A Biological Way of Methane Sensing

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Methane is a significant substance both in the environment and in the industry, thus indicating its presence has a great importance.

We aimed to develop a methane-biosensor medium containing genetically modified lactate producing methanotroph bacteria called Methylococcus capsulatus. During its metabolism, this bacterium produces pyruvate in the presence of methane. After having inserted the lactate-dehydrogenase gene, when an excess amount of methane is present in the environment of the bacteria, pyruvate is converted to have enough lactate for the enzymatic assay to change colour.

In the beginning, the bacteria were cultivated under methane-air mixture. For the genetic modification, we used the lactate-dehydrogenase gene from Bacillus coagulans. First, we put the gene into a specific vector designed especially for M. capsulatus then transform Escherichia coli with this plasmid. Using the method of triparental mating the plasmid was transferred into the M. capsulatus bacteria.

Keywords: methane, *Methylococcus capsulatus*, lactate-dehydrogenase, lactate, enzymatic assay

I. INTRODUCTION

1. About the project

In this year's iGEM competition, our team's project intends to approach problems related to methane leakage and emission. Some of the most serious problems in association with methane are the Earth's increasingly eye-catching climate changes caused by global warming. Methane plays a role in this process as a significant greenhouse gas, by trapping heat in the atmosphere. However, the greenhouse effect is not the only problem methane leakage contributes to. Methane accumulation in closed sites can lead to explosions and, as a result, fatal accidents. For example, this frequently occurs in many of developing countries' households, which use homemade biogas plants for cooking.

To reduce methane emissions, or to recognise its dangerous accumulation, the first step is to find locations where it is leaking. Therefore we aim to create a methane biosensor, a liquid bacterial medium, which changes its colour in the presence of methane. Thus methane leaking is immediately shown by the sensor's colour change easing its use compared to electronic gas sensors.

To create a biosensor we chose methanotroph bacteria, which utilise methane as their sole carbon and energy source. Among these bacteria, we chose *Methylococcus capsulatus* because this is the species, about which we had the most information. Given that the bacteria are methanotroph, the rate of their metabolism can refer to the amount of methane they utilise, which depends on the methane presence in their environment. To detect the rate of their metabolism, we had to find an organic compound that is not used up by their metabolic pathway of methane utilisation but can be set as this pathway product. We observed that pyruvate is an intermediate product of Methylococcus capsulatus' metabolism and it is convertible into lactate. The conversion of pyruvate into lactate is catalysed by an enzyme called lactate dehydrogenase (LDH). If the bacteria are equipped with the gene of LDH, and there is no obstacle of this gene's expression, they will produce lactate during their methane utilisation. Lactate accumulated in the medium then goes through an enzymatic assay, which causes the medium to change colour. Given that the bacteria are methanotrophs, this only occurs in the presence of methane. After all, this was clarified, the project dedicated itself to insert the gene of LDH into Methylococcus capsulatus successfully.

2. Former iGEM projects regarding methane

As all iGEM teams are aiming to solve real-world problems, it might not be surprising that our team is not the only one that focuses on methane. As global warming is becoming one of the most alerting issues of the 21st century and methane is one of its most significant contributors, the reduction of methane emission has become an attractive purpose of some former iGEM participant teams as well. Furthermore, in some cases, synthetic biology allowed these teams' products to provide solutions to problems besides the increasing methane emission as well.

In 2011, the iGEM participant Team METU-Ankara's motivation was to deal with the issue of methane explosions so-called "grisou" as they are one of the main problems of the coal mines. Their project was designed to construct an organism that senses the ambient methane gas and converts it into a potential biofuel source, methanol. Methanol is entrapped by-product of one of the constructs of the project, and then cells are dead by kill switch device of the project to elute methanol. For that purpose, they used E. coli. They also used, among others, the gene methane monooxygenase (MMO), which is related to our project as well. Unfortunately, they could not finish their project.

In 2012, Team JUST-India focused on methane and nitrous oxide emissions in rice agriculture. Their product would have converted methane into carbon-dioxide as well as converting nitrous oxide into nitrate, thus increasing the fertility of the soil. They would have used Methylococcus capsulatus and its MMO genes to convert methane into methanol, which is an inducer of one of Pseudomonas' genes, which converts nitrous oxide into nitrate. However, their webpage provides no information about their results.

Team Braunschweig, an iGEM participant team of 2014, has accomplished their intention of reducing greenhouse emission by their genetically engineered bacterium, 'E. Cowli'. Their modified organism can metabolise methane before its emission in cows' digestive tract without affecting them. They have equipped E. coli with the sMMO multi-enzyme complex of Methylococcus capsulatus and established the gene's stable expression in their product. Thus, methane produced by cows is converted into methanol before its potential emission.

Team UiOslo Norway, which participated in iGEM in 2015, planned to construct an organism, which converts methane into biomass. They intended to provide E. coli with Methylococcus capsulatus' gene, sMMO for the breakdown of methane into methanol, then convert it into biomass using the Ribulose-Monophosphate (RuMP)-pathway and establish this pathway in E.coli. The pathway is found in the methanotroph Bacillus methanolicus. They also wanted to create a filter that makes it possible to capture methane from the atmosphere. Though, they have only accomplished establishing the Ru-MP-pathway in E.coli.

In 2016, Team Maryland aimed to fulfil the plan for the biological removal of methane, which could be implemented into the piping of landfills for ameliorating global climate change. Their E. coli would express Methylococcus capsulatus' sMMO to convert methane into methanol, which by two metabolic pathways would be metabolized into either carbon dioxide. Their results only prove, that the latter pathway is successfully established in their modified organism. However, since this result is enough for their product to be effectively used, their project can be considered successful.

In conclusion, despite the unfinished iGEM projects regarding this topic, many prosperous results indicate that it is worth looking for further synthetic biological methods for the reduction of methane emission. We hope, our project's result will strengthen this statement.

II. BACKGROUND

1. The idea

First, we had the idea to create a methane biosensor using genetically modified bacteria, then came the question how. It was obvious to go for the methane-utilizing bacteria because if they are capable of taking up methane from their environment, we could use them as some detector. After a short research time, our choice became the bacteria Methylococcus capsulatus as it is most researched methanotroph bacterium. It is an obligate aerobe Gram-negative bacterium with coccus shape[1] (figure 3.1). Methylococcus capsulatus is also a thermophilic microbe which optimally lives at 45 °C. Methane-utilizing bacteria serve a unique biological function in earth's environment: they can oxidize the greenhouse gas, methane, as an energy source for growth (figure 3.2). This metabolism is called methanotrophy. Methanotrophy lowers the methane level in earth's atmosphere but does release the less potent greenhouse gas CO_2[2].

