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Investigating Promoter-RBS Strength Variations in Methanosarcina acetivorans in Response to Different Growth Substrates

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TALLINN UNIVERSITY OF TECHNOLOGY
SCHOOL OF ENGINEERING
Department of Chemistry and Biotechnology

INVESTIGATING PROMOTER-RBS STRENGTH VARIATIONS IN *METHANOSARCINA ACETIVORANS* IN RESPONSE TO DIFFERENT GROWTH SUBSTRATES

PROMOOTORI-RBS TUGEVUSE VARIATSIOONIDE UURIMINE *METHANOSARCINA ACETIVORANS'IS* VASTUSENA ERINEVATELE KASVUSUBSTRAATIDELE

MASTER THESIS

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Tallinn 2023

AUTHOR'S DECLARATION

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Abstract:
Methanogenic archaea are strictly anaerobic microorganisms able to convert C1 compounds and acetate into methane to derive energy. *Methanosarcina acetivorans* is considered one of the model organisms for genetic engineering given its metabolic versatility. Despite this advantage, the complete regulatory transcriptional and translational mechanisms that govern its metabolism remain poorly understood. In this study, twelve promoter-RBS combinations from diverse methanogens were selected to test the strength and relevant response in *M. acetivorans*. Two growth environments were selected (Methanol and Trimethylamine), and the gene expression levels were quantified with the help of a β-glucuronidase reporter system. This activity provided a baseline to engineer the expression level by trying eight promoter-RBS combinations from four candidates: *hdr* from *M. mazei*, *mcr* from *Mc. voltae* and *ech* and *vht* from *Mb.* Fusaro. Our results reveal alterations in gene expression in response to the promoter-RBS swaps made (up to a 10-times increased activity), which indicates a regulation in the transcriptional and translational level in *M. acetivorans*. Additionally, eight promoter-RBS combinations were added to the original set of candidates for the *UidA* gene expression. These insights offer a wider perspective on the available tools for metabolic engineering of methanogens.

Keywords Promoter-RBS combination, Growth substrates, *Methanosarcina acetivorans*, Methanogenesis, Transcription, Translation

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Otaniemi, June 2023

Symbols and abbreviations

Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
MCR	Methyl-coenzyme M reductase
mRNA	Messenger RNA
PIC	Pre-Initiation Complex
RBS	Ribosome Binding Site
RNAP	RNA Polymerase
TBP	TATA-Binding Protein
TF	Transcription Factor
TFB	Transcription Factor B
tRNA	Transfer RNA
TSS	Transcription Start Site
UTR	Untranslated Regions

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1 Introduction

Methanogenic archaea are strictly anaerobic organisms that harness energy (and synthesize ATP) through methane production. Most microorganisms belonging to this classification are able to complete this pathway with the uptake and reduction of single carbon compounds (methanol, carbon dioxide, carbon monoxide and methylamines among others) and acetate, even though now there are more substrates known to be useful for the same purpose (Buan, 2018).

The biological production of this gas plays a significant role in the carbon cycle since these organisms use CO_2 and H_2 as reactant to release methane into the environment; This is a greenhouse gas, that even though it contributes to global warming, also has industrial applications on the bio-based energy field (Carr & Buan, 2022).

Methanogenesis takes place under strict anaerobic conditions to ultimately reduce a methyl-coenzyme M molecule into methane, mediated by the methyl-coenzyme M reductase (*MCR*) enzyme. Many methanogens' metabolisms are well-defined in general terms. One way to classify them, is by the catabolic pathway they follow to produce methane. The four ones described until now are the following: (1) methylotrophic methanogenesis using methanol, methylated amines, or methyl thiols, (2) methyl reduction with hydrogen, (3) acetate fermentation, and (4) CO_2 reduction using H_2, formate, or secondary alcohols. On the other hand, there is limited information about gene regulation within these environments (Sowers, 2009).

The aims of this thesis are to (first) investigate how different growth substrates affect the strength of native promoters coming from different methanogenic strains in *Methanosarcina acetivorans*; and (second) provide insights on how the manipulation of one (or more) promoter element(s) can affect the gene expression level. The present work will quantify the strength

of 12 promoters-RBS combinations in the environment of two methylotrophic substrates: methanol and trimethylamine in *M. acetivorans* by. This organism is a versatile methanogen in terms of substrates usage; it is not limited to one carbon source, and this strain can also uptake acetate and follow the aceticlastic pathway to conserve energy (Mand & Metcalf, 2019). *Methanosarcina acetivorans* comes across as a good model system for studying gene regulation, since its' genome encodes the largest number of transcription factors known until now. By understanding the roles that the promoter region and the associated elements play, from the transcriptional and translational level, it is possible to provide new tools for genome engineering in methanogens as well as other applications such as fine tuning of protein levels.

2 Literature review

2.1 Methanogenic archaea

Methanogens are prokaryotic microorganisms that belong to the to the phylum *Euryarchaeota* and Archaea domain (Liu, 2010). They are strict anaerobes and can be found in environments such as marine sediments, in ruminants and landfills, to name a few. These microorganisms thrive in extreme environments regarding temperature (from 1.7 °C to 110 °C), pH (5 to 13) and salinity (Sowers, 2009).

Methanogens are model organisms for archaeal transcription, translation, and gene regulation. *Methanococcus* and *Methanosarcina* are two genera for which these processes have been widely studied, and the engineering tools available have been improved over the years (Leigh et al., 2011). *Methanosarcina* is particularly a good model organism, because even though the growth is slower, their metabolism offers great versatility to carry out complex experiments.
There are multiple classifications for methanogens, and one of these divides them into two groups depending on the (1) absence or (2) presence of cytochromes. For instance, those belonging to the first group utilize H2, formate or secondary alcohols that will function as electron donors to reduce CO2. The hydrogenotrophic route follows several steps, in which H2 is used as electron donor (Berghuis et al., 2019).

On the other hand, the ones that possess cytochromes can accept more substrates such as acetate and some methylated compounds for methanogenesis (in addition to CO2 reduction) (Mand & Metcalf, 2019). This characteristic will affect their metabolic capabilities. *M. acetivorans* belong to the second group and can produce methane using the hydrogenotrophic, aceticlastic or methylotrophic pathways. These are going to be discussed further in this thesis. It is noteworthy that the *M. acetivorans'* genes that are expressed when they grow in a specific substrate will not affect the expression of the ones

needed for a different substrate. This is the reason why both pathways (methylotrophic and aceticlastic) can be studied independently.

2.2 Methanogenesis in *Methanosarcina acetivorans*

Methanogenesis is a form of anaerobic respiration in which methane is the final product. As previously states, the route that each microorganism can take, will differ depending on their morphology, the environment, and specific metabolic preferences. For this purpose, three major pathways of methanogenesis are known depending on the substrates available and the environmental conditions: hydrogenotrophic, methylotrophic, and aceticlastic shown in the figure 1. Undoubtedly, in natural habitats, the growth factors and overall conditions are generally less favourable than the ones in optimized cultures.

