Methanobrevibacter boviskoreani sp. nov., isolated from the rumen of Korean native cattle

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Three strictly anaerobic, methanogenic strains JH1T, JH4 and JH8 were isolated from rumen of the Korean native cattle (HanWoo; Bos taurus coreanae) in South Korea. The colonies were circular, opaque, and slightly yellowish. Phylogenetic analyses of 16S rRNA gene and mcrA (encoding χ subunit of methyl-coenzyme M reductase) sequences confirmed the affiliation of the novel strains with the Methanobacteriales, and Methanobrevibacter wolinii SH7 was the most closely related species. The 16S rRNA gene and mcrA sequence similarities between strains JH1T, JH4 and JH8 and M. wolinii SH7 were 96.2 and 89.0% respectively, and DNA–DNA hybridization of the isolates and M. wolinii DSM 11976T showed a 20% reassociation. Strain JH1T exhibited 92% DNA–DNA relatedness with strains JH4 and JH8, and their 16S rRNA gene and mcrA sequences were identical. Cells stained Gram-positive and were non-motile rods, 1.5–1.8 μm long and 0.6 μm wide. The strains were able to use H2/CO2 and formate. The optimum temperature and pH ranges for growth were 37–40 °C and pH 6.5–7.0. The DNA G+C content of strain JH1T was 28 mol%. Based on data from this study using a polyphasic approach, the three strains represent a novel species of genus Methanobrevibacter, for which the name Methanobrevibacter boviskoreani sp. nov. is proposed. The type strain is JH1T (=KCTC 4102T=JCM 18376T).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and mcrA sequences of strain JH1T are KC608769 and KC865050, respectively. The GenBank/EMBL/DDBJ accession number for the mcrA gene sequence of Methanobrevibacter wolinii SH7 is KC865051.

Two supplementary figures and a supplementary table are available with the online version of this paper.
aim of this research was to isolate methanogens from the Korean native cattle and determine whether these methanogens represent a novel species or are members of the species reported earlier from other parts of the globe (Kumar et al., 2012; Rea et al., 2007). In addition, the pure culture of these isolates will help in subsequent studies such as physiology, biochemistry, etc. We should also expand our knowledge on both dominant and low-abundance species of methanogens to develop a broad spectrum antimethanogenic strategy to reduce methane emissions from ruminants. In the current research, we succeeded in isolating and characterizing a new methanogen isolate from the rumen of Korean native cattle.

Fresh rumen liquor (900 ml) was collected into CO₂-flushed Duran bottles (1 litre), from the ruminally cannulated 13-month-old HanWoo steers. HanWoo steers were maintained on 0.5 kg of rice straw and 4 kg of concentrate (dry matter basis), at the research farm of the Seoul National University, Republic of Korea. Before sealing the bottles, 20 ml l⁻¹ of 2.5 % cysteine hydrochloride (Yakuri) and 2.5 % sodium sulfide (Sigma) were added to keep a strict reduced environment.

For the isolation and purification of hydrogenotrophic methanogens, a novel anaerobic jar with the capacity to bear high pressure, was designed by our research group. The new system contains a butyl rubber stopper located within the cap of the jar and is designed to replace the inside gases with H₂/CO₂ (80 : 20) and pressurize the jar up to 300 kPa using a gassing manifold system (Fig. S1, available in IJSEM Online).

To enrich the methanogen colonies, solid JCM 530 medium with antibiotics was used. The composition of JCM 530 medium is provided in Table S1 and was prepared as per the instructions of the Japan Collection of Microorganisms (JCM; http://www.jcm.riken.jp) with slight modifications. A mixture of filter-sterilized fatty acids (0.5 ml l⁻¹ each of valeric acid, isovaleric acid, x-methylbutyric acid and isobutyric acid; Sigma) and 1.0 ml l⁻¹ of 0.01 % (w/v) filter-sterilized coenzyme M solution (2-mercaptoethanesulfonic acid; Sigma) were supplemented into the medium; 20 µl ml⁻¹ cysteine-hydrochloride (2.5 %, w/v) and sodium sulfide (2.5 %, w/v) from sterilized anoxic stock solutions were added to reduce the medium prior to inoculation. Cultures were grown in 125 ml serum bottles (Wheaton Scientific) containing 30 ml medium or in 18 mm × 125 mm screw-capped tubes (Bellco Glass) with 4 ml medium. The bottles/tubes were then sealed with butyl rubber stoppers and the gas phase, H₂/CO₂ (80 : 20, 200 kPa), was used for routine culturing unless otherwise indicated. Hungate anaerobic techniques were used for isolation and culture of the strains (Hungate, 1969).