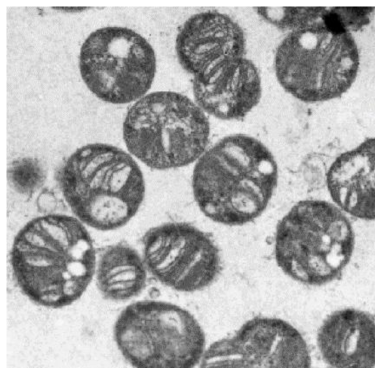


Figure 3.1: Methyloccus capsulatus

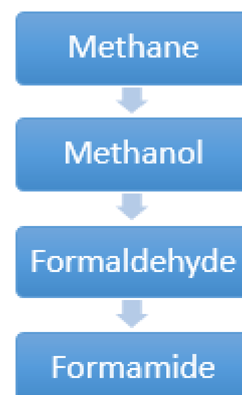


Figure 3.2

Our first idea was to make an Escherichia coli take up methane by producing the enzyme which the Methylococcus capsulatus also has to utilise methane. This enzyme is the methane monooxygenase. There are two well-studied forms of MMOs: the soluble form (sMMO), and the particulate form (pMMO). However, as both types of MMOs are huge and made up of multiple subparts, inserting their gene into an E. coli would have multiple types of plasmids, and we still would have been worried if the subparts would connect the right way in an E. coli. All of this would mean a very low transformation efficiency with a huge risk of not working at all[3].

Following this, we decided instead of using E.coli, in our final product we would use the Methylococcus capsulatus itself. We thought about silencing the gene that codes the enzyme which makes methanol from methane thus methanol could pile up, and we could have used an enzymatic assay to detect methanol but Methylococcus capsulatus *(Bath)* is an obligate methanotroph organism, which means, that we would have needed to insert first a gene that makes our bacteria able to use other carbon and energy source than methane and then we would have needed to silence the methanol dehydrogenase, which would have been very complicated and it would have resulted in an extremely low transformation rate.

Finally, we decided to measure the rate of metabolism of the Methylococcus capsulatus. For this, we had to find an organic compound that is not used in M. capsulatus' metabolism. We observed that M. capsulatus does not produce lactate during its methane utilization thus lactate could be used to monitor the rate of metabolism with its presence. Thus lactate accumulation could indicate methane presence.

2. Transformation method

To transfer our chosen gene into the Methylococcus capsulatus, we needed a transformation method and an appropriate vector. First, we thought about directly transforming the M. capsulatus with the construct, but it turned out that it has an extremely low transformation efficiency, so we choose the way what of triparental mating[3, 4.]

During searching for vectors, we discovered a research that has been conducted in our city[5]. This had a major importance because the article provided us not only various vectors that are specialised to methanotrophic bacteria but we also had the opportunity to consult someone who took part in research concerning modifications genetically on Methylococcus capsulatus and had experience in it.

3. In silico research

We needed an enzyme to catalyse the pyruvate-lactate conversion. Therefore, we searched for the lactate-dehydrogenase (LDH). Since the subject of this study is the *Methylococcus capsulatus (Bath)* bacterium, which is a thermophilic organism (lives optimally at 45 ^{o}C), we thought, it would be worth searching for the sequence of an LDH gene of a thermotolerant organism as well. For this, we used the European Bioinformatics Institute's database (www.ebi.ac.uk) to accomplish the search, and as a result, we found that the bacterium Bacillus coagulans 36D1 has an LDH enzyme, and lives optimally at 50 ^{o}C. The LDH sequence of the bacterium Bacillus coagulans 36D1 was found using the KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html). Opening the enzyme's datasheet, we realized that two enzymes are in connection with this reaction. We chose the L-lactate-dehydrogenase because it was smaller in size and had the usual beginning with ATG bases.

It was also essential to find an appropriate promoter with high efficiency to enable the gene to work. Therefore, we looked for a promoter which can be found originally in Methylococcus capsulatus. The former iGEM team, iGEM12_juit found a nearly 1 000 base pairs long sequence which contained a promoter but they could not determine the exact location of the promoter. We searched to find the 1 000 base pairs long sequence in Methylococcus capsulatus' complete genome. This sequence contained not only the nucleotide sequence between two genes (the Moxy and the mxaF) but did contain a partial part of each of the two genes. The putative Moxy or mxaF used in this study did only contain the nucleotide sequence between the two genes, which must contain the promoter of either the Moxy or the mxaF gene. Unfortunately, the orientation of the promoter is not known because the Moxy and the mxaF genes are in different directions, therefore we could not determine neither the exact orientation of the promoter nor the exact sequence but we managed to approach the exact sequence of the promoter and apply it in such a way that the orientation was not needed to know (figure 5.1).

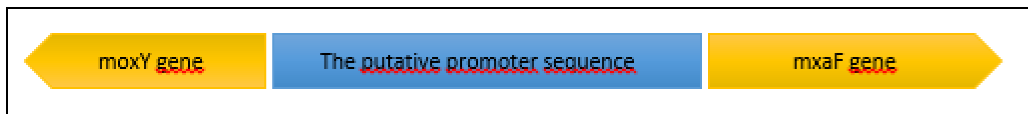


Figure 5.1: The position of the promoter used in this study

Having the sequence of the LDH gene and an appropriate promoter, only a usable vector was needed to transfer the gene and the promoter into Methylococcus capsulatus. An article[5] wrote about the vectors pMHE2, pMHE3, pMHE5, pMHE6 and pMHE7 (figure 5.2). We chose the pMHE5 and pMHE7 vectors because they were available for us in short time.

