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Strain-resolved metagenomics approaches applied to biogas upgrading

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ABSTRACT

Genetic heterogeneity is a common trait in microbial populations, caused by de novo mutations and changes in variant frequencies over time. Microbes can thus differ genetically within the same species and acquire different phenotypes. For instance, performance and stability of anaerobic reactors are linked to the composition of the microbiome involved in the digestion process and to the environmental parameters imposing selective pressure on the metagenome, shaping its evolution. Changes at the strain level have the potential to determine variations in microbial functions, and their characterization could provide new insight into ecological and evolutionary processes driving anaerobic digestion. In this work, single nucleotide variant dynamics were studied in two time-course biogas upgrading experiments, testing alternative carbon sources and the response to exogenous hydrogen addition. A cumulative total of 76,229 and 64,289 high-confidence single nucleotide variants were discerned in the experiments related to carbon substrate availability and hydrogen addition, respectively. By combining complementary bioinformatic approaches, the study reconstructed the precise strain count—two for both hydrogenotrophic archaea—and tracked their abundance over time, while also characterizing tens of genes under strong selection. Results in the dominant archaea revealed the presence of nearly 100 variants within genes encoding enzymes involved in hydrogenotrophic methanogenesis. In the bacterial counterparts, 119 mutations were identified across 23 genes associated with the Wood-Ljungdahl pathway, suggesting a possible impact on the syntrophic acetate-oxidation process. Strain replacement events took place in both experiments, confirming the trends suggested by the variants trajectories and providing a comprehensive understanding of the biogas upgrading microbiome at the strain level. Overall, this resolution level allowed us to reveal fine-scale evolutionary mechanisms, functional dynamics, and strain-level metabolic variation that could contribute to the selection of key species actively involved in the carbon dioxide fixation process.

1. Introduction

Environmental pollution is a significant global concern having a severe impact on the planet. One of the major contributors is the excessive emission of carbon dioxide (CO_2) into the atmosphere. The increasing concentration of CO_2 in the atmosphere has led to a significant rise in global temperatures, causing climate change, and resulting in catastrophic consequences for the environment and human health. The sources of CO_2 pollution are diverse, including the burning of fossil fuels for transportation, electricity, and heating, as well as deforestation, industrial processes, and agriculture. The growth of urbanization and population has also played a role in increasing CO_2 emissions. Reducing CO_2 emissions requires significant changes in energy production and consumption, as well as efforts to promote sustainable practices in agriculture and industry.

The Carbon Capture and Utilization (CCU) concept has gained significant attention in recent years as a crucial solution in mitigating greenhouse gas emissions and addressing climate change (Sabri et al., 2021). CCU can also target gaseous streams derived from industrial processes and convert them into useful products. Among the various applications of CCU, the green biogas generated through the anaerobic digestion of organic matter, including agricultural waste, food waste, and sewage sludge emerges as a highly encouraging and viable source of renewable energy (Deena et al., 2022).

The biological process upon which CCU relies is the Anaerobic Digestion (AD), a microbial-mediated process in which complex compounds such as organic wastes and agricultural residues are converted into methane. Biogas upgrading (BU), the process of removing CO_2, and biogas cleaning from impurities such as hydrogen sulfide, are critical for the wider adoption of biomethane as a replacement for traditional fossil fuels (Angelidaki et al., 2018; Tabatabaei et al., 2020a, 2020b). The removal of unwanted compounds is important to increase biogas energy content, since a methane content around 60–65% corresponds to a

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Abbreviations

Carbon capture and utilization (CCU)
Anaerobic Digestion (AD)
Carbon dioxide (CO_2)
Hydrogen (H_2)
Biogas upgrading (BU)
Syntrophic acetate-oxidizing bacteria (SAOB)
Single-nucleotide variants (SNVs)
Relative abundance (RA)

Lower Calorific Value of 20–25 MJ/m^3-biogas. On the other hand, in biomethane (>95% methane) this metric reaches values up to 36 MJ/m^3-biogas, thus representing a higher fuel standard (Sun et al., 2015a). Moreover, by removing biopollutants such as hydrogen sulfide and siloxanes helps prevent damage to combined heat and power units.

BU technologies are divided into three categories: physical, chemical and biological (Angelidaki et al., 2018). In general, physical approaches are based either on absorption (Bauer et al., 2013), adsorption (Augelletti et al., 2017) or membrane separation (Bauer et al., 2013), all reaching almost pure biomethane. Chemical hydrogenation process, thanks to its high selectivity, allows a complete conversion of H_2 to methane (Jürgensen et al., 2014). However, while both technologies are highly efficient, they necessitate specific materials (e.g., chemicals, pure gasses, membranes) and incur high energy costs for maintaining operational conditions. On the other hand, biological BU represents a more sustainable solution since CO_2 is directly converted into methane, contributing significantly to a circular bio-based economy. More into detail, biological approaches are either photosynthetic (Muñoz et al., 2015; Bahr et al., 2014), where cyanobacteria or microalgae are employed in photobioreactors for biomethane production, or chemoautotrophic (Kougias et al., 2017), in which a pure or mixed microbiota is responsible the conversion of H_2 and CO_2 into methane. To ensure the sustainability of biological upgrading, the H_2 needed should come from renewable sources (Kim et al., 2013). Utilizing surplus electricity from wind or solar panels for water electrolysis and H_2 production became an established solution, known as power to gas.

A key role in the chemoautotrophic production of biogas through the AD of organic matter is played by methanogens. They are fundamental for the CO_2 methanation, and, in particular the hydrogenotrophic archaea use hydrogen (H_2) as an energy source (Lai et al., 2021) to perform the methanogenesis. During the BU process, these microorganisms play a crucial role in regulating H_2 concentration and actively contribute to the removal of sulfur and acetate through a synergistic relationship with sulfate-reducers (Barton and Fauque, 2009) and syntrophic acetate-oxidizing bacteria (SAOB) (Pan et al., 2021; Zhu et al., 2017). The performance and stability of BU are linked to the composition of the microbiome responsible for the process. *De novo* mutations determine the generation of new strains in the population which can be positively selected over time determining an increase in the frequency of the corresponding variant. As a consequence, variants can fluctuate in frequency, and sometimes become fixed over time. Single Nucleotide Variants (SNVs) represent a form of genetic mutation arising from an alteration in the DNA involving a single base pair change. They can be classified according to their effect on the encoded amino acid as synonymous and nonsynonymous.

SNVs can exert a significant influence on the metabolism and on the functional potential of microbes (Garud et al., 2019; Roodgar et al., 2021). In particular, nonsynonymous SNVs are more likely to result in modification at phenotypic level, since they can alter the protein structure and result in gain or loss of functions. Changes of environmental conditions can arise from various factors, imposing selective pressures on the microbiomes during biogas production. Such perturbations can lead to the selection of strains harboring new mutations. For example, the addition of feedstock, the introduction of inhibitory compounds (ammonia, carbon monoxide, etc.) and changes in environmental conditions (pH, temperature, etc.) can drive the selection of new strains with distinct functional properties. These newly emerged strains may display enhanced metabolic capabilities, thereby potentially improving the effectiveness of the biogas generation process.

