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Enabling Ethanologenesis in *Moorella thermoacetica* through Construction of a Replicating Shuttle Vector

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Abstract: Replicating plasmid shuttle vectors are key tools for efficient genetic and metabolic engineering applications, allowing the development of sustainable bioprocesses using non-model organisms with unique metabolic capabilities. To date, very limited genetic manipulation has been achieved in the thermophilic acetogen, *Moorella thermoacetica*, partly due to the lack of suitable shuttle vectors. However, *M. thermoacetica* has considerable potential as an industrial chassis organism, which can only be unlocked if reliable and effective genetic tools are in place. This study reports the construction of a replicating shuttle vector for *M. thermoacetica* through the identification and implementation of a compatible Gram-positive replicon to allow plasmid maintenance within the host. Although characterisation of plasmid behaviour proved difficult, the designed shuttle vector was subsequently applied for ethanologenesis, i.e., ethanol production in this organism. The non-native ethanologenesis in *M. thermoacetica* was achieved via plasmid-borne overexpression of the native *aldh* gene and heterologous expression of *Clostridium autoethanogenum adhE1* gene. This result demonstrates the importance of the developed replicating plasmid vector for genetic and metabolic engineering efforts in industrially important *M. thermoacetica*.

Keywords: replicating plasmid vector; Gram-positive replicon; *Moorella thermoacetica*; acetogen; genetic engineering; gas fermentation; metabolic engineering

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1. Introduction

Acetogens are strictly anaerobic, Gram-positive bacteria with versatile metabolic capabilities, and they have attracted renewed interest recently due to their ability to fix CO_2. Indeed, their Wood-Ljungdahl pathway, thought to be one of the oldest CO_2-fixation pathways, allows them to rely solely on CO_2, CO or a CO_2/CO mixture as their source of carbon and produce the biomass precursor acetyl-CoA, further converted into acetate and species-specific products such as ethanol during gas fermentation [1,2]. Considering their unique metabolic ability to grow both autotrophically and heterotrophically on a wide range of substrates, acetogens stand out as key chassis organisms for the sustainable bioproduction of various target molecules. In fact, extensive research efforts have recently developed and applied diverse genetic engineering strategies [3–6] to eventually harness the synthesis of non-native, value-added target products by acetogens; thereby, developing sustainable alternatives for chemicals and fuels production while reducing greenhouse gas emissions through fixation of CO_2. Reliable and efficient genetic engineering tools are crucial for effective metabolic engineering purposes, and significant progress on tool development in acetogens has already been reported [7]. However, not all acetogens are equally amenable to genetic manipulation techniques. In fact, *Clostridium autoethanogenum* [8], *Clostridium ljungdahlii* and *Acetobacterium woodii* [9] are currently the most studied acetogens, for which reliable genetic tools have been developed and can be

routinely employed for a range of synthetic biology applications [3,5,10–12]. Encouragingly, *Eubacterium limosum* [13], *Clostridium carboxidivorans* [14], and *Thermoanaerobacter kivui* are catching up in terms of genetic engineering possibilities [4,6,15]. Although elaborate genetic manipulation with CRISPR-Cas tools have been achieved in these acetogens [3,4,10,16], it is worth noting that genetic and metabolic engineering efforts in acetogens remain more challenging than for established model organisms such as *Escherichia coli*, partly due to their highly specialised metabolism. Despite the progresses made towards developing reliable genetic engineering tools for acetogens, some species, notably the thermophilic acetogen, *Moorella thermoacetica* [17], remain difficult to manipulate genetically. *M. thermoacetica* is an attractive chassis organism for industrial applications, specifically due to its thermophilic nature, limiting media contamination risks and gas cooling requirements for industrial gas fermentation processes. To date, only one research group has reported successful genetic engineering of this organism, creating a uracil-auxotrophic strain [18] and targeting additional genes for potential metabolic engineering applications [19,20]. While these studies are encouraging, they rely on suicide vectors, i.e., non-replicating plasmid vectors to integrate the desired mutation into the genome. Clearly, this strategy has proven successful for metabolic engineering applications, but excludes any technique that relies on plasmid-borne expression such as CRISPR-based tools. Considering its industrial potential and currently limited toolset for genetic manipulation, we set out to construct a replicating plasmid vector for *M. thermoacetica* to expand its genetic toolkit. Construction of this vector required the identification of a Gram-positive replicon compatible with *M. thermoacetica* to allow successful plasmid maintenance within the population. After identifying and constructing one such replicon, plasmid behaviour was investigated, highlighting the technical difficulties of qPCR for quantifying plasmid copy numbers in the population. We also report the application of the designed shuttle vector for target gene expression, allowing successful non-native ethanologenesis, i.e., ethanol production in *M. thermoacetica*. Therefore, the work reported here describes the first plasmid-based strain engineering effort in this acetogen and further opens the door to additional genetic manipulations.

2. Materials and Methods

2.1. Bacterial Strains

E. coli TOP10 and the created methylation strain (carrying plasmid RBV1-MET; see next section) were grown in Luria-Bertani (LB) medium (L3522 from Sigma-Aldrich, St. Louis, MO, USA), supplemented with 15 g/L of agar for plates at 37 °C. The medium was also supplemented with 500 µg/mL of erythromycin, 50 µg/mL of kanamycin and 100 mM of L-arabinose where appropriate. *Moorella thermoacetica* DSM 521 was grown in MTA medium (0.1 g/L $MgSO_4.7\ H_2O$; 0.5 g/L $(NH_4)_2SO_4$; 0.04 g/L $Fe(NH_4)_2(SO_4)_2$; 2.4 mL/L $Na_2MoO_4.2H_2O$ (0.1% *w:v*); 0.15 mL/L $Na_2SeO_3.5H_2O$ (0.1% *w:v*); 5 g/L tryptone; 5 g/L yeast extract; 7 g/L K_2HPO_4; 4.5 g/L KH_2PO_4; 18 g/L glucose; 10 g/L $NaHCO_3$; 0.3 g/L L-cysteine-HCL.H_2O), supplemented with 200 µg/mL of kanamycin when required. The medium was filter-sterilised and dispensed in serum bottles. The gas headspace was exchanged for 100% CO_2. Cultures were incubated at 55 °C in a static incubator or agitated at 100 rpm as detailed. Growth on plates was performed in AWM medium [21] (0.1 g/L $MgSO_4.7\ H_2O$; 1 g/L yeast extract; 0.4 g/L K_2HPO_4; 0.4 g/L KH_2PO_4; 3 g/L $NaHCO_3$; 0.1 g/L NaCl; 1 g/L NH_4Cl; 1 mL/L trace elements solution; 20 mL/L vitamins solution; 1 mL/L selenite/tungstate solution). pH was adjusted to 7 and 1.5% agar was added. After autoclaving, 20 mM of glucose was added prior to pouring plates. Plates were spread with 50 µL of 4% *w/v* L-cysteine solution 24 h prior to inoculation. Plates were incubated in an anaerobic jar at 55 °C. All chemicals were purchased from Sigma-Aldrich.

