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TAXONOMIC DESCRIPTION

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Anaerosporobacter faecicola sp. nov. isolated from faeces of Korean cow

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Abstract

A novel bacterial isolate designated as strain AGMB01083^T was isolated from Korean cow faeces deposited in the National Institute of Animal Science (Wanju, Republic of Korea). The bacterium is obligate anaerobic, Gram-strain-positive, and motile. Cells are straight or curved rod-shaped, flagella and spores are observed. Growth occurs between 20–40 °C (temperature optimum of 35 °C), at pH 7–9 (pH optimum of 7), and in the presence of 0.5–1.0% (w/v) NaCl. Based on the 16S rRNA gene sequence analysis, the strain belongs to the genus *Anaerosporobacter* and is most closely related to *A. mobilis* HY-37-4^T (=KCTC5027^T, similarity, 95.7%). The DNA G+C content is 36.2 mol%, determined by the whole-genome sequence. The average nucleotide identity value between strain AGMB01083^T and strain *A. mobilis* HY-37-4^T is 75.5%, below the interspecies identity threshold value. The major cellular fatty acids (>10%) of strain AGMB01083^T are $C_{16:0}$, $C_{16:0}$ dimethyl acetal (DMA), and $C_{16:0}$ 3-OH. Based on the phylogenetic, phenotypic, biochemical, chemotaxonomic, and genomic characterization, strain AGMB01083^T is proposed to be a novel species, named *Anaerosporobacter faecicola*, in the genus *Anaerosporobacter*. The type strain is AGMB01083^T (=KCTC 15857^T=NBRC 114517^T).

The microbiome of cattle faeces is important for their health and productivity, as well as food safety and pathogen shedding [1]. The diet is the most important factor that affects the number of bacteria in the faeces, while gender, age, breed, and geographic location exert a relatively small effect [1]. According to a recent metagenome analysis of the 16S rRNA sequences (13663 sequences) isolated from cattle faeces, *Firmicutes* account for 49% of all sequences and represent the largest phylum [2]. *Bacteroidetes* are the second largest phylum and account for 42% of all sequences. *Proteobacteria* account for 6% of all sequences, while minor phyla include *Actinobacteria*, *Fusobacteria*, *Spirochaetes*, and *Tenericutes* [2]. At the family level, the most abundant groups are *Bacteroidaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Ruminococcaceae*, *Succinivibrionaceae*, and *Veillonellaceae* [3]. The genus *Anaerosporobacter* was first proposed by Jeong *et al.* [4], who suggested that *Anaerosporobacter mobilis* be classified within a new genus *Anaerosporobacter* and assigned to the family *Lachnospiraceae*, class *Clostridia*. The family *Lachnospiraceae* presents one of the major taxonomic groups of mammalian intestinal microbiota [5]. Further, intestinal bacteria belonging to this family produce butyrate, which is important for the growth of gut microbes and host intestinal epithelial cells [5]. Cells of species from the genus *Anaerosporobacter* are strictly anaerobic rods with convex, translucent to opaque, grayish-white with erose edges [4]. At the time of writing, the genus *Anaerosporobacter* contains one species with a validly published name (www.bacterio.net/anaerosporobacter.html) [6].

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Keywords: *Anaerosporobacter faecicola*; Faeces; Korean cow; Taxonomy; Novel bacterium.
Abbreviations: AGMB, Animal Gut Microbiome Banking; ALDE, aldehyde; DMA, dimethyl Acetal; KCTC, Korean Collection for Type Cultures.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AGMB01083^T is MT197381. The GenBank/EMBL/DDBJ accession number for the whole genome sequence of strain AGMB01083^T is JAAQRO000000000.
Two supplementary figures are available with the online version of this article.