Previous strain-level studies were exclusively based on cell isolation and phenotypic analysis, however, novel approaches based on metagenomics have been recently developed (Table 1). At the moment, the state of the art is represented by two different strategies: variant calling (Nayfach et al., 2016; Olm et al., 2021; Truong et al., 2017) and strain deconvolution (Luo et al., 2015; Quince et al., 2021; Smith et al., 2022). The first method relies on using shotgun metagenomic reads to track strains by looking for distinct patterns of alleles observed across SNVs within the species. Instead, the second strategy consists in a statistical deconvolution of allele frequencies from shotgun metagenomic data into strain genotypes. These two approaches are complementary and together enable the tracking of strains, assessing their abundance, and observing their evolution while considering their susceptibility to environmental conditions. Moreover, variant analysis allows the evaluation of the metabolic pathways under positive selection, uncovering mechanisms that drive the evolution and dominance of one strain over the others (Roodgar et al., 2021).

Although in recent years many metagenomic studies investigated the AD process (Campanaro et al., 2020), there is still very limited knowledge regarding the number of strains and their evolution. To the best of our knowledge, the sole precedent in the existing literature pertains to a study conducted on the response of gut microbial communities to antibiotic perturbations (Roodgar et al., 2021). The tracking of SNVs trajectories over time enabled a comparison between genetic dynamics and ecological fluctuations occurring at the species level following the imposition of stressors. The genetic changes observed by Rodgar were also in species which did not experience a shift in abundance, highlighting the importance of monitoring the diversity beyond the species-level (Roodgar et al., 2021). For this reason, the study of the impact of SNVs in hydrogenotrophic archaea has the potential to reveal new insights into the genetic heterogeneity of these microorganisms and its impact on BU.

In this study, we aim to investigate the impact of SNVs on the functional properties of hydrogenotrophic archaea in green BU. Two previous experiments were chosen as caste studies: a test of different feedstock substrates (PRJNA999073), and the effect of prolonged H_2 addition to the mixed microbial community (Zhu et al., 2020). The investigation will analyze the strains' responses to these conditions and uncover the potential effects of variants on the metabolic mechanisms. This work aims to offer a more comprehensive insight into the genetic heterogeneity of a metagenome and the impact of mutations on the BU process.

2. Materials and methods

2.1. Datasets used in the analysis

The current work considered two case studies with different set-ups: an experiment previously performed to test different feedstock substrates in batch configuration (PRJNA999073), and another where exogenous H_2 was added to three continuous stirred-tank reactors (Zhu et al., 2020). The shotgun reads were downloaded from SRA, project PRJNA999073 and PRJNA525781. Details regarding the experimental setup and measurements are reported in the original publication.

The first involved an experiment focusing on testing feedstock substrates using a batch configuration. The experimental setup included three different substrates (90% acetate and 10% H_2CO_2, 100% H_2CO_2, and a 1 to 1 M ratio of the two) to study the effect on a microbial community. The inoculum was taken from a trickle-bed reactor, then

Table 1
Summary of state-of-the-art tools available for variant analysis and strain identification in MAGs.

Tool	Type	Algorithm	Tested on	Websites
BCFtools	Variant calling	Probabilistic	Human and mock communities	https://github.com/samtools/bcftools
FreeBayes	Variant calling	Probabilistic	Not available	https://github.com/freebayes/freebayes
MetaSNV	Variant calling	Pool population	Marine and fecal metagenomes	https://github.com/metasnv-tool/metaSNV
Instrain	Variant calling	Pool population	Gut microbiome	https://github.com/MrOlm/inStrain
Mutec2	Variant calling	Probabilistic	Human and mock communities	https://github.com/broadinstitute/gatk
HaplotypeCaller	Variant calling	Probabilistic	Human and mock communities	https://github.com/broadinstitute/gatk
STRONG	Strain identification	Strain deconvolution	Mock communities	https://github.com/chrisquince/STRONG
StrainGE	Strain identification	Strain deconvolution	Gut and mock communities	https://github.com/broadinstitute/strainge
StrainFinder	Strain identification	Maximum-likelihood on SNP frequencies	Gut microbiome	https://github.com/cssmillie/StrainFinder
ConStrains	Strain identification	Strain separation using SNP patterns	Gut and mock communities	https://doi.org/10.1038/nbt.3319
Strainberry	Strain identification	Strain separation using long reads	Mock communities	https://github.com/rvicedomini/strainberry
DESMAN	Strain identification	Frequency count on contigs	Marine and mock communities	https://github.com/chrisquince/DESMAN
StrainPanDA	Strain identification	Pangenome decomposition	Gut microbiome	https://github.com/xbiome/StrainPanDA

cultured in a synthetic medium to produce a simplified community (G0). This community was used to run three parallel batch reactors (G1) and, after 14 days, a second generation (G2) was established by reinoculating the G1 cultures. Samples were collected from the initial G0 community, from each condition at day 13 from G1, and days 7 (TP1) and 9 (TP2) from G2.

The second case study involved the addition of exogenous H_2 to three lab-scale continuous stirred-tank reactors to simulate a BU process (Zhu et al., 2020). They were inoculated with digestate from a full-scale thermophilic biogas plant and operated under thermophilic conditions (55 °C) with a synthetic medium containing acetic acid as the sole carbon source. Once steady state was reached, H_2 gas was added to each reactor at a rate of 1 mL/min. To optimize H_2 utilization, the gas phase was constantly recirculated into the liquid phase using peristaltic pumps. Liquid samples were collected from the three reactors before H_2 addition (TP1), 18 h after (TP2), and 36 days after (TP3).

2.2. Metagenomic data analysis

A previously described genome-centric metagenomic pipeline (Zampieri et al., 2023) was applied to recover the microbial genomes. Reads were filtered with Trimmomatic v0.39 (Bolger et al., 2014) to eliminate adapters or low-quality bases, and checked for contamination with BBDuck v38.93. Paired reads were merged with BBMerge v38.93 (Bushnell et al., 2017). Short-read co-assembly was performed with Megahit v1.29 (Li et al., 2015). Metagenome-assembled genome gain procedure was performed using multiple binning software as previously reported (Zampieri et al., 2023), specifically including Concoct v1.1.0 (Alneberg et al., 2014), MaxBin v2.2.7 (Wu et al., 2016a), MetaBAT1, MetaBAT2 v2.15 (Kang et al., 2019), and VAMB v3.0.2–1 (Nissen et al., 2021). The coverage profiles used for the binning approaches were generated using Bowtie2 v2.4.5 (Langmead and Salzberg, 2012) and SAMtools v1.16.1 (Danecek et al., 2021). MAGs underwent de-replication and were aggregated using dRep v3.4.0. (Olm et al., 2017). To evaluate the quality of the MAGs and determine their relative abundance (RA), CheckM v1.2.1 was employed (Parks et al., 2015). For taxonomic classification, GTDB-Tk v2.1.0 (Chaumeil et al., 2020) was utilized (database version R214). The MAGs were assigned identifiers based on the taxonomic level and binning tool used. Additionally, a progressive number was incorporated into the final name. Alpha-diversity was calculated with the Phyloseq v1.40.0 R package using the mapped reads counts on the MAGs (McMurdie and Holmes, 2013).

