

Expanding the Genetic Toolbox for *Methanothermobacter thermautotrophicus* ΔH

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
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Tübingen
2024

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der
Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:

16.9.2024

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Table of Contents

A.	Abstract (German).....	1
B.	Abstract	2
C.	Importance	3
I.	Background	4
1.	Methanogens.....	4
2.	<i>Methanothermobacter thermautotrophicus</i> Δ H.....	5
2.1.	Power-to-gas and power-to-x.....	7
2.2.	Genetic tools for <i>M. thermautotrophicus</i> Δ H and other methanogens.....	8
3.	Selection markers.....	12
3.1.	Mupirocin	13
3.2.	Puromycin.....	13
3.3.	Simvastatin	14
3.4.	Formate	15
4.	Alternative transformation methods.....	16
4.1.	Electroporation.....	17
5.	Tools for genome editing.....	18
5.1.	Markerless mutagenesis.....	19
5.2.	CRISPR	21
5.3.	Ploidity of <i>M. thermautotrophicus</i> Δ H.....	22
5.4.	Plasmid curing	23
II.	Outline	25
III.	Materials and Methods	26
1.	Methods.....	26
1.1.	Microbial cultivation conditions	26
1.2.	Genetic manipulation of <i>E. coli</i>	27
1.3.	Cloning of Plasmids	34

1.4.	Genetic manipulations of <i>M. thermautotrophicus</i> ΔH	37
1.5.	Genetic analysis of <i>M. thermautotrophicus</i> ΔH	39
2.	Materials	41
2.1.	Strains	41
2.2.	Primers and gBlocks	43
2.3.	Chemicals	46
2.4.	Enzymes and Kits	47
2.5.	Devices	48
IV.	Results	50
1.	Establishment of additional selection markers	50
1.1.	Mupirocin	50
1.2.	Puromycin	55
1.3.	Formate	61
2.	Electroporation as an alternative transformation method	68
2.1.	<i>M. thermautotrophicus</i> ΔH can be transformed with an existing electroporation protocol for <i>Clostridium ljungdahlii</i>	68
2.2.	Optimization of electroporation conditions	69
3.	<i>M. thermautotrophicus</i> ΔH promoters show activity in <i>E. coli</i>	72
4.	Establishing a thermostable CRISPR system for traceless genome editing in <i>M. thermautotrophicus</i> ΔH	77
4.1.	Putative targets for gene editing via CRISPR in <i>M. thermautotrophicus</i> : counterselection markers	78
4.2.	Modular CRISPR module allows quick exchange of parts of the CRISPR system	80
4.3.	First CRISPR plasmids suggest activity of ThermoCas9 in <i>M. thermautotrophicus</i> ΔH	82
4.4.	Successful deletion of the <i>upt</i> gene with plasmid pMVS1112a-CRISPR686	
V.	Discussion	93

1.	Selection markers.....	93
1.1.	Mupirocin	94
1.2.	Puromycin.....	95
1.3.	Formate	97
2.	Electroporation as an alternative transformation method	102
3.	Activity of <i>M. thermautotrophicus</i> Δ H promoters in <i>E. coli</i>	104
4.	Establishing a thermostable CRISPR system for traceless genome editing in <i>M. thermautotrophicus</i> Δ H	106
VI.	Outlook	114
1.	Expanding the genetic toolbox for <i>M. thermautotrophicus</i> Δ H further	114
2.	Applying the genetic toolbox.....	116
VII.	Supplemental information.....	119
1.	Colony PCR of pMVS-1111a:MupR	119
2.	Cloning issues in <i>E. coli</i>	120
3.	Attempts for random mutagenesis of <i>pac</i>	122
4.	Unsuccessful optimization attempts of the electroporation protocol	123
5.	Overview of CRISPR plasmids.....	126
6.	Colony PCR of first CRISPR experiments	127
VIII.	List of abbreviations.....	127
IX.	Contributions of other researchers	129
X.	Bibliography.....	130
XI.	Acknowledgment	138

A. Abstract (German)

Methanothermobacter thermautotrophicus Δ H ist ein thermophiles, methanogenes Archaeon, welches mit Wasserstoff als einziger Elektronenquelle und Kohlenstoffdioxid als einziger Kohlenstoffquelle wächst. Aufgrund seines schnellen und robusten Wachstums ist *M. thermautotrophicus* Δ H seit Jahrzehnten ein Modelorganismus für hydrogenotrophe Methanogenese. In jüngster Zeit gibt es auch ein verstärktes wirtschaftliches Interesse an *M. thermautotrophicus* Δ H für die industrielle Biomethanisierung aufgrund seiner hohen Methanproduktionsraten.

Weitere Grundlagenforschung und industrielle Anwendungen wurden für lange Zeit durch das Fehlen genetischer Werkzeuge verhindert. Das erste genetische System für *M. thermautotrophicus* Δ H wurde jüngst entwickelt (Fink *et al.* 2021). Dies war ein signifikanter Schritt für die Forschung mit *M. thermautotrophicus* Δ H und Methanogenen im Allgemeinen, jedoch sind weitere genetische Werkzeuge vonnöten, um das volle Potential des genetischen Systems auszuschöpfen.