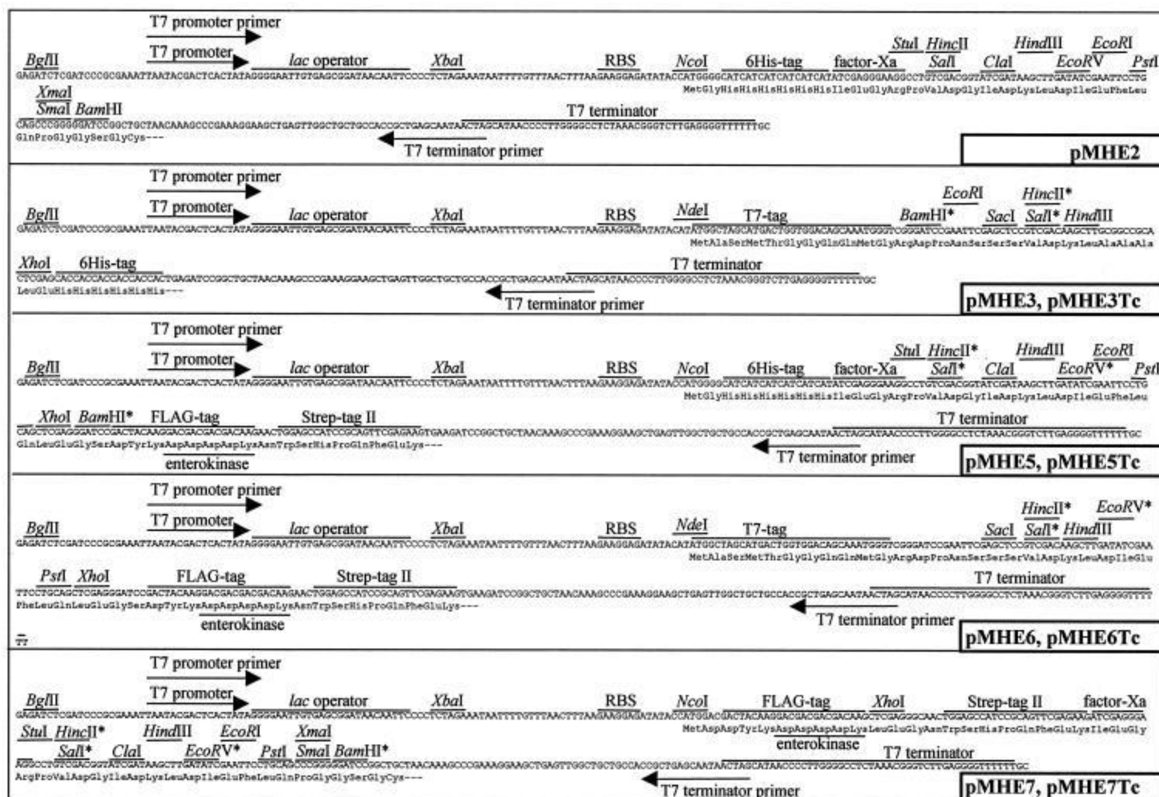


Figure 5.2

The LDH gene was intended to ligate between the NcoI and EcoRV restriction sites. Therefore, it was supplied with a NcoI restriction site and an addition AGTCAGTC nucleotide sequence before and after the NcoI restriction site, to minimalise the possible damage made by the restriction enzymes (figure 6.1). The LDH gene contained a NcoI restriction site in itself. Therefore, we replaced the bases so that the coded amino-acid did not change.

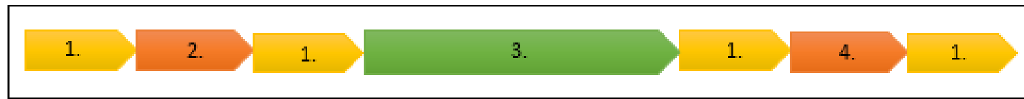


Figure 6.1: The basic structure of the synthesized gene

1. Addition AGTCAGTC bases
2. NcoI restriction site
3. LDH gene
4. EcoRV restriction site

The promoter was intended to ligate between the BglII restriction sites. This could enable the promoter to ligate in both orientations, randomly. The promoter was supplied with BglII restriction sites at both ends and addition AGTCAGTC nucleotides before and after each added AGTCAGTC nucleotides (figure 6.2).

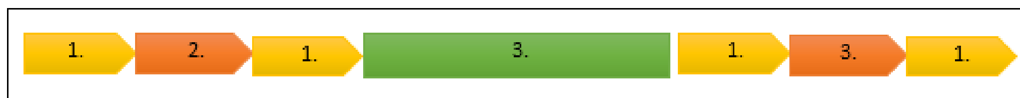


Figure 6.2: The basic structure of the synthesized promoter

1. Addition AGTCAGTC bases
2. BglII restriction site
3. Promoter

III. PROCEDURE

For further information please, visit the Methods page!

1. Plasmid construct

Before beginning the laboratory work, we had to design plasmids *in silico*, which contained an appropriate vector, the gene of the *lactate-dehydrogenase* enzyme (LDH) and an effective promoter (figure 7.1 and figure 7.2, created with Geneious 11). These were the requirements of making the bacteria produce lactate. *For further details of the bioinformatics research, please, click here.*

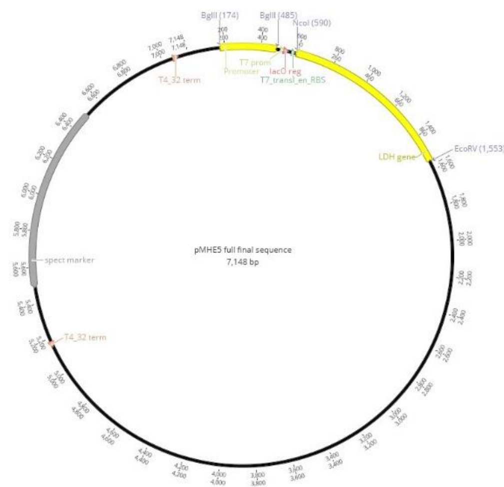


Figure 7.1: Final construct with the pMHE5 vector

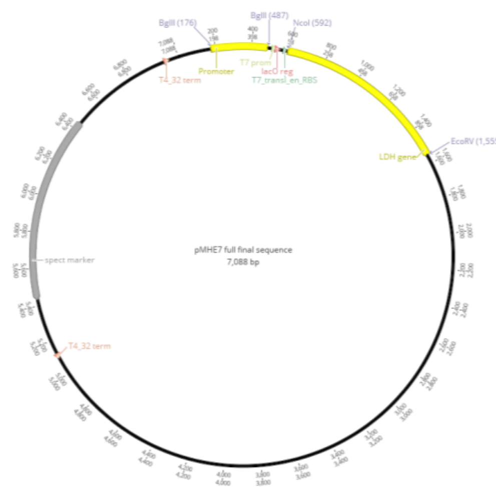
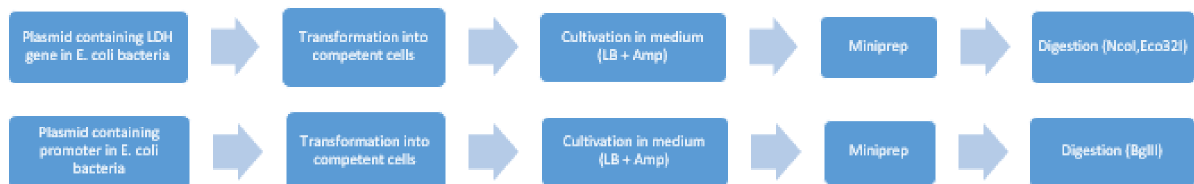


Figure 7.2: Final construct with the pMHE7 vector

When we had the exact sequence of the gene and the promoter, we sent the constructs to be synthesised.

