Methanomassiliicoccus luminyensis gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces

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During attempts to obtain novel, human-associated species of the domain Archaea, a coccoid micro-organism, designated strain B10T, was isolated in pure culture from a sample of human faeces collected in Marseille, France. On the basis of its phenotypic characteristics and 16S rRNA and mcrA gene sequences, the novel strain was classified as a methanogenic archaeon. Cells of the strain were non-motile, Gram-staining-positive cocci that were approximately 850 nm in diameter and showed autofluorescence at 420 nm. Cells were lysed by 0.1 % (w/v) SDS. With hydrogen as the electron donor, strain B10T produced methane by reducing methanol. The novel strain was unable to produce methane when hydrogen or methanol was the sole energy source. In an atmosphere containing CO2, strain B10T could not produce methane from formate, acetate, trimethylamine, 2-butanol, 2-propanol, cyclopentanol, 2-pentanol, ethanol, 1-propanol or 2,3-butanediol. Strain B10T grew optimally with 0.5–1.0 % (w/v) NaCl, at pH 7.6 and at 37 °C. It required tungstate-selenite for growth. The complete genome of the novel strain was sequenced; the size of the genome was estimated to be 2.05 Mb and the genomic DNA G+C content was 59.93 mol%. In phylogenetic analyses based on 16S rRNA gene sequences, the highest sequence similarities (98.0–98.7 %) were seen between strain B10T and several uncultured, methanogenic Archaea that had been collected from the digestive tracts of a cockroach, a chicken and mammals. In the same analysis, the non-methanogenic ‘Candidatus Aciduliprofundum boonei’ DSM 19572 was identified as the cultured micro-organism that was most closely related to strain B10T (83.0 % 16S rRNA gene sequence similarity). Each of the three treeing algorithms used in the analysis of 16S rRNA gene sequences indicated that strain B10T belongs to a novel order that is distinct from the Thermoplasmatales. The novel strain also appeared to be distinct from Methanosphaera stadtmanae DSM 3091T (72.9 % 16S rRNA gene sequence similarity), another methanogenic archaeon that was isolated from human faeces and can use methanol in the presence of hydrogen. Based on the genetic and phenotypic evidence, strain B10T represents a novel species of a new genus for which the name Methanomassiliicoccus luminyensis gen. nov., sp. nov. is proposed. The type strain of the type species is B10T (=DSM 24529T=CSUR P135T).

At the time of writing, only three species in the domain Archaea have been isolated and cultured from microbiota associated with human mucosae. Two of these species, Methanobrevibacter smithii (Miller et al., 1982; Samuel et al., 2007) and Methanosphaera stadtmanae (Miller & Wolin, 1985), were found in the microbiota of the gut but the other species, Methanobrevibacter oralis (Ferrari et al., 1994), was isolated from oral mucosa. Over the last decade, metagenomic and molecular analyses have broadened the spectrum of known human-associated Archaea to include non-methanogenic organisms such as Halobacteria (Oxley et al., 2010) and Crenarchaeota (Rieu-Lesme et al., 2005).
Such analyses have also indicated the presence in humans of several phyotypes that would need to be placed in a new order of Archaea, although, until now, such strains have not been isolated (Mihajlovski et al., 2008, 2010; Scanlan et al., 2008). While investigating the diversity of Archaea in the human gut, by the PCR amplification and sequencing of 16S rRNA genes, we recently detected one such phyotype in a sample of human faeces. This micro-organism, a strict anaerobe that was designated strain B10T, was successfully isolated and cultured. In the present study, which was approved by the Ethics Committee of the Institut Fédératif de Recherche 48 (Marseille, France), the taxonomic affiliation of strain B10T was assessed by using a polyphasic approach. The results indicate that the strain represents a novel species of a new genus of methanogenic Archaea.