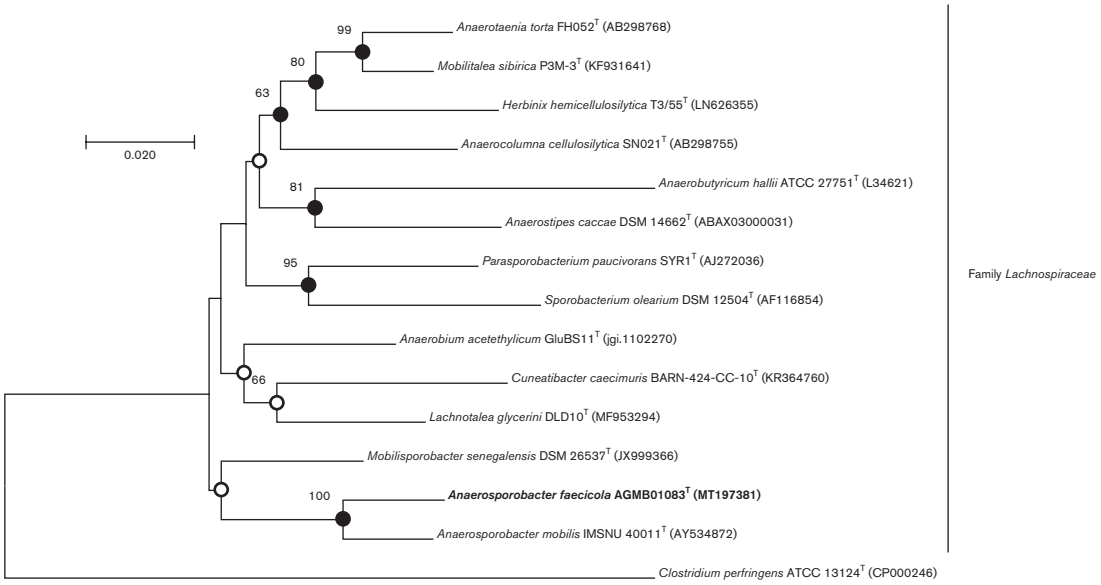

Fig. 1. The phylogenetic relationship between strain AGMB01083^T. And some related species from the family *Lachnospiraceae*. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequence is shown. Bootstrap values >50% were based on 1000 replications, as shown at the branch points. Filled circles indicate that the corresponding nodes (groupings) were recovered in trees generated using the maximum-likelihood and maximum-parsimony methods. Open circles indicate that the corresponding nodes were also recovered by using the maximum-likelihood method. The sequence of *Clostridium perfringens* ATCC 13124^T was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

While metagenomic analyses of faecal bacteria from cattle have been published, few studies report isolation and characterization of bacteria from cattle faeces. Using a polyphasic taxonomic approach, we report the isolation and characterization of a bacterium designated AGMB01083^T recovered from the faeces of cattle. This study furthers the cattle gut microbiome research. The genome sequence of AGMB01083^T was reported in Korean Journal of Microbiology [7]. The analysis suggests that the strain represents a novel species from the genus *Anaerosporobacter*. This study furthers the cattle gut microbiome research.

ISOLATION AND ECOLOGY

Strain AGMB01083^T was isolated from Korean cow faeces, which were deposited by the National Institute of Animal Science (Wanju, Republic of Korea). The isolation was performed under anaerobic conditions, as soon as the sample was collected. The sample was serially diluted in a saline solution 0.85% (w/v) NaCl and spread on tryptic soy agar (BD, NJ, USA) containing 5% (v/v) sheep blood in an anaerobic chamber (Coy Laboratory Products, MI, USA) in an atmosphere of 86% N_2, 7% H_2, and 7% CO_2. The samples were incubated at 37 °C for 48 h, after which single colonies were isolated. After identification by 16S rRNA gene sequencing, taxonomic analysis was performed based on phylogenetic, phenotypic, biochemical, chemotaxonomic, and genomic characterization. The isolate was stored at −80 °C in 10% (w/v) skim milk. *A. mobilis* HY-37-4^T was used as a reference species for the ensuing comparative studies.