2.3. Strain-level metagenomics

The software InStrain v1.6.3 (Olm et al., 2021) was applied to the high quality (Bowers et al., 2017) MAGs of both experiments to perform variant analysis. The InStrain profile module requires a FASTA file containing the MAGs, individual BAM files for each sample, a scaffold-to-bin file, and a gene annotation file. Optional parameters were set as follows: -min_mapq 2, –min_read_ani 0.98 and –min_genome_coverage 1. The most interesting MAGs were selected based on coverage and variant metrics, and a strain deconvolution pipeline was applied using STRONG (Quince et al., 2021). The pipeline used SPAdes for assembly (Prjibelski et al., 2020), CONCOCT for binning (Alneberg et al., 2014), and BayesPaths for graph disentangling (Quince et al., 2021). The default parameters were used for the assembly and binning, while for the Bayesian algorithm, nb_strains was set to 5, max_giter to 4, and nft_runs to 10. The abundances of the deconvoluted strains were defined taking into account the RA of the corresponding MAG.

2.4. Variant selection and phasing

The results obtained from the variant calling using InStrain were processed in order to remove low confidence results. First, all the SNVs located within 150 bp from the 3′ and 5′-end of each scaffold were removed, since in those genomic regions the coverage tends to drop along with the results reliability. Then, all the variants where the difference between the coverage of the SNV and the average coverage of the scaffold exceeded the interval [−100; +100] were discarded. Lastly, the ratio between the number of reads supporting the variant allele and the one supporting the reference allele was computed and all the SNVs where this ratio was lower than 0.15 were discarded. This procedure led to the removal of about 12–20% of the initial number of SNVs detected. After the selection, the variants were clustered together based on their frequency over time. The approach was performed using the scipy.stats library of Python, which is based on a hierarchical clustering algorithm. The Ward distance was used as a metric for calculating variant similarity.

2.5. Gene annotation, metabolic reconstruction and functional impact of the variants

Gene prediction was performed using Prodigal v2.6.3 (Hyatt et al., 2010), and functional annotation was carried out using eggNOG-mapper v2.1.9 (Cantalapiedra et al., 2021). The reference database used was the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2023), consulted for enzyme class, orthology and metabolic pathways. Functions were defined according to the KEGG annotation. The KEGG orthologs obtained from the gene annotations were used to reconstruct the hydrogenotrophic pathway (Evans et al., 2019) for the dominant archaea and the canonical (Westerholm et al., 2016) and alternative (Song et al., 2020; Nobu et al., 2015) Wood-Ljungdahl (WL) pathways for the bacteria. The detected variants were linked to the genes they affect, and the Grantham distance (Grantham, 1974) between the reference amino acids and the mutated ones was evaluated. This metric measures the physical and chemical differences between two amino acids and ranges from 5 to 210. Variants with a distance above 70 were considered to have a medium to high putative impact on the protein.

3. Results and discussion

In this investigation, we considered two discrete case studies pertaining to BU, each possessing its unique experimental configuration. These studies were selected based on their inherent incorporation of two prerequisites essential for strain-resolved metagenomic analysis: first, the implementation of a time-course experimental design encompassing multiple timepoints, second the application of selective pressure capable of shaping the microbiome composition. In both experimental scenarios, the primary impetus driving genetic evolution was a shift in carbon source utilization, compelling the dominant microbial strains to adapt towards a novel equilibrium state.

3.1. Microbial community composition

The average alignment rate of the shotgun reads to the global assembly was around 90% for both case studies, confirming that the microbial community was almost entirely represented. The high alignment rate was probably due to the simple microbial community resulting from the application of the synthetic medium. The community of the first experiment consisted of 47 high-quality MAGs, with Firmicutes accounting for 93% of the total (Supplementary Table 2). The 12 MAGs with a RA higher than 1% accounted on average for 94% of the community (Fig. 1a). The only archaeon present was *Methanothermobacter wolfeii* MA_1, which was also the most abundant species, with a mean RA of 84%. The primary methanogenic pathway for *M. wolfeii* is the reduction of CO_2 to methane, however in the absence of H_2 it is also able to use alternative electron donors such as acetate, formate and methanol for methanogenesis (Lins et al., 2012). The most abundant bacteria were *Sphaerobacter thermophilus* CO_9, *Caldanaerobacter subterraneus* MX_27 and Limnochordia sp. MA_37, each accounting for 2% of RA on average.

The second experiment's assembly and binning process resulted in 50 high-quality MAGs assigned to 13 phyla and Firmicutes was the most represented, accounting for 50% of the community (Supplementary Table 3). There were 31 MAGs with a RA higher than 1%, and together, they accounted for 87–94% of the community in each sample (Fig. 1b). Among the 5 detected archaea, the dominant ones were *Methanosarcina thermophila* MB_65 and *Methanocullus thermophilus* MA_62, both with a RA above 1% in all timepoints. The two archaea have different metabolic preferences and characteristics, with *M. thermophila* preferring acetate as a substrate (Zinder and Anguish, 1992) and *M. thermophilus* being a known hydrogenotrophic (Zhu et al., 2019). This difference in metabolism is reflected in the RA trend, with *M. thermophilus* MA_62 being positively affected by the addition of H_2. Among the bacterial species, Limnochordia sp. MB_100, Bacteroidales sp. VB_122 and *Acetomicrobium* sp. MX_67 showed the highest mean RA, respectively 10%, 8% and 7%.

In both experiments, the environmental parameters are favorable for the establishment of a cooperative coexistence of archaea and bacteria, which interact through the activity of methanogenesis and the conventional (Westerholm et al., 2016) and alternative WL (Song et al., 2020; Nobu et al., 2015) pathways. The high abundance of both *M. wolfeii* MA_1 and *M. thermophilus* MA_62 in their respective communities is likely the result of a symbiotic relationship with acetate oxidizing bacteria. Despite both experiments having acetate as the initial substrate, the microbial composition was markedly diverse (Fig. S1). These disparities primarily arise from variations in the dominant archaeal populations, potentially driven by alternative substrate preferences, as well as differences in the experimental setup and medium composition employed, as previously demonstrated by Sun et al. in their study (Sun et al., 2015b).

In the BU framework it is extremely interesting to note how such dynamics in microbial populations can be observed, despite the overall setup being convergent on the CO_2 methanation process. Furthermore, alpha-diversity highlighted that the first community is much less complex than the second one. Both the Shannon's and Simpson's diversity indexes were higher in the H_2 addition experiment (PRJNA525781), indicating a greater variety of species (Fig. S2). This finding is further supported by a reduced count of observed species in the carbon substrates experiment (PRJNA999073), as determined by the Chao1 index (Supplementary Table 4).