A faecal specimen collected from an 86-year-old healthy man was submitted to the clinical microbiology laboratory, Aix-Marseille-Université, Marseille, France. Although the specimen tested negative for known enteric pathogens, it gave positive results, in real-time PCR for the detection of 16S rRNA (Dridi et al., 2009), for both Methanobrevibacter smithii (cycle threshold (Ct)=35) and a novel phyotype (Ct=28). Enrichment and isolation of the methanogenic archaea in the sample were then attempted using Methanobrevibacter medium (MM) containing (1 l-1) 0.5 g KH2PO4, 0.4 g MgSO4.7H2O, 5 g NaCl, 1 g NH4Cl, 0.05 g CaCl2.2H2O, 1.6 g sodium acetate, 0.5 g cysteine-HCl, 1 g yeast extract, 1 g trypticase peptone, 0.001 g resazurin, 1 ml Widdel trace elements solution and 2 ml tungstate-selenite solution. The tungstate-selenite solution contained (l-1) 0.4 g NaOH, 2 mg Na2SeO3 and 4 mg Na2WO4. 5H2O. The pH of the medium was adjusted to 7.5 with 10 M KOH before the medium was autoclaved, boiled under a stream of O2-free N2 gas and then allowed to cool to room temperature. The medium was then dispensed into Hungate tubes (5 ml medium per tube) or serum bottles (20 ml per bottle) under a stream of N2/CO2 (80:20, v/v) at atmospheric pressure. The tubes and bottles were autoclaved for 20 min at 120 °C before the medium in each vessel was supplemented with sterile NaHCO3 (4 g l-1), Na2S.9H2O (0.5 g l-1), sodium formate (2 g l-1) and vitamin solution (10 ml l-1; Balch et al., 1979); finally, methanol (40 mM) and H2/CO2 [80:20, v/v; at 1 bar (100 kPa) pressure] were added as growth substrates. After inoculation with the faecal specimen (at 10%, v/v), each vessel was incubated at 37 °C with shaking. Positive enrichment cultures were obtained after 1 week. Methane production was detectable 3 days after inoculation. Pure strains were obtained by repeated use of the Hungate roll-tube method (Hungate & Macy, 1973) with H2/CO2 (80:20, v/v; at 1 bar pressure) and basal growth medium that was solidified with 1.5% (w/v) Noble agar (Difco) and supplemented with vancomycin (25 mg l-1) and methanol (40 mM). At 37 °C, regular circular, yellow-blue colonies approximately 0.5–1.0 mm in diameter developed in the agar roll tubes after 1 month. Five strains, designated B10T, B11, B12, B13 and B14, were isolated. These strains, which produced colonies that appeared identical and cells that were undistinguishable by microscopic examination, were found to be phylogenetically similar (16S rRNA gene sequence similarities of 99.8–100%) and all five could use H2 as an electron donor in the presence of methanol as electron acceptor. Strain B10T was selected for further investigation. The purity of strain B10T was verified by incubating it in medium containing yeast extract (1 g l-1), bio-trypticase (1 g l-1) and glucose (20 mM), to check that no fermentative microbial contaminants were present. Purity of the isolate was also confirmed when DNA extracted from it (Dridi et al., 2009) failed to yield any amplicons when run in a PCR based on the universal bacterial 16S rRNA gene primers fD1 and rp2 (Weisburg et al., 1991), which do not amplify archaeal 16S rRNA genes.

Cells of strain B10T are non-motile, Gram-stain-positive, regular cocci that show autofluorescence at 420 nm (Fig. 1). They lyse when placed in 0.1% (w/v) SDS or hypotonic distilled water (Boone & Whitman, 1988). When ultrathin sections were prepared and examined in a transmission electron microscope (Morgagni M 268 D; FEI), following the methods described by Merhej et al. (2008), the cells of strain B10T were found to have mean diameters of about 850 nm. The cell wall consisted of one thin electron-dense layer and one thick transparent layer (140 ± 20 nm) that contained granular material (Fig. 1). Growth of strain B10T was assessed at various pH values, temperatures and salt concentrations in Hungate tubes with basal growth medium containing 40 mM methanol and with H2/CO2 in the gas phase (at 1 bar). All of the analytical assays were performed in duplicate. Growth was measured by directly inserting the tubes into a UV-160A spectrophotometer (Shimadzu) and evaluating OD at 580 nm. Methane production was quantified as described previously (Cord-Ruwisch et al., 1986). The pH was adjusted to the desired value by the addition of anaerobic, sterile solutions containing 10% (w/v) NaHCO3 or 10% (w/v) Na2CO3. During growth, the pH of the medium increased by about 0.1 unit. Growth occurred at pH 7.2–8.4 (optimum pH 7.6). Subsequently, growth of strain B10T was tested at temperatures between 25 and 45 °C in basal growth medium at pH 7.6 (measurements indicated that pH was stable over the temperature range 25–45°C). Optimal growth was observed at 37 °C; no growth occurred at 25 °C or 45 °C. To investigate salt tolerance, NaCl was directly weighed into Hungate tubes to give 0.1–1.5% (w/v). Strain B10T grew in the basal medium in the presence of 0.1–1.5% (w/v) NaCl (optimum 1.0%). Strain B10T did not grow in oxidized medium (indicated by the pink colour of resazurin). To determine its optimal physico-chemical growth conditions and substrate utilization, the novel strain was always subcultured once under the same experimental conditions.

