Comparison of methane yield of a novel strain of Methanothermobacter marburgensis in pure and mixed adapted culture derived from a methanation bubble column bioreactor

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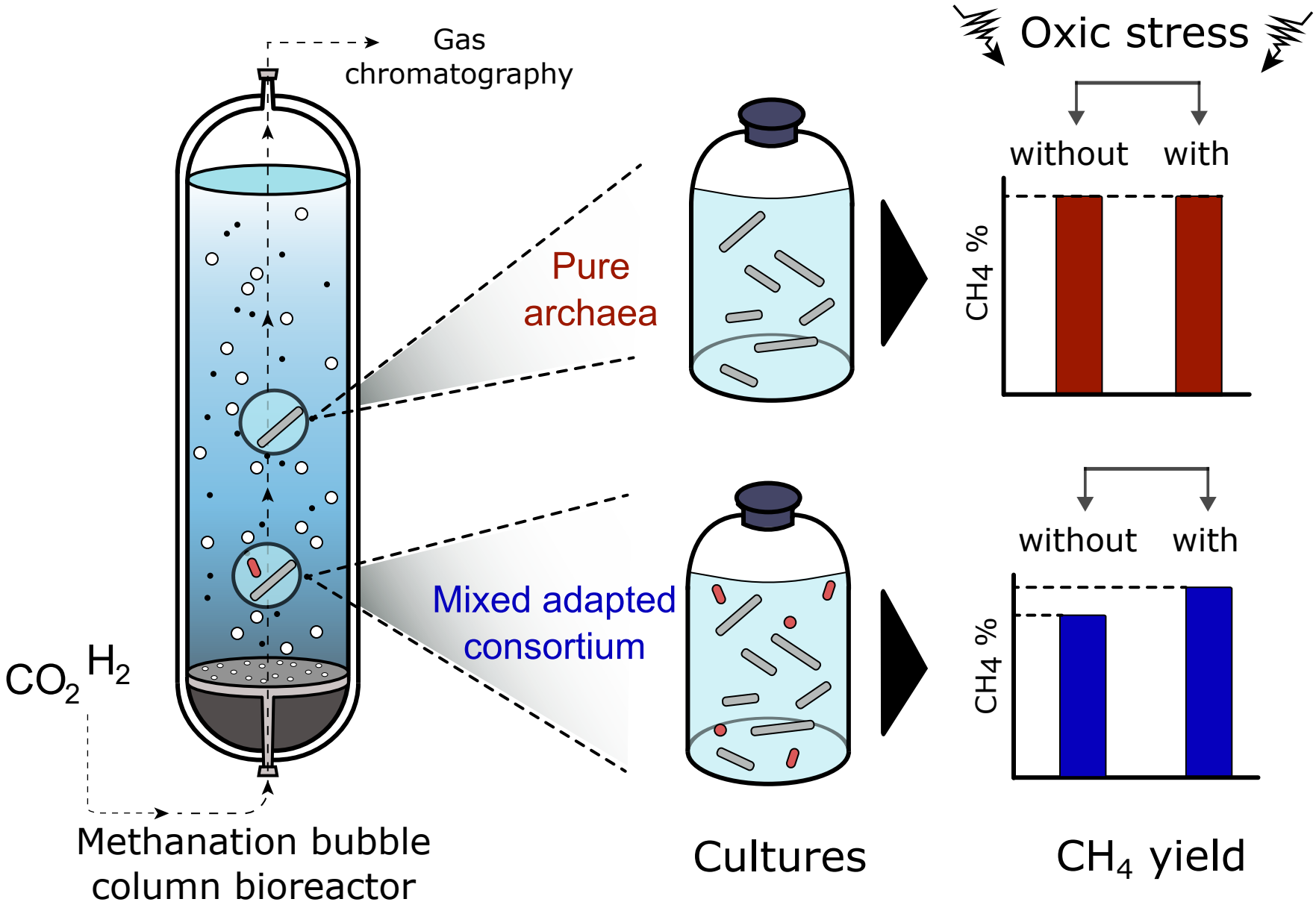
Graphical Abstract (for review)



Highlights

1. Archaeal cell enumeration using flow cytometry

2. MBA03 association with *Methanothermobacter* as an indicator of process stability

3. O_2 exposure impacts CH_4 yield of the consortium but not that of pure strains

4. Two *Methanothermobacter marburgensis* strains with different growth behaviour

Title

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Acknowledgements

The authors would like to acknowledge Hermine Billard and Jonathan Colombet, Plateforme CYSTEM – UCA PARTNER (Clermont-Ferrand, France), for their technical support and expertise. We also thank Pr. Cécile Lepère and Dr. Bernard Ollivier for their thorough reviewing of the manuscript.

Funding sources

This work was sponsored by the French government research program through ANR BIOMINTENS [grant number ANR-20-CE05-0031, 2021].

Title

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Abstract

The ongoing discussion regarding the use of mixed or pure cultures of hydrogenotrophic methanogenic archaea in Power-to-Methane (P2M) bioprocess applications persists, with each option presenting its own advantages and disadvantages. To address this issue, a comparison of methane (CH_4) yield between a novel methanogenic archaeon belonging to the species *Methanothermobacter marburgensis* (strain Clermont) isolated from a biological methanation column, and the community from which it originated, was conducted. This comparison included the type strain *M. marburgensis* str. Marburg. The evaluation also examined how exposure to oxygen (O_2) for up to 240 minutes impacted the CH_4 yield across these cultures. While both *Methanothermobacter* strains exhibit comparable CH_4 yield, slightly higher than that of the mixed adapted culture under non-O_2-exposed conditions, strain Clermont does not display the lag time observed for strain Marburg.

Keywords

Methanothermobacter marburgensis, mixed hydrogenotrophic methanogenic culture, oxygen exposure, multi-omics approaches.

1. Introduction

To limit the rise of global surface temperature to less than 2°C while meeting the increasing energy demand, a significant global energy transition is urgently needed. However, shifting away from polluting fossil fuels to low-carbon solutions requires technological innovation, particularly in renewable energy. Despite substantial progress in wind, solar, and geothermal energies, challenges such as intermittency, variability, geographical limitations, and storage persist (Tong et al., 2021). Power-To-Gas (P2G) concept has emerged as a promising solution allowing the storage of surplus of renewable energy recovered from the electricity sector in the form of gas (*i.e.* dihydrogen (H_2) called P2H and CH_4 called P2M) (Glenk and Reichelstein, 2022). Currently, P2M offers advantages over P2H. It allows converting electricity into chemical energy and uses existing infrastructure. Considering storability, it has a higher energy density (10 kWh/Nm^3 for CH_4 versus 3 kWh/Nm^3 for H_2) and is suitable for long term and large-scale storage (Blanco et al., 2018). P2M systems combine H_2 oxidation and carbon dioxide (CO_2) reduction to produce CH_4 using either physicochemical or biological catalysts (biomethanation). Comparatively, biomethanation processes require lower temperatures and pressures than physicochemical methanation processes and exhibit increased resistance to chemical contaminants including hydrogen sulfide (H_2S), organic acids, or ammonia (Burkhardt et al., 2015).

Hydrogenotrophic methanogenic archaea (HMs), which can use H_2 as a reducing agent for the conversion of CO_2 into CH_4, are key biocatalysts for biomethane (Bellini et al., 2022). They require as much H_2 as the system can provide for CO_2 reduction. Therefore, the competition and sustainable equilibrium between H_2 producers (*e.g.* acetogens) and consumers (*e.g.* HMs) usually result in a very low dissolved H_2 partial

pressure ($p(H_2)$) to maintain a balanced operation of the entire microbiological community. However, numerous abiotic and biotic factors can affect this equilibrium. From a thermodynamical perspective, external H_2 provision strongly favours hydrogenotrophic methanogenesis. But a sudden increment of $p(H_2)$ can enable the homoacetogenic pathway to outcompete the hydrogenotrophic methanogenesis (Treu et al., 2018; Tsapekos et al., 2022). In addition, temperature, pH, H_2/CO_2 ratio, H_2 supply, etc, are all abiotic factors that can influence CH_4 content and microbial community during *in-situ* biological biogas upgrading (Rachbauer et al., 2017; Wahid et al., 2019). Among HMs, the main actors in biomethanation processes comprise members of the genera *Methanoculleus*, *Methanothermobacter*, *Methanobacterium*, or *Methanosarcina*. The relative abundance of these genera in biogas upgrading reactors varies based on factors such as temperature, pH, carbon monoxide (CO), etc (Thema et al., 2021; Xu et al., 2020). In thermophilic conditions, *Methanothermobacter* was shown to be predominant in the mixed cultures due to its favorable growth at higher temperatures (Kaster et al., 2011; Szuhaj et al., 2021). Within this genus, *Methanothermobacter thermautotrophicus* and *Methanothermobacter marburgensis*, largely used as model organisms, have already been implemented as biocatalysts in large-scale industrial processes because they are robust, and reach high cell densities, and CH_4 production rate (Seifert et al., 2014; Pfeifer et al., 2021; Thema et al., 2021, Kaul et al., 2022). Two main approaches can be employed for biomethanation, *i.e.* using pure cultures or enriched mixed cultures, each with its own advantages and drawbacks (Rachbauer et al., 2017; Rafrafi et al., 2021; Rittmann et al., 2018). Indeed, using single self-replicating catalysts would prevent oxidation of H_2 by other hydrogenotrophic microorganisms, thereby avoiding a loss of efficiency in biogas upgrading. It would also allow for better

system variability and behaviour prediction (Martin et al., 2013). On the other hand, using consortia would be more efficient, leading to larger CH_4 yields (Bellini et al, 2022, Paniagua et al., 2022). Other advantages of employing consortia include greater robustness and short recovery time upon starvation/excess input gas rate and oxygenation. However, managing mixed cultures often requires increased control and a thorough understanding of how microbial composition impacts the system (Paniagua et al., 2022). Therefore, despite the growing number of studies in this field, the question of whether pure or mixed cultures are more suitable for biomethanation processes remains unresolved. To address this question, a comparison of the performance of both HM pure cultures and reactor microbiomes from which HMs have been isolated appears essential. This study aims to evaluate the methanation efficiency of a new HM affiliated to the *M. marburgensis* species (strain Clermont) isolated from a bubble column reactor. Its methanogenic performance was compared not only with its native consortium but also with the type strain, *i.e. M. marburgensis* strain Marburg (hereinafter referred to as strain Marburg). This comparison was extended under oxidative stress, a common occurrence in biomethanation processes.

2. Material and Methods

2.1. Laboratory-scale methanation reactor

The mixed adapted culture used in this study was collected from a 3.5 L bubble column reactor, six weeks after its inoculation with 300 mL of digestate from a thermophilic industrial-scale biogas plant treating livestock effluent and agri-food industry wastes that operates between 52 and 54°C (Methelec, Ennezat, France). Briefly, the reactor contained 2.7 L of basal anaerobic (BA) culture medium prepared as previously reported (Bu et al., 2018) and reduced by introducing 0.4 g/L of sodium sulfide

nanohydrate ($Na_2S.9H_2O$). The H_2/CO_2 gas mixture was set at a ratio of 4:1 (v/v) with a mass flowmeter (SLA5800, Brooks Instrument, Hatfield, USA). Flow rates ranged from 0.29 to 0.44 $NL.min^{-1}$. The temperature was set to 55°C using a thermostatic bath (Eco RE1225 silver, Lauda, Königshofen, Germany).

Volatile fatty acids (VFAs) in the six-week mixed adapted culture were determined using a liquid chromatograph (1260 HPLC, Agilent, Santa Clara, USA). The HPLC apparatus was equipped with two columns (Rezex ROA 300 x 7.8 nm, Phenomenex, Torrance, USA) mounted in serial in an oven (50˚C) and coupled with a refractive index detector. The mobile phase was a 2 mM sulfuric acid in ultra-pure water pumped at 0.7 $mL.min^{-1}$ and 70 bars. For the analysis, 2 mL of sample were mixed with 125 µL of $Ba(OH)_2.8\ H_2O$ (0.3 M) and 125 µL of $ZnSO_4.7\ H_2O$ (5% w/v) before a 5 min centrifugation at 10000 g. Samples were filtered through 0.2 µm nylon filters before being injected in the HPLC apparatus.

Measurements of archaeal and bacterial abundance (quantitative real-time PCR), performed on the six-week sample mixed adapted culture, revealed a dominance of archaea over bacteria with an archaea/bacteria ratio of five ($1.0\ 10^9$ archaeal cells/mL versus $2.2\ 10^8$ bacterial cells/mL).