Investigating the microbial communities at the species level in both experiments is crucial, as it reveals the favorable environmental conditions for cooperative coexistence of archaea and bacteria, their symbiotic relationships, and variations in dominant populations. However, the assessment of a species' RA alone is not enough to understand the genetic changes within a given species (Olm et al., 2021). In fact, RA stability is not directly related to the number or to the abundance of the strains, and genetic alterations may still occur leading to the emergence of new strains (Roodgar et al., 2021). This study investigated species-level genetic modifications by examining SNVs in MAGs with a RA of at least 1%, determining the strains and tracking their dynamics through the shifts of SNVs frequency over time. The quantity of species-specific SNVs identified via variant calling closely resembled the findings reported by Rodgar (Roodgar et al., 2021). More specifically, approximately 10,000 SNVs were consistently monitored over time in *Eubacterium eligens*. Similarly, in the reconstructed MAGs of the two case studies, the figures were within the same order of magnitude, ranging between 1000 and 4000 nonsynonymous SNVs for the highly abundant MAGs (Fig. 1).

3.2. Variant selection determined by the shift in carbon substrates availability

In the first case study, a total of 76,229 SNVs were identified considering the subset of MAGs reported in Fig. 1a. The SNVs were further divided into synonymous (56%), nonsynonymous (30%), and intergenic (14%) variations. The variants' distribution revealed substantial differences in the genetic composition of the MAGs analyzed. At the initial stage (G0) most of the nonsynonymous SNVs were associated with *S. thermophilus* CO_9, *M. wolfeii* MA_1 and Limnochordia sp. MA_37, with counts of 2893, 491, and 731, respectively (Fig. 1a). Furthermore, the comparison between RA and the temporal accumulation of SNVs revealed that species with a greater number of variants consistently maintained dominance across all observed generations. Subsequently, nonsynonymous SNVs were analyzed to unveil potential functional impact on key genes involved in methanogenesis and carbon fixation pathways.

In order to evaluate the distribution of variants within the microbiome under investigation, a variant phasing approach was employed by clustering SNVs frequency, and comparing this information with the number of strains and their RA. This approach allows clustering together SNVs that are possibly belonging to the same strain, thus identifying those located on the same bacterial chromosome, and tracking the transmission of genetic variants over time. SNV phasing in *M. wolfeii* MA_1 revealed two distinct clusters, one with high frequency in the first two generations, and the other starting at low frequency (around 0.3) and increasing from G1 to G2 (Fig. 2a). This finding is supported by the strain deconvolution (Fig. 2b): *M. wolfeii* MA_1 was represented by two strains in all reactors, with strain str1 dominating during G0 and G1, then being replaced by str0, which experienced a rapid increase in abundance between G1 and G2. This shift after the environmental modification is likely due to strain str0 having a metabolic advantage over str1.

A total of 248 positively selected SNVs were mapped to the genes to investigate whether the increased abundance of strain str0 was associated with an accumulation of genetic modifications in hydrogenotrophic methanogenesis enzymes (Evans et al., 2019). In reactor A (90% acetate and 10% H_2CO_2), variants having an increased frequency in the population at G2 were found in three crucial proteins: heterodisulfide reductase (*hdrB2*), methyl-coenzyme M reductase (*mcr*), methenyltetrahydromethanopterin cyclohydrolase (*mch*) and coenzyme F420

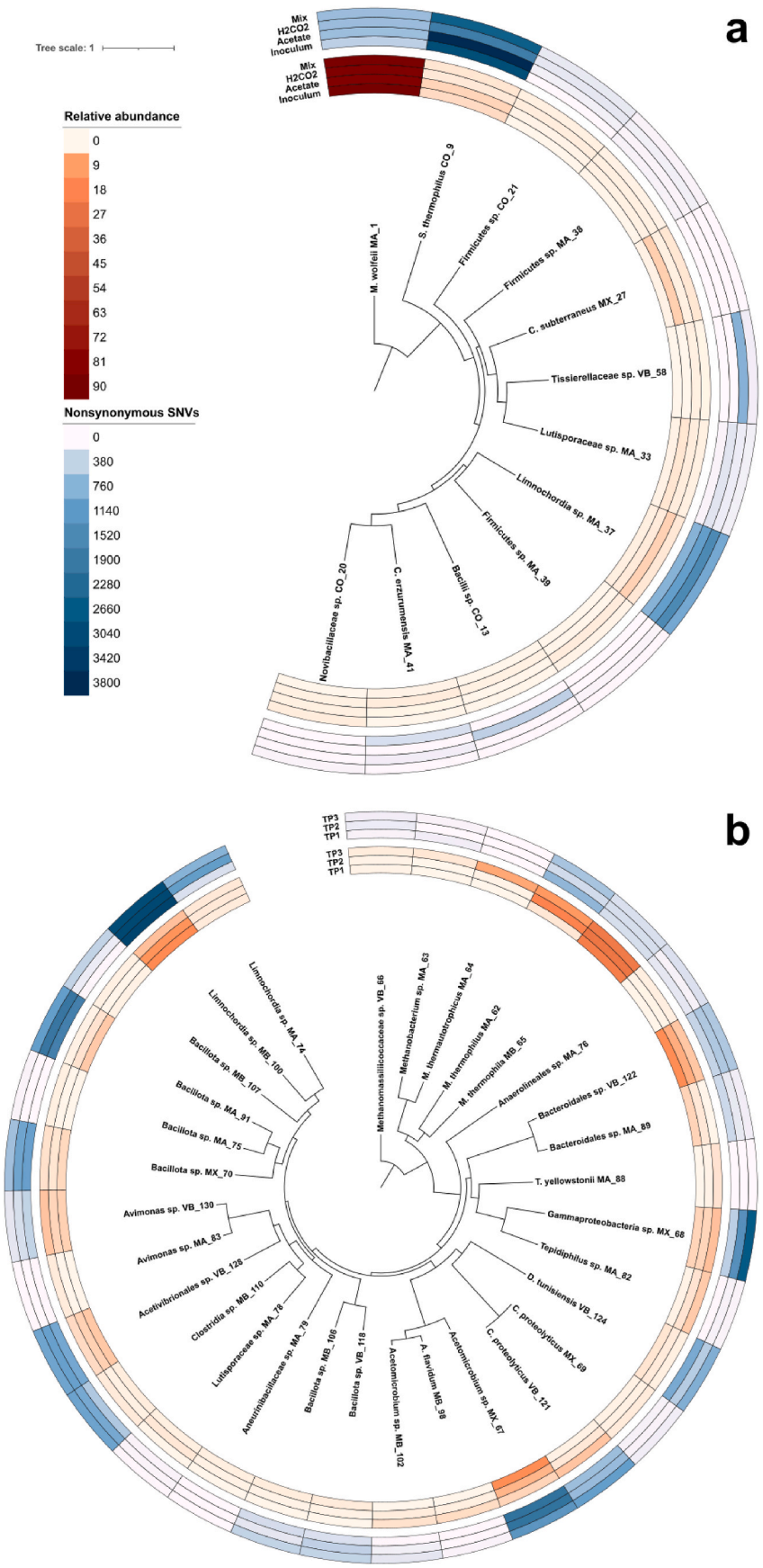

Fig. 1. Overview on the identified microbial taxa, RA across the considered reactors (orange) and corresponding number of nonsynonymous SNVs (blue). Only taxa with RA > 1% are represented in the phylogenetic tree. (a) Results for the experiment in which feedstock conditions were reported as average RA for acetate, H_2CO_2 and Mix. (b) Results for the exogenous H_2 addition experiment were reported as average RA for TP1, TP2 and TP3.