2. Promoter and Gene preparation

We received the synthesised gene and promoter, both in sent in pMA-RQ plasmids. We aimed to transform the plasmids into competent Escherichia coli cells and after amplifying, extract them, and finally, with the help of restriction enzymes extract the LDH gene and the promoter from the plasmids in which they were distributed.



First, the pMA-RQ: *LDH* and the pMA-RQ: *promoter* plasmids were transferred into competent Escherichia coli cells. For this purpose, we used two methods: thermal shock (into E. coli JM109 cells) and electroporation (into E.coli DH5-Alpha cells). The gene and promoter samples were dissolved in nuclease-free water, and then they were added to the competent cells. During thermal shock, 42 °C was applied to the solution, while in the case of high electroporation voltage (1.8 kV) provided the possibility for the plasmids to get into the cells.

When the transformation was ready, we cultivated the cells in Petri-dishes. Both plasmids contained antibiotic resistance features (ampicillin). Therefore, the soils in the Petri-dishes were provided with ampicillin, therefore only successfully transformed cells were able to grow on the solid soils for a day. After that, the colonies were placed into liquid LB medium, which also contained ampicillin, and the samples were incubated for another day.

3. **Miniprep**

After a day, the number of bacteria increased large enough to start the extraction of the plasmids. For this purpose, we used the Thermo Scientific GeneJET Plasmid Miniprep Kit. During the extraction, we first filled the medium containing the bacteria into Eppendorf tubes and centrifuged them. After removing the supernatant, a resuspension solution was added to the bacteria and then we vortexed them. Next, we added lysis solution and, after a few minutes, neutralisation solution. Now, that plasmids were removed from the cells, after centrifugation, the supernatant was filled into Thermo Scientific spin columns. We added wash solution two times to the samples, and finally, by adding elution buffer and centrifugation, the plasmids were extracted into Eppendorf tubes.

4. **Digestion of the promoter and the gene**

To extract the gene and promoter from the pMA-RQ plasmids, we had to digest the constructs using restriction enzymes. At the ends of the gene there were NcoI and Eco32I (EcoRV) restriction sites, therefore, when we applied Thermo Scientific FastDigest NcoI and Thermo Scientific FastDigest Eco32I restriction enzymes, the gene became separated from the plasmid. Likewise, at both ends of the promoter, there were BglII restriction sites. Therefore the promoter was cut with Thermo Scientific FastDigest BglII. The successfulness of the digestion was controlled with agarose gel running (figure 8.1 and figure 8.2). The density of the samples shows that the E. coli transformation was more effective using thermal shock than using the method of electroporation. Because of this reason, the only thermal shock was used in the further studies.

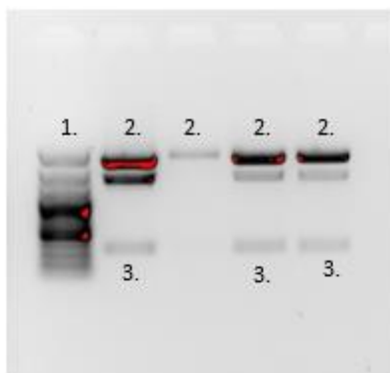


Figure 8.1: Promoter samples. The second sample did not contain the promoter

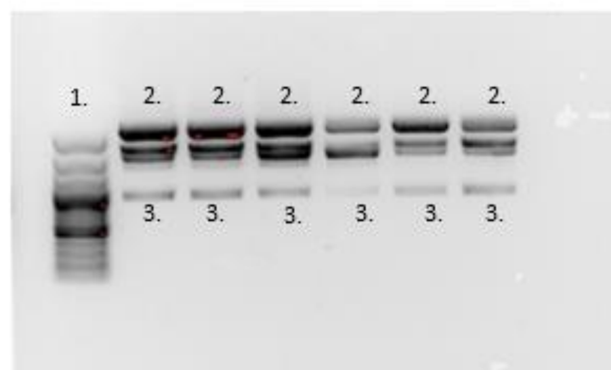


Figure 8.2: The first three samples beside the ladder are from
E. coli JM109 cells (transformed using thermal shock), and the next three samples are from E. coli DH5-Alpha cells

1. Thermo Scientific GeneRuler 100 bp Plus DNA Ladder
2. Undigested plasmids
3. Promoter/gene fragments

5. Gel extraction

When both the gene and the promoter were cut from the plasmid, we inserted them into agarose gel and let them run. Because of the size differences, the gene and promoter got further than the other parts of the plasmids. As a consequence, we were able to cut out only the gene and promoter from the agarose gel with a blade.

Our next task was to extract the necessary DNA fragments from the agarose gel for further use. Therefore, we applied the Thermo Scientific GeneJET Gel Extraction Kit. First, we dissolved the gel in Binding buffer at high temperature (60 ^{o}C), then transferred the liquid into Thermo Scientific purification columns. Then, we applied wash solution and finally, the DNA was extracted with elution buffer into new Eppendorf-tubes.

6. The vectors

We received the vectors pDSK5, pMHE5, and pMHE7[i] from the Biological Research Centre of the Hungarian Academy of Sciences in Escherichia coli bacteria. First, we had to decide which vector would be the best option for inserting the promoter and the LDH gene in it. The promoter had BglII restriction sites at both ends, while the gene had NcoI and Eco32I (EcoRV) restriction sites at the ends. To examine our plasmid, we decided to digest them with these three restriction enzymes.

There were three Escherichia coli samples (each of them contained one type of the three vectors) with many colonies, from which we first had to extract, and then amplify them. To be able to digest the plasmids, we placed colonies into a liquid LB medium, containing antibiotic: bacteria with carrying pDSK5 were kanamycin resistant, while bacteria carrying pMHE5 and pMHE7 plasmids were streptomycin resistant.

