**Proteomics Analysis: Description of Results**

The significant reduction of the lag phase time in the assays with pure cultures of *Methanobacterium formicicum* in the presence of activated carbon (AC) led to further investigation on protein level as an attempt of understanding these observations. Samples of the pure culture without material and the pure culture with 0.5 g/L AC were taken in three different times, namely during the initial methane production phase, the middle of the exponential phase and in the end of the exponential phase for each condition (with and without AC, respectively).

The *M. formicicum* strain used in these assays was DSM 1535 and the proteome the corresponding database was used for proteomics analysis. The proteome of this strain possess 2392 proteins (<https://www.uniprot.org/proteomes/UP000032423>) from which 684 proteins were detected in the present analysis. The analysis of the proteins detected was done based on the following criteria: first, it was identified the proteins only detected in each condition (with or without AC) based on the peptide spectrum count, which means the spectra identified were present in one condition and absent in the other one; and then it was listed the proteins whose peptide spectra were detected in both conditions (with or without AC) but in which the level of expression/detection was different (up and down expression/detection). The analysis of the differences in proteins expression was performed with less of 10 % of uncertainty and between 10 % and 20 % of uncertainty.

From the analysis with less than 10 % of uncertainty, a total of 15 proteins were identified as being differentially expressed comparing both conditions (with and without AC). To facilitate the identification, the name of the proteins was put in italic.

In Table 1 are listed the proteins whose spectra were only detected during the proteomics analysis in the assay with the pure culture without AC. With an uncertainty less than 10 %, the following 9 proteins were identified as only present in the condition without the material:

* 5 proteins are related to information storage and processing, namely: *30S ribosomal protein S4*, *50S ribosomal protein L15* and *30S ribosomal protein S19e* with a role in translation, ribosomal structure and biogenesis; *Transcription initiation factor IIB (TFIIB)* and *2-aminoadipate transaminase* with a role in the transcription process;
* 1 protein is associated with cellular processes and signalling: *Signal recognition particle 54 kDa protein (SRP54)* with a role in intracellular trafficking, secretion, and vesicular transport;
* 1 protein is related to metabolism: *NH(3)-dependent NAD(+) synthetase* with a role in coenzyme transport and metabolism, whose pathway include cofactor biosynthesis; NAD(+) biosynthesis; NAD(+) from deamido-NAD(+) (ammonia route): step 1/1;
* The other 2 proteins (*Putative pantothenate synthetase*; *Ribonuclease J (RNase J)*) are poorly characterized according to the respective COG information.

**Table 1 –** Proteins identified as only being detected in the assay of the pure culture of M. formicicum without AC, with an uncertainty less than 10 %

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| --- | --- | --- | --- | --- |
| **Protein names** | **COG general functional category** | **COG functional category** | **COG protein description** | **Uncertainty** (%) |
| 30S ribosomal protein S4 | INFORMATION STORAGE AND PROCESSING | Translation, ribosomal structure and biogenesis | Ribosomal protein S4 and related proteins | 0.01 |
| Signal recognition particle 54 kDa protein (SRP54) | CELLULAR PROCESSES AND SIGNALING | Intracellular trafficking, secretion, and vesicular transport | Signal recognition particle GTPase | 0.02 |
| Transcription initiation factor IIB (TFIIB) | INFORMATION STORAGE AND PROCESSING | Transcription | Transcription initiation factor TFIIIB, Brf1 subunit/Transcription initiation factor TFIIB | 0.03 |
| 50S ribosomal protein L15 | INFORMATION STORAGE AND PROCESSING | Translation, ribosomal structure and biogenesis | Ribosomal protein L15 | 0.53 |
| Putative pantothenate synthetase | POORLY CHARACTERIZED | Function unknown | Uncharacterized protein conserved in archaea | 1.04 |
| Ribonuclease J (RNase J) (EC 3.1.-.-) | POORLY CHARACTERIZED | General function prediction only | Predicted hydrolase of the metallo-beta-lactamase superfamily | 1.04 |
| NH(3)-dependent NAD(+) synthetase (EC 6.3.1.5) | METABOLISM | Coenzyme transport and metabolism | NAD synthase | 9.71 |
| 2-aminoadipate transaminase (EC 2.6.1.39) | INFORMATION STORAGE AND PROCESSING | Transcription | Transcriptional regulators containing a DNA-binding HTH domain and an aminotransferase domain (MocR family) and their eukaryotic orthologs | 9.71 |
| 30S ribosomal protein S19e | INFORMATION STORAGE AND PROCESSING | Translation, ribosomal structure and biogenesis | Ribosomal protein S19E (S16A) | 9.71 |