2.2. Microbial community analysis of the mixed adapted culture

Two mL of the six-week mixed adapted culture were centrifuged at 10000 g at room temperature (RT) for 10 min and total genomic DNA (gDNA) was extracted from the pellet using a Nucleo Spin Soil kit in accordance with manufacturer's instructions (Machery Nagel, Düren, Germany). Then, gDNA was quantified using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, USA). Subsequently, the V3-V4 hypervariable region of the bacterial 16S ribosomal RNA (rRNA) genes was

amplified using the primer set F343 (5'-CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG-3') - R784 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT-3') (Carmona-Martinez et al., 2015) while the V4-V5 region of the archaeal 16S rRNA genes was amplified with the primer set F504-519 (5'-CTTTCCCTACACGACGCTCTTCCGATCTCAGCMGCCGCGGKAA-3') and R910-928 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTCCCGCCWATTCCTTTAAGT-3') (Braga Nan et al., 2020), primers containing adapters and barcodes for Miseq sequencing. PCR reactions contained Taq^{TM} TaKaRa Premix, 1 µM of each primer, 200 µM of each deoxynucleoside triphosphate (dNTP), 0.625 U TaKaRa Taq polymerase (TakaRa Inc., France), nuclease free-H_2O and 50 to 100 ng gDNA template in a total volume of 50 µL. For both bacteria and archaea, after a denaturation step of 1 min at 98°C, PCR steps at 98°C for 1 min, 59°C for 40 s, and 72°C for 1 min were repeated 35 times, followed by an elongation step at 72°C for 10 min in a Mastercycler® thermal cycler (Eppendorf, Hamburg, Germany). Amplicon size was checked by Agilent High Sensitivity DNA Kit on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Amplicon libraries were prepared and sequenced by the GenoToul platform (Toulouse, France) with an Illumina MiSeq sequencer to generate 2 x 300 bp paired-end reads. Bacterial and archaeal reads were separately processed using a homemade bioinformatics pipeline. Briefly, paired reads were merged using the VSEARCH v2.18.0 (Rognes et al., 2016) and then trimmed and filtered with Cutadapt (Martin, 2011) to minimize the effects of random sequencing errors as follows: (i) only merged reads with a length 200-500 bp were kept, (ii) paired reads with sequencing errors in primers were discarded and (iii) primer sequences and nucleotides with Phred quality

scores upper than 30 were trimmed. Deletion of chimeric sequences and clusterization were carried out using VSEARCH (Rognes et al., 2016) and operational taxonomic units (OTUs) that accounted for <0.005% of the total set of sequences were discarded (Bokulich et al., 2013). The taxonomic assignment was performed against the SILVA v138.1 SSU NR99 database using the global alignment script in VSEARCH (Pruesse et al, 2007; Rognes et al., 2016).

2.3. Hydrogenotrophic methanogenic archaea isolation and analytical procedures

A serum vial (Dutscher, Bernolsheim, France) containing 50 ml of BA medium (110 mL in capacity) was inoculated with an aliquot of the six-week mixed adapted culture (10% v/v). After BA medium autoclaving, and prior to its inoculation, dinitrogen (N_2) filling the head space was replaced by H_2/CO_2 (4:1, v/v) gas mixture from a gas cylinder (Westfalen, France) at 2 bars. Liquid cultivation was conducted at 55°C in the dark, without agitation.

HM isolation was performed *via* successive dilution to extinction series in Hungate tubes (16.5 mL capacity) containing 5 mL of either liquid or solid BA medium (2% agar w/v; roll-tube technique (Hungate, 1969)). Headspace composition was determined by gas chromatography (3000A MicroGC, Agilent Technologies, Santa Clara, CA, USA) equipped with two capillary columns (one MS-5A column associated with a backflush injector and one PorapLOT Q column associated with a standard injector) and Soprane software v3.5.2 for the analysis. The microGC used argon as gas carrier and the temperature of the columns and injectors were at 50 and 60°C, respectively.

The purity of HMs was checked by microscopic observations (Leica DM IRB inverted microscope equipped with a Hamamatsu C13440 camera and Zen Blue v3.1 software) and by adding yeast extract (2 g/L) and glucose (20 mM final concentration) to the BA

medium to confirm the absence of fermentative bacteria. Subsequently, HM taxonomic identification was performed by 16S rRNA gene sequence amplification using the universal primer set Arch21F-1492R (Nakagawa et al., 2006) followed by sequencing (Eurofins Genomics, Cologne, Germany). For the ultrastructural characterization, the archaeal cells in 1% (v/v) formaldehyde fixed samples were collected by centrifugation at 20000 g for 20 min at 14°C directly onto 400-mesh electron microscopy copper grids covered with carbon-coated Formvar film (AO3X, Pelanne Instruments, Toulouse, France). Particles were over contrasted with 2% uranyl salts and rinsed three times in distilled deionized water before being dried at RT. Subsequently, the characterization was performed with a transmission electron microscope using a JEOL 2100 (Akishikma, Tokyo, Japan; Plateforme CYSTEM, UCA Partner, Clermont-Ferrand, France). The microscope was operated at 80 kV, and the images were recorded with an Gatan CMOS RIO 9 camera (Gatan Ametek, Pleasaton, USA) at 3072 x 3072 pixels. Since all HMs belonged to the same strain, one representative, called *Methanothermobacter marburgensis* strain Clermont (hereafter referred to as strain Clermont), was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection (accession number DSM 34405).

2.4. Cultivation procedures

The hydrogenotrophic and methanogenic activities of strain Clermont, the mixed adapted culture, and *M. marburgensis* strain Marburg obtained from DSMZ (DSM 2133), which is the closest relative of strain Clermont (99.5% 16S rRNA gene identity), were investigated under reduced conditions. Cultures were conducted in serum vials containing 50 mL of BA medium as described above (Paragraph 2.3). All inoculations were performed with the same number of archaeal cells, *i.e.* 5.7 x 10^5 cells. Briefly, an

aliquot of 1 mL of pure or mixed cultures at the end of the exponential growth phase was diluted to 1:10 and directly used for flow cytometry analysis (BD LSR Fortessa X-20; BD Biosciences, CA, USA). Two lasers (Violet, 405 nm, 50 mW and Blue, 488 nm, 60 mW) were used for F420 cofactor excitation (autofluorescence of archaeal cells) and morphological characterization, respectively. The threshold was set at 200 on the F420 cofactor parameter. Data were acquired during 60 sec at a constant flow rate of 29.85 µL/min and processed using FACSDivA 9 software (BD Biosciences).

Cultures subjected to oxidative stress were inoculated and grown in medium devoid of chemical reducing agents, namely $Na_2S.9H_2O$ and resazurin, generally used to reinforce anaerobiosis and detect any potential oxidation, to prevent O_2 reduction during the exposure. Indeed, their presence would have reduced the effective amount of O_2 during oxic stress. Anaerobiosis prior to oxic stress was confirmed by microGC. After reaching the end of the exponential growth phase in anaerobic conditions, the cultures were transferred to sterile beakers in a laminar flow hood and placed under high stirring speed to be exposed to atmospheric levels of O_2 (21%) for durations of 0, 10, 30, 60, 90, 120, and 240 min. The quantity of dissolved O_2 measured in the medium was 10 mg/L ± 0.32 mg/L from 1 min up to 240 min of exposure (portable oximeter, Laqua 200 series, Horiba Scientific, Japan). Subsequently, fresh BA medium containing a reducing agent was inoculated as above. The absence of O_2 was checked by gas chromatography before incubation.

All experiments were conducted in biological triplicates. During growth, gas composition in the headspace was monitored daily by gas chromatography. Statistical analysis of CH_4 yield during growth was conducted using the Student t-test, one-way or two-way ANOVA (culture × incubation days) followed by Tukey's test under normality

and homoscedasticity assumption. The level of significance was set at α=0.05. All statistical analyses were performed using the R Stats package v4.2.2.

2.5. DNA extraction for whole genome sequencing of the *M. marburgensis* strain Clermont

A culture of strain Clermont (50 mL) in exponential growth was concentrated by centrifugation for 15 min at 10000 g at RT. gDNA was extracted from the pellet using a standard phenol–chloroform method (Biderre-Petit et al, 2024) before quantification on a Qubit Fluorometer (ThermoFisher Scientific, USA) by using Qubit™ dsDNA HS assay kit in accordance with manufacturer's instructions. Subsequently, 300 ng of gDNA were sequenced using Illumina HiSeq technology (2 × 150 bp; Eurofins Genomics, Constance, Germany). Raw paired-end reads were quality-filtered with fastp v0.23.4 (Chen et al., 2018). *De novo* assembly of whole genome sequencing data was performed using Unicycler v0.5.0 (Wick et al., 2017) with default settings. In the following step, the short contigs with viral/transposable elements (BLASTX search against non-redundant database) and fragmented rRNA operons (barrnap v0.9 and parameter "--kingdom arc", https://github.com/tseemann/barrnap) were filtered out.

2.6. Phylogenomic tree construction

Representative genomes of *Methanothermobacterium, Methanonatronarchaeia, Archaeoglobi, Methanobacteria, Methanococci, Methanomicrobia, Methanopyri, Thermococci,* and *Halobacterium* were downloaded from the National Center for Biotechnology Information (NCBI) RefSeq. Subsequently, a genome-based phylogenetic tree was generated with the program GToTree v1.8.2 as reported in Biderre-Petit et al. (2024), using an archaea-specific gene set composed of 73 markers. Individual gene alignments were concatenated to construct a species tree using IQ-

TREE v2.2.3 with the evolution model LG+F+I+R10 and parameters "-B 2000 --alrt 2000 --bnni" (Minh et al., 2020). *Halobacterium* was used as an outgroup.

2.7. Pan-genomic analysis and other genome characterizations

Pan-genomic analysis of the Marburg clade (*i.e.* including the strains Marburg, Clermont and their closest relatives-strain KEPCO-1 (assembly accession GCA_008033705.1), strain K4 (GCA_022014235.1), strain THM2 (GCA_009917665.1), bin GMQ_75_MeOH_H2_bin_21 (GCA_030055425.1) and bin JZ-3_D_bin_25 (GCA_030055435.1); seven genomes in total) was carried out using the "pan-genomics workflow of anvi'o v7.1 (Delmont and Eren, 2018). The average nucleotide identity (ANI) was also calculated through anvi'o and to complete the results, *in silico* DNA-DNA hybridization (*is*DDH) was computed using Genome-to-Genome Distance Calculator v2.1 with formula 2 as previously recommended (Meier-Kolthoff et al., 2013). Genome synteny was visualized by NGenomeSyn v1.41 (He et al., 2023).

3. Results and discussion

3.1. Overview of microbial community diversity in a bubble column reactor

Metabarcoding procedure was used to separately address the archaeal and bacterial diversity present in the six-week adapted culture. Although less abundant than archaea, bacteria showed much higher diversity, with 833 OTUs (19403 reads in total) versus 42 OTUs (28482 reads), respectively, which is consistent with what is generally described for mixed cultures (Xu et al., 2020). For the archaea, the four most abundant OTUs (>1% relative abundance in the sample) covered 96.1% of the community (See supplementary material). *Methanothermobacter* genus represented 98.5% of the

archaeal community in terms of reads, followed by *Methanobacterium* (1.2%), *Methanomassiliicoccus* (0.09%) and *Methanoculleus* (0.01%) (Fig. 1A). This finding closely aligns with previous research, which has shown that *Methanothermobacter* is the dominant genus in the hydrogenotrophic methanogenic consortium of *ex-situ* methanation systems at 55°C (Xu et al., 2020). Within this genus, 98.9% of the reads are affiliated to the *M. marburgensis* species (>97% 16S rRNA gene sequence identity). Regarding bacteria, the most represented phyla in terms of reads were *Bacillota* (ex *Firmicutes*, 91.8% of total bacterial reads) followed by *Pseudomonadota* (ex *Proteobacteria*, 6.8%) (Fig. 1B), in agreement with previous studies (Bassani et al., 2015; Campanaro et al., 2020). The 11 most abundant OTUs (>1% relative abundance in the sample) covered 48.7% of the bacterial community (See supplementary material) and mostly affiliated with the genus *Haloplasma* (35.2% of all bacterial reads) and the class *Limnochordia* (33.2%) in *Bacillota*.

The most abundant taxon, *i.e. Haloplasma,* is currently represented by only one representative, a halophilic bacterium isolated from a deep-sea brine lake, named *H. contractile*. The growth limit of this bacterium was determined at 44°C (Antunes et al, 2008), which is not in accordance with the temperature used in this study, *i.e.* 55°C. However, although SILVA classifies this predominant taxon in the genus *Haloplasma*, the sequence used as reference and which shows 99% identity with OTUs affiliated to *Haloplasma* (accession number FN436037, see supplementary material), displays only up to 89% sequence similarity with *Haloplasma* using BLAST N against the nucleotide database in NCBI. This may be due to the low number of sequences representative of the genus *Haloplasma* and also more generally of the family and order. Indeed, the order Haloplasmatales currently includes a single family -Haloplasmataceae- which

includes a single genus and species: *H. contractile*. This most likely not only leads to an inaccurate affiliation at the genus level, but also potentially at the family level. Consequently, the representatives that will be discovered for this group of Haloplasmatales will most certainly allow significant changes to the current description made for the single type species.

The second most abundant taxon, the *Limnochordia* class, is frequently observed in full-size and laboratory-scale thermophilic biogas reactors (Campanaro et al., 2020). The main representative in this class was MBA03 (14.6% of all bacterial reads). Laguillaumie et al. (2022) suggested that MBA03, referenced as a carbohydrate fermentative taxon, would grow on lysis products and prevent side products, such as VFAs, from accumulating in the reactor. The low amount of VFAs measured in the reactor could therefore be explained, at least in part, by the MBA03 abundance. This low quantity also indicates that hydrogenotrophic methanogenesis has not shifted towards homoacetogenesis. Moreover, MBA03 association with *Methanobacterium* was described as an indicator of process stability (Laguillaumie et al., 2022). Its association with *Methanothermobacter* could therefore still be such an indicator.

In addition to MBA03, two other bacteria known to be syntrophic acetate oxidizing, *i.e.* *Tepidiphilus* (*Pseudomonadota*) and norank order D8A-2 (*Bacillota*), showed significant relative abundance, with 2.9% and 3.6% of all bacterial reads, respectively. These results are in line with previous studies, which showed that these taxa were abundant in thermophilic samples and worked synergistically with HMs, providing the substrates they need towards biogas production (Tang et al., 2008; Xu et al., 2020). An anaerobic digestion system seeded from manure samples (which is comparable to what was used for the bioreactor, *i.e.* a biogas plant treating livestock effluent) and running at

55°C (Sun et al., 2015), was shown to be populated with similar microbial taxa. This supports the view that temperature, but also inoculum, are crucial variables in determining the structure of microbial consortia in hydrogenotrophic methanogenic mixed cultures (Xu et al., 2020).