The colonies formed on solid JCM 530 medium were picked and transferred, to the tubes containing 4 ml of liquid medium, in the Coy anaerobic chamber (Coy Laboratory Product). The bottles were pressurized with H₂/CO₂ (80 : 20, 200 kPa), and incubated at 37 °C with shaking (160 r.p.m.) for 2–4 days. Genomic DNA was purified from the liquid cultures using the protocol of Fujimoto et al. (2004) and the DNA templates were used to obtain partial 16S rRNA gene sequences by PCR with primers 344F (5’-ACGGGGGCGACGCGGCGGGA-3’) and 1492R (5’-GCTACCTTGTTCAGACCTT-3’); numbers of primers refer to positions relative to the Escherichia coli 16S rRNA gene (Madrid et al., 2001). When the isolated colonies were confirmed as methanogens by 16S rRNA gene sequencing, the liquid cultures maintained at 4 °C were serially diluted in JCM 530 medium and streak plated again, in the Coy anaerobic chamber. This process was repeated three times and purity of the final isolated strains was further confirmed. The Archaeal specific primers, 1F (5’-TCYGKTTGATCCYGSCRGA-3’; Y: C/T, R: G/A), 344F, 1100R (5’-TGGTTGGCTGTCGCCGTG-3’), and the universal primer 1492R were used to obtain partial 16S rRNA gene sequences of the final isolated strains. Cultures were maintained by repeated sub culturing in JCM 530 medium every 7 days using a 10 % (v/v) inoculum. Long-term preservation followed the glass-capillary method of Hippe (1991).

Phenotypic characterization of strains JH1T, JH4 and JH8 included determination of catabolic substrates utilization [H₂ + CO₂ and formate (50 mM); acetate (50 mM); ethanol (20 mM); methanol (50 mM) and methylamines (20 mM)] under N₂-CO₂ and supplement requirements (yeast extract + peptone; coenzyme M; fatty acid solution). To assess the requirement of yeast extract (2.0 g l⁻¹) and peptone (2.0 g l⁻¹), each was omitted from the medium. Coenzyme M (2-mercaptoethanesulfonic acid) and fatty acid solution were tested at concentrations of 0.01 and 2.0 mg ml⁻¹, respectively. The fatty acid solution contained valeric, isovaleric, 2-methylbutyric and isobutyric acid at 2.5 % (v/v) each.

The optimum pH for growth was measured in basal medium with the addition of sterile 1 M HCl, 10 % (w/v) NaHCO₃ or 8 % (w/v) Na₂CO₃ solutions to obtain the required pH from 4 to 8.5. The pH remained stable and did not change by more than 0.2 during the growth experiments. The growth of all strains was also checked at temperatures of 25–55 °C. Growth in 2 % bovine bile (ox bile) and at different concentrations of sodium chloride (0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 0.5 M) was also assessed. Tolerance to 10 % (w/v) SDS, hypotonic solution and antibiotics (mg l⁻¹; chloramphenicol, 10; bacitracin, 10; polymyxin, 10; vancomycin, 2; erythromycin, 1; and puromycin 0.02) was also tested.

All experiments were performed at least in duplicate with 4 ml medium inoculated with 5 % (v/v) culture in the exponential growth phase. Growth was measured by recording the OD₆₅₀ (Spectronic 20D +; ThermoFisher Scientific).

Cell morphology, size, motility and the Gram reaction (Gerhardt et al., 1994) were determined using light, phase-contrast (DS-Ri; Nikon) and transmission electron microscopy (CM-20; Philips). For transmission electron microscopic analysis, cells were cultured for 2 weeks at 37 °C on solid JCM 530 medium, and were stained with
1 % (w/v) phosphotungstic acid. Preparations for negative staining were performed as previously described by Lai et al. (2000).

Mid-exponential-phase cells of strains JH1\(^T\), JH4, JH8 and reference strains Methanobrevibacter wolinii DSM 11976\(^T\) and Methanobrevibacter smithii DSM 861\(^T\) were harvested and cell walls were extracted using the method of König (1995). Protein and carbohydrate concentration of the cell wall and intracellular fractions were determined using a protein assay (Bio-Rad) and glycoprotein carbohydrate estimation kit (Pierce), respectively. Standards used in the assay were provided with the respective kit.