On the other hand, Table 2 contains the proteins that were identified as only present in the condition with activated carbon. Only 2 proteins (*Anaerobic ribonucleoside-triphosphate reductase* and *CTP synthase (Cytidine 5'-triphosphate synthase) (UTP--ammonia ligase)*) related with metabolism, specifically with a role in nucleotide transport, were directly associated with the assay of the pure culture in contact with AC with less of 10 % of uncertainty. The protein CTP synthase (EC 6.3.4.2) is involved in the following pathway: pyrimidine metabolism; CTP biosynthesis via de novo pathway; CTP from UDP: step 2/2.

**Table 2 –** Proteins identified as only being detected in the assay of the pure culture of M. formicicum in the presence of 0.5 g/L of AC, with an uncertainty less than 10 %

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| --- | --- | --- | --- | --- |
| **Protein names** | **COG general functional category** | **COG functional category** | **COG protein description** | **Uncertainty** (%) |
| Anaerobic ribonucleoside-triphosphate reductase (EC 1.17.4.2) (Anaerobic ribonucleoside-triphosphate reductase NrdD) | METABOLISM | Nucleotide transport and metabolism | Oxygen-sensitive ribonucleoside-triphosphate reductase | 0.00 |
| CTP synthase (EC 6.3.4.2) (Cytidine 5'-triphosphate synthase) (Cytidine triphosphate synthetase) (CTP synthetase) (CTPS) (UTP--ammonia ligase) | METABOLISM | Nucleotide transport and metabolism | CTP synthase (UTP-ammonia lyase) | 9.71 |

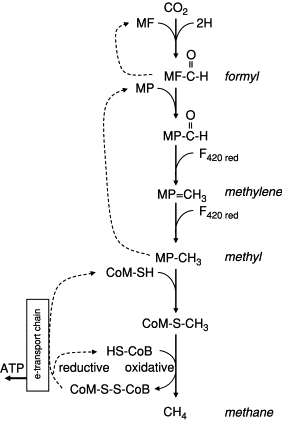
In Table 3 is possible to see the proteins that are common to both conditions but with different levels of detection. For the analysis with less than 10 % of uncertainty, only 4 proteins were identified as being present in both conditions, which are the following:

* 1 protein is uncharacterized, but the analysis reveals that this protein is under expressed in the condition with AC and its function is related to cellular processes and signaling, specifically with cell wall, membrane and/or envelope biogenesis, being descripted by COG information as “Putative peptidoglycan-binding domain-containing protein”;
* The other 3 proteins, which are *CoB--CoM heterodisulfide reductase subunit A HdrA2 (CoB-CoM heterodisulfide reductase iron-sulfur subunit A)* and *V-type ATP synthase alpha chain* *(V-ATPase subunit A)* (both with a role in energy production and conversion) and *Methyl-coenzyme M reductase subunit alpha* (with a role in coenzyme transport and metabolism) are related to metabolism and they were overexpressed (/over detected) in the condition with AC. The *Methyl-coenzyme M reductase subunit alpha* protein is involved in the following pathway: one-carbon metabolism; methyl-coenzyme M reduction; methane from methyl-coenzyme M: step 1/1.

**Table 3 –** Proteins identified as being detected in both conditions, namely in the assay of the pure culture of M. formicicum with and without 0.5 g/L of AC, with an uncertainty less than 10 %