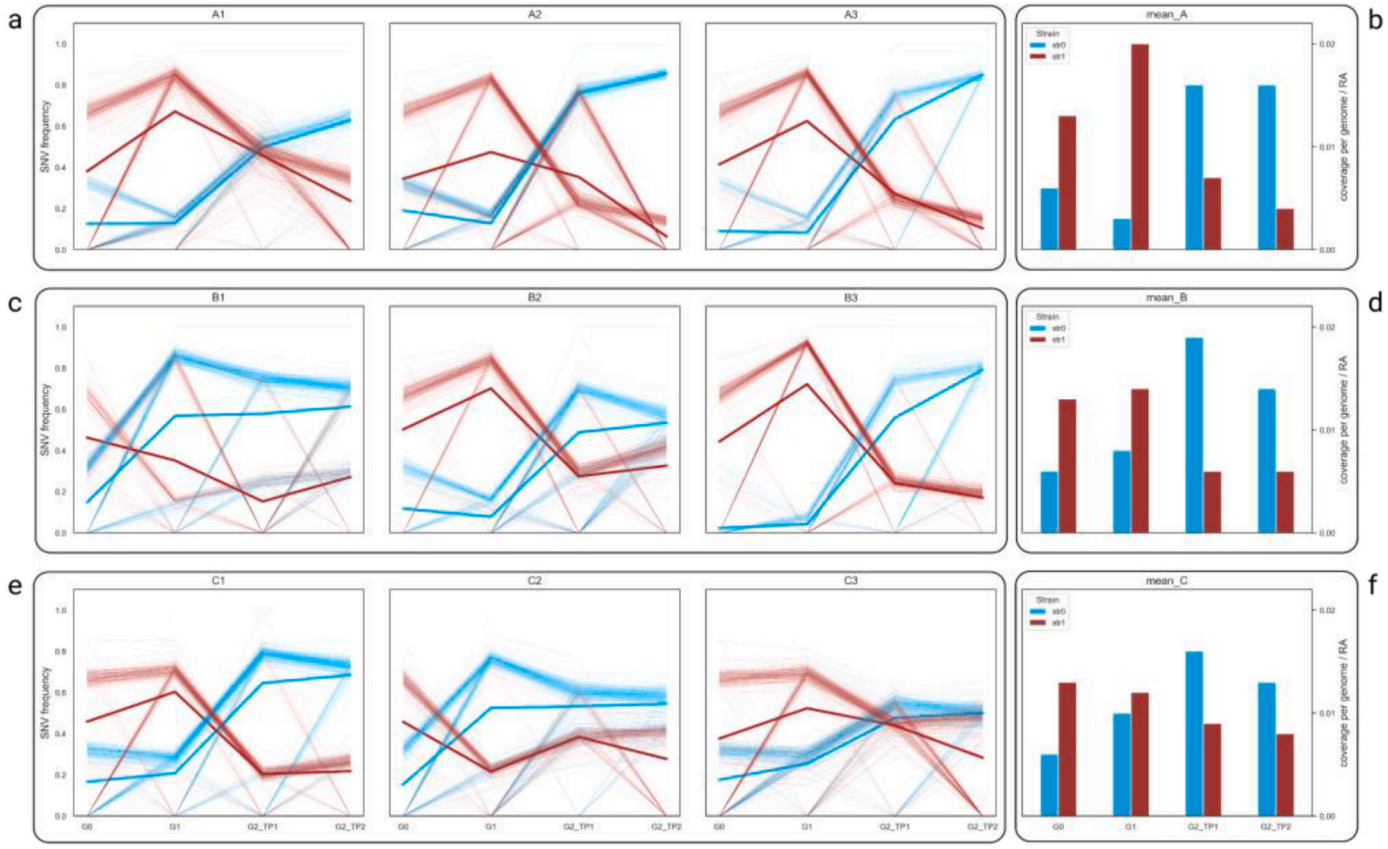

Fig. 2. Frequency of nonsynonymous SNVs over time and strain deconvolution results for *M. wolfeii* MA_1. (a,c,e) SNVs frequency at different time points for the reactors fed with 90% acetate and 10% H_2CO_2 (A1-A3), for those fed with 100% H_2CO_2 (B1–B3) and for those fed with 50% acetate and 50% H_2CO_2 (C1.C3). (b,d,f) Strains relative abundance for reactors A, B and C. The abundance was weighted by the RA of the MAG and the average value for the three replicates was reported.

hydrogenase (*frh*) (Fig. 3). Additionally, three genes encoding subunits of energy-converting hydrogenase A (*ehaL*, *ehaG*, *ehaR*) were also harboring SNVs (Fig. 3). This hydrogenase plays a role in energy conservation, catalyzing the H_2-dependent reduction of the ferrodoxin (Lie et al., 2012), thus providing electrons to fix CO_2. In reactor B (100% H_2CO_2), alongside the genes identified in reactor A, additional genes were linked to SNVs, including formylmethanofuran dehydrogenase (*fwd*), the V/A-type H^+/Na^+-transporting ATPase (*atpv*A), and another subunit of heterodisulfide reductase (*hdrA2*) (Fig. 3). In reactor C (1:1 acetate and H_2CO_2) the affected genes were identical to those identified in reactor B, plus one extra subunit of methyl-coenzyme M reductase (*mcrD*) and V/A-type ATPase (*atpv*I).

The hydrogenotrophic pathway was under selective pressure in all reactors, since several genes aforementioned had a dN/dS ratio >1 (Supplementary Table 5), suggesting the presence of one or more SAOB capable of converting acetate through the WL pathway (Nobu et al., 2015). Reactors A and C showed 5% of positively selected SNVs associated with genes involved in the putative syntrophic metabolisms of Limnochordia sp. MA_37 (Fig. S3). Specifically, variants were located in both acetate kinase (*ackA*) and formate dehydrogenase (*fdh*) genes (Fig. 3). Additionally, SNVs were identified in the enzymes involved in the conversion of pyruvate to 5,10-methenyltetrahydrofolate, including pyruvate formate lyase (*pfl*, *pflA*), pyruvate-ferredoxin/flavodoxin oxidoreductase (*por*), L-serine dehydratase (*sda*) and methylenetetrahydrofolate dehydrogenase (*folD*) (Fig. 3).

It is worth speculating that the presence of these variants may be associated with the existence of a syntrophic relationship between *M. wolfeii* MA_1 and Limnochordia sp. MA_37. Moreover, a potential syntrophy between *M. wolfeii* and Limnochordia spp. has already been suggested by flux balance analysis applied to BU (De Bernardini et al., 2022). Overall, results highlight the potential impact of SNVs in enhancing the methanogenic capability of *M. wolfeii* MA_1, as indicated by a Grantham distance exceeding 70 in several instances (Supplementary Table 6). However, further investigations will be needed to clarify the mechanistic impact of variants on protein functions and the activity of the hydrogenotrophic pathway.