2.2. Methylation Strain

The three genes, Moth_1671, Moth_1672, and Moth_2281 [18], coding for *M. thermoacetica* methyltransferases involved in restriction-modification systems, were amplified from *M. thermoacetica* genomic DNA with Q5® High-Fidelity DNA polymerase (New Eng-

land Biolabs, Ipswich, MA, USA) using the primers listed in Table 1. Moth_1671 and Moth_1672 were cloned into the backbone vector RBV1 with HiFi Master Mix (New England Biolabs) while Moth_2281 was added with SacI and XhoI into RBV1-1671/2. The three genes were controlled with the arabinose-inducible promoter, P_{BAD} [22]. The *E. coli* strain carrying the modified RBV1-MET plasmid (Supplementary Material Figure S1) was used as the methylation strain for plasmid in vivo pre-methylation prior to transformation into *M. thermoacetica*. To do so, strains carrying RBV1-MET and the desired shuttle vector were cultivated in LB supplemented with 500 µg/mL of erythromycin, 50 µg/mL of kanamycin, and 100 mM of L-arabinose.

Table 1. List of primers used in this study. Low-case characters represent restriction sites.

Primer	5′-to-3′ Sequence
1671_Fwd	AAGTTGGGTTTCGGAGGC
1671_Rev	ATTACATGGGAAATGGGTTTTGTACTGCG
1672_Fwd	AAACCCATTTCCCATGTAATAACGGAGG
1672_Rev	CCTGCCTGTTTCCTTCCTTCACCTCATTAAC
2281_Fwd	ATATATGAGCTCAACAGGCAGGTAACCAATAGAATG
2281_Rev	ATATATCTCGAGAAAACTACCCCCACATAG
KAN_Fwd	ATATATggccggccGGACGGTTGCCAAGTAC
KAN_Rev	ATATATgtttaaacACTAAAACAATTCATCCAGTAAAA
T. pet_Fwd	ATATATggcgcgccGAATGTGGTTAGTGTGATTAG
T. pet_Rev	ATATATggccggccTTAACCATATCCCACTAGTTC
C. bes_Fwd	ATATATggcgcgccACCGTGAGCATTCTGGACAGGT
C. bes_Rev	ATATATggccggccATTCCCATGAGCCCACGAACAGT
T. therm_Fwd	ATATATggcgcgccCTGCAGTAAATTAATTAACAGTTTT
T. therm_Rev	ATATATggccggccGGTCTCTCAGGAGCC
N. therm_Fwd	ATATATggcgcgccAGGCGTTTCCTCCCAC
N. therm_Rev	ATATATggccggccTTATTTAACACAATTATCATCCTCCAAG
Ori_Fwd	ATATATggcgcgccTTTTCCCTCCTACATAAAAATATCT
Ori_Rev	ATATATggccggccCCGTAGATAAATAGCGGATT
P*ffh*_Fwd	ATATATggccggccAATAAACAAGCCGCAGGTTAC
P*ffh*_Rev	CATTTTAGCCATATGCCTGTTACACCCCCGGTTT
KAN_Fwd_P*ffh*	CGGGGGTGTAACAGGCATATGGCTAAAATGAGAAT
aldh_Fwd	GTGGACAAGGTAAAAGTGGCTG
aldh_Rev	TCATGCTACCCCTTCCTTTACCTG
adhE1_Fwd	ATGAAAGTTACAAACGTAGAA
adheE1_Rev	TTACTTTTCTTCATCTTCTACA

2.3. Shuttle Vector Construction

The thermostable kanamycin gene, fused to *M. thermoacetica* native P_{G3PD} promoter [20], was synthesised by Integrated DNA Technologies, amplified with Q5® High-Fidelity DNA polymerase with the primers listed in Table 1 and cloned into a pMTL80000 vector (pMTL84141) [23] with FseI and PmeI, creating the pMTLK plasmid vector. Genomic DNA from the different species selected as donor strains for candidate replicons, as described in the Results section, was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The replicons were amplified by PCR using the primers listed above (Table 1) and cloned into pMTLK using FseI and AscI. The resulting plasmids were pre-methylated to protect them from *M. thermoacetica* restriction-modification systems prior to transformation by propagating them in the *E.coli* methylation strain created in this work. The list of plasmids created in this work can be found in Table 2.

Table 2. List of plasmids created with their respective features. Further details on the specific features can be found in the Results section.

Plasmid	Gram +ve Replicon	Gram – ve Replicon	Selection Marker	Additional Feature
1	Replicon from pUB110	ColE1	P_{G3PD}-*kanR*	-
2 (pMTLK-Tp)	Replicon from pRKU1	ColE1	P_{G3PD}-*kanR*	-
3	Replicon from pBAS2	ColE1	P_{G3PD}-*kanR*	-
4	Replicon from pNB2	ColE1	P_{G3PD}-*kanR*	-
5	Replicon from pNTHE02	ColE1	P_{G3PD}-*kanR*	-
6	*M. thermoacetica* chromosomal origin	ColE1	P_{G3PD}-*kanR*	-
7	Replicon from pRKU1	ColE1	P_{G3PD}-*kanR*	*P. thermoglucosidasius* toxin-antitoxin system
8 (pMTLK$_{ffh}$)	Replicon from pRKU1	ColE1	P_{ffh}-*kanR*	-
9	Replicon from pRKU1	ColE1	P_{ffh}-*kanR*	P_{G3PD}-*aldh*
10	Replicon from pRKU1	ColE1	P_{ffh}-*kanR*	P_{G3PD}-*adhE1*

2.4. Transformation of M. thermoacetica

To make electrocompetent cells, *M. thermoacetica* cells were harvested from a 100 mL culture at OD_{600} ~0.4–0.5 by centrifugation at 7800 rpm and 4 °C for 10 min, and resuspended in 50 mL of ice-cold filter-sterilised SMP buffer (1 mM sodium phosphate pH 5.8, 1 mM $MgCl_2$ and 270 mM sucrose). Two additional wash steps were performed, after which cells were resuspended in 2 mL of SMP buffer supplemented with 10% DMSO. Competent cells were stored in screw-cap tubes at −80 °C or used immediately. For the optimised transformation of *M. thermoacetica*, 1 µg of DNA was mixed with 100 µL of competent cells in 1-mm cold cuvettes. Cells were electroporated at 1.8 kV, 350 Ω, and 25 µF and resuspended immediately in 55 °C MTA medium. Recovery was performed in 5 mL of MTA medium at 55 °C for 48 h in CO_2-filled Hungate tubes. Recovery cultures were used to inoculate liquid selective medium to select for transformants.

