FINAL PROJECT REPORT

THE ROLE OF THE HYDROGENASE ENZYMES IN THE METHANE PRODUCTION OF HYDROGENOTROPHIC METHANOGENS

Project number: FK 123902
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The aim of the present study was to determine the expression levels of the hydrogenases and other related genes, the examination of the expression profile of the hydrogenotrophic methanogens in the presence of various hydrogen concentrations. This study was also dedicated to unveil the response of the biogas producing anaerobic mixed microbial consortium, with special emphasis on methanogens to the presence of hydrogen.

Background of the study

Anaerobic digestion is one of the most promising among the various bioenergy production processes. The regulatory roles of the H_2 levels and interspecies H_2 transfer have been recognized as a significant element in the concerted action of the complex microbial community (Bagi et al., 2007). Our hypothesis is based on H_2 being an important player in the activity and methane production of the methanogens. One of the rate-limiting factors in methanogenic consortia is the actual level of H_2 in the system. The presence of too much H_2 inhibits the acetogenic bacteria that generate H_2 in the system, whereas limiting H_2 has an adverse effect on an important group of methanogens, the hydrogenotrophic methanogens. In natural ecosystems, a very low partial pressure of H_2 is maintained, which may be a limiting factor for the methanogens. We demonstrated earlier that reductant accessibility is a regulating element in biogas production and presented data supporting the hypothesis that the introduction of H_2-producing bacteria into a natural biogas-generating consortium appreciably increases the efficacy of biogas production both in batch fermentations and in scaled-up anaerobic digestion. The relationship between the acetogens and methanogens is syntrophic, supported by a process called interspecies hydrogen transfer or interspecies electron flow. The actual H_2 concentration has been shown to determine the composition of the methanogenic community. The methanogenic archaea are a highly specialized group of microbes as they produce CH_4, both a useful energy source and a powerful greenhouse gas. The hydrogenotrophic methanogens use H_2 to reduce CO_2 to CH_4, while the acetotrophic methanogens split acetate to CH_4 and CO_2. The expressions of up to 10% of the total proteins in a hydrogenotrophic methanogen were reported to change in response to a H_2 limitation,

indicating that the H_2 availability is sensed by the methanogens and that this gas has a major effect on their physiology. The effect of H_2 on the expression of genes takes part in the methanogenesis but other genes, not directly involved in H_2 metabolism, has not been systematically examined yet. The exploration of the different environmental conditions, which affect the expression of these genes could improve our knowledge concerning molecular redox mechanisms in environmental microbiology in general. It is astonishing to note the complexity of the molecular machinery, which handles the simplest molecule, H_2.

Results

The two main points of the research plan were as follows:

(1) Transcriptomic investigation of pure methanogenic strains cultivated under various conditions (gas supply and carbon source).

(2). Metagenomic, metatransciptomic and RT-PCR analysis of the biogas producing mixed natural consortia incubated under various gases.

(1) Transcriptomic investigation of pure methanogenic strains cultivated under various conditions (gas supply and carbon source).

First the optimization of the cultivation of methanogenic strains was carried out. Growing methanogens in laboratory is a very challenging task because they need special cultivation media and strictly anaerobic conditions. They can utilize only a few low energy content substrates, therefore they grow slowly and reach only low cell density. The optimization of the cultivation conditions to increase the cell density was needed for sufficient amount of DNA and RNA isolation. Six methanogenic strains have been purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures): *Methanocaldococcus fervens, Methanothermococcus okiwanensis, Methanococcus voltae, Methanoculleus burgensis, Methanoculleus thermophilus* and *Methanothermobacter marburgensis*. The selection criteria were to work with both strict and facultative hydrogenotrophic strains. The strict hydrogenotrophic strains can utilize only CO_2/HCO_3^- as a carbon source, the facultative hydrogenotrophic strains also consume formate and/or acetate. The cultures were first grown in laboratory reactors (Braun CT5-2) under strict control (pH, temperature, redox potential, gas concentrations) and anaerobic conditions using the recommended DSM media and gas mixture of H_2 and CO_2. The volume of the liquid phase was 5 L, the headspace of the fermentor was 2 L. The optimization of the growth parameters of the strains was carried out. The methanogens were also cultivated on various carbon sources (formate, acetate and CO_2) and under different H_2 and CO_2 concentrations. We failed to grow *Methanothermococcus okiwanensis, Methanoculleus burgensis* and *Methanothermobacter marburgensis* to generate enough cell mass for DNA and RNA isolation. Cultivation

was successful in the case of *Methanocaldococcus fervens, Methanococcus voltae* and *Methanoculleus thermophilus.* The pure cultures have been maintained in anaerobic serum bottles.

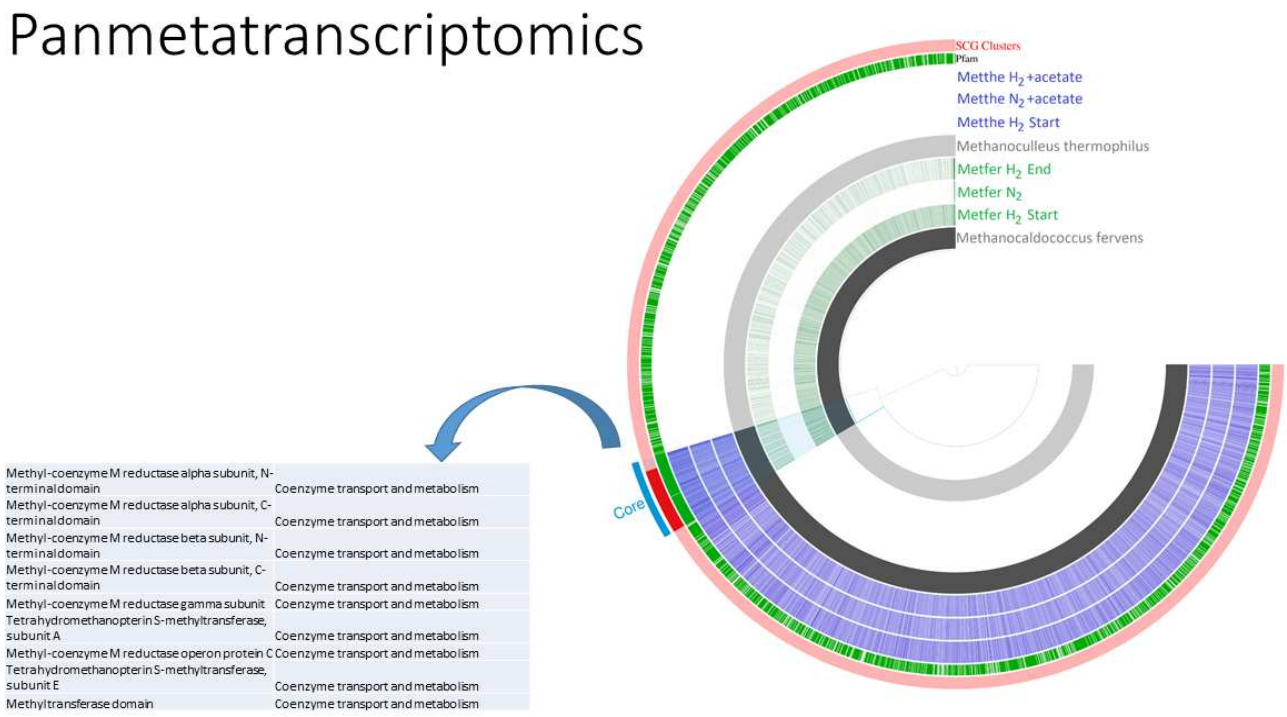
The next step was the optimization of the RNA isolation method as no reliable method was available for the methanogenic Archaea. The RNA extractions were carried out with the Zymo Research Soil/Fecal RNA kit (R2040, Zymo Research, Irvine, CA, United States). After lysis (bead beating), the Zymo Research kit protocol was followed. The DNA contamination was removed by Thermo Scientific RapidoutTM DNA removal kit (K2981, Thermo Fisher Scientific, Waltham, MA, United States). Before metatranscriptome sequencing, rRNA was depleted from metaRNA by using the Gram+/Gram- depletion kit in 60:40 ratio (RiboMinus A15020 Life Technologies, United States). The mRNA library was prepared from samples with the appropriate quality and quantity using the mRNA Sample Prep kit (Illumina, USA). Sequencing was performed using the Illumina V2 chemistry (2 × 250 bp) and applying the MiSeq paired-end mode. Agilent Tapestation system was used to control the quality of the isolated RNA. The RIN values were >9, which indicates that the RNA samples are suitable for sequencing.

In the next experiments our aim was to determine the transcriptomic activity of two thermophilic hydrogenotrophic methanogens (*M. fervens*, *M. thermophilus*) induced by H_2/CO_2 addition and withdrawal circumstances. The two strains can utilize and activate distinct methanogenesis pathways in the presence of various carbon sources. The whole procedure was repeated three times, the transcriptomic analysis contains the data of three biological parallels. We observed previously in mixed enriched hydrogenotrophic culture a relatively fast (<12-14 hours) and flexible response to the H_2/CO_2 (Szuhaj et al., 2016), but transcriptomic results have not been reported yet. The two selected strains are able to utilize CO_2 via the autotrophic pathway, but *M. thermophilus* can also consume formate in a heterotrophic pathway (Narihiro et al., 2016). Comparison of these two hydrogenotrophic strains reveals response of the microbes at gene level to the H_2/CO_2 "turn on-turn off" situations both in the presence (Na-acetate, Na-formate) and the lack of alternate organic substrate.

Panmetatranscriptomics, similar to the pangenomics, provides detailed information about core and accessory genes in closely related genomes (Delmont and Eren, 2018), and the results are based on the transcriptomic activity of the genes. Pantranscriptomics offers the possibility to understand the transcriptomic changes across various environments on single or clustered gene level. In this case the H_2 withdrawal changed the initial environment in both fermentations and Na-acetate and Na-formate were injected as novel organic substrate in the *M. thermophilus* medium. For both hydrogenotrophic organisms, the main substrate (H_2/CO_2) removal predestines the shift in the hydrogenotrophic gene expression and the alternation of the methanogenic metabolism.