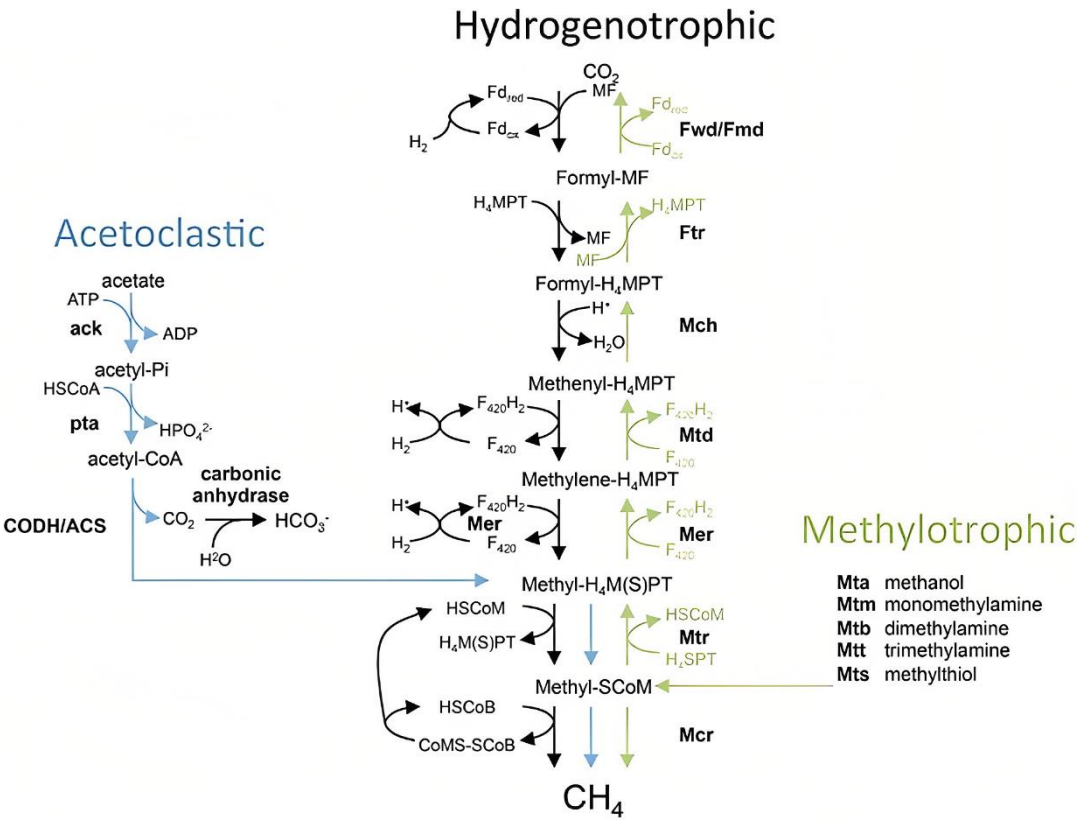

Fig 1. Pathways followed by M. acetivorans for methanogenesis in blue and green. (Lambie et al., 2015)

According to literature, temperature and substrate concentration are two of the most important factors for energy obtention, whereas pH and pressure have a moderate effect if modified. The production of these substrates is done by a range of microorganisms in two general steps; First, the decomposition of organic matter (polysaccharides, proteins, nucleic acids, etc.) is carried out by fermentative bacteria and fungi present in the environment. These will subsequently be hydrolysed into formate, alcohols, hydrogen, carbon dioxide and acetate (methanogenic substrates) (Formolo, 2010). In the second step, methanogens will take these products and convert them into methane and carbon dioxide. Methanogenic substrates are classified in three categories depending on the metabolic pathway they follow: CO2, methylated compounds, and acetate (Table 1).

Table 1. Methanogenic substrates and reactions. Modified from (Liu & Whitman, 2008).

Substrate	**Reaction**	**Organisms**
CO2	$4H_2 + CO_2 -> CH_4 + 2H_2O$	Most methanogens
	$4H_2 + CO_2 -> CH_4 + 2H_2O$	Hydrogenotrophic methanogens
CO	$4CO + 2H_2O -> CH_4 + 3CO_2$	*Methanothermobacter* and *Methanosarcina*
Methylated compounds	$4CH_3OH -> 3CH_4 + CO_2 + 2H_2O$	*Methanosarcina* and other methylotrophic methanogens
	$(CH_3)_3N + 6H_2O -> 3CH_4 + 2CO_2 + 2H_2O$	
Acetate	$CH_3COOH -> CH_4 + CO_2$	*Methanosarcina* and *Methanosaeta*

For this thesis' purposes, *Methanosarcina acetivorans*, from the *Methanosarcinales* order is employed. The species belonging to this order are the most diverse, regarding metabolic pathways, and this specific organism can use either single-carbon compounds like methanol, mono-, di- and trimethylamines as growth substrates, or two-carbon ones such as acetate (Galagan et al., 2002). Both pathways share the last steps, when methyl-CoM is reduced to methane. The overview for these pathways is shown in Figure 1.

2.2.1 Methylotrophic pathway

The second pathway introduced is one of the most studied among methanogenesis. It begins when the methyl group from the methylated compounds is transferred by a (substrate-specific) methyltransferase, before being transferred for a second time, forming Methyl-SCoM. Subsequently, this molecule will enter the methanogenesis pathway and be furtherly reduced to methane with help of the *MCR* enzyme (Liu & Whitman, 2008). The specific methyltransferases needed for each substrate are listed in the figure 1, under the methylotrophic pathway. This study focuses on the growth on methanol and trimethylamine conditions.

2.2.2 Aceticlastic pathway

Finally, the pathway for acetate catabolism begins with the formation of acetyl-CoA, with the mediation of phosphotransacetylase and acetyl kinase enzymes in *Methanosarcina*. The C-C and C-S bonds of this molecule are then cleaved by the acetyl-CoA decarbonylase/synthase complex. Next, the methyl group is transferred to H4MSPT, and follows the same steps to furtherly bind that same methyl group to HS-CoM through methyltransferases. Finally, methyl-SCoM can be converted to methane with the help of a methyl reductase (Sowers, 2009).

2.3 Gene expression in *Methanosarcina acetivorans*

Gene expression in the archaeal domain is a field that is constantly expanding, given to the complexity of the mechanisms involved. However, there are key aspects that allow us to understand better their metabolism and how it is modulated. Gene regulation, more specifically in the translation step is the most common in archaea for two main reasons. It is a rate-limiting stage and it gives the cell enough time to respond to changes compared to the transcription step (Brenneis & Soppa, 2009). Furthermore, regulation at this stage is a mechanism involved in cell survival and stimuli responses.

Global regulation happens when translation initiation factors are changed whereas mRNA specific regulation refers to the modification of protein complexes usually present in the UTRs of the target mRNA by different means (Gebauer & Hentze, 2004). The discovery of archaeal transcription mechanism, together with the transcription machinery (coupled system) were two milestones in this field. These two processes have some similarities with eukaryotic mechanisms (such as the RNA polymerase composition and the same basal transcription initiation and elongation factors) and with bacterial ones (transcription unit organization and mRNAs basic elements) (Blombach et al., 2019).