3.2. Isolation and genome sequencing of *M. marburgensis* strain Clermont

The strain isolated from the bioreactor belonged to the genus *Methanothermobacter* and showed >99.5% identity with strain Marburg 16S rRNA gene sequence (accession number NR_102881.1). It was named *M. marburgensis* strain Clermont and deposited in the DSMZ collection (DSM 34405). This strain was rod-shaped (~5 µm long and 0.6 µm wide) and non-motile (See supplementary material).

The draft genome of strain Clermont (~330-fold coverage), featuring six contigs (from ~21.9 to 776.9 kb), had a total length of ~1.72 Mb, a N50 contig length of 593 kb, and a G+C content of 48.7%. The number of coding DNA sequences was 1805 with two 16S-23S rRNA gene clusters, three 5S rRNA genes and 37 transfer RNA (tRNA) genes. No extra-chromosomal genetic elements were detected. Genome fragmentation was mainly due to the high conservation degree between rRNA operons and transposase sequences that hamper the assembly tool to resolve these loci. Phylogenomic analysis confirmed the close relationship of strain Clermont with strain Marburg but also with three other *Methanothermobacter* strains (*i.e.* KEPCO-1, THM_2, and K4) and two bins (*i.e.* GMQ_75_MeOH_H2_bin_21 and JZ-3_D_bin_25). They all formed a clade (hereinafter referred to as Marburg clade) within the genus *Methanothermobacter* (Fig. 2A). Their genomes revealed a high degree of synteny (Fig. 2B). Based on ANI and *is*DDH values, strains Clermont, Marburg, KEPCO_1 and THM_2 formed a single species (ANI ≥96% and DDH ~70%; thresholds proposed for species definition

(Lindsey et al., 2023)) while strain K4 and the bins represented three novel *Methanothermobacter* species (Fig. 2C).

At the pan-genome level, the Marburg clade comprised 2076 gene clusters (GCs) with 1564 (75.3%) forming the core genome (shared by all seven genomes), 324 (15.6%) constituting the accessory genome (specific to a subset of genomes) and 188 (9.1%) being unique to a single genome (Fig. 2C). The core genome contained the full suite for proteins encoded to carry out the hydrogenotrophic pathway. Members of this clade can assimilate acetyl-coA *via* the CO-methylating acetyl-CoA synthase from methyl-tetrahydromethanopterin. In strain Marburg, this complex was also shown to play a key role in CO oxidation (Diender et al., 2016). Moreover, the presence of all genes involved in carboxydotrophic methanogenesis in all Marburg clade genomes suggests they are also able to grow with CO as the sole carbon source. A protein of the carbonic anhydrase family (Cah), known to potentially convert bicarbonate into bioavailable CO_2, was also present. Finally, as strains Clermont and Marburg were unable to grow on formate as an energy source (data not shown), the function of the formate dehydrogenase (FdhAB) is likely to reduce CO_2 to formate for its use in the purine synthesis (Kaster et al, 2011).

3.3. Comparison of CH_4 yields between the pure cultures-strains Clermont and Marburg-, and the mixed adapted culture

3.3.1. CH_4 yield during growth under reduced conditions

The isolation of a new strain for the species *M. marburgensis* (strain Clermont) from a reactor microbiome enabled to compare CH_4 yield not only between two strains of the same species but also between strain Clermont and the community from which it was isolated. In this respect, a flow cytometry method based on the cofactor F420

362 fluorescence (Lambrecht et al., 2017) was used for quantification and each culture was
363 inoculated with 5.7 x 10^5 archaeal cells. The maximum specific growth rates (μ_{MAX})
364 were 0.017, 0.03 and 0.02 h^{-1} for strain Clermont, Marburg and the mixed adapted
365 culture, respectively.
366 Methanogenic activity was observed at one-day post-inoculation for strain Clermont
367 and the mixed adapted culture (Fig. 3). CH_4 yield increased linearly until total H_2
368 conversion, reaching a maximum value after six days of growth, corresponding to
369 54.3% ± 1.4% CH_4 in the gas fraction for strain Clermont and to 49.3% ± 2.6% for the
370 mixed adapted culture ($p<0.05$). This aligns with the view that the use of unique, self-
371 replicating catalysts would avoid a loss of efficiency in biogas upgrading due to H_2
372 oxidation by other hydrogenotrophic microorganisms (Martin et al, 2013).
373 For strain Marburg, methanogenic activity was observed three days post-inoculation,
374 *i.e.* with a two-day lag phase compared with strain Clermont. Total H_2 conversion was
375 observed after eight days of growth, resulting in a maximum of 53.9% ± 2.4% CH_4 in
376 the gas fraction, a proportion similar to that obtained for strain Clermont (Fig. 3,
377 $p>0.05$). Consequently, the medium used in this study (*i.e.* BA medium), which is that
378 used for the isolation of strain Clermont, favoured the growth behaviour of the latter but
379 not its CH_4 yield. As the two pure strains are genetically very close, one explanation of
380 the two-day delay for strain Marburg may be, in part, attributed to their accessory
381 genomes. Indeed, the genome of strain Clermont contains 121 genes that are not present
382 in strain Marburg while the genome of strain Marburg contains 49 genes not found in
383 strain Clermont, all mostly organized into clusters (the largest contained 34 genes for
384 strain Clermont while 11 genes, for strain Marburg; See supplementary material).
385 Among these accessory genes, those encoding glycosyltransferases associated with the

synthesis and glycosylation of cellular surface proteins (*e.g* RafB, WcaA, WcaE) were more abundant in strain Clermont (See supplementary material). Kaster et al. (2011) suggested that these protein families might be partially responsible for the observed differences in growth rate phenotype between strain Marburg and *M. thermautotrophicus*. If this hypothesis proves to be true, it could also partly account for the observed phenotypic differences between strains Clermont and Marburg.

3.3.2. CH_4 yield following oxidative stress

As methanation reactors can experience episodic oxygenation (accidents, maintenance operations), it is essential to assess the HMs ability to maintain CH_4 yield after exposure to O_2. This has never been done for *Methanothermobacter* species, either in pure or mixed cultures. Interestingly, the results showed that O_2 exposure had no impact on the pure strains (*i.e.* strains Clermont and Marburg) as they exhibited the same CH_4 yield levels when all H_2 was consumed (*e.g.* 51.2% ± 1.6% and 55.3% ± 2.3%, respectively; after 240 min of O_2 exposure, Fig. 4B), whatever the time of exposure to O_2, *i.e* from 0 min up to 240 min ($p>0.05$; See supplemental material). The O_2 resistance capacity of these strains is suspected to be mediated by the presence in their genome of energy-free reactive oxygen species (ROS) scavengers of various protection enzymes, *i.e.* superoxide dismutase (SOD), superoxide reductase (SOR), $F_{420}H_{20}$ oxidase (FprA), peroxiredoxin (PRX), and rubrerythrin (Rbr) (Fig. 5), as previously reported for other methanogens (Liu et al., 2022). However, no catalase-encoding gene was found, similar to what was observed by Lyu and Lu (2018) in the Class I methanogens (*i.e*. *Methanobacteriales, Methanocellales and Methanopyrales*) to which *Methanothermobacter* spp. belongs. In all Marburg clade genomes, most ROS scavengers co-localize with genes encoding protection enzymes like rubredoxin (Rub,

410 electron providers to SOD and SOR), ferritin (FtnA, iron detoxifier during transient O_2)
411 (Fig. 5) and F390-synthetase which is thought to have a regulatory function in O_2 stress
412 response (Vermeij et al., 1997). As most of these genes are up-regulated during the
413 growth of strain Marburg on CO, it was hypothesized that they respond to redox stress
414 in general, and not just to O_2 stress, or are regulated by universal stress proteins
415 (Diender et al., 2016). Moreover, strain Marburg still shows the same two-day lag
416 compared with strain Clermont. Its growth behaviour is therefore not altered by oxic
417 stress either.
418 Conversely, exposure to O_2 has an impact on CH_4 yield by the mixed adapted culture.
419 Indeed, it exhibited a slight increase from the sixth day of incubation, when all H_2 was
420 converted, with 50.1% ± 1.6% CH_4 yield at 240 min of O_2 exposure (Fig. 4A) versus
421 46.4% ± 2.9% at 0 min ($p<0.02$; See supplementary material), thereby reaching the
422 level of the pure cultures (Fig. 4B). This gap in CH_4 yield could be associated with the
423 inhibition of hydrogenotrophic microorganisms, other than strain Clermont, present in
424 the mixed adapted culture.
425

426 **4. Conclusions**

427 Although methanogenesis is well studied, gaps remain in the understanding of
428 biomethanation, particularly regarding the choice between pure and mixed cultures in an
429 energy bioprocess. Comparative analysis of bioenergetic performances between strain
430 Clermont and its native consortium shows that the pure strain outperforms the
431 consortium in CH_4 yield under reduced conditions. However, this is no longer the case
432 after exposure to O_2. Furthermore, although both pure strains show the same CH_4 yield,

strain Clermont displays a higher reaction speed than the type strain of its species under the culture conditions used in this study.

E-supplementary data of this work can be found in online version of the paper.

Data availability

The raw reads of the 16S rRNA gene sequencing and genomic data were deposited at the NCBI database under the BioProject PRJNA1044399. *M. marburgensis* strain Clermont was deposited in the DSMZ German Collection of Microorganisms under accession number DSM 34405. Although the strain is not in the DSMZ catalogue or website, it is available on demand.

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Figure captions:

Figure 1: **Relative abundance of microbial taxa inferred from Illumina MiSeq sequencing of 16S rRNA genes. (A)** Archaeal abundance at the genus level from the V4-V5 16S rRNA region of the 16S rRNA gene. The pie chart on right indicates abundance of minor genera (<2% of total archaeal reads). **(B)** Bacterial abundance at the phylum level from the V3-V4 16S rRNA region of the 16S rRNA gene. The pie charts on right show the proportion of the different classes and genera constituting the phyla *Bacillota* and *Pseudomonadota*.

Figure 2: Comparative genomics of strain Clermont. (A) Phylogenomic tree of major archaeal clades based on a 73 genes core set using GToTree v1.8.2. ***On left***: Known major clades, including *Methanothermobacter* (dark pink) are collapsed and shown as wedges of different colors. *Halobacteriales* was placed as outgroup. Bar, 0.3 substitution per amino acid position. ***On right***: Decollapsed *Methanothermobacter* wedge showing the position of strain Clermont (in red) within this genus. Bar, 0.05 substitution per amino acid position. **(B)** Collinearity analysis among assemblies of the seven genomes forming the Marburg clade using MUMmer v4.0.0rc1 and visualized using NGenomeSyn. **(C)** Anvi'o representation of the pan-genome of the Marburg clade. Gene clusters (n = 2076) were ordered according to a hierarchical clustering of their presence/absence (inner dendrogram). Rings show the presence (filled) or absence (undashed) of the gene clusters in each genome. Single copy core and other core: gene clusters present in all seven *Methanothermobacter* genomes. Gene clusters exclusively present in a unique genome are indicated by a number: 1. strain K4, 2. strain THM_2, 3. JZ-3_D_bin_25, 4. strain Kepco-1, 5. GMQ_75_MeOH_H2_bin_21, 6. strain Marburg,

7. strain Clermont. To the right is given an ANI percentage identity heatmap; red: 100% identity; light red: values ranging from 96% to 97%; white: values <96%.

Figure 3: **Methane yield under reduced conditions in batch culture for pure strains (*i.e.* Marburg and Clermont) and the mixed adapted culture.** An asterisk denotes a significant difference ($p < 0.05$) between strain Marburg versus the two other cultures (days 3 to 6), and between the mixed culture versus pure cultures (days 8 and 18). The error bar indicates the standard error (n=3).

Figure 4: Methane yield in batch cultures under oxidative stressed conditions (240 min of exposure to O_2). (A) Comparison over time between strain Clermont and mixed adapted culture. **(B)** Comparison over time between strain Clermont and strain Marburg. An asterisk denotes a significant difference ($p < 0.05$) between cultures over time. The error bar indicates the standard error (n=3).

Figure 5: **Schematic representation of oxidative stress protection enzymes detected in the Marburg clade genomes. (A)** Gene-loci in the genome of strain Marburg. **(B)** Potential cellular responses to oxidative stress.

Title

Comparison of methane yield of a novel strain of *Methanothermobacter marburgensis* in pure and mixed adapted culture derived from a methanation bubble column bioreactor

Abstract

The ongoing discussion regarding the use of mixed or pure cultures of hydrogenotrophic methanogenic archaea in Power-to-Methane (P2M) bioprocess applications persists, with each option presenting its own advantages and disadvantages. To address this issue, a comparison of methane (CH_4) yield between a novel methanogenic archaeon belonging to the species *Methanothermobacter marburgensis* (strain Clermont) isolated from a biological methanation column, and the community from which it originated, was conducted. This comparison included the type strain *M. marburgensis* str. Marburg. The evaluation also examined how exposure to oxygen (O_2) for up to 240 minutes impacted the CH_4 yield across these cultures. While both *Methanothermobacter* strains exhibit comparable CH_4 yield, slightly higher than that of the mixed adapted culture under non-O_2-exposed conditions, strain Clermont does not display the lag time observed for strain Marburg.

Keywords

Methanothermobacter marburgensis, mixed hydrogenotrophic methanogenic culture, oxygen exposure, multi-omics approaches.

