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Isolation and characterization of *Methanosphaera* sp. ISO3-F5, a member of a novel and widespread species of rumen methanogens growing with methanol plus hydrogen

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ABSTRACT

Rumen methanogens predominantly fall into two physiological groups: hydrogenotrophs which use hydrogen (H_2) to reduce carbon dioxide (CO_2) to methane (CH_4), and methylotrophs which use H_2 to reduce methanol and methylamines as substrates for methanogenesis. We used a dilution to extinction approach to isolate two hydrogenotrophic *Methanocatella* spp. and four cultures of methylotrophic methanogens from sheep rumen contents. Three of the methylotrophs were stable mixed cultures containing methanogens belonging to different lineages of the order *Methanomassiliicoccales* and one was a pure *Methanosphaera* culture. *Methanosphaera* sp. ISO3-F5 has a comparatively large genome (2.68 Mb) comprised of two replicons, a chromosome and a mega-plasmid. The genome has an average G + C content of 30.5 % and encodes 2360 putative protein-coding genes. Cells of ISO3-F5 have a spherical shape, 0.6–1.2 μm in diameter, usually occurring in pairs or loose clumps, and have no flagellum. Cells stain Gram positive, have a single thick cell wall and divide by the formation of a cross wall. The optimum temperature for growth was 39°C to 42°C and the optimum pH was 6.7–6.8. Acetate was required for growth, but CH_4 was not produced from acetate, formate, ethanol, methylamine, or isopropanol with or without H_2/CO_2. Volatile fatty acids and rumen fluid were also found to enhance the growth of ISO3-F5, while coenzyme M did not. ISO3-F5 produced CH_4 from methanol in the presence of H_2 and the genes encoding the necessary methanogenesis pathway have been identified. Based on morphological, physiological, and genomic characteristics, ISO3-F5 is a new species of the genus *Methanosphaera*. Our study shows that simple isolation methods allowed us to culture diverse and significant members of the rumen methanogen community.

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1. Introduction

Ruminants depend on the diverse microbial community in their forestomach (rumen) to ferment ingested plant polysaccharides, releasing short-chain volatile fatty acids (VFAs; acetate, propionate and butyrate) that are used by the animal for maintenance and growth. Other fermentation end-products, including H_2, methanol, and methylamines, are not used by the host animal, but act as energy sources for rumen hydrogenotrophic and methylotrophic methanogens, leading to atmospheric CH_4 emissions (Mizrahi et al., 2021). Hydrogenotrophs have the ability to use H_2 to reduce CO_2 to CH_4, while rumen methylotrophic methanogens use simple methyl-containing compounds like methanol and methylamines and reduce these to CH_4 with H_2. CH_4 produced from enteric fermentation in ruminant livestock is a significant source of agricultural greenhouse gases, and the rumen and ruminant methanogens are seen as the main targets for interventions (Khairunisa et al., 2023). As a result, development of technologies that can practically mitigate CH_4 emissions has become a focus of international research (Beauchemin et al., 2020; Mizrahi et al., 2021; Reisinger et al., 2021).

The most common methanogens in the rumen, and other gut environments (Protasov et al., 2023), are members of the family *Methanobacteriaceae*, which includes both hydrogenotrophs (*Methanobrevibacter*, *Methanocatella*, and *Methanarmilla*) and methylotrophs (*Methanosphaera*), and members of the order *Methanomassiliicoccales*, which are methylotrophs able to use a range of simple methyl-containing compounds (Seedorf et al., 2014; Seedorf et al., 2015; Poehlein et al., 2018; Thomas et al., 2022). Members of the *Methanobacteriaceae* generally dominate the methanogen population found in the rumen (Seedorf et al., 2015; Poehlein et al., 2018). Hydrogenotrophic *Methanobrevibacter* and *Methanocatella* spp. often make up > 75 % of the population in sequence-based studies, with methylotrophic *Methanosphaera* contributing > 10 % of the population (Seedorf et al., 2015). While many culture-independent studies (Henderson et al., 2015; Seedorf et al., 2015; Patra et al., 2017) have enumerated rumen methanogen populations, most of our knowledge of these organisms has come from investigation of a very limited number of cultured isolates (Leahy et al., 2010; Poehlein et al., 2018; Seshadri et al., 2018).

Members of the genus *Methanosphaera* are found in many gut environments, and in a study of the gut microbiome of 250 animal species (Thomas et al., 2022) three *Methanosphaera* clades (related to *M. stadtmanae*, *M. cuniculi* and ISO3-F5) were associated with different mammal taxa. Despite their prevalence, the genus *Methanosphaera* remains poorly characterized (Hoedt et al., 2018). The two formally described species (*M. stadtmanae* from humans and *M. cuniculi* from rabbits) are methylotrophs that only use methanol and H_2 as substrates for methane formation (Miller and Wolin, 1985; Biavati et al., 1988; Fricke et al., 2006; Thauer et al., 2008), and other human and rumen isolates (PA5, BMS) show the same metabolism (Hoedt et al., 2018). However, a *Methanosphaera* strain WGK6 isolated from the foregut of a Western Grey Kangaroo was also able to use ethanol as a source of reducing power for methanogenesis and growth. Recently, a new *Methanosphaera* species isolated from a human fecal sample has been proposed (Pilliol et al., 2024), *Candidatus* Methanosphaera massiliense sp. nov., that is proposed to be of mammalian zoonotic origin and resembles *Methanosphaera* sp. WGK6. The ability to use ethanol was linked to the presence of genes for alcohol and aldehyde dehydrogenases (Hoedt et al., 2016), and homologues of these genes have also been found in some rumen *Methanarmilla* cultures (*M. boviskoreani* and *M. wolinii*) and in uncultured *Methanosphaera* detected in MAGs assembled from rumen metagenome data (Hoedt et al., 2016; Kelly et al., 2019; Li et al., 2023).

In the rumen, methanol is derived from methoxy groups released from dietary pectins by the action of pectin methylesterases (Kelly et al., 2019; Carbone et al., 2023) and its availability is therefore influenced by diet and other members of the rumen microbiota (Henderson et al., 2015; Palevich et al., 2019). *Methanosphaera* strains show lower H_2 thresholds than species of *Methanobacteriaceae* that grow with H_2 plus CO_2 (Kim, 2012; Feldewert et al., 2020), but the dominance of the latter physiological group suggests that the population balance is determined by the availability of methyl compounds rather than the dissolved H_2 concentration. Studies in both cattle (Manafiazar et al., 2021; Smith et al., 2022) and sheep (Kittelmann et al., 2014; Shi et al., 2014) have demonstrated a correlation between elevated *Methanosphaera* relative abundance and reduced methane output. It appears that when the fermentation on a specific diet shifts to produce less H_2, it is the hydrogenotrophs that are most affected, and the methylotrophs therefore increase as a proportion of the methanogen population.

Here we describe the isolation of several methanogen cultures from the rumen of a single sheep and focus on the metabolic and genomic characterization of a rumen *Methanosphaera* isolate (ISO3-F5), which is the first cultured representative of a novel *Methanosphaera* species.

2. Materials and methods

2.1. Sample collection and processing

All rumen samples used for cell counts and isolation experiments were collected from a ruminally-fistulated wether sheep (Romney cross, 9 years old, 80 kg, animal identifier 5472) grazing on a perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) mixed pasture. The use of this sheep for sample collection was approved by the AgResearch Grasslands Animal Ethics Committee and complied with the Code of Ethical Conduct for the Use of Animals in Research, Testing and Teaching, as prescribed in the Animal Welfare Act of 1999 and its amendments. Samples were collected 2 h after the animal was taken from the pasture. A glass container flushed with CO_2 was used to collect the sample (500 g) which was immediately brought to the laboratory for further processing.

2.2. Direct microscopic cell counts

Five milliliters of rumen sample were added to 15 mL of 4 % paraformaldehyde (PFA), mixed, then incubated for 3–4 h at 4°C in the dark. To count cells with F_{420} fluorescence, these fixed rumen samples were serially diluted in 2-fold steps in phosphate-buffered saline (PBS). PBS contained, per liter, 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4, adjusted to pH 7.4, and autoclaved for 15 min at 121°C. Ten microliter aliquots of the diluted samples were added to 10 wells each of a Teflon-coated microscope slide (Erie Scientific Company, Ramsey, Minnesota, USA). The slides were dried at 39°C for approximately 30 min. A small volume of Vectashield anti-fading solution (Vector Laboratories Inc., Burlingame, California, USA) was placed on each well, and a cover slip (24 × 40 mm) was placed on top of the slide. The surplus anti-fade was removed with a tissue. The edges of the coverslip were sealed with nail varnish, and the varnish was allowed to dry before microscopy under UV illumination using a DM2500 microscope (Leica Microsystems, Wetzlar, Germany) and Filter System D (355–425 nm band pass-excitation filter, 455 nm dichromatic mirror, 470 nm long pass-suppression filter). Dilutions that yielded approximately 30–100 cells per field were used, and 30 fields were counted per well. The field of view that was counted was calculated to have an area of 0.0363 mm^2, so that each field represented approximately 1/779 of the 10 μL sample that was distributed over the well (i.e., over 28.274 mm^2).