On the same line, the characterization of archaeal promoters helped to the characterization of gene regulatory networks. Studies have focused on the design of methods to find these pathways on model archaeal organisms. One example of this is the utilization of the *uidA* gene as a reporter gene; First in *Methanococcus voltae*, and then in *Methanosarcina* (Rother et al., 2011). This genetic system has the advantage of saving time, effort, and materials to test different conditions. Furthermore, the quantification of the activity can be done using the appropriate substrate (Rother et al., 2011).

2.3.1 Transcription

There are three main steps in the archaeal transcription cycle: (1) Initiation, (2) Elongation and (3) Termination. This mechanism has been thoroughly studied *in vitro*, which makes it easier to collect more information. However, transcriptional regulation within methanogenic archaea is not fully understood yet.

For the transcription process to begin, a pre-initiation complex (PIC) needs to be formed. At first, two elements get recruited, which are the transcription factor B (TFB) and the TATA-binding protein (TBP) (they recognize the B-recognition element and TATA-box, respectively). *Methanosarcina acetivorans* possesses one of the former and three of the latter, which gives

the possibility of a different gene expression mechanisms depending on the complex formed (Galagan et al., 2002). They get attached to the promoter region, followed by the RNA polymerase (RNAP) and the transcription factor E. The binding of the first two elements to the promoter produces a bend in the DNA, giving the right orientation for the TFE to ease the conversion from the closed complex to the open complex. When the change is produced, the template strand can be inserted to the active site of the RNAP. The transcription start site (TSS) is mainly determined by the initiator promoter element. At the beginning of the transcription, short "abortive transcripts" are synthesized and DNA scrunching (compression created when the RNAP stays stationary causing tension in the DNA molecule) takes place (Kapanidis et al., 2006). When the interaction with the promoter ends, the synthesis of RNA will proceed.

When it comes to transcription in archaea, we can make a distinction between basal transcription and regulated transcription. Basal transcription refers to the general transcription factors (TFs) needed for the RNA polymerase to work. It requires basic factors mentioned before: the TFB and the TBP. On the other hand, the regulated transcription focuses on all the other TFs. Archaeal factors resemble to the bacterial ones in the sense that they depend on environmental stimuli to either release or stimulate TF–DNA binding interactions. On the other hand, eukaryotes and archaea are similar in the sense that they need to recruit activators (TBP & TFB in the case of archaea) close or in between the promoter region.
More recently, some regulators specific to archaea have been described, that can regulate several metabolic pathways in response to the cell redox status (Martinez-Pastor et al., 2017).

2.3.2 Translation

Archaeal translation process has similarities with other domains in the general picture. However, the molecular mechanisms and specific elements can differ significatively from each domain. In archaea, the initiation is an important step because the rate and efficiency are influenced by the elements interacting. This process takes place within a complex composed of a small ribosomal subunit (mRNA), an initiation (tRNA) and initiation factors. Once the initial codon on a mRNA is selected, the translation factors are released, and the ribosome that will begin elongation is formed. (Schmitt et al., 2020). Once the ribosome is fully formed, the two steps that follow are elongation and termination, which has similar factors to the ones employed by eukaryotes. The tRNAs are recruited to the active site of the ribosome by a molecule homologous to the eEf1α, and the ribosome shifting is mediated by a similar element (Bell & Jackson, 1998).For termination, the recognition of a stop codon takes place. This is mediated by (again, the eukaryotic-like) “eRF” transcription factor. Within this domain, transcription and translation is coupled process, meaning that the translation of the transcripts is initiated before the transcript is finished (French et al., 2007).

It is interesting to mention that even though the machinery used by archaea is very similar to the one used by eukaryotes, there are certain factors that also resembles a bacterial mechanism. For example, it has been elucidated that in both archaea and bacteria, transcription and translation are coupled (physically by the RNAP and the first translating ribosome) even though it has been far more studied in the second case (French et al., 2007). Furthermore, some additional elements are found in archaea that can be compared to the functions they perform in a bacterial model organism. For instance, it has been reported that *E. coli* NusG has the role of connecting RNAPs and ribosomes, and it has been seen that archaeal Spt4/5 can cover the same function (Blombach et al., 2019). One of the factors that are fundamental for the regulation of the translation process is the ribosome binding site.

In archaea, the ribosome binding site (RBS) is the sequence located upstream of the start codon (ATG) where the translation process initiates; It recognizes the ribosome and facilitates the union between the ribosome and mRNA. Most archaeal genomes have a conserved 5′-GGTG-3′ RBS sequence and it is an interesting region to modify and try to optimize and balance gene regulation (Omotajo et al., 2015).

Furthermore, RBS engineering has been a popular method to optimize gene expression in bacteria for a longer time. According to literature, it has direct influence in translation efficiency. Since only small changes are needed (less than 10 bp), it has become a useful and efficient method to up- or down-regulate translation of proteins (Oesterle et al., 2017).
It is noteworthy that even though archaeal translation is thoroughly described in literature, there is still a lack of understanding of (1) some of the specific mechanisms across species and (2) how can these elements be manipulated for gene regulation.

2.3.3 Untranslated regions

A relevant site to consider within the gene regulation in all organisms, including methanogens, is the 5' and 3' untranslated regions in the mRNA. These are fragments that are not translated into a protein since the sequence is placed before and after the coding sequence of the gene. In eukaryotes, the 5' and 3'-UTRs have biological functions such as translational efficiency and stress response (Hinnebusch et al., 2016).
On the other hand, the UTRs in archaea has been far less studied but it is known that they play essential roles in transcriptional and translational regulation; There is evidence stating that the 3'-UTR affects the direction of gene regulation while 5'-UTR has an influence in translational efficiency and overall regulation. For instance, in *Methanococcoides burtonii,* elements that are involved in cold adaptation are found upstream of an RNA helicase. On the same line, literature suggests that the length of the UTRs variates depending

on the strain (or even be absent) and even though the regulation mechanism is still not completely defined, deletion strains have shown its influence. In *Methanosarcina spp*., long 5'-UTRs are associated with methanol methyltransferase (MTA) regulation (Sowers, 2009). These sequences and how they can affect protein expression are going to be further discussed in this work.

2.4 Genome Editing in *Methanosarcina acetivorans.*

Formerly, gene edition in M. acetivorans was a complex and time-consuming task, given the complexity of the organism and the limited tools available (Nayak & Metcalf, 2017). However, technological advancements and extensive research within the Archaeal domain have made possible to develop more strategies for this purpose. For instance, homologous and site-specific recombination in *Methanosarcina* have been widely used methods to insert modifications of interest into the chromosome (Guss et al., 2008). Subsequently, the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system was developed. It uses a guide RNA to lead the Cas9 nuclease to the target site, making it highly specific and suitable for different organisms. (Ran et al., 2013). Similarly, another type of CRISPR system (Cas12A) has successfully been employed for genome editing *in Methanococcus* and *Methanosarcina* with favourable results. The ability to form multiple guide RNAs from a single transcript makes this option especially appealing for genome editing (Yan et al., 2017).

