Report of the Short-Term Scientific Mission (STSM)

STSM title:	**Transcriptomic study of the metabolism of rumen methanogens**
Reference:	ECOST-STSM-FA1302-160516-076649
STSM dates:	16/05/2016 – 17/06/2016
Visitor:	**Dr Livia Kolesar Fecskeova**; fecskeova@saske.sk
Home Institution:	Institute of Animal Physiology, Slovak Academy of Sciences, Kosice, Slovakia
Host:	**Dr Milka Popova**; milka.popova@clermont.inra.fr
Host Institution:	Institute National de la Recherche Agronomique, Clermont-Ferrand, France

Objective and the purpose of STSM

At the host institution Dr Milka Popova has a comprehensive ongoing-project that employs four pure cultures of rumen methanogens and aims to collect their complete biochemical and physiological characteristics and genome-wide expression profiles for combination with already available genomic data to predict a genome-scale metabolic network. Part of this project is an RNA-Seq experiment in which differential gene expression profiles of these methanogens in two growth phases will be generated and compared with focus on the transcripts of methanogenesis. I joined the group for this transcriptomic study.

Our goal was to extract high quality, intact RNA with high RIN (preferably 8 and higher) in sufficient yield (min. 3 µg needed for the RNA sequencing). We targeted two different growth stages of the microbial cultures – the **mid-log** phase when microbes were actively producing methane and the **early stationary** when H_2 was completely depleted from the culture and no active methanogenesis occurred. We needed RNA from several biological replicates from each microbial culture and growth phase and prepare it for sequencing.

Description of the work carried out

During my stay we followed a previously stated work plan. During the first week I got training on the anaerobic cultivation techniques, prepared the necessary amount of medium and started to grow my cultures and look after them. During the second, third and fourth week I was mainly collecting RNA and DNA samples. During the last week quantitative and qualitative analyses were done.

Four anaerobic mono-cultures of *Methanobrevibacter smithii, Methanobrevibacter ruminantium, Methanobrevibacter wolinii* and *Methanobacterium formicium* were grown in liquid batch media under the overpressure of H_2/CO_2 (80%:20%) at 39°C. Microbial growth was monitored

mainly by measures of methane production and H_2 consumption on an Agilent microGC gas analyser. We also followed the optical density of the cultures and changes in inner pressure inside the tube.

RNA and DNA extraction:

Microbial cultures were split in half and pelleted: one half was used for immediate RNA extraction, other half was saved for DNA extraction at -20°C.

During the first and second week trial RNA extractions were carried out from three actively growing microbial cultures and slight modifications in the extraction protocols were compared. We compared the yields with and without DNA digestion, with and without the use of liquid N_2 and also compared the yields from different amounts and growth stages of the cultures. RNA extraction using the RNeasy Mini kit from Qiagen with on-membrane DNase digestion without the need of using liquid N_2 gave us high quality and high concentration total RNA. The protocol included physical lysis using bead beating. RNAses were inactivated by β-mercaptoethanol in the lysis buffer. The concentration of RNA was measured by Nanodrop and its quality, integrity and DNA contamination was assessed on the Bioanalyzer (Agilent) RNA Nano 6000 microchip (Figures).

DNA was extracted using a standard protocol with chemical and physical lysis, chloroform-phenol-isoamylalcohol extraction and isopropanol precipitation. DNA yield was quantified on the Qubit fluorometer.

Two of our cultures (*M. smithii* and *M. formicium*) were growing actively, so all the extractions were completed by the end of the third week. Work with the other two microbial cultures were a bit delayed as for some time they showed no sign of growing and no methane production was noted. Eventually after preparing new media *M. ruminantium* started to grow actively and extractions could be completed in several biological replicates. Unfortunately, *M. wolinii* showed no sign of growth during my whole stay, despite we used up all the available stock cultures.