1. Introduction

To limit the rise of global surface temperature to less than 2°C while meeting the increasing energy demand, a significant global energy transition is urgently needed. However, shifting away from polluting fossil fuels to low-carbon solutions requires technological innovation, particularly in renewable energy. Despite substantial progress in wind, solar, and geothermal energies, challenges such as intermittency, variability, geographical limitations, and storage persist (Tong et al., 2021). Power-To-Gas (P2G) concept has emerged as a promising solution allowing the storage of surplus of renewable energy recovered from the electricity sector in the form of gas (*i.e.* dihydrogen (H_2) called P2H and CH_4 called P2M) (Glenk and Reichelstein, 2022). Currently, P2M offers advantages over P2H. It allows converting electricity into chemical energy and uses existing infrastructure. Considering storability, it has a higher energy density (10 kWh/Nm^3 for CH_4 versus 3 kWh/Nm^3 for H_2) and is suitable for long term and large-scale storage (Blanco et al., 2018). P2M systems combine H_2 oxidation and carbon dioxide (CO_2) reduction to produce CH_4 using either physicochemical or biological catalysts (biomethanation). Comparatively, biomethanation processes require lower temperatures and pressures than physicochemical methanation processes and exhibit increased resistance to chemical contaminants including hydrogen sulfide (H_2S), organic acids, or ammonia (Burkhardt et al., 2015).

Hydrogenotrophic methanogenic archaea (HMs), which can use H_2 as a reducing agent for the conversion of CO_2 into CH_4, are key biocatalysts for biomethane (Bellini et al., 2022). They require as much H_2 as the system can provide for CO_2 reduction. Therefore, the competition and sustainable equilibrium between H_2 producers (*e.g.* acetogens) and consumers (*e.g.* HMs) usually result in a very low dissolved H_2 partial

pressure ($p(H_2)$) to maintain a balanced operation of the entire microbiological community. However, numerous abiotic and biotic factors can affect this equilibrium. From a thermodynamical perspective, external H_2 provision strongly favours hydrogenotrophic methanogenesis. But a sudden increment of $p(H_2)$ can enable the homoacetogenic pathway to outcompete the hydrogenotrophic methanogenesis (Treu et al., 2018; Tsapekos et al., 2022). In addition, temperature, pH, H_2/CO_2 ratio, H_2 supply, etc, are all abiotic factors that can influence CH_4 content and microbial community during *in-situ* biological biogas upgrading (Rachbauer et al., 2017; Wahid et al., 2019). Among HMs, the main actors in biomethanation processes comprise members of the genera *Methanoculleus*, *Methanothermobacter*, *Methanobacterium*, or *Methanosarcina*. The relative abundance of these genera in biogas upgrading reactors varies based on factors such as temperature, pH, carbon monoxide (CO), etc (Thema et al., 2021; Xu et al., 2020). In thermophilic conditions, *Methanothermobacter* was shown to be predominant in the mixed cultures due to its favorable growth at higher temperatures (Kaster et al., 2011; Szuhaj et al., 2021). Within this genus, *Methanothermobacter thermautotrophicus* and *Methanothermobacter marburgensis*, largely used as model organisms, have already been implemented as biocatalysts in large-scale industrial processes because they are robust, and reach high cell densities, and CH_4 production rate (Seifert et al., 2014; Pfeifer et al., 2021; Thema et al., 2021, Kaul et al., 2022). Two main approaches can be employed for biomethanation, *i.e.* using pure cultures or enriched mixed cultures, each with its own advantages and drawbacks (Rachbauer et al., 2017; Rafrafi et al., 2021; Rittmann et al., 2018). Indeed, using single self-replicating catalysts would prevent oxidation of H_2 by other hydrogenotrophic microorganisms, thereby avoiding a loss of efficiency in biogas upgrading. It would also allow for better

system variability and behaviour prediction (Martin et al., 2013). On the other hand, using consortia would be more efficient, leading to larger CH_4 yields (Bellini et al, 2022, Paniagua et al., 2022). Other advantages of employing consortia include greater robustness and short recovery time upon starvation/excess input gas rate and oxygenation. However, managing mixed cultures often requires increased control and a thorough understanding of how microbial composition impacts the system (Paniagua et al., 2022). Therefore, despite the growing number of studies in this field, the question of whether pure or mixed cultures are more suitable for biomethanation processes remains unresolved. To address this question, a comparison of the performance of both HM pure cultures and reactor microbiomes from which HMs have been isolated appears essential. This study aims to evaluate the methanation efficiency of a new HM affiliated to the *M. marburgensis* species (strain Clermont) isolated from a bubble column reactor. Its methanogenic performance was compared not only with its native consortium but also with the type strain, *i.e. M. marburgensis* strain Marburg (hereinafter referred to as strain Marburg). This comparison was extended under oxidative stress, a common occurrence in biomethanation processes.

2. Material and Methods

2.1. Laboratory-scale methanation reactor

The mixed adapted culture used in this study was collected from a 3.5 L bubble column reactor, six weeks after its inoculation with 300 mL of digestate from a thermophilic industrial-scale biogas plant treating livestock effluent and agri-food industry wastes that operates between 52 and 54°C (Methelec, Ennezat, France). Briefly, the reactor contained 2.7 L of basal anaerobic (BA) culture medium prepared as previously reported (Bu et al., 2018) and reduced by introducing 0.4 g/L of sodium sulfide

nanohydrate ($Na_2S.9H_2O$). The H_2/CO_2 gas mixture was set at a ratio of 4:1 (v/v) with a mass flowmeter (SLA5800, Brooks Instrument, Hatfield, USA). Flow rates ranged from 0.29 to 0.44 $NL.min^{-1}$. The temperature was set to 55°C using a thermostatic bath (Eco RE1225 silver, Lauda, Königshofen, Germany).

Volatile fatty acids (VFAs) in the six-week mixed adapted culture were determined using a liquid chromatograph (1260 HPLC, Agilent, Santa Clara, USA). The HPLC apparatus was equipped with two columns (Rezex ROA 300 x 7.8 nm, Phenomenex, Torrance, USA) mounted in serial in an oven (50˚C) and coupled with a refractive index detector. The mobile phase was a 2 mM sulfuric acid in ultra-pure water pumped at 0.7 $mL.min^{-1}$ and 70 bars. For the analysis, 2 mL of sample were mixed with 125 µL of $Ba(OH)_2.8\ H_2O$ (0.3 M) and 125 µL of $ZnSO_4.7\ H_2O$ (5% w/v) before a 5 min centrifugation at 10000 g. Samples were filtered through 0.2 µm nylon filters before being injected in the HPLC apparatus.

Measurements of archaeal and bacterial abundance (quantitative real-time PCR), performed on the six-week sample mixed adapted culture, revealed a dominance of archaea over bacteria with an archaea/bacteria ratio of five ($1.0\ 10^9$ archaeal cells/mL versus $2.2\ 10^8$ bacterial cells/mL).

2.2. Microbial community analysis of the mixed adapted culture

Two mL of the six-week mixed adapted culture were centrifuged at 10000 g at room temperature (RT) for 10 min and total genomic DNA (gDNA) was extracted from the pellet using a Nucleo Spin Soil kit in accordance with manufacturer's instructions (Machery Nagel, Düren, Germany). Then, gDNA was quantified using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, USA). Subsequently, the V3-V4 hypervariable region of the bacterial 16S ribosomal RNA (rRNA) genes was

amplified using the primer set F343 (5'-CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG-3') - R784 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT-3') (Carmona-Martinez et al., 2015) while the V4-V5 region of the archaeal 16S rRNA genes was amplified with the primer set F504-519 (5'-CTTTCCCTACACGACGCTCTTCCGATCTCAGCMGCCGCGGKAA-3') and R910-928 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTCCCGCCWATTCCTTTAAGT-3') (Braga Nan et al., 2020), primers containing adapters and barcodes for Miseq sequencing. PCR reactions contained Taq^{TM} TaKaRa Premix, 1 µM of each primer, 200 µM of each deoxynucleoside triphosphate (dNTP), 0.625 U TaKaRa Taq polymerase (TakaRa Inc., France), nuclease free-H_2O and 50 to 100 ng gDNA template in a total volume of 50 µL. For both bacteria and archaea, after a denaturation step of 1 min at 98°C, PCR steps at 98°C for 1 min, 59°C for 40 s, and 72°C for 1 min were repeated 35 times, followed by an elongation step at 72°C for 10 min in a Mastercycler® thermal cycler (Eppendorf, Hamburg, Germany). Amplicon size was checked by Agilent High Sensitivity DNA Kit on 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Amplicon libraries were prepared and sequenced by the GenoToul platform (Toulouse, France) with an Illumina MiSeq sequencer to generate 2 x 300 bp paired-end reads. Bacterial and archaeal reads were separately processed using a homemade bioinformatics pipeline. Briefly, paired reads were merged using the VSEARCH v2.18.0 (Rognes et al., 2016) and then trimmed and filtered with Cutadapt (Martin, 2011) to minimize the effects of random sequencing errors as follows: (i) only merged reads with a length 200-500 bp were kept, (ii) paired reads with sequencing errors in primers were discarded and (iii) primer sequences and nucleotides with Phred quality

scores upper than 30 were trimmed. Deletion of chimeric sequences and clusterization were carried out using VSEARCH (Rognes et al., 2016) and operational taxonomic units (OTUs) that accounted for <0.005% of the total set of sequences were discarded (Bokulich et al., 2013). The taxonomic assignment was performed against the SILVA v138.1 SSU NR99 database using the global alignment script in VSEARCH (Pruesse et al, 2007; Rognes et al., 2016).

2.3. Hydrogenotrophic methanogenic archaea isolation and analytical procedures

A serum vial (Dutscher, Bernolsheim, France) containing 50 ml of BA medium (110 mL in capacity) was inoculated with an aliquot of the six-week mixed adapted culture (10% v/v). After BA medium autoclaving, and prior to its inoculation, dinitrogen (N_2) filling the head space was replaced by H_2/CO_2 (4:1, v/v) gas mixture from a gas cylinder (Westfalen, France) at 2 bars. Liquid cultivation was conducted at 55°C in the dark, without agitation.

HM isolation was performed *via* successive dilution to extinction series in Hungate tubes (16.5 mL capacity) containing 5 mL of either liquid or solid BA medium (2% agar w/v; roll-tube technique (Hungate, 1969)). Headspace composition was determined by gas chromatography (3000A MicroGC, Agilent Technologies, Santa Clara, CA, USA) equipped with two capillary columns (one MS-5A column associated with a backflush injector and one PorapLOT Q column associated with a standard injector) and Soprane software v3.5.2 for the analysis. The microGC used argon as gas carrier and the temperature of the columns and injectors were at 50 and 60°C, respectively.

The purity of HMs was checked by microscopic observations (Leica DM IRB inverted microscope equipped with a Hamamatsu C13440 camera and Zen Blue v3.1 software) and by adding yeast extract (2 g/L) and glucose (20 mM final concentration) to the BA

medium to confirm the absence of fermentative bacteria. Subsequently, HM taxonomic identification was performed by 16S rRNA gene sequence amplification using the universal primer set Arch21F-1492R (Nakagawa et al., 2006) followed by sequencing (Eurofins Genomics, Cologne, Germany). For the ultrastructural characterization, the archaeal cells in 1% (v/v) formaldehyde fixed samples were collected by centrifugation at 20000 g for 20 min at 14°C directly onto 400-mesh electron microscopy copper grids covered with carbon-coated Formvar film (AO3X, Pelanne Instruments, Toulouse, France). Particles were over contrasted with 2% uranyl salts and rinsed three times in distilled deionized water before being dried at RT. Subsequently, the characterization was performed with a transmission electron microscope using a JEOL 2100 (Akishikma, Tokyo, Japan; Plateforme CYSTEM, UCA Partner, Clermont-Ferrand, France). The microscope was operated at 80 kV, and the images were recorded with an Gatan CMOS RIO 9 camera (Gatan Ametek, Pleasaton, USA) at 3072 x 3072 pixels. Since all HMs belonged to the same strain, one representative, called *Methanothermobacter marburgensis* strain Clermont (hereafter referred to as strain Clermont), was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection (accession number DSM 34405).

2.4. Cultivation procedures

The hydrogenotrophic and methanogenic activities of strain Clermont, the mixed adapted culture, and *M. marburgensis* strain Marburg obtained from DSMZ (DSM 2133), which is the closest relative of strain Clermont (99.5% 16S rRNA gene identity), were investigated under reduced conditions. Cultures were conducted in serum vials containing 50 mL of BA medium as described above (Paragraph 2.3). All inoculations were performed with the same number of archaeal cells, *i.e.* 5.7 x 10^5 cells. Briefly, an

aliquot of 1 mL of pure or mixed cultures at the end of the exponential growth phase was diluted to 1:10 and directly used for flow cytometry analysis (BD LSR Fortessa X-20; BD Biosciences, CA, USA). Two lasers (Violet, 405 nm, 50 mW and Blue, 488 nm, 60 mW) were used for F420 cofactor excitation (autofluorescence of archaeal cells) and morphological characterization, respectively. The threshold was set at 200 on the F420 cofactor parameter. Data were acquired during 60 sec at a constant flow rate of 29.85 µL/min and processed using FACSDivA 9 software (BD Biosciences).

Cultures subjected to oxidative stress were inoculated and grown in medium devoid of chemical reducing agents, namely $Na_2S.9H_2O$ and resazurin, generally used to reinforce anaerobiosis and detect any potential oxidation, to prevent O_2 reduction during the exposure. Indeed, their presence would have reduced the effective amount of O_2 during oxic stress. Anaerobiosis prior to oxic stress was confirmed by microGC. After reaching the end of the exponential growth phase in anaerobic conditions, the cultures were transferred to sterile beakers in a laminar flow hood and placed under high stirring speed to be exposed to atmospheric levels of O_2 (21%) for durations of 0, 10, 30, 60, 90, 120, and 240 min. The quantity of dissolved O_2 measured in the medium was 10 mg/L ± 0.32 mg/L from 1 min up to 240 min of exposure (portable oximeter, Laqua 200 series, Horiba Scientific, Japan). Subsequently, fresh BA medium containing a reducing agent was inoculated as above. The absence of O_2 was checked by gas chromatography before incubation.