Cells of strain JH1\(^T\) and Methanobrevibacter wolinii DSM 11976\(^T\) were harvested during late-exponential phase (OD\(_{600}\)=0.8) and intact genomic DNA (total >1 mg from 6 litres of culture; 400 ml in 1 litre Duran bottles, modified according to Balch & Wolfe (1976) \(\times 15\) bottles and pressurized with \(\text{H}_2/\text{CO}_2; 80:20\)) was extracted and purified. After freezing and thawing cells for more than three times, the rigid cell wall was disrupted by glass beads as previously described (Fujimoto et al., 2004). Later, the intact genomic DNA was hooked up by a glass rod (Marmur & Doty, 1962). The quality of DNA was assessed by measuring \(A_{260}/A_{280}\) ratio on NanoDrop 2000 spectrophotometer (Thermo Scientific). The DNA G+C content was determined by HPLC using a YMC-Triat C18 column (150 \(\times\) 4.6 mm) at 30 \(^\circ\)C with a mobile phase of 0.5 M \(\text{NH}_4\text{H}_2\text{PO}_4/\text{acetonitrile} (20:1)\).

The DNA of E. coli KCTC 2441\(^T\) was used as the reference (Mesbah et al., 1989; Tamaoka & Komagata, 1984). DNA–DNA hybridization was carried out using the micro dilution-well technique, with photobiotin for DNA labeling (Ezaki et al., 1989; Lee et al., 2013).

For 16S rRNA gene and mcrA sequencing, genomic DNA was extracted as described above, and genes were amplified according to Luton et al. (2002). The closest known strains of the isolates JH1\(^T\), JH4 and JH8 were determined using the BLASTN algorithm and EzTaxon-e server 2.1 (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The sequences of closely related species were retrieved from the NCBI GenBank database. Multiple sequence alignments were analysed with CLUSTAL W (Thompson et al., 1994). The maximum-likelihood inference method (Tamura & Nei, 1993) with the MEGA 5.1 software package (Tamura et al., 2011) was used to reconstruct phylogenetic trees. Methanobrevibacter mobile DSM 1539\(^T\) (GenBank accession number AF414044) was used as an outgroup. Phylogeny was further confirmed by deduced MrcA amino acids. The robustness of each tree was evaluated from 1000 resamplings.

Three pure cultures of methanogens were obtained, designated JH1\(^T\), JH4 and JH8. The polyphasic approach used to characterize the three novel strains revealed that they were affiliated to the genus Methanobrevibacter. Cells of all three strains were non-motile and rod-shaped, 1.5–1.8 \(\mu\)m long and 0.6 \(\mu\)m wide. Negatively stained electron micrographs also revealed the rod-shaped structure; flagella could not be detected in any of the three strains (Fig. S2).

Strains JH1\(^T\), JH4 and JH8 utilized \(\text{H}_2/\text{CO}_2\) and formate/\(\text{CO}_2\) for growth but not acetate, methanol, ethanol or methylamines (Table 1). It is reported that the ability to utilize only \(\text{H}_2/\text{CO}_2\) and in some cases formate and \(\text{CO}_2\) is a characteristic feature of the genus Methanobrevibacter. Rea et al. (2007) also reported that Methanobrevibacter millerae ZA-10\(^T\) and Methanobrevibacter olleyae KM1H5-1P\(^T\) were able to utilize formate plus \(\text{CO}_2\). Results for nutritional requirements, growth in bile salt, tolerance to NaCl, SDS and antibiotics, and temperature and pH ranges for growth for all three novel strains are also detailed in Table 1. Growth was stimulated by all the nutritional factors tested (yeast extract, coenzyme M and fatty acids) except acetate and peptone.

Growth for all three strains (JH1\(^T\), JH4 and JH8) was observed at 35–45 \(^\circ\)C with an optimum at 37–40 \(^\circ\)C. The growth temperature for many members of the genus Methanobrevibacter ranges from 26 to 46 \(^\circ\)C, with optimum in the range of 34–46 \(^\circ\)C. The pH range for growth was between pH 5.5 and pH 8.0, with an optimum around pH 6.0–7.0. At pH 5.0 and pH 8.5 no growth was observed. However, certain strains of species of the genus Methanobrevibacter, isolated from the rumen, were reported to grow at pH 5.0 (Rea et al., 2007). Results of growth at different NaCl concentrations (Table 1) showed that all strains could survive at all NaCl concentrations tested (0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5 M). The results obtained are in accordance with Rea et al. (2007) who reported that different strains of species of the genus Methanobrevibacter could tolerate up to 0.45 M (2.6 %) NaCl. Similar results were also obtained by Kumar et al. (2012). In addition, no difference was observed in the growth pattern at all NaCl concentrations tested compared to the control. Thus, the novel strains could tolerate NaCl up to 0.5 M.