2.5. Genomic DNA Extraction

The GenElute™ Bacterial Genomic DNA (Sigma-Aldrich) and the DNeasy® blood and tissue (Qiagen, Hilden, Germany) kits were used for genomic DNA extraction from *M. thermoacetica*. gDNA extraction was also performed with a phenol:chloroform method when needed. Briefly, pellets were first resuspended in 180 µL of PBS supplemented with 10 mg/mL of lysozyme (Sigma-Aldrich) and incubated at 37 °C for 30 min. 25 µL of Proteinase K (Qiagen), 85 µL of H_2O and 110 µL of a 10% *w*/*v* SDS solution were added to samples, followed by a 30 min incubation at 65 °C. 400 µL of a phenol:chloroform mix (Sigma-Aldrich) was added to the samples and mixed by inversion. After centrifugation at 13,000× *g* for 3 min, the top layer was carefully transferred to a new tube. 5 µL of RNase was added to the samples and incubated at room temperature for 30 min. 40 µL of a 3 M sodium acetate solution and 800 µL of ice-cold 100% ethanol were added to the samples and mixed gently by inversion before incubation at −80 °C for 30 min. After a 15-min centrifugation step, the samples were washed with 1 mL of 70% ethanol. Pellets were dried at room temperature for 45 min and resuspended in 50 µL of distilled water. In addition to the lysozyme-mediated lysis advised for gDNA kits, additional lysis methods were explored, including using Bugbuster® protein extraction (EMD Millipore™) as per the manufacturer's protocol or sonication (5 cycles of '30 sec on, 30 sec off' of high-intensity sonication at 4 °C). For boiled lysates, 1 mL of culture was incubated at 95 °C for 10 min and used as a template for further applications.

2.6. Fluorescence Measurement by qPCR

Plasmid copy number was investigated by qPCR using a LightCycler 480 thermal cycler (Roche Life Sciences, Indianapolis, IN, USA) and the LuminoCt SYBR Green qPCR ReadyMix (SigmaAldrich), as per the manufacturer's instructions. 5 µL of 1:100 diluted

genomic DNA was used per 20-µL qPCR reaction. Each reaction also contained 1 µL of forward primer, 1 µL of reverse primer (both from 0.5 µM stocks; final concentration 0.025 µM), 3 µL of H_2O, and 10 µL of LuminoCT ReadyMix. For each genomic DNA sample, three technical replicates were analysed by qPCR. Primers were designed using the IdtDNA online tool (https://eu.idtdna.com/scitools/Applications/RealTimePCR, accessed on 4 October 2022), with a target amplicon length of 100 bp. The qPCR programme used is shown in Table 3. Fluorescence was measured during the extension of each cycle as well as continuously at 95 °C at the end of the programme to create melt curves. The software provided with the LightCycler 480 was used to determine CT (threshold cycle) values for reference and target genes in each genomic DNA sample. Copy number was calculated with the LightCycler480 software, using the $2^{-\Delta\Delta CT}$ method [24]. The housekeeping genes, *rpoB* and *gyrA* were used as chromosomal targets while the Gram-positive replicon and the *kanR* genes were the targets on the plasmid. Primers used for qPCR are listed in Table 4.

Table 3. qPCR programme used in this study.

Cycle Step and Description	Temperature (°C)	Duration	Program Cycle	Data Capture
Initial denaturation	95	10 min	-	-
Denaturation	95	20 s	-	-
Annealing	55	20 s	-	-
Extension	72	20 s	Return to step 2 (x 50)	Single
Melting	72	-	-	Continuous
Hold	30	-	-	-

Table 4. List of qPCR primers used in this work.

Primer	5′-to-3′ Sequence
rpoB_Fwd	GAAGACCTCCTGTTCCTTAACC
rpoB_Rev	CAAAGAGCGGGATATGACCTAC
gyrA_Fwd	AACCATCACCGCTGTTATCC
gyrA_Rev	GCCCAGTGAGGTCTTCTTTAC
Replicon_Fwd	GCAAGTGTGGAAGTGGTTATG
Replicon_Rev	CCTCTGGCGGTATCTCAATAAT
Kan_Fwd	CTGGGAAGAAGACACTCCATTTA
Kan_Rev	GCCACTTACTTTGCCATCTTTC

2.7. Protein Quantification

To quantify protein concentration in cell lysates for estimating lysis efficiency, a bicinchoninic acid (BCA) assay was performed with the Pierce™ BCA Protein Assay kit (ThermoFisher, Waltham, MA, USA), following instructions provided by the manufacturer.

2.8. Ethanol Production

To test if the designed shuttle vector could be used for plasmid-mediated gene expression applications, expression of two target genes in *M. thermoacetica* was explored. First, to prevent unwanted recombination between multiple copies of the same promoter, P_{G3PD} driving the *kanR* gene was replaced by *M. thermoacetica* native promoter, P*ffh* by fusing this promoter and the *kanR* gene in a HiFi reaction (New England Biolab; primers listed in Table 1) and cloning the fragment into pMTLK with FseI and PmeI, creating pMTLK*ffh*. This step was required as previous experiments (data not shown) showed recombination between two copies of the P_{G3PD} promoter carried on the same vector when used to express *kanR* and a target gene, respectively. The genes, *aldh* (accession number AKX94582.1) and *adhE1* (accession number ALU38082.1) were amplified by PCR from *M. thermoacetica* and *Clostridium autoethanogenum* genomic DNA, respectively and cloned into pMTLK*ffh*

with NotI and NheI (Table 2). Their expression was driven by the P_{G3PD} promoter. Once transformed into *M. thermoacetica*, transformants were confirmed by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). To measure ethanol production, transformants were grown in the MTA medium in a static or shaking incubator. OD600 was measured at each timepoint. At the same timepoints, 1 mL of culture was centrifuged at 13,000× *g* for 5 min. Supernatants were stored in screw-cap tubes at −20 °C for subsequent analysis. Ethanol concentration was measured with a Varian ProStar HPLC system equipped with a RID (Refractive Index Detector) operated at 30 °C and an Aminex HPX-87H column (1300 × 7.8 mm, particle size 9 µm) (Bio-Rad Laboratories, Hercules, CA, USA) kept at 30 °C. Slightly acidified water was used (0.005 M H_2SO_4) as mobile phase with a flow rate of 0.5 mL/ min.

3. Results

3.1. E. coli Methylation Strain for In Vivo Plasmid Pre-Methylation

Similar to many bacteria, *M. thermoacetica* carries protective restriction-modification (RM) systems that recognise and cleave foreign DNA based on associated methylation patterns to protect itself against invading DNA [25–27]. These defence mechanisms must be considered during genetic engineering to prevent them from targeting and degrading introduced plasmids in the host. In fact, different methods to bypass RM systems have already been described [7]. A methylation strain for *M. thermoacetica* was previously built by expressing three *M. thermoacetica* genes [18], coding for methyltransferases in RM systems to enable in vivo plasmid pre-methylation in *E. coli*; thereby protecting target plasmids from recognition and cleavage in *M. thermoacetica.* According to the REBASE database [28], the *M. thermoacetica* strain DSM 521 used in this work carries identical RM systems to the *M. thermoacetica* ATCC 39073 strain used by Kita et al. (2013). Therefore, the same strategy was applied here with the same three genes were used in the *E. coli* methylation strain to allow for partial pre-methylation. Pre-methylated plasmids were digested with ApaI, an isoschizomer of Moth_2281 [29], to test for successful methylation (Supplementary Material Figure S2). Optimising the pre-methylation process was not explored further in this work, as it was reasoned that partial methylation was acceptable to prevent plasmid degradation at a sufficient level.