To count total cells, PFA-fixed rumen samples were serially diluted in 2-fold steps in PBS that contained 5 μg of 4',6-diamidino-2-phenylindole dihydrochloride per milliliter (DAPI; Fluka Chemie, Buche, Switzerland) and 1 mg of acridine orange (Calbiochem, Burlington, Massachusetts, USA). After two hours the samples were washed three times with PBS, each time harvesting the cells by centrifugation at 8000 × *g* for 5 min and then resuspending in PBS. These preparations were then aliquoted

onto slides and counted as described above, using UV illumination with Filter System A (340–380 nm band pass-excitation filter, 400 nm dichromatic mirror, 425 nm long pass-suppression filter).

2.3. Dilution to extinction to isolate methanogens

We used the dilution to extinction method (Button et al., 1993; Kenters et al., 2011) to dilute rumen contents in growth media so that individual culture tubes were inoculated with small numbers of viable methanogen cells. Several experiments were set up in a similar manner, using slightly different dilution steps, replicate cultures at each dilution step, media, and medium additives (Tables S2 to S7). Forty grams of sheep rumen contents were blended with 360 mL of RM02 medium (Kenters et al., 2011) or BY+ (see below) under anaerobic conditions using a Waring blender (Waring Products Inc., Torrington, CT, USA) with a glass chamber and tight-fitting rubber lid. Two different blending procedures were used, designated as short (HI setting for 20 s) or long (HI setting for four 20 s bursts with 60 s between bursts). Five millilitres of blended rumen samples were transferred from the blender, using a 16-gauge hypodermic needle and a sterile plastic syringe, into serum vials containing the same growth medium under a gas phase of 100 % CO_2. Further serial dilutions were then made, using sterile syringes and needles, to serum vials or Hungate tubes (Bellco Glass, Vineland, New Jersey, USA) containing the same medium, to produce suspensions with different amounts of rumen contents. Culture vessels were closed with butyl rubber stoppers. These dilutions were used to inoculate Hungate tubes containing the same medium supplemented with substrates (20 mM sodium acetate, 60 mM sodium formate and 20 mM methanol). The cultures to be incubated were pressurized with H_2:CO_2 (80:20 vol/vol) to 140 kPa over the 101 kPa CO_2 already in the vessels, and incubated at 39°C in the dark with shaking (200 rpm). All processing and transfers were carried out under a stream of O_2-free CO_2 gas.

BY^+ medium was prepared by mixing solution A (17 % [v/v]), solution B (17 % [v/v]), rumen fluid (30 % [v/v]), $NaHCO_3$ (5 g/L [w/v]), yeast extract (2 g/L [w/v]), 5 drops of resazurin (0.1 % [w/v]), L-cysteine-HCl (0.5 g/L [w/v]) and selenite/tungstate solution (Tschech and Pfennig, 1984) (0.1 % [v/v]). All components except L-cysteine-HCl were mixed thoroughly, boiled under O_2-free 100 % CO_2, and cooled in an ice bath while being bubbled with 100 % CO_2. Once the solution had cooled, the L-cysteine-HCl was added, and the media dispensed into Hungate tubes under 100 % CO_2. These were sterilized by autoclaving for 20 min at 121°C and stored in the dark for at least 24 h before use. Salt solution A was prepared by mixing NaCl (6 g/L [w/v]), KH_2PO_4 (3 g/L [w/v]), $(NH_4)_2SO_4$ (1.5 g/L [w/v]), $CaCl_2{\cdot}2\,H_2O$ (0.79 g/L [w/v]) and $MgSO_4{\cdot}7\,H_2O$ (1.2 g/L [w/v]) in distilled water. Salt solution B was prepared by dissolving $K_2HPO_4{\cdot}3\,H_2O$ (7.86 g/L [w/v]) in distilled water.

The presence of methanogens in the enrichment cultures was determined by gas chromatography. Headspace gas (0.3 mL) from the culture tube was taken using a sterile syringe and needle and injected into an Aerograph 660 (Varian Associates, Palo Alto, CA, USA) fitted with a Porapak Q 80/100 mesh column (Waters Corporation, Milford, MA, USA) and a thermal conductivity detector operated at 100°C. The column was operated at room temperature with N_2 as the carrier gas at 12 cm^3/min. Tubes from terminal methane-positive dilutions were subcultured into the same medium with the same substrates and the methane production was monitored. DNA was extracted from the successful subcultures and was used for archaeal 16S rRNA gene PCR to identify the methanogens present using the methanogen-targeted primers 915af and 1386r (as described below in section 2.7).

2.4. Isolation of Methanosphaera sp. ISO3-F5

Tubes from terminal methane-positive dilutions were sub-cultured into the same medium with the same substrates but supplemented with the antibiotics streptomycin (10 μg/mL), ampicillin (10 μg/mL) and vancomycin (86.7 μg/mL)). This process removed most of the bacteria from the enrichment cultures, which were then given repeated heat treatment (50°C for 30 min immediately after inoculation in each subculturing) to eliminate surviving bacteria. Finally, and as described by Jeyanathan (2010), a novel *Methanosphaera* isolate (ISO3-F5) was obtained in pure culture (Table S4). The purity of the *Methanosphaera* ISO3-F5 isolate was confirmed by observing the live cells under phase contrast and epifluorescence microscopy for cell morphology and F_{420} fluorescence. To further confirm its purity, the culture of ISO3-F5 was inoculated into RM02 medium with 0.5 mL of GenRFV solution (Kenters et al., 2011) to check for bacterial growth under both anaerobic and aerobic conditions. As an additional check DNA was extracted from the purified ISO3-F5 culture and PCR was performed using with bacterial universal 16S rRNA gene primers 27 f and 1492r (Skillman et al., 2004).

2.5. Electron microscopy

Electron microscopic examination was carried out at the Manawatū Microscopy and Imaging Centre (MMIC), Massey University, Palmerston North, New Zealand, using a Philips CM10 Transmission Electron Microscope (Philips, Eindhoven, The Netherlands) with SIS Morada high-resolution digital imaging (Olympus, Soft Imaging Solutions GmbH, Germany) operated at 60 kV. For negative staining, a drop of concentrated cells was placed on formvar-coated copper grids and allowed to settle for 2–6 min and blotted dry. The sample was then covered with a drop of 2 % uranyl acetate (in water) and left for 6–10 min. The grid was drained well and allowed to dry before observation. For transmission electron microscopy (TEM), ISO3-F5 cells were fixed with 3 % glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. Secondary fixing was performed with osmium tetroxide and 0.1 M phosphate buffer. This was followed by a buffer wash (0.1 M phosphate buffer), acetone series dehydration, and setting in 50/50 acetone/resin (Procure 812, ProSciTech, Qld, Australia). TEM sections were cut on a diamond knife and these ultra-thin sections were collected onto copper grids and double stained with saturated uranyl acetate in 50 % (v/v) ethanol for 4 min and lead citrate for 4 min. The stained sections were observed by electron microscopy at 60 kV.

2.6. Substrate utilization and growth experiments

ISO3-F5 was tested for its ability to grow and produce methane using combinations of the following substrates (20 mM final concentration unless stated): H_2/CO_2 (200 kPa; 80:20, v/v), sodium acetate, sodium formate, methanol, ethanol, isopropanol, and methylamine. These were added to Hungate tubes containing RM02 medium supplemented with yeast extract (1 g/L) and vitamin mixture (0.1 mL/10 mL medium) (Kenters et al., 2011). The experiment was continued for 3 serial transfers to eliminate the carry-over effects of the initial inocula. Growth was followed every day by measuring culture density at 600 nm by inserting the culture tubes directly into an Ultrospec 1100 pro UV/Vis spectrophotometer (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Substrate solutions were prepared anaerobically in serum vials sealed with a butyl rubber stopper and an aluminum cap (Thermo Fisher Scientific) and autoclaved for 20 min at 121°C. These solutions were stored at room temperature in the dark and were added to the media on the day of inoculation. All the experiments were conducted with three replicates.