M. fervens, being an obligate hydrogenotrophic methanogen, showed active gene expression of the core and also at the accessory level (1st sample – Metfer H_2 start, Metther H_2 start) in the suitable H_2/CO_2 rich environment (Figure 1.). The genes, which were located in the core region, i.e. directly involved in

hydrogenotrophic methanogenesis, were highly expressed as the result of H_2/CO_2 exposure, relative to the accessory genes. After the 1st sampling, the headspaces of the reactors were replaced with N_2. The 2nd sampling, 1 hour after changing the gas phase to N_2, was expected to expose the expression of the genes in the *M. fervens* upon H_2 starvation. Studies demonstrated that the gene expression level in Bacteria reach the maximum in most cases within 1 hour (Golding et al., 2005). The absence of the H_2 resulted inhibition in the gene expression all around the genome, not just in the core genes, i.e. the genes involved in direct H_2 utilization. An important result of this set of experiments was the fast response of the microbial community to the diminishing gaseous substrates. This is a sign of quick turn-on and turn-off response by the methanogens, which is important for practical Power-to-Gas applications. Upon replacing the gas phase again to the initial H_2/CO_2 headspace, the transcriptomic activity of *M. fervens* was reactivated (3rd sampling point). The expression of the genes after 1 hour almost reached the previous level.



Methyl-coenzyme M reductase alpha subunit, N-terminal domain	Coenzyme transport and metabolism
Methyl-coenzyme M reductase alpha subunit, C-terminal domain	Coenzyme transport and metabolism
Methyl-coenzyme M reductase beta subunit, N-terminal domain	Coenzyme transport and metabolism
Methyl-coenzyme M reductase beta subunit, C-terminal domain	Coenzyme transport and metabolism
Methyl-coenzyme M reductase gamma subunit	Coenzyme transport and metabolism
Tetrahydromethanopterin S-methyltransferase, subunit A	Coenzyme transport and metabolism
Methyl-coenzyme M reductase operon protein C	Coenzyme transport and metabolism
Tetrahydromethanopterin S-methyltransferase, subunit E	Coenzyme transport and metabolism
Methyltransferase domain	Coenzyme transport and metabolism

Figure 1. The pan-genome and metatranscriptome of *M. fervens* (Metfer), *M. thermophilus* (Metthe).

The innermost ring dispalys the total genome of *M. fervens* (black). The next 3 layers represents the coverage of the genes which were annotated in the genome (the color darkness increasing by the coverage of the genes) at the 3 sampling points. The 4th layer represents the total genom of *M. thermophilus* (black). The next 3 layers represent the coverage of the genes which was annotated in the genome (the colour darkness increasing by the coverage of the genes) at the 3 sampling points. The green ring indicates the gene clusters in which at least one gene was functionally annotated using Pfams. In the outermost ring, the red bands represent the distribution of SCGs (genes, that are present in both organisms and overlap) of the two archaea. The blue band marks the position of core genes in the two genomes. The core genes, which were activated in the methanogenesis are highlighted in the table and their main roles in the metabolism are indicated.

The genome level transcript results clearly indicated that the withdrawal of the H_2/CO_2 (2nd sampling) negatively affected the expressions of the core and the accessory genes in *M. fervens*. The lack of the available substrate exerted the inhibiting effect on the entire life of *M. fervens*, the most pronounced changes were apparent among the methanogenesis related genes (Figure). Some of these enzymes are methanogenesis marker proteins, such as those playing important role in the regulation of the hydrogenotrophic methane formation (methylenetetrahydromethanopterin (methylene-H_4MPT) reductase, -hydrogenase, coenzyme F_{420} reducing hydrogenase, F_{420} hydrogenase, CoB-CoM heterodisulfide reductase, F_{420}-dependent methylene-H_4MPT dehydrogenase, formate dehydrogenase, formylmethanofuran dehydrogenase, methyl coenzymeM reductase, tetrahydromethanopterin S-methyltransferase). Correlation in the scale of the inhibition or in the reactivation after the readjustment of the H_2/CO_2 rich headspace (3rd sample) could not be observed. The restoration of the methanogenic activity at transcriptomic level corroborates the rapid response of the metabolism in the obligate autotrophic *M. fervens*.

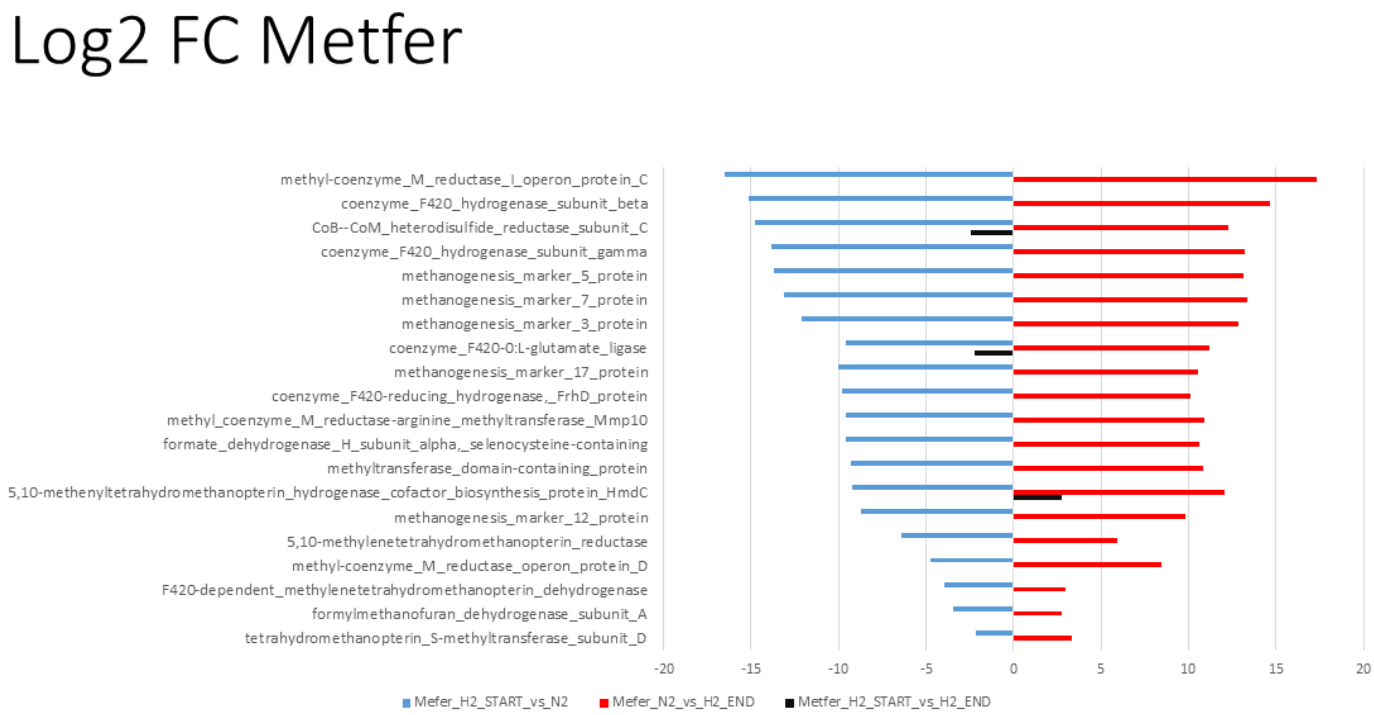


Figure 2. Significant (-2≥log$_2$FC≤2) gene expression changes in core transcriptome of *M. fervens* (Metfer).
Blue bars: expression changes between 1st (H2_START) and 2nd (N2) sampling points; red bars: expression changes between 2nd (N2) and 3rd (H2_END) sampling points; black bars: expression changes between 1st (H2_START) and 3rd (H2_END) sampling points. $log2FC = log2\left(\frac{relative\ abundance\ sample\ 1}{relative\ abundance\ sample\ 2}\right)$

The H_2 metabolism of *M. thermophilus* differs from that of *M. fervens*. *M. thermophilus* is a type strain, it is a hydrogenotrophic archaeon able to utilize also formate in a heterotrophic pathway (Maestrojuan et al., 1990; Rivard and Smith, 1982). To reveal the similarities and the differences among the obligate and the facultative autotrophic transcriptomic response to the H_2/CO_2 supply/withdrawal, the same experimental protocol was applied except that when the headspace replacement for N_2 was carried out, organic carbon source was also added. The injected Na-formate prevented the inhibition of the core and accessory expression, thus significant changes did not appear. Formate, the alternative substrate and reductant source compensated the absence of the H_2 for the hydrogenotrophic metabolims, therefore the methanogenesis related genes remained highly expressed. In contrast to the *M. fervens*, the *M. thermopilus* genome scale transcript results did not show remarkable changes upon the withdrawal of H_2/CO_2 (Figure 1.). The detailed analysis of the core gene expressions revealed significant transcriptional changes (Figure 3.). The removal of the H_2/CO_2 and concomitant addition of organic substrates (Na-acetate, Na-formate) increased the expression of a nucleotide binding protein and the S-adenosylmethionine decarboxylase. S-adenosylmethionine decarboxylase is a polyamine that functions as an activated methyl donor for cells to modify RNA, DNA, proteins, lipids and cofactors and has an alternate function in spermidine biosynthesis (Kim et al., 2000). The change of the headspace significantly decreased the expression of several hypothetical proteins, such as domain of unknown function (DUF) 2180 protein, histone family protein, heat repeat domain-containing protein and Hsp20/alpha crystallin family protein. Most of these genes code for proteins of unknown or poorly understood functions. Heat shock proteins are responsible of the environmental stresses. Heat shock proteins also act as chaperons, protecting target proteins from denaturation, aggregation and inactivation. Repeated supply of H_2/CO_2, elevated the expression of the genes coding for unknown proteins, protein domains (hypothetical proteins, domain of unknown function (DUF) 2180 protein, PD40 domain-containing protein, PRC-barrel domain-containing protein, histone family protein) and glycosyltransferase. Unfortunately, the present knowledge about their physiological roles do not allow drawing any conclusion about their role in the metabolism of *M. thermopilus*. Nevertheless, these data point to hitherto uncovered metabolic pathways, related to H_2 metabolism in archaea, which demand further systematic studies. The transient elevated partial pressure of H_2/CO_2 inhibited the expression of ketoisovalerate ferredoxin oxidoreductase and S-adenosylmethionine decarboxylase. Ketoisovalerate ferredoxin oxidoreductase catalyses the coenzyme A-dependent oxidation of branched-chain 2-ketoacids coupled to the reduction of ferredoxin (Heider et al., 1996). This is an additional sign of the involvement of distant metabolic routes in the H_2 metabolism and presumably that of the intracellular redox balance in hydrogenotrophic archaea.