3.2. Transformation of the Candidate Shuttle Vectors into M. thermoacetica

When creating the plasmid shuttle vector for *M. thermoacetica*, the thermostable kanamycin resistance gene, *kanR* [30] was controlled by *M. thermoacetica* native promoter, P_{G3PD} as previously reported [18]. In addition to appropriate selective pressure, a replicon (i.e., the origin of replication and any replication-related genes) is crucial to maintain the plasmid within the population, allowing plasmid replication. This element must be compatible with the chosen host organism for successful plasmid maintenance. Unfortunately, no straight-forward method to predict compatibility has been established to date; instead, a trial-and-error method is used. To do so, several candidate replicons were selected based on their previously reported compatibility with thermophilic organisms or their species of origin (Table 5). In addition to the candidate replicons, *M. thermoacetica* chromosomal origin was used as a potential plasmid replicon. This approach has, in fact, proven to be successful in transforming non-model organisms [31], as it ensures compatibility between the host and the plasmid; it, however, may lead to unwanted recombination events between the plasmid and the chromosome. To test compabitility of the chosen candidates, the replicon was amplified from their respective plasmid (Table 5) and cloned into a shuttle vector, which also carried a Gram-negative replicon and a selection marker.

Table 5. List of candidate Gram-positive replicons tested in *M. thermoacetica*. The replicons were amplified from genomic DNA with the primers listed in Table 1 and cloned into pMTLK with FseI and AscI. Accession numbers are provided for replicons that have not been previously reported for transformation.

Species	Plasmid	References/Accession Numbers
Staphylococcus aureus	pUB110	*Geobacillus thermoglucosidasius* [32]
Thermotoga petrophila	pRKU1	*Thermotoga* sp. [33]
Caldicellulosiruptor bescii	pBAS2	*Clostridium thermocellum* [34]
Thermoanaerobacterium thermosaccharolyticum	pNB2	Accession number: NC_004979.1
Natranaerobus thermophilus	pNTHE02	Accession number: CP001036.1
M. thermoacetica chromosomal origin	-	Accession number: CP012369 REGION: 2527442.1545

During transformation optimization, a major limitation arose since transformants could not be successfully grown on selective solid plates; thus, preventing isolation of pure colonies. In fact, growth of the wild-type strain on solidified MTA medium was not successful. Instead, an alternative medium, used for the other acetogen *Acetobacterium woodii* [35], led to successful growth on plates of the wild-type strain. While the optimized growth conditions were also applied for transformant selection on plates, no colony were obtained regardless of the tested replicon. Different approaches were investigated to optimize transformant growth on plates, such as plating a higher number of cells or growing the cells in liquid selective cultures first, but did not resolve this issue. It is possible that an unknown effect of plasmid maintenance impacted either *M. thermoacetica* metabolism or growth pattern, preventing selection of transformants on plates. To overcome this challenge, previous reports relied on the "roll-tube" method for transformant selection [18,20]. However, application of this technique in this work led to a significant number of false transformants, as previously reported elsewhere [18]. As such, subsequent transformant selection was performed in selective liquid cultures, which clearly is not ideal, especially for experiments where a large number of colonies must be screened. To do so, selective cultures were routinely inoculated with a 1:100 inoculum from the recovery cultures. Serial dilutions were also explored but did not lead to successful growth in selective medium. However, despite this limitation, the replicon from *Thermotoga petrophila* pRKU1 plasmid showed positive results. This replicon allowed successful plasmid maintenance in the *M. thermoacetica* population while the other candidates did not lead to selection of any transformants; therefore, suggesting that they are not compatible with *M. thermoacetica*. Both the Gram-positive replicon and the *kanR* gene could be amplified by PCR from selective cultures of *M. thermoacetica* (Figure 1a) and were sequenced to confirm they were indeed pMTLK-Tp. *T. petrophila* pRKU1 is a cryptic 850-bp mini-plasmid shown to replicate via the rolling-circle method [36] and thought to encode only the Rep protein. Notably, the constructed shuttle vector (pMTLK-Tp; Figure 2) could be maintained for five passages in selective cultures (Figure 1b). It was also attempted to extract the plasmid from *M. thermoacetica* cultures and retransform this extracted vector into *E. coli*. Although transformation efficiency in *E. coli* was low due to poor plasmid yield during extraction, successful colonies were isolated (Figure 1c,d). Therefore, we report successful transformation of *M. thermoacetica* with a shuttle vector; therefore, opening the door to perform additional and more complexnugenetic manipulation techniques in this organism.