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| --- | --- | --- | --- | --- | --- |
| **Protein names** | **COG general functional category** | **COG functional category** | **COG protein description** | **Uncertainty** (%) | **Level of detection/ expression (?)** |
| Uncharacterized protein | CELLULAR PROCESSES AND SIGNALING | Cell wall/membrane/envelope biogenesis | Putative peptidoglycan-binding domain-containing protein | 0.00 | Under expressed in AC |
| CoB--CoM heterodisulfide reductase subunit A HdrA2 (CoB-CoM heterodisulfide reductase iron-sulfur subunit A) (EC 1.8.98.1) | METABOLISM | Energy production and conversion | Heterodisulfide reductase, subunit A and related polyferredoxins | 0.03 | Overexpressed in AC |
| **Methyl-coenzyme M reductase subunit alpha (EC 2.8.4.1)** | METABOLISM | Coenzyme transport and metabolism | Methyl coenzyme M reductase, alpha subunit | 6.49 | Overexpressed in AC |
| V-type ATP synthase alpha chain (EC 7.1.2.2) (V-ATPase subunit A) | METABOLISM | Energy production and conversion | Archaeal/vacuolar-type H+-ATPase subunit A | 9.71 | Overexpressed in AC |

The proteins related with metabolism identified on Table 3 were overexpressed/ over detected when the hydrogenotrophic culture was in contact with 0.5 g/L of AC. These proteins play a role on methane production from carbon dioxide reduction (Figure 1), and they overexpression/over detection makes sense, since the main observations during the assays are the reduction of the lag phase duration (almost absent) followed by a quick initial methane production in comparison with the assay of the pure culture without the material.



**Figure 1 –** “Outline of the biochemistry of methane formation from CO2⧸H2. CO2 is activated with a methanofuran-containing enzyme (MF) with the reduction of the carbon to the level of formyl. The formyl carbon is transferred to an enzyme containing methanopterin (MP) with reduction steps to the methylene and methyl levels. The methyl carbon is transferred to a coenzyme M (CoM)-containing enzyme, forming CoM-S-CH3. Methane is formed as this complex combines with HS-CoB, forming also the heterodisulfide CoM-S-S-CoB. ATP is conserved as this complex is reduced back to the sulfide-bonded coenzymes. Modified from Madigan et al. (2003), with inspiration from Thauer (1998).” <https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/methanofuran>

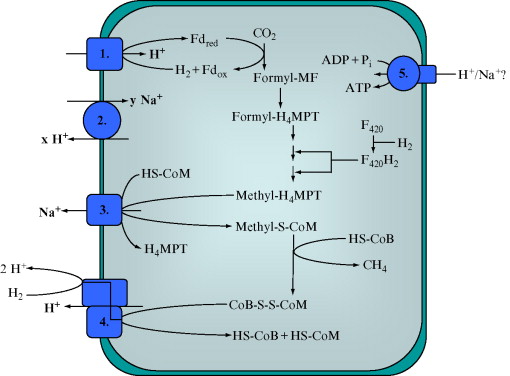


Figure 12.1. Ion currents during methanogenesis from H2 + CO2. Please note that this model only describes the pathway in M. mazei and M. barkeri. M. acetivorans does not contain hydrogenases. (1) Ech/Eha hydrogenase; (2) Na+/H+ antiporter; (3) methyl-H4MPT coenzyme M methyltransferase; (4) H2: heterodisulfide oxidoreductase system; (5) A1AO ATP synthase; MF, methanofuran; H4MPT, tetrahydromethanopterin; CoM-SH, coenzyme M; CoB-SH, coenzyme B; Fd, ferredoxin. <https://www.sciencedirect.com/topics/agricultural-and-biological-sciences/methanofuran>

When the analysis is extended to an uncertainty between 10 % and 20 %, more 19 proteins are identified as being differentially detected comparing the conditions with and without AC.

Table 4 shows 9 proteins that only were detected in the assay without AC for a level of uncertainty between 10 % and 20 %. The following proteins and their functions were identified:

* 2 proteins related with metabolism with a role on amino acid transport and metabolism: *ATP phosphoribosyltransferase (ATP-PRT) (ATP-PRTase)* (pathway: amino-acid biosynthesis; L-histidine biosynthesis; L-histidine from 5-phospho-alpha-D-ribose 1-diphosphate: step 1/9) and *Carbamoyl-phosphate synthase small chain* *(Carbamoyl-phosphate synthetase glutamine chain)* (pathway: Amino-acid biosynthesis; L-arginine biosynthesis; carbamoyl phosphate from bicarbonate: step 1/1.; pathway: Pyrimidine metabolism; UMP biosynthesis via de novo pathway; (S)-dihydroorotate from bicarbonate: step 1/3);
* 1 protein also related with metabolism but with a role in energy production and conversion: *Malate dehydrogenase*;
* 1 protein associated with cellular processes and signaling with a role in posttranslational modification, protein turnover, chaperones: *ATP-dependent protease S16 family (Archaeal Lon protease)*;
* 1 protein also associated with cellular processes and signaling but with a role in signal transduction mechanisms: *Histidine kinase/response regulator hybrid protein (Signal transduction histidine kinase)*;
* 2 proteins associated with information storage and processing, in which the *exosome subunit (Ribosome maturation protein SDO1 homolog)* has a role in translation, ribosomal structure and biogenesis and the *DNA ligase (Polydeoxyribonucleotide synthase [ATP])* that has a role in replication, recombination and repair;
* And finally, 2 proteins poorly characterized, where one of them is a *CBS domain-containing protein*.