3.3. Variants selection in continuous stirred tank reactors upon exogenous H_2 addition

In the second case study, 64,289 SNVs were identified in MAGs of Fig. 1b: 55% were classified as synonymous, 31% nonsynonymous and 13% intergenic. *M. thermophilus* MA_62 and *M. thermophila* MB_65, the two dominant archaea, displayed distinct behaviors both in terms of RA and SNVs dynamics. *M. thermophilus* MA_62 had a high number of SNVs fluctuating over time, ranging between 400 and 1100, while *M. thermophila* MB_65 accumulated around 300 variants in total (Fig. 1b). The SNV frequency for both archaea suggested the presence of multiple strains with different fitness depending on the reactor condition. One group of variants declined in frequency after TP1, while the second one showed an increase from TP1 to TP3. Additionally, de-novo SNVs with high frequency at TP3 were also evidenced (Fig. 4a and c).

The strain deconvolution for *M. thermophilus* MA_62 confirmed the previous findings, with strain str1 being dominant at TP1, but its abundance decreased after the addition of H_2. On the contrary, the abundance of strain str0 rapidly increased after H_2 addition (Fig. 4a). These findings evidenced that H_2 favored the hydrogenotrophic metabolism of *M. thermophilus* MA_62, resulting also in the selection of a new strain, as already proposed by Treu et al. in a previous study (Treu et al., 2018). On the other hand, *M. thermophila* MB_65 did not indicate any

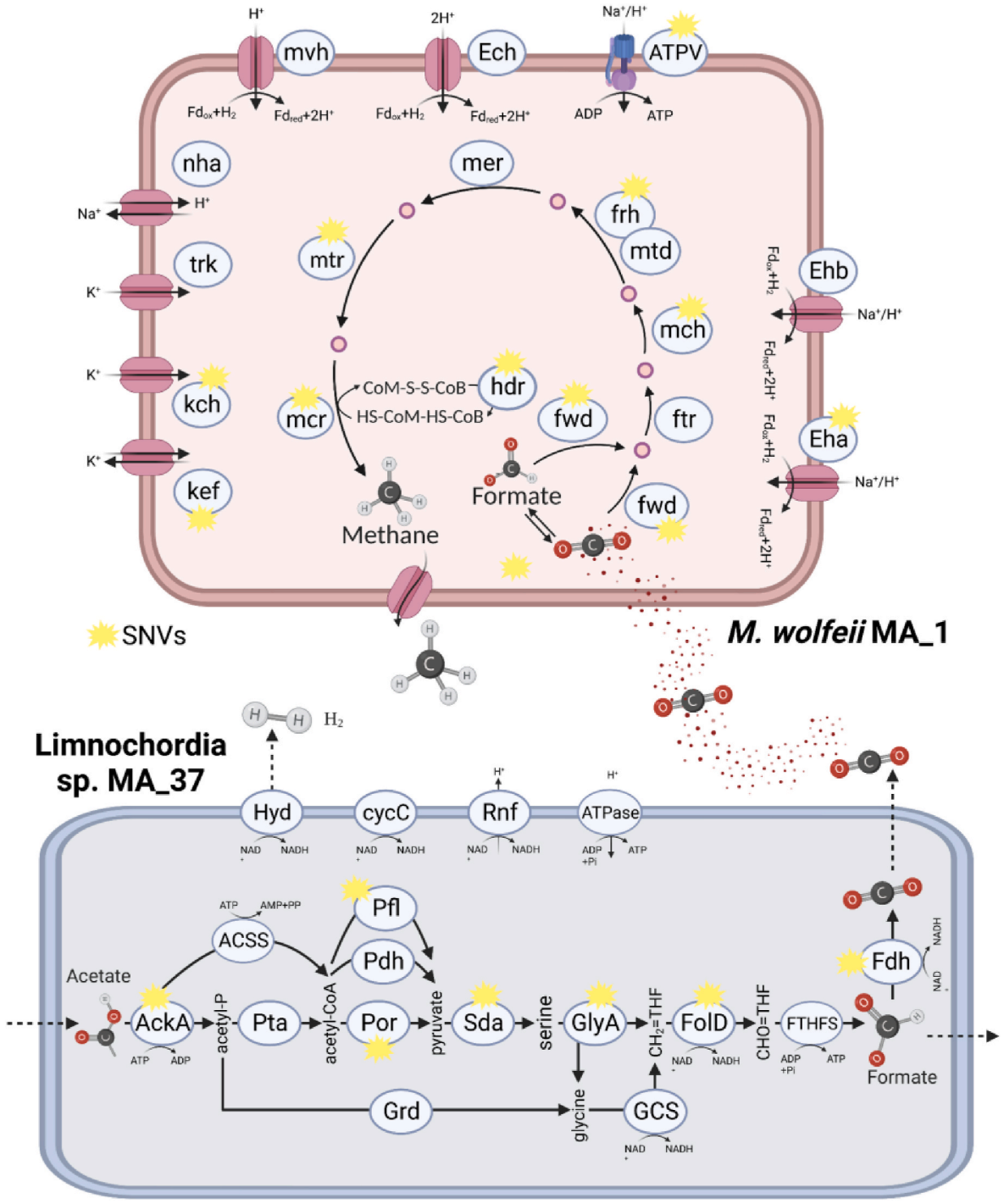

Fig. 3. Genes impacted by SNVs in *M. wolfeii* MA_1 and in the putative SAOB, Limnochordia sp. MA_37. Genes where SNVs have been mapped are highlighted with a yellow identifier. Their association with specific pathways and putative metabolic exchanges is schematically represented in figure. Methane, hydrogen, formate and CO_2 molecules are reported showing their role in the putative syntrophic association.

signs of strain selection. The abundance of the single strain detected remained stable (Fig. 4d), suggesting that the addition of H_2 had no impact on its fitness. This outcome can be explained by the preference of *M. thermophila* for acetate as a substrate to perform methanogenesis (Zinder and Anguish, 1992). Moreover, the shift in frequency observed at TP2, which was not as pronounced as in *M. thermophilus* MA_62, likely resulted from other factors such as random fluctuations or competition with SAOB for acetate utilization.