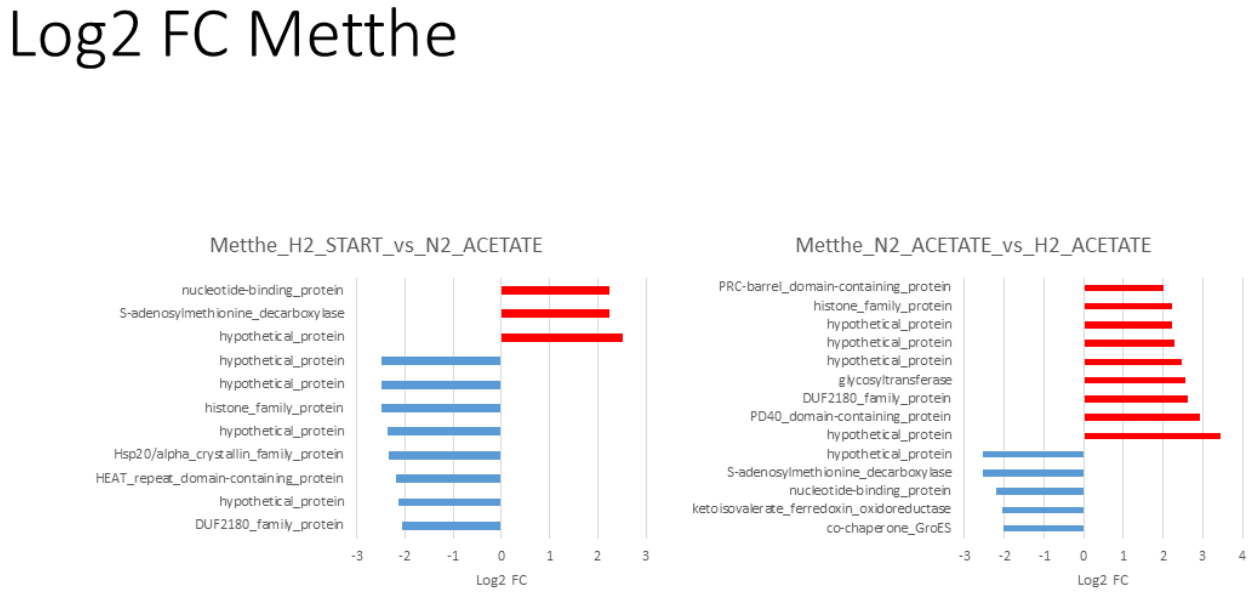


Figure 3. Significant (-2≥log$_2$FC≤2) gene expression changes in core transcriptome of *M. thermopilus* (Metthe).

a., Negative (blue bars) and positive (red bars) expression changes between 1st (H2_START) and 2nd (N2_ACETATE) sampling points; Negative (blue bars) and positive (red bars) expression changes between 2nd (N2_ACETATE) and 3rd (H_ ACETATE) sampling points.

The investigation of the mRNA predicted COGs (Clusters of Orthology Groups of proteins) helps to track the changes of vertical evolutionary descent connected genes. Despite of the distinct fermentation environments, the COGs' changes were similar in both organisms (Figure 4.).

The most spectacular change was the decrease of the expression of category H genes as the response to H_2 withdrawal in *M. fervens*. The coenzyme transport and metabolism genes (COGs H) expressed the majority of the initial annotated sequences (25.66%) in the entire transcriptomic activity of *M. fervens*, which decreased to 15.98% upon diminishing H_2. The genes of the H category regulate coenzyme transport and metabolism, so the major group of these genes are coding methanogenic (methyl-coenzyme M reductase (mcr)) (Thauer, 1998) and especially hydrogenotrophic methanogenrsis related enzymes (tetrahydromethanopterin S-methyltransferase (mtr)) (Wagner et al., 2018). After restoring H_2/CO_2 feeding, the metabolism of the microorganisms was quickly reactivated and the gene activity of the COGs group H surpassed the previous detected coverage (36.64%).

The coenzyme transport and metabolism (COGs H) group was less dominant in *M. thermophilus* following similar treatment. These genes, which are responsible for the hydrogenotrophic metabolism, were in similar abundance range (10.08%) as the C (energy production and conversion, 13.14%), K (transcription,

10.85%), R (predicted general functions, 11.22) COGs. Along the 2nd (9.29%) and 3rd (8.99%) sampling no remarkable change were detected presumably due to the activation of the heterotrophic methanogenic pathway via the organic substrate addition. The readjustment of the H_2/CO_2 headspace did not increase the transcriptomic activity of the genes in this category.

COG category C contains the majority of the core genes of hydrogenotrophic methanogenesis. The coverage of this category was elevated despite of the changing environment. In comparison with the H category, expression of genes in category C remained stable throughout the entire fermentation, and the H_2 withdrawal slightly elevated the relative coverage of these genes from 9.03% to 11.56% in *M. fervens* and from 13.14% to 16.13% in *M. thermophiles*, respectively.

The genes of the COG category C are responsible for the energy production and conversion (Tatusov et al., 2001) and with the genes of the category H are the most active groups in methanogens (Gilmore et al., 2017). Formylmethanofuran dehydrogenase, coenzyme F_{420}-reducing hydrogenase/dehydrogenase and F_{420}-dependent methylene-tetrahydromethanopterin dehydrogenase (Mtd) core hydrogenotrophic methanogenic genes of COGs category C were annotated in the two archaea.

Formylmethanofuran dehydrogenase is the first enzyme of the hydrogenotrophic methanogenesis pathway. This enzyme catalyzes the binding of CO_2 to the amino group of methanofuran, forming formylmethanofuran (BERTRAM et al., 1994). Coenzyme F_{420}-reducing hydrogenase is responsible for the formation of reduced coenzyme F_{420} ($F_{420}H_2$), which is the electron donor of the methenyl-tetrahydromethanopterin (methenyl- H_4MPT) reduction. Additionally the $F_{420}H_2$ is one of the electron sources for the F_{420} H_2-dependent methylene-tetrahydromethanopterin dehydrogenase (mtd), which is the electron donor of the methenyl-H_4MPT to methylene-tetrahydromethanopterin (methylene-H_4MPT) alongside of $F_{420}H_2$ (Hendrickson and Leigh, 2008). The tetrahydromethanopterin S-methyltransferase (mtr) is a membrane associated enzyme complex, which catalyzes a Na^+ translocation dependent methyl transfer from methyl-tetrahydromethanopterin (methyl-H_4MPT) to coenzyme M (CoM-SH), creating methyl-coenzyme M (CH_3-S-CoM). Methyl-coenzyme M (CH_3-S-CoM) is a precursor molecule of the heterodisulfide complex (CoB-S-S-CoM) formation. The methyl-coenzyme M reductase (mcr) catalyzes the reduction of methyl-coenzyme M (CH_3-S-CoM) with coenzyme B (CoB-SH) (Wagner et al., 2018).

The COGs analysis correlated with the expression results of the total genome (Figure 1). The COGs analyses revealed the detailed changes in both methanogens. The total transcript changes proved the rapid inhibition in homoautotrophic *M. fervens* when H_2/CO_2 depleted. Although apparently the majority of the genes were affected, *M. fervens* was able to restore its expressional activity after replenishing H_2/CO_2. To the contrary, the hydrogenotrophic methanogenesis activities in *M. thermophilum* did not go through similar remarkable changes. The available organic substrates kept the hydrogenotrophic gene expression at stable level in the absence of H_2/CO_2.

No hydrogenotrophic methanogenesis related significant ($-2 \geq \log_2 FC \leq 2$) COG changes occurred in *M. fervens* in I (lipid transport and metabolism), N (cell motility), P (inorganic ion transport and metabolism), U (intracellular trafficking, secretion, and vesicular transport), X (nuclear structure) and in *M. thermophilum* in B (chromatin structure and dynamics). These COGs are therefore not directly connected with the hydrogenotrophic metabolism.

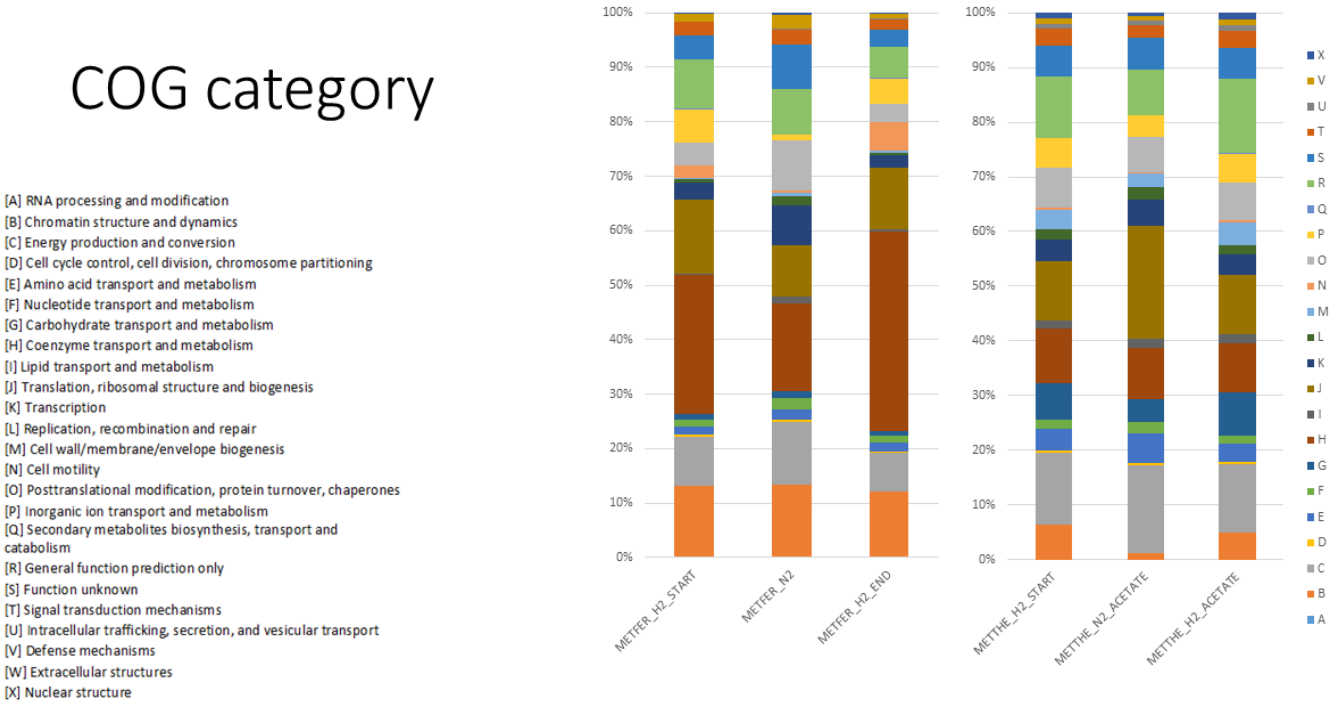


Figure 4. Transcriptomic changes in *M. fervens* (Metfer), *M. thermophilum* (Metthe) based on the COG categories.