Experiments for possible growth factor requirements including rumen fluid (RF), yeast extract (YE), 2-mercaptoethanesulfonic acid (CoM), vitamins (Vit) and volatile fatty acids (VFA) were performed using RM02 medium supplemented with the substrate mix of acetate (20 mM), methanol (20 mM) and H_2/CO_2 (80:20). The requirements for growth factors in the additions were determined by the single or multiple omissions of the components. The experiment continued for 3 serial transfers to remove the carry-over effect of the initial inocula. Growth was measured every day by spectrophotometer at 600 nm. The RF, YE

and VFA solutions were prepared anaerobically and sterilized by autoclaving. CoM solution was prepared anaerobically, and filter sterilized into N_2-filled sterile serum vials. VFA solution (100 mL) was prepared by mixing the following components in distilled water and adjusting the pH to 7.5 with NaOH: 17 mL of acetic acid (3 M), 6 mL of propionic acid (0.8 M), 4 mL of butyric acid (0.4 M), 1 mL of isobutyric acid (0.1 M), 1 mL of valeric acid (0.1 M), 1 mL of isovaleric acid (0.1 M) and 1 mL of D, L-α-methylbutyric acid (0.1 M). Above solutions were added to the media to obtain final concentrations of 5 % (v/v) RF, 1 g/L (w/v) YE, 10 μM 2-mercaptoethanesulfonic acid (CoM), 1 % (v/v) VFA and 1 % (v/v) Vit. All the experiments were conducted with three replicates.

The effect of NaCl on the growth of ISO3-F5 was determined using RM02 supplemented with NaCl ranging from 0.1 M to 0.5 M with 0.05 M increments. Acetate (20 mM), methanol (20 mM) and H_2/CO_2 (80:20) were included as substrates with the addition of yeast extract. The pH optimum for the growth of ISO3-F5 was determined using 2 different media adjusted to different pH values; RM02 medium and RM02 medium amended with a stock solution of 500 mM MOPS (3-[N-morpholino] propane sulfonic acid) designated as RM02-MOPS. For the RM02 medium, different pH values were obtained by using HCl (5 M) and NaOH (5 M) solutions. For the RM02-MOPS medium, 500 mM MOPS solution was prepared with different pH values using NaOH (5 M) and added to RM02 media at 1 mL/10 mL medium. Methanol (20 mM), acetate (20 mM), and H_2/CO_2 (80:20) were used as the substrates along with YE (1 g/L w/v) and vitamins (0.1 mL/10 mL medium). The pH measurement of the pH-adjusted media was taken at 37°C to minimize the temperature effect. Growth was measured every day by measuring culture density at 600 nm by spectrophotometer.

The effect of temperature on the growth of ISO3-F5 was determined in RM02 media with temperatures ranging from 30°C to 46°C. Growth was measured every day by measuring culture density at 600 nm by spectrophotometer. The resistance of ISO3-F5 cells to sodium dodecyl sulphate (SDS) (0.5 % and 2 % w/v), lysozyme (1 mg/mL and 10 mg/mL), and proteinase K (1 mg/mL and 10 mg/mL) were determined by adding these solutions to actively growing cultures and examining the cells after 2 h with a Leica DM2500 microscope under 100 × oil phase contrast objective.

The effects of antibiotics on the growth of ISO3-F5 were determined using the following antibiotics: ampicillin, kanamycin, penicillin, erythromycin, tetracycline, streptomycin, vancomycin, chloramphenicol, lincomycin and bacitracin. Susceptibility was determined by transferring 0.5 mL of an exponentially growing culture into 9.5 mL of fresh medium containing 5, 10, 25 and 50 mg/L of each antibiotic and incubating at 39°C with shaking in the dark. Duplicate cultures were incubated for 2 weeks, and the effects of the antibiotics were determined by comparing the growth rates with control cultures containing no antibiotics by monitoring OD_{600}. The Gram reaction of ISO3-F5 was determined by Hucker's modification of the Gram stain method and cells were observed using a DM2500 microscope under 100 × oil phase contrast objective.

2.7. Archaeal genomic DNA isolation and identification

DNA for genomic sequencing was extracted from a pure culture of *Methanosphaera* sp. ISO3-F5 as described by Palevich et al. (2024). Briefly, the Qiagen Genomic-tip kit for the 20/G size extraction (Qiagen, Hilden, Germany) including RNase treatment was used following the manufacturer's instructions. DNA quantity, quality and integrity were assessed on a Qubit Fluorometry (Invitrogen, Carlsbad, CA), Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific), using the High Sensitivity DNA LabChip Kit on the Bioanalyzer 2100 (Agilent Technologies) and agarose gel electrophoresis respectively. To confirm strain identity, the 16S rRNA gene was amplified from the purified high molecular weight genomic DNA using the AmpliTaq Gold 360 master mix (Applied Biosystems) with methanogen-targeted primers 915af (5'-AGGAATTGGCGGGGGAGCAC-3') (Watanabe et al., 2004) and 1386r (5'-GCGGTGTGTGCAAGGAGC-3') (Skillman et al., 2004). The PCR cycling conditions used were 93°C for 3 min, 30 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final cycle of 72°C for 2 min (Jeyanathan et al., 2011). The partial 16S rRNA marker gene sequence was sequenced using the same primers on an ABI 3730 DNA system (Applied Biosystems), with strain identity confirmed (GenBank accession no. KF697734) via alignment to the NCBI 16S rRNA database using BLAST v2.9.0 (Altschul et al., 1990).

2.8. Genome sequencing, assembly and annotation

Genome sequencing of *Methanosphaera* sp. ISO3-F5 (Palevich et al., 2024) was performed by the NovogeneAIT Genomics Singapore Pte. (Novogene) using the Sequel II (PacBio) platform. For PacBio, thirty micrograms of genomic DNA was fragmented using g-TUBEs (Covaris) and size-selected for fragment size of > 20 kb using the BluePippin size-selection system (Sage Science). The DNA fragments were end-repaired to construct multiplexed SMRTbell DNA template libraries with pulsed-field gel electrophoresis (PFGE) and a Bioanalyzer used to assess quality and fragment size estimation. The SMRTbell DNA CLR template prep kit v2.0 (PacBio) was used with standard protocols (30-h movie) for library preparation according to the manufacturer's protocol and sequenced using two single-molecule real-time (SMRT) cells on the Sequel II v8.0 system with continuous long read (CLR) mode. Data QC, demultiplexing, filtering and statistical analysis of the raw sequencing reads (also called the polymerase reads) generated from the PacBio platform were processed using the SMRTLink v8.0 software with the parameter minLength set to 50 and all other parameters kept at default. The postprocessing also removed the hairpin adapter sequences from the polymerase reads to produce subreads and subreads with a length of < 50 bp were filtered out.

Circular long read sequences from PacBio were assembled *de novo* using Flye v2.9.1 (Kolmogorov et al., 2019). The genome was deposited and initially annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v5.0 (Tatusova et al., 2016). CheckM v1.2.2 (Parks et al., 2015) was used to assess the quality of the genome assembly by confirming species designation, genome completeness and contamination. Further annotations were determined by the DOE Joint Genome Institute (JGI) genome annotation pipeline v.5.1.13 (Chen et al., 2020). The *Methanosphaera* sp. ISO3-F5 genome assembly was deposited in NCBI Assembly database, along with raw sequence data in SRA under BioProject accession number PRJNA937785 (Palevich et al., 2024).

2.9. Comparative genomics

2.9.1. Genome data sets

The data set supporting the conclusions of this article are available through the IMG portal (https://img.jgi.doe.gov/). We compared the genome of *Methanosphaera* sp. ISO3-F5 to 5 closely related genomes (IMG Ids: 2651869595 (*Methanosphaera* sp. BMS), 2595698213 (*Methanosphaera* sp. WGK6), 637000163 (*M. stadtmanae* MCB-3^T (DSM 3091)), 2754412591 ((*M. stadtmanae* PA5) and 2802429591 (*M. cuniculi* 1R-7^T (DSM 4103)). Functional analyses were performed within the Integrated Microbial Genomes Expert Review (IMG-ER) platform (Markowitz et al., 2009; Grigoriev et al., 2011) and the Blast2GO (Götz et al., 2008) suite. In addition, all publicly available genome sequences representing cultivated *Methanobacteriaceae* strains (n = 82) were recovered from NCBI (detailed in Supplementary Table S16).