7. Miniprep

After a day, the number of bacteria increased large enough to start the extraction. For this purpose, we used the Thermo Scientific GeneJET Plasmid Miniprep Kit following the method described in III/3.

8. Control digestion and gel electrophoresis

After the pDSK5, pMHE5 and pMHE7 vectors were digested with Thermo Scientific FastDigest NcoI, Thermo Scientific FastDigest Eco32I, and Thermo Scientific FastDigest BglII restriction enzymes, gel electrophoresis was accomplished. According to the results (figure 10.1, figure 10.2, figure 10.3), only the pMHE5 and pMHE7 vectors were applicable for us because only these two types of plasmids contained the necessary restriction sites in the necessary number (only once).

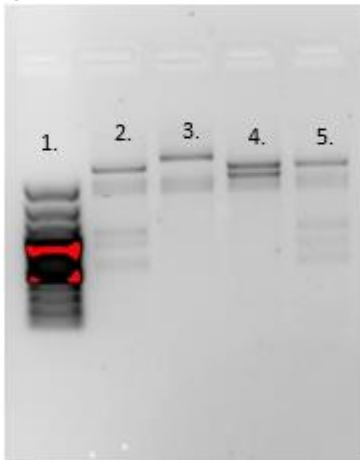


Figure 10.1: pDSK5 vector

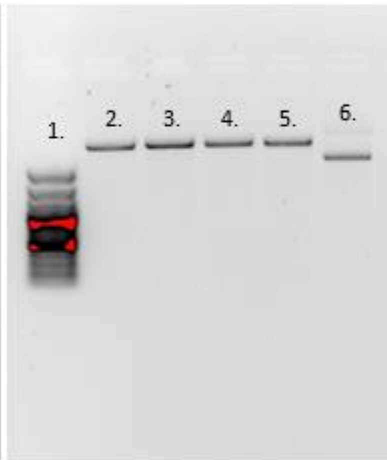


Figure 10.2: pMHE5 vector

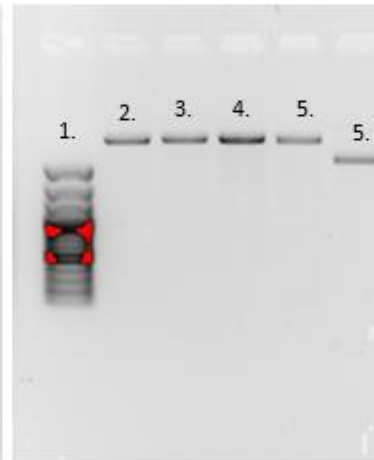


Figure 10.3: pMHE7 vector

1. Thermo Scientific GeneRuler 100 bp Plus DNA Ladder
2. Digested with Thermo Scientific FastDigest NcoI
3. Digested with Thermo Scientific FastDigest BglII
4. Digested with Thermo Scientific FastDigest Eco32I
5. Digested with Thermo Scientific FastDigest NcoI, BglII and Eco32I
6. Undigested plasmids

9. Gene ligation

Now, we had two types of plasmids, pMHE5 and pMHE7, the gene and the promoter. The next step was to assemble the final plasmid. We decided to ligate first the gene and the pMHE5 / pMHE7 plasmids because the gene had bigger size than the promoter. Therefore it was less likely that that the ligation would be successful.

First, the plasmids were digested with Thermo Scientific FastDigest NcoI and Thermo Scientific FastDigest Eco32I restriction enzymes. After that, Thermo Scientific T4 DNA Ligase was applied. We ligated together with the plasmids and the LDH gene, both having ends cut with NcoI and Eco32I. Then, we added the T4 DNA ligase, and after incubation, we the plasmid probably contained the gene. The experiments were carried out with the pMHE5 and pMHE7 vectors.

In this step, it was important that the ends of the LDH gene made the gene-oriented because it had a determined direction. Therefore it must have been inserted into the plasmid in the right direction. The orientation was due to that at the beginning of the gene, there is the sticky end, and at the end of the gene, there is the blunt end (figure 10.4).

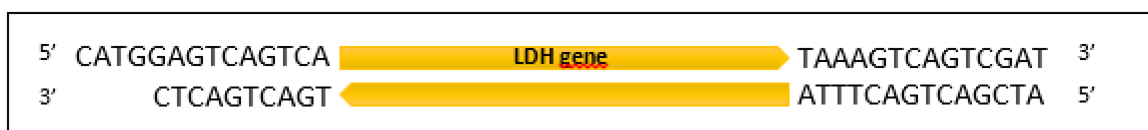


Figure 10.4: The ends of the synthetized LDH gene after digesting it with Thermo Scientific FastDigest NcoI (at the beginning) resulting sticky end, and Thermo Scientific FastDigest Eco32I (at the end) resulting blunt end

10. Transformation

The plasmids, which probably contained the LDH gene, were transferred into Escherichia coli JM109 competent cells with thermal shock. We cultivated the bacteria using the same method as described in III/2. When the transformation was ready, we the cells were placed in Petri-dishes. Both plasmids contained antibiotic resistance features (ampicillin). Therefore, the soils in the Petri-dishes were provided with ampicillin, therefore only successfully transformed cells were able to grow on the solid soils for a day. After that, the colonies were placed into liquid LB medium, which also contained ampicillin, and the samples were incubated for another day.

11. Miniprep, digestion and gel electrophoresis

After a day, we extracted the plasmid from the bacteria with Thermo Scientific GeneJET Plasmid Miniprep Kit (see III/3.) and checked the results with gel electrophoresis. We digested the samples with Thermo Scientific FastDigest NcoI and Thermo Scientific FastDigest Eco32I, and the two parts of the plasmid (the LDH gene and the initial pMHE5/pMHE7) could be seen in the gel (figure 11.1). It was an important step because we could not only control whether the ligation of the gene and the plasmid was successful but also ensure that later we will work only with the successfully ligated plasmid constructs.