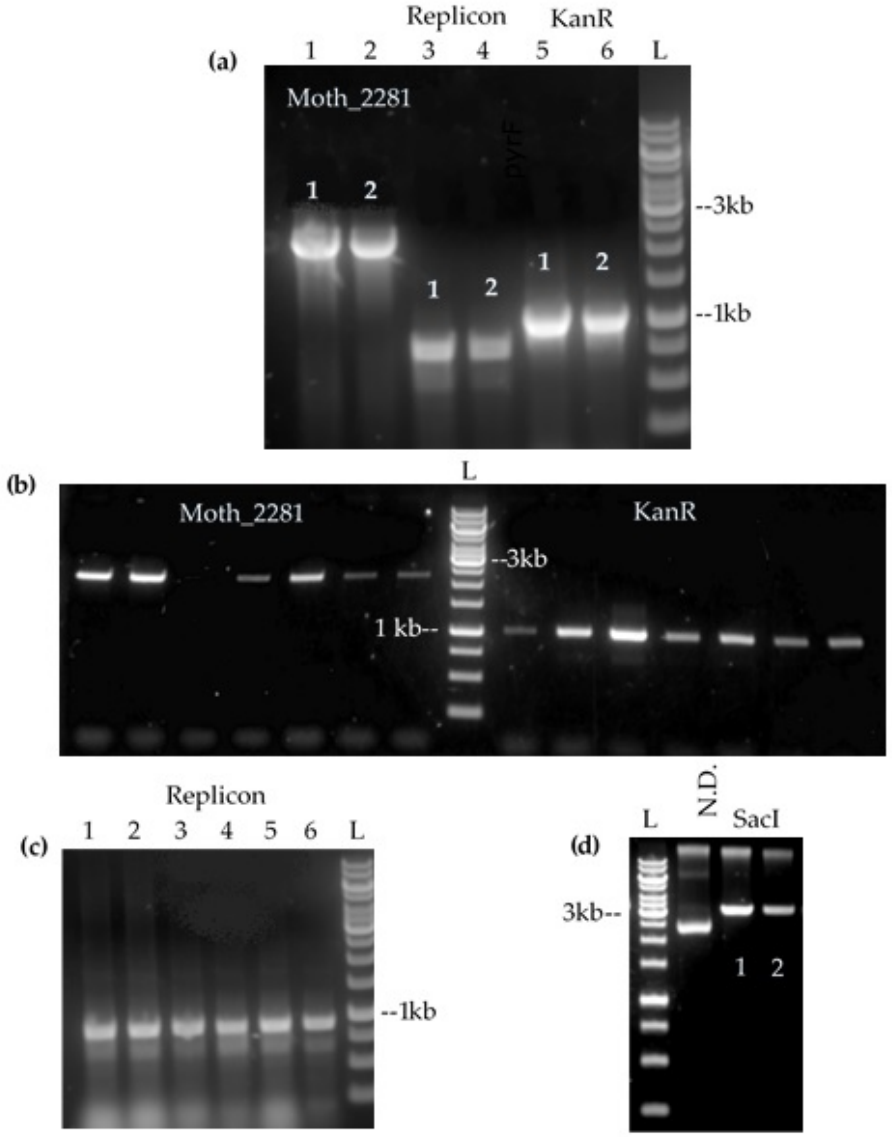

Figure 1. (**a**) Colony PCR of two *M. thermoacetica* transformants after transformation. Primers amplifying the Gram-positive replicon (Replicon) and the *kanR* gene (KanR) were used to check presence of the pMTLK-Tp plasmid. As a control, primers amplifying the gene Moth_2281 on the chromosome were used. Two individual transformants are shown. (**b**) Colony PCR of *M. thermoacetica* transformants after 5 generations in selective cultures with primers amplifying Moth_2281 and *kanR*. (**c**) Colony PCR of the Gram-positive replicon of plasmid pMTLK-Tp in *E. coli* after plasmid extraction from *M. thermoacetica* transformants and retransformation into *E. coli*. Six isolated colonies are analysed here. (**d**). Plasmid from colonies 1 and 2 from (**c**) was extracted and digested for further analysis with the unique cutter SacI. N.D.: non-digested control. L: molecular ladder.

Figure 2. The designed plasmid shuttle vector pMTLK-Tp contains *T. petrophila* pRKU1 replicon and the thermostable *kanR* gene, expressed with the P_{G3PD} promoter for plasmid maintenance and selection, respectively, in *M. thermoacetica*. It also harbours the ColE1 Gram-negative replicon and a multiple cloning site (MCS) for easy downstream cloning.

3.3. Characterisation of Plasmid Behaviour

To further investigate plasmid behaviour of the designed shuttle vector, we set to estimate plasmid copy numbers using quantitative PCR (qPCR), a technique widely used for this application (30). However, initial qPCR results showed a very low ratio of plasmid DNA/chromosomal DNA, suggesting that only a subpopulation of cells carried the plasmid. Considering these results, plasmid instability, leading to a significant number of wild-type cells remaining in cultures, was suspected. To mitigate this issue, an improved selective pressure was explored by increasing kanamycin concentration or using the alternative antibiotic neomycin, for which resistance is encoded by the same *kanR* gene [37]. Unfortunately, this strategy did not improve the measured plasmid DNA to chromosomal DNA ratios (data not shown). Instead, a toxin-antitoxin system from *Parageobacillus thermoglucosidasius* (genes BCV53_19475 and BCV53_19480 on the plasmid pNCI001 (accession number CP016623) was added to the plasmid to induce post-segregational killing in plasmid-free cells. The antitoxin, which neutralizes the toxin, is degraded more rapidly than the toxin in plasmid-free cells, leading to cell death, as previously described [38,39]. Similar to other strategies, this approach did not impact measured fluorescence intensity by qPCR. Although these experiments did not confirm that the toxin-antitoxin system is functional in *M. thermoacetica*, its addition to the plasmid did not impact plasmid stability, therefore questioning whether the observed low ratios were caused by plasmid instability or by other unknown mechanisms. Thus, it was hypothesised that cell aggregation or biofilm formation was possibly protecting wild-type *M. thermoacetica* cells from the selective pressure, as observed in some pathogens [40]. Although very limited literature is available on biofilm formation or any quorum sensing-mediated behaviour in *M. thermoacetica*, it can perform microbial electrosynthesis by capturing electrons from an electrode and forming cell aggregation or biofilm [41,42]. As cell dispersion or biofilm prevention methods are very species-specific, only agitation was explored to prevent the suspected phenotype, reasoning that mechanical force through agitation would limit cells forming any unwanted structure.

While agitation was suspected to partly alleviate this problem, as indicated by higher OD600 reached and faster growth under selective pressure, this observation was not reflected in the qPCR results; thus, suggesting that the initial qPCR method based on genomic DNA (gDNA) extraction might not be appropriate for plasmid copy number quantification. In fact, it has previously been reported that gDNA extraction is not sufficiently reproducible for qPCR applications [43,44]. Instead, crude lysates, obtained by boiling culture samples at 95 °C for 10 min to release DNA, have been proposed as more reproducible qPCR templates. To further investigate this, the crude lysate method and three different gDNA extraction methods (GenElute™ Bacterial Genomic DNA (Sigma-Aldrich), the DNeasy® blood and tissue (Qiagen) and a phenol-chloroform method) were tested as well as additional lysis techniques, including treatment with Bugbuster® (EMD Millipore™, Burlington, MA, USA), sonication or a combination of both, to exclude inefficient lysis as the cause of poor gDNA extraction. The results showed that boiled samples were the most appropriate templates for qPCR quantification (Figure 3), measuring an average of around 100 plasmid copies per cell. This outcome is in agreement with previous studies which suggested that boiled crude lysates are superior samples than gDNA for qPCR analysis.