2.9.2. Phylogenomics and taxonomy

Taxonomic and phylogenomic assignments were explored using three distinct whole-genome-based methods as previously described (Palevich et al., 2024). Initially, pairwise comparison of nucleotide-level similarity between the genomic coding regions to calculate Average Nucleotide Identity (ANI) (Varghese et al., 2015) and the select *Methanosphaera* genome sequences were compared using the Genome

Taxonomy Database Toolkit (GTDB-Tk) v2.3.0 (Chaumeil et al., 2019). Default parameters were used for each software tool. The *Methanosphaera* genome sequences were uploaded to the Type (Strain) Genome Server v387 (TYGS, https://tygs.dsmz.de) for pairwise digital DNA-DNA hybridization (dDDH) analysis to calculate digital DDH values (Meier-Kolthoff and Göker, 2019) and confidence intervals of the distance formula d_4 were calculated using the Genome BLAST Distance Phylogeny v3.01 (GBDP, https://ggdc.dsmz.de) under recommended settings (Meier-Kolthoff et al., 2013). Phylogenomic species-tree reconstruction was generated with one hundred pseudo-bootstrap replicates with clustering based on established thresholds for delineating species (DDH > 70 %) and subspecies (DDH > 79 %), respectively. A balanced minimum evolution tree was inferred based on the resulting distance matrix using FastME v2.1.4 with SPR postprocessing (Lefort et al., 2015) and visualized using Geneious Prime (v2023.0.1) software. The resulting phylogenetic tree was drawn to scale, with the branch lengths being in the same units as those of the functional distances used to infer the distribution tree under recommended settings. In addition, a phylogenomic species-tree reconstruction for the *Methanobacteriaceae* strains was generated using the same parameters (detailed in Supplementary Fig. S2 and Supplementary Table S16). Metagenome-assembled genomes (MAGs) were not included in this study and only genomes of cultured isolates have been analysed.

2.9.3. Genome alignment and clustering

The ISO3-F5 circular map or genome atlas diagram was generated using GenoVi v0.2.16 software (Cumsille et al., 2023). Default parameters were used for each software tool. Genome synteny alignments of the amino acid sequences for the *Methanosphaera* genomes were generated and compared using the PROmer pipeline of the MUMmer v3.0 software (Kurtz et al., 2004) with *Methanosphaera* sp. ISO3-F5 as a reference genome using standard parameters. The general statistics and levels of completeness for each genome assembly are detailed in Table 3. The synteny dot plot diagrams reveal regions of exact identity between the pair of genomes compared and thus are an indicator of DNA similarity or conservation between the two genomes. Whenever the two sequences agree, a coloured line or dot is plotted. Colour codes: Blue, forward sequence; red, reverse sequence. Principal component analysis (PCA) was performed using the "Genome Clustering" function on the IMG-ER analysis platform to compare the *Methanosphaera* genomes based on several genomic features including the genome size and the number of genes belonging to each Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) category profiles. Dimensionality reduction was performed using the PCA analysis method with the clustering conducted using HDBSCAN v0.8.33 (McInnes et al., 2017) with default parameters.

2.9.4. Gene family evolution

To determine losses and gains of gene homologs and investigate orthologous gene (OG) family groupings, the IMG annotated protein-coding gene sequences from each of the *Methanosphaera* genomes were concatenated and subjected to an OrthoFinder v2.5.2 analysis with default settings (Emms and Kelly, 2019). If two proteins within a genome met the designated cutoff, they were clustered into one OG family. OG families that included protein sequences from all genomes in the comparison were designated a core OG family and representative of the accessory genome. Subset or variable OG families consisted of genes shared between species groups or were found to be present among 2–5 of the genomes within the comparison. Unique OG families represent subsets of genes identified to be specific within individual genomes.

3. Results

3.1. Isolation of methanogens

The total count of DAPI-stained prokaryotic cells in fresh rumen contents collected from a rumen-fistulated sheep feeding on ryegrass-white clover pasture was 1.67×10^{10} cells per mL of contents (Supplementary Table S1). This value is within the range of cell counts from rumen contents of cattle and sheep (Warner, 1962; Leedle et al., 1982). We also counted cells that fluoresced blue under UV illumination, indicative of methanogens containing F_{420}, from the same sheep on four separate days at least one month apart. The counts were 1.98×10^8 to 3.53×10^8 cells per mL of contents. Based on a count of approximately 3×10^8 methanogens cells per mL of fresh rumen contents, we then estimated a range of dilutions for culturing by serial dilution that were likely to result in inocula that contained few to single viable methanogen cells (Supplementary Table S2). The dilutions extended below that required for single cells, which increased the likelihood that cultures that grew did so from a single viable methanogen.

Rumen contents from the same sheep were blended and used for the cell counts for two different lengths of time and serially diluted each of these into two media to obtain viable counts of methanogens (experiment 1, Supplementary Table S2). The tubes were incubated for 30 days, and positive tubes were identified by the appearance of methane in the headspace. The most-probable numbers of viable cells stabilised after 10 d (Supplementary Fig. S1A). We found that the viable cell counts were greater using BY^+ rather than RM02, and greater with the longer blending time. We repeated the experiment (experiment 2, Supplementary Table S3) using only the longer blending time, and again found that more tubes were positive for methane formation with BY^+ medium compared to RM02 medium (Supplementary Fig. S1B).

A series of four similar experiments were then set up to obtain stable methanogen cultures, again from the same sheep (experiments 3–6; Supplementary Tables S4 to S7, Supplementary Fig. S1B). Methane positive tubes were repeatedly subcultured into the same medium, and six stable cultures were obtained that continued to produce methane after repeated subculture: ISO3-F5, ISO4-G1, ISO4-G11, ISO4-G16, ISO4-H5, and ISO5-H6. We were able to obtain stable bacteria-free cultures of three of the six cultures (Table 1). These were a member of the genus *Methanosphaera* (ISO3-F5) and two of the genus *Methanocatella* (formerly part of the genus *Methanobrevibacter*). The other three cultures were stable but mixed, each containing two cell types: non-fluorescent cocci and curved rods. When cultured on BY^+ with glucose but no added hydrogen or methanol, only the curved rods grew, and their 16S rRNA gene sequences indicated they were *Succinivibrio* spp. that originated from the rumen inoculum. Amplification of 16S rRNA genes from the bicultures using primers targeting archaea resulted in products from members of the *Methanomassiliicoccales*. These represent possibly three different genera of this order (Seedorf et al., 2015). Our simple isolation method thus enabled us to isolate cultures of phylogenetically very different rumen methanogens.

3.2. Morphology of Methanosphaera sp. ISO3-F5

Phase contrast and epifluorescence microscopy confirmed the purity of isolate ISO3-F5. Cells stained Gram positive (Doetsch, 1981) and were spherical in shape, 0.6–1.2 μm in diameter (mean 0.82 ± 0.196 μm [n = 30]), usually occurring in pairs or loose clumps (Fig. 1A). They displayed the blue fluorescence typical of F_{420}-containing methanogens under UV illumination (Fig. 1B). Spores and motile cells were never observed by phase-contrast microscopy of living cultures. No flagella or surface structures were visible by EM examination of negatively stained cells (Fig. 1C). Electron microscopy of thin sections revealed that the cells divided by the formation of a cross wall (Fig. 1D). The cleavage furrows were clearly visible in dividing cells. The cell envelope appeared to consist of a thick single layer (17.4 ± 3.8 nm) and its surface was smooth. All the cells were surrounded by layer that was less electron-dense that the cells or surrounding embedding medium.

Table 1
Methanogen cultures obtained through serial dilution of rumen contents.

Culture	Identity	Cell morphology[a]	16S rRNA gene[b]	Genome assembly[b]	Reference[c]
ISO3-F5	*Methanosphaera* sp.	Fluorescent cocci[d]	KF697734	CP118753[e] CP118754[f]	This report
ISO3-H6[g]	*Methanocatella* sp.[h]	Fluorescent coccobacilli[d]	KF697730	NA[i]	Unpublished
ISO4-G1	Methanomassiliicoccales	Non-fluorescent cocci and curved rods	KF697737	CP013703	Kelly et al. (2016)
ISO4-G11	Methanomassiliicoccales	Non-fluorescent cocci and curved rods	KF697736	NA	Unpublished
ISO4-G16	*Methanocatella* sp.	Fluorescent coccobacilli[d]	KF697729	NA	Unpublished
ISO4-H5	Methanomassiliicoccales	Non-fluorescent cocci and curved rods	KF697735	CP014214	Li et al. (2016)

[a] Morphology of cells when grown in BY^+ with H_2/CO_2 (200 kPa), acetate (20 mM), formate (60 mM) and methanol (20 mM).
[b] GenBank accession numbers for methanogen 16S rRNA gene and/or genome assembly.
[c] References reporting the methanogen genome description.
[d] No growth was observed in BY^+ supplemented with glucose.
[e] GenBank accession number of the ISO3-F5 chromosome complete sequence (Palevich et al., 2024).
[f] GenBank accession number of the ISO3-F5 megaplasmid (pNP174) complete sequence (Palevich et al., 2024)
[g] Also designated D5 by Kim (2012).
[h] Formerly part of the genus *Methanobrevibacter* (Protasov et al., 2023).
[i] NA, not available.