2.5 Application of Promoter and RBS in gene expression in Methanogens

The core promoters are short sequences (100–1,000 bps) in the DNA, where the RNA polymerase can bind and initiate the transcription of a gene (Le et al., 2019). It has been stated that the promoter region will determine (on sequence and conformation) the correct binding of the RNA polymerase.

Moreover, beyond the core promoter, this sequence has elements that are close or within the main sequence, that will greatly affect how a certain gene is regulated. There are multiple examples, especially in bacteria, of how changing the promoter specificity regarding the RNAP will affect gene expression (Decker & Hinton, 2013).

Similarly, methanogens use a range of promoters to regulate gene expression. While there is still a lack of information, several have been well characterized along with the relevant regions that participate in gene regulation. For instance, previous studies have tested promoter strength in *Methanococcus maripaludis* to facilitate metabolic engineering and flux balancing (Bao et al., 2022).
Additionally, the studies in *Sulfolobus solfataricus* and *Sulfolobus islandicus* promoters were essential to identify the sequences that could be engineered to achieve modulated gene expression (Peng et al., 2011).
Finally, in *Methanosarcina acetivorans* a LacZ reporter system was designed to regulate translational initiation. This demonstrated the possibility of fine-tuning protein levels in this strains (Karim et al., 2018).

3 Research material and methods

The experimental part was composed by two main parts. First, a *uid*A gene-based reporter system was used to measure the activity of each of previously selected promoter candidates. After analysis, a swap of promoter elements (ribosome binding site) was made to assess their influence on gene expression at the transcriptional and translational level.

3.1 Growth media conditions/strains

High Salt medium was prepared in anaerobic 100 ml serum bottles sparged with a gas mix consisting of 80% N2/20% CO2 at 37 °C. It was further supplemented with Methanol (125 mM), Trimethylamine (50 mM) or Sodium Acetate (120 mM) (Rother et al., 2005) as growth substrates, depending on each culture purposes. Finally, puromycin at a concentration of 2 µg/ml was added to select for strains carrying the puromycin transacetylase (Bose & Metcalf, 2008).

The vector used for transformation in E. *coli* was the pNB730 (Shea et al., 2016) containing: the native promoter of the enzyme, the *uid*A gene and the gene encoding for puromycin acetyltransferase. Additionally, it contains a multiple cloning site for gene cloning. The plasmids were subsequently inserted into the *M. acetivorans' genome.*
Methanosarcina acetivorans C2A was grown under anaerobic conditions at 37°C in high-salt medium, as previously described (Sowers et al., 1993). Growth was monitored until the optical density (OD 600) was between 0.45-0.65.

3.2 Promoter-RBS system amplification and plasmid construction

After the promoter regions were selected, they were fused with the starting sequence encoding the *uidA* gene (β-glucuronidase). These were further

inserted into the M. *acetivorans*' genome. The same procedure was used for the Promoter-RBS swap (figure 2).

Escherichia coli Top 10 competent cells were used for plasmid construction. HiFi Gibson assembly was used for all the RBS and candidates' constructions, using the manufacturer's protocol (*NEBuilder® HiFi DNA Assembly Master Mix | Gene Assembly | NEB*, n.d.). Cells were then transformed by subjecting them to heat shock for overnight growth.

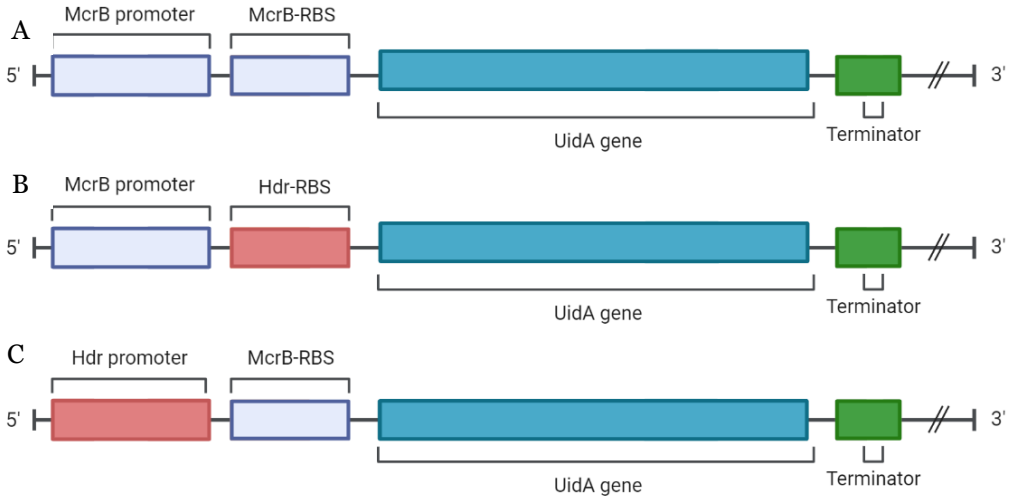

Fig 2. Representative scheme of the promoter-RBS combinations for the (A) sequence of *Methanosarcina acetivorans mcrB* promoter-β-glucuronidase fusion including the original RBS. (B) Sequence of *Methanosarcina acetivorans mcrB* promoter-β-glucuronidase fusion which includes the *hdr* RBS in the place of the original RBS. (C) Sequence of *Methanosarcina acetivorans hdr* promoter-β-glucuronidase fusion which includes the *mcrB*-RBS combination in the place of the original RBS.

3.3 Colony PCR and transformation in M. acetivorans

After overnight growth, 8 to 10 transformants (in *E. coli*) were selected for screening by colony PCR with primers covering the *uidA* sequence and a region complementary to the vector, using Sapphire Amp Fast PCR master mix. Conditions for the PCR cycles were the following: Initial denaturation at 95°C for 30 seconds, followed by 32 amplification cycles of 98°C for 5 seconds, 56°C for 7 s, and 72°C for 10 seconds. The DNA sequences of all the constructs

were confirmed first by Colony PCR and then by DNA sequencing. Primers are shown in table 2. A second confirmation was made by DNA sequencing.

Table 2. Primers used for assessing transformants.

Primer name	Description
730Sequencing F	5'-CCTGGCGTTACCCAACTTAATC-3'
Veri u*idA*-R	5'-CGGTAATCACCATTCCCGGCGGGATA-3'

For the final transformation in *M. acetivorans*, a polyethylene glycol (PEG)-mediated transformation procedure was used (Metcalf et al., 1997). Obtained strains were further grown in the appropriate substrate.

3.4 β-Glucuronidase Activity Assay

For activity measurements, the following method was adapted from previously described methods in literature. *Methanosarcina acetivorans* strains containing the desired promoter and *uidA* gene were cultivated until the OD600 was between the range of 0.45-0.65. The cells were lysed by addition of 50 mM phosphate buffer saline (PBS) buffer (containing Na2HPO4 and NaH2PO4), pH 7.0. Protease inhibitor was added; The lysate was centrifuged, and the supernatant further diluted with PBS. The final volume was incubated for 15 minutes along with 4-nitrophenyl-β-*D*-glucuronide. 400 µL of 200 mM Na2CO3 were added to stop the reaction. Finally, OD405 was measured for each sample.