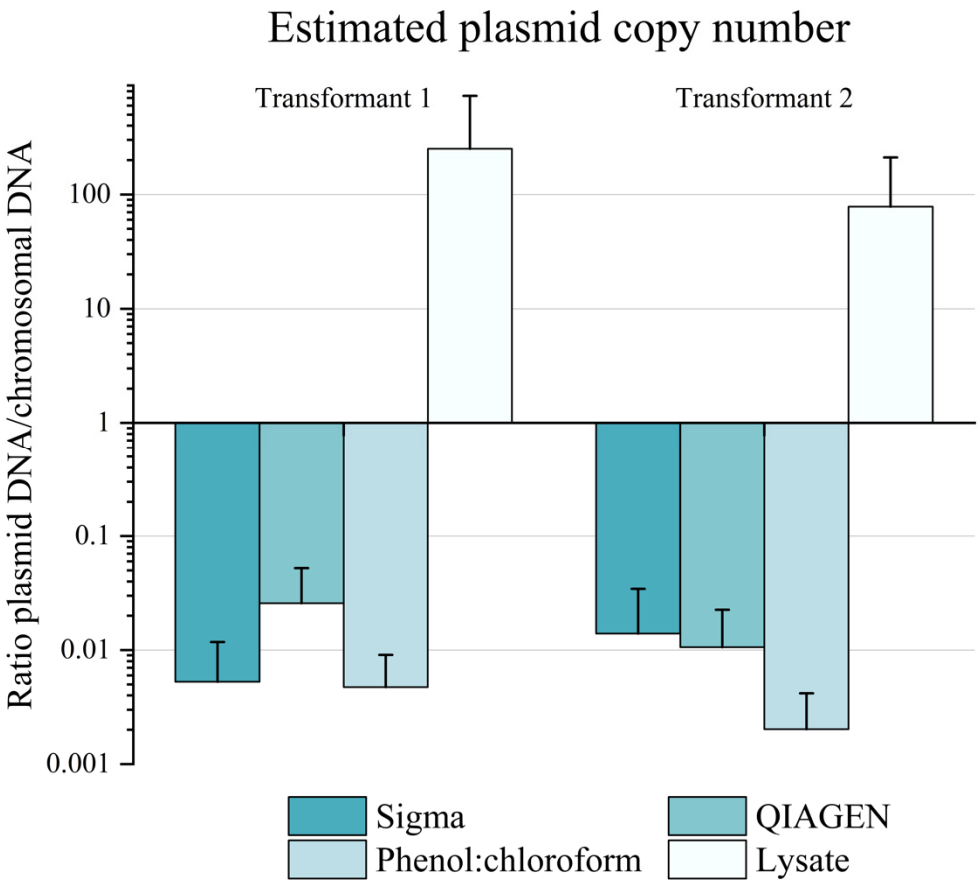

Figure 3. Estimated plasmid copy numbers using qPCR-measured fluorescence. Three genomic DNA (gDNA) extraction methods were explored and compared to boiled crude lysates, highlighting that gDNA might not be an appropriate template for qPCR, as previously observed. Error bars represent standard deviation (n = 12).

In addition, these experiments also highlighted the resistance of *M. thermoacetica* cells to lysis, potentially due to its unique cell wall structure that is evolved to resist high growth temperatures. The different lysis methods were tested for releasing DNA before gDNA extraction with the Sigma kit, and released proteins were quantified with a bicinchoninic acid (BCA) assay (ThermoFisher). Interestingly, boiling seemed to be the most efficient

lysis method. This observation was surprising as, although it was expected that enzyme-based lysis methods might not perform well in lysing thermophilic Gram-positive bacteria, mechanical methods such as sonication were expected to yield acceptable results since they are not affected by the cell wall structure. Lysates treated with Bugbuster followed by sonication yielded similar ratios to boiled lysates according to the qPCR results on gDNA extracted with the Sigma kit (Figure 4), although lysis efficiency seemed reduced according to the protein concentration measured in the BCA assay (Figure 5), further highlighting that gDNA extraction is not suitable to use for qPCR-based plasmid copy number estimation. In addition, it can be excluded that the plasmid is not retained during gDNA extraction as both manufacturers (Sigma and Qiagen) assure that the gDNA kits can be used to extract small fragments. In addition, similar ratios were obtained with a phenol:chloroform extraction (Figure 3) although this method is independent on DNA fragment size, which further supports that the obtained results were not caused by plasmid loss during gDNA extraction. However, gDNA extraction efficiency has a direct impact on the number of plasmid copies retained within the samples and can influence qPCR results and other applications, as previously observed for other species; thus, further confirming that gDNA might not be an adequate template for qPCR.

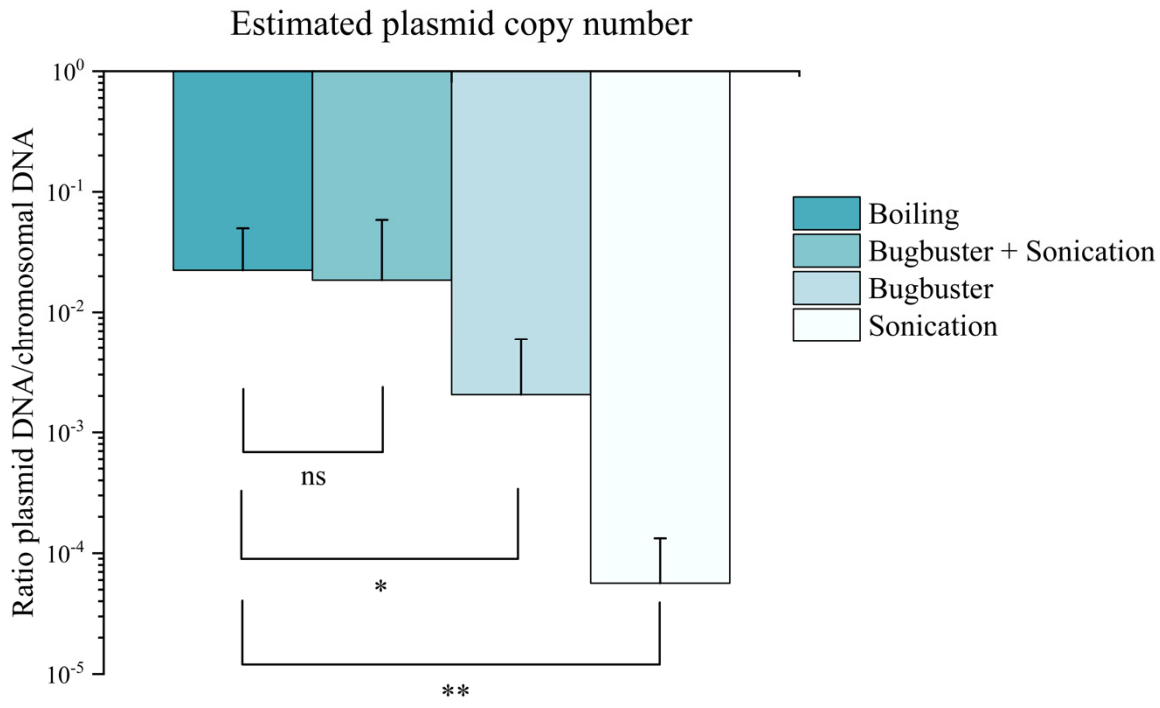

Figure 4. Estimated plasmid copy numbers, as measured by qPCR fluorescence, in gDNA samples obtained by using different lysis methods. Boiling and Bugbuster followed by sonication led to similar results. Error bars represent standard deviation (n = 12). Statistical significance was calculated with a two-tailed t-test: n.s. = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$.

To further investigate efficiency of the lysis method for gDNA extraction, protein quantification by the BCA assay was performed from lysed samples as only proteins released from lysed cells are quantified in this assay, which can be used to infer lysis efficiency [45]. This step also confirmed the qPCR results, suggesting that boiling remained the best lysis method for *M. thermoacetica*, whereas all the other methods explored showed suboptimal efficiency (Figure 5). In addition, the BCA assay was performed on wild-type and transformants cells to investigate whether the plasmid interfered with the lysis efficiency, which was not reflected in the results described here. As such, both the qPCR and protein quantification experiments indicated that boiling cells was the most efficient lysis method for *M. thermoacetica*.