Additionally, the putative impact of SNVs on the methanogenic activity of *M. thermophilus* MA_62 at high H_2 concentrations was investigated. More specifically, nonsynonymous SNVs having an increased frequency at TP2 were mapped back to the genes. On average 12% of these variants were affecting hydrogenotrophic pathway genes (Evans et al., 2019). Three crucial enzymes carrying SNVs were identified (Fig. 5): methyl-coenzyme M reductase (*mcr*), coenzyme F420 hydrogenase (*frh*), and formylmethanofuran dehydrogenase (fwd). Moreover, variants were also mapped on a subunit of the multicomponent Na^+:H^+ antiporter (*mnhD*). This electrochemical potential-driven transporter is responsible for creating the proton gradient necessary for ATPase activity, thus helping in regulating cell homeostasis (Ito

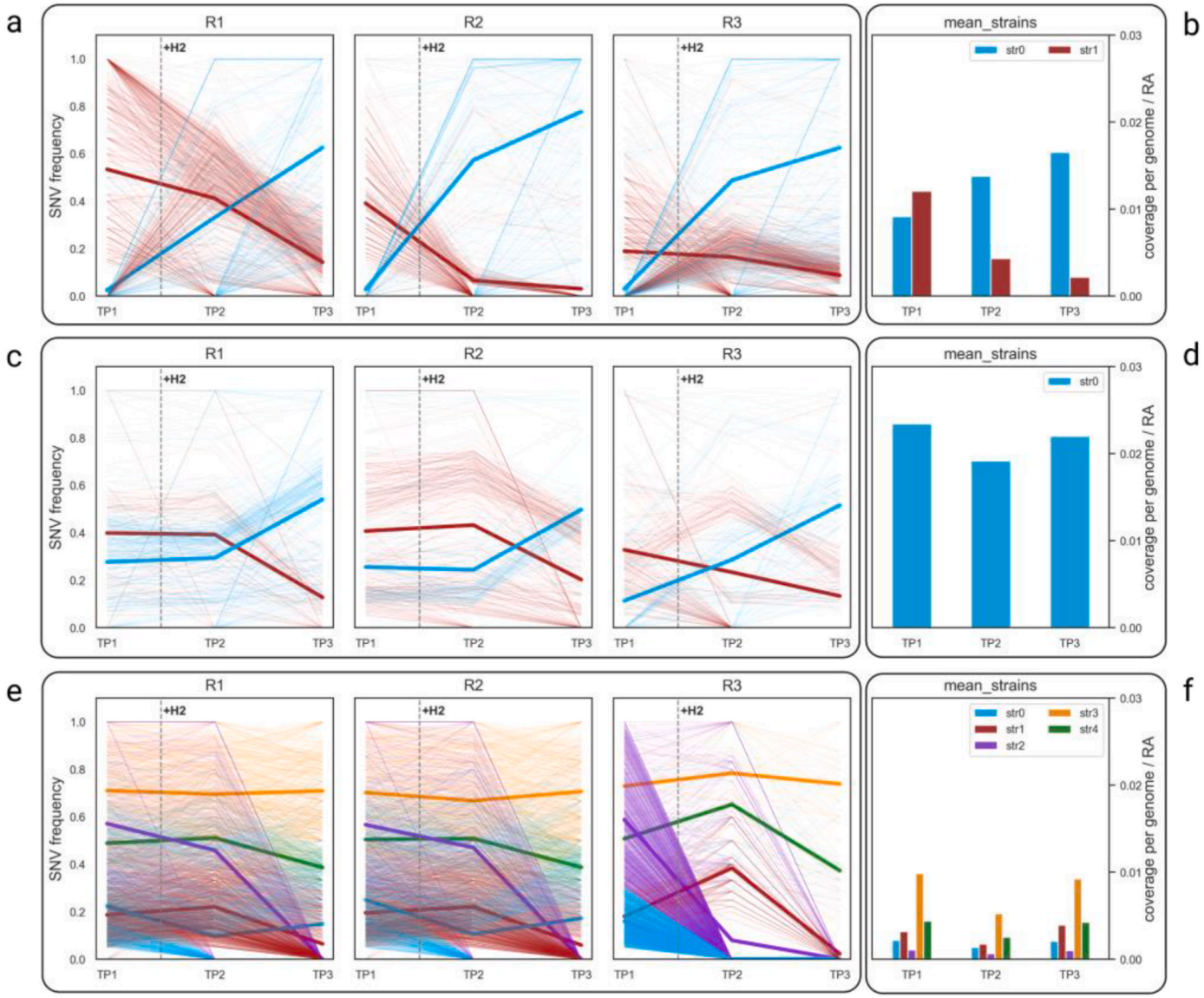

Fig. 4. Frequency of nonsynonymous SNVs over time and strain deconvolution results for *M. thermophilus* MA_62, *M. thermophila* MB_65 and *Acetomicrobium* sp. MX_67. (a,c,e) SNVs frequency at different time points for the reactors R1 (A1-A3), R2 (B1–B3) and R3 (C1–C3). (b,d,f) Strains relative abundance for reactors R1, R2 and R3. The relative abundance of the strains was normalized taking into account the RA of the MAG; the average value for the three replicates was reported in figure.

et al., 2017).

Overall, results suggest that SNVs could play a crucial role in the enhanced methanogenic capability of *M. thermophilus* MA_62. This is evidenced by several genes, i.e. *mcr*, exhibiting a dN/dS ratio >1 (Supplementary Table 5) and multiple variants with Grantham distances above 100 (Supplementary Table 6). These findings highlight the importance of understanding the underlying mechanisms of short- and long-term archaeal adaptation to changing environments. As already suggested in previous studies (Treu et al., 2018; Wu et al., 2016b), an increase in network modularity over time indicates segregation into finer niches and specialized functional units, resulting in decreased overall interactivity.

Variants were also investigated in *Acetomicrobium* sp. MX_67, since manual genome inspection unveiled the presence of a complete gene set for canonical and alternative WL pathways. *Acetomicrobium* spp. have previously been proposed to play a pivotal role within acetate-fed AD systems, particularly in conjunction with *Methanoculleus* spp. (Li et al., 2022). Moreover, a metatranscriptomic analysis validated the active expression of genes associated with acetate metabolism (Singh et al., 2023), thereby reinforcing the hypothesis of its role as candidate SAOB. Specifically, they exhibited on average 3% of positively selected SNVs affecting genes associated with this metabolic route (Nobu et al., 2015). This includes the gene encoding acetate kinase (*ackA*), which converts formate to acetyl-P, as well as the enzymes involved in the synthesis of pyruvate from acetyl-CoA (Fig. 5): pyruvate dehydrogenase (*pdhA*) and pyruvate formate lyase (*pfl*, *pflA*). Additionally, variants were identified across all components of the glycine cleavage system (Ren et al., 2022), a multi-subunit complex working in combination with the WL pathway to fuel the hydrogenotrophic methanogenesis (Fig. 5). Finally, variants were also detected in methylenetetrahydrofolate dehydrogenase (*folD*) and formate-tetrahydrofolate ligase (*fhs*), genes encoding enzymes involved in formate production (Fig. 5). These results suggest that *Acetomicrobium* sp. MX_67 could probably act as SAOB, establishing a syntrophic relationship with *M. thermophilus* MA_62 and competing for acetate with *M. thermophila* MB_65.