3.4.1 Calibration curve for β-glucuronidase activity

An enzyme calibration curve was constructed to compare the activities from the selected candidates with the specific activity of a commercial β-glucuronidase. This measurement was taken by using β-glucuronidase from E. *coli* K12 (Product no. 3707580001, Sigma-Aldrich).

A stock solution was prepared by adding 5 µl of pure enzyme into 1995 µl of PBS buffer. Six further dilutions were made accordingly, to reach the desired OD range between 0 and 1. Each tube was incubated for 15 minutes, along with the enzyme substrate (4-Nitrophenyl β-D-glucuronide). Finally, 400 µL of 200 mM Na2CO3 were added to stop the reaction and the absorbance at 405 nanometres was measured for each sample. Concentration vs absorbance was plotted to calculate the accuracy of the measurements. Finally, activity and OD405 was plotted to obtain the final curve. The resulting curve is shown in the figure 3., giving a conversion factor of 157 nmol/minute.

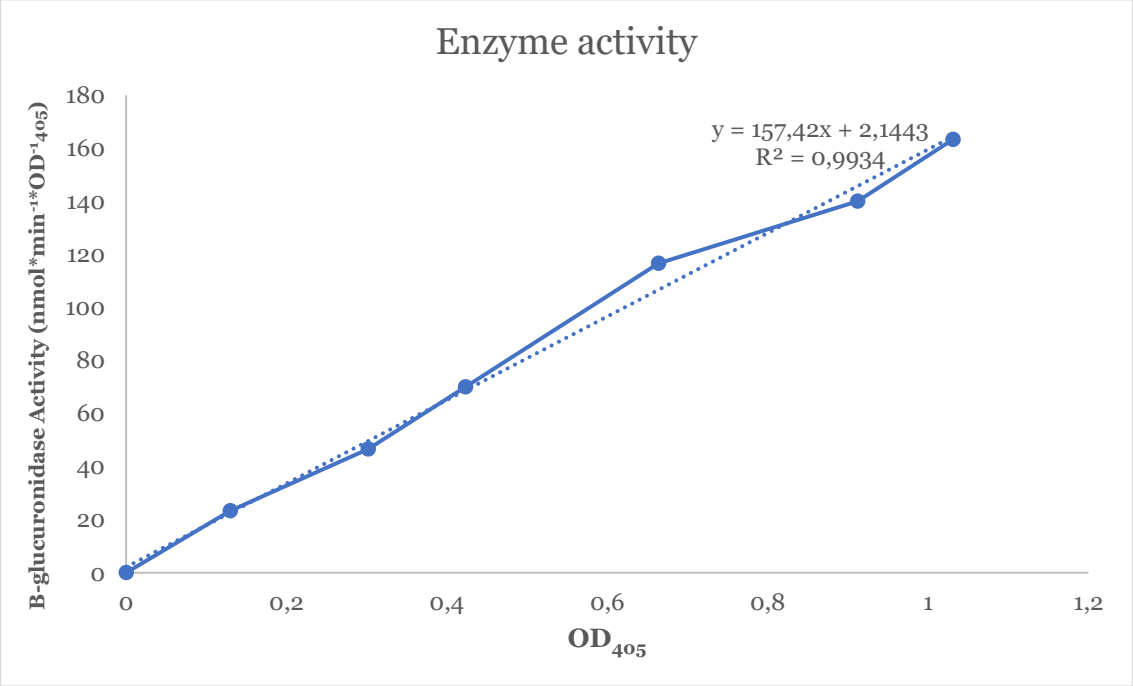

Fig 3. Standard curve, activity of β-glucuronidase. The conversion factor obtained was 157,42 nmol * min^{-1}.

The unit definition was defined in this study and for comparative purposes as the following:

The unit of β-glucuronidase activity is the enzyme activity that increases the rate of release of 4-nitrophenol from 4-nitrophenyl-β-D-glucuronide (4NPG) at a pH 7.0 by 1 mmol.

3.4.2 Cell-lysis time efficiency test

SDS-PAGE was run to verify the efficiency of the lysis time and the location of the protein within the reaction tube for the previously described protocol. The cell was lysed for four different periods (5, 10, 20 and 40 minutes). Subsequently, supernatant, and resuspended cell debris were loaded to verify protein presence. As it can be seen on the figure 4, the enzyme of interest was present in the supernatant (lanes 2, 4, 6 and 8) and the cell debris remained empty, which indicates that (1) the lysis time employed in the activity assay is effective, and (2) that the protein stays in the supernatant and not in the cell debris.

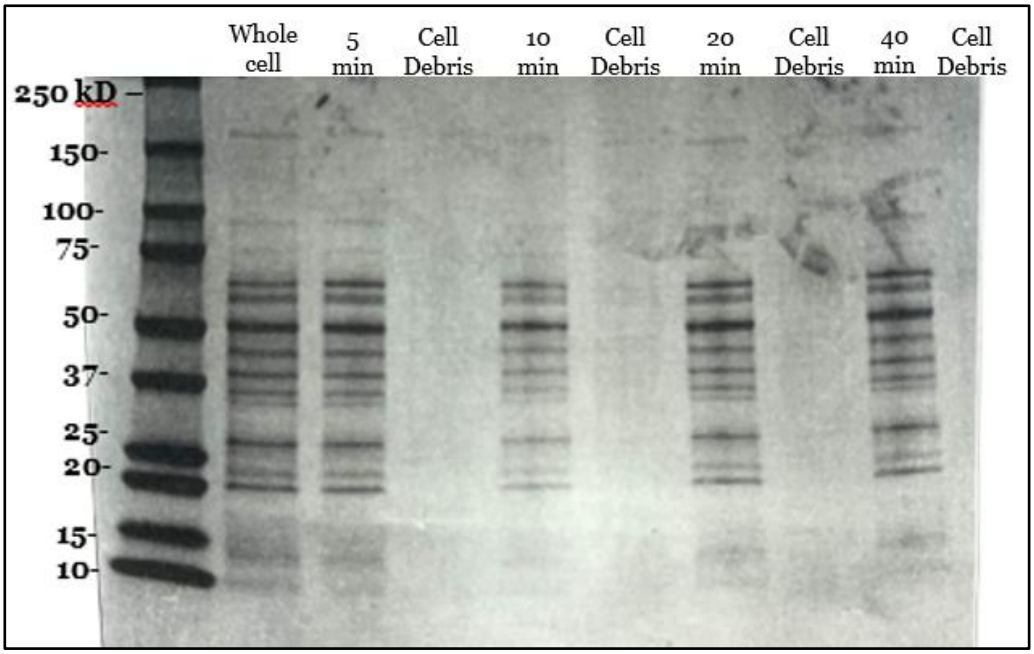

Fig 4. SDS-PAGE. Beta-glucuronidase (GUS) protein expression *in Methanosarcina acetivorans*. The first lane (from left to right) shows the ladder and the whole cells were used as a control in the secon lane.