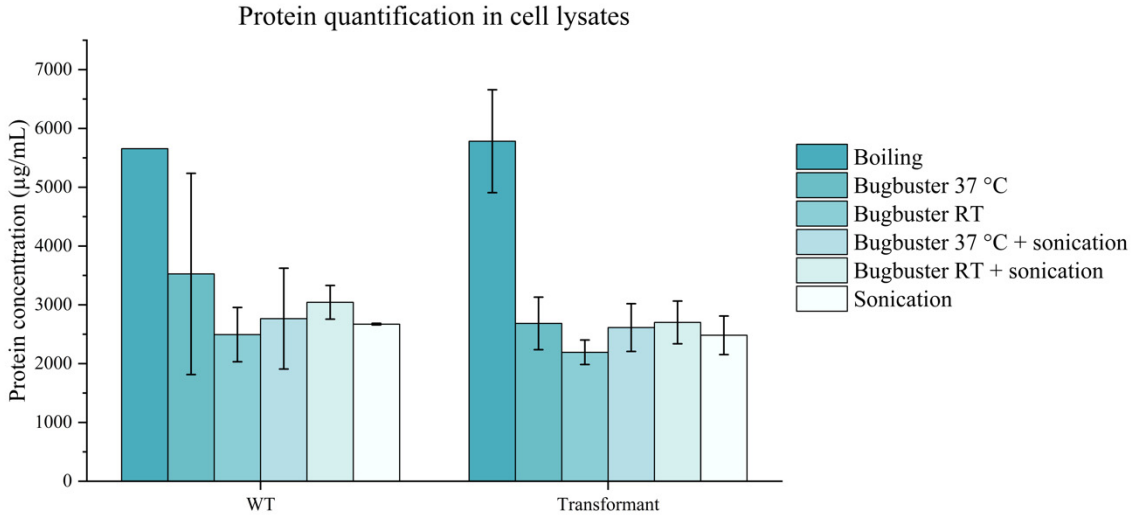

Figure 5. Total protein concentration in cell lysate samples, quantified by the BCA assay. Different lysis methods were investigated on wild-type (WT) cells and transformant cells to exclude that the plasmid was impacting lysis efficiency. Error bars represent standard deviation (n = 3). RT = room temperature.

3.4. Ethanol Production

Although ambiguous results were observed from the qPCR results, it seemed that the designed shuttle vector was maintained within the population at high copy numbers. To investigate whether this plasmid could be used for target gene expression, introducing ethanologenesis, i.e., ethanol production in *M. thermoacetica* was attempted (Figure 6). This compound was chosen because (1) it can easily be quantified from supernatant samples (2) its heterologous production had previously been explored in *M. thermoacetica* through genome-integration of its native aldehyde dehydrogenase gene (*aldh*) driven by the P_{G3PD} promoter, therefore ensuring metabolic compatibility [46].To test ethanol bioproduction using the designed shuttle vector, the P_{G3PD} promoter driving *kanR* expression was replaced by the native constitutive P*ffh* promoter derived from the signal recognition particle protein, Ffh, allowing the expression of two target genes with P_{G3PD}. This promoter change at the *kanR* gene was required to prevent any unwanted recombination events between multiple copies on the P_{G3PD} promoter on the same vector, which had been observed during previous experiments. The native aldehyde dehydrogenase gene, *aldh*, previously overexpressed in *M. thermoacetica* for ethanol synthesis [46], was cloned in the shuttle vector for plasmid-based expression. In addition, the bi-functional aldehyde-alcohol dehydrogenase gene, *adhE1* from the acetogen, *Clostridium autoethanogenum* was also expressed in *M. thermoacetica*, through plasmid-borne expression. Although thermostability of *adhE1* was unclear, this gene is central to ethanol production in *C. autoethanogenum* [5] and it was, thus, reasoned that it might lead to higher ethanol production than the native *aldh*. The measured ethanol production, in fact, confirmed this hypothesis as higher ethanol production was achieved with *adhE1* (Figure 6a). In addition, agitation enhanced growth and ethanol yield (Figure 5b), partly due to better diffusion of CO_2 within the medium, leading to improved performance of transformants. However, from these results, it cannot be concluded whether agitation may prevent cell clumping. From a metabolic engineering point of view, the results reported here are encouraging because they show that plasmid-borne heterologous gene expression is possible in *M. thermoacetica*. Interestingly, the production behaviour was surprising as most ethanol was produced in mid or late stationary phase (Figure 6a), even though most nutrients are expected to be depleted in stationary phase [47]. Considering that the P_{G3PD} promoter is native to *M. thermoacetica*, any regulatory mechanism at this promoter would also impact ethanol production. Although unlikely for the G3PD gene, upregulation of specific genes in stationary phase has been reported in other organisms [48], which might

explain the production pattern observed here. Other metabolic patterns such as the excess of cofactors might occur in stationary phase, leading to the observed production behaviour. In addition, the yields reported here (3.5 mg ethanol/g glucose and 5.5 mg ethanol/g glucose for *aldh* and *adhE1*, respectively) are obviously insufficient for industrial applications and more strain engineering efforts would be needed to improve ethanol production in the engineered strain. However, the described approach was designed mostly as a proof-of-concept research to investigate if plasmid-borne gene expression was feasible in *M. thermoacetica*. In addition, the ethanol yields obtained in this study are similar to previously reported [46], which further excludes any detrimental impact of plasmid-borne gene expression in *M. thermoacetica* compared to chromosomal integration.