16S rRNA PHYLOGENY

Genomic DNA was extracted using the phenol:chloroform:isoamyl alcohol method from cells grown on RCM agar [8]. The extracted DNA was used as a template to amplify the 16S rRNA gene. The nearly-complete 16S rRNA gene sequence was amplified using the universal primer pair: 785F (5′-GGATTAGATACCCTGGTA-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′). The amplified 16S rRNA gene was sequenced (Macrogen Inc., Republic of Korea). The complete 16S rRNA sequence was assembled by comparing with the whole-genome sequence, and analysed using EZBioCloud database [9] and GenBank/EMBL/DDBJ databases (http://www.ncbi.nlm.nih.gov/blast). After multiple-alignments of 16S rRNA gene sequences using CLUSTAL W [10], phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA) 7.0 software [11]. Evolutionary distances were calculated according to Kimura's two-parameter model [12]. Phylogenetic trees based on the 16S rRNA gene sequence were reconstructed by using the neighbour-joining (NJ) [13], maximum-likelihood (ML) [14], and maximum-parsimony (MP) [15] algorithms, with bootstrap analysis [16] based on 1000 re-samplings.

Comparative analysis of the 16S rRNA gene sequence (1520 bp) revealed that strain AGMB01083^T is closely related

Table 1. Differential characteristics of strain AGMB01083^T and type strain of a phylogenetically related species from the genus *Anaerosporobacter*

Strains: 1, *Anaerosporobacter faecicola* AGMB01083^T; 2, *Anaerosporobacter mobilis* KCTC 5027^T.

Characteristic	1	2
Source	Faeces of Korean cow	Forest soil
Cell morphology	Straight or curved rods	Slightly curved rods*
Size (μm)	1.6–2.96×0.3–0.5	0.2–0.4×2–4*
Growth pH range	7–9	7–10
Growth NaCl range (%)	0.5–1.0	0.5–2.0
Enzyme activity		
Alkaline phosphatase	w+	+
Pyroglutamic acid Arylamidase	–	+
Acid production		
Glucose	w+	+
Mannose	w+	–
Glycerol	w+	–
Rhamnose	+	–
Fermentation end products	S, p, e	S, e
DNA G+C content (mol%)	36.2	34.9*

*Data from Jeong *et al.* [4].
Unless otherwise noted, all data were obtained in the current study. +, Positive; –, negative; W+, weakly positive. E, ethanol; P, propionate; S, succinate. The upper- and lower-case letters indicate the major and minor fermentation end products, respectively.

to *A. mobilis* HY-37-4^T (=KCTC 5027^T, similarity, 95.7%). In phylogenetic trees reconstructed using the NJ, ML, and MP methods, strain AGMB01083^T formed a robust cluster within the genus *Anaerosporobacter* (Fig. 1).

GENOME FEATURES

For the DNA G+C content determination and genomic analysis, whole-genome sequencing was performed using the NovaSeq 6000 system (Illumina, San Diego, CA, USA) at Macrogen Inc. The average nucleotide identity (ANI) value was calculated using the ChunLab's online ANI calculator with the OrthoANI algorithm [17]. Digital DNA–DNA hybridization (dDDH) was performed using a genome-to-genome distance calculator GGDC 2.1 [18]. The average amino acid identity (AAI) was determined using the AAI calculator tools (http://enve-omics.ce.gatech.edu/aai/) [19]. Protein sequences for AAI analysis were obtained from the NCBI database (www.ncbi.nlm.nih.gov/protein/). For further phylogenomic analysis, the up-to-date bacterial core gene (UBCG) pipeline was used [20]. Whole-genome analysis was performed using the CLgenomics software and UniProt database (https://www.uniprot.org).