In dieser Arbeit präsentieren wir wichtige Ergänzungen zum genetischen Werkzeugkasten für *M. thermautotrophicus* Δ H. Wir haben mehrere potenzielle Selektionsmarker, basierend auf Mupirocinresistenz, Puromycinresistenz und Formiatprototrophie untersucht. Wir konnten zeigen, dass das Formiatdehydrogenase (*fdh*) Operon aus *M. thermautotrophicus* Z-245 das Wachstum von *M. thermautotrophicus* Δ H mit Formiat als alternativem Substrat ermöglicht. Diese Eigenschaft kann auch verwendet werden, um rekombinante Zellen direkt aufgrund ihrer Formiatprototrophie zu selektionieren. Damit ist das *fdh* Operon der zweite Selektionsmarker überhaupt und der erste Selektionsmarker ohne Wachstum von spontanresistenten Wildtyp-Zellen.

Zusätzlich untersuchten wir alternative Transformationsmethoden. Zuvor war *M. thermautotrophicus* Δ H nur durch Konjugation transformierbar. Hier konnten wir Elektroporation als alternative Transformationsmethode etablieren. Außerdem haben wir Werkzeuge zur Genomeditierung untersucht. Bis jetzt basierten alle Genomeditierungen in *M. thermautotrophicus* Δ H auf homologer Rekombination. Wir etablierten ein CRISPR-System basierend auf einer thermostabilen Cas9 (ThermoCas9) und konnten zeigen, dass dieses System für markerlose Genomeditierungen in *M. thermautotrophicus* Δ H geeignet ist.

B. Abstract

Methanothermobacter thermautotrophicus Δ H is a thermophilic, methanogenic archaeon that grows with hydrogen as the sole electron and carbon dioxide as the sole carbon source. Due to its fast and robust growth, *M. thermautotrophicus* Δ H has been a model microbe for hydrogenotrophic methanogenesis for decades. Recently, there has been increased commercial interest in *M. thermautotrophicus* Δ H in industrial biomethanation due to its high methane production rates.

However, a lack of genetic tools hindered further basic research and industrial applications for a long time. The first genetic system for *M. thermautotrophicus* Δ H was recently established (Fink *et al.* 2021). While this was a significant step for research with *M. thermautotrophicus* Δ H and methanogens in general, additional genetic tools are required to fully unlock the potential of the genetic system.

In this study, we present important additions to the genetic toolbox for *M. thermautotrophicus* Δ H. So far, the only selection marker was a thermostable neomycin resistance cassette. We investigated several potential selection markers based on mupirocin resistance, puromycin resistance, and formate prototrophy. We could show that the formate dehydrogenase (*fdh*) operon from *M. thermautotrophicus* Z-245 enables *M. thermautotrophicus* Δ H to utilize formate as an alternative growth substrate. Also, we could show that this can be directly used to select recombinant cells based on their formate prototrophy, thus establishing the *fdh* operon as the second selection marker and as the first one with no spontaneous growth of wild-type cells under selection pressure.

In addition, we also investigated alternative transformation methods. Previously, *M. thermautotrophicus* Δ H was only transformable *via* conjugation. Here, we expanded the available transformation methods by establishing an electroporation protocol. Furthermore, we investigated tools for genome editing. Until now, all genome editing in *M. thermautotrophicus* Δ H was based on homologous recombination. We established a CRISPR system based on a thermostable Cas9 (ThermoCas9) and could show that this system is suitable for traceless genome editing in *M. thermautotrophicus* Δ H.

C. Importance

The fight against climate change is the major challenge of our time. While an important focus has to be put on reducing carbon dioxide emissions, methane cannot be neglected. While methane constitutes only a tiny fraction of the atmosphere (around 1.8 parts per million [ppm]), concentrations have more than doubled since pre-industrial times, and methane is considered to be responsible for one-third of global warming caused by greenhouse gas emissions (IPCC 2021). Besides methane emissions from natural wetlands, anthropogenic sources, such as fossil fuels and agriculture (especially cattle and rice farming), are the major sources of methane emissions and are steadily on the rise (Van Amstel 2012, Jackson *et al.* 2021). Approximately 71% of atmospheric methane is produced biologically by methanogenic archaea (methanogens) (Holmes *et al.* 2016). A thorough understanding of methanogens is, therefore, critical to establish strategies for the reduction of global methane emissions. Species of the genus *Methanothermobacter* have been extensively applied as model microbes for methanogenesis and the involved enzymes have been studied in much detail (Thauer *et al.* 2008, Thauer 2015).

Hydrogenotrophic methanogens are also employed in industrial processes. In the power-to-gas process, renewable methane is produced with carbon dioxide from industrial off-gasses and hydrogen from water electrolysis. If renewable energies power the process, the resulting methane has a drastically lower carbon footprint than natural gas from fossil sources (Thema *et al.* 2019). Strains of *Methanothermobacter* are commonly employed for this process (Thema *et al.* 2021). Prospectively, the process could be further improved and expanded by employing microbes that produce additional products besides methane (power-to-x).

Thus, further investigation of methanogenesis and possible industrial applications would greatly benefit from genetic tools. A genetic system for *M. thermautotrophicus* ΔH was recently established (Fink *et al.* 2021). Based on these results, we aim to build a versatile genetic toolbox with the required tools for basic research and industrial applications.