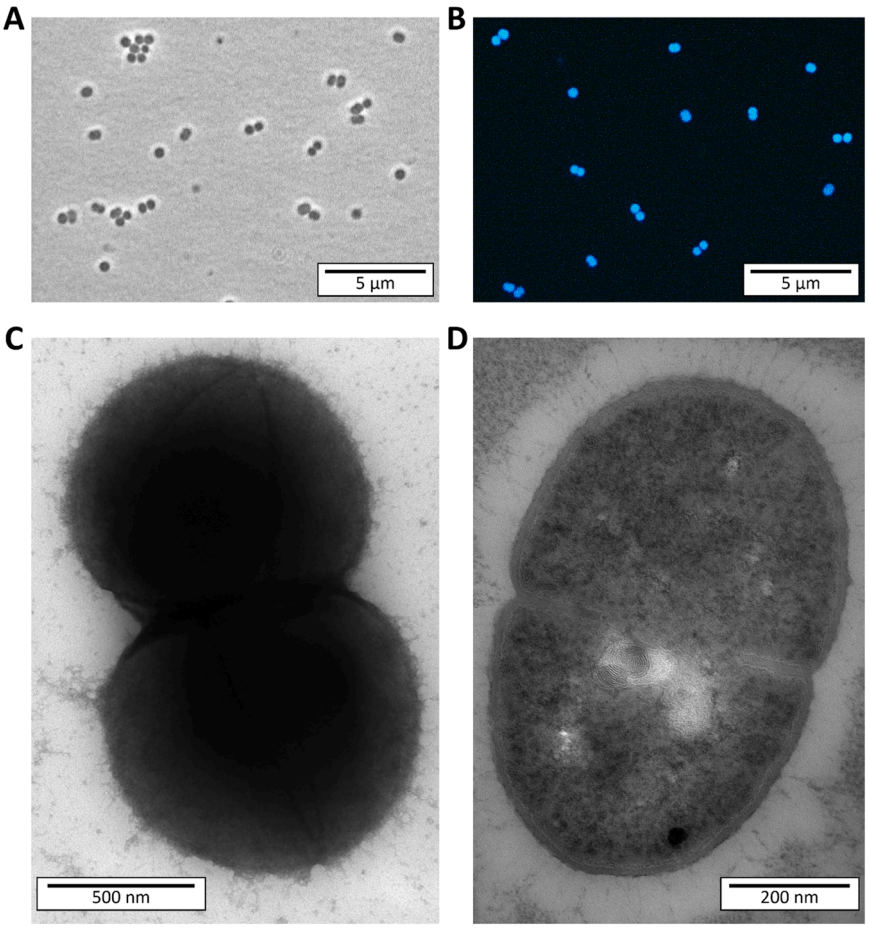

Fig. 1. Morphology of ISO3-F5. Photomicrographs of cells observed under phase contrast (**A**) and epifluorescence microscopy showing autofluorescence under UV illumination (**B**) Electron photomicrograph of negatively stained dividing cells of ISO3-F5 (**C**). Ultrastructure of ISO3-F5 shown in a thin section through dividing cells with the cleavage furrow and cross-wall formation clearly visible (**D**). Scale bars are included in each panel.

3.3. Substrate requirements, cell lysis and antibiotic sensitivity

Growth of ISO3-F5 only occurred when methanol, acetate, and either H_2/CO_2 or formate were supplied together (Fig. 2). No growth occurred with formate (20 mM), ethanol (20 mM), isopropanol (20 mM), or methylamine (20 mM) as the sole substrate or in different combinations with other potential substrates. ISO3-F5 required yeast extract for growth (Table 2). Rumen fluid or a volatile fatty acid mixture were not required, but they each increased the growth yield of ISO3-F5 (Figs. 3A and 3B). Growth was not affected by the inclusion or omission of co-enzyme M (Fig. 3C) or the vitamin mixture (Fig. 3D), but the requirement for yeast extract means that the vitamin requirements of ISO3-F5 could not be accurately determined. Cultures typically reached a peak optical density 7–9 days after inoculation, followed by a marked optical density decrease. ISO3-F5 grew over a temperature range of 34–44°C, with the greatest optical density at 39–42°C (Fig. 3E). No growth was

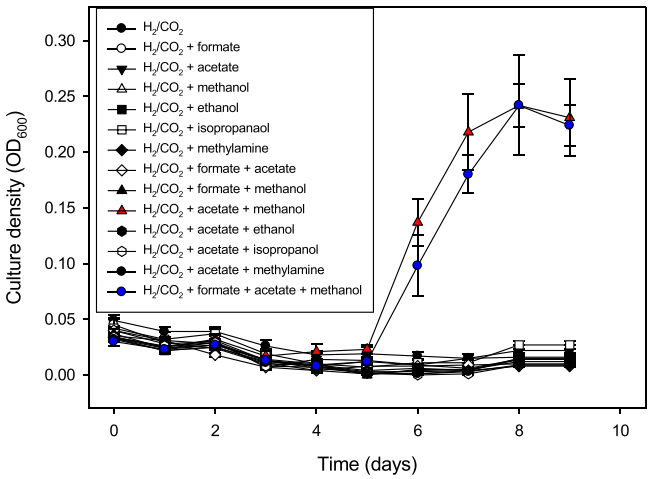

Fig. 2. Growth of ISO3-F5 with different substrates, measured as optical density (OD) at 600 nm. RM02 medium containing yeast extract (1 g/L) and vitamin mixture was used in this experiment. The concentration of each substrate was always 20 mM, except H_2:CO_2 (80:20), which was added at 200 kPa. Each point corresponds to the mean of 4 replicates, and the vertical bars represent one standard error on either side of the mean.

Table 2
Combinations of growth supplements tested for their effects on the growth of ISO3-F5. The results are from the third of three serial transfers to eliminate carryover of supplements from the inoculum.

Additions	Growth
RF + CoM + VFA + YE + Vit + Subs	+ + +
CoM + VFA + YE + Vit + Subs	+ + +
VFA + YE + Vit + Subs	+ + +
YE + Vit + Subs	+ +
Vit + Subs	−
CoM + VFA + Vit + Subs	−
RF + YE + Subs	+ + +
RF + VFA + Vit + Subs	−
RF + Vit + Subs	−
CoM + YE + Vit + Subs	+ +
YE + Subs	+ +

+ + + very good growth, + + good growth, + little growth, − no growth. The growth supplements tested were rumen fluid (RF), yeast extract (YE), coenzyme M (CoM), vitamin mixture (Vit) and volatile fatty acid mixture (VFA). Substrates (Subs) added were the same in all the combinations: acetate (20 mM), methanol (20 mM) and H_2:CO_2 (80:20; 200 kPa).

observed at 30°C and 46°C. The greatest optical density at 39°C was at pH 6.7–6.8 (Fig. 3F). ISO3-F5 tolerated up to 0.40 M NaCl. Cells were resistant to lysis by sodium dodecyl sulfate (SDS; 2 % w/v), lysozyme (1 mg/mL) and proteinase K (1 mg/mL). Antibiotic susceptibility tests showed that ISO3-F5 was resistant to the following antibiotics (50 mg/L): ampicillin, kanamycin, penicillin, erythromycin, tetracycline, streptomycin, and vancomycin. It was susceptible (5 mg/L) to chloramphenicol, lincomycin and bacitracin.

3.4. Genome characteristics

The genome of ISO3-F5 consisted of a 2,506,044-base pair (bp) chromosome (30.87 % G+C) and a 174,118 bp megaplasmid (29.02 % G+C), giving a genome size of 2,680,162 bp (Palevich et al., 2024). A total of 2,421 genes were predicted, of which 2,360 were protein-coding genes. The properties of the ISO3-F5 genome and comparison with the genome sequences of other *Methanosphaera* isolates are shown in Table 3. The size of the ISO3-F5 genome is similar to those of other *Methanosphaera* from ruminants (from isolates or assembled from metagenomic data), and these are generally larger than those from other animal hosts (Hoedt et al., 2018).

We used Genome BLAST Distance Phylogeny (GBDP) employing both 16S rRNA gene and whole-genome sequence-based pairwise methods and including genome-wide identity indexes using digital DNA: DNA hybridization (dDDH), along with average nucleotide identity (ANI) analyses to compare the phylogeny of cultivated *Methanobacteriaceae* strains. This analysis showed that all *Methanosphaera* strains clustered separately from other methanogen genera (Supplementary Fig. S2). Our comparisons of the ISO3-F5 genome with the most closely related *Methanosphaera* isolates (Table 1 and Fig. 5A) also support previous reports on the differentiation of *Methanosphaera* isolates (Hoedt et al., 2018). These analyses indicate that ISO3-F5 represents an uncharacterized clade or novel species within the genus *Methanosphaera*.