All experiments were conducted in biological triplicates. During growth, gas composition in the headspace was monitored daily by gas chromatography. Statistical analysis of CH_4 yield during growth was conducted using the Student t-test, one-way or two-way ANOVA (culture × incubation days) followed by Tukey's test under normality

and homoscedasticity assumption. The level of significance was set at α=0.05. All statistical analyses were performed using the R Stats package v4.2.2.

2.5. DNA extraction for whole genome sequencing of the *M. marburgensis* strain Clermont

A culture of strain Clermont (50 mL) in exponential growth was concentrated by centrifugation for 15 min at 10000 g at RT. gDNA was extracted from the pellet using a standard phenol–chloroform method (Biderre-Petit et al, 2024) before quantification on a Qubit Fluorometer (ThermoFisher Scientific, USA) by using Qubit™ dsDNA HS assay kit in accordance with manufacturer's instructions. Subsequently, 300 ng of gDNA were sequenced using Illumina HiSeq technology (2 × 150 bp; Eurofins Genomics, Constance, Germany). Raw paired-end reads were quality-filtered with fastp v0.23.4 (Chen et al., 2018). *De novo* assembly of whole genome sequencing data was performed using Unicycler v0.5.0 (Wick et al., 2017) with default settings. In the following step, the short contigs with viral/transposable elements (BLASTX search against non-redundant database) and fragmented rRNA operons (barrnap v0.9 and parameter "--kingdom arc", https://github.com/tseemann/barrnap) were filtered out.

2.6. Phylogenomic tree construction

Representative genomes of *Methanothermobacterium, Methanonatronarchaeia, Archaeoglobi, Methanobacteria, Methanococci, Methanomicrobia, Methanopyri, Thermococci,* and *Halobacterium* were downloaded from the National Center for Biotechnology Information (NCBI) RefSeq. Subsequently, a genome-based phylogenetic tree was generated with the program GToTree v1.8.2 as reported in Biderre-Petit et al. (2024), using an archaea-specific gene set composed of 73 markers. Individual gene alignments were concatenated to construct a species tree using IQ-

TREE v2.2.3 with the evolution model LG+F+I+R10 and parameters "-B 2000 --alrt 2000 --bnni" (Minh et al., 2020). *Halobacterium* was used as an outgroup.

2.7. Pan-genomic analysis and other genome characterizations

Pan-genomic analysis of the Marburg clade (*i.e.* including the strains Marburg, Clermont and their closest relatives-strain KEPCO-1 (assembly accession GCA_008033705.1), strain K4 (GCA_022014235.1), strain THM2 (GCA_009917665.1), bin GMQ_75_MeOH_H2_bin_21 (GCA_030055425.1) and bin JZ-3_D_bin_25 (GCA_030055435.1); seven genomes in total) was carried out using the "pan-genomics workflow of anvi'o v7.1 (Delmont and Eren, 2018). The average nucleotide identity (ANI) was also calculated through anvi'o and to complete the results, *in silico* DNA-DNA hybridization (*is*DDH) was computed using Genome-to-Genome Distance Calculator v2.1 with formula 2 as previously recommended (Meier-Kolthoff et al., 2013). Genome synteny was visualized by NGenomeSyn v1.41 (He et al., 2023).

3. Results and discussion

3.1. Overview of microbial community diversity in a bubble column reactor

Metabarcoding procedure was used to separately address the archaeal and bacterial diversity present in the six-week adapted culture. Although less abundant than archaea, bacteria showed much higher diversity, with 833 OTUs (19403 reads in total) versus 42 OTUs (28482 reads), respectively, which is consistent with what is generally described for mixed cultures (Xu et al., 2020). For the archaea, the four most abundant OTUs (>1% relative abundance in the sample) covered 96.1% of the community (See supplementary material). *Methanothermobacter* genus represented 98.5% of the

archaeal community in terms of reads, followed by *Methanobacterium* (1.2%), *Methanomassiliicoccus* (0.09%) and *Methanoculleus* (0.01%) (Fig. 1A). This finding closely aligns with previous research, which has shown that *Methanothermobacter* is the dominant genus in the hydrogenotrophic methanogenic consortium of *ex-situ* methanation systems at 55°C (Xu et al., 2020). Within this genus, 98.9% of the reads are affiliated to the *M. marburgensis* species (>97% 16S rRNA gene sequence identity). Regarding bacteria, the most represented phyla in terms of reads were *Bacillota* (ex *Firmicutes*, 91.8% of total bacterial reads) followed by *Pseudomonadota* (ex *Proteobacteria*, 6.8%) (Fig. 1B), in agreement with previous studies (Bassani et al., 2015; Campanaro et al., 2020). The 11 most abundant OTUs (>1% relative abundance in the sample) covered 48.7% of the bacterial community (See supplementary material) and mostly affiliated with the genus *Haloplasma* (35.2% of all bacterial reads) and the class *Limnochordia* (33.2%) in *Bacillota*.

The most abundant taxon, *i.e. Haloplasma*, is currently represented by only one representative, a halophilic bacterium isolated from a deep-sea brine lake, named *H. contractile*. The growth limit of this bacterium was determined at 44°C (Antunes et al, 2008), which is not in accordance with the temperature used in this study, *i.e.* 55°C. However, although SILVA classifies this predominant taxon in the genus *Haloplasma*, the sequence used as reference and which shows 99% identity with OTUs affiliated to *Haloplasma* (accession number FN436037, see supplementary material), displays only up to 89% sequence similarity with *Haloplasma* using BLAST N against the nucleotide database in NCBI. This may be due to the low number of sequences representative of the genus *Haloplasma* and also more generally of the family and order. Indeed, the order Haloplasmatales currently includes a single family -Haloplasmataceae- which

includes a single genus and species: *H. contractile*. This most likely not only leads to an inaccurate affiliation at the genus level, but also potentially at the family level. Consequently, the representatives that will be discovered for this group of Haloplasmatales will most certainly allow significant changes to the current description made for the single type species.

The second most abundant taxon, the *Limnochordia* class, is frequently observed in full-size and laboratory-scale thermophilic biogas reactors (Campanaro et al., 2020). The main representative in this class was MBA03 (14.6% of all bacterial reads). Laguillaumie et al. (2022) suggested that MBA03, referenced as a carbohydrate fermentative taxon, would grow on lysis products and prevent side products, such as VFAs, from accumulating in the reactor. The low amount of VFAs measured in the reactor could therefore be explained, at least in part, by the MBA03 abundance. This low quantity also indicates that hydrogenotrophic methanogenesis has not shifted towards homoacetogenesis. Moreover, MBA03 association with *Methanobacterium* was described as an indicator of process stability (Laguillaumie et al., 2022). Its association with *Methanothermobacter* could therefore still be such an indicator.

In addition to MBA03, two other bacteria known to be syntrophic acetate oxidizing, *i.e.* *Tepidiphilus* (*Pseudomonadota*) and norank order D8A-2 (*Bacillota*), showed significant relative abundance, with 2.9% and 3.6% of all bacterial reads, respectively. These results are in line with previous studies, which showed that these taxa were abundant in thermophilic samples and worked synergistically with HMs, providing the substrates they need towards biogas production (Tang et al., 2008; Xu et al., 2020). An anaerobic digestion system seeded from manure samples (which is comparable to what was used for the bioreactor, *i.e.* a biogas plant treating livestock effluent) and running at

55°C (Sun et al., 2015), was shown to be populated with similar microbial taxa. This supports the view that temperature, but also inoculum, are crucial variables in determining the structure of microbial consortia in hydrogenotrophic methanogenic mixed cultures (Xu et al., 2020).

3.2. Isolation and genome sequencing of *M. marburgensis* strain Clermont

The strain isolated from the bioreactor belonged to the genus *Methanothermobacter* and showed >99.5% identity with strain Marburg 16S rRNA gene sequence (accession number NR_102881.1). It was named *M. marburgensis* strain Clermont and deposited in the DSMZ collection (DSM 34405). This strain was rod-shaped (~5 µm long and 0.6 µm wide) and non-motile (See supplementary material).

The draft genome of strain Clermont (~330-fold coverage), featuring six contigs (from ~21.9 to 776.9 kb), had a total length of ~1.72 Mb, a N50 contig length of 593 kb, and a G+C content of 48.7%. The number of coding DNA sequences was 1805 with two 16S-23S rRNA gene clusters, three 5S rRNA genes and 37 transfer RNA (tRNA) genes. No extra-chromosomal genetic elements were detected. Genome fragmentation was mainly due to the high conservation degree between rRNA operons and transposase sequences that hamper the assembly tool to resolve these loci. Phylogenomic analysis confirmed the close relationship of strain Clermont with strain Marburg but also with three other *Methanothermobacter* strains (*i.e.* KEPCO-1, THM_2, and K4) and two bins (*i.e.* GMQ_75_MeOH_H2_bin_21 and JZ-3_D_bin_25). They all formed a clade (hereinafter referred to as Marburg clade) within the genus *Methanothermobacter* (Fig. 2A). Their genomes revealed a high degree of synteny (Fig. 2B). Based on ANI and *is*DDH values, strains Clermont, Marburg, KEPCO_1 and THM_2 formed a single species (ANI ≥96% and DDH ~70%; thresholds proposed for species definition

(Lindsey et al., 2023)) while strain K4 and the bins represented three novel *Methanothermobacter* species (Fig. 2C).

At the pan-genome level, the Marburg clade comprised 2076 gene clusters (GCs) with 1564 (75.3%) forming the core genome (shared by all seven genomes), 324 (15.6%) constituting the accessory genome (specific to a subset of genomes) and 188 (9.1%) being unique to a single genome (Fig. 2C). The core genome contained the full suite for proteins encoded to carry out the hydrogenotrophic pathway. Members of this clade can assimilate acetyl-coA *via* the CO-methylating acetyl-CoA synthase from methyl-tetrahydromethanopterin. In strain Marburg, this complex was also shown to play a key role in CO oxidation (Diender et al., 2016). Moreover, the presence of all genes involved in carboxydotrophic methanogenesis in all Marburg clade genomes suggests they are also able to grow with CO as the sole carbon source. A protein of the carbonic anhydrase family (Cah), known to potentially convert bicarbonate into bioavailable CO_2, was also present. Finally, as strains Clermont and Marburg were unable to grow on formate as an energy source (data not shown), the function of the formate dehydrogenase (FdhAB) is likely to reduce CO_2 to formate for its use in the purine synthesis (Kaster et al, 2011).

3.3. Comparison of CH_4 yields between the pure cultures-strains Clermont and Marburg-, and the mixed adapted culture

3.3.1. CH_4 yield during growth under reduced conditions

The isolation of a new strain for the species *M. marburgensis* (strain Clermont) from a reactor microbiome enabled to compare CH_4 yield not only between two strains of the same species but also between strain Clermont and the community from which it was isolated. In this respect, a flow cytometry method based on the cofactor F420

fluorescence (Lambrecht et al., 2017) was used for quantification and each culture was inoculated with 5.7 x 10^5 archaeal cells. The maximum specific growth rates (μ_{MAX}) were 0.017, 0.03 and 0.02 h^{-1} for strain Clermont, Marburg and the mixed adapted culture, respectively.

Methanogenic activity was observed at one-day post-inoculation for strain Clermont and the mixed adapted culture (Fig. 3). CH_4 yield increased linearly until total H_2 conversion, reaching a maximum value after six days of growth, corresponding to 54.3% ± 1.4% CH_4 in the gas fraction for strain Clermont and to 49.3% ± 2.6% for the mixed adapted culture ($p<0.05$). This aligns with the view that the use of unique, self-replicating catalysts would avoid a loss of efficiency in biogas upgrading due to H_2 oxidation by other hydrogenotrophic microorganisms (Martin et al, 2013).

For strain Marburg, methanogenic activity was observed three days post-inoculation, *i.e.* with a two-day lag phase compared with strain Clermont. Total H_2 conversion was observed after eight days of growth, resulting in a maximum of 53.9% ± 2.4% CH_4 in the gas fraction, a proportion similar to that obtained for strain Clermont (Fig. 3, $p>0.05$). Consequently, the medium used in this study (*i.e.* BA medium), which is that used for the isolation of strain Clermont, favoured the growth behaviour of the latter but not its CH_4 yield. As the two pure strains are genetically very close, one explanation of the two-day delay for strain Marburg may be, in part, attributed to their accessory genomes. Indeed, the genome of strain Clermont contains 121 genes that are not present in strain Marburg while the genome of strain Marburg contains 49 genes not found in strain Clermont, all mostly organized into clusters (the largest contained 34 genes for strain Clermont while 11 genes, for strain Marburg; See supplementary material). Among these accessory genes, those encoding glycosyltransferases associated with the

synthesis and glycosylation of cellular surface proteins (*e.g* RafB, WcaA, WcaE) were more abundant in strain Clermont (See supplementary material). Kaster et al. (2011) suggested that these protein families might be partially responsible for the observed differences in growth rate phenotype between strain Marburg and *M. thermautotrophicus*. If this hypothesis proves to be true, it could also partly account for the observed phenotypic differences between strains Clermont and Marburg.