The results represent the coverage of the COGs relative to the total genes annotated in the genomes at the 3 sampling points. 1st (H2_START), 2nd (N2 and N2_ACETATE), 3rd (H2_END and H2_ACETAT)

The behavior of *Methanococcus voltae* was also examined. This species is heterotrophic, strictly anaerobic, mesophilic, and able to produce methane from H_2/CO_2 or formate. It was cultivated under N_2 and H_2 gas phase. The fold changes of the individual genes were determined and we have identified several genes, which had elevated expression in the absence of H_2. Figure 5 indicates the genes, which showed the most noticeable changes. These genes take part mainly in transcription (transcriptional regulator, ribosomal proteins) and in methanogenesis (subunit of formylmethanofuran dehydrogenase, coenzyme-B sulfoethylthiotransferase, coenzyme F430synthase).

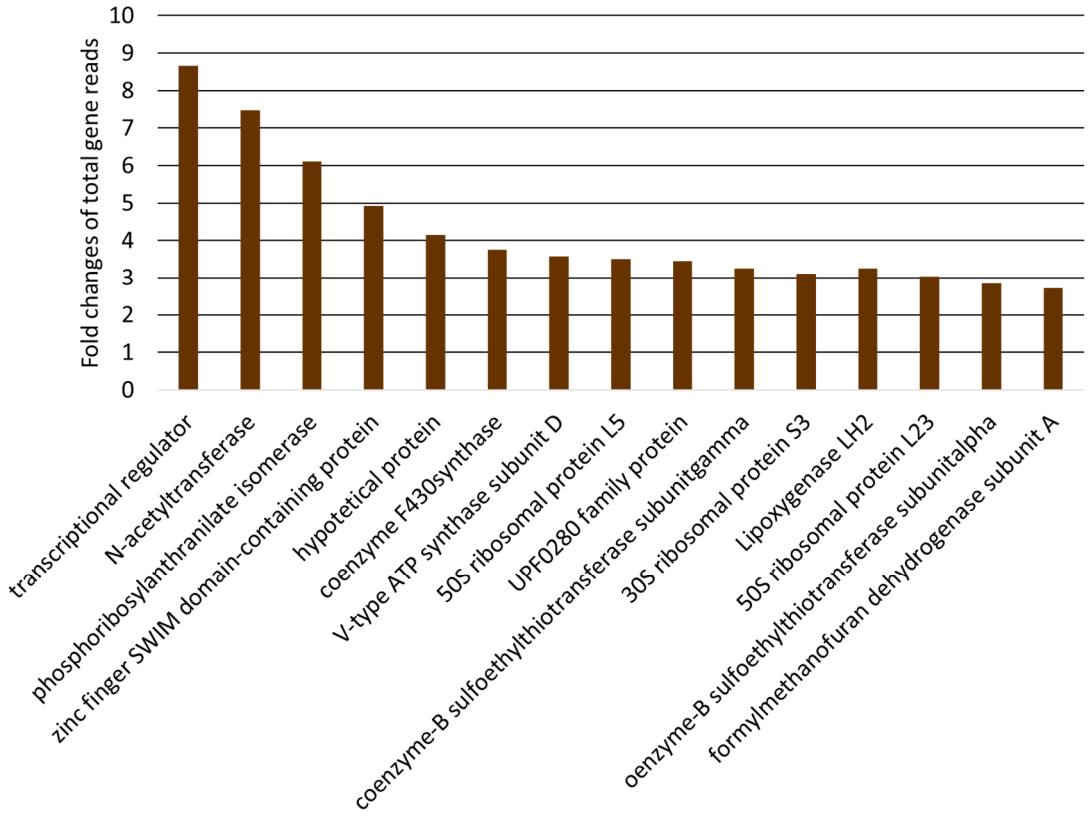


Figure 5. Genes having increased expression upon withdrawal of hydrogen in *M. voltae*

The mRNA sequences were analyzed using the PATHVIEW visualization software. Although the expression of most enzymes involved in methanogenesis decreased under N_2, enzymes that catalyze the final step in the formation of methane and enzymes that take part in the conversion of formate and acetate significantly increased in *M. voltae*. This suggests the turn-on of an escape route for the surviving methanogens, the cells adopted to the new environment, the expression of the enzymes which utilize energy from formiate are increased. The cells were able to grow under these conditions.

Conclusions

The studies presented above contributed to our understanding of the fine details of molecular regulation of methanogenesis, a complex process playing important roles in environmental microbiology as well as renewable energy production and storage. The specific novel conclusions can be briefly summarized as follows:

1. The experimental system developed, i.e. gas fermentation combined with pangenomic and transcriptomic studies is suitable to study the complex and sensitive methanogenesis pathways.
2. In autotrophic methanogens, e.g. *Methanocaldococcus fervens*, H_2/CO_2 is sufficient and effective regulator of methane biosynthesis. The entire metabolism of these hydrogenotrophic methanogens is affected, in the absence of H_2/CO_2 the microbes are practically completely “switched off” and

remain in a sort of hibernated state. This indicates that H_2/CO_2 regulates the entire metabolism, probably via generalized redox regulation of the main energy production and utilization pathways. Moreover, the microbes can be easily and quickly "switched on" and their metabolism reactivated upon restoring H_2/CO_2. From practical point of view these findings have importance in the Power-to-Gas renewable electricity conversion and storage systems because the results confirmed that hydrogenotrophic methanogens are effective, flexible and easily manageable biological devices to convert the intermittently produced "green" electricity, generated by photovoltaics and/or wind power, to storable and transportable bio-methane.

3. In methanogens, e.g. *Methanoculleus thermophilus* that can also utilize acetate/formate as carbon and reductant source, H_2/CO_2 is not indispensable for active metabolism. These microbes grow and function vigorously both in the presence and absence of H_2/CO_2. On the one hand this means that they cannot be "switched-on and switched-off", by using H_2/CO_2 as the single regulatory element in the Power-to-Gas process. On the other hand, these methanogens seem outstandingly suitable for converting acetate/formate to biomethane. This may offer a bypass to avoid the obstacle of extremely low solubility of H_2, which limits the efficacy of the Power-to-Gas conversion and renewable electricity storage process. Future studies are needed to explore efficient conversion of H_2/CO_2 to formate and to understand additional details of gas metabolism in microbes.

(2). Metagenomic, metatransciptomic and RT-PCR analysis of the biogas producing mixed natural consortia incubated under various gases.

From both basic research and practical applications points of view the understanding of CO_2/H_2 fermentation of a mixed anaerobic community represents a great challenge. During the optimization of the total RNA isolation from the biogas forming natural consortium we were succeed to purify good quality total RNA, which could be used for both qPCR and metatranscriptomic analyses. These methods were used to study the diversity of the entire cellular activities within the community, to quantify the expression levels of selected genes coding for enzymes of key metabolic processes and to monitor how these levels change under various conditions. Therefore we made metatranscriptional investigations in addition to qPCR, which resulted a much more complex picture and more valuable results. The interaction between the acetogens and other H_2 producers and the methanogens could be investigated during the metatranscriptomic analysis. qPCR analysis were done for validation of the metatranscriptomic data.

Our primary objective was to examine the initial response of the methanogenic archaea and other members of the consortia, and assess the genes that are the first up- or down-regulated ones by the H_2 injection. Anaerobic digestions (AD) were carried out in continuously stirred tank reactors. The fermentation volume was 5,000 mL, leaving a headspace of 2,000 mL. The apparatus can be fed continuously or intermittently via a piston type delivery system, the fermentation effluent is removed through an air-tight overflow. The

reactors are equipped with a spiral strip mixing device driven by an electronic engine. An electronically heated jacket surrounds the cylindrical stainless steel body, electrodes for the measurement of pH and redox potential are inserted through the reactor wall, in sealed sockets. The device can be drained at the bottom. The evolved gas leaves the reactor through the top plate, where ports for gas sampling and the delivery of liquids by means of syringes through silicone rubber septa are also installed. Gas volumes are measured with thermal mass flow devices (DMFC SLA5860S, Brooks). A fresh sample from an industrial scale mesophilic biogas plant, fed with pig slurry and maize silage mix (Zöldforrás Biogas Plant, Szeged, Hungary) was used as an inoculum, i.e. the microbial community adopted to heterogeneous substrate degradation. The reactors were flushed with N_2 to ensure anaerobic conditions and were closed air tight. During the experiment the digesters were fed twice a day with synthetic medium in which only alpha-cellulose was added as a carbon source at a loading rate of 1 g oDM L^{-1} day^{-1}. The reactors operated under mesophilic conditions, at 37°C.

A constant value of VOA/TIC is a reliable indicator of a stable mesophilic fermentation process. Each experiment started with a 20-days long start-up period in order to adapt the microbial community to the alpha-cellulose substrate. During this period the average VOA and TIC values stabilized at VOA=1.1 g L-1 and the TIC=14 g CaCO3 L^{-1}. Because of the relatively low substrate loading rate, the VOA/TIC ratios were moderate, which allowed balanced operations. The biogas productivity of the digesters was also stable: 650 mLN biogas alpha-cellulose g^{-1} day^{-1} were produced with 53% of CH_4 content. The first samples for DNA and RNA analysis were taken on day 20 from the stabilized reactors. After sampling the digesters were flushed with H_2 gas from a gas cylinder for 10 min and 2 hours later the second sampling was carried out. This protocol was repeated after 2 months of continuous reactor operation. The reactors displayed stable operation during the course of the experiment. The daily biomethane production varied by <10%. The H_2 injection took place on days 15 and 71. The reactors responded with a sudden increase in daily CH_4 evolution by 20-25% at both time points, which lasted for 1-2 days. The CH_4 content of the biogas was 53% throughout the experimental period. Afterwards the reactors returned to their previous biomethane production levels. It is worth noting that the microbial community responded exactly the same manner to the H_2 spike 2 months apart, which indicates the robustness, reproducibility and quick response time by the microbial community. Assuming H_2 saturation of the liquid phase by the H_2 bubbling for 10 min, we estimated that more than 95% of the injected H_2 was converted to CH_4 by the community within 16-24 hours, although the amount of available dissolved H_2 decreased rapidly during the second half of the H_2 consumption phase. This is in line with the observations of Szuhaj et al (M Szuhaj et al., 2016), who found in fed-batch H_2 feeding experiments at much lower size that the injected H_2 was completely consumed after 16-24 hours and suggests that competing H_2 consuming reactions did not interfere significantly. The H_2 injection apparently did not alter markedly the cumulative biomethane production curve, which showed a straight line throughout the experiment.