Cells were not lysed in bile (2.0 %), SDS (10 %) or hypotonic solution, and intact cells were observed under phase-contrast microscope. All three strains (JH1\(^T\), JH4 and JH8) could tolerate the antibiotics bacitracin and vancomycin but were completely inhibited by chloramphenicol, polymyxin and puromycin. However, Methanobrevibacter wolinii could tolerate only chloramphenicol and was susceptible to other antibiotics.

The amount of cell-wall protein of the total protein for strain JH1\(^T\) was 85.3 \(\pm\) 0.3 %, and, for the reference strains Methanobrevibacter wolinii DSM 11976\(^T\) and Methanobrevibacter smithii DSM 861\(^T\) was 86.0 \(\pm\) 0.3 % and 85.6 \(\pm\) 0.3 %, respectively. The sugar content of the cell wall of strain JH1\(^T\) was 24.7 %, and of Methanobrevibacter wolinii DSM 11976\(^T\) and Methanobrevibacter smithii DSM 861\(^T\) was 39.1 % and 26.8 %, respectively. The result for Methanobrevibacter smithii DSM 861\(^T\) is in corroboration with the reports of Kandler & König (1985) and Rea et al. (2007), who reported 85.9 % protein and 27 % carbohydrate content in the cell wall. The carbohydrate content of the cell...
The wall of strain JH1<sup>T</sup> was very similar to that of *Methanobrevibacter smithii* DSM 861<sup>T</sup>, however it varied considerably to that of *Methanobrevibacter wolinii* DSM 11976<sup>T</sup>. The DNA G+C content of strain JH1<sup>T</sup> was 28 mol%, which was well within the range (26–38 %) given for the species of genus *Methanobrevibacter* (Miller & Lin, 2002) (Table 1). DNA–DNA hybridization analysis revealed only 20 % DNA–DNA reassociation between strain JH1<sup>T</sup> and *Methanobrevibacter wolinii* DSM 11976<sup>T</sup>.

Phylogenetic analyses revealed that the novel strains were members of the family *Methanobacteriaceae*. Comparative 16S rRNA gene sequence analysis using the EzTaxon-e server 2.1 showed that strains JH1<sup>T</sup>, JH4 and JH8 have the highest sequence similarity (96.2 %) with *Methanobrevibacter wolinii* SHT (Fig. 1). A similarity of less than 98 % in a 16S rRNA gene sequence is generally considered evidence for separate species (Keswani & Whitman, 2001). Furthermore, deduced amino acid sequences from mcrA gene analyses showed 89 % similarity between all the isolated strains and

### Table 1. Comparison of characteristics (morphological, biochemical and physiological properties) of strain JH1<sup>T</sup> and some members of the genus *Methanobrevibacter*

| Strains: 1, JH1<sup>T</sup>; 2, *Methanobrevibacter wolinii* DSM 11976<sup>T</sup>; 3, *Methanobrevibacter ruminantium* M1T<sup>T</sup>; 4, *Methanobrevibacter gottschalkii* HO<sup>T</sup>; 5, *Methanobrevibacter thaueri* CWT; 6, *Methanobrevibacter smithii* PS<sup>T</sup>. Data for taxa 1 and 2 are from this study, data for taxa 3–6 are from Rea et al. (2007) and Miller & Lin (2002). Data for strain JH1<sup>T</sup> also represents characteristics of strains JH4 and JH8. ND, No data available; H<sub>2</sub> + CO<sub>2</sub>; f, formate; Ye, yeast extract; P, peptone; c, Coenzyme M; FA, fatty acids; Ba, bacitracin; Va, vancomycin; Chl, chloramphenicol. +, Growth (OD<sub>600</sub> is >0.1); −, no growth (OD<sub>600</sub> is <0.1). |

