BioSFerA
Biofuel for biotravels

H2020-LC-SC3-2018-2019-2020
EUROPEAN COMMISSION
Innovation and Networks Executive Agency

Biofuels production from **S**yngas
Fermentation for **A**viation and maritime use
Grant Agreement No 884208

Deliverable D3.2

Metabolic engineering of acetogenic bacteria for efficient syngas fermentation and production of acetate

Document Details

Due date	M18-Sep 21
Actual delivery date	31/03/2022 (M24)
Lead Contractor	CSIC
Version	Final
Prepared by	CSIC
Input from	-
Reviewed by	CERTH

Document Details

x	PU - Public
	PP - Restricted to other programme participants (including the EC)
	RE - Restricted to a group specified by the consortium (including the EC)
	CO - Confidential, only for members of the consortium (including the EC)

Disclaimer of warranties

This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 884208. This document reflects only the author´s view and INEA is not responsible for any use that may be made of the information it contains.

This document has been prepared by BioSFerA project partners as an account of work carried out within the framework of the EC-GA contract no 884208.

Neither Project Coordinator, nor any signatory party of BioSFerA Project Consortium Agreement, nor any person acting on behalf of any of them:

a. makes any warranty or representation whatsoever, express or implied,
    i. with respect to the use of any information, apparatus, method, process, or similar item disclosed in this document, including merchantability and fitness for a particular purpose, or
    ii. that such use does not infringe on or interfere with privately owned rights, including any party's intellectual property, or
    iii. that this document is suitable to any particular user's circumstance; or
b. assumes responsibility for any damages or other liability whatsoever (including any consequential damages, even if Project Coordinator or any representative of a signatory party of the BioSFerA Project Consortium Agreement, has been advised of the possibility of such damages) resulting from your selection or use of this document or any information, apparatus, method, process, or similar item disclosed in this document.

Abbreviations

ALE	Adaptive Laboratory Evolution
AF	Scrubbing water after filtration
AR	Scrubbing water after reformer
BHI	Brain Heart Infusion medium
CIB	Centro de Investigaciones Biológicas
DSM	German Collection of Microorganisms
FOA	Fluoroorotic acid
Gm	Gentamycin
IPTG	Isopropyl-β-D-thiogalactoside
Km	Kanamycin
OD	Optical Density
Syngas	Synthesis gas
RCA	Reinforced Clostridial (Agar) medium
TAG	Triacylglyceride
TGB	Tryptone Glucose Beef medium
TSB	Tryptic Soy Broth medium
YT	Yeast Tryptone medium
YTF	Yeast Tryptone Fructose medium

Contents

Executive Summary

The main objective of Work Package 3 (WP3) is to develop at lab scale a two-stage biological gas-to-liquid process for the conversion of CO_2/CO/H_2 (syngas) produced by gasification into medium (C14) and long (C16-18) chain triacylglycerides (TAGs) that will be upgraded to biofuels.

Deliverable D3.2 has covered two major goals:

a) To identify the best acetogenic bacterial strain able to produce acetate based on the produced syngas composition and the presence of possible inhibitors.
b) To genetically modify the selected acetogenic bacterial strains in order to increase its acetate production.

Several acetogenic strains were tested as putative candidates to transform syngas into acetate by fermentation (i.e., *Clostridium ljungdahlii, Clostridium autoethanogenum, Acetobacterium woodii, Moorella thermoacetica* DSM 521, and *Moorella thermoacetica* DSM 2955).

After reforming of syngas, the acetogenic bacteria can survive well to the syngas contaminants contained in the scrubbed water (AR water). However, the scrubbed water derived only after syngas filtration (AF water) is highly toxic since it allows only a limited bacterial growth after a large dilution. Therefore, syngas must be reformed to be used as carbon source in the first stage fermentation process.

Moorella thermoacetica has been selected as the most useful strain to produce acetate from syngas. We have tested the *Moorella* tolerance to different concentrations of several pollutants present in the reformed syngas, such as NH_3 (ammonia), HCN (hydrogen cyanide), H_2S (hydrogen sulphide) and benzene. *Moorella* shows a significant tolerance to all of them. Nevertheless, the most critical contaminant appears to be H_2S due to the high concentration in the reformed syngas. Some options to create more tolerant strains by adaptive laboratory evolution (ALE) are envisioned.

Based on metabolic studies we have proposed the modification of *Moorella* by the deletion and overproduction of some specific genes involved in the acetic acid metabolism. A modular synthetic cassette has been designed to allow the creation of 8 different plasmids to create 8 modified *Moorella* strains. Thus, modified *Moorella* strains have been created by using these cassettes and they were tested for the

production of acetate. Under the tested conditions the transformants showed similar acetate production to the wild type strain, indicating that the modifications do not improve the acetate yield. The reasons for such behaviour must be investigated by using different genetic and biochemical tools in order to propose further modifications.

1. Introduction

The main objective of Work Package 3 (WP3) is to develop at lab scale a two-stage biological gas-to-liquid process for the conversion of CO_2/CO/H_2 (syngas) produced by gasification into medium (C14) and long (C16-18) chain triacylglycerides (TAGs) that will be upgraded to biofuels. The first stage of the biological process involves the production of acetate from syngas fermentation and, to fulfil this aim, D3.2 has covered two major goals:

a.- To identify the best acetogenic bacterial strain able to produce acetate based on the produced syngas composition and the presence of possible inhibitors.

b.- To genetically modify the selected acetogenic bacterial strains in order to increase its acetate production.

Several anaerobic bacteria named acetogenic (e.g., *Clostridium*, *Acetobacterium*, *Eubacterium*) are able to transform syngas into useful chemicals (e.g., acetate, ethanol, lactate, butanediol, etc.) through the Wood–Ljungdahl pathway (Merli et al., 2021; García & Galán, 2022). Since acetogenic bacteria can secrete this mixture of compounds during syngas fermentation, metabolic engineering and synthetic biology are powerful tools to increase the acetate production and reduce the spectrum of unwanted by-products. In this way, the main product of gas fermentation, i.e., acetate, can be used as substrate for a second fermentation stage to produce TGAs.

Depending on the feedstock and the gasification conditions, syngas might contain apart from CO, CO_2 and H_2 a number of toxic contaminants (e.g., H_2S, COS, CS_2, NOx, NH_3, HCN, C_2H_4, tar, etc.) that can inhibit the fermentation process when the amount of these toxic compounds exceeds certain limits. Therefore, it is necessary to select acetogenic bacteria that not only can not only produce high amounts of acetate, but also can tolerate high concentrations of these toxic compounds. The use of the tolerant bacteria will facilitate the gasification of different feedstock in different operational conditions that can render variable amounts of contaminants since it will not be necessary to carry out complex and costly cleaning processes of syngas.

On the other hand, an efficient transformation of syngas into acetate requires to funnel the carbon metabolism to the secretion of acetate as the main or unique product. This means that the production of undesired by-products such as acetone, lactate, ethanol, and other alcohols, must be reduced or eliminated by creating mutant strains with a reduced capacity to secrete these undesired by-products. The technologies currently

used to improve the producer strains are mainly based on conventional random mutagenesis and selection procedures that are grounded in typical trial and error methodologies. The aim of this project is to apply a more rational approach by implementing the construction of recombinant strains based on the use of new tools and technologies provided by systems and synthetic biology. This approach should allow us to construct complex mutants that will have an extremely low probability of being generated by random mutagenic approaches. Thus, the aim was to develop and use systems metabolic engineering tools to improve the capacities of the selected acetogenic strains.

2. Screening and selection of acetogenic bacterial strains

Several acetogenic strains were tested as putative candidates to transform syngas into acetate by fermentation. The strains tested were:

1.- *Clostridium ljungdahlii* (CIB collection)
2.- *Clostridium autoethanogenum* (CIB collection)
3.- *Acetobacterium woodii* WB1 DSM 1030
4.- *Moorella thermoacetica* DSM 521
5.- *Moorella thermoacetica* DSM 2955

These strains were cultured in anaerobiosis in different liquid media such as RCA, BHI, TSB, TGB and YTF to determine their growing capacities (Figure 1). *Acetobacterium* was cultivated at 30 ºC, *Clostridium* species were cultured at 37 ºC; however, *Moorella* is a thermophilic bacterium that grows at 65 ºC. The results of these experiments are shown in Table 1.

Figure 1. Bottles used to culture anaerobic bacteria.

Table 1. Growth of acetogenic bacteria in liquid media determined by measuring the Optical Density (O.D.) at 600 nm at the corresponding time (h). (-) Means that the strain does not grow. Bottles (100 mL) were filed with 80 mL of media using 20 mL of head space filled with N_2 (1.8 atm). Culture media (RCA, BHI, TSB, TGB, YTF) are described in the annexes.

Strains/Media	RCA	BHI	TSB	TGB	YTF
Clostridium ljungdahlii	0.8 (72 h)	0.8 (48 h)	-	-	1.5 (24 h)
Clostridium autoethanogenum	-	-	-	-	3.0 (48 h)
Acetobacterium wodii	-	-	0.6 (24 h)	-	-
Moorella thermoacetica DSM521	-	-	1.0 (24 h)	-	-
Moorella thermoacetica DSM2955	-	-	0.8 (24 h)	-	-

The results of table 1 indicate that although the clostridial strains are able to grow on several rich media, YTF medium appears to be the best medium to grow these acetogenic clostridial strains. On the other hand, *Acetobacterium* and *Moorella* show a narrower range of possibilities to use rich media. In fact, this is the first time that *Moorella* was able to grow in a rich medium such as TSB.

Based on these data we have tested the production of acetate in the three best growing strains using syngas (40:40:10:10. $CO:H_2:CO_2:N_2$) as carbon source in 100 mL bottles. *C. autoethanogenum* was cultured in YT medium. *M. thermoacetica* DSM 2955 and *M. thermoacetica* DSM 521 were cultured in TSB medium. The results of these experiments are shown in table 2.

The production of acetate was determined using the Acetic Acid Assay Kit (Megazyme) or by HPLC (see annexes).

Table 2. Production of acetate from syngas using the acetogenic strains in YT (*Clostridium*) and TSB (*Moorella*) media.

Strain	OD_{600} (time)	Acetate (g/L)
Clostridium ljungdahlii	0.62 (7 days)	1.5
Clostridium autoethanogenum	0.61 (7 days)	1.6
Moorella thermoacetica DSM521	1.05 (7 days)	2.6
Moorella thermoacetica DSM2955	1.18 (7 days)	2.7

The results of table 2 suggest that *Moorella* strains are better acetate producers than *Clostridium* strains, at least under this culture conditions. Figure 2 shows that *C. autoethanogenum* was able to consume more than 50% of CO while CO_2 concentration increased. It is important to notice that these experiments are performed using sealed bottles and that the behaviour in a bioreactor can be different.

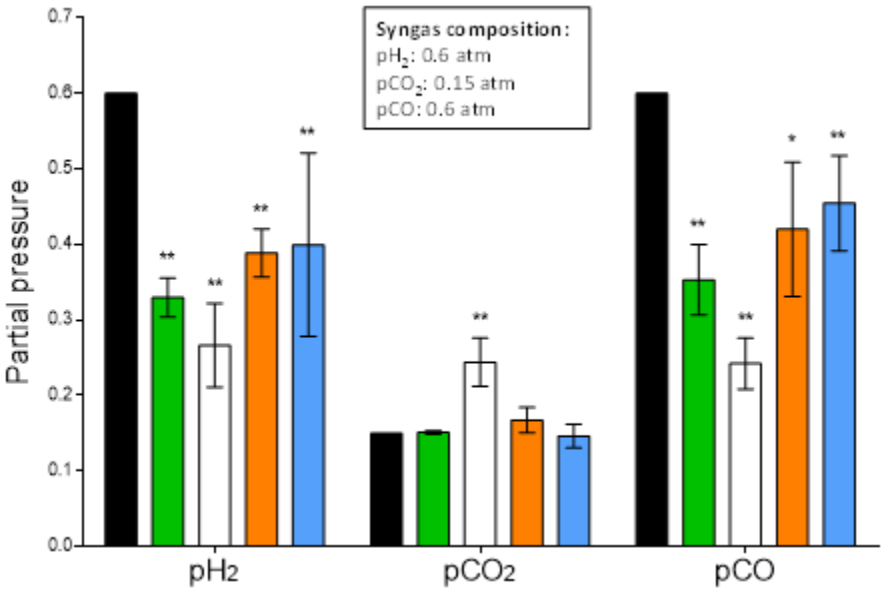
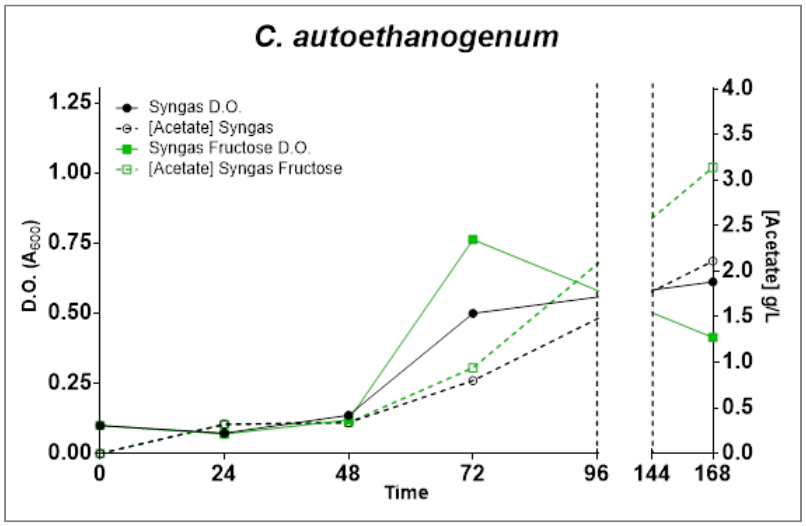


Figure 2. Consumption of gases and production of acetate by acetogenic strains. Syngas (100 mL head space, 1.8 atm initial pressure). *Clostridium* was cultured in YT medium plus 3 mM cysteine. *Moorella* was cultured in TSB medium plus 3 mM cysteine. Colour code: **Black**: initial pressure, **Green**: *Clostridium ljungdahlii*, White, *Clostridium autoethanogenum*, **Orange**: *Moorella thermoacetica* DSM521, **Blue**: *Moorella thermoacetica* DSM2955.