I. Background

1. Methanogens

All methanogens are strictly anaerobic archaea (Lyu *et al.* 2018). They produce methane from various substrates. The most relevant types of methanogenesis are: **1)** hydrogenotrophic methanogenesis, where carbon dioxide is reduced to methane with different electron donors such as hydrogen, formate, or certain alcohols; **2)** methylotrophic methanogenesis, where methyl groups of different methylated compounds, including methanol, methylamines, and methylsulfides are disproportionated into carbon dioxide and methane; and **3)** acetoclastic methanogenesis, where the methyl group of acetate is reduced to methane with the electrons from the oxidation of the carbonyl group (Thauer *et al.* 2008). Methanogens can be found ubiquitously in natural and artificial anaerobic environments, such as deep-sea hydrothermal vents, freshwater sediments, the digestive tracts of ruminants and termites, rice paddy fields, sewage treatment plants, and anaerobic digesters (Lyu *et al.* 2018).

Methanogens are not a monophyletic group but form two different clades within the kingdom of Euryarcheota, with several distinct orders (Adam *et al.* 2017). The first clade, called class I methanogens, contains the orders Methanobacteriales, Methanococcales, and Methanopyrales, and most members are mesophilic or thermophilic hydrogenotrophic methanogens (Garcia *et al.* 2022). Of particular research interest are the genera *Methanococcus* and *Methanothermobacter*. *Methanococcus* species were the first methanogens to be genetically accessible and, therefore, played a key role in the genetic investigation of hydrogenotrophic methanogenesis (Bertani *et al.* 1987, Sarmiento *et al.* 2011). On the other hand, the enzymes involved in hydrogenotrophic methanogenesis were extensively studied in the genus *Methanothermobacter* (Thauer 2015, Thauer 2019).

The second clade, called class II methanogens, contains the orders Methanosarcinales, Methanomicrobiales, and Methanocellales. Here, the genus *Methanosarcina* is of particular research interest because different species of this genus can perform hydrogenotrophic, methylotrophic, or acetoclastic methanogenesis

(Carr *et al.* 2022) and have been genetically accessible for more than 25 years (Metcalf *et al.* 1997).

Two additional orders were recently reported outside the two clades, Methanomassiliicoccales and Methanonatronarchaeales (Aouad *et al.* 2019, Cozannet *et al.* 2020). In addition, recent discoveries suggest that methanogenesis, or at least the genes that encode involved enzymes, is by no means limited to these eight orders but can be found all over the archaeal domain in metagenome-assembled genomes from environmental samples (Garcia *et al.* 2022).

2. *Methanothermobacter thermautotrophicus* Δ H

Methanothermobacter thermautotrophicus Δ H is a rod-shaped, non-motile archaeon that grows optimally in a minimal salt medium with an atmosphere of 80% molecular hydrogen gas (hereafter referred to as hydrogen) and 20% carbon dioxide at 65 °C. It was first isolated from sludge from a sewage treatment plant in Illinois in 1972 (Zeikus *et al.* 1972). *M. thermautotrophicus* Δ H is a chemolithotrophic microbe that can grow with hydrogen and carbon dioxide as sole energy and carbon sources and ammonia and sulfide as nitrogen and sulfur sources, respectively (Wasserfallen *et al.* 2000).

M. thermautotrophicus Δ H conserves energy *via* methanogenesis in the Wolfe cycle, a metabolic pathway in which carbon dioxide is stepwise reduced to methane (Figure 1A). Carbon dioxide is first reduced and bound to methanofuran, producing formylmethanofuran. Next, the formyl group is transferred to tetrahydromethanopterin (H₄MPT) and stepwise reduced to methyl-tetrahydromethanopterin (CH₃-H₄MPT). The methyl group is further transferred to coenzyme M by methyl-H₄MPT/coenzyme M methyltransferase. Methyl-CoM is then reduced by methyl-CoM reductase, producing methane and the heterodisulfide CoM-S-S-CoB. The heterodisulfide is reduced by electron-bifurcating hydrogenase–heterodisulfide reductase complex, which couples the exergonic reduction of the heterodisulfide with the endergonic reduction of ferredoxin with electrons from hydrogen. The reduced ferredoxin can then be used by formylmethanofuran dehydrogenase again, completing the cycle. The methyl-H₄MPT/coenzyme M methyltransferase is membrane-associated and couples the exergonic methyl-group transfer to the translocation of sodium ions across the membrane. The resulting gradient is used by an A₀A₁-ATP synthase to catalyze ATP

synthesis (Thauer 2012). The ATP yield is low, with approximately 0.5 moles of ATP per mole of methane (Thauer *et al.* 2008).

While the carbon dioxide that is fixated during the Wolfe cycle is rereleased as methane, some carbon dioxide also needs to be assimilated into biomass. *M. thermautotrophicus* Δ H achieves this *via* the Wood-Ljungdahl pathway, which was first described in acetogenic bacteria (Wood *et al.* 1986). In *M. thermautotrophicus* Δ H, the pathway is linked to methanogenesis (Figure 1B). In the large enzyme complex CO dehydrogenase/acetyl-CoA synthase (CODH/ACS), the methyl group from methyl-H₄MPT, an intermediate from the Wolfe cycle, is first loaded onto an iron-sulfur protein and, from there, onto the central nickel ion of the CODH/ACS complex. A second carbon dioxide molecule is reduced to carbon monoxide and also loaded onto the nickel ion. Afterward, one molecule of acetyl-CoA is synthesized from CoA and the methyl- and carbonyl group, which are coordinated to the nickel ion (Fuchs 2011). Acetyl-CoA is the central metabolic intermediate in *M. thermautotrophicus* Δ H (Simpson *et al.* 1993).