4. Conclusions and prospects

This study provided relevant information about the role of SNVs in promoting the adaptation of microbial species to environmental changes

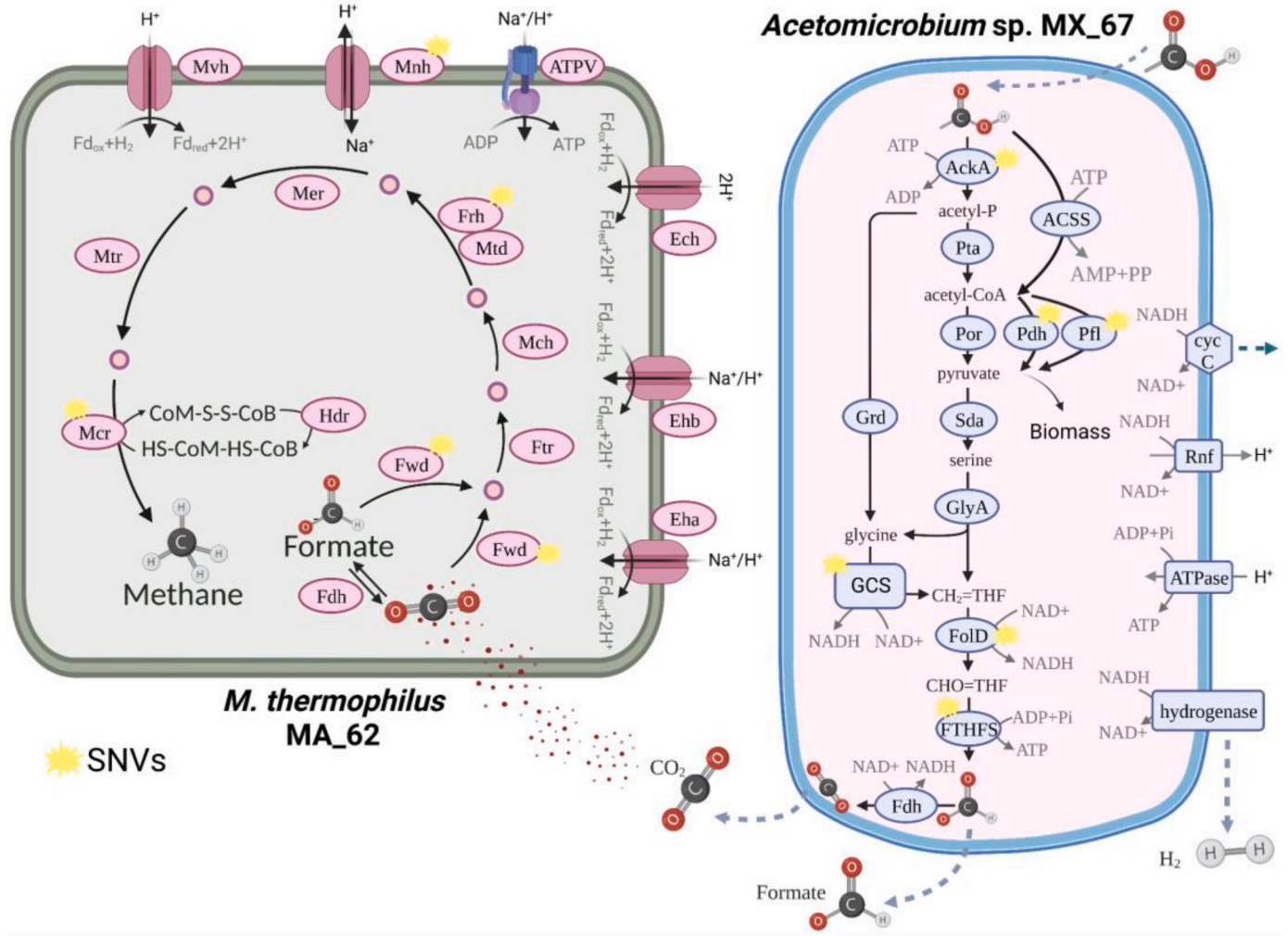

Fig. 5. Genes impacted by SNVs in *M. thermophilus* MA_62 and the putative SAOB *Acetomicrobium* sp. MX_67. Genes where SNVs have been mapped are highlighted in yellow. Their association with specific pathways or transport functions is schematically represented in figure. Methane, hydrogen, formate and CO_2 molecules are reported showing their putative role in the syntrophic association.

that occur in reactors for biogas production. The results obtained aided the understanding of how nucleotide variants of genes involved in essential pathways are a fundamental and still poorly explored component. More specifically, this investigation led to the conclusion that delving the dynamic behavior in the AD microbiome at the strain level is crucial since microbial diversity plays a key role, fostering positive interactions among microorganisms, and leading to niche differentiation over time.

The innovative approach proposed in the current study made it possible to connect the frequency of SNVs with the microbial strains to which individual variants are linked. To mitigate the occurrence of false-positive variants, a tailored filtering step was implemented, resulting in the successful removal of approximately 12–20% of spurious SNVs, varying by reactor. The presence of selective pressure and the accumulation of variants were observed among certain species of archaea and bacteria, indicating their potential role in shaping the community structure and dynamics of mixed cultures involved in BU. The impact of SNVs on the methanogenic ability of hydrogenotrophic archaea was demonstrated, specifically in *M. wolfeii* and *M. thermophilus* species, highlighting the importance of considering the impact of genetic variations in cellular processes and energy metabolism for BU. The application of this knowledge can lead in the future to an optimization of the methanation process, nonetheless a forthcoming life cycle assessment analysis will be imperative to quantify potential environmental impacts and ensure the method's feasibility (Aghbashlo et al., 2022).

In conclusion, exploring SNVs in hydrogenotrophic methanogens plays a pivotal role in enhancing our comprehension of metabolic shifts within the microbiota, which are responsible for their proper functioning. Moreover, unveiling the most efficient and resistant strain can be used to establish a "gold standard" microbial community through isolation experiments and then integration in operating biogas plants, maximizing the CCU capability of the microorganisms and ensuring survival in the face of unbalanced environmental parameters. This would enable the development of a system that is more efficient in CO_2 fixation and less susceptible to failure in the event of unforeseen occurrences, such as pH acidification or sudden ammonia level spikes.

Future research should prioritize a more comprehensive analysis of the variants' impact on the proteins involved, potentially involving the reconstruction of the three-dimensional structures of the enzymes under investigation (Jumper et al., 2021). Given the current prominence of this topic in the metagenomics field, novel methodologies are actively under development. These include the exploration of extra-long Oxford Nanopore reads (Chen et al., 2022) or single-cell technologies (Zheng et al., 2022) to retrieve strain-level phased variants. The biotechnological significance of this research will contribute to optimizing the BU methanation process and unleashing the complete potential of green biogas as a renewable and sustainable energy resource.

CRediT authorship contribution statement

Gabriele Ghiotto: Conceptualization, Investigation, Software, Formal analysis, Data curation, Visualization, Writing – original draft. **Guido Zampieri:** Data curation, Visualization, Writing – review & editing. **Stefano Campanaro:** Conceptualization, Supervision, Resources, Funding acquisition, Writing – review & editing. **Laura Treu:** Conceptualization, Supervision, Resources, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Laura Treu reports financial support was provided by European Climate Infrastructure and Environment Executive Agency.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117414.

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