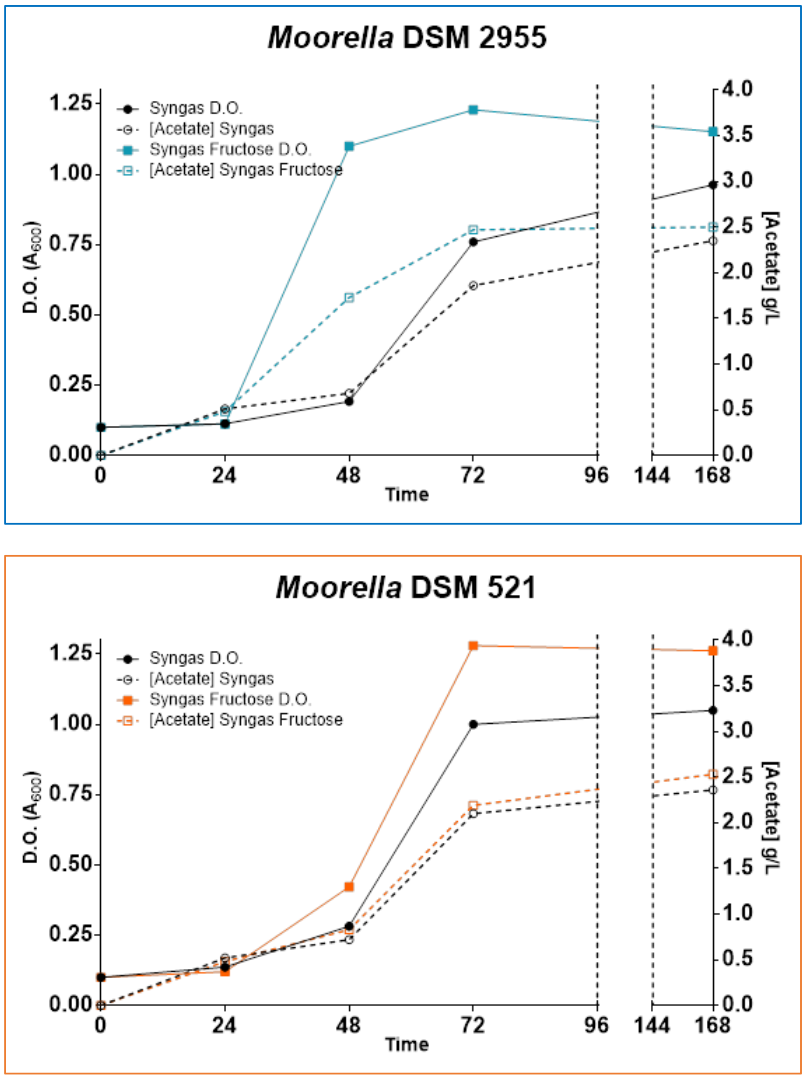


Figure 3. Production of acetate along with the growth curves of acetogenic strains growing in fructose and syngas. *C. autoethanogenum* was cultured in YT medium. *M. thermoacetica* DSM 2955 and *M. thermoacetica* DSM 521 were cultured in TSB medium.

The acetate production of selected acetogenic strains was also followed by the growth curve in different growing conditions with syngas and fructose as carbon source. Figure 3 shows that the addition of fructose increases the biomass, but does not increase significantly the production of acetate in the presence of syngas.

3. Identification of the best acetate producer strains based on the tolerance to the contaminants present in syngas

One of the aims of WP3 is to determine the effects of contaminants of syngas in the viability of bacterial strains used for syngas fermentation. We have initially tested the

effect of the contaminants of syngas retained in scrubbing water obtained after filtration of syngas (AF) or after a reforming process (AR) (Figure 4). It is worth to mention that AF and AR samples were obtained from VTT in the framework of bench scale gasification tests (T3.1) as was described under D3.1.

Figure 4. Samples of AR and AF scrubbing waters.

To test the effect of syngas contaminants on the growth of acetogenic strains, *Clostridium* strains were cultured in YTF medium while *Moorella* strains were cultured in TSB medium for 72 h. AR and AF fractions were added to the media in different proportions. The results of these experiments are shown in Table 3.

Table 3. Toxicity analysis of AR and AF scrubbing waters added to the culture media. NG = no growth. The OD_{600nm} of the culture was determined after 72 h.

Strain / Medium OD_{600nm}	YTF/TSB in control water	YTF/TSB in 100% AR water	YTF/TSB in 100% AF water	YTF/TSB in 50% AF water	YTF/TSB in 20% AF water
C. ljungdahlii	3.0	2.7	NG	NG	0.3
C. autoethanogenum	3.1	2.8	NG	NG	0.3
M. thermoacetica DSM 521	1.0	0.8	NG	NG	0.3
M. thermoacetica DSM 2955	1.1	0.8	NG	NG	0.3

In addition, we have also tested the AF toxicity using two model aerobic bacteria, i.e., *Escherichia coli* W and *Pseudomonas putida* KT244 growing in LB medium in the presence of 50% AF. Under aerobic conditions these bacteria were not able to grow in this medium (data not shown), strongly reinforcing the observation about the high toxicity of AF on bacteria even under aerobic conditions.

The results of these experiments show that after reforming of syngas the acetogenic bacteria can survive well to the syngas contaminants even using media containing the AR water that contains even higher concentrations of contaminants than those present in the syngas. However, the AF water is highly toxic since it allows only a limited bacterial growth after a large dilution. Therefore, syngas must be reformed to be used as carbon source in the first stage of the fermentation process.

On the other hand, we have tested the tolerance of acetogenic bacteria to specific contaminants still present in the syngas after reforming. Figure 5 shows the growth of the acetogenic bacteria in the presence of 9.35 mM NH_4Cl, 2.5 mM benzene or 6.5 mM Na_2S. The results indicate a clearly different response of the strains depending on the contaminants. Interestingly, it can be observed that in general the strains can be adapted to the contaminants after a lag phase. This observation suggests that most probably the strains can be evolved by adaptation (ALE) to create mutants more tolerant to the contaminants if required. In any case, the *Moorella* strains appear to be more resistant to the contaminant than the *Clostridium* strains.

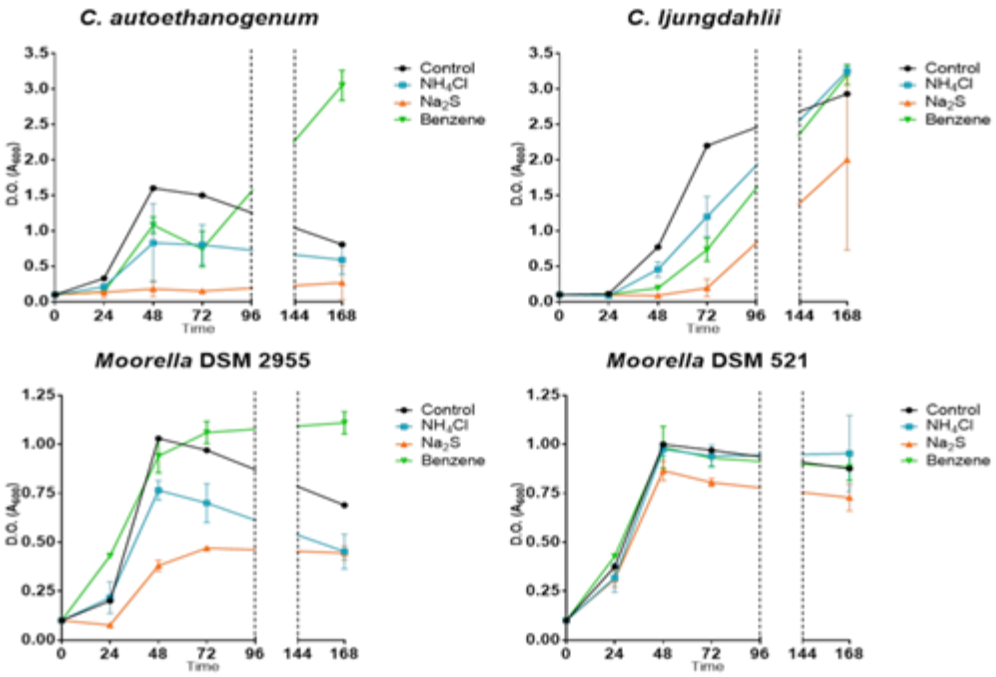


Figure 5. Effect of syngas contaminants on the growth of acetogenic bacteria. The final concentration of contaminants was: 9.35 mM NH_4Cl, 2.5 mM benzene and 6.5 mM Na_2S.

To test in more detail the limits of tolerance of *Moorella* strains to syngas contaminants after reforming we carried out other experiments using different concentrations of the contaminants in cultures in TSB medium. The results of these experiments can be observed in Figure 6.

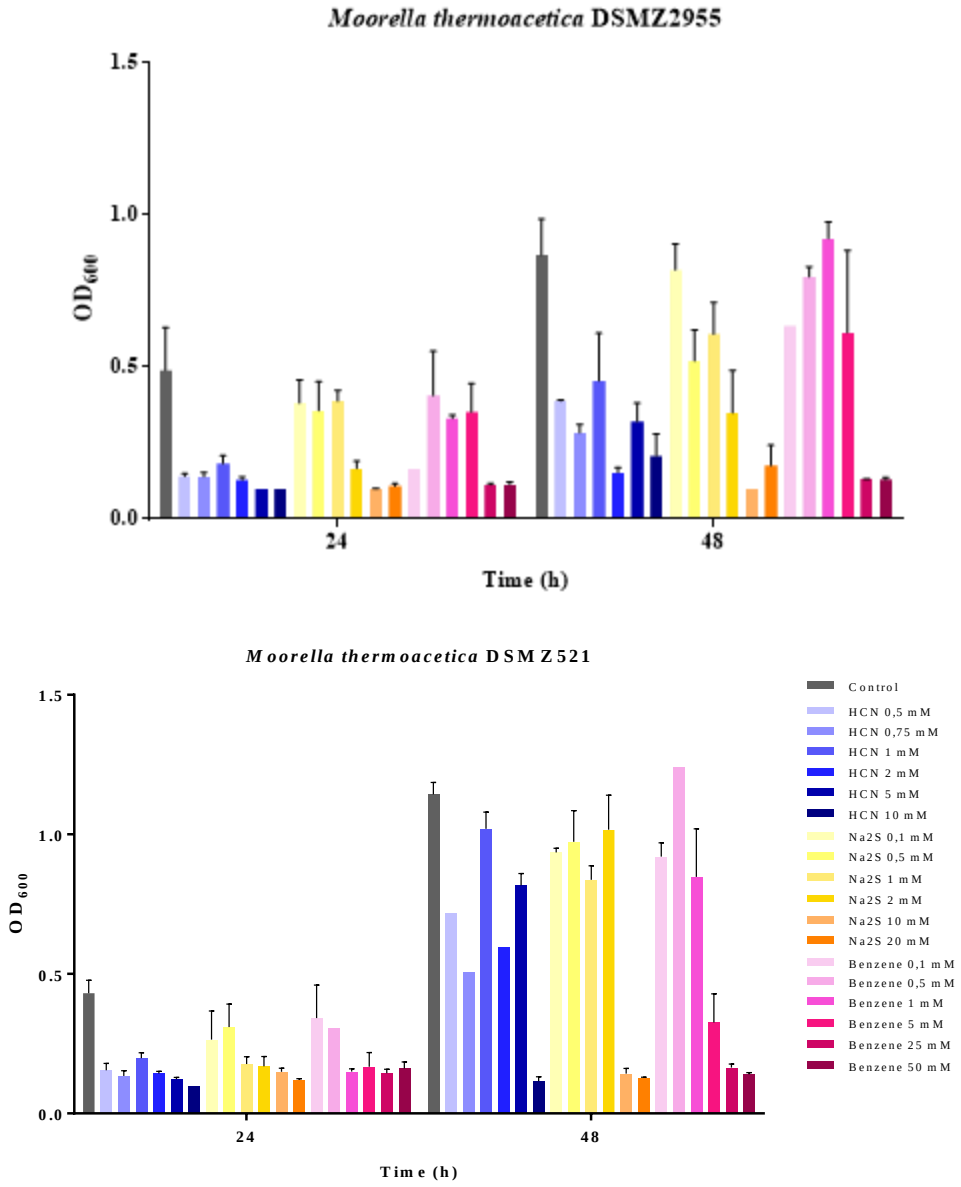


Figure 6. Effect of syngas contaminants (HCN, benzene and H_2S (Na_2S) on the growth of *Moorella* strains.

The results of these experiments indicate that *M. thermoacetica* DSM 521 is more resistant than *M. thermoacetica* DSM 2955 to HCN and Na_2S (equivalent to neutralized H_2S), because it grew with 1 mM HCN and up to 2 mM Na_2S. On the other hand, both strains grew in the presence of 1 mM benzene. We have also determined that *Moorella* strains are able to grow even with 1 M NH_4Cl (data not shown).

Considering these experiments, we can conclude that the concentration of H_2S is the most critical contaminant factor for bacterial growth. According to the contents of syngas contaminants provided in deliverable D3.1, and assuming that H_2S of syngas

is accumulated in the culture medium as Na_2S after its neutralization, in the case of *M. thermoacetica* DSM 521, it will be possible to provide up to 100 L of syngas per L of culture without any significant risk to the viability of the strain. The *M. thermoacetica* DSM 2955 would tolerate well only up to 50 L of syngas. Therefore, in continuous cultures it will be important to handle the putative accumulation of Na_2S in the culture medium.

4. Improving the selected strains using metabolic engineering.

According to the previous results we decided to select *M. thermoacetica* strains as the preferred strains to carry out the production of acetate from syngas. Moreover, there is an extensive literature that supports this selection (Sakai et al.,2005; Hu et al., 2016; Bengelsdorf et al. 2018; Redl et al., 2020). Therefore, we have developed a metabolic engineering strategy to improve the production of acetate by modifying the metabolism of these strains using genetic and metabolic engineering tools. To fulfil this aim we had to implement different tools that are described below.

4.1 Construction of *E. coli* recombinant strains expressing three DNA methylases from *M. thermoacetica*

Due to the DNA restriction systems that bacteria use to avoid bacteriophages attack, it is usually very difficult to transform a bacterial strain with exogenous DNA that has not been previously methylated. Because of that we have constructed an *E. coli* strain carrying three DNA methylase genes of *M. thermoacetica* encoded by four genes (Jensen et al., 2019).

The genes *MOTHA-c17610 (hsdS*), *MOTHA-c17620 (hsdM), MOTHA-c24230* and *MOTHA-c23800* of *M. thermoacetica* encoding the three DNA methylases have been synthesized and cloned in the vector pIZ2 that has the replication *pBBR1*-origin. The resulting plasmid named pIZMethylases was transformed in *E. coli* DH10B to generate *E. coli* DH10B (pIZMethylases). This recombinant strain is resistant to gentamycin and expresses the DNA methylases under the control of the IPTG (Isopropyl-β-D-thiogalactoside) inducible *Ptac* promoter. The sequences and maps of the genes and plasmids are shown in the Annex.

The strain *E. coli* DH10B (pIZMethylases) is used as a host to methylate the plasmids that are going to be used to transform *M. thermoacetica* as described below. These

plasmids are derived from plasmid *pK18mobsacB* that confers resistance to kanamycin and has the replication *ColE1*-origin that is compatible with the replication *pBBR1*-origin of pIZMethylases. This allows to maintain both plasmids in the same strain.

Thus, the recombinant *E. coli* DH10B strains carrying plasmids pIZMethylases and pK18*mobsacB* derivatives that have to be methylated were constructed *ad hoc* (see annexes) for each designed recombinant event. The strains were cultured in LB medium (Gm-Km) at 37 ºC and the methylated plasmids extracted and purified from these strains were used for the transformation experiments.

4.2 Construction of plasmids used to modify *M. thermoacetica*

To improve the production of acetate in *M. thermoacetica* we have considered the acetate metabolism of this strain that is described in Figure 7.

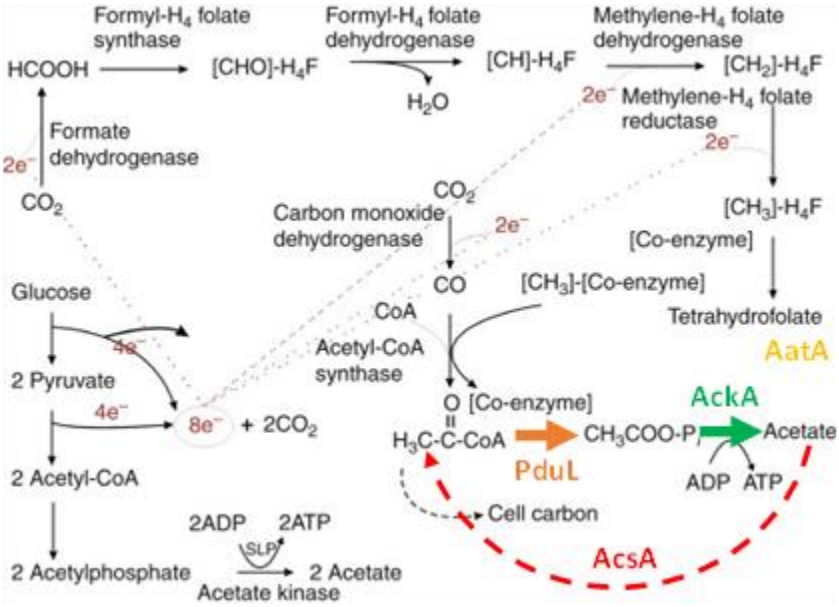


Figure 7. Acetate metabolism of *M. thermoacetica*

To construct this strain, we designed a recombinant cassette carrying the regions up and down of the *acsA* gene to delete it. Within these regions, a gene cluster composed by the GmR, *pduL*, *ackA* and *aatA* genes, all of them controlled by the strong constitutional promoter of the glyceraldehyde 3-phosphate dehydrogenase gene from *Moorella* were inserted. In this way, the *acsA* gene will be replaced by the genes we want to insert in *Moorella*.