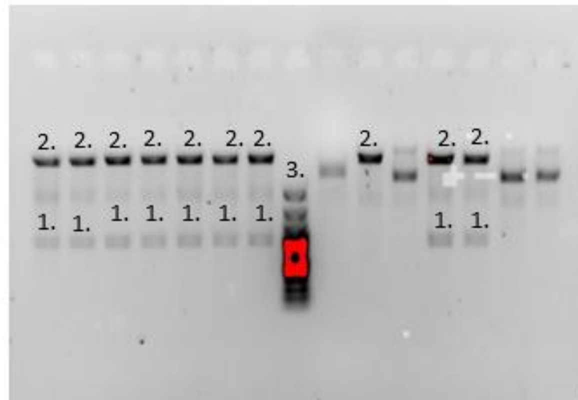


Figure 11.1: The digested putative plasmid-LDH constructs pMHE5-LDH plasmids on the left side and pMHE7-LDH constructs on the right side

1. Gene fragments
2. Undigested plasmids
3. Thermo Scientific GeneRuler 100 bp Plus DNA Ladder

12. Promoter ligation

We accomplished digested the ligated pMHE5 and pMHE7 plasmids containing the LDH gene with the Thermo Scientific FastDigest BglII. Having digested the plasmids, Thermo Scientific T4 DNA ligase was added to the sample. Since we did not know in which direction the promoter should have been inserted, the insertion of the promoter could occur randomly because at both ends of the promoter there was the same sticky end restriction sites. Consequently, nearly half of the promoters were inserted in one direction, while the other half in the other direction. As a result, we had the final plasmid, containing both the LDH gene and the promoter.

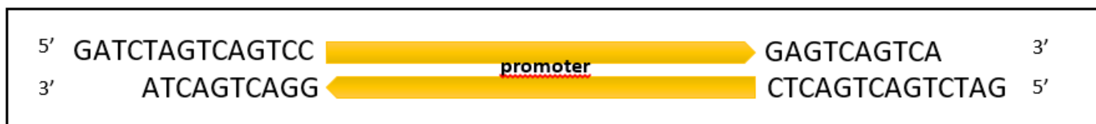


Figure 12.1: The ends of the synthetized promoter after digesting it with Thermo Scientific FastDigest BglII resulting sticky

13. Control digestion

The ligated samples were digested with Thermo Scientific FastDigest BglII, Thermo Scientific FastDigest NcoI and Thermo Scientific FastDigest Eco32I to control whether the ligation process was successful. The samples were incubated for 12 hours to enhance the efficiency of the digestion.

14. Gel electrophoresis

The digested samples were placed into agarose gel, and a gel electrophoresis was accomplished. The result of the gel electrophoresis (figure 12.2 and figure 12.3) showed that there were two samples which were sure to contain both the LDH gene and the promoter.

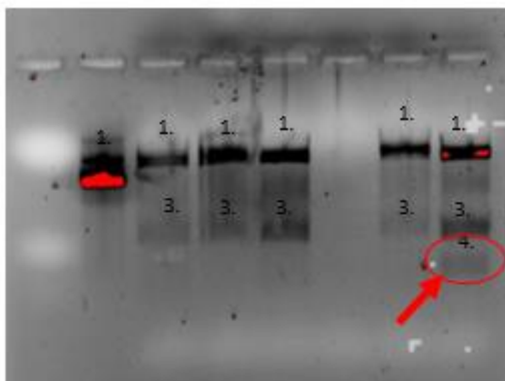


Figure 12.2: the digested plasmids

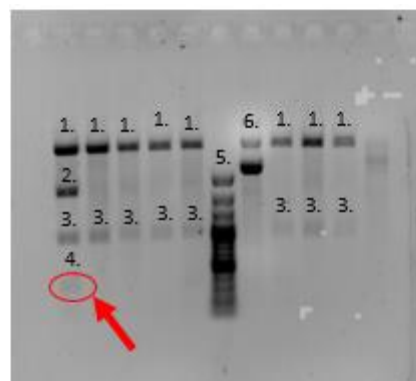


Figure 12.3: the digested plasmids

1. Undigested sample
2. Linearized vector
3. Gene fragment
4. Promoter fragment (pointed by the arrows)
5. Thermo Scientific GeneRuler 100 bp Plus DNA Ladder
6. Undigested plasmid

15. Cultivation of Methylococcus capsulatus

The Methylococcus capsulatus (Bath) bacteria were cultivated on solid LB soil containing nitrate mineral salts (NMS), under methane-air (1:1) mixture in glass flasks. The supply of methane was ensured by a balloon (figure).

1. We attach a balloon to a tube which supplies the methane gas from a methane-containing bottle.
2. The balloon is blown up by the gas. The volume of the balloon is almost the same as the volume of a bottle containing the Methylococcus bacteria.
3. We close the neck of the balloon with a clip.
4. The balloon is removed into the bacteria-containing tank.
5. Finally, we open the way between the balloon and the tank.

Methylococcus capsulatus bacteria are cultivated on NMS (Nitrate mineral salts) medium at 45°C.

The components of NMS medium are:

ATCC medium: 1306 Nitrate mineral salts medium (NMS)

$MgSO_4 \cdot 7H_2O$ ....1.0 g
$CaCl_2 \cdot 6H_2O$ ....0.20 g
Chelated Iron Solution (see below).......2.0 ml
KNO_3...................................1.0 g
Trace Element Solution (see below).......0.5 ml
KH_2PO_4 ....0.272 g
$Na_2HPO_4 \cdot 12H_2O$..........................0.717 g
Purified Agar (e.g., Oxoid L28).........12.5 g
Distilled deionized water................1.0 L

Adjust pH to 6.8. Autoclave at 121C for 15 minutes.

Chelated Iron Solution:
Ferric (III) ammonium citrate*...........0.1 g
EDTA, sodium salt........................0.2 g
HCl (concentrated).......................0.3 ml
Distilled deionized water..............100.0 ml

*0.05 g of Ferric (III) chloride may be substituted.

Use 2.0 ml of this chelated iron solution per liter of final medium.

Trace Element Solution:
EDTA................500.0 mg
$FeSO_4 \cdot 7H_2O$ ....200.0 mg
$ZnSO_4 \cdot 7H_2O$ ....10.0 mg
$MnCl_2 \cdot 4H_2O$ ....3.0 mg
H_3BO_3 ....30.0 mg
$CoCl_2 \cdot 6H_2O$ ....20.0 mg
$CaCl_2 \cdot 2H_2O$ ....1.0 mg
$NiCl_2 \cdot 6H_2O$ ....2.0 mg
$Na_2MoO_4 \cdot 2H_2O$..........3.0 mg
Distilled water........1.0 L

Autoclave at 121C for 15 minutes.