Q-PCR : 1. Quantification of the 16S rRNA gene

2. DNA contamination in RNA samples

SYBR Green based qPCR was run targeting the archaeal 16S rRNA gene. We used DNA templates for quantification of this gene and RNA templates for monitoring DNA contamination in them. Even though no trace of DNA contamination was seen in any of the Bioanalyzer profiles, qPCR showed amplification from the RNA samples. For this reason RNA samples were further subjected to DNA digestion by the Turbo Dnase kit (Thermo Fisher) and concentrated using the RNA Clean & Concentrator (Zymo Research). Another qPCR will follow to confirm all contaminating DNA was eliminated and samples will be sent to Illumina sequencing facility in Roy J. Carver, Biotechnology Center at the University of Illinois (Urbana, Illinois, USA).

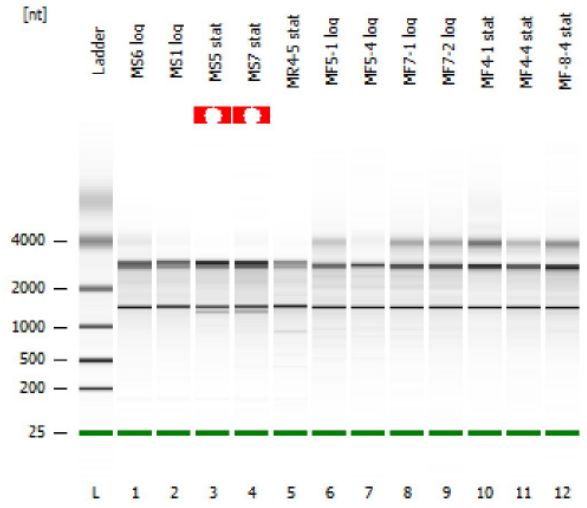
Output from the Bioanalyzer result

2100 expert_Prokaryote Total RNA Nano_DE54107264_2016-06-13_14-34-47.xad Page 1 of 15

Assay Class: Prokaryote Total RNA Nano
Data Path: \\I...rokaryote Total RNA Nano_DE54107264_2016-06-13_14-34-47.xad

Created: 6/13/2016 2:34:46 PM
Modified: 6/13/2016 2:58:39 PM

Electrophoresis File Run Summary



Instrument Information:

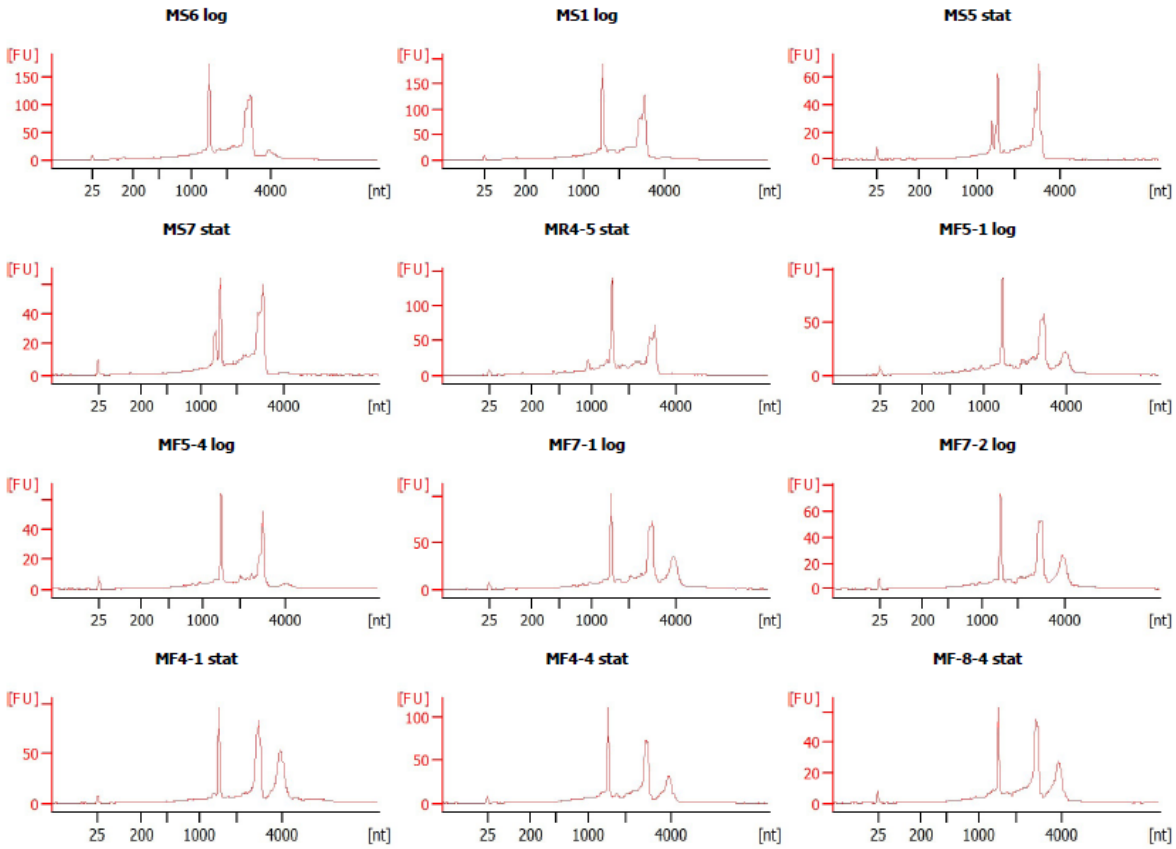
Instrument Name: DE54107264 Firmware: C.01.069
Serial#: DE54107264 Type: G2939A