The modular design of our recombinant cassette allows us to construct different cassettes with different gene combinations. In all cases, the *acsA* gene will be deleted and the acetate cannot be transformed back into acetyl-CoA.

Cassette 1.- ACSA-Gm^R-*pduL*-*ackA*-*aatA*-ACSA: this is a complete cassette which carries the genes encoding the phosphotransacetylase, the acetate kinase and the acetate exporter. This strain should accumulate a higher concentration of acetate inside the cell that should be exported to the medium.

Cassette 2.- ACSA-Gm^R-*pduL*-*ackA*-ACSA: this cassette lacks the transporter, so this strain should accumulate a higher concentration inside the cell. Then, the only way to prevent acetate toxicity is by increasing its own mechanisms to export this compound or by growing slower in order to manage acetate accumulation.

Cassette 3.- ACSA-Gm^R-*pduL*-ACSA: this cassette only carries the acetate exporter, so virtually all the acetate produced inside the cell should be exported. This is another strategy to increase the metabolic flux inside the cell towards acetate without overexpressing any other gene of the acetate pathway, and at the same time avoiding toxicity problems.

Cassette 4.- ACSA-Gm^R-ACSA: this cassette would generate an insertion mutant in which *acsA* is only replaced by the GmR encoding gene. This mutant should also generate a higher amount of acetate since it cannot be transformed back to acetyl-CoA.

The insertion of the gene cassettes will create the following changes in the metabolism (**Figure 8**).

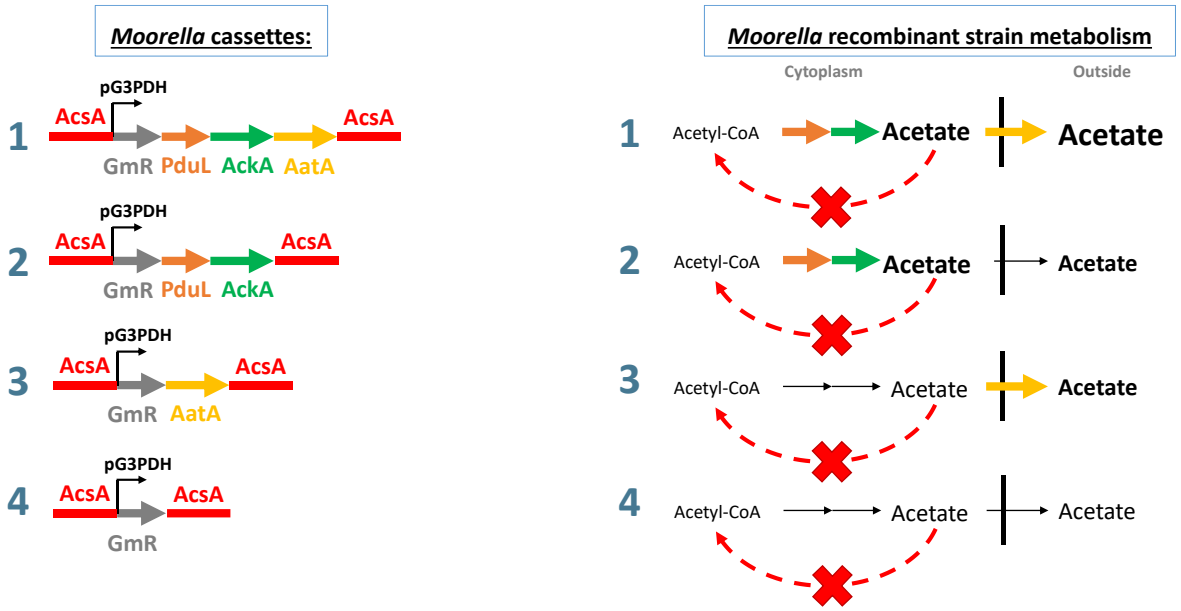


Figure 8. Insertion cassettes created to inactivate the *acsA* gene that encodes the acetyl-coenzyme A synthetase. The *pduL*, *acaK* and *aatA* genes encode the phosphate propanoyltransferase, acetate kinase, and acetate exporter, respectively. Gm^R encodes the gene that confers resistance to gentamicin. The genes are expressed under the control of the P_{G3PDH} promoter. The size of the arrows and the bold letter indicates higher acetate production.

However, we are not sure if the deletion of *acsA* gene will have a huge impact in the central metabolism of *Moorella*. To solve this eventual problem, we have also designed the cassettes to change the flanking regions by the homologous regions to the *pyrF* locus.

Cassette 5.- PYRF-Gm^R-*pduL*-*ackA*-*aatA*-PYRF: this cassette is equivalent to cassette 1, but inserted in *pyrF*.

Cassette 6.- PYRF-Gm^R-*pduL*-*ackAk*-PYRF: This cassette is equivalent to cassette 2, but inserted in *pyrF*.

Cassette 7.- PYRF-Gm^R-*pduL*-PYRF: This cassette is equivalent to cassette 3, but inserted in *pyrF*.

Cassette 8.- PYRF-Gm^R-PYRF: This cassette is equivalent to cassette 4 and will generate an insertion mutant in which *pyrF* is only replaced by the GmR encoding gene.

All these mutants would be uracil auxotroph and resistant to the uracil analogous 5-FOA (5-fluoroorotic acid). The insertion of these cassettes will create the following changes in the metabolism (Figure 9).

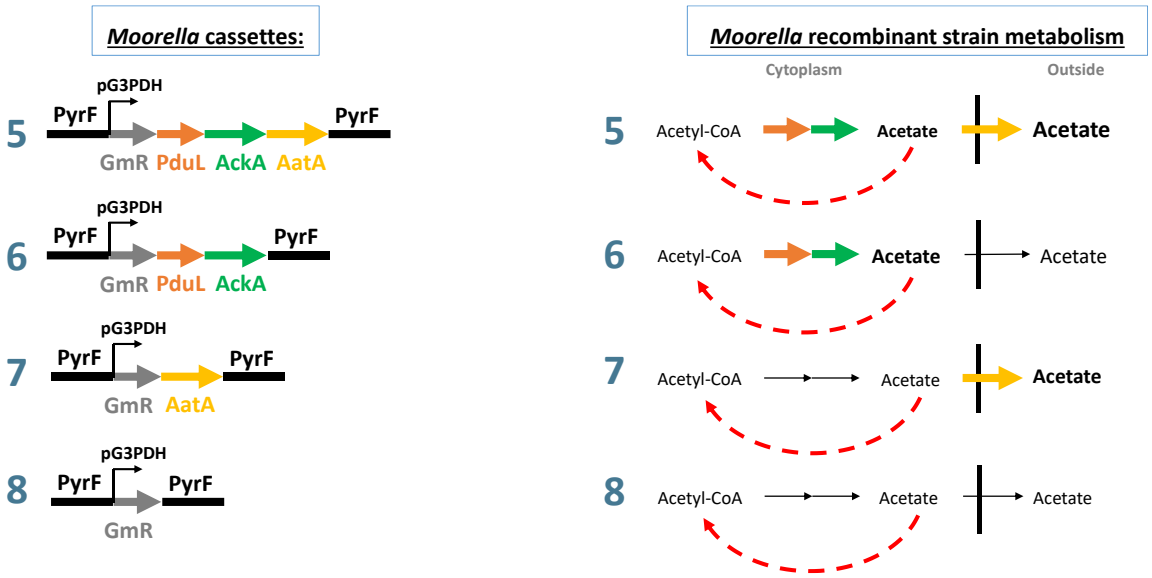


Figure 9. Insertion cassettes to inactive the *pyrF* gene that encodes the orotidine 5'-phosphate decarboxylase. The *pduL*, *acaK* and *aatA* genes encode the phosphate propanoyltransferase, acetate kinase, and acetate exporter, respectively. Gm^R encodes the gene that confers resistance to gentamicin. The genes are expressed under the control of the P_{G3PDH} promoter. The size of the arrows and the bold letter indicates higher acetate production.

These mutant strains would have lower acetate production than the previous ones because in this case the acetyl-CoA synthetase (AcsA) is still functional and some acetate will be transformed back into acetyl-coA.

The genes used to modify *M. thermoacetica* have been synthesized according to the codon usage of *M. thermoecetica* (see annexes) and cloned into plasmid pK18mobSacB originating the plasmids described in Table 4.

Table 4. Plasmids generated to modify *M. thermoecetica*

Plasmid	Genotype
pK18ACSACassette Complete (1)	Δ*acsA*, Gm^R, overexpression of *pduL* (phosphate propanoyltransferase), *acaK* (acetate kinase) and *aatA* (acetate exporter) with P_{G3PDH} promoter.
pK18ACSACassette PudL-AckA (2)	Δ*acsA*, Gm^R, overexpression of *pduL* (phosphate propanoyltransferase) and *acaK* (acetate kinase) with P_{G3PDH} promoter.

pK18ACSACassetteACSA AatA (3)	Δ*acsA*, Gm^R, overexpression of *aatA* (acetate exporter) with P_{G3PDH} promoter.
pK18ACSACassette GmR (4)	Δ*acsA*, Gm^R
pK18PYRFCassette Complete (5)	Δ*pyrF*, Gm^R, overexpression of *pduL* (phosphate propanoyltransferase), *acaK* (acetate kinase) and *aatA* (acetate exporter) with P_{G3PDH} promoter.
pK18PYRFCassette PudL-AckA (6)	Δ*pyrF*, Gm^R, overexpression of *pduL* (phosphate propanoyltransferase) and *acaK* (acetate kinase) with P_{G3PDH} promoter.
pK18PYRFCassette AatA (7)	Δ*pyrF*, Gm^R, overexpression of *aatA* (acetate exporter) with P_{G3PDH} promoter.
pK18PYRF cassette GmR (8)	Δ*pyrF*, Gm^R

4.3 Transformation of *M. thermoacetica* with recombinant plasmids.

The plasmids shown in table 4 were used to transform *M. thermoacetica* by electroporation (Figure 10) after being previously methylated as described in 4.1.

Figure 10. Electroporator.

The *M. thermoacetica* cells were electroporated following the protocol described in Annexes and according to published procedures (Iwasaki et al., 2013; Kita et al., 2013a,b; Rahayu et al., 2017; Jensen et al., 2019; Kato et al., 2021). The transformants were selected after the electroporation using a liquid culture medium containing Gm (gentamycin).

Using this protocol and the plasmids described in table 4 we have been able to obtain the following transformants:

1.- *M. thermoacetica* DSM 521 pK18ACSACassette complete (1)
2.- *M. thermoacetica* DSM 521 pK18ACSACassette AatA (3)
3.- *M. thermoacetica* DSM 2955 pK18ACSACassette PudL-AckA (2)
4.- *M. thermoacetica* DSM 2955 pK18ACSACassette AatA (3)
5.- *M. thermoacetica* DSM 2955 pK18PYRFCassete Complete (5)

Although the low efficiency of the transformation procedure has not allowed to obtain all possible transforms, the collection of obtained transformants provide enough material to test the behaviour of these new strains concerning the acetate production. Nevertheless, the transformants are under production to this study (see future actions).

4.4 Analysis of the acetate production by *M. thermoacetica* transformed strains.

To determine the acetate produced by the *M. thermoacetica* transformed strains, these strains were cultured in TSB medium containing gentamycin and the productions have been compared with the acetate production of wild type strain in TSB medium without gentamycin (Table 5).

Table 5. production of acetate by the *M. thermoacetica* strains. Experiments were done by triplicate.

3 days culture	
Strain	Acetate (g/L)
M. thermoacetica DSM2955	6.72 ± 0.43
M. thermoacetica DSM2955 pK18PYRFcassette complete (5)	7.38 ± 0.08
M. thermoacetica DSM2955 pK18ACSAcassette PudL-AcaK (2)	7.14 ± 0.62
M. thermoacetica DSM2955 pK18ACSAcassette aatA(3)	6.91 ± 0.03
M. thermoacetica DSM521	7.83 ± 0.04
M. thermoacetica DSM521 pK18ACSAcassette complete (1)	6.51 ± 0.01
M. thermoacetica DSM521 pK18ACSAcasssette aatA (3)	6.77 ± 0.08
7 days culture	
Strain	Acetate (g/L)
M. thermoacetica DSM2955	7.37 ± 0.31
M. thermoacetica DSM2955 pK18PYRFcassette complete (5)	7.76 ± 0.21
M. thermoacetica DSM2955 pK18ACSAcassette PudL-AcaK (2)	7.65 ± 0.16
M. thermoacetica DSM2955 pK18ACSAcassette aatA (3)	7.77 ± 0.05
M. thermoacetica DSM521	8.06 ± 0.01
M. thermoacetica DSM521 pK18ACSAcassette complete (1)	7.25 ± 0.10
M. thermoacetica DSM521 pK18ACSAcasssette aatA (3)	7.16 ± 0.20

The results of these experiments show that the modified strains of *Moorella* resistant to gentamycin do not increase the production of acetate when compared to the wild type strain, at least in the tested conditions. Since there are many metabolic reasons that can explain such behaviour, these reasons will be investigated in order to take future actions. It is worth to mention that the current results performed in bioreactor have shown that the production of acetate is already very high in the wild type strain of *Moorella* and very close to the theoretical maximum yield, and perhaps the bottlenecks in the acetate production are not the expression of the proposed genes. Because of that, we have to change our metabolic approach to better identify these bottlenecks.

5. Conclusions

1.- *Moorella thermoacetica* has been selected as the most useful strain to produce acetate from syngas.

2.- For the first time, we have been able to grow *Moorella* in a rich medium like TSB that allows the production of high concentrations of biomass.

3.- It has been determined that syngas has to be reformed to avoid the high concentration of contaminants that appear in the scrubbing water only after filtration of syngas.

4.- The *Moorella* tolerance to different concentrations of several pollutants present in the reformed syngas, such as NH_3 (ammonia), HCN (hydrogen cyanide), H_2S (hydrogen sulphide) and benzene has been tested. *Moorella* shows a significant tolerance to all of them. The most critical contaminant appears to be H_2S due to the high concentration in reformed syngas. Some options to create more tolerant strains by adaptive laboratory evolution (ALE) are envisioned.

5.- A recombinant *E. coli* carrying three DNA methylases from *Moorella* has been created to favour the transformation of *Moorella* strain using the methylated plasmids.

6.- Based on metabolic studies we have proposed the modification of *Moorella* by the deletion and overproduction of some genes. To fulfil this task a modular synthetic cassette has been designed that allows the creation of 8 different plasmids to create 8 modified *Moorella* strains.

7.- Several modified *Moorella* strains have been created by using the cassettes and tested for the production of acetate. Under the tested conditions the transformants showed similar acetate production, indicating that the modifications do not improve the

acetate yield. The reasons for such behaviour must be investigated by using different genetic and biochemical tools in order to propose further modifications.