3.3.2. CH_4 yield following oxidative stress

As methanation reactors can experience episodic oxygenation (accidents, maintenance operations), it is essential to assess the HMs ability to maintain CH_4 yield after exposure to O_2. This has never been done for *Methanothermobacter* species, either in pure or mixed cultures. Interestingly, the results showed that O_2 exposure had no impact on the pure strains (*i.e.* strains Clermont and Marburg) as they exhibited the same CH_4 yield levels when all H_2 was consumed (*e.g.* 51.2% ± 1.6% and 55.3% ± 2.3%, respectively; after 240 min of O_2 exposure, Fig. 4B), whatever the time of exposure to O_2, *i.e* from 0 min up to 240 min ($p>0.05$; See supplemental material). The O_2 resistance capacity of these strains is suspected to be mediated by the presence in their genome of energy-free reactive oxygen species (ROS) scavengers of various protection enzymes, *i.e.* superoxide dismutase (SOD), superoxide reductase (SOR), $F_{420}H_{20}$ oxidase (FprA), peroxiredoxin (PRX), and rubrerythrin (Rbr) (Fig. 5), as previously reported for other methanogens (Liu et al., 2022). However, no catalase-encoding gene was found, similar to what was observed by Lyu and Lu (2018) in the Class I methanogens (*i.e*. *Methanobacteriales, Methanocellales and Methanopyrales*) to which *Methanothermobacter* spp. belongs. In all Marburg clade genomes, most ROS scavengers co-localize with genes encoding protection enzymes like rubredoxin (Rub,

electron providers to SOD and SOR), ferritin (FtnA, iron detoxifier during transient O_2) (Fig. 5) and F390-synthetase which is thought to have a regulatory function in O_2 stress response (Vermeij et al., 1997). As most of these genes are up-regulated during the growth of strain Marburg on CO, it was hypothesized that they respond to redox stress in general, and not just to O_2 stress, or are regulated by universal stress proteins (Diender et al., 2016). Moreover, strain Marburg still shows the same two-day lag compared with strain Clermont. Its growth behaviour is therefore not altered by oxic stress either.

Conversely, exposure to O_2 has an impact on CH_4 yield by the mixed adapted culture. Indeed, it exhibited a slight increase from the sixth day of incubation, when all H_2 was converted, with 50.1% ± 1.6% CH_4 yield at 240 min of O_2 exposure (Fig. 4A) versus 46.4% ± 2.9% at 0 min ($p<0.02$; See supplementary material), thereby reaching the level of the pure cultures (Fig. 4B). This gap in CH_4 yield could be associated with the inhibition of hydrogenotrophic microorganisms, other than strain Clermont, present in the mixed adapted culture.

4. Conclusions

Although methanogenesis is well studied, gaps remain in the understanding of biomethanation, particularly regarding the choice between pure and mixed cultures in an energy bioprocess. Comparative analysis of bioenergetic performances between strain Clermont and its native consortium shows that the pure strain outperforms the consortium in CH_4 yield under reduced conditions. However, this is no longer the case after exposure to O_2. Furthermore, although both pure strains show the same CH_4 yield,

strain Clermont displays a higher reaction speed than the type strain of its species under the culture conditions used in this study.

E-supplementary data of this work can be found in online version of the paper.

Data availability

The raw reads of the 16S rRNA gene sequencing and genomic data were deposited at the NCBI database under the BioProject PRJNA1044399. *M. marburgensis* strain Clermont was deposited in the DSMZ German Collection of Microorganisms under accession number DSM 34405. Although the strain is not in the DSMZ catalogue or website, it is available on demand.

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Figure captions:

Figure 1: **Relative abundance of microbial taxa inferred from Illumina MiSeq sequencing of 16S rRNA genes. (A)** Archaeal abundance at the genus level from the V4-V5 16S rRNA region of the 16S rRNA gene. The pie chart on right indicates abundance of minor genera (<2% of total archaeal reads). **(B)** Bacterial abundance at the phylum level from the V3-V4 16S rRNA region of the 16S rRNA gene. The pie charts on right show the proportion of the different classes and genera constituting the phyla *Bacillota* and *Pseudomonadota*.

Figure 2: Comparative genomics of strain Clermont. (A) Phylogenomic tree of major archaeal clades based on a 73 genes core set using GToTree v1.8.2. ***On left***: Known major clades, including *Methanothermobacter* (dark pink) are collapsed and shown as wedges of different colors. *Halobacteriales* was placed as outgroup. Bar, 0.3 substitution per amino acid position. ***On right***: Decollapsed *Methanothermobacter* wedge showing the position of strain Clermont (in red) within this genus. Bar, 0.05 substitution per amino acid position. **(B)** Collinearity analysis among assemblies of the seven genomes forming the Marburg clade using MUMmer v4.0.0rc1 and visualized using NGenomeSyn. **(C)** Anvi'o representation of the pan-genome of the Marburg clade. Gene clusters (n = 2076) were ordered according to a hierarchical clustering of their presence/absence (inner dendrogram). Rings show the presence (filled) or absence (undashed) of the gene clusters in each genome. Single copy core and other core: gene clusters present in all seven *Methanothermobacter* genomes. Gene clusters exclusively present in a unique genome are indicated by a number: 1. strain K4, 2. strain THM_2, 3. JZ-3_D_bin_25, 4. strain Kepco-1, 5. GMQ_75_MeOH_H2_bin_21, 6. strain Marburg,

7. strain Clermont. To the right is given an ANI percentage identity heatmap; red: 100% identity; light red: values ranging from 96% to 97%; white: values <96%.

Figure 3: **Methane yield under reduced conditions in batch culture for pure strains (*i.e.* Marburg and Clermont) and the mixed adapted culture.** An asterisk denotes a significant difference ($p < 0.05$) between strain Marburg versus the two other cultures (days 3 to 6), and between the mixed culture versus pure cultures (days 8 and 18). The error bar indicates the standard error (n=3).

Figure 4: Methane yield in batch cultures under oxidative stressed conditions (240 min of exposure to O_2). (A) Comparison over time between strain Clermont and mixed adapted culture. **(B)** Comparison over time between strain Clermont and strain Marburg. An asterisk denotes a significant difference ($p < 0.05$) between cultures over time. The error bar indicates the standard error (n=3).

Figure 5: **Schematic representation of oxidative stress protection enzymes detected in the Marburg clade genomes. (A)** Gene-loci in the genome of strain Marburg. **(B)** Potential cellular responses to oxidative stress.

Figure

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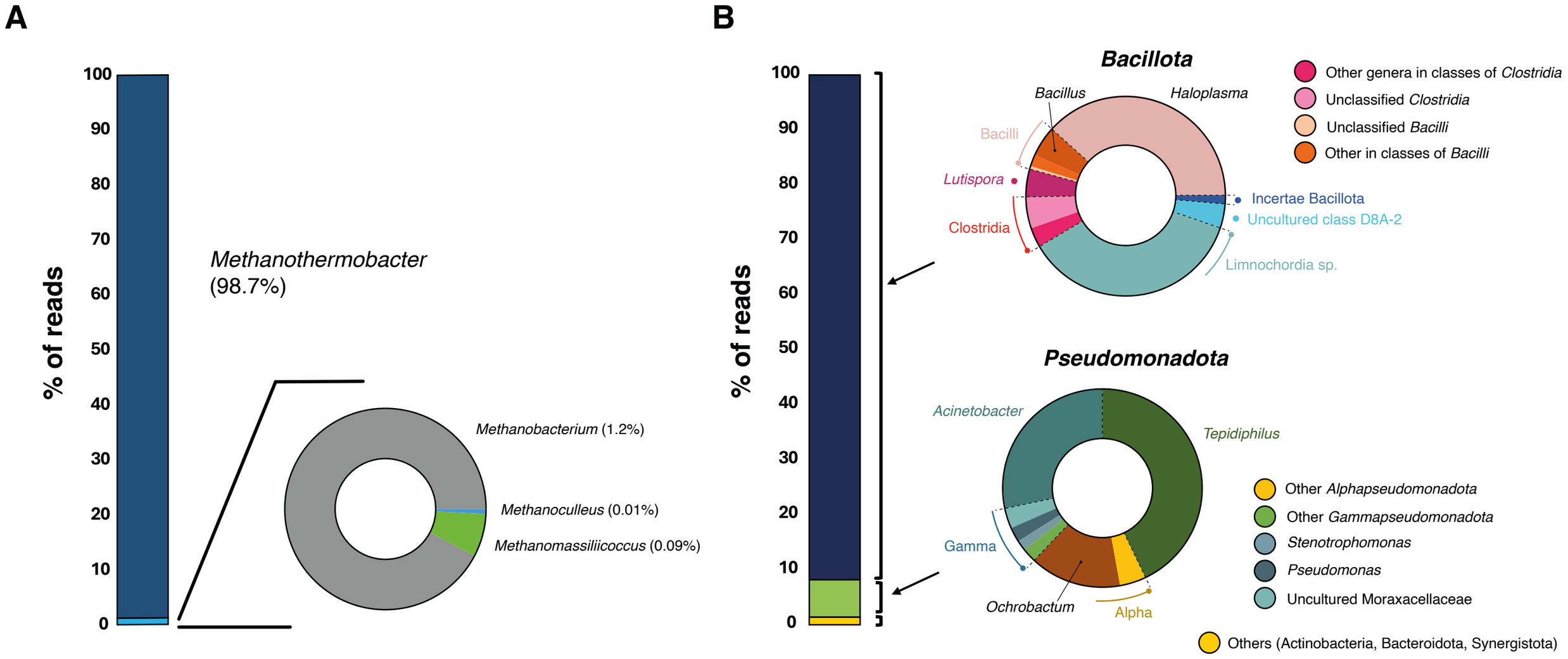