M. thermautotrophicus Δ H and the closely related species *Methanothermobacter marburgensis* have been extensively studied as model microbes for hydrogenotrophic methanogenesis (Thauer *et al.* 2008, Thauer 2015). Especially outstanding is their contribution to the understanding of the distinct enzymes of methanogenesis, most notably the methyl-CoM reductase (MCR) (studies are summarized in Thauer (2019)). For older literature, it should be noted that the genus *Methanothermobacter* was reclassified in 2000. Before that time, *M. thermautotrophicus* Δ H and *M. marburgensis* were grouped together with their mesophilic *Methanobacterium* relatives, considered members of the same species, and named *Methanobacterium thermoautotrophicum* Δ H and *Methanobacterium thermoautotrophicum* Marburg, respectively (Wasserfallen *et al.* 2000). The importance of *M. thermautotrophicus* Δ H as a model microbe is also highlighted by the fact that the genome was published as one of the first archaeal genomes (Smith *et al.* 1997).

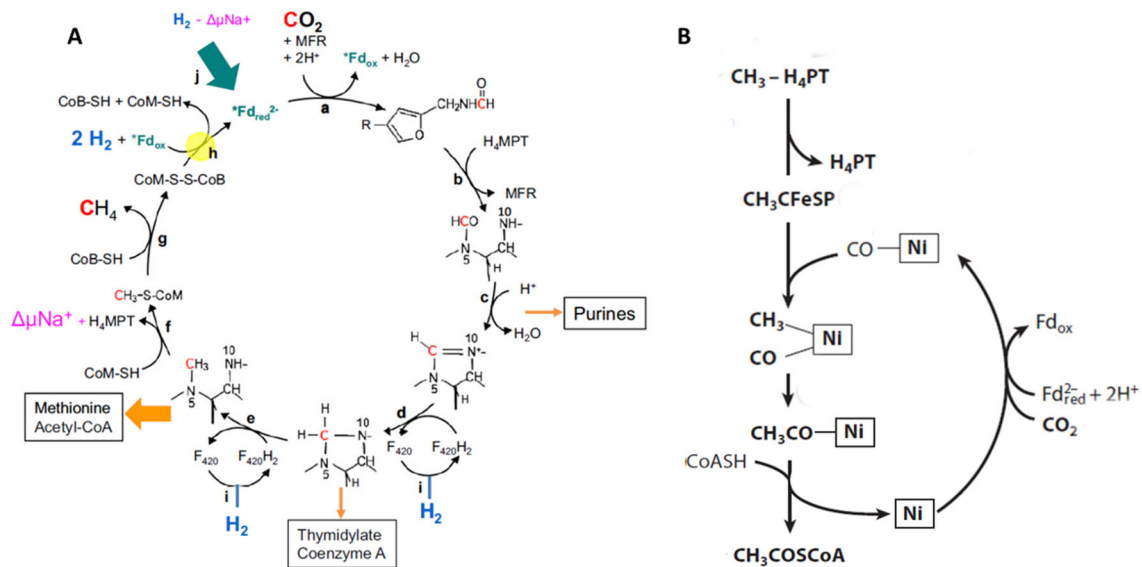


Figure 1. A: Methanogenesis pathway in *M. thermautotrophicus* ΔH (Wolfe cycle): F_{420} , coenzyme F_{420} ; Fd , specific ferredoxin; H_4MPT , tetrahydromethanopterin; MFR, methanofuran; $\Delta\mu\text{Na}^+$, electrochemical sodium ion potential. Enzymes: a, formylmethanofuran dehydrogenase; b, formylmethanofuran/ H_4MPT formyl transferase; c, methenyl- H_4MPT cyclohydrolase; d, methylene- H_4MPT dehydrogenase; e, methylene- H_4MPT reductase; f, methyl- H_4MPT /coenzyme M methyltransferase; g, methyl-coenzyme M reductase; h, electron-bifurcating hydrogenase–heterodisulfide reductase complex; i, F_{420} -reducing hydrogenase; j, energy-converting hydrogenase catalyzing the sodium motive force-driven reduction of ferredoxin with hydrogen. Figure from Thauer (2012). B: Acetyl-CoA biosynthesis in *M. thermautotrophicus* (Wood-Ljungdahl pathway). After steps a–e from Figure A, the methyl-tetrahydromethanopterin ($\text{CH}_3\text{-H}_4\text{MPT}$) is used for acetyl-CoA synthesis by CO-dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex. Figure adapted from Fuchs (2011).

2.1. Power-to-gas and power-to-x

Besides being used as a model microbe to understand and investigate methanogenesis, there is also industrial interest in microbes of the genus *Methanothermobacter*. The main industrial application for *Methanothermobacter* spp. is the power-to-gas process in which electrolytically produced hydrogen together with carbon dioxide from a suitable source (e.g., power plants, large-scale fermenters, or cement factories) is chemically or biologically converted to methane (i.e., renewable natural gas). As long as the required electric power is produced by renewable means, this renewable natural gas has a low overall carbon footprint because it consumes a considerable amount of the carbon dioxide that combustion produces (Thema *et al.* 2019). When compared to chemical catalysis, biological power-to-gas processes have several advantages. For example, they can be performed at lower temperatures and

are less sensitive to gas impurities (Schiebahn *et al.* 2015). Due to the fast growth and high methane production rates, *M. thermautotrophicus* strains are already employed as biocatalysts in these systems (Martin *et al.* 2013, Thema *et al.* 2021).