6. Future actions

In order to improve the results obtained under WP3 we propose the following future actions that will be reported in an updated version of D3.2 or even within other deliverables of WP3 such as D3.4 “Optimization of syngas fermentation process parameters for acetate production (1st stage)”:

1.- We will develop protocols for ALE evolution in order to improve the tolerance of *Moorella* to syngas contaminants. This will allow the growth of cells under continuous culture operational conditions in case of having a high accumulation of these contaminants in the culture medium due to the recirculation of high amounts of syngas.

2.- We will analyse in deep by using genetic and biochemical protocols the reasons why the created transformants do not increase the production of acetate in order to propose further actions and metabolic changes. It is important to identify the bottlenecks of acetate production under extreme fermentation conditions. In this way we will use omic tools to try to identify these bottlenecks.

3.- We will test the possibility of increasing the overproduction of enzymes in *Moorella* by increasing the copy number of the genes introduced in the strain by using plasmids. Although to the best of our knowledge the transformation of *Moorellla* with plasmids has not been tested yet, we believe that the plasmids used to transform *Clostridium thermocellum* (Groom et al., 2016), a closely related strain (Rabemanolontsoa et al., 2017), will be useful for this aim. Thus, we will test the usefulness of these plasmids to create new *Moorella* recombinants. In case of success, the use of plasmids will facilitate the studies in order to reach new metabolic improvements.

7. References

- Bengelsdorf FR, Beck MH, Erz C, Hoffmeister S, Karl MM, Riegler P, Wirth S, Poehlein A, Weuster-Botz D, Dürre P. Bacterial Anaerobic Synthesis Gas (Syngas) and CO(2)+H(2) Fermentation. Adv Appl Microbiol. 2018;103:143-221.
- García JL, Galán B. Integrating greenhouse gas capture and C1 biotechnology: a key challenge for circular economy. Microb Biotechnol. 2022 Jan;15(1):228-239.

- Groom J, Chung D, Olson DG, Lynd LR, Guss AM, Westpheling J. Promiscuous plasmid replication in thermophiles: Use of a novel hyperthermophilic replicon for genetic manipulation of *Clostridium thermocellum* at its optimum growth temperature. Metab Eng Commun. 2016 Jan 29;3:30-38.
- Hu P, Chakraborty S, Kumar A, Woolston B, Liu H, Emerson D, Stephanopoulos G. Integrated bioprocess for conversion of gaseous substrates to liquids. Proc Natl Acad Sci U S A. 2016 Apr 5;113(14):3773-8.
- Iwasaki Y, Kita A, Sakai S, Takaoka K, Yano S, Tajima T, Kato J, Nishio N, Murakami K, Nakashimada Y. Engineering of a functional thermostable kanamycin resistance marker for use in *Moorella thermoacetica* ATCC39073. FEMS Microbiol Lett. 2013 Jun;343(1):8-12.
- Jensen TØ, Tellgren-Roth C, Redl S, Maury J, Jacobsen SAB, Pedersen LE, Nielsen AT. Genome-wide systematic identification of methyltransferase recognition and modification patterns. Nat Commun. 2019 Aug 19;10(1):3311.
- Kato J, Takemura K, Kato S, Fujii T, Wada K, Iwasaki Y, Aoi Y, Matsushika A, Murakami K, Nakashimada Y. Metabolic engineering of *Moorella thermoacetica* for thermophilic bioconversion of gaseous substrates to a volatile chemical. AMB Express. 2021 Apr 23;11(1):59.
- Kita A, Iwasaki Y, Sakai S, Okuto S, Takaoka K, Suzuki T, Yano S, Sawayama S, Tajima T, Kato J, Nishio N, Murakami K, Nakashimada Y. Development of genetic transformation and heterologous expression system in carboxydotrophic thermophilic acetogen *Moorella thermoacetica*. J Biosci Bioeng. 2013a Apr;115(4):347-52.
- Kita A, Iwasaki Y, Yano S, Nakashimada Y, Hoshino T, Murakami K. Isolation of thermophilic acetogens and transformation of them with the *pyrF* and *kan*(r) genes. Biosci Biotechnol Biochem. 2013b;77(2):301-6.
- Merli G, Becci A, Amato A, Beolchini F. Acetic acid bioproduction: The technological innovation change. Sci Total Environ. 2021 Dec 1;798:149292.
- Rabemanolontsoa H, Van Nguyen D, Jusakulvjit P, Saka S. Effects of gas condition on acetic acid fermentation by *Clostridium thermocellum* and *Moorella thermoacetica* (*C. thermoaceticum*). Appl Microbiol Biotechnol. 2017 Sep;101(17):6841-6847
- Rahayu F, Kawai Y, Iwasaki Y, Yoshida K, Kita A, Tajima T, Kato J, Murakami K, Hoshino T, Nakashimada Y. Thermophilic ethanol fermentation from lignocellulose hydrolysate by genetically engineered *Moorella thermoacetica*. Bioresour Technol. 2017 Dec;245(Pt B):1393-1399.

- Redl S, Poehlein A, Esser C, Bengelsdorf FR, Jensen TØ, Jendresen CB, Tindall BJ, Daniel R, Dürre P, Nielsen AT. Genome-Based Comparison of All Species of the Genus *Moorella*, and Status of the Species *Moorella thermoacetica* and *Moorella thermoautotrophica*. Front Microbiol. 2020 Jan 17;10:3070.
- Sakai S, Nakashimada Y, Inokuma K, Kita M, Okada H, Nishio N. Acetate and ethanol production from H_2 and CO_2 by *Moorella* sp. using a repeated batch culture. J Biosci Bioeng. 2005 Mar;99(3):252-8.

Annexes

Protocol for electroporation of *M. thermoacetica*

The *M. thermoacetica* strains were cultured in the TSB medium and harvested by centrifugation (6,000 g for 10 min at RT) at an optical density at 600 nm (OD_{600}) of 1. The cells were then washed and resuspended in sterile water.

The methylated pk18mob plasmids carrying the cassettes were extracted and purified from the transformants of *E. coli* DH10B (pIZMethylases, pk18mobcassettes) strains. These plasmids were used for the transformation experiments

Plasmids were introduced into the *M. thermoacetica* strains by electroporation using a BIO-RAD Gene Pulser 220V (Model nº: 1652077). Plasmid DNA (2-5 µg) was added to 200-µl cell suspensions in an electroporation cuvette with a 2-mm gap. The cells were pulsed at 1.5 kV, 600 Ω, and 25 µF and immediately inoculated into 20 ml of TSB medium with CO_2 (20%) plus H_2 (80%). The cell suspension was then incubated at 55°C for 24 h and inoculated into a 20 ml of TSB medium with CO_2 (20%) plus H_2 (80%) and 10 µg/ml gentamycin during 5-7 days. The culture was inoculated into the same medium and now the clones were able to grow in 48 h.

Analysis of acetate by HPLC

Acetate concentration was measured by HPLC (Agilent 1260 Infinity II) using a refractive index detector and an Aminex HPX-87H column 300mm x 7.8 mm (BIO-RAD), at 55°C. Ultrapure water containing 5 mM sulfuric acid was used as the mobile phase at a flow rate of 0.5 ml/min.

RCA Medium

Reinforced Clostridial Agar (RCA) is an enriched non-selective medium intended for the cultivation of *Clostridia* and other anaerobic and facultative bacteria from various clinical and non-clinical specimens.
Composition:

- Tryptone 10 g/L
- HM peptone / Beef extract 10 g/L
- Yeast extract 3 g/L
- Dextrose (Glucose) 5 g/L
- Sodium chloride 5 g/L
- Sodium acetate 3 g/L

- Starch, soluble 1 g/L
- L-cysteine hydrochloride 0.5 g/L
- Agar 13.5 g/L

Final pH (at 25°C) 6.8±0.2

BHI Medium

Brain Heart Infusion Broth is recommended for the cultivation of fastidious pathogenic microorganisms. Brain Heart Infusion Broth has been used as culture medium for *Streptococcus pneumonia*, *Listeria monocytogenes*, *Leishmania promastigotes* and *Clostridium difficile*.

Composition:

- Beef heart (infusion from 250g), 5 g/L
- Calf brains (infusion from 200g), 12.5 g/L
- Disodium hydrogen phosphate, 2.5 g/L
- D(+)-glucose, 2 g/L
- Peptone, 10 g/L
- Sodium chloride, 5 g/L

Final pH (at 25°C) 7.4±0.2

TSB Medium

Tryptic Soy Broth (TSB) is a nutritious medium that will support the growth of a wide variety of microorganisms, especially common aerobic and facultatively anaerobic bacteria.

Composition:

- Casein peptone (pancreatic), 17 g/L
- Dipotassium hydrogen phosphate, 2.5 g/L
- Glucose, 2.5 g/L
- Sodium chloride, 5 g/L
- Soya peptone (papain digest.), 3 g/L

Final pH (at 25°C) 7.3±0.2

TGB Medium

Thioglycolate Broth with Resazurine is a nutritious medium for sterility testing of biologicals and for cultivation of aerobic and anaerobic organisms. Any increase in the oxygen content is indicated by a color change of redox indicator resazurin to red.

Composition:

- Agar, 0.75 g/L

- Casein enzymic hydrolysate, 15g/L
- L-cystine, 0.50 g/L
- Dextrose, 5.50 g/L
- Resazurine, 0.001 g/L
- Sodium chloride, 2.5 g/L
- Sodium thioglycolate, 0.50 g/L
- Yeast extract, 5 g/L

Final pH (at 25°C) 7.1±0.2

YTF Medium

Routine medium growth for *Clostridium ljungdahlii* DSM 13528

Composition:

- Yeast extract, 10 g/L
- Bacto tryptone 16 g/L
- Sodium chloride 4 g/L
- Fructose 5 g/L
- L-cystine, 0.50 g/L

Final pH (at 25°C) 5.8 ± 0.2

YT Medium

YTF medium without fructose

LB medium

Miller's LB is a highly-referenced microbial growth medium used for the cultivation of *E. coli*. This nutrient-rich microbial broth contains peptides, amino acids, water-soluble vitamins, and carbohydrates.

Composition:

- Tryptone 10g/L
- NaCl 10 g/L
- Yeast Extract 5 g/L

Final pH (at 25°C) 6.5 - 7.2

Sequences of DNA methylase *MOTHA-c17620 hsdM* gene from *Moorella thermoacetica* codon optimized for expression in *E. coli*.

```
ATGACTGAAAACACAAATATGGATCTATCAACGCTGGAGAACTGGCTGTGGGAAGCTGCGTG
CGTCATCCGCGGTGCAGTGGACGCCCCGAAGTATAAAGACTACATCCTGCCCCTGATTTTCC
TGAAACGCCTCTCCGATGTTTTCGAGGACGAAATCGCTCGTTTGGCGGAAGAGATCTTTGAC
```

AGCATCGAAGAAGCTCTGAAGCAGGTTGAGGAGGACCACGCCCTCGTGCGCTTCTACATTCC
GCCTCAGGCACGTTGGGATGCAATCAGCCGCCAAACTACGAACATCGGCGAATATTTGACTT
CCGCTGTGCGTGCTGTGGCACGGGAAAACCCGAAGCTGCATGGTATTTTTGAAAATATCGAC
TTCAATGCGCAGATGGCGGGTCAGCCGGTGATTGATAACGAcCGTCTTTACAACCTCATTCA
AGTTTTGTCGCGCCATCGTCTGGGTCTGAAGGACGTCGAGGTGGACATCCTCGGCCGCGCCT
ATGAGTACCTGTTGAGAAAGTTTGCGGAGGGCCAGGGCCAAAGCGCAGGGGAATTtTATACC
CCGCGCGAGGTTACCTGGCTTATGGCGTACCTGTTAGAGCCGCGTCCGGGTGACGAGATCTA
CGACCCGGCCTGCGGTTCTGGTGGCCTGTTGATTAAAAGCGTGTTGGCGCTGAAGGAGACGT
ACGGCGACGATCCGCGTATTGCACCGGTTAAAATCTATGGTCAAGAAATCTTGTACACGACC
TTCGCCATGGCTAAGATGAACGCATTTATCCATGATCTGGAGGCGGATATCCGTCTGGGTGA
TACCATGGCGCGTCCGGCGTTTACCAATCCAGATGGTTCACTGCGTACCTTCGACAAAGTGA
CCGCGAATCCGATGTGGAATCAAAAATTCCCGCTGCCGCTATACGAGGAGGACCCGTTTGAC
CGTTTCAAATTTGGCGGCATTCCACCGGCATCTAGCGCGGATTGGGGTTGGATACAGCACAT
GTTTGCCTCGCTGAAAGAAGGTGGCAAGATGGCGGTTGTTCTGGATACCGGTTCCGTCAGCC
GCGGCTCCGGTAATCAGGGTAGCAATCGTGAGCGCGATATTCGTAAGGTGTTCGTGGAGAAC
GATCTGGTTGAATGTGTGATCCTGCTGCCGGAAAACATGTTCTACAACACCACCGCGCCAGG
CATCATTATGGTTATCAACAAAGCAAAAAAACACCCGGCGGAAATTCTTCTGATTAACGCgA
GCAAACTATTCACCAAAGGTCGTCCGAAAAACTATATGGAAGACGAGCACATTAAGCAGGTC
TATAGCATCTACCGCGAATGGCGTGAAGAAGAGGGCCTGAGCAAGATTATTCCGGTAGAGGA
GGCGGCGCGTAATGACTATAACCTGTCTCCGAGCCGTTATGTTAGCATTAACGGCAAGGAGG
AATACCGTCCGATTGAAGAGATCTTGGTGGAACTGGCGGAGGTTGAAGAGGAACGTCAAGCG
GTTGACAAAGAGTTGAATGATATCTTGGGGAAGCTGGGTTTTGGTGGCTGGCTGAACGGCTA
A

Sequences of DNA methylase *MOTHA_c17610 hsdS* gene from *Moorella thermoacetica* codon optimized for expression in *E. coli*.

ATGGGAAAAGAAGTAAATGAAGTTAAGGAGGGCTATAAGGAAACGGAGATCGGCGTTTTGCC
AGAAGACTGGGAGGTTGTTCGTCTGGGTAAGGTGTTTGAAGAAGTTGATCGTCGCGTGAATA
ACGTGAAGAACGCCGCGAGCCTGCCGGTGCTCTCCCTGACTAAAAACAACGGCATTATCCCT
CAGACCGAACGTTTTAAAAAGCGCATTGCGACGGACGACCTCAGTAACTATAAGGTGGTCTA
CAAGAAAGAGCTGGTTTACAATCCGTATGTGATCTGGGAGGGTGCCATTCATATTCTGAATC
GCTTGGAGGCGGGTTTAGTTAGCCCGGTTTACCCGGTCTTATCTGTTAACAAAAAGGTGGCC
GACGCATACTTCTTTGATTTCTGGCTGCGCACCCCGAGCGCAATTAAGGCGTACAGCCGTTA
TGCAAGCGGTGCTGTGAACCGCCGTCGTGCTATCCGTAAGACCGATTTCAAAAATATCGATG
CTCCGTTACCTCCGCTGCACGAACAGCGCAAAATTGCGTACGTGCTGAGCACCATTCAAAGA
GCGATCCAACTGCAaGATAAAGTTATTGCGGCAACCCGTGAGCTGAAAAAGTCCCTGATGCG
CCACTTGTTCACCTATGGTCCGGTgCCGGTGGACCAGATCGACCGTGTTCCGTTGAAAGAGA
CAGAGATCGGCATGGTTCCGGAACATTGGGAGGTTGTTCGGCTGCGTGAAGTGGCAGACTTT
ACCAAGAAGCCGCGTGGTCTGAACTATTCTGGCAATATACCATTCATTCCGATGGAACTGAT
CCCCATCGGCCGTGTCAACATTCAAAAATACATCATCAAACCGTCGAGCGAAATTTCGAGCG
GGGTGTACTGCGAACAAGGTGATCTGCTCTTGGCTAAAATCACCCCGTCCTTTGAGAACTAC
AAACAGGGTATTATCTCACAGATCCCGAAACCGTTTGCGTTTGCGACCACTGAGGTGTACCC
GATCAAGGCGCGTAAGGACTTCCTGGAGATTCTGTATTTGTTCTATTACTTGCTGATTCCGC
AGGTTCGCCAGGATATCGCTGGTAAGATGGAAGGCACCACGGGTCGTCAACGCATTAGCAAA
AGCGTAATCCAGAATTACCTGATCCCGATCCCGCCGTTGTCTGAACAACGCCAAATCGCGCG
TTTCCTGATTACCGTTGATAAAAAAATTGAAGCCGAAGAGTATCGTAAGTCCACCCTGCAAA
GCCTGTTCCAGACGATGCTGCACCTGCTTATGACCGGCAAGGTGCGCGTGAAAGACCTTGAG
GTCAAAGAGGACGCGCTGCGTCAGTAA

Sequences of DNA methylase *MOTHA_c24230* gene from *Moorella thermoacetica* codon optimized for expression in *E. coli*.