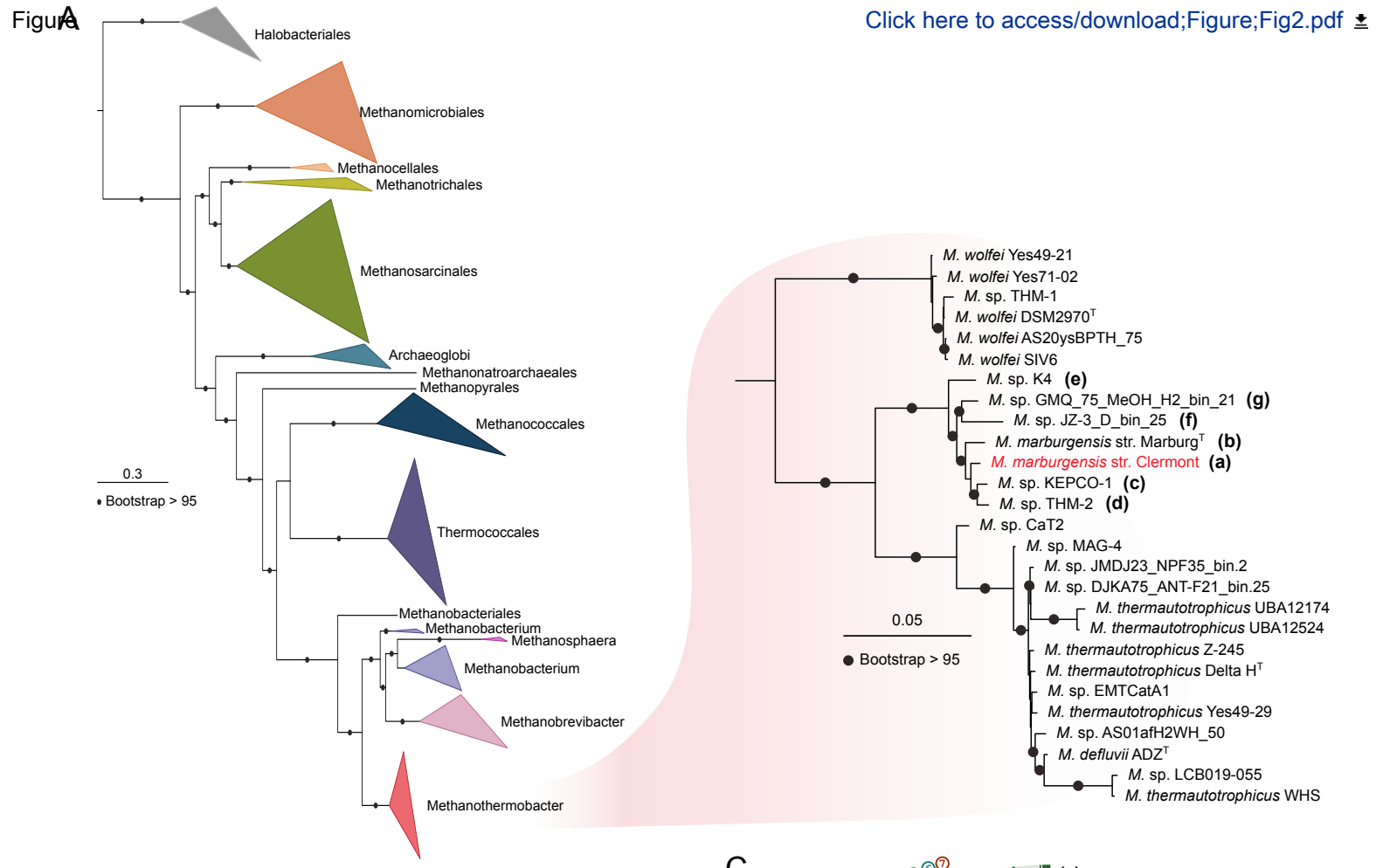

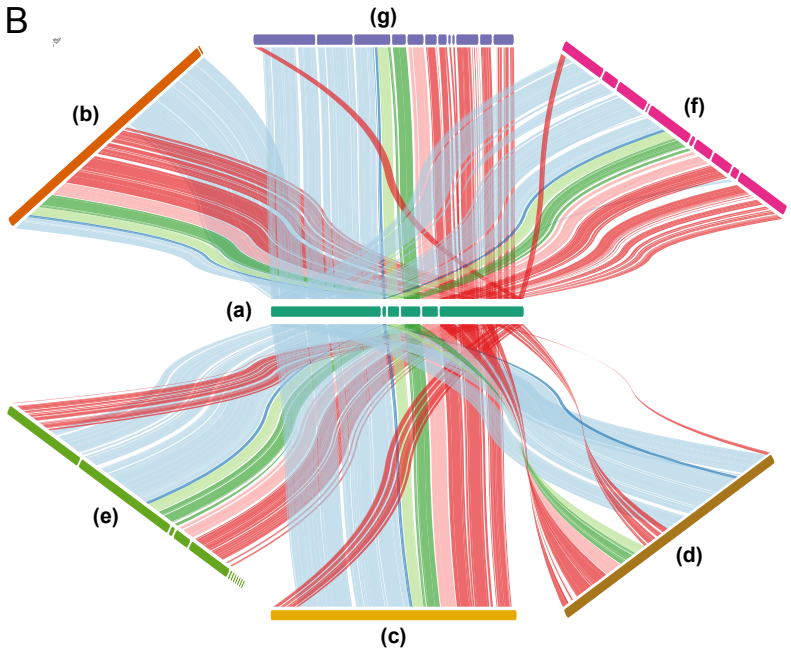

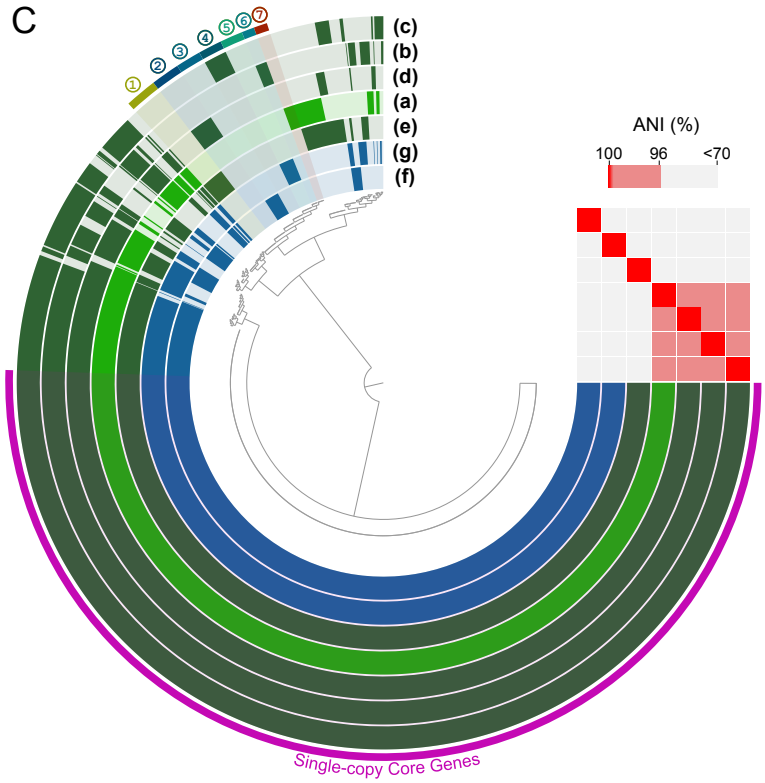

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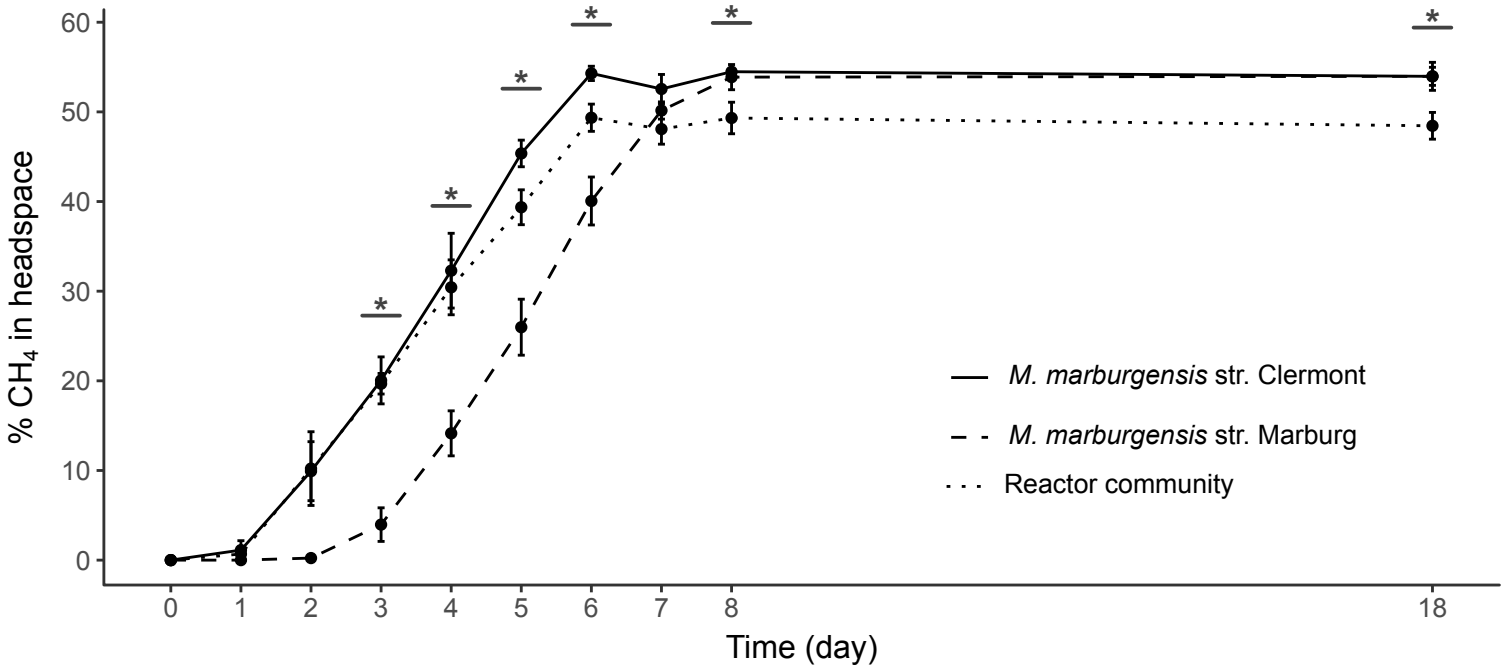

Figure 3

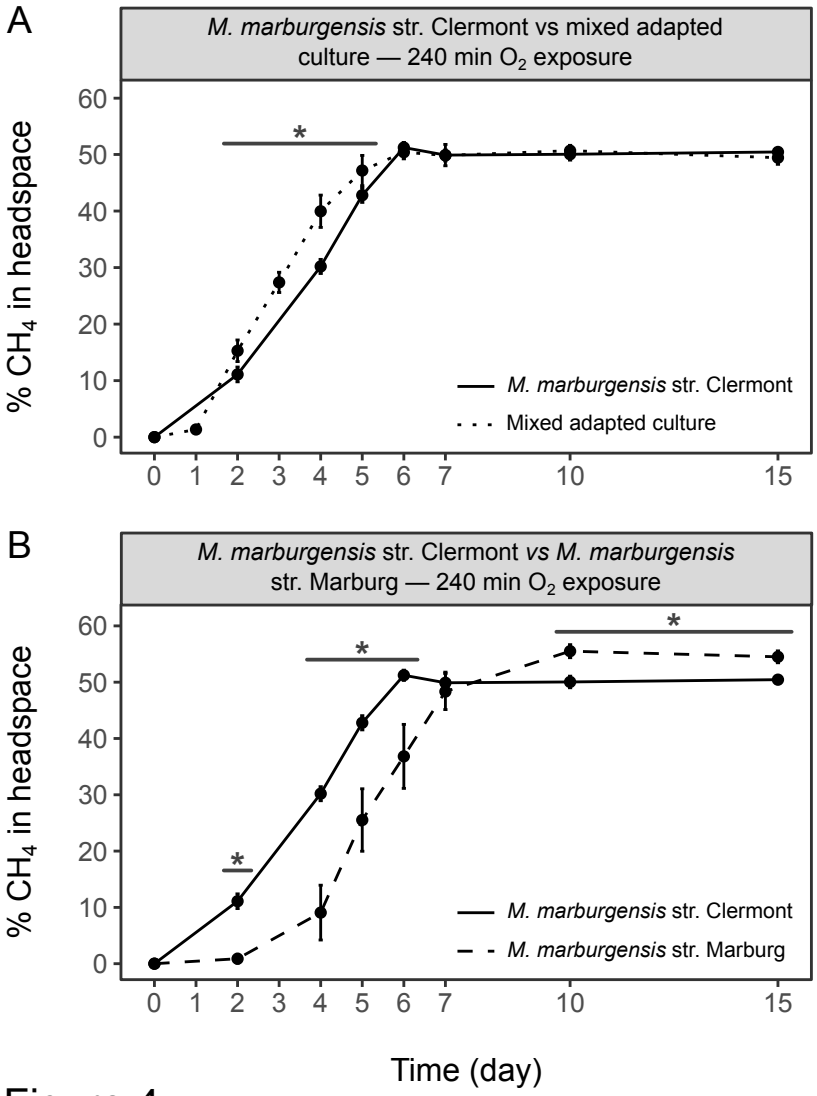

Figure 4

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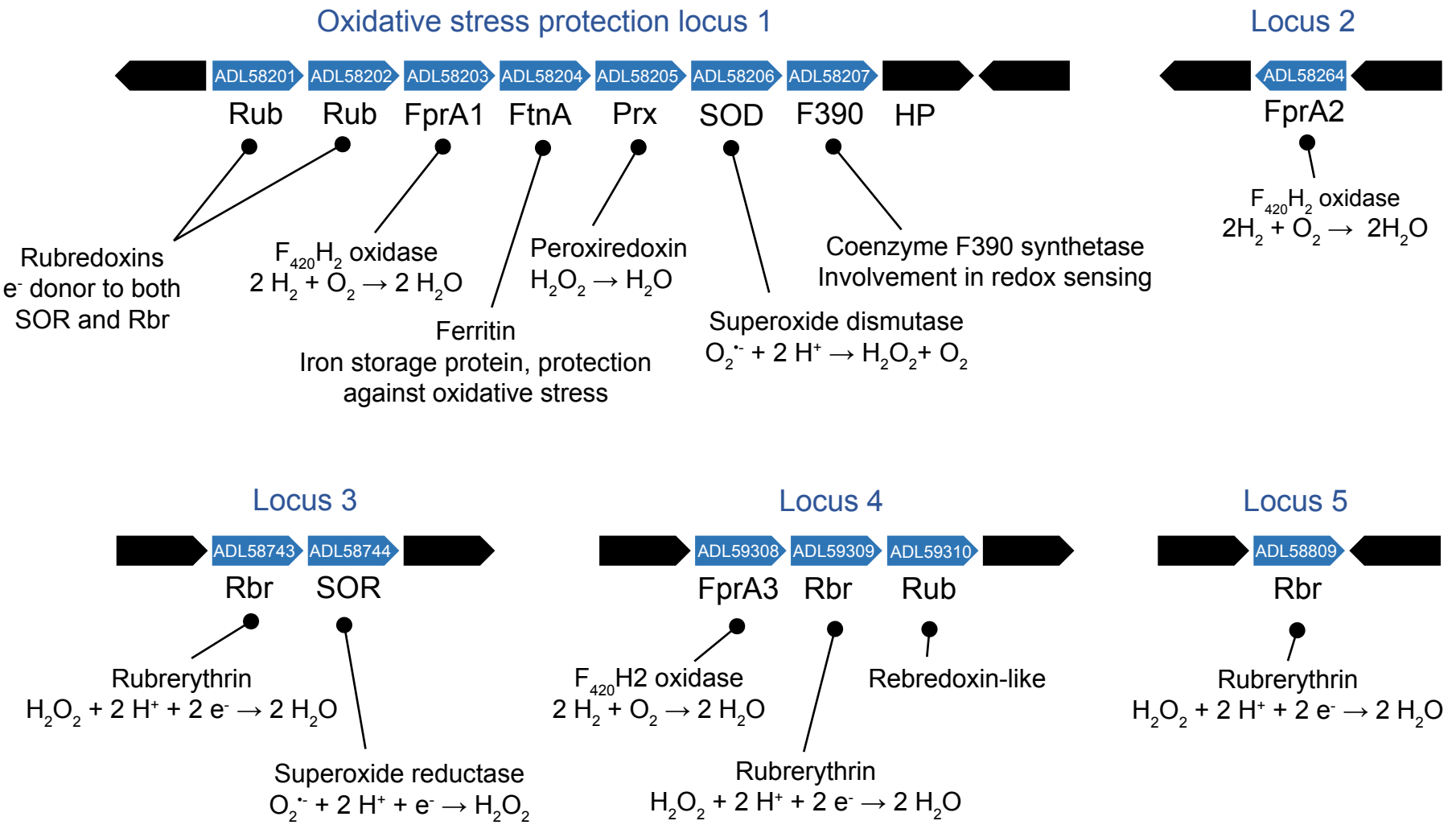