Renewable natural gas from power-to-gas processes can be an important step towards emission-neutral fuel and energy production. However, upgrading the process to produce value-added chemical compounds from carbon dioxide and hydrogen (*i.e.*, power-to-x) has the potential for an even more significant economic and ecological benefit. A particularly interesting group of chemicals for power-to-x with *M. thermautotrophicus* would be isoprene and terpenoids because the cell membrane of *M. thermautotrophicus*, like all archaeal cell membranes, is composed of a terpene layer, which is synthesized from isoprenoid monomers (Koga *et al.* 2007). The possibility of the production of isoprene and the terpenoids was already demonstrated in hydrogenotrophic methanogens: Isoprene was produced in *Methanosarcina barkeri* (Aldridge *et al.* 2021), and the terpenoid geraniol was produced in *Methanococcus maripaludis* (Lyu *et al.* 2016). However, both strains are mesophiles, and due to comparably faster growth and higher methane production rates, only thermophilic methanogens have been adopted for power-to-gas technology to date (Thema *et al.* 2021).

2.2. Genetic tools for *M. thermautotrophicus* Δ H and other methanogens

The power-to-gas process provides an industrial application that can contribute to the fight against climate change and the dependence on fossil fuels. However, methane and water are the sole metabolic end products for wild-type *M. thermautotrophicus* Δ H. Therefore, a genetic system and a versatile genetic toolbox are required to allow the production of value-added chemicals and transit from power-to-gas to power-to-x applications. While *Methanothermobacter* spp. have been subject to research for more than 40 years, genetic tools were absent. Only two publications demonstrated genetic modifications in *M. marburgensis*, one based on viral transduction (Meile *et al.* 1990) and one on natural competence (Worrell *et al.* 1988). Both systems had to rely on genetic material from spontaneous-resistant *M. marburgensis* strains to select for successful DNA transfer, and no transfer of foreign DNA was demonstrated. Thus,

neither system has resulted in tools for genetic experiments, and no further research based on these systems has been published since then.

In contrast, genetic tools were successfully established for the species of the two orders Methanococcales (*Methanococcus* spp.) (Bertani *et al.* 1987) and Methanosarcinales (*Methanosarcina* spp.) (Metcalf *et al.* 1997). Naturally, further genetic research focused on these two orders, and now, versatile genetic toolboxes, including several selection markers (e.g., neomycin, puromycin, mupirocin) and tools for genome editing (e.g., markerless mutagenesis and CRISPR), are available for both orders (Enzmann *et al.* 2018). The selection markers and tools for genome editing that could be applicable to *M. thermautotrophicus* Δ H are discussed in detail below.

Additional genetic systems for thermophilic methanogens were recently established (Susanti *et al.* 2019, Fonseca *et al.* 2020, Lie *et al.* 2022). *Methanocaldococcus jannashii*, a hyperthermophilic ($T_{\text{opt}} = 85^{\circ}\text{C}$) methanogen from the order *Methanococcales*, was shown to be transformable with integrative plasmids by heat-shock treatment (85°C) of previously cold-adapted (65°C) cells with a simvastatin resistance cassette (section I.3.3) as selection marker (Susanti *et al.* 2019). In contrast, the closely related strain *Methanocaldococcus* sp. FS406-22 was transformed with tools similar to the ones from the mesophilic relative *M. maripaludis*. Non-replicative plasmids carrying a thermostable neomycin resistance as a selection marker were transformed *via* polyethylene glycol (PEG)-mediated transformation (Lie *et al.* 2022). In addition, a genetic system for *Methanoculleus thermophilus* was established. The strain was naturally competent and transformed with linearized integrative plasmids carrying the same thermostable neomycin resistance used in *Methanocaldococcus* sp. FS406-22 (Fonseca *et al.* 2020).

Finally, more than 30 years after the initial work, a genetic system for *M. thermautotrophicus* Δ H was recently established in our lab (Fink *et al.* 2021). The system is based on modular *E. coli* – *M. thermautotrophicus* shuttle-vectors that contain four basic modules, comprised of origins of replication (*ori*) and selection markers for both microbes (Figure 2). For *M. thermautotrophicus* Δ H, the replicon is the entire cryptic plasmid pME2001 from *M. marburgensis* (Luo *et al.* 2001), and the selection marker is a thermostable neomycin resistance cassette (Hoseki *et al.* 1999) – the same selection marker as in *M. thermophilus* and *Methanocaldococcus* sp. FS406-22, discussed above. These four modules of the basic shuttle vector, pMVS-

V1, are each flanked by rare 8-bp recognition sequences for specific restriction enzymes. A fifth module (application module) was added for further genetic applications, which can be used to include any additional genetic cargo in the transferred plasmid. Plasmids are named according to a standardized nomenclature: pMVS1111a:promoter of cargo-cargo. MVS stands for *Methanothermobacter* vector system, and the four numbers represent (in order): **1)** the replicon for *E. coli*; **2)** the selection marker for *E. coli*; **3)** the replicon for *M. thermautotrophicus* Δ H; and **4)** the selection marker for *M. thermautotrophicus* Δ H. When I commenced the work for this dissertation, only one variant had been established for each of the modules.