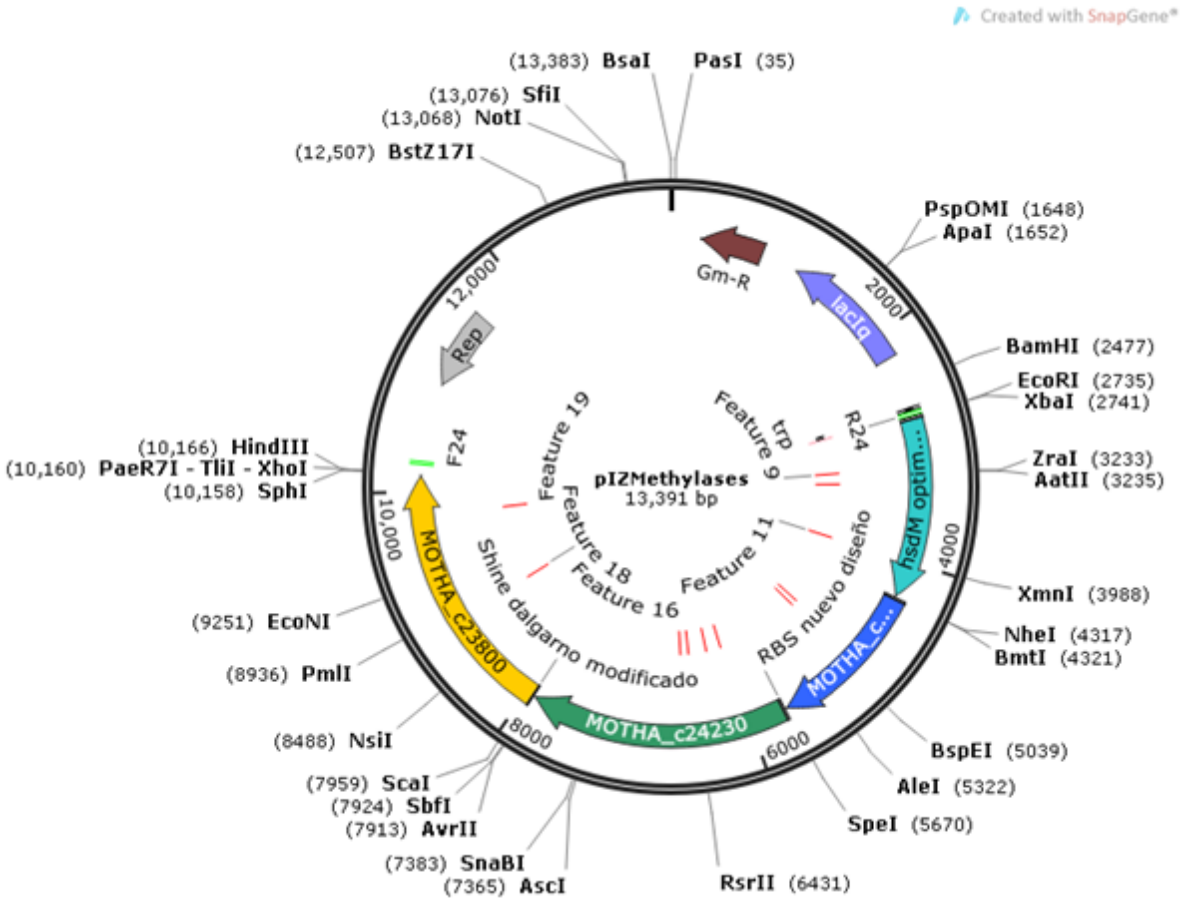
ATGACAATTGAAAGGAATTTTGATATAGCTTTCGTCGCGGACCTCGCGCTTCACGAAAAGCA
AATCCAACAGAATTATAGACCTATTATCGCGGTGCACAAATGGTTTGCTCGTCGCCCTGGTA
CTCTGTTTCGTAGCCTGTTGCTGGCGGAGTTCGCTCAGGGCAACTTGGCCGATAATTACTAC
CGTTCACATAACTTTCAGGGTTTGAAGGTAGCGGACCCCTTCATGGGTGGCGGCACCCCGCT
GATCGAGGCGAACCGCTTGGGTTGTCACATCCTGGGCTACGATATCAACCCGATGGCTTACT
GGATCGTTCGTGAGGAGATTGAACATCTGAACCTCGAAGCgTACCAACAAGCAGCACGGGAA
GTTGGCTTCTTCCTGGAAGAAAAGGTGGGTCCGTTTTATCGTACGCGCTGCCCGATTTGCGG
CCGTCAAGACGCGTTGGTTAAATACTTCTTATGGGTTAAGGTTCACCGGTGTCACAACTGCG
GCCGTGAGTTCGATCTGTTCCCGGGCTACGTGCTGGCGCAGAAGGGcCGACATCCGAAAGAT
GTTATTATCTGCTCGACCTGTGGTTCTCTGAACGAGGTGGGCGACAAGCGCAACCCGGGCCA
CTGCCACAACTGCGGTGAAGAGTTGAAAACCAAAGGCCCGGCCGGTCGCAACCAATGCCCGT
GCCCGCATTGTGGTGTGCGTAATAGCTACCCGGACCCGGAGAGCGGTCCGCCGGGTCATCGT
ATGGTTGCTATCGAATATCACTGtAGCTACTGCAAACCGGAGCACCGCGGCCGTTTTTTTAA
AAAACCGGATGCCGACGACCTGGCGAAATTCGCTACTGCGGTCGGTACGTGGGAAGCGCTGC
AaCCGCAGTTTGTGCCGGAGGAGAAGATTCCGGCAGGCGACGAGACAAACCGTCTGCATCGT
TGGGGTTACCGCTATTATCGTGAGATGTTTAACGAGCGCCAATTGCTGGGTTTGGAGCTGCT
GGCGCGTAAAATCAGCCAGCAACCGGATGAGCGCATTAAGAACGCCCTTGCGACCAATCTGT
CCGATCTGCTGCGCTACCAAAACATGTTATGCCGTTATGATCCATATGCGTTGAAATCCTTG
GACATCTTTTCCGTGCACGGCTTTCCGGTCGGTCTGATCCAATGCGAATCTAACATGCTGGG
GATTCCAGGTGGTAAAACGGGCCTGAATATCGGTTCCGGTGGCTGGACCAACATCGTGGACA
AGTACCTGAAGGCGAAACACTATTGCCAGTGGCCGTTCGAAATCCGCCATGTTAATGGTCGC
AAACGTCAGCTGTGGATTAAGGGCGAGTGGATTGGCGAACGCAGACAAGGAATGACCCAGCA
GCGTGAGGTTGATCTCCGCTGCGCAAGCGCAACCACTGCGTTTCTGAAGCCGAGCAGCTTAG
ACGCCGTTCTGACCGATCCGCCTTATTTCGCGAATGTTCAGTATGCAGAACTGATGGACTTC
TGCTATGTTTGGCTGCGTCGTCTGGTGGGTGCCTCCAATCCAGTCTTCACCCCGCGTACGAC
CCGCAACCCAGAAGAGTTGACCGGTAATACCACGATGAGCCGTGGCATCGACGACTTCACCG
GTGGACTGAGCCGTGTGTTTAGCAACATGGCTGCTGCTCTTAAGCCGGGCGCGCCATTCGTG
TTTACGTACCACCATAATCGTCTGGAGGCCTATTACCCGGTTGCTGTGGCGTTGCTTGATGC
GGGTCTGGCATGTACCGCAACCCTGCCGTGTCCGGCTGAAATGGCGGCATCGATCCATATTA
ACGGCACCGGCAGCAGCATCATTGATACCGTGTTCGTGTGCCGTACGACCGGCGTCGTGTCT
CGTCGTCTGCTCGTGAAAGAACCGGAACAGATTGCGGCGCTGATCATCAAAGAACTAGAGGA
ACTTGAGAAAGGTGGCGTTCCGGTTACCCGCGGTGACACCCGATGTATTATCTATGGTCACC
TGATACGTTTGGCTGTTTGGTATCTTCGCGCAACCTGGGACAAAAATCTGTCTTGGGATAAG
AAGTTCGCCCTCATTGCGCGTATGATTGATGAGCTGGGTGGCGCGGGCGCGATTGAAACCTA
CTTGGAAGAGAACGGTGTCCAGCTGAAGACCCGTCGCGAAACCATTGTATGTGAAGGCGAAT
CTGAATACGGCGCAGGTGGTGACGAAGTGTCCTTTTAA

Sequences of DNA methylase *MOTHA_c23800* gene from *Moorella thermoacetica* codon optimized for expression in *E. coli*.

ATGCCCATGCCAGTACTATCAGTTGAACAACGTAACAAGCTGGAAAGAACAGTTGTTGAAGC
GCGCGATGTCGCGGAGGCCGGTGCGAAAGCCGCGTTGGAGGCGCTTGCGGTGCATCACCACG
AGCCGTACTCTCATATGACCCCGGAGCAGCGCCGTTTGAGAAATCACCTGAGAGCCAGAGCG
CGCCAACTGGGCGACCGTCAAGACCAGCGTGGCAAGATGGAAATCACGCATCTCATTCAAGA
GTGGGCATATGAACATTGGCATCGTATGTTGTTCGCCCGGTTCCTGGCAGAGAACGACCTGC

TGATCGAACCGGAGATGGGTGTGGCGGTTAGCCTGGAAGAATGCGGCGAACTGGCCCAAGAA
GAGGGCACCGATCTGTGGACGCTGGCGTCTCGTTTCGCTCAGCAAATGCTGCCCCAGATATT
TAGACCTGATGACCCGGTGCTGCAAGTGACCTTCGCGCGTGAATACCAGCTTAAGCTGGAAC
AGCTTTTGAACGACTTGGAGCCGGGTATCTTCAAGGCCTCCGATGCATTAGGCTGGGCGTAC
CAGTTTTGGCAGAGCAAACGTAAGAAACAAGTCAATGAAAGCGGAAACAAAATCGGTGCGGA
CGAATTGCCAGCAGTTACGCAGCTGTTCACCGAACCGTACATGGTTAACTTTCTGATTCATA
ATACGATTGGCGCCTGGTATGCGGGTAAGGTGCTGGCGGAGAACCCGCAATTGGCTGGCCAG
GCAGAGTCCGAAGAGGAGCTGCGCCGTGCTGTGGCGTTGCCGGGCGTTACTTGGGATTACCT
GCGTTTCGCCCGCTCTGGTGACGGCGAAGGTCCGTGGCGTCCGGCAGCTGGCACTTTTGAAG
GTTGGCCTCAACGTGCAGCGGAGCTGAAGATCCTGGAcCCGTGCTGCGGTAGCGGCCACTTT
CTGGTCGCGACCTTTTATCACCTAGTGCCGATCCGCATGGCAGAGGAGGGCCTGACCGCACG
TGAAGCCTGCGACGCTGTGCTGAGCGATAATCTGCATGGTCTGGAGATCGATGAACGTTGTA
CCCAGATCGCAGCGTTTGCTCTCGCGTTAGCGGCTTGGACCTACCCGGGTGCGGGTGGTTAT
CGTCCGCTGCCGGGTCTGAGGATTGCTTGCTCGGGTATCGCGCCAAATACCAAAAAAGAAAA
CTGGCTGGCCCTAGCGGGTGACGACGAACGTCTGCGAAACGGTATGGCACGTCTGTACGATT
TGTTCCGCGAAGCACCGATCTTAGGCTCTCTGATTGATCCGGGGTCCGCACTTGAGAACAAC
CTGATCGAGGCTGGCTTTGATGAACTGCGTCCGCTGTTGGAGAAAGTAATGAGCGCAGAGAA
GGACGACTACGAGCAGCATGAGCTCGGCGTAGCCGCGTGCGGTATCGCTGATGCGGTTCAGA
TTCTTTCCGGCCGTTATCATTTGGTCATTACGAACGTGCCGTACCTGGCTCGTGGCAAGCAG
GGTGCTGGTCTGAAGAACTATCTGGATACCCACCACGCGCGCTCTAAGCAGGACCTGGCCAC
CGCGTTCATCGAGCGCAATCTGATGCTGTGTCTGGAGGGTGGTAGTACCGCGCTGGTGACGC
CGCAACAATGGTTGTTCCAAACCGGTTATAGCAAGATCCGTAAAAAACTGCTGCGTGACCTG
CGTTGGGAGCTCCTGGCCATCTTGGGCGAGCACGCGTTCCGCAGCTCTGAAGCGGCTGGTGC
GTTCCCGGCGATTTATGTTTGTAGCAAACTCTTGCCGAAAGATAACCACCTGTTTTTTAGCA
TCAATGTTTCCGAGCGCAGCTCAGCGTGCGACAAAGACTTGTACCTGCAaCACGCTTCGCTG
AATAAGATGCAGCAAATCAGCCAGCTTAAGAACCCGGAGCACATTATTATCACCAATACCTT
CAGCGGCTTTCGCGTGTTTGGTGACTACGCAGATTGTTATCAAGGTATTAGCACCGGTGACA
ACCCGCGTTTCTGCATTAAATTCTGGGAACTGCCATCGATCCTGCCGGGCTGGGAACGCTTC
CAGACCCCGCCTGAGGAAACCCAATATTACGGCGGCCGTGAACACCTGATCCGTTGGGAGGA
GCGCGCCAGCATACAAGAGTTAGGCGCGATTCGTGGTAAAAATGCGTGGGGTAAGTGGGGCA
TCGTTATTGGTGTTGACTGCCAGTAA

Map of plasmid pIZMethylases



Sequences of the genes used to modify *M. thermoacetica*

Sequence of homologous region up to *pyrF* (*MOTHA_c09230*) gene

CGCGGCCTCCTCAATATTCAATTTGTCATCCACCGGGGCGAGGTCTACGTCCTGGAGGTAAA
TCCCCGTTCCAGCCGCACGGTACCCTACCTCTCCAAGATTACCGGCGTGCCCATGGTGGCCC
TGGCCACCAACGTCATGCTGGGCAAAAGCCTGCCGGAGCAGGGCTACCGGGGCGGCTTAATG
CCGCCGCCGGATTTTACCGCCGTAAAGGTCCCCGTCTTTTCCTTCGGCAAGCTGTTGCAGGT
GGACACCTCCCTGGGACCGGAGATGAAGTCCACCGGCGAGGTAATGGGGATTGATCCCGTCT
TCGAACGCGCCCTCTATAAAGGCCTGGTAGCCGCCGGCTGCTCCATCCCCCATCACGGCACC
CTGCTGGCGACCATCGCCGATAAGGACAAGGCGGAAGCAGTGCCCATCATCAAGGGCTTTGC
CGAACTGGGCTTCCAGGTGGTGGCTACCGCCGGCACCGCCGGCGCCCTGGCCGCAGCGGGAC
TCTTCGTAGAGAGGGTGGGGAAGATCCGCGAGGGTTCGCCCCACATTATCGACTATATCCGG
GAAGGGAAGGTCCACTTTGTCCTCAACACCCTCACCAGGGGCAAGATGCCCGGCCGGGACGG
TTTTAAGATCCGCCGCGCCGCGGCCGAACTGGGCATCCCCTGCCTGACTTCCCTGGATACGG
CCCGGGCCCTGCTCAAAGTCCTCCAGTCCCTGAAGTCCGGCGACGGGTTTAACCTCAAACCC
CTGCAGGAGTATGTACCCCTTTCCCGTCCTTAACGGAGGAGCGCCAAATCGCCTCCGCCCCA
CCCCGGCAGGAGCAGCAGCCCGCGGCTGCACCGGCCGGGCGGTTTCCCGGCCGGCCCTTCAA
GCACCAGGCGAGATGGCCGGGCCGCCGCCATTTAGCATATCAAGAGCGCCGGAAGGGAAGGG
CTTTTCCGGTTTTTACCGGTCGGGGTTAAGCCTGACTTAAGGGCCGGTACCGGACCCTCCCC