After sampling total RNA and DNA were isolated with the Zymo Research Soil/Fecal RNA and DNA kit. During sequencing, paired-end libraries were prepared for the metagenome and metatranscriptome samples using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (Cat.Num.: E7645L). Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq® 500/550 High Output Kit v2. After the sequence result were available we used a custom bioinformatics workflow for the downstream analysis of the genes and pathways of each MAGs. This involved primarily the SqueezeMeta (Tamames and Puente-Sánchez, 2019) pipeline, which can jointly analyze metangenome (MG) and metatranscriptome (MTR) sequencing data, amended with a more extensive binning procedure, a subsequent pathway enrichment analysis and statistical evaluation of the log2FC of the gene expressions of the MAGs between the H_2 and N_2 MTR samples. In order to gain higher statistical confidence in the results, we used biological duplicates separated by a two-month interval in CSTR AD reactors. The following important considerations were also adopted: 1.) The metagenomes of the samples separated by just a two-hour time-window, i.e. before H_2 addition and 2 hours later, to reveal whether the community composition did change in 2 hours. No perceivable changes occurred in the AD community, therefore the observed variations in the metatranscriptome were not biased by the changes in the community composition. 2.) qPCR tests of a handful of selected genes validated the results from the metatranscriptomics pipeline. 3.) A distinct bioinformatics pipeline (humann2 (Franzosa et al., 2018)) corroborated our sequence analysis results. First we established that the composition of the microbial community did not change significantly (Figure 5), therefore the different reproduction rates of the various taxa did not disturb the picture of early functional response.

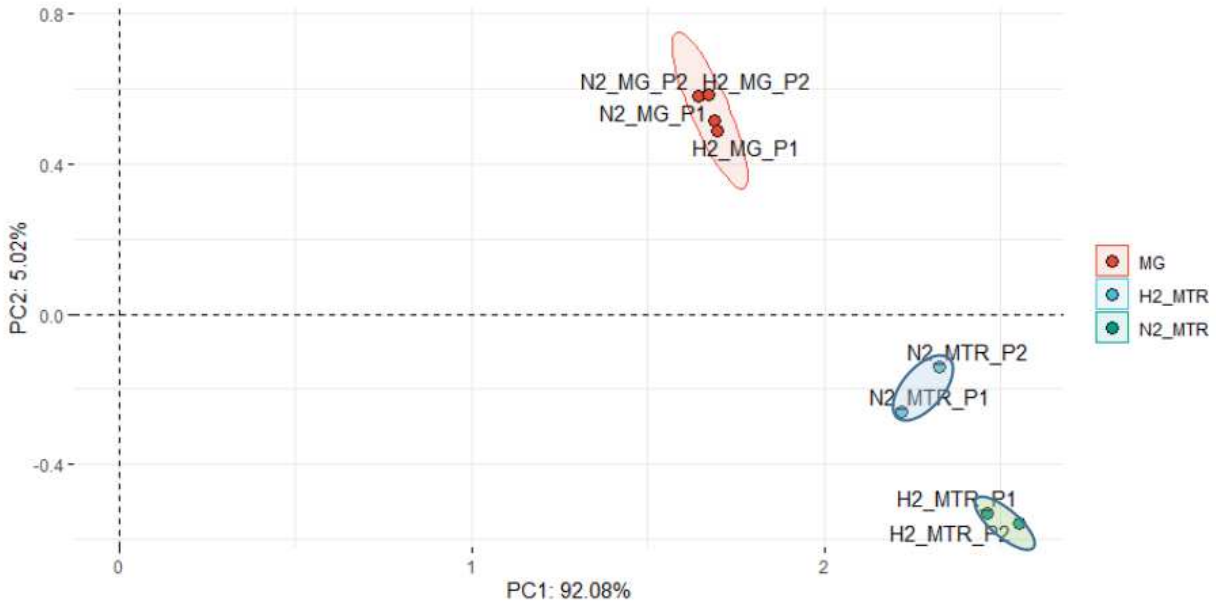


Figure 6. PCA biplot of the rlog-transformed (regularized-logarithm transformation) total gene expressions, i.e. copy number in the MG sample, of each MAG in each sample.

Up-to-date metagenomic and metatranscriptomic methods were employed to determine the biochemical events taking place as the result of H_2 administration. The reproducibility of the system was tested by repeated H_2 injections 2 months apart. Practically identical results were obtained (Figure 6). Four metagenome sequencing datasets were combined to assemble a fairly large number of 436 bins (84 bins: 7 Archaea, 61 Bacteria and 16 unclassified bins). The non-H_2-adapted, "raw" biogas forming microbial community was essentially the same in structure and composition as the ones sampled previously from the same industrial biogas plant fed with manure and maize silage (Wirth et al., 2019). This community switched to H_2 consumption and biomethane production almost immediately following H_2 injection, although feeding of the entire community with alpha-cellulose substrate continued as before. We interpret that this behavior indicated the presence of sufficient hydrogenotrophic methanogenesis activity in the "raw" biogas community, i.e. in the large scale biogas plant effluent. This observation could be very important in the Power to biomethane (P2bioCH4) process (Götz et al., 2016). The diverse, "raw" anaerobic communities can be used in switching on P2bioCH4 without a lengthy adaptation and enrichment period. This allows a quick and efficient turn-on and turn-off response by the mixed methanogenic community. The microbial community composition rearranges upon long-term exposure to H_2 (and CO_2), particularly when no other organic substrate is available for the community (Ács et al., 2019). The metatranscriptomic responses to the H_2 treatments separated two months apart were very similar to each other indicating that

the metabolic pathways were flexibly restored after switching on and off the P2bioCH4 operational mode. A thorough analysis of the differences between the H_2 treated metatranscriptomes and corresponding controls identified the early events in the microbial communities brought about by H_2.

A typical microbial community flourished in our biogas digesters, which indicated that the synthetic medium containing only cellulose as a carbon source proved to be a good model system for the metatranscriptomic investigations. A comparison of the DNA and mRNA-based omics data clearly indicated that the community compositions were very similar in all cases except for the H2-MTR samples (Figure 6). The overall Archaea gene abundance in N2_MTR samples was 22.9±13.4% whereas in N2-MG or H2-MG samples the same values were 22.5±2.4%, respectively. This observation corroborates that i.) all reactors worked under the same conditions maintaining the same microbial community; ii.) the microbial communities did not change perceptibly within 2 hours as expected; iii.) the observations were highly reproducible after 2 months. Striking changes took place in the transcriptome-based community composition when H_2 was offered to the reactors' microbial community. Approximately two times more Archaeal genes were activated 2 hours after the H_2 injection (H2_MTR=45.06±4.4%) compared to the N_2 supplied reactors. This demonstrates a rapid response to the appearance of excess H_2 by the microbial community. The elevation of the total number of transcribed Archaeal genes (H2_MTR samples) was mainly attributed to representatives of the genus *Methanobacterium* (bins 35 and 51), which increased from 8.6% to 30% of all bins abundance. *Methanobacteria* are hydrogenotrophic methanogens. The second in contribution was the order *Methanomicrobiales*. The genera *Methanoculleus* and *Methanosarcina* both belong in this order. Overall *Methanomicrobiales* showed an increase from 2.3% to 13.9% upon H_2 exposure. Remarkably, the genus *Methanosarcina* effectively ceased to express genes to near zero upon H_2 dispensation (Figure 7). *Methanosarcina* are known to possess genes coding for all three methanogenic pathways, i.e. hydrogenotrophic, acetotrophic and methylotrophic methanogenesis (e.g. 8, 16). Members of the genus *Methanoculleus* are solely hydrogenotrophic methanogens. H_2 exposure apparently turns on the activity of the hydrogenotrophic methanogenesis in *Methanoculleus* but turns off the hydrogenotrophic pathway in *Methanosarcina.*