From these proteins, 3 proteins are associated with metabolism, 2 proteins are related with cellular processes and signalling, 2 proteins are linked to information storage and processing and other 2 proteins whose function is poorly characterized.

**Table 4 –** Proteins identified as only being detected in the assay of the pure culture of M. formicicum without AC, with an uncertainty between 10 % and 20 %

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| --- | --- | --- | --- | --- |
| **Protein names** | **COG general functional category** | **COG functional category** | **COG protein description** | **Uncertainty**  (%) |
| ATP phosphoribosyltransferase (ATP-PRT) (ATP-PRTase) (EC 2.4.2.17) | METABOLISM | Amino acid transport and metabolism | ATP phosphoribosyltransferase | 10.34 |
| ATP-dependent protease S16 family (Archaeal Lon protease) (EC 3.4.21.-) | CELLULAR PROCESSES AND SIGNALING | Posttranslational modification, protein turnover, chaperones | Predicted ATP-dependent protease | 10.89 |
| Malate dehydrogenase (EC 1.1.1.37) | METABOLISM | Energy production and conversion | Malate/lactate dehydrogenases | 18.68 |
| Exosome subunit (Ribosome maturation protein SDO1 homolog) | INFORMATION STORAGE AND PROCESSING | Translation, ribosomal structure and biogenesis | Predicted exosome subunit | 18.96 |
| Carbamoyl-phosphate synthase small chain (EC 6.3.5.5) (Carbamoyl-phosphate synthetase glutamine chain) | METABOLISM | Amino acid transport and metabolism | Carbamoylphosphate synthase small subunit | 18.96 |
| DNA ligase (EC 6.5.1.1) (Polydeoxyribonucleotide synthase [ATP]) | INFORMATION STORAGE AND PROCESSING | Replication, recombination and repair | ATP-dependent DNA ligase | 18.96 |
| CBS domain-containing protein | POORLY CHARACTERIZED | General function prediction only | FOG: CBS domain | 18.96 |
| Uncharacterized protein | POORLY CHARACTERIZED | General function prediction only | Uncharacterized protein (ATP-grasp superfamily) | 18.96 |
| Histidine kinase/response regulator hybrid protein (Signal transduction histidine kinase) | CELLULAR PROCESSES AND SIGNALING | Signal transduction mechanisms | Signal transduction histidine kinase | 19.03 |

Table 5 shows 4 proteins that were detected in the assay of the pure culture in contact with 0.5 g/L of activated carbon and they are the following:

* *Arginine--tRNA ligase (Arginyl-tRNA synthetase) (ArgRS)*, a protein that in general is associated within the information storage and processing category and more specifically its function is related with translation, ribosomal structure and biogenesis;
* 1 uncharacterized protein was detected and it is related within cellular processes and signaling category and its function is associated with posttranslational modification, protein turnover, chaperones;
* *5'-deoxyadenosine deaminase (5'-dA deaminase)*, a metabolism protein with a role in nucleotide transport, whose pathway include amino-acid biosynthesis; S-adenosyl-L-methionine biosynthesis;
* And another uncharacterized protein whose function is unknown.