The genomic DNA G+C content of strain *A. mobilis* HY-37-4^T is 34.9 mol% [4]. Based on the whole-genome sequence, the genomic DNA G+C content of strain AGMB01083^T is 36.2 mol%, lower than that of the reference strain (Table 1). The ANI value between AGMB01083^T (JAAQRO000000000) and the closely-related *A. mobilis* HY-37-4^T (FRCP00000000) was 75.5%, which is lower than the 95–96% cut-off for species demarcation. The dDDH and AAI value between *A. faecicola* AGMB01083^T and *A. mobilis* HY-37-4^T was 21.9 [19.6–24.3%] and 73.7%, respectively. In the UBCG-based phylogenomic tree, *A. faecicola* AGMB01083^T was identified as the closest relative to *A. mobilis* HY-37-4^T, which supports 90 UBCGs (Fig. 2).

The genome of strain AGMB01083^T is 4589105 bp [7]. It contains the following annotated sequences: 3981 coding sequences, 27 rRNA genes (5S, 16S, and 23S), and 70 tRNA genes. Based on the whole-genome analysis, the strain AGMB01083^T encodes most genes involved in the flagellar assembly, which agrees with the observation that the cells of strain AGMB01083^T were flagella observed with the electron scanning microscope. These genes encode the flagellar biosynthesis protein (*flhA*), negative regulator of flagellin synthesis (*flgM*), flagellar basal-body rod protein (*flgB*, *flgC*, and *flgG*), flagellar hook protein (*flgE*), flagellar hook-associated protein (*flgK*, *flgL*, and *fliD*), chemotaxis protein (*motA* and *motB*), and others. Also, cells have several antibiotic resistance genes gene, such as metallo-beta-lactamase class B (*blaB*), bla regulator protein (*blaR1*), beta-lactamase class C (*ampC*), ATP-binding cassette (*abcA*), beta-N-acetylhexosaminidase (*nagZ*), oligopeptide transport system ATP-binding protein (*oppD* and *oppF*), penicillin-binding protein 1A (*mrcA*) and penicillin-binding protein 2 (*mrdA*).

PHYSIOLOGY AND CHEMOTAXONOMY

Cell morphology was observed using a phase-contrast microscope (Eclipse 80i, Nikon, Japan) and a scanning electron microscope (Quanta 250 FEG, FEI, USA). For the analysis, the cells were pre-grown on reinforced clostridial medium (RCM) (MB cell, Republic of Korea; 10.0 g peptone, 5.0 g NaCl, 10.0 g beef extract, 3.0 g yeast extract, 5.0 g dextrose, 1.0 g starch, 0.5 g L-cysteine hydrochloride monohydrate, and 3.0 g sodium acetate per 1 litre) supplemented with agar (15.0 g per 1 litre) for 24–48 h. Gram-staining was performed according to the manufacturer's instructions of the Gram-stain kit (Difco) and cells were fixed with methanol [21]. The growth was observed for 48 h at 37 °C under aerobic, microaerophilic (in a CO_2 incubator in the presence of 5% O_2), and anaerobic conditions (in an anaerobic chamber, Coy's LAB). The optimum growth conditions were determined by incubating at 37 °C and at a temperature range of 10 to 50 °C (at 5 °C intervals), and at pH 5, 6, 7, 8, 9, 10, and 11. For salt tolerance determinations, the cells were incubated in NaCl solutions from 0.5–5.0%