ATATTCACTCCGCTTACACTCCGTTTTTTGAACTATAAGATCATAAAGCGATATTTAAGGGC
TTCTGGCCTGCTTGCCAACACTAATGTACCTGCAGGAGATG

Sequence of homologous region down to *pyrF* (*MOTHA_c09230*) gene

AATAGAGATGGTGAAATAATAACTTCGGCCTGCTTTCATGCTTGATAATTTTTGTCATGTAG
GGCTACAATGATAGTAACAGGTGATGACACGATGGAACGAATTAACTTTATCAATACCCGGG
AGTTTAAAAATAGAGCAACCCAAATCTTGAGGCAGGTACAAAAAGACCAGGTTATTATTATA
ACCAATCGCGGTAAACCTGTAGCCACTTTAAAAGGTTTCAATCCACGTGACCTGGTTGTTGC
AGAAGATAGACATGATAGCCTTTACCAGCATTTGCGGCAACAAATTTTAAAAGAAAGTCCAG
AACTGGCTGCCAGGGATACCAGGCAAATCGCCACTGATTTTGAAAAGATAACAGCTAAAATG
AGAAAACAGATTGCCTACAGGACCTGGGAAGAAATGGACCGGCACTTAAAAGGGGATCCTTA
TGATCTTACTGGATACTAATATTTTTATAATCGATCGTTTTTTTCCAGGGGATAGTCATTAC
GCTATAAACAAGGAATTCATTCAAGAGTTATCGCGGCTCGAGGCGGGGTTTTCTATTTTTTC
GTTATTAGAACTTACCGGCATTGCTTCTTTTAACCTTTCAGCCAAAGAATTGCAGCAATGGT
TGTTTGATTTTGCCTCCGTTTATCCTATTCGTATTCTTGATCCCTATGATTTAAAGATTGAT
TCTGCCAAGGAATGGTATACTAAATTTTTGCAGGAACTAATGGCAAAAGTTACCCACCAAAT
GACTTTTGGCGACGCTATTTTTTTACGTGAAGCTGAAGGTTATCAAGTAGAGTATATTATTA
GCTGGAATAAGAAACATTTTCTTTCACGTACGACAATCAAAGTGCTCAACCCTGAAGAGT

Sequence of homologous region up to *acsA* (*MOTHA_c23020*) gene

GCCGGGCCAGAAGAAAGACCTTGACCTCGGCCCCCCGGTTCAGGAGATGGCGGGCGACCACC
AGGCCGTCGCCGCCATTGTTGCCTTTACCACAGAAGATTAAAATCCGGCGGTTGGCTACCTG
GCCCTGAAAGTGGCGCTCGATGGATTCTACCACCCGCAAGCCGGCGTTTTCCATTAAGACGA
TACTGGGTATCATGTACTCGCTGGACGCCAGGCGATCCAGCTGTCCCATCTCTGCTGCCGTT
ACCAGGTACATGGCTTTAACCCTCTTCCGAAAATGAACTTTCAACCAGAGCTACCGCTGCGG
CGTAAGCGTGGCAGTGGGAGAGACTGACGGTTATCTCACCCACCCCCAGTTCCCGGGCCAGC
TGTCGGGCCCGGTTATGGAGGATTACCCGCGGTCGCCCACCCTGTTCCCTGGTGATTTCAAT
ATCTTGCCAGGAACAACGGCCCAGCCCTATCCCCAGGGCCTTCATTACCGCCTCCTTGGCCG
CGAAACGAGCTGCCAGGGAAGCCCCGGGCCGGTGCCGGGCCAGGCAATAGGCTACCTCCGCC
GGTGTAAATACCCTGGCCAGGAGGCGGGGGTGGCGTTTTATAGAGCGCTCCAAACGGCTGAT
TTCAATGATGTCTATCCCGGCCTCTAGCATAATCCCATCCTAACCATTTACCTGCTCCTTAA
TTCCCTTAAAGCTTATTCGGCAACAATGTTGAAAAACCTTCTCTTTTTGTAGAAATTAACTG
GCCGGCAGGACCACTAACAACGCTACATGCATCTGTGCGGCCCAGGGACCCGGTCCGGGCGG
CGAAACAACCCGGGTGCGCCCCGAAAAGGACGGCTAAGGCCGCGGACAGGTAAGCCAGGGCC
GTTACCTCCTCCCCTGCCCGCTCAGTCCCCCCTATTTACATTTAAGGCCGTCCGGGCGACCT
TTAACGCCGAAAGCCTTTCCTTTTTTTAGTCCCGGTCTTAAGATAGAAATACACGAAGGGAG
TGACCTCA

Sequence of Homologous region down to *acsA* (*MOTHA_c23020*) gene

ATTTTCTTATTATCTATCTAATATTAAGGAGGGCGGGTATGGGCCGTAAGGTTTACCTGCTT
TTTGCTACTTTAACCATCAGCGAAGTTCTGACGGCAGTACTTTTTTTCCGCGTACCGCCCTT
CCCGGTTTTCCTTGCCTTCGTGGTTTTGAACGCCCTGGGAGCAGGCCTGGCAATATACTTCC
AGGGGCTATCCTGTTCTTTACCCCAGCGGCCGATAGAGGAAGACGCCAATTTTAATACTGAC
AGTATTTTTGTCTCCACCCTGCAGATTGCCGACCAGACCCTGCCGGTTTTACGCGGCGGGTT
AAATAAAACTACGGCGGCCAAAACGGCCGATATAATCCAGGACCTCACCCAAGTGCCGGCCA
TAGCCATAACCGACCGGGAGCGGGTCCTGACCTTCCTGGGCGTAGGATGCGACCAGCACCAG
GCGGGGGACCGGATTCTCACGGAAGCCACCAAAGAGGTCATTGCCACCGGGCGCATGAAGGT
TGTCCACACTCCCCAGGGCCTCTGCTGCCCCCGGTATGGTACAGGCTGCAATTGTCCCCTGA

AATCGGCGGTTATCGTCCCCCTGAAGTGCCGGGATGAGATTGTCGGCGCCCTCAAACTCTAC
CAGACCAGGGAGGGGGTTTTCCAGCCGGAGATTGTCCGCCTGGCCGTCGGCCTGGCCAATTT
GTTGAGCCTGCAGATAGAGCTTTCCGAGTTGGACCGCCAGCGCCAGCTCCTCACCGAGGCGC
GGCTGGAGGCCCTCAACGCCCAGATTAACCCTCACTTCTTCTTTAATACCCTGAACACCATC
ATTAGCTTCAGCCGGACGGACCCGGAGCGGGCCCGGCGCCTGCTCATCCGCCTGGCCAAACT
CTTCCGGCAAACCTTGAGCCGCCACGGCAGCTTGACTACCCTGCAGGAAGAGCTGGATTGTG
TTCGTACCTACCTGGTACTGGAAAAGGCACGCTTCGGCAATAAATTCCGCTACCTTGAGGAC
GTGCCGCCGGAACTGCTGGAGTACCATATTCCAGTCCTCAGCCTGCAACCCCTGGTGGAAAA
TGCCGTTAACCATGGTCTGTTGGCCAAAGAAGGGCGGGGCACGGTCAAGGTATCCGGCCGCC
TGGCGGGGCACGAACTCCACCTGGCCGTCAAGGACGACGGCGTCGGCATCCCGGCCGAAAAG
ATCAACCTGGTGACGCAACCGG

Sequence of P_{G3PDH} promoter

GGACGGTTGCCAAGTACCGGGACGAGCAGGGCATCCCGGCGGCGGCTAAAAGAAAACGATAT
TAGTTAAGAAGGATTTTGACCATTTGTGTTGAATAGATAGTGTTTGACGGTACAATCTCCGG
CAATTAGCAATATATCATAATAAATCCTGATTGGGTTAGGAATAATATCAAAAGCCAAGGAG
CCTGAAAGCGGTGGGGGTTGACGCTGCAGGAATTTAACCCTTGCCGTTACAATAAATATAAG
GAGGAGTACA

Sequence of *GmR* codon optimized for expression in *M.thermoacetica*

ATGCTGCGGAGCAGCAACGACGTCACCCAGCAGGGCAGCCGGCCCAAAACCAAACTGGGCGG
CAGCAGCATGGGCATCATtCGGACCTGCCGGCTGGGCCCCGACCAGGTCAAAtcCATGCGGG
CCGCCCTGGACCTGTTTGGCCGGGAATTTGGCGACGTCGCCACCTACAGCCAGCACCAGCCC
GACAGCGACTACCTGGGCAACCTGCTGCGGAGCAAAACCTTTATCGCCCTGGCCGCCTTTGA
CCAGGAAGCCGTCGTCGGCGCCCTGGCCGCCTACGTCCTGCCCCGGTTTGAACAGCCCCGGA
GCGAAATCTACATCTACGACCTGGCCGTCAGCGGCGAACACCGGCGGCAGGGCATCGCCACC
GCCCTGATtAACCTGCTGAAACACGAAGCCAACGCCCTGGGCGCCTACGTCATCTACGTCCA
GGCCGACTACGGCGACGACCCCGCCGTCGCCCTGTACACCAAACTGGGCATCCGGGAAGAAG
TCATGCACTTTGACATCGACCCCAGCACCGCCACCTAA

Sequence of *pduL* codon optimized for expression in *M.thermoacetica*

ATGAAACTGAAACAGCCCGGCATCGTCGCCGGCGTCAGCAACCGGCACGTCCACCTGAGCCG
GGAAGACGTCGAAGTCCTGTTTGGCAAAGGCTACACCCTGACCCCCATCAAAGACCTGGGCC
AGCCCGGCCAGTTTGCCTGCCAGGAAACCGTCATCATCGTCGGCCCCAAAGGCGCCATCGAA
AAAGTCCGGGTCCTGGGCCCCGAACGGAAAGAAACCCAGGTCGAAATCAGCCTGACCGACGC
CTTcAAACTGGGCGTCAAACCCCCCGTCCGGGACAGCGGCGACCTGGACAACACCCCCGGCA
TCGTCATCGTCGGCCCCAAAGGCAGCGTCATCAAAGACCGGGGCGTCATCATCGCCAAACGG
CACATCCACATGCACACCAGCAACGCCGAAAAATACGGCGTCAAAGACAAACAGATCGTCAA
AGTCCTGGTCGAAAAAGAAGGCCGGCGGCTGATCTTTGACGACGTCCTGATCCGGGTCAGCG
AAAAATACGCCCTGGAATTTCACGTCGACACCGACGAAGCCAACGCCGCCCTGCTGAAAACC
GGCGACCTGGTCTACATCATCGAAGAATAA

Sequence of *acaK* codon optimized for expression in *M.thermoacetica*

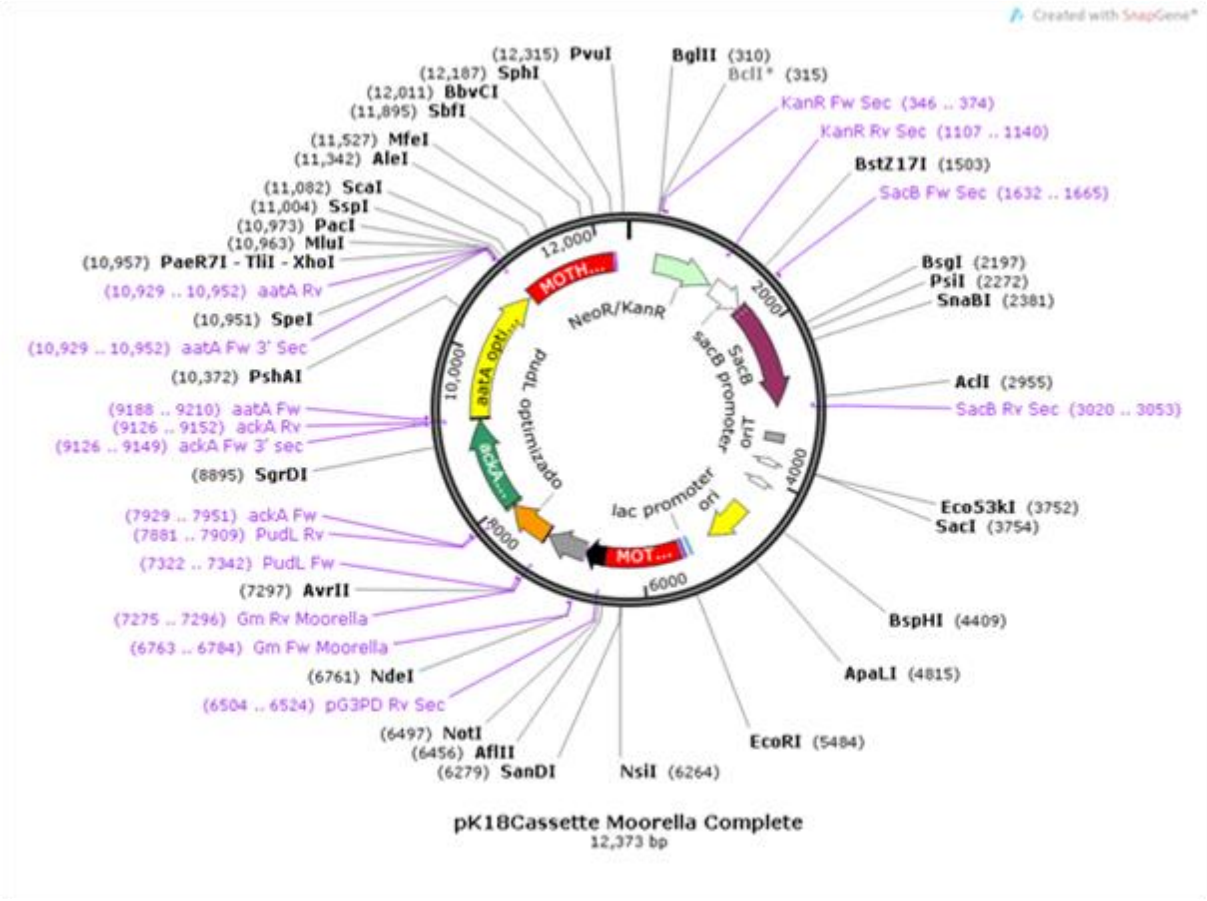
ATGATCGTCCTGGTCGTCAACAGCGGCAGCAGCAGCATCAAATACCAGCTGCTGGACATGGA
CAACGAAAAAGTCCTGTGCAAAGGCCTGGCCGAACGGATCGGCATCCCCGGCAGCCGGATCG
TCCACAAAAAAGCCGGCGAAAAATTTATCGTCGAAAAACCCATGCCCAACCACGACGAAGCC
CTGAAAATCGTCCTGGAAGTCCTGAAAGACGAAAAACTGGGCGCCATCAAAGACTTcAAAGA
AATCGACGCCGTCGGCCACCGGGTCGTCCACGGCGGCGAAAAATTTAGCGGCAGCGTCCTGA