4 Results and discussion

4.1 Promoter-RBS systems selected from methanogens

Finally, the promoters for the enzymes expressed in different strains of methanogens is described in the table 3.

Table 3. The native promoter's proteins assayed for β- glucuronidase activity.

Promoter	Enzyme	Strain	Function	Reference
P*mcr*	Methyl coenzyme M reductase	*(Mb*. Fusaro*) (Mtc. Okinawensis) (M. mazei)*	Reduction of methyl-coenzyme M to methane	(Rother et al. 2005)
P*mta*	Methanol methyltransferase	*(M. acetivorans* C 2A*)*	Transfers the methyl group from methanol to coenzyme M	(Bose & Metcalf, 2008)
P*hdr*	Heterodisulfide reductase	*(Mb*. Fusaro*) (Mc. voltae) (M. mazei)*	Reduction of CoM-S-S-HTP and the oxidation of H-S-HTP and H-SCoM to CoM-S-S-HTP	(Kulkarni et al., 2018)
P*mtr*	Methyl-H4MPT methyltransferase	*(Mb*. Fusaro*) (Mc. voltae)*	Methyl transfer from methyl coenzyme M to H4MPT	(Welander & Metcalf, 2008)
P*vht*	Methanophenazine-dependent hydrogenase	*(Mb*. Fusaro*)*	Reduction of Methanophenazine with electrons from H2 in methylotropic pathway	(Hoerr et al., 2021)
P*acs*	Acetyl coenzyme A (CoA) synthase	*(Mb*. Fusaro*)*	Activation of acetate to acetyl-coenzyme A	(Jetten et al., 1989)
P*ech*	Ferredoxin-dependent hydrogenase	*(Mb*. Fusaro*)*	Electron transport from Fd(red) into the respiratory chain	(Welte et al., 2010)
P*mtr*	Tetrahydromethanopterin-CoM methyltransferase	*(Mc. voltae)*	Transfer of methyl (H4MPT) at the end of WL pathway to CoM-SH	(Adam et al., 2022)
P*acs*/CODH	CO dehydrogenase/acetyl CoA synthase multienzyme complex	*(Mb*. Fusaro*)*	Catalysis of CO oxidation/CO2 reduction and cleavage/synthesis of acetyl-CoA	(Matschiavelli et al., 2012)
P*mcr*	Methyl coenzyme M reductase	*(Mc. voltae)*	Reduction of methyl-coenzyme M to methane	(Klein et al., 1988)

The methyl-coenzyme M reductase (Mcr) is one of the most relevant enzymes in methanogenic microorganisms and it is the only one present in all types of methanogenesis. It catalyses the final step of methanogenesis to complete the production of methane in *Methanosarcina acetivorans* metabolism: reduction of methyl-coenzyme M to methane (Chen et al., 2020). This reaction is common to all metabolic pathways for methanogenesis, indicating that these genes are essential for survival (in the case of M. *acetivorans*, it encodes only one set). Furthermore, There have been studies to investigate which are the elements that interact directly with the promoter region and how they relate to its gene regulation (Shinzato et al., 2008). The promoter corresponding to this enzyme from *M. barkeri* Fusaro was chosen as a control to assess the candidate's activities and will be further discussed in this work.

In the second part of the experimental method and after assessing the activities of the first promoters, eight different *uidA* fusions were constructed from four out of twelve candidates*: hdr* (M. *mazei*), *mcr* (Mc. *voltae*), *ech* (*Mb*. Fusaro) and (*Mb*. Fusaro); All with the purpose of comparing the activity in *M. acetivorans* C2A. The first set of plasmids contained the original promoter, the *uidA* gene and the RBS sequence corresponding to the native *mcrB*. The second batch was composed of the native *mcrB* promoter from *Mb*. Fusaro, the native RBS and the *uidA* gene. The obtained strains that were subjected to the described activity assay are listed in table 4.

Table 4. Plasmids from (native and exchanged) promoters and RBS.

Plasmid	**Relevant genotype**
730P*hdr*-RBS	pNB730 with P*hdr* (*Mc. mazei*) + RBS*mcrB*
730P*vht*-RBS	pNB 730 with P*ech* (*Mb*. Fusaro) + RBS*mcrB*
730P*ech*-RBS	pNB 730 with P*mcr* (*Mb*. Fusaro) + RBS*mcrB*
730P*mcr*Vol-RBS	pNB 730 with P*mcr* (Mc. *voltae*) + RBS*mcrB*
730P*mcrB*-RBS*hdr*	pNB 730 with P*mcr*B (*Mb*. Fusaro) + RBS*hdr*
730P*mcrB*-RBS*vht*	pNB 730 with P*mcr*B (*Mb*. Fusaro) + RBS*vht*

730P*mcrB*-RBS*ech*	pNB 730 with P*mcr*B (*Mb*. Fusaro) + RBS*ech*
730P*mcrB*-RBS*vol*	pNB 730 with P*mcr*B (*Mb*. Fusaro) + RBS*vol*

4.2 Promoter-RBS strengths in different growth substrates

The β-glucuronidase reporter system was used to measure enzymatic activity from 12 native promoters from different methanogen strains in two growth substrates.

A fusion of the *uid*A gene and the promoter from M. *barkeri* Fusaro (P*mcr*) was employed as a control since it has been well described in literature that *mcr* is a constitutive gene as well as highly expressed (Rother et al., 2005). The figure 5 shows the result of the strains' strength grown in both substrates. The reporter gene expression was found to be significantly higher when employing the P*mcr* from *M. mazei* in both methanol and trimethylamine compared to the control. This promoter will operate differently according to the host characteristics and the growth environment (Gendron & Allen, 2022). A the same time, the difference between substrates is consistent with the ones previously reported which is between 25-30% (Rother et al., 2005).