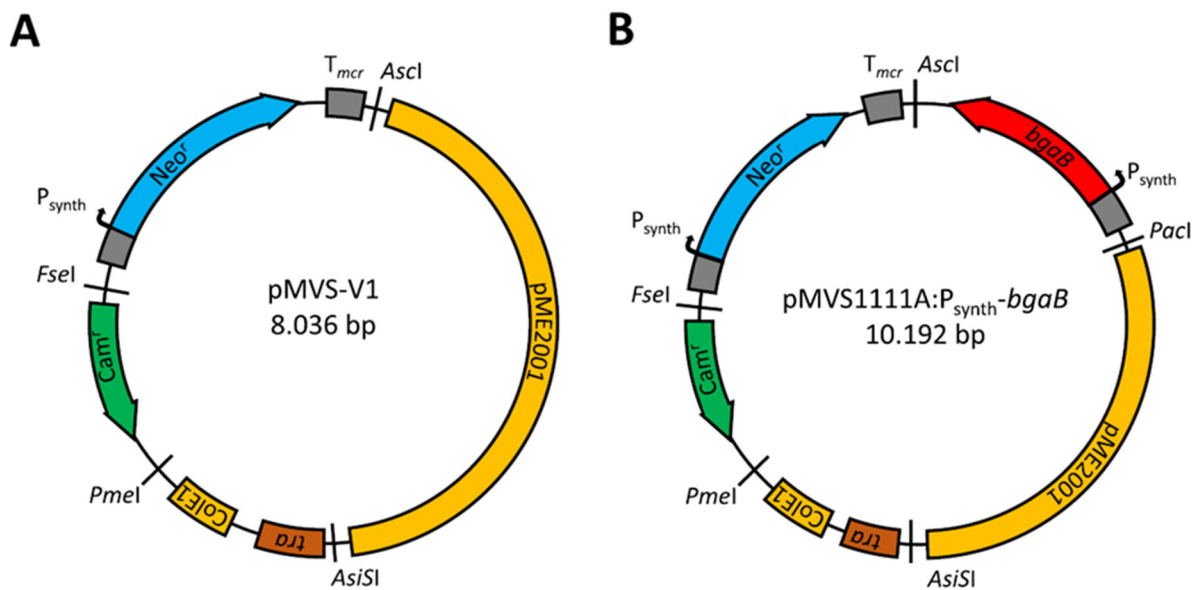


Figure 2. A: Plasmid map of basic *M. thermautotrophicus* – *E. coli* shuttle vector (pMVS-V1); B: Plasmid map of shuttle vector with application module (exemplified by shuttle vector carrying thermostable β -galactosidase). pME2001, cryptic plasmid for *M. marburgensis* (*ori* for *Methanothermobacter*); CmpR, chloramphenicol resistance for *E. coli*; P_{synth}, promoter for *M. thermautotrophicus* Δ H; T_{mcr}, Terminator for *M. thermautotrophicus* Δ H. Pacl, Ascl, Fsel, Pmel, AsiSI: recognition sites of restriction enzymes flanking the different modules of the shuttle vector. Figure from Fink *et al.* (2021).

Plasmid transfer was achieved *via* conjugation. For this, early stationary cultures of *E. coli* S17-1 culture carrying the shuttle vector and wild-type cultures of *M. thermautotrophicus* Δ H were plated on LB-MS plates and incubated overnight anoxically at 37°C in an H₂/CO₂ (80/20 vol-%) atmosphere. The next day, the spots were washed off and transferred to liquid medium with no antibiotics for recovery. After four hours, the cells were transferred to selective medium. In case of a successful conjugation, growth would be visible after about two days, and the grown cultures were

then plated to obtain single colonies, which were further analyzed (Figure 3). In addition, several putative promoters were assayed with the help of a thermostable β -galactosidase, yielding three constitutive promoters of different strengths (from weakest to strongest, P_{synth} , P_{synthBRE} , and P_{hmtB}).

It was also demonstrated that the genetic system could be used for gene deletion *via* homologous recombination (HR) (Fink *et al.* 2023). An integrative plasmid with one kbps homologous regions on both sides of the neomycin selection maker and no *ori* for *Methanothermobacter* was transferred to *M. thermautotrophicus* Δ H with the previously established conjugation protocol. With this plasmid, the *mth60* operon encoding the fimbriae of *M. thermautotrophicus* Δ H was successfully deleted. The resulting strain was used to investigate the role of the fimbriae in cell-cell connections (Fink *et al.* 2023).

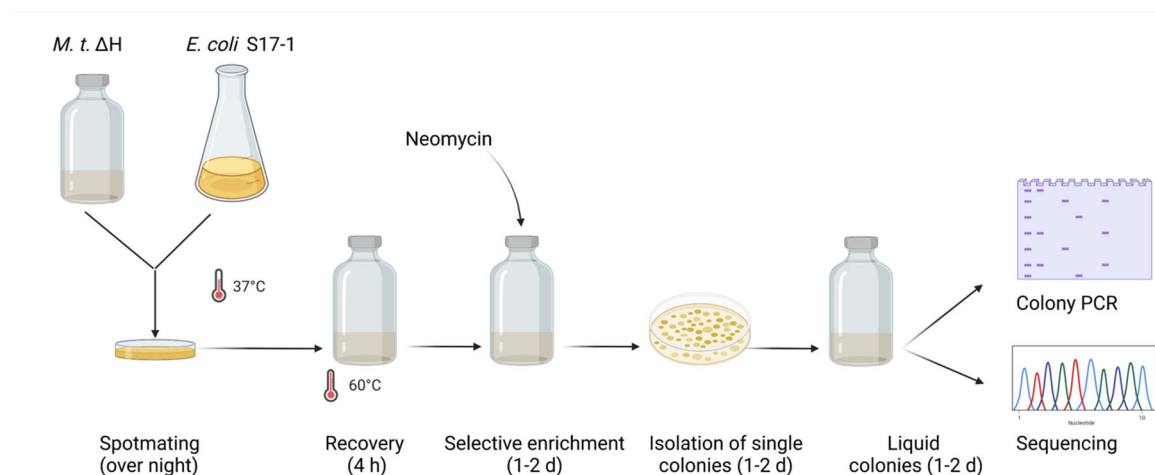


Figure 3. Interdomain conjugation protocol for *M. thermautotrophicus* Δ H (Fink *et al.* 2021). Figure created with Biorender.com.