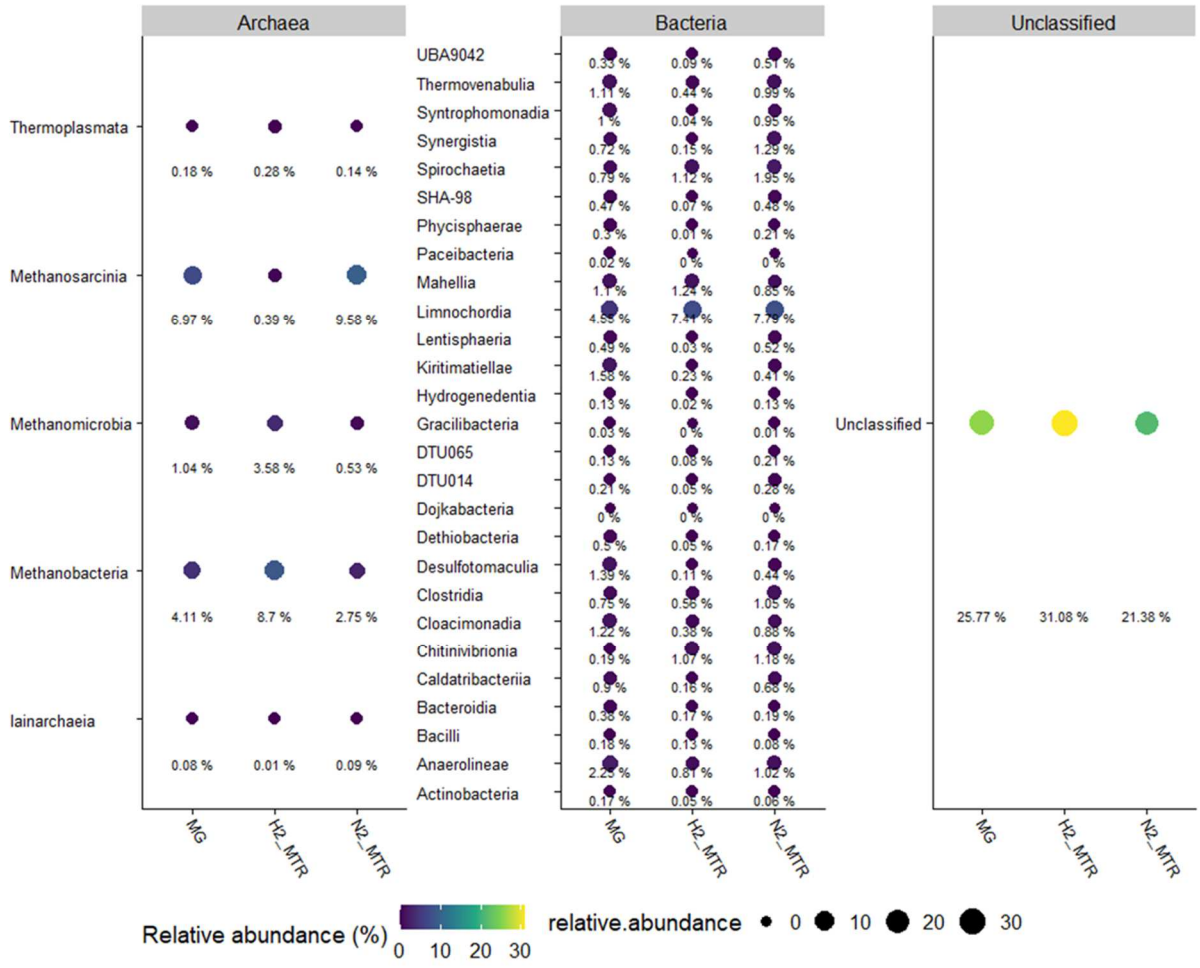


Figure 7. Relative abundances of Archaea and Bacteria bins.

Changes in the expression levels in methanogenesis genes

The contig assembly and ORF prediction/annotation workflow yielded 219,353 KEGG orthology annotated (KO) ORFs. Out of these 98,791 ORFs were binned in the refined MAGs. The remaining 120,562 ORFs were used for the community-level pathway analysis. The changes in the expression levels of the genes involved in the various methanogenesis related metabolic pathways and modules were examined according to KEGG annotation. The results indicated that the methanogenesis pathway was primarily affected as the result of H_2 injection. The upregulation of differentially expressed (DE) genes was the highest in this pathway (48) and in the associated modules (Figure 8). It is noteworthy that some other carbon metabolism associated pathways were also affected, such as Glycolysis/Gluconeogenesis and Propanoate metabolism, which suggest that acetogenic and acetate utilizing microbes were also affected by the specifically altered environment. H_2 is known to inhibit acetogenic microbes (Batstone et al., 2006), thus their response to the H_2 addition is not surprising. The RNA polymerase pathway also changed significantly, this was due to triggered transcription machinery, which had to adapt to the demands of increased transcriptional activity as a response to the altered environment.

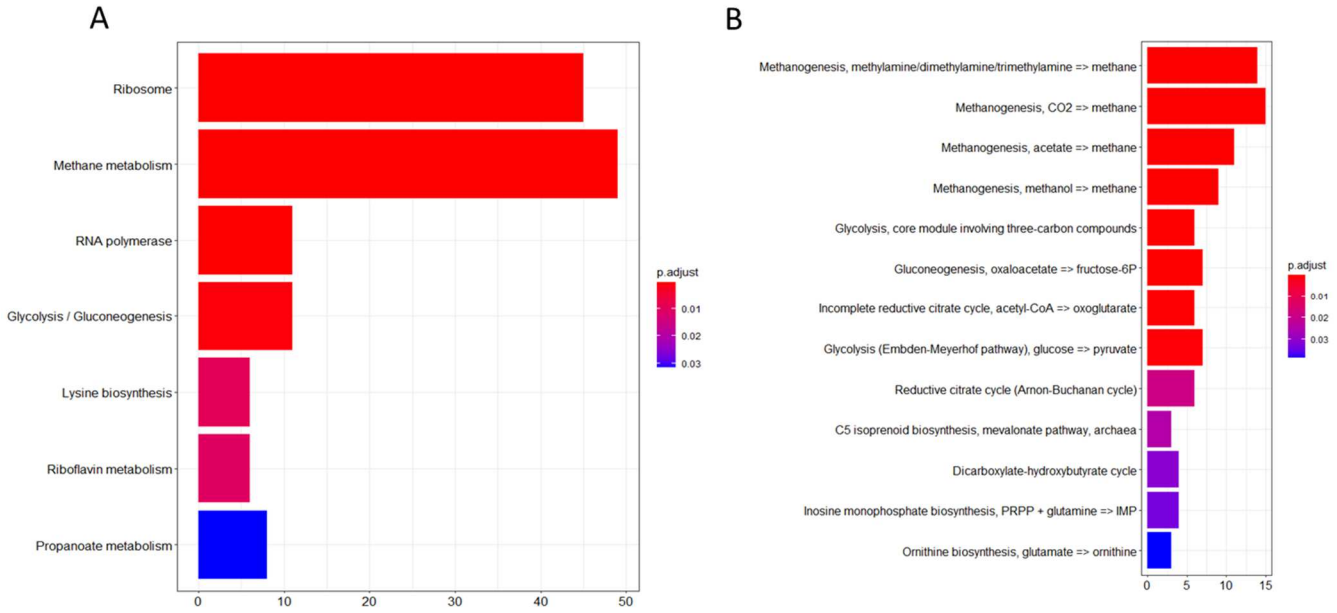


Figure 8. A.) Results of KEGG Module enrichment analysis (left), and B.) KEGG Pathway (right). The pathways, which were significantly different between N2_MTR and H2_MTR samples are presented. X-axis indicates the number of KEGG IDs found as significantly different in the given pathway (listed along the Y axis). P-adjust stands for corrected P values.

Bins harboring more than five KEGG methanogenesis (map00680) pathway genes were plotted in Figure 8. The two bins identified as belonging in the genus *Methanobacterium* (bin_35 and bin_51) and 232 bin_6 of *Methanoculleus bourgensis* showed a very similar response, most of their map00680 genes are expressed at log2 fold change (log2FC) higher than 2, i.e. four-times higher expression. Two additional *Methanoculleus* bins (bin_60 and bin_66), a low and a medium quality MAG according to CheckM, were identified but not presented in Figure 8. In the hydrogenotrophic strains, the expression level of numerous genes increased shortly after H_2 injection, which indicated that the several metabolic pathways responded to the increased H_2 concentrations. The log2FC values of the genes ENO (phosphopyruvate hydratase, EC 4.2.1.11), COF (7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase, EC 4.3.1.32), and COM (sulfopyruvate decarboxylase, EC 4.1.1.79) were the largest in M. bourgensis. The ENO enzyme takes part in the biosynthesis of the Coenzyme B, which is an essential molecule in the final step of the methanogenesis. The COF enzymes are responsible for the synthesis of the other important coenzyme, the Coenzyme F420. The COM enzyme catalyze the 3-sulfopyruvate = 2-sulfoacetaldehyde reaction, which is an intermediate step of synthesis of the third important coenzyme, the Coenzyme-M (Grochowski and White, 2010). These results clearly indicated that the cells increased the synthesis of all coenzymes, which are involved in methanogenesis to support the quick conversion of H_2 and CO_2 to CH_4. In bins belonging *Methanobacterium* strains, the expression level of the enzymes MFN (tyrosine decarboxylase, EC 4.1.1.25), ADC (aspartate 1-decarboxylase, EC 4.1.1.11), FMD/FWD (formylmethanofuran

dehydrogenase, EC 1.2.99.5), AKS (methanogen homocitrate synthase, EC 2.3.3.14 2.3.3), COM increased. These enzymes also play an important role in the hydrogenotroph methanogenesis pathway. The MFN and ADC enzymes are normally involved in the methanofuran biosynthesis pathway, when they catalyze the L-tyrosine = tyramine reaction. The FMD/FWD redox enzyme complex contains a molybdopterin cofactor and numerous [4Fe-4S] clusters in order to catalyze the reversible reaction the formyl-methanofuran synthesis from methanofuran, which is an important methanogenesis step in CO_2 conversion and the oxidation of coenzyme-M to CO_2. The reaction is endergonic and is driven by coupling the soluble CoB-CoM heterodisulfide reductase via electron bifurcation. The AKS enzyme also takes part in the synthesis of Coenzyme-B. Overall, the results indicated that the hydrogenotrophic methanogenic cells activated the majority of the key enzymes in the methanogenesis pathway to consume more effectively the additional H_2. It is important that the genes of the MCR enzymes (methyl-coenzyme M reductase, EC265 2.48.4.1.) showed lower expression in all hydrogenotrophic bins. The MCR enzymes (methyl-coenzyme M reductase) catalyze the final step of the methanogensis. One of the potential considerations explaining this observation was that 2 hours was perhaps not enough for redirecting this section of methanogenesis pathways. If the local substrate availability did not increase significantly, the cells did not need to increase the transcriptional activity of the MCR enzymes. Almost all genes in *Methanosarcina honorobensis* showed decreased expression in the presence of H_2. This strain has been described as acetotrophic, which also grew on methanol, dimethylamine, trimethylamine, dimethylsulfide and acetate but not on monomethylamine, H_2/CO_2, formate, 2-propanol, 2-butanol or cyclopentanol (Shimizu et al., 2011). The expression levels of MCR, ACS (acetyl-CoA decarbonylase/synthase, EC 3.1.2.1) and FAE (5,6,7,8-275 tetrahydromethanopterin hydro-lyase, EC 4.2.1.147) significantly decreased. The ACS enzyme is responsible for the conversion of acetate to acetyl-CoA, which is a typical step in the acetotrophic methanogenesis pathway. The next enzyme, FAE generates 5,10-Methylene-THMPT from formaldehyde, an important compound, intermediate of methanogenesis. The substantial decrease in the transcriptional response of *M. honorobensis* to H_2 injection corroborated that this strain is unable to utilize H_2, but indicated an active inhibitory role of H_2 on acetotrophic methanogenesis. This implicates a hitherto unrecognized tight regulatory role of H_2 on diverse pathways coupled to methanogenesis.

