginosum* P2st^T (= DSM 2956^T), AF095265; *Methanosphaera stadtmanae* MCB-3^T (= DSM 3091^T), M59139. Bar, 2 nucleotide substitutions per 100 nucleotides.

of the cell wall are shown in Fig. 1 (b). No flagella were observed by electron microscopy. The cell fluorescence examined by epifluorescence microscopy at 420 nm faded after about 30 s. On the basis of specific growth rates derived from optical density measurements in basal medium with H_2/CO_2 as the substrate, the optimum growth temperature was between 37 and 42 °C. Growth and methane production were not detected at 25 or 50 °C. The optimum pH for growth at 37 °C in basal medium was 7.2. No growth occurred below pH 5.9 or above pH 8.2. The doubling time under optimal growth conditions was 7.5 h. The only substrate that supported growth was H_2/CO_2 . Measurable growth of strain C^T was not detected in basal medium (gas phase: N_2/CO_2 , 80:20, v/v) containing 5 or 10 mM formate, acetate, methanol, ethanol, 1-propanol, isobutanol, 1-butanol, 1-pentanol, methylamine or trimethylamine. However, methane

production was observed from 2-propanol, from 2-butanol and from cyclopentanol (10 mM), in contrast with the methane production that occurred in control medium containing no alcohol. Strain C^T grew in basal medium without yeast extract, bio-Tryptase or vitamins (in an H_2/CO_2 atmosphere) and could be transferred indefinitely in the absence of vitamins. The DNA base composition was 39.5 ± 0.3 mol % G + C (mean of three determinations). The complete 16S rRNA gene sequence of strain C^T (1453 bp) was determined by direct sequencing of PCR-amplified 16S rDNA. Phylogenetic analysis revealed that strain C^T was a member of the family *Methanobacteriaceae* of the order *Methanobacteriales* (Boone & Mah, 1989), and the closest relatives were *Methanobacterium palustre* (similarity 95.4%), *Methanobacterium oryzae* (94.9%), *Methanobacterium subterraneum* (94.8%), *Methanobacterium ivanovii* (94.5%), *Methanobacterium espanolae*, *Methanobacterium uliginosum* (both 94.4%), *Methanobacterium bryantii* (94.3%) and *Methanobacterium formicicum* (94.1%). A dendrogram depicting these relationships is shown in Fig. 2.

On the basis of 16S rRNA gene sequences and other characteristics of non-motile, rod-shaped, methanogenic species that can use H_2/CO_2 as a growth and methanogenic substrate, strain C^T is related to the genus *Methanobacterium*. With its optimum growth temperature of 37–42 °C, it is clearly distinct from the thermophilic species of *Methanobacterium* and *Methanothermobacter*, which also generally have a DNA base compositions greater than 40 mol % G + C (Boone *et al.*, 1993; Wasserfallen *et al.*, 2000). Strain C^T differs from the mesophilic species *Methanobacterium alcaliphilum* (Worakit *et al.*, 1986), *Methanobacterium espanolae* (Patel *et al.*, 1990), *Methanobacterium ivanovii* (Jain *et al.*, 1987), *Methanobacterium uliginosum* (König, 1984) and *Methanobacterium oryzae* (Joulain *et al.*, 2000) in its ability to utilize 2-propanol or 2-butanol as hydrogen donors for the reduction of CO_2 to produce methane. The optimum pH for growth of strain C^T (pH 7.2) is also quite different from those of *Methanobacterium alcaliphilum* (pH 8.1–9.1), *Methanobacterium uliginosum* (pH 6.0–8.5) and *Methanobacterium espanolae* (pH 5.6–6.2). Strain C^T differs from *Methanobacterium formicicum* and *Methanobacterium palustre*, both of which can use formate as the sole source of carbon and energy for growth (Bryant & Boone, 1987; Zellner *et al.*, 1989). Formate is not utilized by *Methanobacterium bryantii* strain M.o.H.^T and its utilization by strain M.o.H.G. is not unequivocal (Benstead *et al.*, 1991; Zellner & Winter, 1987). Studies have shown that some mesophilic and thermophilic strains of *Methanobacterium* that were originally described as being unable to use formate can grow on formate under certain conditions (Benstead *et al.*, 1991; Touzel *et al.*, 1992).

It has been proposed that members of the same genus that share less than 97% 16S rRNA similarity should be regarded as separate species (Stackebrandt &

Goebel, 1994). On the basis of this criterion, as well as the phenotypic characteristics, we propose strain C^T as a new species of the genus *Methanobacterium*, namely *Methanobacterium congolense* sp. nov.

Description of *Methanobacterium congolense* sp. nov.

Methanobacterium congolense (con.go.len'se. N.L. adj. *congolense* from the Congo, where the type strain was isolated).

Colonies are circular, white, opaque, raised at the centres, have rough surfaces and entire margins and are up to 1 mm in diameter. The cells are rods, 0.4–0.5 × 2–10 µm, occurring individually or in pairs, rarely in chains and small aggregates. Gram-positive. Non-motile. Methanogenic. Obligatory member of the *Euryarchaeota* of the domain *Archaea*. H₂/CO₂ is the only substrate that supports growth. Methane is produced from 2-propanol/CO₂, 2-butanol/CO₂ and cyclopentanol/CO₂ without growth. Formate, acetate, methanol, ethanol, 1-propanol, isobutanol, 1-butanol, pentanol, methylamine and trimethylamine are not used. Growth factors such as yeast extract, bio-Tryptase and vitamins are not required. The optimum growth temperature is between 37 and 42 °C. No growth occurs at 25 or 50 °C. The optimum pH is 7.2; no growth occurs at pH 5.9 or 8.2. The doubling time under optimal growth conditions is 7.5 h. The DNA base composition is 39.5 ± 0.3 mol% G + C (as determined by HPLC). Isolated from an anaerobic digester used for the treatment of cassava peel in the Congo (Central Africa). The type strain is C^T (= DSM 7095^T = OCM 779^T).

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