Despite differences in genome size of the *Methanosphaera* isolates, synteny analysis revealed segments that show a conserved gene order (Fig. 5B), but also demonstrate that a large section of the ISO3-F5 genome contains genes not found in other isolates (Supplementary Table S13), most of which can only annotated as encoding hypothetical proteins. Although the genomes of ISO3-F5 and BMS show regions of gene synteny, their 16S rRNA gene homology (96.9 %) indicates they belong to different species. Both cultures have large genomic regions encoding genes that have no matches in the NCBI database and are distinguished from the other cultured *Methanosphaera* species by their large genome size. An additional comparison was performed based on principal component analysis (PCA) analysis using KEGG orthology (KO) functional profiling (Fig. 5C). This revealed that ISO3-F5 and BMS grouped together, possibly due to functional similarity determined by their rumen origin, while the human gut isolates (*M. stadtmanae* MCB-3^T and PA5) also grouped together. *Methanosphaera* sp. WGK6 and *M. cuniculi* 1R-7^T had significantly different profiles and separated from the other isolates. To evaluate gene ancestry and evolution among *Methanosphaera*, OrthoFinder was used to identify orthologous gene or protein families and their corresponding orthogroup (OG) affiliations (Supplementary Table S14). A total of 2,551 OGs were found, of which 1,111 represented the gene families shared among all *Methanosphaera* genomes (Fig. 5D), representing the core genome set of genes encoding housekeeping, methanogenesis pathways and transport functions (Supplementary Table S15).

4. Discussion

Methanogenic archaea capable of utilizing a variety of methyl-containing compounds are major contributors to CH_4 production in many anaerobic environments (Thomas et al., 2022). Two groups of obligate hydrogen-dependent methylotrophic methanogens occur commonly in the rumen. Members of the genus *Methanosphaera* convert methanol to CH_4, while members of the order *Methanomassiliicoccales* are more versatile and can use methanol, methylamines and possibly other methyl-containing compounds as methanogenic substrates (Söllinger and Urich, 2019; Mesquita et al., 2023). Their similar metabolism suggests that these two groups would compete for methanol but metatranscriptomic studies have shown that the majority of transcripts from the methanol-utilizing pathway derive from *Methanosphaera* (Söllinger et al., 2018; Pitta et al., 2022). Since methanol availability is likely dependent on bacteria that express pectin methylesterases able to act on methoxylated pectins (Kelly et al., 2019; Carbone et al., 2023), it will be important to investigate how this cross-feeding relationship is established. While the importance of methylotrophic methanogens in the rumen has been established via culture-independent studies, our understanding of their physiology, biochemistry, and interaction with other members of the rumen microbiota has been limited by a lack of cultured representatives. Here, we characterise the *Methanosphaera* isolate, ISO3-F5, and report its genome sequence.

Overall, the total counts methanogens recovered from fresh rumen

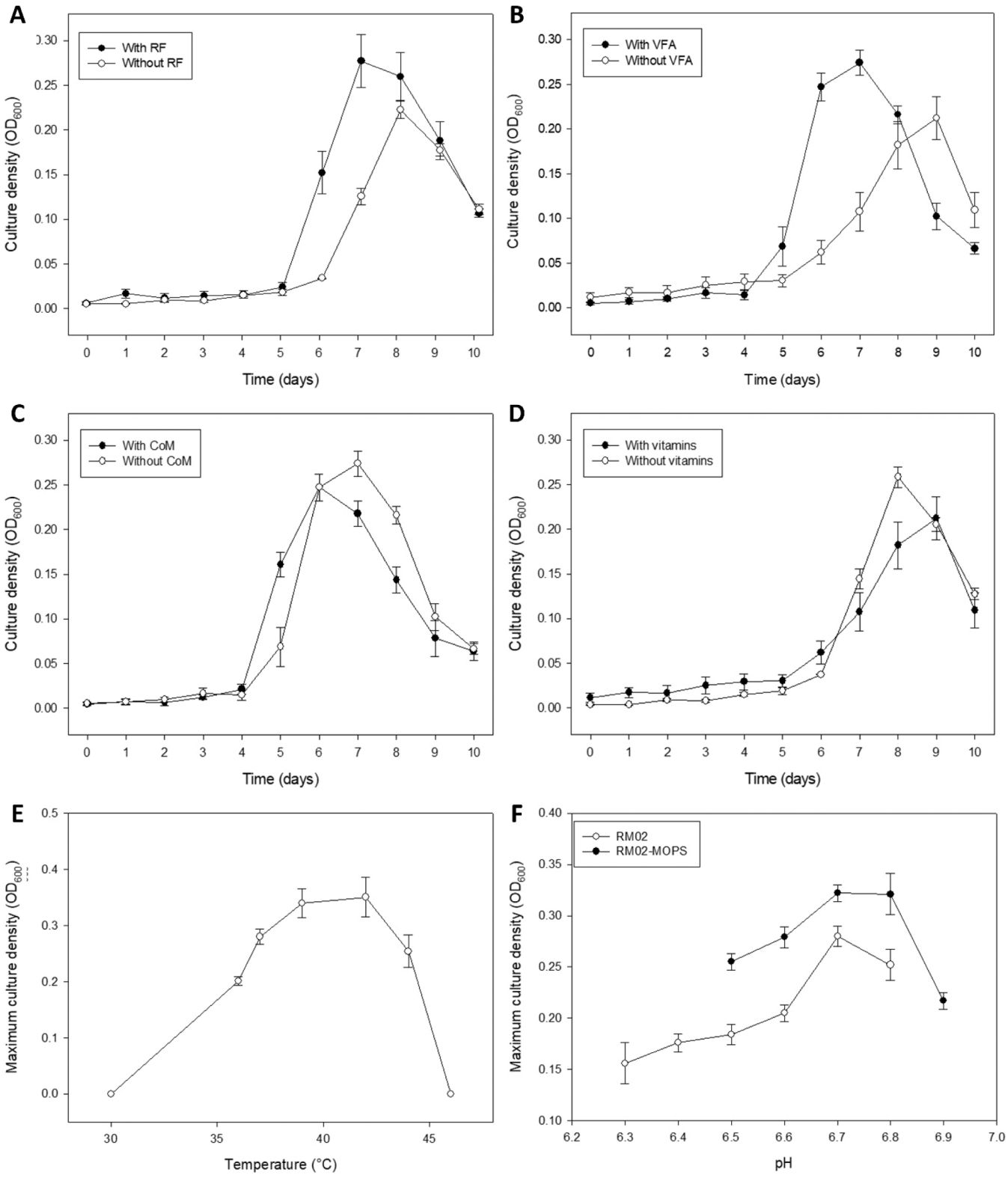

Fig. 3. Growth requirements of ISO3-F5 determined by measuring optical density (OD) at 600 nm. Growth curves of ISO3-F5 with and without 5 % (v/v) rumen fluid (**A**), 1 % (v/v) volatile fatty acid mixture (**B**), 10 μM coenzyme M (CoM, **C**) and 1 % (v/v) vitamin mixture (**D**). RM02 medium contained yeast extract (1 g/L w/v) and substrates (20 mM acetate, 20 mM methanol and 200 kPa H_2:CO_2, 80:20) was used in this experiment. Effect of incubation temperature (**E**) and pH (**F**, in two different media) on maximum culture density. Each point is the mean of 4 replicates and the vertical bars represent the standard error.

Table 3
Comparison of the genome features of ISO3-F5 and other cultured *Methanosphaera* spp.

Metadata						
Assigned taxonomy	*Methanosphaera* spp.			*M. stadtmanae*		*M. cuniculi*
Strain identifier	ISO3-F5	BMS	WGK6	PA5	MCB−3^T	1R−7^T
Isolation source	Ovine rumen	Bovine rumen	Kangaroo foregut	Human faeces		Rabbit rectum
Isolation country	New Zealand	Australia	Australia	Australia	Germany	Italy
Genome project information						
Status	Complete	Complete	Draft	Draft	Complete	Draft
GenBank ID	CP118753–4	CP014213	JRWK01	NGJK01	CP000102	LMVN01
BioSample ID	33419672	4445725	3108761	6928224	2604180	4229037
Assembly ID	34480035	3268005	1729965	3265405	12545	2287195
BioProject ID	937785	310024	263968	386222	15579	300716
GOLD ID	734154	119560	107741	242114	406	322644
Reference	Palevich et al., 2024	Hoedt et al., 2018	Hoedt et al., 2016	Hoedt et al., 2018	Fricke et al., 2006	Gilmore et al., 2017
Genome statistics (NCBI)						
Assembly size (bp)	2680,162	2868,093	1729,155	1701,400	1767,403	1928,201
Genome coverage (×)	327.0	60.0	68.0	285.0	10.0	102.0
G+C content (%)	30.5	33.0	27.5	27.5	27.5	28.0
Contigs	2	1	37	143	1	29
Contig N50 (bp) | L50	2506,044 | 2	2868,093 | 1	154,076 | 5	30,025 | 16	1767,403 | 1	138,173 | 6
CheckM scores (%)[a]	99.2 | 1.6	98.9 | 2.2	98.5 | 0.4	98.9 | 0.0	99.5 | 0.0	98.2 | 0.9
Genome annotations (IMG)						
Total genes | PCGs	2421 | 2360	2258 | 2204	1643 | 1592	1597 | 1554	1592 | 1536	1685 | 1647
No. of rRNA operons | tRNAs	3 | 40	2 | 40	3 | 43	1 | 38	4 | 42	1 | 36
PCGs with function prediction	1755	1500	1192	1177	1036	1135
PCGs connected to MetaCyc | KEGG	558 | 654	550 | 653	480 | 570	505 | 597	523 | 616	476 | 562
PCGs with enzymes | KO	619 | 1102	616 | 1116	535 | 917	555 | 933	580 | 980	523 | 878
PCGs with COG | Pfam	1741 | 1646	1282 | 1581	1067 | 1252	1058 | 1226	1062 | 1233	1037 | 1175
CRISPR arrays	1	4	1	2	3	1
Taxonomy						
NCBI taxon ID	1452353	1789762	1561964	339860		1077256
GTDB-Tk species taxon ID	*M. sp017431845*	*M. sp003268005*	*M. sp001729965*	*M. stadtmanae*	*M. stadtmanae*	*M. cuniculi*