TCGACGACGAAGTCATCAAAGCCATCGAAGAATTTAGCTACCTGGCCCCCCTGCAtAACCCC
CCCAACCTGATGGGCATCAAAGCCATtATGAAACTGCTGCCCGGCGTCCCCAACATCGGCGT
CTTTGACACCGCCTTTCACGCCAAAATGCCCGAAAAAGCCTACCTGTACGCCATCCCCTACG
AATTTTACGAAAAATACAAAATCCGGCGGTACGGCTTTCACGGCACCAGCCAtCGGTACGTC
AGCAAACGGACCGCCGAAATCCTGGGCCTGGACTACAACAAAGCCAAAATCGTCACCGTCCA
CCTGGGCAACGGCGCCAGCATCGCCGCCGTCAAAAACGGCAAAAGCGTCGACACCAGCATGG
GCTTTACCCCCCTGGAAGGCCTGGTCATGGGCACCCGGAGCGGCGACCTGGACCCCAGCATC
GTCACCTTTCTGATGGAAAAAGAAGGCCTGAGCGCCGAAGAAGTCTACACCATCCTGAACAA
AAAAAGCGGCGTCCTGGGCCTGAGCAAAAACTTTAGCAGCGACATGCGGGACATCGAAGACA
AAGCCCTGGAAAACGACCCCCTGTGCCGGCTGGTCCTGGACATCTACGAATACCGGATCGCC
AAATACATCGGCGCCTACGCCGCCGCCATGAACGGCGTCGACGCCATCAGCTTTACCGCCGG
CGTCGGCGAAAACAGCCCCATCACCCGGGAAGAAATCTGCGTCAACTACCTGAGCTTTCTGG
GCATCAAAATCGACAAAGAAAAAAACAACGTCAAAGGCGAAGAACGGATCATCAGCACCCCC
GACAGCAAAGTCAAAGTCCTGGTCGTCCCCACCAACGAAGAACTGATGATCGCCCGGGACAC
CAAAGAAATCATCGAAAAAGGCCTGAAACAGCTGGAATACTAA

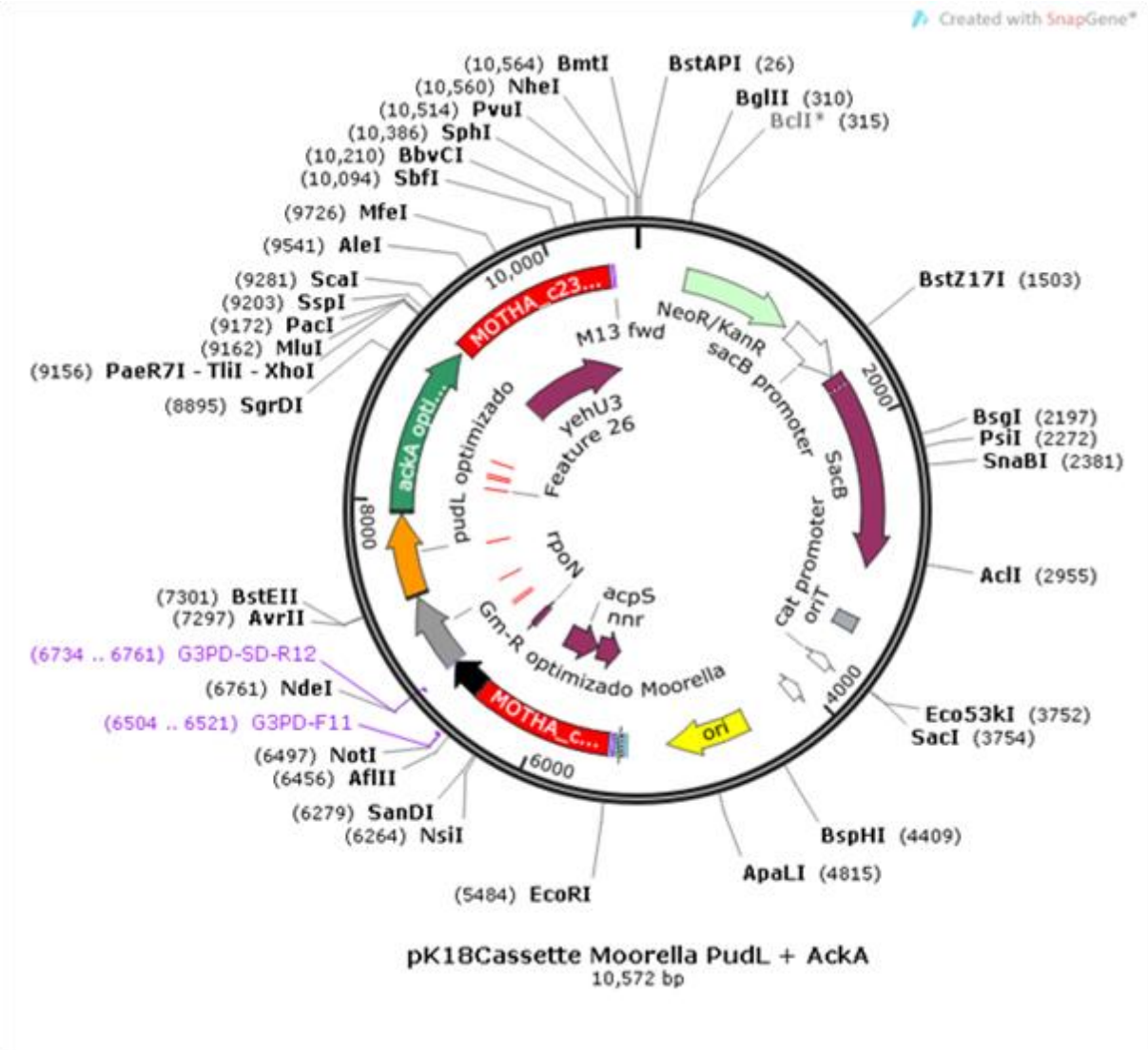
Sequence of *aatA* codon optimized for expression in *M.thermoacetica*

ATGGCCCACCCCCCCCTGCTGCAtCTGCAGGACATCACCCTGAGCCTGGGCGGCAACCCCCT
GCTGGACGGCGCCGGCTTTGCCGTCGGCCGGGGCGAACGGCTGTGCCTGGTCGGCCGGAACG
GCAGCGGCAAAAGCACCCTGCTGAAAATCGCCGCCGGCGTCATCCAGCCCGACAGCGGCAGC
GTCTTTGTCCAGCCCGGCGCCAGCCTGCGGTAtCTGCCCCAGGAACCCGACCTGAGCGCCTA
CGCCACCACCGCCGACTACGTCGTCGGCCAGATCGGCGACCCCGACATGGCCTGGCGGGCCA
CCCCCCTGCTGGACGCCCTGGGCCTGACCGGCCGGGAAAGCACCCAGAACCTGAGCGGCGGC
GAAGGCCGGCGGTGCGCCATCGCCGGCGTCCTGGCCGCCGCCCCCGACGTCCTGCTGCTGGA
CGAACCCACCAACCACCTGGACATGCCCACCATCGAATGGCTGGAACGGGAACTGCTGAGCC
TGGGCGCCATGGTCATCATCAGCCACGACCGGCGGCTGCTGAGCACCCTGAGCCGGAGCGTC
GTCTGGCTGGACCGGGGCGTCACCCGGCGGCTGGACGAAGGCTTTGGCCGGTTTGAAGCCTG
GCGGGAAGAAGTCCTGGAACAGGAAGAACGGGACGCCCACAAACTGGACCGGAAAATCGCCC
GGGAAGAAGACTGGATGCGGTACGGCGTCACCGCCCGGCGGAAACGGAACGTCCGGCGGGTC
CGGGAACTGGCCGACCTGCGGACCGCCCGGAAAGAAGCCATCCGGGCCCCCGGCACCCTGAC
CCTGAACACCCAGCTGCGGCCCCACCGGAAACTGGTCGCCGTCGCCGAAGACATCAGCAAAG
CCTGGGGCGAAAAACAGGTCGTCCGGCACCTGGACCTGCGGATtCTGCGGGGCGACCGGCTG
GGCATCGTCGGCGCCAACGGCGCCGGCAAAACCACCCTGCTGCGGATGCTGACCGGCCTGGA
CCAGCCCGACAGCGGCACCATCAGCCTGGGCCCCAGCCTGAACATGGTCACCCTGGACCAGC
AGCGGCGGACCCTGAACCCCGAACGGACCCTGGCCGACACCCTGACCGAAGGCGGCGGCGAC
ATGGTCCAGGTCGGCACCGAAAAACGGCACGTCGTCGGCTACATGAAAGACTTTCTGTTTCG
GCCCGAACAGGCCCGGACCCCCGTCAGCGCCCTGAGCGGCGGCGAACGGGGCCGGCTGATGC
TGGCCTGCGCCCTGGCCAAACCCAGCAACCTGCTGGTCCTGGACGAACCCACCAACGACCTG
GACCTGGAAACCCTGGACATtCTGCAGGACATGCTGGCCAGCTGCGAAGGCACCGTCCTGCT
GGTCAGCCACGACCGGGACTTTCTGGACCGGGTCGCCACCAGCGTCCTGGCCACCGAAGGCG
ACGGCAACTGGATCGAATACGCCGGCGGCTACAGCGACATGCTGGCCCAGCGGCACCAGAAA
CCCCTGACCACCGCCAGCGTCGTCGAAAACGAACCCACCAAACCCAAAGAAACCACCGCCGC
CCGGGGCCCCACCAAAAAACTGAGCTACAAAGACCAGTTTGCCCTGGACAACCTGCCCAAAG
AAATGGAAAAACTGGAAGCCCAGGCCGCCAACTGCGTCAAAAACTGGCAGATCCAGATtTAC
ATGGAAAAAACCCCCCGGAGCCTGCGGAACTTTCGGCTGATCTACCGGAGCAGCAAACAGAG
CTGGCAGAACCTGAAAAACGCCGGCTGGAACTGGAAATGA

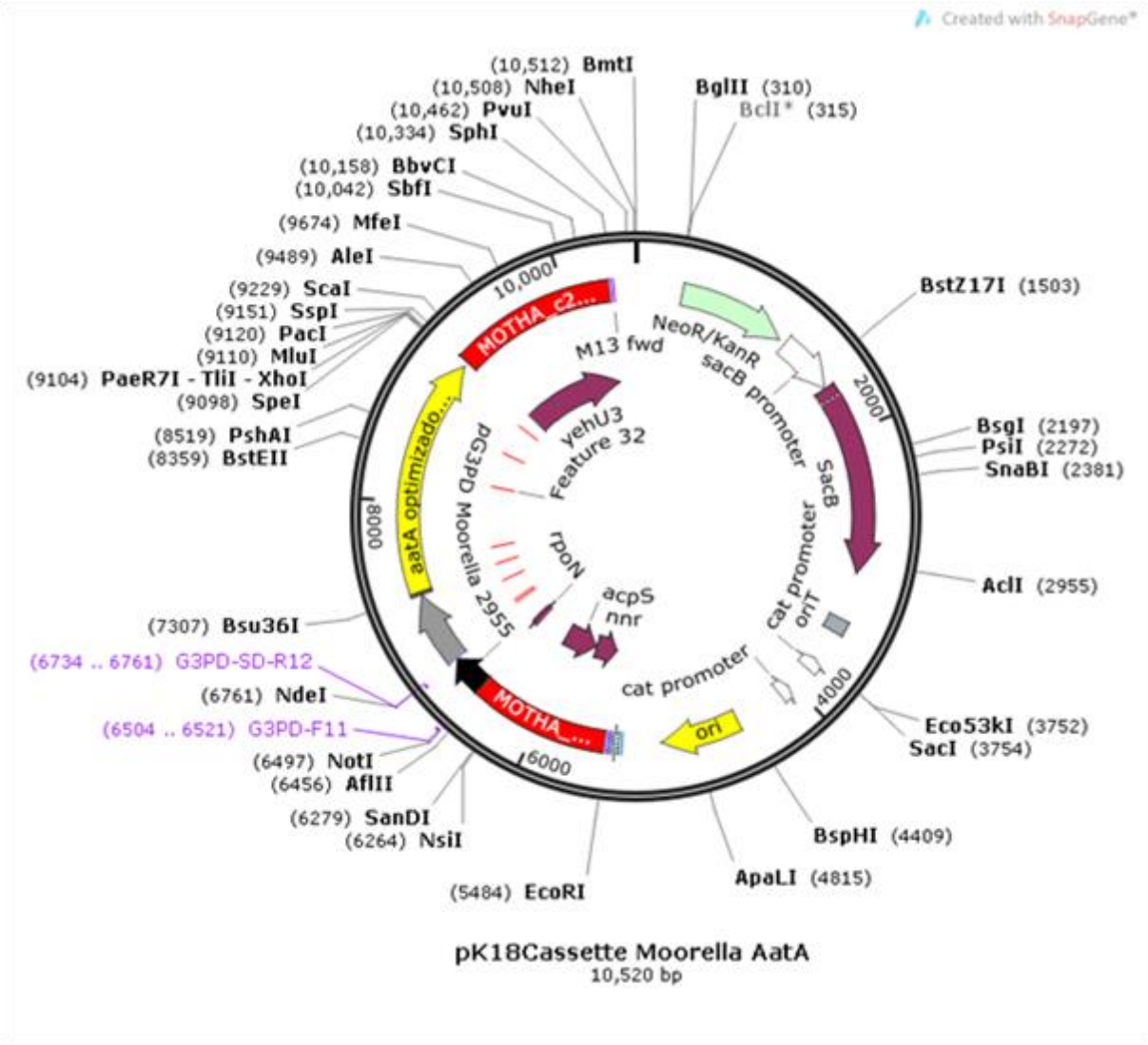
Map of plasmid pK18ACSAcassette Complete (1)


Created with SnapGene®
(12,315) PvuI
(12,187) SphI
(12,011) BbvCI
(11,895) SbfI
(11,527) MfeI
(11,342) AleI
(11,082) ScaI
(11,004) SspI
(10,973) PacI
(10,963) MluI
(10,957) PaeR7I - TliI - XhoI
(10,929 .. 10,952) aatA Rv
(10,951) SpeI
(10,929 .. 10,952) aatA Fw 3' Sec
(10,372) PshAI
(9188 .. 9210) aatA Fw
(9126 .. 9152) ackA Rv
(9126 .. 9149) ackA Fw 3' sec
(8895) SgrDI
(7929 .. 7951) ackA Fw
(7881 .. 7909) PudL Rv
(7322 .. 7342) PudL Fw
(7297) AvrII
(7275 .. 7296) Gm Rv Moorella
(6763 .. 6784) Gm Fw Moorella
(6761) NdeI
(6504 .. 6524) pG3PD Rv Sec
(6497) NotI
(6456) AflII
(6279) SanDI
BglII (310)
BclI* (315)
KanR Fw Sec (346 .. 374)
KanR Rv Sec (1107 .. 1140)
BstZ17I (1503)
SacB Fw Sec (1632 .. 1665)
BsgI (2197)
PsiI (2272)
SnaBI (2381)
AclI (2955)
SacB Rv Sec (3020 .. 3053)
Eco53kI (3752)
SacI (3754)
BspHI (4409)
ApaLI (4815)
EcoRI (5484)
NsiI (6264)
12,000
2000
4000
6000
8000
10,000
MOTH...
NeoR/KanR
SacB
sacB promoter
oriT
ori
lac promoter
MOT...
pudL optimizado
aatA opti...
ackA...
pK18Cassette Moorella Complete
12,373 bp