In order to determine the pattern of substrate utilization, a sterile stock solution of each substrate was added to basal medium to give a final concentration of 10 mM (trimethylamine, 2-butanol, 2-propanol, cyclopentanol, 2-pentanol, ethanol, 1-propanol and 2,3-butanediol; all with an
atmosphere of \(\text{N}_2/\text{CO}_2\), 20 mM (acetate), 40 mM (formate and methanol) or 40 mM (methanol) with an atmosphere of \(\text{H}_2/\text{CO}_2\). Hydrogen oxidation was tested using \(\text{H}_2/\text{CO}_2\) (80 : 20, v/v; at 1 bar) in the gas phase with and without 2 mM acetate as a carbon source. Formate oxidation was also tested with and without 2 mM acetate. These tests indicated that strain B10T oxidized \(\text{H}_2\) as the only energy source by reducing methanol to methane. Strain B10T did not use acetate, trimethylamine, 2-butanol, 2-propanol, cyclopentanol, 2-pentanol, ethanol, 1-propanol or 2,3-butadienol under an atmosphere of \(\text{N}_2/\text{CO}_2\). Furthermore, formate could not be used as an energy source. Yeast extract and tungstate-selenite were required for growth with \(\text{H}_2\) and methanol.

The genomic DNA G+C content of strain B10T, 59.93 mol%, was evaluated using the strain’s complete genome sequence, which was obtained by pyrosequencing on a GSFLX system (Roche 454) as previously described (Margulies et al., 2005). The estimated size of the strain’s complete genome was 2.05 Mb. Two of the genes of strain B10T were amplified in PCR: 16S rRNA by using the Met86F (5′-GCTCAGTAACACGTGG-3′) and Met1340R (5′-CGGTGTGTGCAAGGAG-3′) primers (Wright & Pimm, 2003) and mcrA by using the mcrA-F (5′-GGTGGTG- TMMGATTACACARTGYGCWACAGC-3′) and mcrA-R (5′-TCATTGCRTAGTTWGRTAGTT-3′) primers (Luton et al., 2002). Sterile distilled water was used as a negative control in each PCR. Although mcrA-F and mcrA-R were also used for the mcrA gene sequencing, an additional internal primer pair – B10-dir (5′-AGCCGCCGGCTAAYAC-3′) and B10-rev2 (5′-CYGGCGTGTGAVTCCAATT-3′) – was used for sequencing of the 16S rRNA gene. The PCR products were purified and sequenced by using a BigDye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems) and a 3130 genetic analyzer (Applied Biosystems). The sequences were analysed with the Seqscape program (Applied Biosystems) and similarity values were determined with the BLAST program (blast.ncbi.nlm.nih.gov). The 16S rRNA and mcrA gene sequences of the novel strain were aligned with those of various archaea using the CLUSTAL W program. 16S rRNA gene sequence-based phylogenetic trees were reconstructed by the neighbour-joining and maximum-likelihood methods using version 4.2 of the MEGA software package (Kumar et al., 2008), and the MrBayes algorithm.

The phylogenetic analysis based on the 16S rRNA gene sequence of strain B10T (1434 bp) indicated that the novel strain belonged to the Archaea but its closest, cultivated phylogenetic relative, ‘Candidatus Aciduliprofundum boonei’, which has been placed in the order Thermoplasmatales, showed only 83.0 % 16S rRNA gene sequence similarity. However, strain B10T showed higher 16S rRNA gene sequence similarities (98.0–98.7 %) with several uncultured, methanogenic archaea previously retrieved from the digestive tracts of various animals, including wallabies (Evans et al., 2009), humans (Mihajlovski et al., 2008, 2010), pigs (Nehmé et al., 2009), sheep (Saengkerdsub et al., 2007) and chickens (Wright et al., 2006). In the phylogenetic trees based on 16S rRNA gene sequences, i.e. those reconstructed with the neighbour-joining (Fig. 2), maximum-likelihood (Fig. S1, available in IJSEM Online) or MrBayes algorithms (Fig. S2), strain B10T was clustered with these uncultured archaea in a group that was distinct from members of the order Thermoplasmatales. Members of the order Thermoplasmatales could also be differentiated from strain B10T by several phenotypic characteristics (Table 1). The current members of the order Thermoplasmatales are sulfur-reducing, thermoacidophilic archaea that have been isolated from extreme environments (Reysenbach et al., 2006), whereas strain B10T is a methanol-reducing, mesophilic methanogen isolated from a clinical sample. Strain B10T was able to produce methane under anaerobic conditions. This observation is in agreement with the detection of an mcrA gene (GenBank accession no. HQ896500) in the strain, as this gene encodes the alpha subunit of the methyl-coenzyme M reductase that plays a crucial role in methanogenesis (Hallam et al., 2003). It appears that strain B10T and its uncultured phylogenetic relatives belong to a new order within the methanogenic Archaea that is different from the order Thermoplasmatales.