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>Cell shape</td>
<td>Rods</td>
<td>Coccolithi</td>
<td>Coccolithi</td>
<td>Coccolithi</td>
<td>Coccolithi</td>
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<td>Cell width (μm)</td>
<td>0.6</td>
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<td>0.7</td>
<td>0.7</td>
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<td>Cell length (μm)</td>
<td>1.5–1.8</td>
<td>1.1–1.4</td>
<td>0.8–1.8</td>
<td>0.9</td>
<td>0.6–1.2</td>
<td>ND</td>
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<tr>
<td>DNA G+C content (mol%)&lt;sup&gt;*&lt;/sup&gt;</td>
<td>28 (LC)</td>
<td>33 (LC)</td>
<td>31 (T&lt;sub&gt;mo&lt;/sub&gt;)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>29 (T&lt;sub&gt;mo&lt;/sub&gt;)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>30 (T&lt;sub&gt;mo&lt;/sub&gt;)&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>37</td>
<td>38</td>
<td>37</td>
<td>37</td>
<td>ND</td>
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<td>Optimum pH</td>
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<td>NaCl range (M)</td>
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<td>Cell-wall protein (%)</td>
<td>85.3</td>
<td>86.0</td>
<td>85.2</td>
<td>ND</td>
<td>ND</td>
<td>85.6&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>Cell-wall glycoprotein (%)</td>
<td>24.7</td>
<td>39.1</td>
<td>44.0</td>
<td>ND</td>
<td>ND</td>
<td>26.8&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>Nutritional requirements</td>
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<td>H, F, Ye, P, C</td>
<td>H, Ye, P</td>
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<td>−</td>
<td>−</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Growth with SDS (10 %)</td>
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<td>+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Tolerance to antibiotics</td>
<td>Ba, Va</td>
<td>Chl</td>
<td>ND</td>
<td>ND</td>
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<sup>*</sup>Determined by: LC, HPLC; T<sub>mo</sub> thermal denaturation.
<sup>†</sup>Data obtained in this study.

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**Fig. 1.** Bootstrapped maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences. *Methanomicrobium mobile* DSM 1539<sup>T</sup> was used as an outgroup. Bootstrap values >50 % (of 1000 cycles) are indicated. Bar, 0.05 substitutions per site.
Methanobrevibacter wolinii SH² (Fig. 2). However, the NCBI database showed that the 16S rRNA gene sequence of strain JH1T had 99% similarity with Methanobrevibacter sp. strain AbM4, isolated from sheep abomasum. This strain first appeared in an unpublished work and to date there is no published report on the characterization of strain AbM4. Recently Methanobrevibacter sp. AbM4 was reported in the diversity analysis of methanogens in the rumen of beef cattle with respect to high or low feed efficiencies (Zhou et al., 2009). In 2010, the same group reported the dominance of Methanobrevibacter sp. AbM4 in the cattle fed high-energy diet using cultivation-independent approaches (Zhou et al., 2010).

Based on phylogeny, strains JH1T and AbM4 could be similar. However, due to the lack of a pure culture of AbM4, taxonomy based on its physiology and genome could not be analysed. Therefore, the polyphasic approach taken to characterize the three novel strains herein indicates that these strains (JH1T, JH4 and JH8) should be classified as representing a separate novel species of the genus Methanobrevibacter. For which the name Methanobrevibacter boviskoreani sp. nov. is proposed. The result of this study expands our knowledge on rumen methanogens from the Korean peninsula.

**Description of Methanobrevibacter boviskoreani sp. nov.**

Methanobrevibacter boviskoreani (bo.vis.ko.re.a’ni. L. masc. n. bovis, bovis cattle; N.L. adj. koreanus Korean; N.L. gen. n. boviskoreani of Korean cattle, in recognition of the first isolation of a ruminal methanogen from Korean native cattle).

Cells stain Gram-positive, occur singly or in pairs or chains and are rod-shaped (1.5–1.8 × 0.6 µm). Cells are non-motile and resistant to lysis by 2% bile, 10% SDS and hypotonic solution. Catabolic substrates include H₂/CO₂ and formate plus CO₂, but not acetate, ethanol, methanol or methylamines. Requires yeast extract for growth and is stimulated by coenzyme M and volatile fatty acids. Mesophilic. The optimum pH and temperature for growth are pH 6.5–7.0 and 37–40 °C. The maximum salt (NaCl) tolerance for growth is 0.5 M. Resistant to vancomycin and bacitracin, but susceptible to puromycin, polymyxin and chloramphenicol.

The type strain, JH1T (=KCTC 4102T=JCM 18376T), was isolated from the rumen content of Korean native cattle (Bos taurus coreanae). The DNA G+C content of the type strain is 28 mol%.

**Acknowledgements**

This work was supported by the National Research Foundation of Korea (NRF) (grant no. OGM0211212), funded by the Korean government (MEST), and partially supported by grants from MEST (RBMI4351112 & NMM0101232) and grants from the KRIIBB Research Initiative Program (KCM1051312, KGM4111342).

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