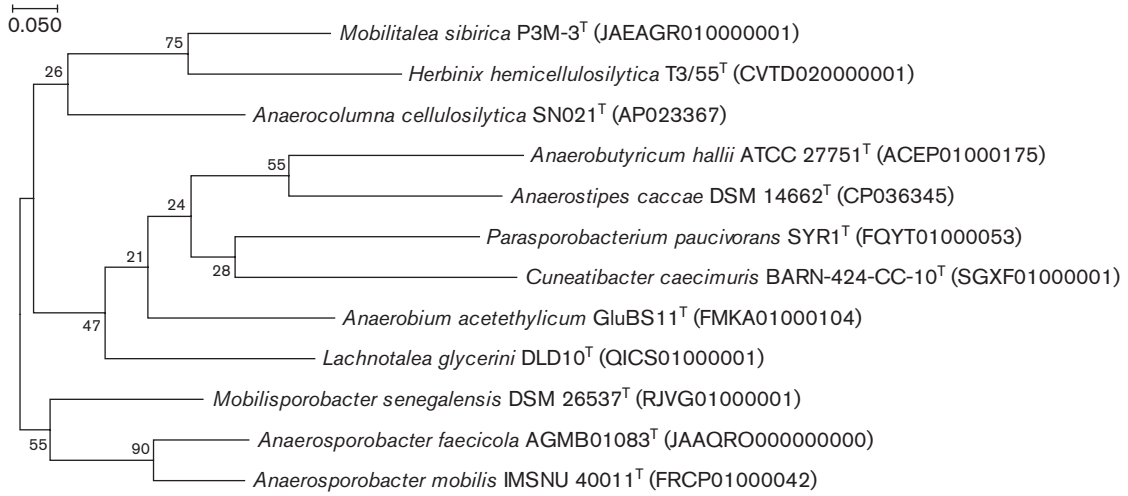

Fig. 2. The phylogenomic tree of strain AGMB01083^T and related taxa based on 92 bacterial core genes. The core genes were *alaS, argS, aspS, cgtA, coaE, cysS, dnaA, dnaG, dnaX, engA, ffh, fmt, frr, ftsY, gmk, hisS, ileS, infB, infC, ksgA, lepA, leuS, ligA, nusA, nusG, pgk, pheS, pheT, prfA, pyrG, recA, rbfA, rnc, rplA, rplB, rplC, rplD, rplE, rplF, rplI, rplJ, rplK, rplL, rplM, rplN, rplO, rplP, rplQ, rplR, rplS, rplT, rplU,rplV, rplW, rplX, rpmA, rpmC, rpmI, rpoA, rpoB, rpoC, rpsB, rpsC, rpsD, rpsE, rpsF, rpsG, rpsH, rpsI, rpsJ, rpsK, rpsL, rpsM, rpsO, rpsP, rpsQ, rpsR, rpsS, rpsT, secA, secG, secY, serS, smpB, tig, tilS, truB, tsaD, tsf, uvrB, ybeY* and *ychF*. The numbers (maximum value is 92) at the nodes indicate the Gene Support Index (GSI). Bar, 0.05 substitutions per position.

NaCl (at 0.5% intervals). Cell growth was confirmed using a DU 700 UV-visible spectrophotometer (Beckman Coulter, CA, USA). Oxidase activity was determined based on purple colour formation using an oxidase reagent (bioMérieux, #55635), and catalase activity was verified based on bubble formation using a catalase reagent (bioMérieux, #55561). Cell motility was confirmed by using a semi-solid agar (RCM with 0.3% (w/v) agar). For the KOH test, the formation of viscous and mucoid strings was observed after 15 s [22]. To verify spore formation, Schaeffer–Fulton method with malachite green was used [23]. The biochemical properties of strain AGMB01083^T were analysed using the API 20A and Rapid ID 32A strips (bioMérieux, Marcy-l'Étoile, France). End products of fermentation were determined in cell-free supernatants of cells cultured for 2 d in RCM broth (MB cell) using high-performance liquid chromatography (Shimadzu, Kyoto, Japan) equipped with Aminex Organic Acid Columns (Bio-rad, CA, USA).

The physiological and chemotaxonomic properties of strain AGMB01083^T were compared with those of the closely-related reference *A. mobilis* HY-37-4^T. The cells of strain AGMB01083^T were obligately anaerobic, Gram-stain-positive, spore-forming (Fig. S1, available in the online version of this article), and motile. They were long straight or curved rods (1.6–3.0×0.3–0.5 µm in size), and formed filamentous (Fig. S1), translucent white colonies with curled margins. Growth occurred in the temperature range of 20–40 °C (optimum of 35 °C) on RCM agar, at pH 7–9 (optimum pH 7) in RCM broth, and in the presence of 0.5–1.0% (w/v) NaCl. (Table 1). As a result of the KOH test, the cells showed no viscous and mucoid strings that support the Gram-positive nature of the cell wall. Strain AGMB01083^T utilized amino acids, such as L-arginine and glutamic acid, and polysaccharides, such as sucrose, maltose, D-xylose, and L-arabinose. No catalase or oxidase activity was detected in strain AGMB01083^T. As to the fermentation end products, the two strains analysed in the current study also contained succinate and ethanol. Further, strain AGMB01083^T produced propionate, while the strain *A. mobilis* HY-37-4^T did not. As shown in Table 1, strain AGMB01083^T produced succinate as the main fatty acids supporting the close phylogenetic relationship of these two organisms.