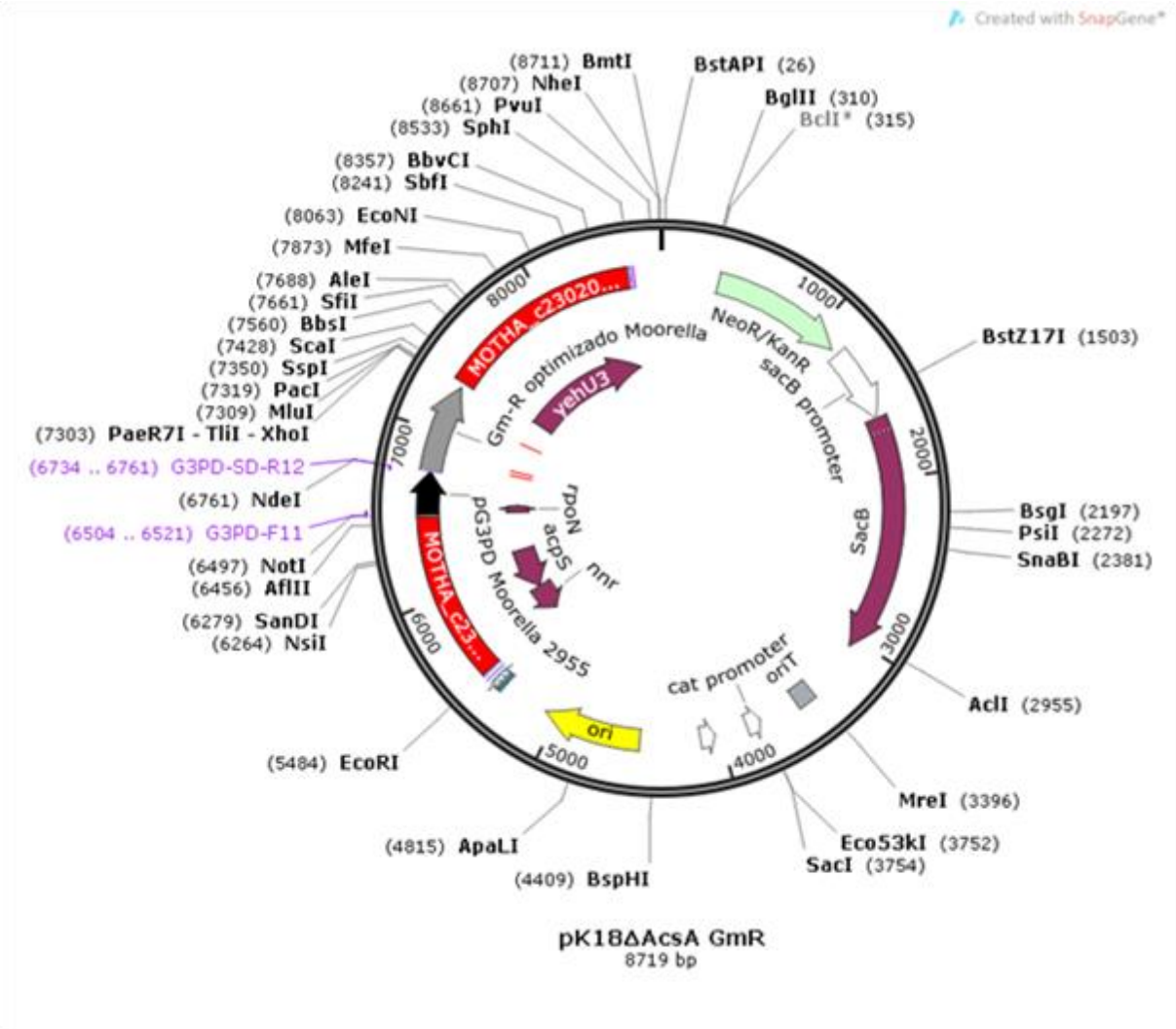
Map of plasmid pK18ACSAcassette PudL-AckA (2)



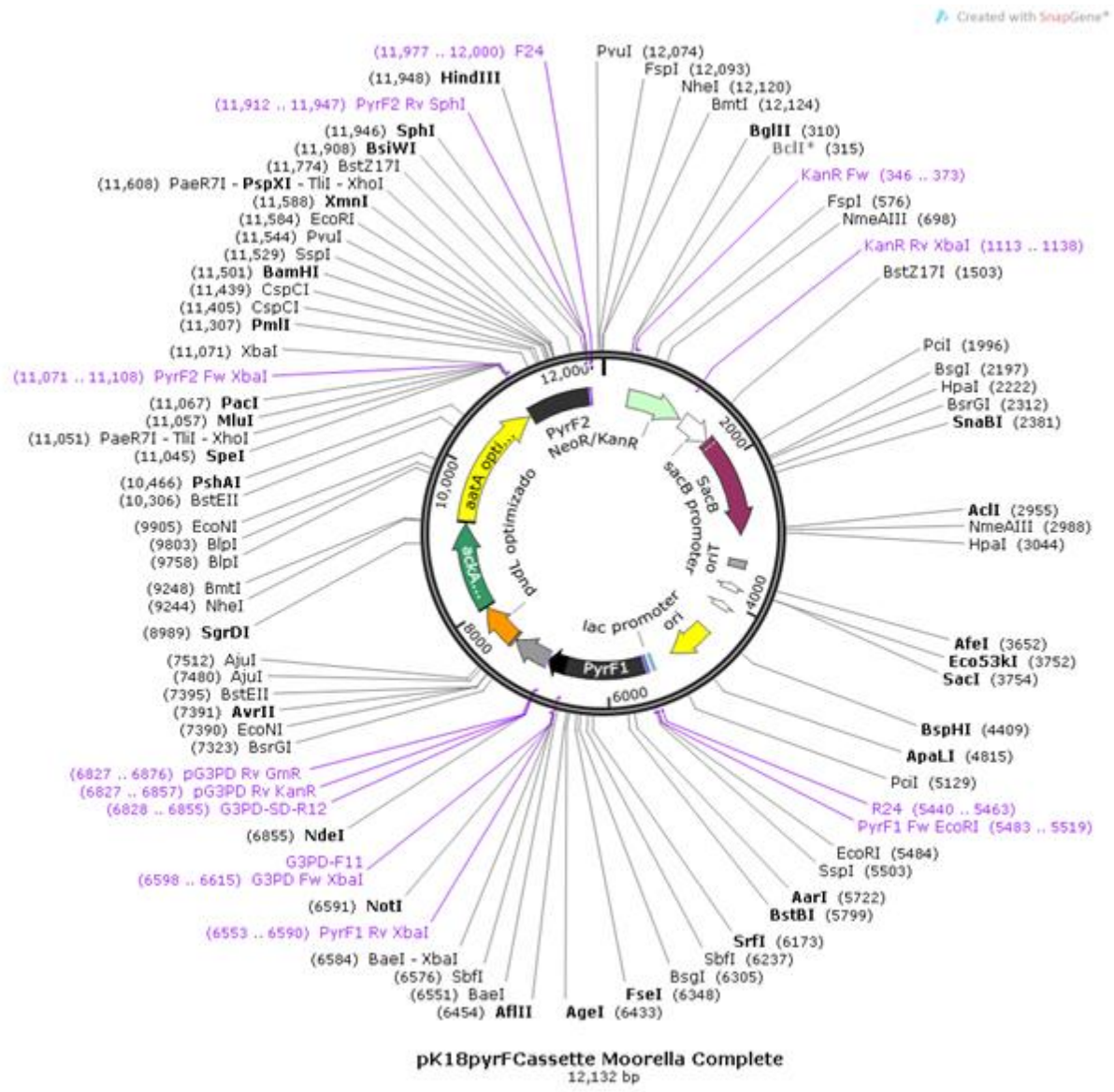
Map of plasmid pK18ACSAcassette AatA (3)



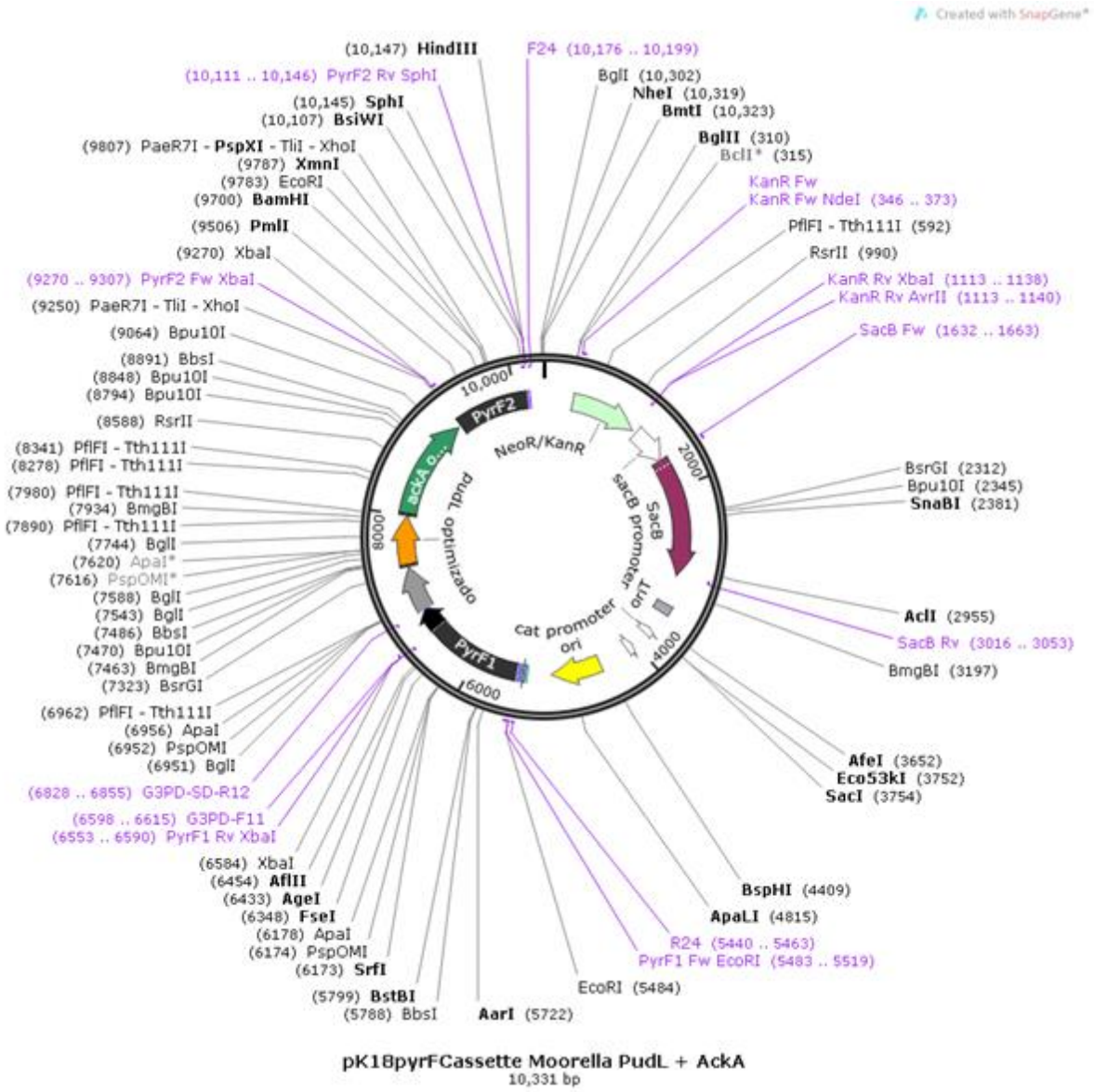
Map of plasmid pK18ACSAcassette GmR (4)



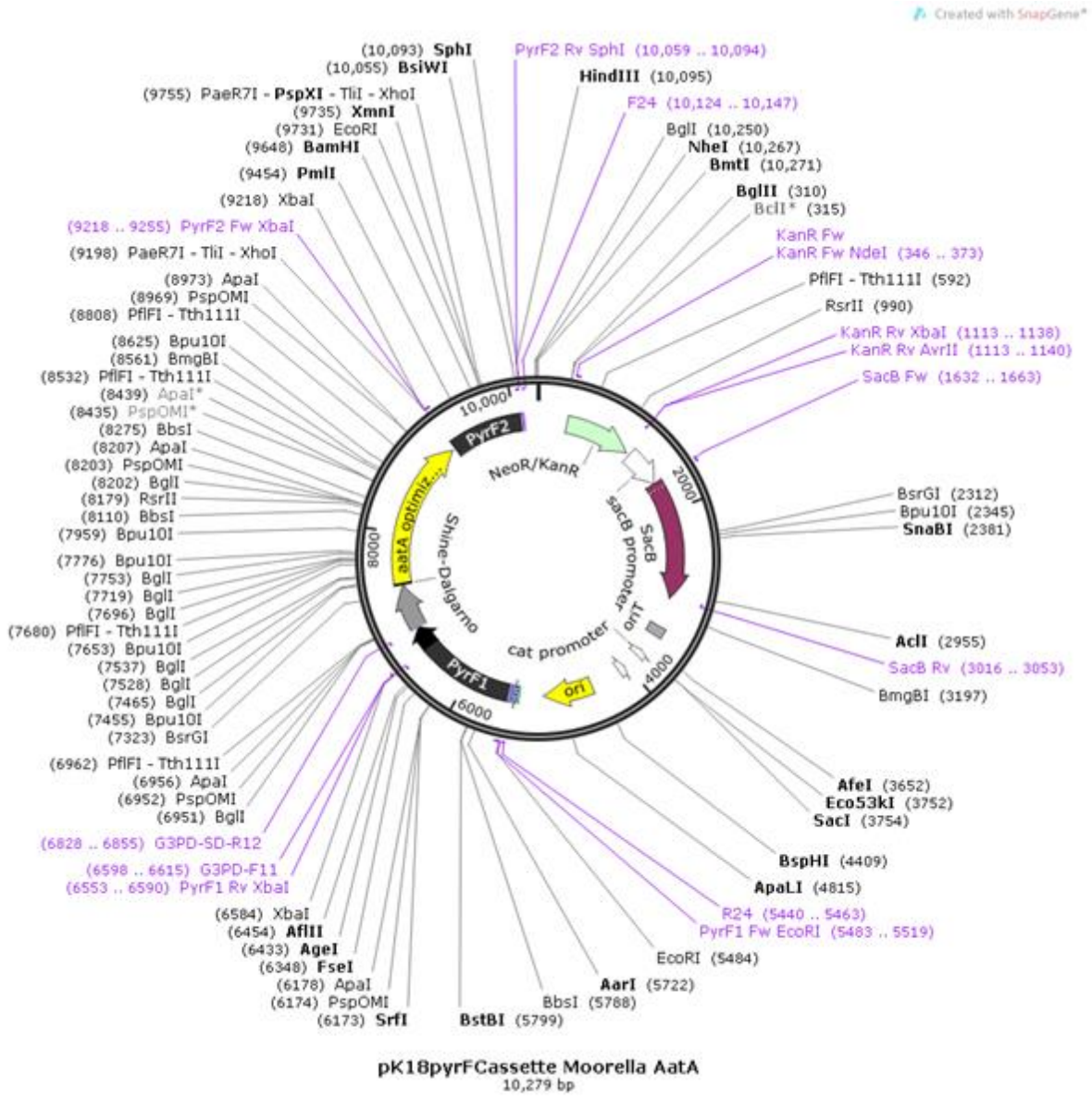
Map of plasmid pK18PYRFcassette Complete (5)



Map of vector plasmid pK18PYRFcassette PudL-AckA (6)



Map of plasmid pK18PYRFcassette AatA (7)



pK18pyrFCassette Moorella AatA
10,279 bp

Map of plasmid pK18PYRFcassette GmR (8)