Strain B10T shows 75.65 % 16S rRNA gene sequence similarity with the type strain of *Methanobrevibacter smithii*, its most closely related methanogen within the order Methanobacteriales. Two major differences distinguish strain B10T from the other three cultured archaea that were isolated from humans (i.e. *Methanobrevibacter smithii*, *Methanosphaera stadtmanae* and *Methanobrevibacter oralis*): it requires both a slightly more alkaline pH and tungstate-selenite for its growth. Tungstate-selenite has been found essential for the growth of several other methanogenic archaea but none of these were isolated from humans (Belay et al., 1986; Jones & Stadtman, 1977, 1981). Strain B10T and *Methanosphaera stadtmanae* share the ability to oxidize \(\text{H}_2\) and reduce methanol to methane. This ability is not seen in members of the order Methanosarcinales (e.g. *Methanosarcina*
species), which can, however, still use methanol and other methylotrophic compounds as energy sources (Kendall & Boone, 2006). In contrast to *Methanosphaera stadtmanae*, which only has a thick electron-dense layer as its cell wall (Miller & Wolin, 1985), the cell wall of strain B10T consists of one thin electron-dense layer and one thick transparent layer. Moreover, the genomic DNA G+C content reported for *Methanosphaera stadtmanae* (25.8 mol%) is much lower than that of strain B10T (59.93 mol%) (Table 1).

Based on the phenotypic, molecular and phylogenetic data presented here, strain B10T represents a novel species of a new genus within the methanogenic *Archaea* for which the name *Methanomassiliicoccus luminyensis* gen. nov., sp. nov. is proposed.

**Description of *Methanomassiliicoccus* gen. nov.**

*Methanomassiliicoccus* [Me.tha.no.mas.si.li.i.coc cus]. N.L. pref. *methano-* pertaining to methane; L. n. *Massilia* Latin name for Marseille; N.L. masc. n. *coccus* (from Gr. masc. n. *kokkas* grain, seed) coccus; N.L. masc. n. *Methanomassi liicoccus* a methane-forming coccus from Marseille].

Cells are non-motile, regular, Gram-stain-positive cocci with a diameter of about 850 nm. Methanogenic and obligatory anaerobic member of the domain *Archaea*. Mesophilic (25–45 °C) and slightly alkaliphilic, with growth occurring with NaCl at 0.1–1.0 % (optimum 1.0 %). Produces methane from H₂ + methanol. The type species is *Methanomassiliicoccus luminyensis*.

**Description of *Methanomassiliicoccus luminyensis* sp. nov.**

*Methanomassiliicoccus luminyensis* (lu.mi.ny.en'sis. N.L. masc. adj. *luminyensis* belonging to Luminy, the place where the type strain was isolated).

In addition to the characters described for the genus, the species is characterized by the following properties. Cells lyse in 0.1 % (w/v) SDS or hypotonic distilled water. Produces

![Fig. 2. A maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain B10T and 36 members of the domain *Archaea*. Bootstrap values ≥ 90% (based on 1000 replications) are shown at branching points. Bar, 0.05 substitutions per nucleotide position.](http://ijs.sgmjournals.org)
methane by reducing methanol with hydrogen as the electron donor, although it cannot produce methane when hydrogen or methanol is the sole energy source. Cannot produce methane from formate, acetate, trimethylamine, 2-butanol, 2-propanol, cyclopentanol, 2-pentanol, ethanol, 1-propanol or 2,3-butanediol, even with CO2 in the atmosphere.

The type strain, B10T (=DSM 24529T=CSUR P135T), was isolated from human faeces collected in Marseille, France. The genomic DNA G+C content of strain B10T is 59.93 mol%.

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Methanosphaera stadtmanae gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. *Arch Microbiol* 141, 116–122.