Assay Information:

Assay Origin Path: C:\Program Files\Agilent\2100 bioanalyzer\2100 expert\assays\RNA\Prokaryote Total RNA Nano Series II.xsy
Assay Class: Prokaryote Total RNA Nano
Version: 2.5
Assay Comments: Total RNA Analysis ng sensitivity (Prokaryote)

Chip Information:

Chip Lot #: TH28BK20
Reagent Kit Lot #: 1537
Chip Comments:



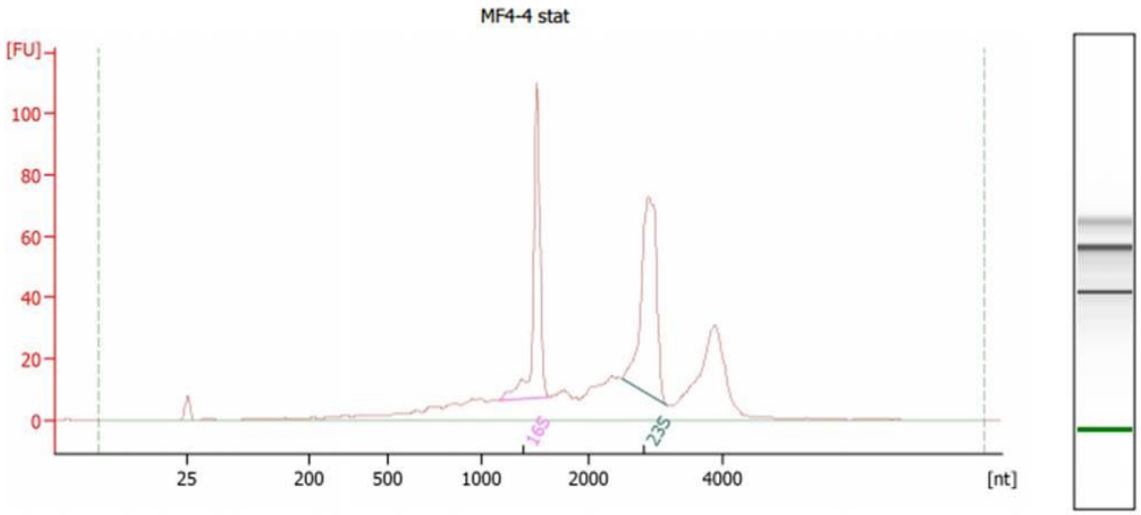
Total RNA profile of one of the samples.

Sharp peaks for 16S (~ 1500 nt in length) and 23S (~ 2800 nt in length). No sign of DNA contamination (lack of large peak at > 5000 nt). The third peak (at 4000 nt) might be due to insufficient RNA denaturation and thus migration of secondary structure.

2100 expert_Prokaryote Total RNA Nano_DE54107264_2016-06-13_14-34-47.xad Page 14 of 15

Assay Class: Prokaryote Total RNA Nano
Data Path: \\I...rokaryote Total RNA Nano_DE54107264_2016-06-13_14-34-47.xad
Created: 6/13/2016 2:34:46 PM
Modified: 6/13/2016 2:58:39 PM

Electropherogram Summary Continued ...