Cellular fatty acid profiles were determined for AGMB01083^T cells and reference species grown on RCM agar for 24 h. Cellular fatty acids were saponified, methylated, and extracted according to the instructions of Chemical Analysis System (MIDI, DE, USA). The fatty acids were analysed using gas chromatography (GC-2010, Shimadzu) and the Sherlock Chromatographic Analysis System software package with the Anaerobe Database version 6.4. Isoprenoid quinones were extracted and analysed according to methods of Shin *et al.* [24]. The polar lipids were determined by two-dimensional thin-layer chromatography (TLC) using the procedures described by *Komagata* and *Suzuki* [25], and the polar lipid profiles were identified by spraying with 50% H_2SO_4, ninhydrin, molybdenum blue spray reagent and α-naphthol reagents. The major cellular fatty acids (>10%) of strain AGMB01083^T were $C_{16:0}$ (21.3%), $C_{16:0}$ DMA (25.9%), and $C_{16:0}$ 3-OH (24.0%). The strain *A. mobilis* HY-37-4^T analysed in the current study contained $C_{16:0}$ (25.6%), $C_{16:0}$ DMA (22.5%), and $C_{16:0}$ 3-OH (18.4%) as the main fatty acids. The detailed cellular fatty acid profiles of strain AGMB01083^T and the reference strain are shown in Table 2. No respiratory

Table 2. Cellular fatty acid profiles (% of total) of strain AGMB01083^T and type strain of a closely-related species from the genus *Anaerosporobacter*

Strains: 1, *Anaerosporobacter faecicola* AGMB01083^T; 2, *Anaerosporobacter mobilis* KCTC 5027^T. All data were obtained in the current study. Values are percentages of total cellular fatty acids. TR, trace amount (<1%); –, not detected. Only fatty acids accounting for more than 1% of the total in at least one strain are shown. The major components (>10%) are highlighted in bold.

Fatty acid	1	2
$C_{10:0}$	TR	TR
$C_{11:0}$ DMA	TR	TR
$C_{12:0}$	TR	TR
$C_{14:0}$	1.7	TR
$C_{14:0}$ DMA	1.3	TR
$C_{14:1}$ *cis* 9	TR	TR
anteiso-$C_{15:0}$	TR	TR
$C_{16:0}$	**21.3**	**25.6**
$C_{16:0}$ DMA	**25.9**	**22.5**
$C_{16:0}$ ALDE	9.1	8.3
$C_{16:0}$ -3OH	**24.0**	**18.4**
$C_{16:1}$ *cis* 7	TR	TR
$C_{16:1}$ *cis* 9	1.5	1.7
$C_{16:1}$ *cis* 9 DMA	1.9	2.0
$C_{17:0}$ DMA	TR	TR
$C_{17:0}$ -3OH	TR	TR
$C_{18:0}$	TR	TR
$C_{18:0}$ DMA	TR	TR
$C_{18:0}$ ALDE	TR	TR
$C_{18:1}$ DMA	TR	TR
$C_{18:1}$ *cis* 9	TR	1.8
$C_{18:1}$ *cis* 9 DMA	TR	1.2
$C_{18:1}$ *cis* 11 DMA	TR	3.1
$C_{19:0}$ *cyc* 11, 12 DMA	1.3	TR
Summed features*		
1	1.2	TR
4	1.0	1.2
5	3.0	TR
6	TR	1.1
7	TR	TR
8	TR	1.3
10	1.8	4.1