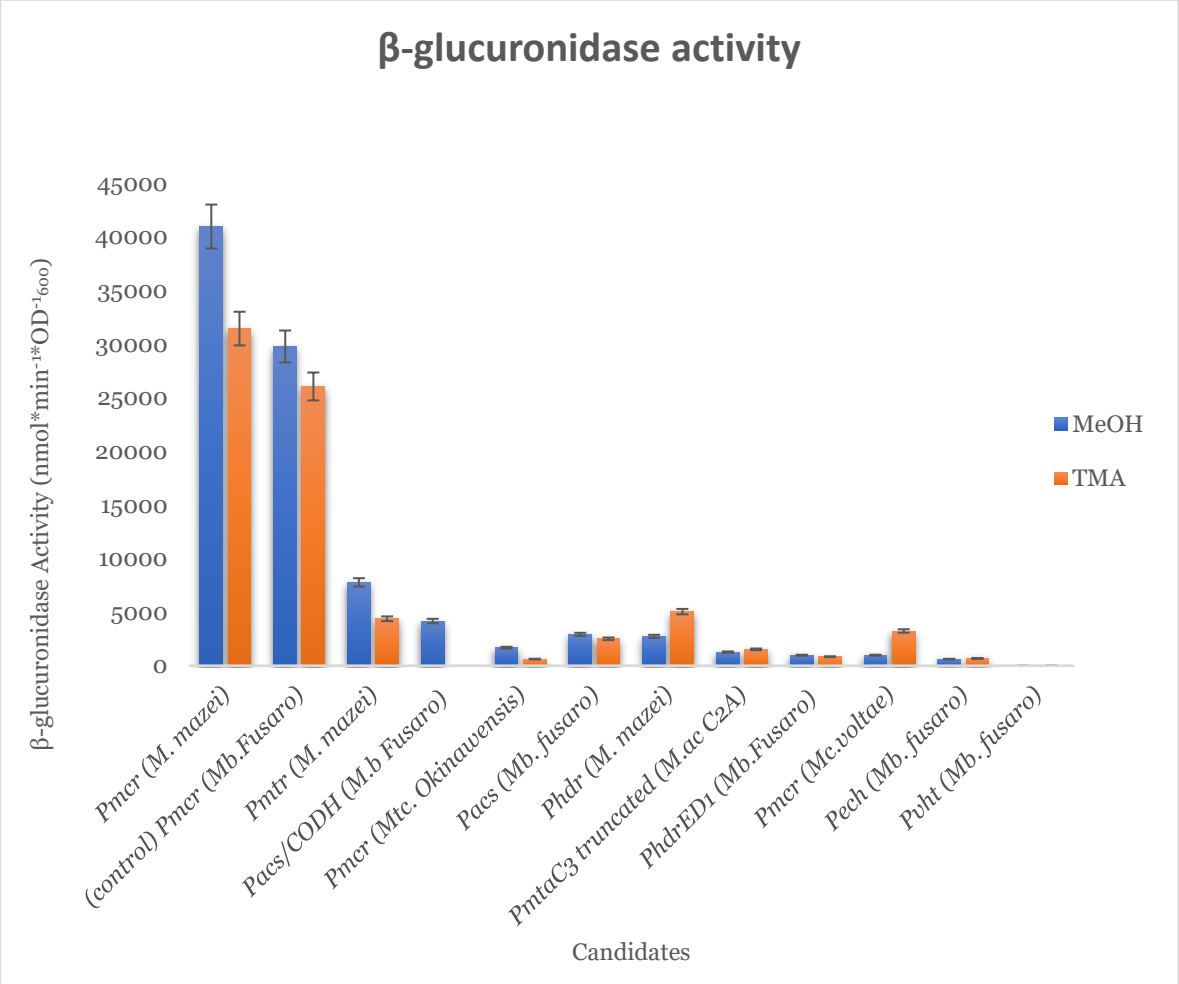

Fig 5. β-glucuronidase activity in MeOH and TMA. Units in nmol per minute. Error bars represent the standard deviation.

A second interesting result is the shown inactivity of *vht* (nondetectable). It has been established that *M. acetivorans* is a type of methanogen that even though it has the machinery to express hydrogenases, they do not play a role in hydrogen metabolism during methanogenesis in the methanol and trimethylamine growth conditions (Hoerr et al., 2021). This hypothesis is supported by Guss et al., by experimentally concluding that the hydrogenase operons in M. acetivorans are not active unless an appropriate substrate is present (acetate); Therefore, inhibiting any activity in these growth conditions (Guss et al., 2005).

As it has been stated before, the MCR sequence is highly conserved in all methanogens and according to literature (Rother et al., 2005), the expression

in methanol is consistently higher than the other substrates tested. However, as it can be seen in the activity results, the promoter of MCR from *M. mazei* shows a higher activity compared to the control and at the same time, both are significantly higher than the MCR promoter from *M. okinawensis*. This can be attributed to several factors. One possible explanation is that the utilized promoter (regulatory) elements associated with the original host are different, affecting protein levels.

Finally, it is noteworthy to mention the results obtained from the *acs*/CODH promoter. As described before, the *acs*/CODH complex is mainly expressed in the aceticlastic environment. We can see in the figure 5 that the activities of this candidate, together with the Acs one are low on methylotrophic growth substrates given the anabolic role the enzyme plays under this condition (Matschiavelli et al., 2012).

4.3 Promoter engineering for strength variations by RBS swapping

Even though the exact mechanisms by which archaeal regulators act on protein expression are partially unknown, it has been reported that the modification of these elements (e.g., RBS, 5' and 3'- UTRs) will influence the overall gene regulation. As previously mentioned in this work, translation initiation in archaea (among other factors) essentially involves the RBS sequence; Furthermore, it has been reported that this element has the potential to increase the transcript stability and therefore influence RNAP binding protein production and regulation (Karim et al., 2018). For the first round, four weak promoters were chosen to exchange either the promoter sequence or the RBS for one that has proven to be strong (control). The figure 6 shows the results of this exchange.