Overall Results for sample 11 : MF4-4 stat

RNA Area:	615.0	rRNA Ratio [23s / 16s]:	1.3
RNA Concentration:	364 ng/µl	RNA Integrity Number (RIN):	8.4 (B.02.08)

Fragment table for sample 11 : MF4-4 stat

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
16S	1,151	1,636	101.5	16.5
23S	2,491	3,176	128.9	21.0

RNA-Seq

When it comes to transcriptomic studies through RNA deep sequencing, it is all or nothing - meaning that the experiment requires thorough planning, precise design and the choice of the right options. During my stay I was involved in discussions and email communications regarding the conditions of sequencing (depth, rRNA depletion, library preparation) and requirements for the RNA supply (concentration, integrity, purity).

The usual protocol for RNA-Seq in case of transcriptomic studies includes an rRNA elimination step as these molecules count up to 95-98% of the total RNA sample. Prokaryotic rRNA depletion is carried out by probes that bind to the given ribosomal RNA with high specificity. In our case ribosomal RNA depletion was of a special concern due to non-existence of specific probes for Methanobrevibacterial rRNA.

Literature states that for differential gene expression studies 2-3 million reads of mRNA per sample are essential to follow the change with high statistical significance - in case biological replicates are available (Haas et al; 2012). However, the same paper mentions that with as low as 30,000 non-rRNA fragments per sample it was possible to identify over 180 genes whose abundance differed more than 2-fold between late exponential and early stationary phases ($P < 1\times10\text{-}5$) (*E. coli*).

Based on advice and literature data we decided to include a depletion step, but this will not be specific and depletion may fail. Still, even in that case sequencing will produce several hundred thousands of mRNA reads that will be enough to follow a differential gene expression with regard on transcripts of methanogenesis (Poulsen et al., 2013).

rRNA depletion using the RiboZero Bacteria kit (Epicentre), complete cDNA library preparations and the sequencing itself will be done at Biotechnology Center (Illinois, USA) on the HiSeq2000 sequencer in paired-end 160 nucleotide long reads. HiSeq sequencers produce up to 400 million reads per lane. Prepared libraries from four microbes in two different growth phases, each in at least two biological replicates, totally: (4x2)x2=16 samples, will be multiplexed in one lane resulting in 5-10 million reads per sample. Bioinformatic analysis of the raw transcriptomic data will be the objective of another Methagene short mission.

Conclusion and the big picture

Combination of generated transcriptomic data with available complete genomic sequences, biochemical and physiological data will be used for genome-scale metabolic modeling that will be used to predict a complete, species-specific and condition-specific metabolic network, under the assumed simplest conditions (mono-cultures with no interaction and influence of other organisms obtaining energy for growth only by reducing CO_2 to CH_4). Furthermore, all the collected information including growth rates, methane production and its kinetics, differential gene expressions in mid-log and stationary phases with reference cell number quantification can be integrated into a stoichiometric

model that would lead to metabolism quantification and comparison of the methanogens´ affinity for substrates and their efficiency of methane production. This metabolic model can be used for metabolic engineering of methanogens in favor of methane production/emission mitigations at its very core.

Acknowledgments

This short-term mission had mainly a learning-collaborative character. It provided me with lots of new information and experiences and a comprehensive introduction to the world of RNA and transcriptomics. Also, I believe I was an asset for the host institution. Results produced during this STSM will be used in a publication, where Methagene COST Action will be acknowledged. In the near future communications will continue as I would like to follow the outcome of RNA sequencing and further future collaborations will be discussed.

In the end I would like to acknowledge the COST Action FA1302 and thank for providing a financial support for this great opportunity. I would like to thank my host Dr Milka Popova for welcoming me and also thank all the members of the group for their help during my stay.

Confirmation by the host institution

See attachment

Haas BJ, Chin M, Nusbaum C, Birren BW, Livny J. How deep is deep enough for RNA-Seq profiling of bacterial transcriptomes? BMC Genomics. 2012;13:734. doi: 10.1186/1471-2164-13-734.

Poulsen M, Schwab C, Jensen BB et al. Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen Nat Commun. 2013;4:1428.
doi: 10.1038/ncomms2432.