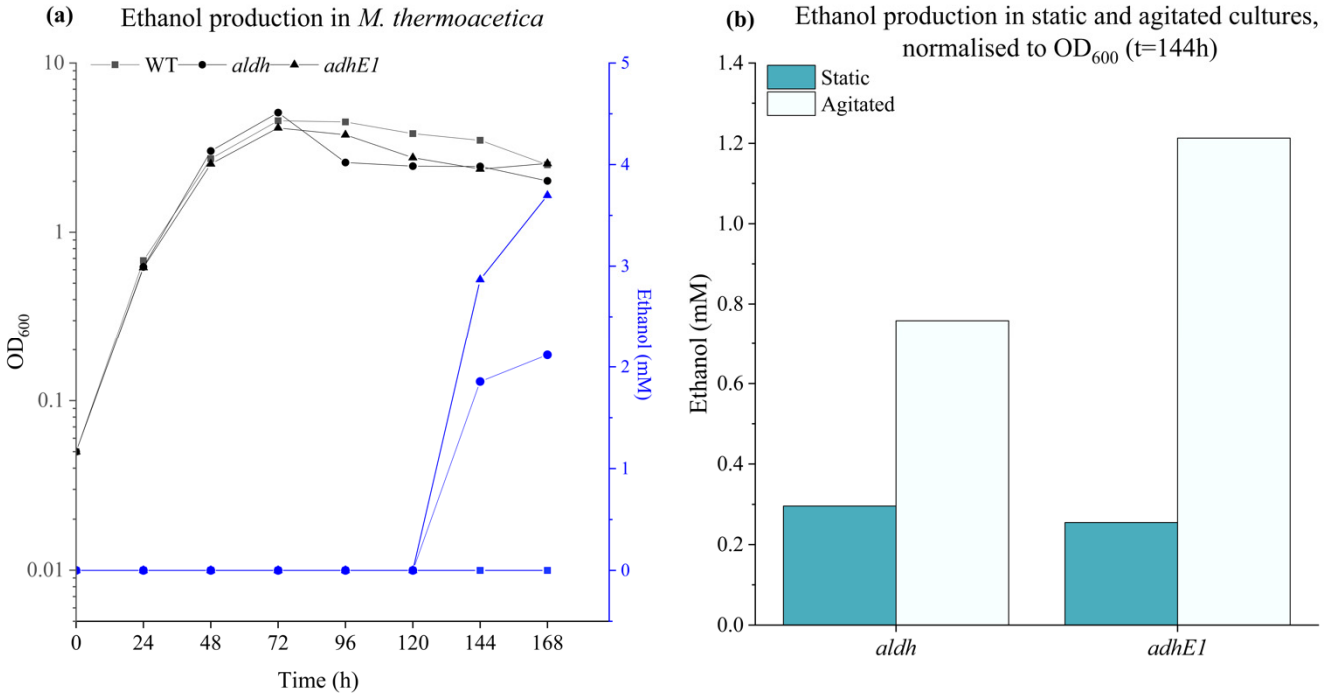

Figure 6. Comparison of ethanol production in *M. thermoacetica* under different conditions. (**a**) Ethanol production in *M. thermoacetica* by expressing *aldh* and *adhE1* genes. Ethanol was only detected in the late stationary phase. OD_{600} and ethanol concentration (mM) are represented in black and blue, respectively. Square: WT; circle: *aldh* mutant; triangle: *adhE1* mutant (**b**) Ethanol production at t = 144 h in static and agitated *M. thermoacetica* cultures. No ethanol was detected in the wild-type strain.

4. Discussion

The work described here set out to develop and characterise a self-replicating shuttle vector for *M. thermoacetica*, allowing plasmid maintenance within the population and plasmid-borne target gene expression in the host. A compatible replicon was identified from *T. petrophila* and allowed construction of a shuttle vector for *M. thermoacetica*, leading to successful transformation. Interestingly, during transformation, *M. thermoacetica* transformant selection could not be performed on solid medium on plates, although an optimised solid medium had been developed during this work for growth of the wild-type strain. It can be hypothesised that *M. thermoacetica* is not very well adapted to grow on plates as, in fact, previous transformant selection process was not performed on plates but on solidified medium in Hungate tubes [18,20]. In addition, to our knowledge, very limited reports have been published on growth of the wild-type strain on solid medium, further highlighting that *M. thermoacetica* cannot easily be grown on plates. In fact, medium optimisation in this work was challenging as plates with a solid medium having an identical composition to the liquid medium did not support growth. In addition, other unknown processes might hinder growth on plates when maintaining the plasmid. Although this considerable

limitation could not be overcome in this work and imposed transformant selection in liquid cultures instead, the developed transformation protocol was robust enough to lead to a successful transformation in all subsequent attempts mentioned in this study. Furthermore, unclear results were unfortunately gathered when characterising plasmid behaviour, specifically regarding quantification of plasmid copy numbers. Plasmid stability did not stand out as problematic; instead, a potential cell aggregation behaviour was suspected to help propagate a significant number of wild-type cells in selective cultures. Although mechanical agitation partly resolved this issue, qPCR results on gDNA samples proved particularly difficult to reproduce, in accordance with some other studies [44,49]. Although qPCR has been widely used for quantification of plasmid copy numbers, qPCR results are notoriously difficult to reproduce [50], questioning whether other methods might be more accurate when studying plasmid behaviour, as previously discussed [51]. qPCR fluorescence is also highly dependent on primer design and gene targets used. The *kanR* gene and the Gram-positive replicon were chosen as the plasmid targets as it was reasoned that these two elements were essential for plasmid maintenance and would therefore better reflect plasmid behaviour. Similarly, two housekeeping genes, commonly used in qPCR studies [52], were used for chromosomal fluorescence quantification, although, as DNA was the target here, chromosomal targets should not influence qPCR results, as long as they are single-copy genes. It is worth mentioning that additional primers and target genes were explored in this work (data not shown) but led to similar results. Nonetheless, despite an uncharacterized plasmid behaviour, the qPCR quantification on boiled crude lysates suggests that the designed shuttle vector is, in fact, a high copy-number plasmid in *M. thermoacetica*. While investigating plasmid copy number, the analyses also highlighted that lysis efficiency seemed suboptimal for *M. thermoacetica* with multiple lysis methods, probably due to having a unique cell wall structure adapted for its specific growth conditions. Although boiling was shown to be efficient in releasing DNA or proteins, this method cannot be performed for other applications as such high temperatures denature most proteins. For example, if a reporter gene is expressed in *M. thermoacetica* to measure promoter activity, boiling could not be used to lyse cells because the reporter protein would be deactivated. Instead, a thermostable in vivo reporter system such as flavin-based fluorescent reporters might be more appropriate when testing genetic elements in *M. thermoacetica*. As such, inefficient lysis with the other methods tested here is a major drawback for different genetic applications and more work is needed to resolve this issue. Other lysis methods such as lysis beads might be promising due to their mode of action but were not explored here.

Nonetheless, despite the several limitations described above, it was later shown that the designed shuttle vector was functional for target gene expression, leading to ethanol production in *M. thermoacetica* through plasmid-borne expression of the native *aldh* gene and *C. autoethanogenum adhE1* gene, albeit with low ethanol yields. While further strain engineering would be required to establish an ethanol-producing *M. thermoacetica* strain at industrial standards, this work shows that the designed shuttle vector is functional in *M. thermoacetica* and can be applied for metabolic engineering of this industrially important chassis.

5. Conclusions

Acetogens are particularly attractive chassis organisms for industrial gas fermentation processes, allowing the bioproduction of a range of value-added chemicals while simultaneously fixing CO_2. While great progresses have been achieved towards developing fast and efficient genetic manipulation techniques for several acetogens, the thermophilic acetogen, *M. thermoacetica* remains poorly amenable to genetic modifications. Development of sustainable bioprocesses for chemicals and fuels production is highly dependent on the availability of efficient and expanded genetic and metabolic engineering toolboxes to improve and modify strains for viable and cost-effective industrial applications. Thus, the engineered replicating shuttle vector for *M. thermoacetica* developed in this study will not only expand and enhance its genetic toolbox, but also facilitate and accelerate strain

engineering efforts in this versatile acetogen for industrial applications. While further vector optimization and characterization are required to improve the performance of the developed plasmid vector, it has been shown to be effective in the host cell factory through implementing non-native ethanologenesis and can be applied for a range of strain engineering purposes to fully unlock its industrial potential through the development of additional genetic tools and metabolic engineering strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8110585/s1, Figure S1: Plasmid map of RBV1-MET used in the methylation strain. Figure S2: The pre-methylated engineered vector was digested with ApaI, an isoschizomer of Moth_2281, to test for successful methylation.

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Data Availability Statement: The authors declare that the data supporting the findings of this study are available within the article and its supplementary material.

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