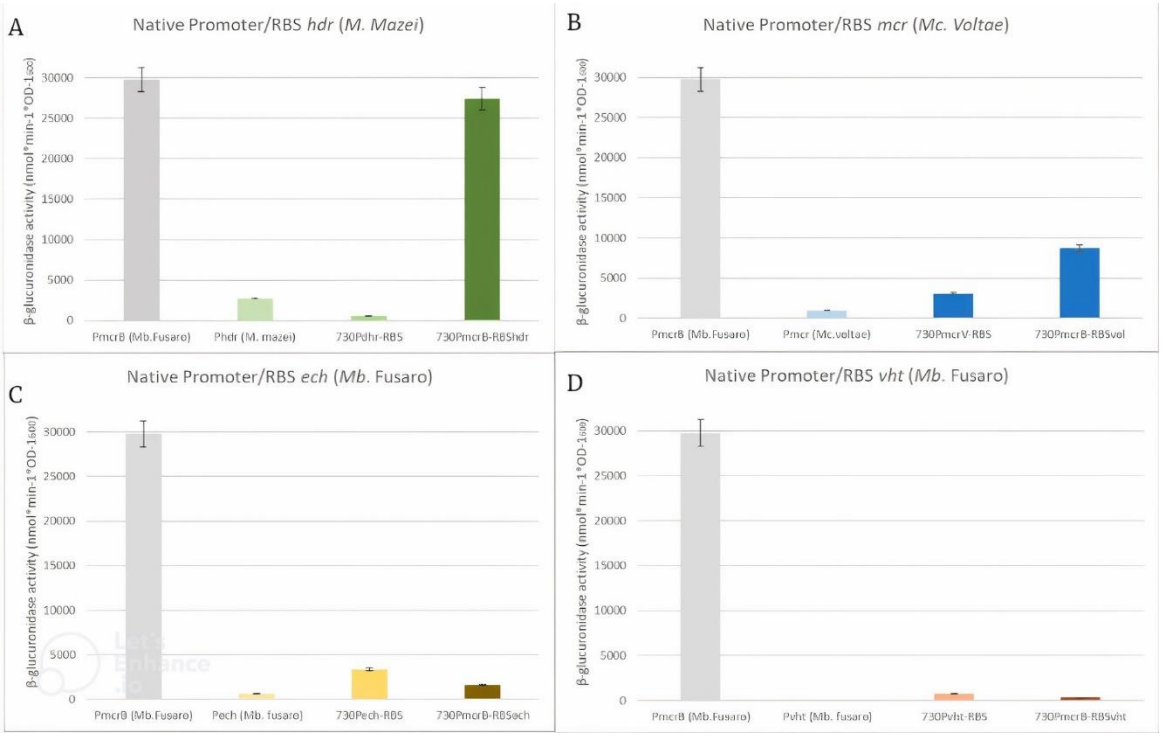

Fig 6. β-glucuronidase activity in MeOH for constructs combining the exchange between (native/*mcrB*) promoter and (native/*mcrB)* RBS-*uidA* from *(A) hdr (M. mazei), (B) mcr (Mc. voltae), (C) ech (Mb.* Fusaro*)* and (D) *vht (Mb.* Fusaro*)*. The control candidate was added in the first place of each graph for reference.

As it can be seen in the Figure 6A, the *hdr* promoter presented itself as a weak promoter compared to the control. When the Mcr ribosome binding site was introduced instead of the native one (and keeping the *hdr* promoter), the activity decreased. However, in the next scenario the *mcrB* promoter was used, and the activity increased more than ten times with respect of the original promoter. This was the biggest difference out of the pool of candidates assessed, giving a new strong promoter-RBS combination.

In the case of the second promoter (*mcr*) from Mc. *voltae,* the behavior was remarkably like the first set of strains; With the difference that in this case, exchanging the original RBS to the *mcr*B one, did increase the activity, similarly as the original promoter and native RBS combination (Figure 6 B).
In the graph C, it is reported that the Ech candidate was initially a weak promoter, and the results present a different behavior with respect to the earlier

two cases. This promoter is taken from *Mb.* Fusaro genome since M. *acetivorans* does not encode this hydrogenase. Here, the native promoter with the Mcr's RBS showed the highest increase between the two fusions. As previously mentioned, Ech promoter and enzyme are mainly used in the aceticlastic pathway; Given that all these strains were tested in methanol, the effect of the RBS in this assembly could be further analyzed.

Finally, the vht promoter expressing the *uidA* gene showed no activity that can be reported in either substrate. It has been established that M. *acetivorans* don't express this enzyme even though they have operons present in the DNA (Guss et al., 2009). However, it is noteworthy that the two strains constructed, showed an increase in the activity. In this case, the native promoter and the *McrB*'s RBS gave the highest activity, doubling the one using the inverse sections.

5 Conclusions and future perspectives

5.1 Promoter-RBS strength in acetate environment

In summary, the work presented employed molecular biology and genetic engineering methods to reliably measure the strength of 12 different promoters. Those have been quantified via a β-glucuronidase reporter system on methylotrophic growth substrates. The ongoing work aims to include the strength of the same candidates, with acetate as the growth substrate. This could be of interest, especially with the hydrogenases' promoters.
Moreover, the 5'-untranslated regions are yet to be tested. These results are of interest since it will give a broader perspective on the available tools and how these modifications will affect *M. acetivorans'* gene expression.

5.2 Applications

This research, in conjunction with the ongoing experiments in acetate will expand the tools for gene expression tuning. As seen in figure 7, the promoter strength range for the expression level of UidA gene has been extended on the two methylotrophic substrates conditions. These results open the door to expanding this research line to other strains, substrates, and engineering methods. Gene expression determines the function of proteins that will be essential for growth and metabolic processes.

Furthermore, the fact that methanogens can survive in extreme environments talks about the complexity and robustness of their metabolism. By fine-tuning gene expression, we can manipulate the organism's response to substrates and other environmental conditions. If we can take advantage of this machinery, the academic and industrial applications could grow exponentially; For instance, regulating protein expression levels by metabolic engineering for the production and optimization of organic chemicals from single-carbon compounds (Karim et al., 2018).

Another example is the production of archaeal lipids with characteristics (pH and thermal resistance) appealing for biotechnological applications. Furthermore, the understanding of the archaeal engineering toolbox would be helpful regarding the heterologous expression of proteins and added-value compounds in *M. acetivorans* (Rother et al., 2001).

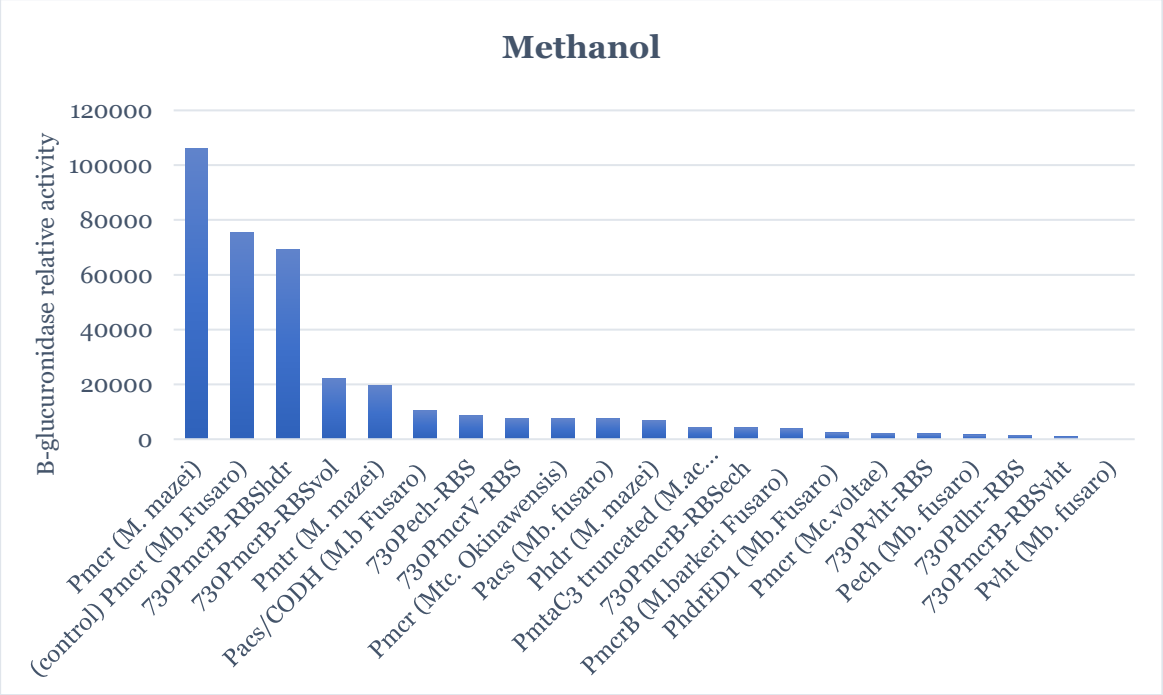

Fig 7. Promoter strengths from the first 12 native enzymes and from the Promoter-RBS combinations

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