*Summed features: 1, $C_{13:1}$*cis* 12 and/or $C_{14:0}$ ALDE; 4, $C_{15:2}$; 5, $C_{15:0}$ DMA and/or $C_{14:0}$–3OH; 6, *anteiso*-$C_{15:0}$–3-OH and/or $C_{16:1}$*cis* 7 DMA; 7, $C_{17:2}$ and/or $C_{17:1}$*cis* 8; 8, $C_{17:1}$*cis* 9 and/or $C_{17:2}$; 10, $C_{18:1}$*cis*11/*trans*9/*trans*6; 11, iso-$C_{17:0}$ 3-OH and/or $C_{18:2}$ DMA.

quinones were detected in strain AGMB01083^T and the reference strain. The polar lipid of strain AGMB01083^T were one unidentified aminophosphoglycolipid, two unidentified aminophospholipid, five unidentified aminoglycolipid, six unidentified lipid, four unidentified phospholipid, one unidentified aminolipid and one unidentified glycolipid (Fig. S2).

The phylogenetic tree analysis based on the 16S rRNA gene sequence revealed that strain AGMB01083^T clusters with *Anaerosporobacter mobilis* species but forms a separate lineage. This indicates that the novel strain belongs to the genus *Anaerosporobacter*, in the family *Lachnospiraceae* in the class *Clostridia*. However, the genotypic, phenotypic, and chemotaxonomic characterization presented in the current study highlighted its differences from the closely-related *A. mobilis* strain. Taken together, strain AGMB01083^T represents a novel species in the genus *Anaerosporobacter*, for which the name *Anaerosporobacter faecicola* sp. nov. is proposed.

DESCRIPTION OF *ANAEROSPOROBACTER FAECICOLA* SP. NOV.

Anaerosporobacter faecicola sp. nov. (fae.ci'co.la. L. n. *faex, -cis*, yeast, faeces; L. suff. *-cola*, inhabitant, dweller; N.L. masc. n. *faecicola*, inhabiting faeces).

Cells are obligately anaerobic, Gram-stain–positive, and long straight or short rod-shaped (1.6–3.0×0.3–0.5 µm in size). Colonies cultured for 24–48 h on RCM agar are small, circular, with curled margin, and translucent white. The cells are mobile and filamentous. Growth occurs at 20–40 °C (optimum at 35 °C) and pH 7–9 (optimum at pH 7). Based on API 20A analysis, the cells are negative for the hydrolysis of urea and indole formation, and positive for the hydrolysis of aesculin ferric citrate. The cells utilize lactose, sucrose, maltose, salicin, D-xylose, L-arabinose, glycerol, cellobiose, D-mannose, raffinose, D-rhamnose, and trehalose. The cells do not utilize D-mannitol, melezitose, or D-sorbitol. Based on API rapid ID 32A analysis, the cells are negative for urease, β-galactosidase-6-phosphate, β-glucuronidase, *N*-acetyl-β-glucosaminidase, nitrate reduction, indole formation, proline arylamidase, phenylalanine arylamidase, and pyroglutamic acid arylamidase; the cells are positive for arginine dihydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, α-arabinosidase, acetyl-β-glucosaminidase, the fermentation of mannose and raffinose, glutamic acid decarboxylase, α-fucosidase, arginine arylamidase, leucyl glycine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, and serine arylamidase. The cells produce succinate, propionate, and ethanol as fermentation end products. The major cellular fatty acids (>10%) are $C_{16:0}$, $C_{16:0}$ DMA, and $C_{16:0}$ 3-OH. No respiratory quinones were detected. A member of the family *Lachnospiraceae*. The genomic DNA G+C content is 36.2 mol%, calculated based on the whole-genome sequence.

The type strain, AGMB01083^T (=KCTC 15857^T=NBRC 114517^T), was isolated from the faeces of Korean cow. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AGMB01083^T is MT197381. The GenBank/EMBL/DDBJ accession number for the whole-genome sequence of strain AGMB01083^T is JAAQRO000000000.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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