While establishing a genetic system was already a breakthrough for *Methanothermobacter* research, additional tools for genome editing are required to turn the genetic system into a versatile genetic toolbox. The most essential tools are additional selection markers and tools for genome editing that do not leave the selection marker behind in the genome. Alternative transformation methods could also be beneficial. The following section will review promising candidates in more detail.

3. Selection markers

Selection markers are essential for genetic work because they allow the selection of genetically modified cells from the cell populations. The current genetic system for *M. thermautotrophicus* ΔH is based on a neomycin selection marker (Fink *et al.* 2021). However, complex genetic work, such as several subsequent genetic modifications, requires selection pressure for each step. Therefore, the selection marker that is used either needs to be removed again or a different selection marker needs to be applied. Also, the growth of spontaneously neomycin-resistant wild-type cells is visible after approximately five days (Fink *et al.* 2021). Exconjugant growth is already visible after two days. Therefore, spontaneously resistant wild-type usually does not interfere with exconjugant selection. However, in the case of genetic alterations that lead to growth defects, there could be the issue that slow-growing exconjugants are overgrown by spontaneously resistant wild-type cells. Thus, the genetic system would greatly benefit from a selection marker for which spontaneous growth of wild-type cells does not occur.

The most common type of positive selection marker is antibiotic resistance. Two requirements must be met to use antibiotic resistance as a selection marker: **1)** the wild-type organism needs to be sensitive toward the antibiotic; and **2)** the selection marker needs to confer resistance to the antibiotic in the respective organism. However, this severely reduces the number of putative selection markers for *M. thermautotrophicus* ΔH . Common cell wall-targeting antibiotics, such as ampicillin and vancomycin, do not work due to differences in cell wall structure (Meyer *et al.* 2014). Other common antibiotics, such as tetracycline and erythromycin, also do not have an effect on *M. thermautotrophicus* ΔH (Hilpert *et al.* 1981). Other possible antibiotics are effective, but there is no selection marker available that would be applicable to *M. thermautotrophicus* ΔH . While chloramphenicol is highly effective against methanogens, chloramphenicol resistance is conferred by an enzyme that converts chloramphenicol to acetyl-chloramphenicol, which is equally toxic to methanogens (Beckler *et al.* 1984). Also, most selection markers originate from mesophilic microbes and are not functional at 60°C. Despite the limitations, a few antibiotic resistances could still work in *Methanothermobacter* and will be discussed in more detail.

3.1. Mupirocin

Mupirocin is an antibiotic produced by *Pseudomonas fluorescens* (Fuller *et al.* 1971), which has a unique way of inhibiting microbial growth by inhibiting the isoleucine-tRNA synthetase (Hughes *et al.* 1978). It has been shown effective against *M. marburgensis*, where 10 µg/mL mupirocin inhibited growth for at least 50 hours (Kiener, Rechsteiner *et al.* 1986). Also, a spontaneous resistant strain (minimal inhibitory concentration, MIC, 100 µg/mL) was generated by chemical mutagenesis (Kiener *et al.* 1986, Rechsteiner *et al.* 1986). The resistance could be transferred between strains *via* phages (Meile *et al.* 1990). It was shown that the resistant strain had a mutated isoleucine tRNA synthetase gene (*ileS*), which led to an exchange of the glycine residue at position 590 for an aspartate residue (G590D) in the enzyme (Jenal *et al.* 1991). Similar results were obtained for *M. barkeri*. Five mutations in the native IleS enzyme were characterized, conferring different levels of mupirocin resistance. By transforming the mutated *ileS* genes into *Methanosarcina acetivorans*, it was demonstrated that the mutated *ileS* genes conferred resistance to mupirocin. The combination of several distinct mutations further increased the resistance to mupirocin. Despite a gene identity of 90%, no recombination with the native *ileS* of *M. acetivorans* was observed (Boccazzi *et al.* 2000). It should also be noted that one of the five reported mutations that were observed was the same as the one reported for *M. marburgensis*. The high sensitivity of *Methanothermobacter* spp. to mupirocin and the availability of a selection marker based on an intrinsic, and therefore, thermostable gene makes mupirocin a promising candidate for selection.

3.2. Puromycin

Puromycin is an antibiotic produced by the actinomycete *Streptomyces alboniger* (Tercero *et al.* 1996). It inhibits protein biosynthesis by serving as a tyrosyl-tRNA analog. During protein biosynthesis, puromycin can bind to the ribosomal A-site, and the nascent polypeptide chain is loaded onto the free amino group of puromycin, leading to premature translation termination (Figure 4, Aviner (2020)). Resistance towards puromycin is conferred by puromycin acetyltransferase (encoded by the *pac*-gene), which acetylates the free amino group of the O-methyltyrosine moiety of puromycin (Sugiyama *et al.* 1985). Early on, puromycin was established as a selectable marker for *Methanococcus* (Gernhardt *et al.* 1990) and *Methanosarcina*

(Metcalf *et al.* 1997). Its use in mesophilic methanogens makes it a promising candidate as a selection marker in *M. thermautotrophicus* Δ H. So far, there has been no report on its use in thermophiles, but it was reported that the Pac enzyme is stable for at least twelve minutes at 70°C *in vitro* (Paik *et al.* 1985).