**Table 5 –** Proteins identified as only being detected in the assay of the pure culture of M. formicicum in the presence of 0.5 g/L of AC, with an uncertainty between 10 % and 20 %

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| --- | --- | --- | --- | --- |
| **Protein names** | **COG general functional category** | **COG functional category** | **COG protein description** | **Uncertainty (%)** |
| Arginine--tRNA ligase (EC 6.1.1.19) (Arginyl-tRNA synthetase) (ArgRS) | INFORMATION STORAGE AND PROCESSING | Translation, ribosomal structure and biogenesis | Arginyl-tRNA synthetase | 13.59 |
| Uncharacterized protein | CELLULAR PROCESSES AND SIGNALING | Posttranslational modification, protein turnover, chaperones | La protein, small RNA-binding pol III transcript stabilizing protein and related La-motif-containing proteins involved in translation | 18.96 |
| 5'-deoxyadenosine deaminase (5'-dA deaminase) (EC 3.5.4.41) (5'-methylthioadenosine deaminase) (MTA deaminase) (EC 3.5.4.31) (Adenosine deaminase) (EC 3.5.4.4) (S-adenosylhomocysteine deaminase) (SAH deaminase) (EC 3.5.4.28) | METABOLISM | Nucleotide transport and metabolism | Cytosine deaminase and related metal-dependent hydrolases | 18.96 |
| Uncharacterized protein | POORLY CHARACTERIZED | Function unknown | Uncharacterized conserved protein | 18.96 |

Table 6 allows to observe the main protein differences at level detection by comparing both conditions (with and without AC). It was detected 6 proteins divided in the following COG general categories:

* **Metabolism**: *F420-non-reducing hydrogenase subunit A*, with a role in energy production and conversion (Coenzyme F420-reducing hydrogenase, alpha subunit) and *Methyl-coenzyme M reductase subunit alpha* with a role in coenzyme transport and metabolism (Methyl coenzyme M reductase, alpha subunit), whose pathway include one-carbon metabolism; methyl-coenzyme M reduction; methane from methyl-coenzyme M: step 1/1. Both proteins were overexpressed/ over detected in the condition with AC;
* **Cellular processes and signaling**: *Proteasome subunit beta (20S proteasome beta subunit) (Proteasome core protein PsmB)* with a role in posttranslational modification, protein turnover, chaperones. This protein was under expressed/ under detected in the condition with AC;
* **Information storage and processing**: *Replication factor-A domain-containing protein* associated with replication, recombination and repair (single-stranded DNA-binding replication protein A (RPA), large (70 kD) subunit and related ssDNA-binding proteins) and this protein was overexpressed/ over detected in the condition with AC; *30S ribosomal protein S4e* (under expressed/ under detected in the condition with AC) and *Elongation factor 2 (EF-2)* (overexpressed/ over detected in the condition with AC) both with a role in translation, ribosomal structure and biogenesis.

**Table 6 –** Proteins identified as being detected in both conditions, namely in the assay of the pure culture of M. formicicum with and without 0.5 g/L of AC, with an uncertainty between 10 % and 20 %

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Protein names** | **COG general functional category** | **COG functional category** | **COG protein description** | **Uncertainty (%)** | **Level of detection/ expression (?)** |
| F420-non-reducing hydrogenase subunit A (EC 1.12.99.-) | METABOLISM | Energy production and conversion | Coenzyme F420-reducing hydrogenase, alpha subunit | 11.59 | Overexpressed in AC |
| Proteasome subunit beta (EC 3.4.25.1) (20S proteasome beta subunit) (Proteasome core protein PsmB) | CELLULAR PROCESSES AND SIGNALING | Posttranslational modification, protein turnover, chaperones | 20S proteasome, alpha and beta subunits | 15.03 | Underexpressed in AC |
| Methyl-coenzyme M reductase subunit alpha (EC 2.8.4.1) | METABOLISM | Coenzyme transport and metabolism | Methyl coenzyme M reductase, alpha subunit | 16.94 | Overexpressed in AC |
| Replication factor-A domain-containing protein | INFORMATION STORAGE AND PROCESSING | Replication, recombination and repair | Single-stranded DNA-binding replication protein A (RPA), large (70 kD) subunit and related ssDNA-binding proteins | 18.96 | Overexpressed in AC |
| 30S ribosomal protein S4e | INFORMATION STORAGE AND PROCESSING | Translation, ribosomal structure and biogenesis | Ribosomal protein S4E | 18.96 | Underexpressed in AC |
| Elongation factor 2 (EF-2) | INFORMATION STORAGE AND PROCESSING | Translation, ribosomal structure and biogenesis | Translation elongation factors (GTPases) | 18.96 | Overexpressed in AC |