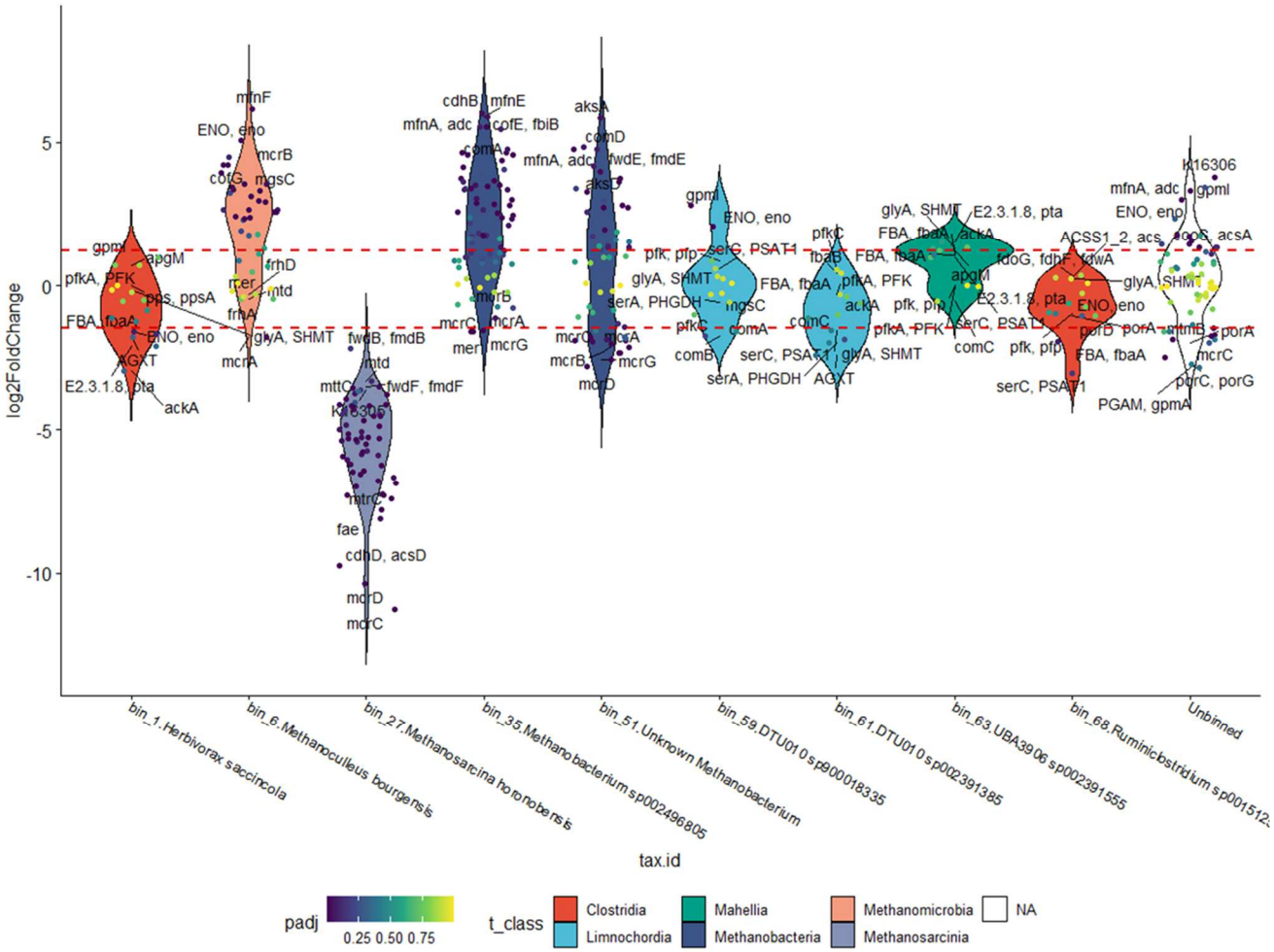


Figure 9. Violin plot of genes (small dots) involved in the peripherial methanogenesis KEGG688 pathway (map00680) in each bin (arranged on the X-axis) and the unbinned gene collection. Only bins, which contain at least 5 methanogenesis genes are plotted. Filling colors indicate taxonomy at Class level. Each dot represents a KEGG orthologue (KO) in the respective bin. Colors of the dots indicate the p-value of the log2FC difference between N2_MTR and H2_MTR samples. Horizontal dashed red lines mark the log2FC thresholds for significantly different KOs (respective p-value <0.05).

Interactions between methanogenesis and other metabolic processes

In addition to the methanogenesis pathways in the archeal bins, we identified 9 additional pathways that were expressed differently as the early response of the microbiota to H_2 injection. Figure 10 presents the Archaea and Bacteria bins that indicate substantial up- or down regulation of several KEGG pathways. It is clear that H_2 addition rapidly caused gene expression changes in the Archaea, i.e. bin_6, bin_27, bin_35 and bin_51, since the Ribosome, RNA polymerase and Methanogenesis pathways were altered mainly in these bins In the case of Archaea, one *Methanoculleus* bin (bin_6) and the two *Methanobacteria* bins (bin_35 305 and bin_51) responded with elevated gene expression in all pathways, while the *Methanosarcina* (bin_27) and *Iainarchaeia* (bin_18) responded with a substantial and general loss of transcripts, i.e. biological activity, in them Interestingly, the three *Methanoculleus* bins responded differently to the H_2 injection. Apparently, the entire metabolic activity, including all KEGG orthologs,

were tuned up in bin_6 (classified as *M. bourgensis*), whereas only Ribosomal activity, RNA transport and Lysine biosynthesis was strongly upregulated in bin_60 and hardly any change in metabolic activity took place in bin_66 representing presumably a different strain of *M. bourgensis*. Although their overall gene expression did increase (log2FC of 2.19 and 2.37, respectively), thus the observed differences might as well indicate a slower response by bin_60 and bin_66 and perhaps further H_2 addition would have triggered a response more similar to that of the abundant *M. bourgensis* (bin_6). If further experiments corroborate this situation, than the observation may indicate the time resolution limit of H_2 triggered transcription and metabolic changes. It seems that the whole RNA machinery must be altered for responding to a significant change in the environment. Indeed, almost all genes (including the subunits of RNA polymerase for instance) from these pathways were highly expressed in the *Methanomicrobia* and *Methanobacteria* bins, and 64% of them with a log2FC of 2 or higher (p-value of 0.05 or lower). The early response to H_2 injection by *Methanosarcina horonobensis* (bin_27) was quite the opposite as the expression of all investigated KEGG orthologs and metabolic pathways were hindered significantly, i.e. up to 33%. Other carbon metabolism-related pathways that showed an overall significant difference in the pathway enrichment analysis were "carbon fixation" pathways in prokaryotes and "glycolysis / gluconeogenesis", which showed a similar pattern. For example the folD gene of the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway) was transcribed vigorously in bin_6 (*M. bourgensis*) (log2FC =3.7). The relative enrichment of Methanogenesis, acetate => methane was overall the highest in this bin (mean log2FC =3.55), this can be linked to the elevated acetotrophic methanogenesis, as there were no other major difference between the expression change in these pathways. Interestingly though, the methanogenesis CO_2 to methane module did not increase drastically (nor did the methylotrophic module), with the exception of a handful of genes showing log2FC higher than 2, including methenyltetrahydromethanopterin cyclohydrolase gene in bin_6 and bin_35 (log2FC = 2.56 and 3.49, respectively), and some others with smaller but still significant differences, including the F420-non-reducing hydrogenase iron-sulfur subunit gene of bin_6 (log2FC = 1.32, p-value=0.04).

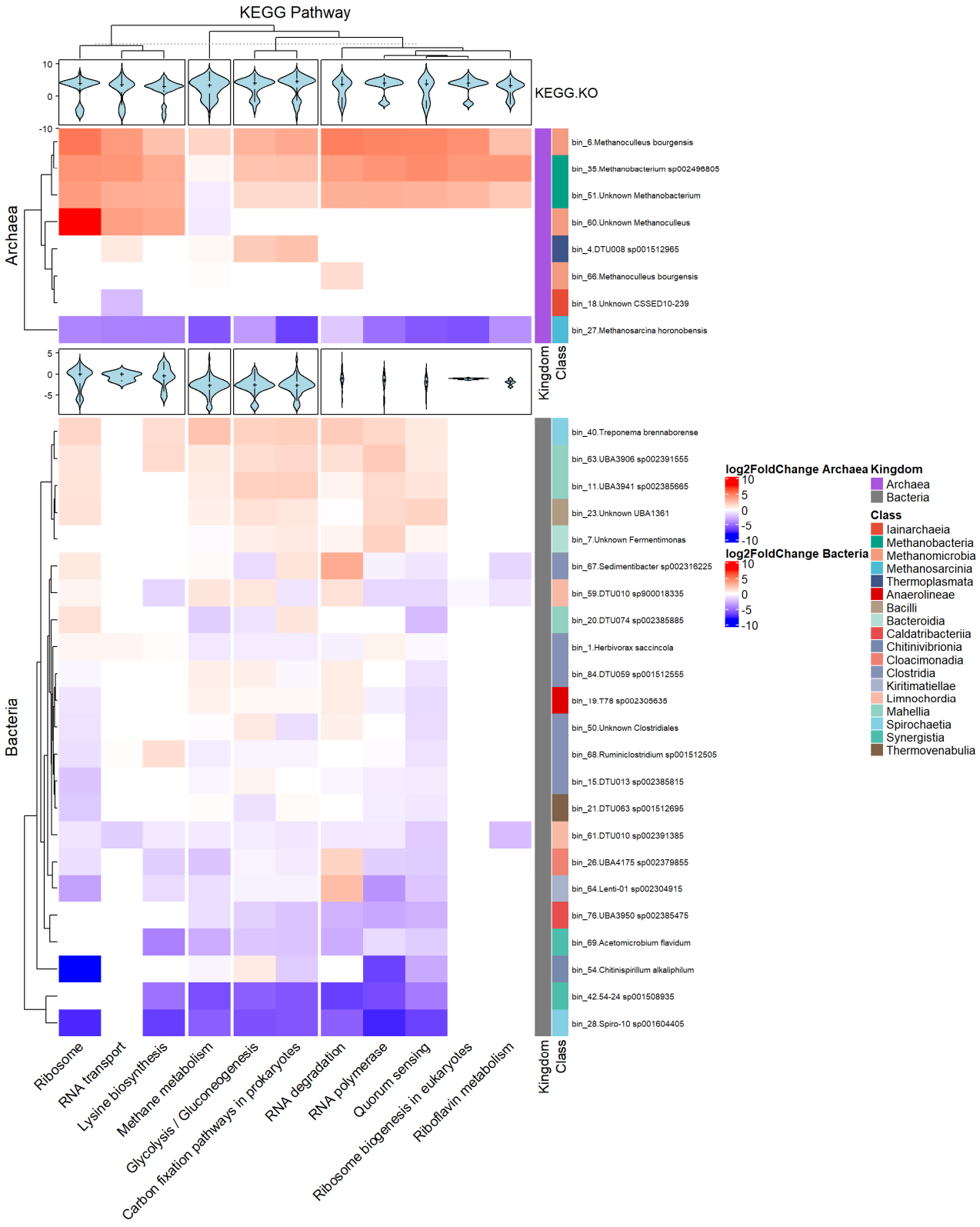


Figure 10. Heatmap of significantly various KEGG Pathways in bins that harbor a total of at least 10 genes in any of these pathways or modules. Top panel shows Archaeal, while the bottom panel shows Bacterial bins. Filling colors are according to the log2FC of all the genes in that pathway/module in the given bin. Violin plots represent log2FC values of every gene participating in the given pathway/module.