Abbreviations: CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; PCG, protein coding sequences. [a]CheckM assessment of genome quality (% completeness | % contamination).

contents were in agreement with estimates made using different methods that methanogens make up 0.3–3 % of prokaryotes in rumen contents (Morvan et al., 1996; Janssen and Kirs, 2008). These counts were relatively constant, although are underestimates of total methanogen numbers because members of the *Methanomassiliicoccales*, part of the rumen methanogen community, do not display obvious fluorescence under UV illumination (Paul et al., 2012). Two of the three *Methanomassiliicoccales* isolated were eventually purified from their bacterial partner and their genomes have been described elsewhere (Kelly et al., 2016; Li et al., 2016). Studies of sheep in New Zealand estimated that members of *Methanomassiliicoccales* make up about 10 % of the rumen methanogen community (Seedorf et al., 2015), and this was also found in the same sheep we used in our studies (Henderson et al., 2013).

The genome of ISO3-F5 contains a full complement of genes enabling methanol reduction with H_2 to CH_4, like those found in *M. stadtmanae* MCB-3^T (Fricke et al., 2006; Thauer et al., 2008). The first step is formation of methyl-coenzyme M from methanol and coenzyme M catalyzed by three proteins: methanol:coenzyme M methyltransferase (mtaA), methanol—corrinoid protein Co-methyltransferase (mtaB), and methanol corrinoid protein (mtaC). Two additional genes encoding the subunits of a methyltransferase corrinoid protein reductive activase (mapAB) are also required. In the ISO3-F5 genome, these genes are clustered (mtaA-mtaB1-mtaC1-mtaB2-mtaC2-mapA-mapB), but there is also another set of mtaBC genes as well as four other mtaA genes. Genes encoding the final steps for CH_4 formation (methyl-coenzyme M reductase, heterodisulfide reductase, and non-F_{420}-reducing hydrogenase) as well as the genes required for coenzyme M biosynthesis are all present in the genome. In addition to the non-F_{420}-reducing hydrogenase, the ISO3-F5 genome also encodes a F_{420}-reducing hydrogenase (frh) and a membrane-bound energy converting hydrogenase complex (ehbA-T). Like *M. stadtmanae* MCB-3^T, ISO3-F5 lacks the genes for molybdopterin biosynthesis. Genes required for CH_4 production from methylamine or alcohols other than methanol were not identified, which agrees with the outcomes of the substrate use tests. The ISO3-F5 genome encodes an acetyl coenzyme A synthetase and genes for the reductive carboxylation of acetyl-CoA to pyruvate homologous to those described from *M. stadtmanae* MCB-3^T (Fricke et al., 2006), indicating a mechanism for acetate assimilation. We could not identify an acetate transporter (Supplementary Tables S8–12), but we found that acetate was required for growth.

ISO3-F5 contained a megaplasmid (pNP174), which is the first to be detected in *Methanosphaera* species (Fig. 4). Three genes on the megaplasmid (encoding small and large primase subunits and an MCM family replicative DNA helicase) may have specific roles in plasmid replication, but functional predictions for most of the genes present will require more investigation. The ISO3-F5 genome also contains an integrated 55 kb prophage, but functional predictions can only be made for an integrase, a terminase and a peptidoglycan hydrolase-like protein that may act as a lytic enzyme. It remains to be determined if the drops in optical density seen in stationary phase cultures (Fig. 3A and D) are related to phage activity. There is a single CRISPR array with genes for eight CRISPR-associated proteins and 57 spacer sequences. Similar to other rumen methanogens (Leahy et al., 2010), the ISO3-F5 genome encodes numerous adhesin-like proteins predicted to be anchored to the cell surface. The function of these proteins is not known but many are very large (Gupta and Seedorf, 2024), with the largest being 9,508 aa (28,527 bp). The larger genomes of rumen *Methanosphaera* sp. ISO3-F5 and BMS had the highest numbers of variable and unique gene families. Additional functional annotation of ISO3-F5, which had the largest accessory genome ($n = 297$), revealed that a significant proportion of genes were annotated as hypothetical proteins, proteins of unknown function or genes containing domains of unknown function, with the functional accessory genome of ISO3-F5 represented by 88 genes.

Only one rumen *Methanosphaera* culture has previously been reported (BMS) (Hoedt et al., 2018), and ISO3-F5 is the first cultured representative of a novel *Methanosphaera* species which microbial community analyses have shown to be one of the most abundant and prevalent methanogens found in the rumen. Analysis of 16S rRNA gene