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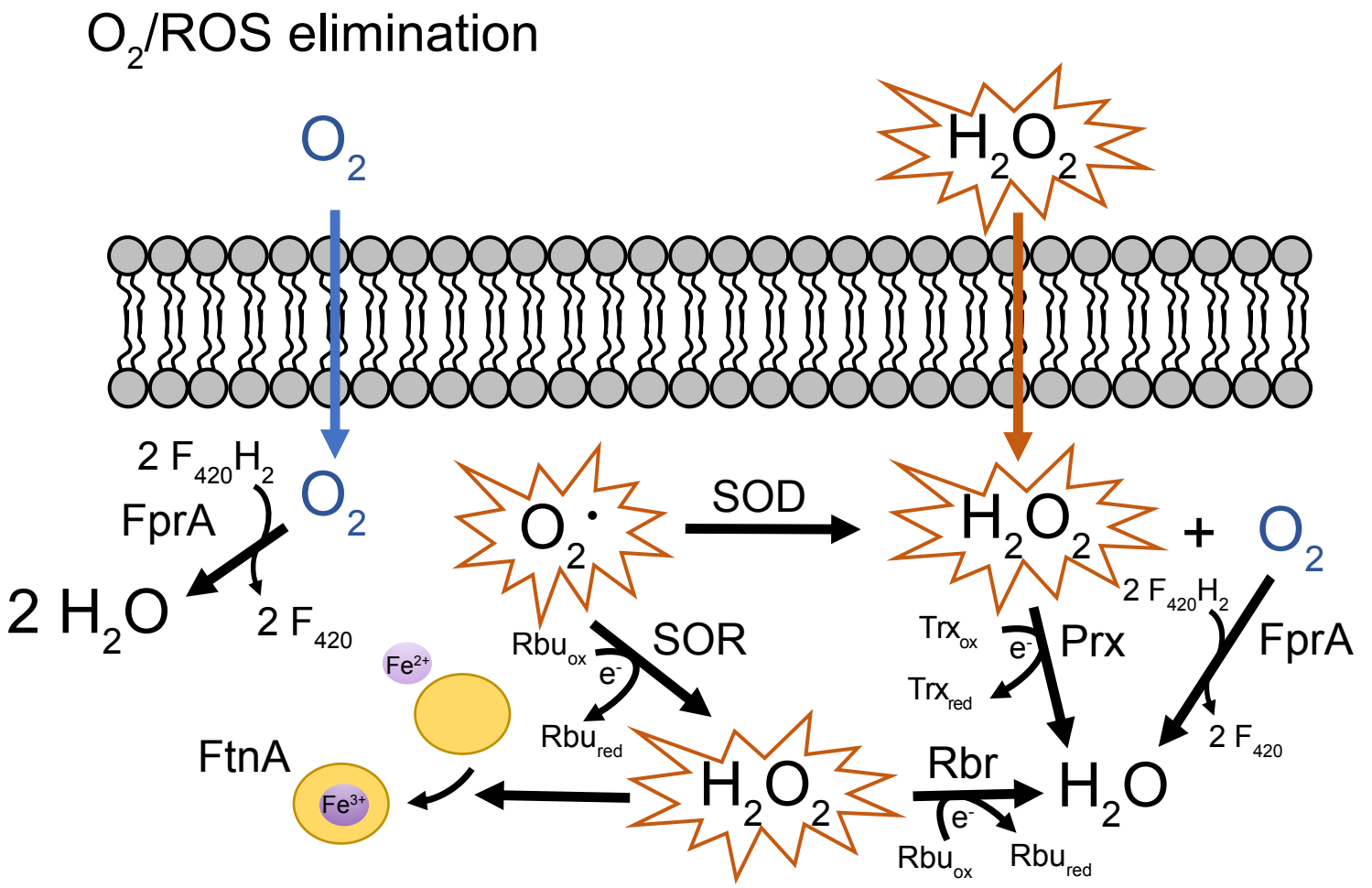

Figure 5

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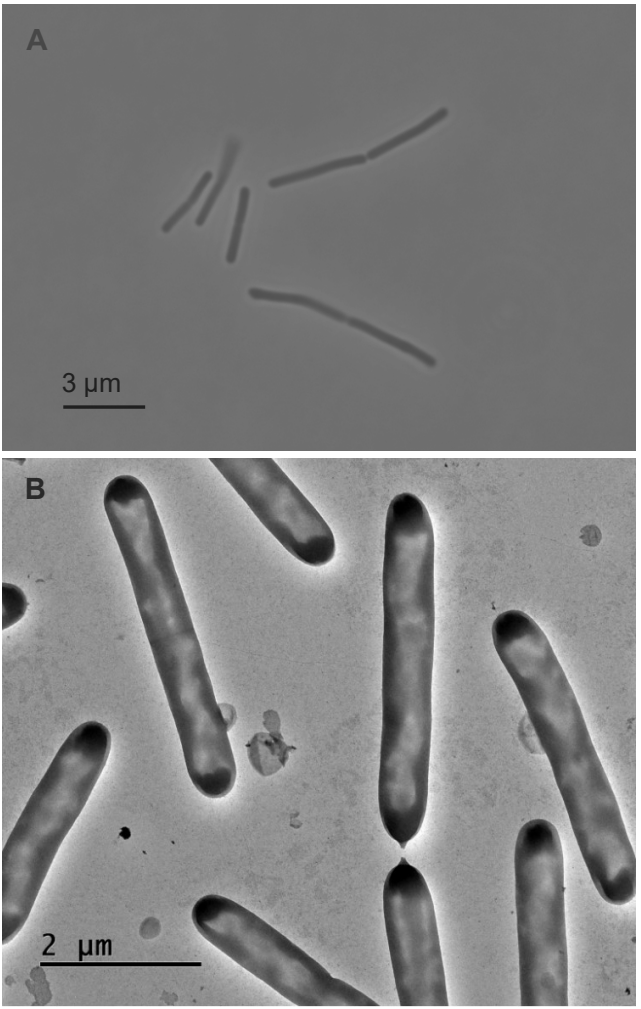

Figure S1: Morphology of *Methanothermobacter marburgensis* strain Clermont in optical microscopy (A) and transmission electron microscopy (B).

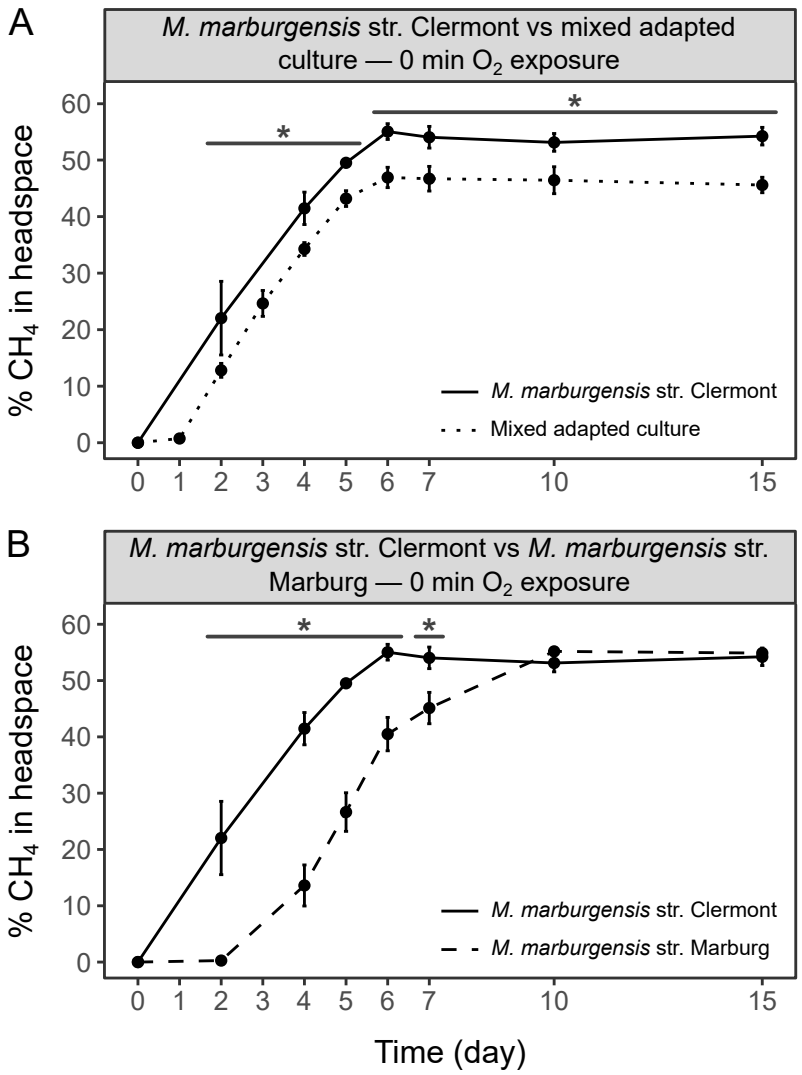

Figure S2: Methane production in batch cultures at the first level (0 min of exposure to O_2) of the experiment oxidative stressed conditions. (A) Comparison over time between strain Clermont and the mixed adapted culture. (B) Comparison over time between strain Clermont and strain Marburg. Asterisks denote a significant difference ($p < 0.05$) between cultures over time. The error bar indicates the standard error (n=3).

Figure

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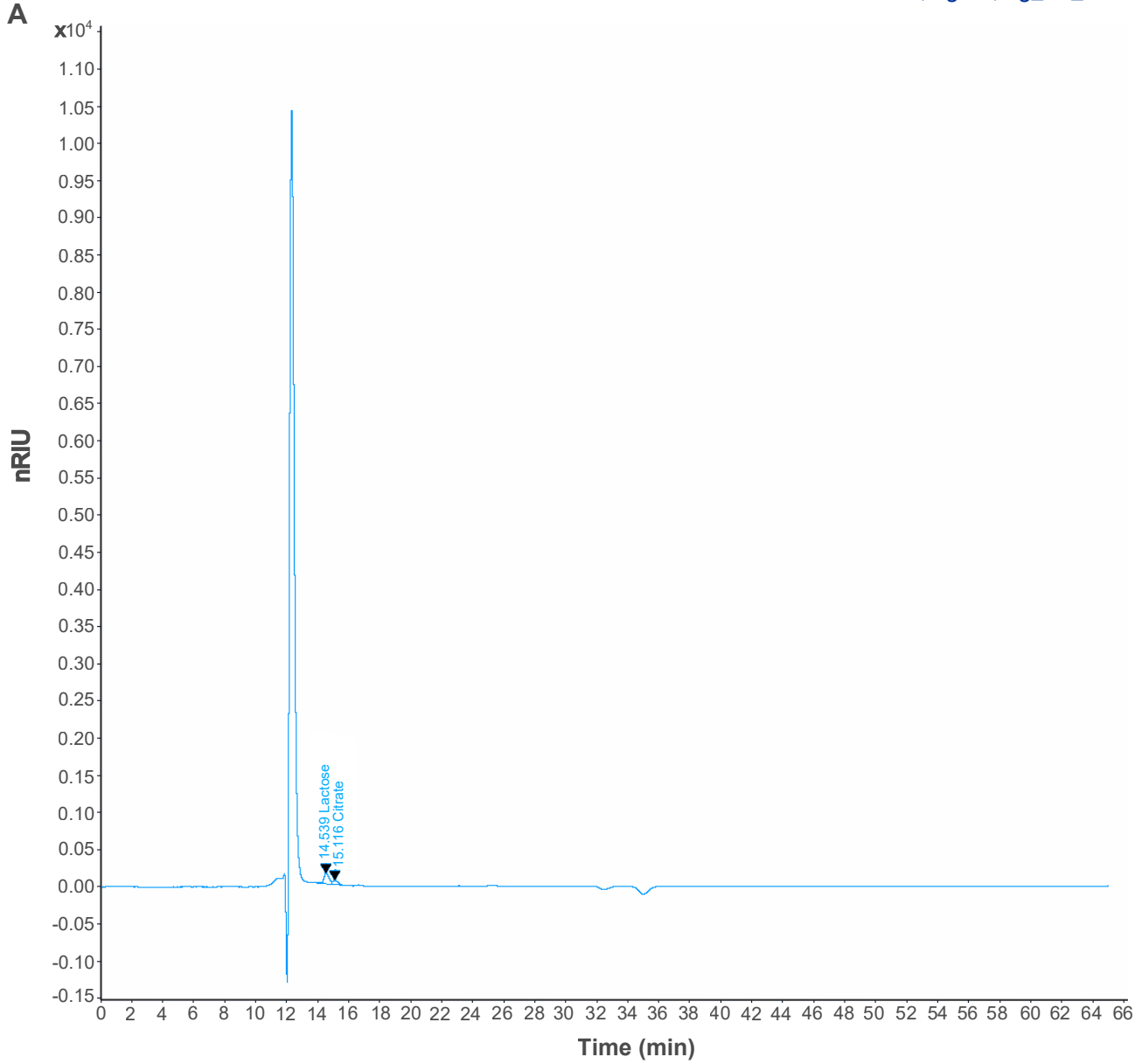
A



B

Age	Citrate	Lactate	Acetate	Propionate	Butyrate	Succinate	Ethanol	Lactose	Glucose
0	0.017	0	0	0.054	0	0	0	0.033	0
43	0.015	0	0	0.017	0	0	0	0.032	0
46	0.015	0	0	0	0	0	0	0.036	0
50	0.016	0	0	0	0	0	0	0.036	0
54	0.014	0	0	0	0	0	0	0.032	0

Figure S3: Organic acids, ethanol, lactose, and glucose concentrations measured by liquid chromatography in mixed adapted cultures. (A) Chromatogram for the six-week mixed adapted culture used in the study (age 46 days post inoculation of bubble column reactor with digestate sample). (B) Concentrations for mixed adapted cultures of different ages (0 to 54 days post inoculation) including that used in the study (in blue, 46 days).

Declaration of interests

☐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.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Pierre Fontanille has patent #Procede de biomethanation in situ Number FR2211561 pending to neither licensee nor assignee. The authors declare that the strain Methanothermobacter marburgensis strain Clermont presented in this work was deposited according to the Budapest Treaty (rule 11.2, option 1) for the purposes of patent (filed on 7 november 2022). The authors declare that they have no known competing interests. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.