Figure 13.1: Preparing the DIY Methylococcus capsulatus cultivating bottle

16. Transformation of Methylococcus capsulatus

Methylococcus capsulatus (Bath) bacteria were transformed using the method of triparental mating[3, 4]. First, the final plasmid was transferred into E. coli JM109 lambda-pir competent cells with thermal shock (see III/2.). To the transformation, E. coli K12 HB101 helper cells were used to enhance transformation efficiency.

17. Lactate assay kit

Based on the homepage of the product; Catalog No. MAK064

Procedure

Lactate Standards for Colorimetric Detection Dilute
Ten mL of the 100 nmole/mL Lactate standard with 990 mL of Lactate Assay Buffer to generate a one nmole/mL standard solution. Add 0, 2, 4, 6, 8, and 10 ml of the 1 nmole/mL Lactate standard into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmol/well standards. Add Lactate Assay Buffer to each well to bring the volume to 50 mL. Lactate Standards for Fluorometric Detection Prepare a one nmole/mL standard solution as for the colourimetric assay. Dilute ten mL of the one nmole/mL standard solution of 990 mL of the Lactate Assay Buffer to make a 0.01 nmole/mL standard solution. Add 0, 2, 4, 6, 8, and 10 mL of the prepared 0.01 nmole/mL standard solution into a 96 well plate, generating 0 (blank), 20, 40, 60, 80, and 100 pmole/well standards. Add Lactate Assay Buffer to each well to bring the volume to 50 mL. Sample Preparation Both the colourimetric and fluorometric assays require 50 mL of sample for each reaction (well). Tissue or cells can be homogenised in 4 volumes of the Lactate Assay Buffer. Centrifuge the samples at 13,000 g for 10 minutes to remove insoluble material. Samples should be deproteinized with a ten kDa MWCO spin filter to remove lactate dehydrogenase. The soluble fraction may be assayed directly. Serum samples (0.5–10 mL/assay) can be assayed directly by adding in duplicate to 96 well plate. If lactate dehydrogenase activity is present, samples should be deproteinized with a ten kDa MWCO spin filter. Bring samples to a final volume of 50 mL/well with Lactate Assay Buffer. For unknown samples, it is suggested to test several sample volumes to make sure the readings are within the standard curve range. Note: Lactate Dehydrogenase (LDH) will degrade lactate. There, samples containing LDH (such as culture medium or tissue lysate) should be kept –80 °C for storage, and filtered through a ten kDa cut-off spin filter. Complete medium containing FBS should be deproteinized due to high LDH content.

Lactate Standards for Fluorometric Detection:

Prepare a one nmole/mL standard solution as for the colourimetric assay. Dilute 10 mL of the 1 nmole/standard solution with 990 mL of the Lactate Assay Buffer to make a 0.01 nmole/mL standard solution. Add 0, 2, 4, 6, 8, and 10 mL of the prepared 0.01 nmole/mL standard solution into a 96 well plate, generating 0 (blank), 20, 40, 60, 80, and 100 pmole/well standards. Add Lactate Assay Buffer to each well to bring the volume to 50 mL.

Sample Preparation:

Both the colourimetric and fluorometric assays require 50 mL of sample for each reaction (well). Tissue or cells can be homogenized in 4 volumes of the Lactate Assay Buffer. Centrifuge the samples at 13,000 ´ g for 10 minutes to remove insoluble material. Samples should be deproteinized with a 10 kDa MWCO spin filter to remove lactate dehydrogenase. The soluble fraction may be assayed directly. Serum samples (0.5–10 mL/assay) can be assayed directly by adding in duplicate to 96 well plate. If lactate dehydrogenase activity is present, samples should be deproteinized with a 10 kDa MWCO spin filter. Bring samples to a final volume of 50 mL/well with Lactate Assay Buffer. For unknown samples, it is suggested to test several sample volumes to make sure the readings are within the standard curve range.

Note: Lactate Dehydrogenase (LDH) will degrade lactate. There, samples containing LDH (such as culture medium or tissue lysate) should be kept –80 °C for storage, and filtered through a 10 kDa cut-off spin filter. Complete medium containing FBS should be deproteinized due to high LDH content.

Assay Reaction

1. Set up the Master Reaction Mix according to the scheme in Table 1. 50 mL of the Master Reaction Mix is required for each reaction (well).

Reagent	Master Reaction Mix
Lactate Assay Buffer	46 ±L
Lactate Enzyme Mix	2 ±L
Lactate Probe	2 ±L

2. Master Reaction MixAdd 50 mL of the Master Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 30 minutes at room temperature. Protect the plate from light during the incubation.
3. For colourimetric assays, measure the absorbance at 570 nm (A570). For fluorometric assays, measure fluorescence intensity (lex = 535/lem = 587 nm).

Calculations

The background for either assay is the value obtained for the 0 (blank) lactate standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate lactate standards to plot a standard curve. The amount of lactate present in the samples may be determined from the standard curve. Note: A new standard curve must be set up each time the assay is run.

IV. RESULTS

1. The Escherichia coli JM109 and DH5-Alpha colonies appeared in the Petri-dishes after transforming them with the synthesised pMA-RQ: promoter and pMA-RQ: LDH plasmids in III/2. There were two samples, in which no colonies were observable, and one sample got unsterile.
2. The digestion of the pMA-RQ: promoter and the pMA-RQ: gene plasmids resulted that the promoter and the pMA-RQ vector (figure 16.1), and in the other case the gene and the pMA-RQ vector (figure 16.2) were successfully separated from each other. Only one sample did not contain the wanted DNA fragment.

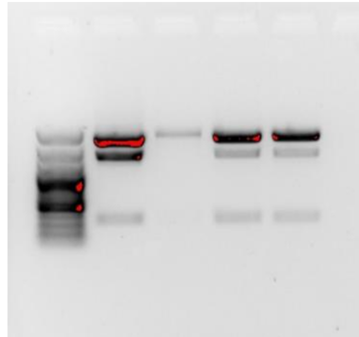


Figure 16.1: pDSK5 vector

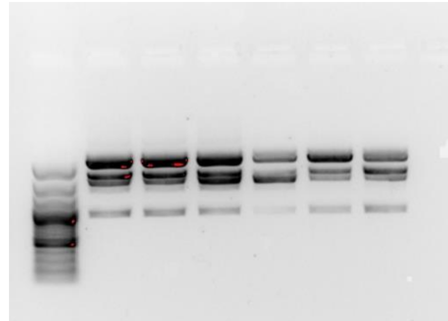


Figure 16.2: pMHE5 vector

3. The gel electrophoresis, after digesting the pDSK5, pMHE5 and pMHE7 vectors showed that for our purposes only the pMHE5 and pMHE7 plasmids could be used since only this vectors contain the necessary restriction sites (NcoI, BglII and EcoR32I) in the necessary amount (only once). The results of the restriction analysis are shown in figure 16.3, figure 16.4 and figure 16.5.