Changes in gene expression levels in bacterial bins

Some genes involved in, or related to elements of the methanogenesis pathway could be found in bacterial bins as well, e.g. *Herbivorax saccincola, Ruminiclostridium* sp001512505, two unknown *Limnochorida* and a *Mahellia* bin. However, when inspecting the change of the methanogenesis- related KEGG orthologs in the bins, it becomes clear that these genes showed significant difference only in a few cases, i.e. their log2FC values are spread between the variance lines. This means that while they are involved in the overall methanogenesis, and closely related metabolic pathways (which are included in the KEGG map00680 pathway), they did not respond to the H_2 provision change. This is substantially different from the behavior of the Archaea bins, which clearly express their genes differently as a respond to H_2 injection. In the case of Bacteria, the RNA-machinery pathways (ko03010) showed an overall decrease in gene expression, with the exception of bin_40 (*Treponema brennaborense*), bin_8 (*Fermentimonas massiliensis*), bin_11 (*UBA3941_sp002385665*) and bin_7 (*Unknown Fermentimonas*). These bins had low abundance, though they showed an increase in the MTR samples. These pathways seem to be up-regulated in bin_40 and in bin_11 (mapped in class *Mahellia*, order *Caldicoprobacterales*). Most of the small and large ribosomal subunits showed log2FC of 2 or higher. Another member of the family *Treponemataceae* (*bin_28 Spiro-10 sp001604405*) showed a clear downregulation in all discussed pathways. In AD, *Treponema* behave like the homoacetogenes, they consume H_2 and CO_2 to produce acetate, hence they may compete with hydrogenotrophic methanogens (Kotsyurbenko et al., 2001), although not very efficiently. We identified only two methanogenesis related genes in bin_28 and bin_40 (formate- tetrahydrofolate ligase and methylenetetrahydrofolate reductase NADPH), bin_40 showed an overall activity increase (log2FC =2.216), indicating either that this pathway would become more active at a later time-point, or these bacteria utilize alternative catabolic activities. Essential genes of the Wood-Ljungdahl (WL) pathway were apparently not expressed in bacterial bins in a recent study (Zhu et al., 2020). In contrast, in the present work we identified several bins harboring these genes, including bin_7 (*Unknown Fermentimonas*), bin_8 (*Fermentimonas massiliensis*) and bin_20 (*DTU074 sp002385885*), although all of them showed low abundance (~0.3-1%). Interestingly, bin_20 exhibited an overall decrease, but the expression of its WL pathway genes increased. This can be attributed to the elevation of the transcriptional activity of only two genes, the fhs gene (formate-tetrahydrofolate ligase) and the folD gene (methylene-tetrahydrofolate oxidase), which are important in WL pathway (log2FC = 6.31 and 3.14, respectively). This response to H_2 is thus the opposite to that of bin_40, suggesting that as acetogenic methanogenesis increased, it might have tried to compete with the Archaea for the acetate. The other two potential homoacetogens, which increased their transcriptional activity (log2FC = 1.40 and 2.56, respectively), apparently included the fhs and folD genes as well.

qPCR validation of the transcriptomic data

11 genes were selected for testing the metatranscriptomic data by Real-Time quantitative polymerase chain reaction (RT-qPCR). The genes were selected to cover a broad range of genes displaying various gene expression levels according to the metatranscriptomic data. Genes participating in methanogenesis as well as others involved in general in cell metabolism were included. Based on the log2FC (Figure 9) most of the examined genes showed consistent results with the metatranscriptomic data (Figure 11), although in several cases their fold change was slightly lower than derived from the metatranscriptomic analysis. Despite these minor differences, the RT-qPCR data clearly corroborated the MTR results.

Name of the encoded protein	Genes	Bin	log_2 fold change in metatranscriptome	qPCR log_2 fold change
methyl-CoM reductase beta subunit	*mcrB*	bin_27	-6.24	-8.79
(4-{4-[2-(gamma-L-glutamylamino)ethyl]phenoxymethyl}furan-2-yl)methanamine synthase	*mfnF*	bin_6	3.23	2.57
7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase	*cofG*	bin_6	4.20	0.98
Ribosomal protein L10	*rplJ*	bin_6	6.48	4.77
Coenzyme F420 hydrogenase subunit alpha	*frhA*	bin_27	-4.66	-5.75
enolase	*eno*	bin_6	5.06	4.19
glycerinaldehide 3-phosphate dehydrogenase	*gapA*	bin_59	3.65	3.01
peptid nickel transport system substrate binding protein	*oppA*	bin_59	-1.45	-2.93
pyruvate, phosphate dikinase	*ppdK*	bin_1	-1.52	-1.37
acetyl-CoA decarboxylase/synthase	*cdhC*	bin_27	-6.87	-8.84
methyl-CoM reductase gamma subunit	*mcrC*	bin_35	-1.58	-1.83

Figure 11. Genes and qPCR primers used in this study

Conclusions

In this study the response of the metanogenic strains and the mixed biogas microbial community to the presence of H_2 was examined. Metagenomic and metatranscriptomic analyses have been carried out to determine the changes of the expression levels of the different genes take part in the methanogenesis. The results indicated that the microbial community responded instantaneously to the presence of H_2. In mixed cultures the activity of acetotrophs reduced significantly. In addition, the metabolic activity of numerous bacterial strains changed substantially as a response to H_2. Clearly, the excess H_2 does not only affect the methanogenesis pathways in Archaea, rather the microbial community respond with a complete gene expression profile change, which seems to be rather selective. H_2 (and dissolved CO_2) is readily converted to CH_4 by both direct (hydrogenotrophic) and indirect (homoacetogenesis and subsequent acetotrophic) methanogenesis. Our results suggest that the second route is unlikely the predominant one in the early response of the microbial community to H_2 addition at least under mesophilic conditions, since the acetotrophic pathways reacted sluggishly, while the gene transcription of the hydrogenotrophic route increased dramatically after a very short period of extensive H_2 feeding. This predicts that under this operation conditions the physiological readiness of the hydrogenotrophic methanogen members of the community will determine the reactor response rate upon switch-on of the H_2 addition. Interestingly, this study revealed an extensive reaction to the transient H_2 stress within the Bacteria community as well although Bacteria cannot directly generate CH_4 from H_2 as many Archaea do. Many of these Bacteria

possess the complete or partial enzyme sets for the Wood-Ljungdahl pathway. These and the homoacetogens are probably the best candidates for syntrophic community interactions between members of the distinct kingdoms of Archaea and Bacteria. The details of these interactions in the complex anaerobic environment and consequences to stabilize robust and vigorous P2bioCH4 microbial communities during long term P2G operation should be the subjects of future studies. Nevertheless, the transcriptional activity of the primary potential syntrophic bacterial partners (bin_1 (*Herbivorax saccincola*), bin_68 (*Ruminococcus sp*)., and unidentified bins_59, _61, _63, see Fig. 4) did not change substantially upon H_2 exposure. This may mean that either there is enough syntrophic capacity already in the non-adapted, "raw" community to support increased hydrogenotrophic methanogen activity or the syntrophic partners respond slowly to the sudden H_2 burst appearing in the microbial environment.

The results of this study indicate a more global regulatory role of H_2 in the life of anaerobic communities than assumed earlier. The syntrophic interactions contribute to the stability and metabolic activity of the hydrogenotrophic methanogens. This, together with the non-sterile operation conditions and continuous supply of inexpensive catalyst, underlines the benefits of using mixed communities in the P2bioCH4 process instead of pure hydrogenotrophic cultures.

Publications based on the project:

Due to the COVID pandemic the planned conference in 2020 was deleted (Biogas Science 2020, Uppsala, Sweden).

Márk, Szuhaj; Roland, Wirth; Gergely, Maróti; Balázs Kakuk; Gábor, Rákhely; Kornél L., Kovács; Zoltán, Bagi. Pangenomic reconstitution and exploration of H_2 metabolic pathways in methanogens. (Manuscript ready for submission).

Balázs, Kakuk; Roland, Wirth; Gergely, Maróti; Márk, Szuhaj; Gábor, Rákhely; Krisztián, Laczi; Kornél L., Kovács; Zoltán, Bagi. Early response of methanogenic archaea to H_2 as evaluated by metagenomics and metatranscriptomics. Microbial Cell Factories, Manuscript submitted (manuscript ID: MICF-D-21-00083) (2021)

Roland, Wirth; Bernadett, Pap; Dénes, Dudits; Balázs, Kakuk; Zoltán, Bagi; Prateek, Shetty; Kornél L., Kovács; Gergely, Maróti. Genome-centric incestigation of anaerobic digestion using sustainble second and third generation substrates. Journal of Biotechnology, Manuscript submitted (manuscript ID: JBIOTEC-D-21-00385) (2021)

Norbert, Ács; Márk, Szuhaj; Roland, Wirth; Zoltán, Bagi; Gergely, Maróti; Gábor, Rákhely; Kornél L., Kovács. Microbial Community Rearrangements in Power-to-Biomethane Reactors Employing Mesophilic Biogas Digestate. Frontiers Energy Res. 7:132, (2019)

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