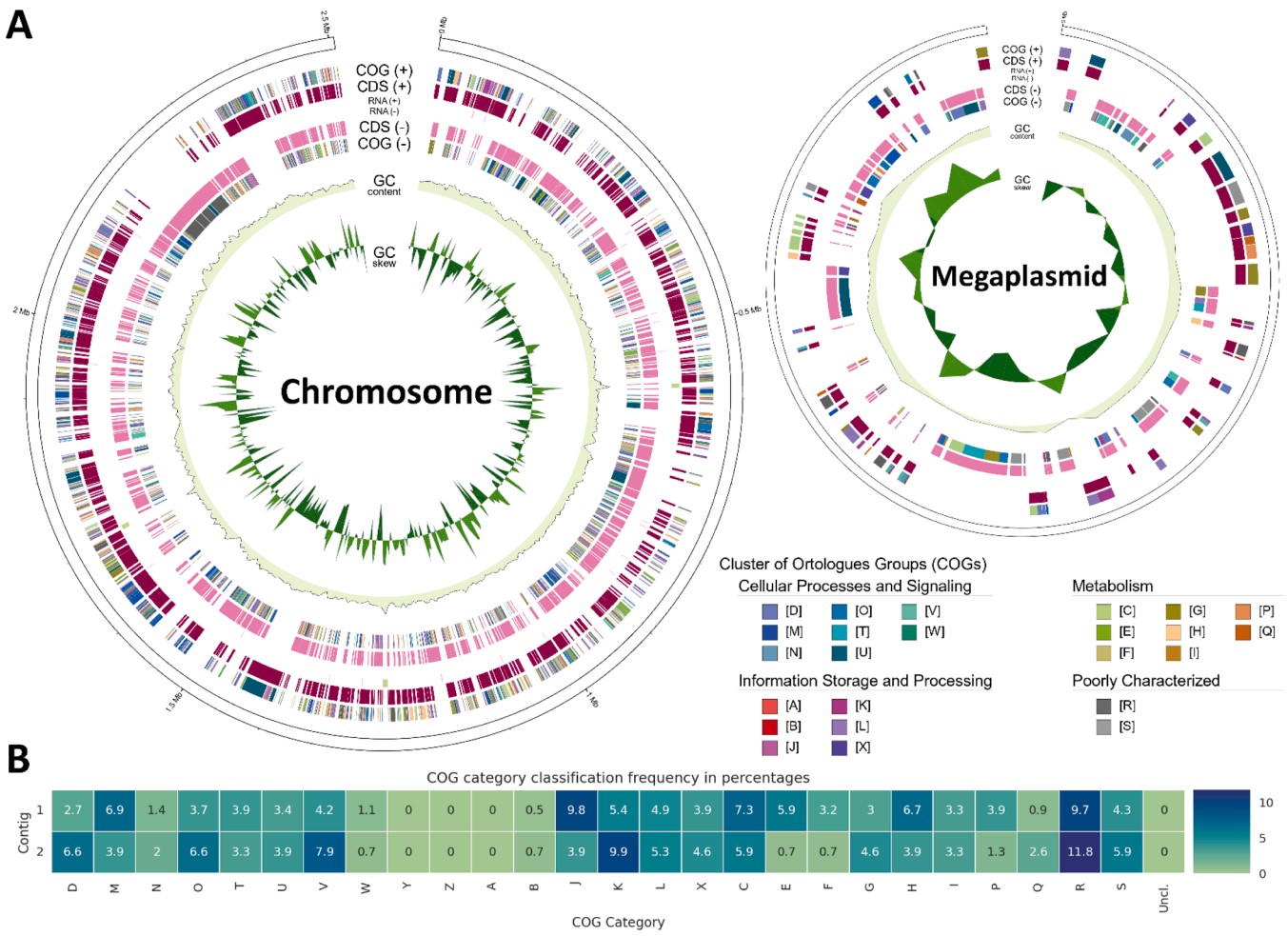

Fig. 4. Genome atlas and functional profile for ISO3-F5. Circular representations of the ISO3-F5 chromosome (left, contig 1) and megaplasmid (right, contig 2) are not represented to scale (**A**). Labeling of genomic features from outside to the inside: Contigs; COGs on the forward strand; CDS, tRNAs (pink), and rRNAs (green) on the forward strand; CDS, tRNAs (pink), and rRNAs (green) on the reverse strand; COGs on the reverse strand; GC content; GC skew. Heatmap representing the percentage frequency for classification of proteins into Clusters of Orthologous Groups of proteins (COGs) categories of the ISO3-F5 genome (**B**). Uncl., unclassified.

amplicons obtained from 252 sheep and cattle rumen samples detected close relatives of ISO3-F5 in 94.8 % of samples at a mean relative abundance of 8.2 % (Seedorf et al., 2015). A larger study with more diverse animal species ($n = 704$) reported ISO3-F5 relatives in 97.4 % of samples at a mean relative abundance of 5.8 % (Henderson et al., 2015). In both studies, a second *Methanosphaera* species (termed *Methanosphaera* group 5) was also recorded as both prevalent and abundant in rumen samples. There is no cultured representative from this group. The species that ISO3-F5 belongs to has also been associated with other regions of the ruminant digestive tract (Ban et al., 2022) and with the gut microbiota of other animal species (Thomas et al., 2022).

5. Conclusion

Methanogenic archaea are key members of the rumen microbiota, and their production of CH_4 has placed them at the centre of research aimed at reducing greenhouse gas emissions from ruminant livestock. While culture-independent studies have described the complement of methanogens in the rumen, our understanding of their physiology, biochemistry, and interaction with other members of the rumen microbiota has been limited by a lack of cultured representatives. This is especially the case for the methylotrophic methanogens, members of the genus *Methanosphaera*, which use methanol, and members of the order *Methanomassiliicoccales*, which can use both methanol and methylamines. *Methanosphaera* sp. ISO3-F5 is a representative of a novel *Methanosphaera* species that has been reported to be both abundant and prevalent in culture-independent studies of the rumen and detected in other mammalian gut environments. Our study shows that simple isolation methods allowed us to culture diverse and significant members of the rumen methanogen community.

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CRediT authorship contribution statement

Jeyamalar Jeyanathan: Writing – original draft, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Nikola Palevich**: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kerri Reilly**: Investigation. **Faith P. Palevich**: Investigation. **Paul H.**

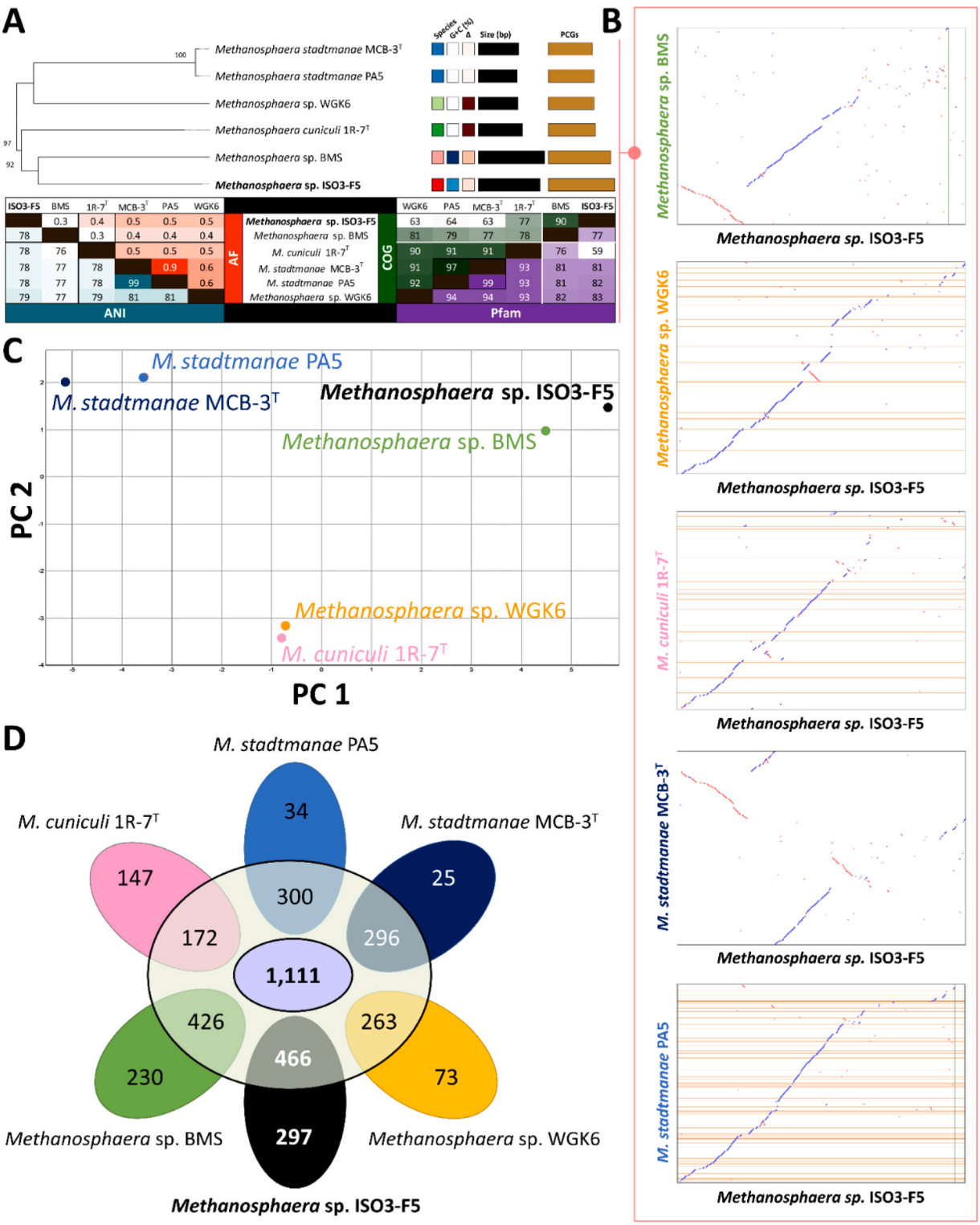

Fig. 5. Functional distributions and phylogenomic comparisons of *Methanosphaera* genomes. Phylogenomic differentiation of cultivated *Methanosphaera* sp. strains (upper, **A**). Consensus phylogenomic species-tree reconstruction with type species indicated with a superscripted T, *Methanosphaera* sp. ISO3-F5 in bold and average branch support (%) shown. Pairwise genome clustering comparison based on ANI, AF, Pfam and COG domains analyses of *Methanosphaera* genomes (lower, **A**). Genome synteny alignments with *Methanosphaera* sp. ISO3-F5 as the reference (**B**). Principal component analysis (PCA) plot of predicted genome wide KEGG orthology (KO) functional profiles of selected *Methanosphaera* isolates (**C**). Flower plot of accessory (or unique, outer petal), variable (or subset, inner petal), and core (center) orthogroup (OG) gene families in genomes of *Methanosphaera* spp. (**D**).

Maclean: Validation, Software, Investigation. **Dong Li**: Investigation. **Eric Altermann**: Investigation. **Caroline C. Kim**: Investigation. **Inge Maartje van Scheepstal**: Investigation. **Simone O. Hoskin**: Supervision, Investigation. **William J. Kelly**: Writing – review & editing, Writing – original draft, Supervision, Investigation. **Sinead C. Leahy**: Investigation. **Graeme T. Attwood**: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Ron S. Ronimus**: Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Gemma Henderson**: Supervision, Investigation. **Peter H. Janssen**: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization..

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.microb.2024.100210.

Data availability

Data will be made available on request.

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