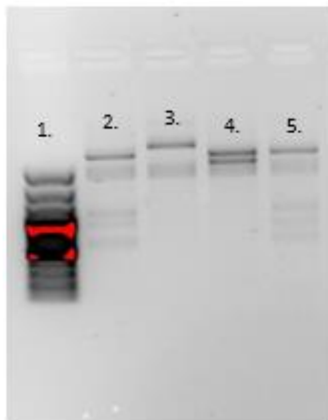


Figure 16.3: pDSK5 vector

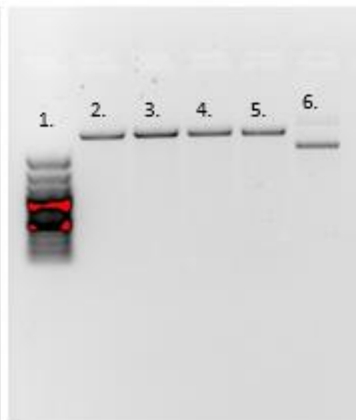


Figure 16.4: pMHE5 vector

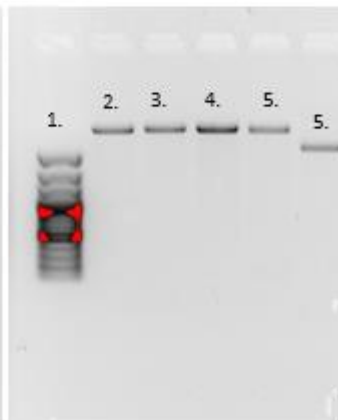


Figure 16.5: pMHE7 vector

7. Thermo Scientific GeneRuler 100 bp Plus DNA Ladder
8. Digested with Thermo Scientific FastDigest NcoI
9. Digested with Thermo Scientific FastDigest BglII
10. Digested with Thermo Scientific FastDigest Eco32I
11. Digested with Thermo Scientific FastDigest NcoI, BglII and Eco32I
12. Undigested plasmid

4. The gel electrophoresis, after digesting the pMHE5: LDH and pMHE7: LDH showed that the LDH gene was successfully ligated with the plasmids (figure 17.1).

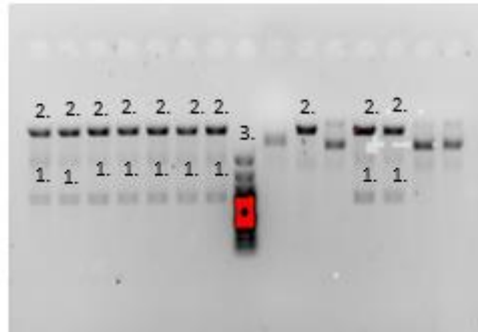


Figure 17.1: The digested putative plasmid-LDH constructs pMHE5-LDH plasmids on the left side and pMHE7-LDH constructs on the right side

4. Gene fragments
5. Undigested plasmids
6. Thermo Scientific GeneRuler 100 bp Plus DNA Ladder

5. The gel electrophoresis, after digesting the final constructs, resulted in two samples which contained both the LDH gene and the promoter.

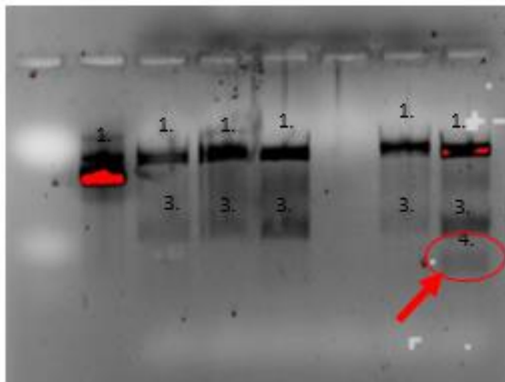


Figure 17.2: the digested plasmids

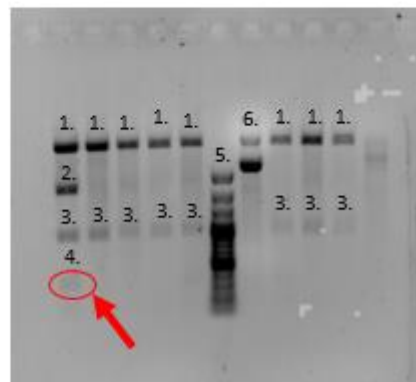


Figure 17.2: the digested plasmids

1. Undigested sample
2. Linearized vector
3. Gene fragment
4. Promoter fragment (pointed by the arrows)
5. Thermo Scientific GeneRuler 100 bp Plus DNA Ladder
6. Undigested plasmid

V. CONCLUSIONS

1. We designed *in silico* a possible promoter and a gene which have been synthesised
2. We managed to design a plasmid construct in silico containing the pMHE7 modular broad-host-range expression vector[5], the gene of the lactate-dehydrogenase (LDH) and the chosen promoter.
3. To the end of the project, we also succeeded in assembling these parts in vitro.
4. We have developed a DIY equipment in which the Methylococcus capsulatus bacteria could be cultivated under methane-air mixture.

VI. ACKNOWLEDGEMENTS

We would like to say special thanks to Gábor Rákhely, Ph.D., head of Department of Biotechnology of the Hungarian Academy of Sciences. He provided us valuable help during the planning and carrying out the experiments. He showed us the way of plasmid designation and also arranged us to be able to use the modular broad-host-range expression vectors, which were baselines in our project. Andrea Borbola, the assistant manager of the school laboratory, also meant a valuable help for us. She supported our project with her practised pieces of advice and was always there, even on Sunday afternoons, when we needed help. We would like to say thank for Lori Giver PhD and Sergey Stolyar, Ph.D. for giving us priceless help in cultivating and transforming the bacteria. Thank you also for Biomatters Ltd. for providing us with the molecular biology designing software, Geneious, which was a really helpful tool in our project. Thank you for the Szeged Scientists Academy for ensuring us the possibility to take part in the iGEM 2017 competition.

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