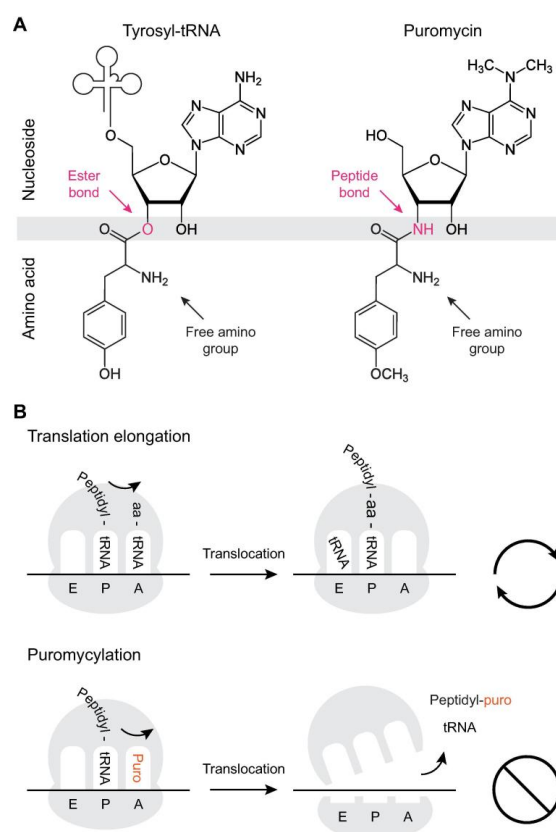


Figure 4. A: Structure of tyrosyl-tRNA and puromycin. Puromycin is a structural analog of tyrosyl-tRNA. Puromycin acetyltransferase (Pac) acetylates puromycin at the marked free amino group. B: Mode of action of puromycin. The nascent peptide chain is loaded onto puromycin during protein biosynthesis, leading to premature termination. Figure from Aviner (2020).

3.3. Simvastatin

Simvastatin and the closely related lovastatin are not antibiotics but statins, a type of cholesterol-lowering medication. Statins, such as simvastatin, lower cholesterol levels by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)-reductase, a central enzyme of the mevalonate pathway in humans, which produces the terpenoid precursors for cholesterol biosynthesis (Pedersen *et al.* 2004). Archaea possess the mevalonate pathway because they do not synthesize cell membrane lipids from fatty acids but from terpenoids, such as geranylgeranyl pyrophosphate and geranyl farnesyl

pyrophosphate, which stem from the mevalonate pathway (Koga *et al.* 2007). Inhibition of this pathway should, therefore, be lethal for archaea, and indeed, selection based on resistance to simvastatin was already established in the archaeal orders of Thermococcales and Sulfolobales (Farkas *et al.* 2013). In this case, the selection marker is not a classical resistance gene as with antibiotics but based on overproduction of the native HMG-CoA reductase (Matsumi *et al.* 2007). Unfortunately, mutants spontaneously resistant to simvastatin or lovastatin already arose after a few days in both orders (Farkas *et al.* 2013). As previously mentioned, this selection marker was recently established for the hyperthermophilic methanogen *M. jannashii* (Susanti *et al.* 2019), making it the only selection marker in thermophilic methanogens besides the already established neomycin. The sensitivity of *M. thermautotrophicus* ΔH towards simvastatin and lovastatin was already tested: 21.5 $\mu\text{g/mL}$ of simvastatin inhibits growth for at least 48h. In contrast, lovastatin did not have any inhibiting effect on growth at the same concentration. However, even for simvastatin, spontaneously resistant cells appeared after three days, which is even faster than the previously established neomycin selection (Fink *et al.* 2021).

3.4. Formate

Resistance to antibiotics is the most common type of positive selection marker. However, other strategies can also be employed. For example, genes that enable growth on alternative substrates and are not present in the wild-type can be implemented. While *M. thermautotrophicus* ΔH can only utilize hydrogen as an electron source, some *Methanothermobacter* spp. and *Methanobacterium* spp. are able to utilize formate as an alternative electron source (Nölling *et al.* 1993). These strains were formerly known as *Methanobacterium (thermo)formicum*. However, genetic analysis revealed that they are closer related to non-formate utilizing strains than to each other. During the reclassification of all *Methanobacterium* species, each strain was grouped with their non-formate utilizing close relatives (Wasserfallen *et al.* 2000). The strain *M. thermautotrophicus* Z-245, which is closely related to *M. thermautotrophicus* ΔH , can grow on formate as well as on hydrogen/carbon dioxide (Nölling *et al.* 1993). Genetic analysis revealed that the strain contains an operon, *fdhCAB*, which shows high homology to the formate dehydrogenase operon from the formate-utilizing *Methanobacterium formicum* JF-1. At the same time, the surrounding genes are homologous to *M. thermautotrophicus* ΔH (Figure 5, Nölling *et*

al. (1997)). The formate dehydrogenase of *M. formicicum* JF-1 is a two-subunit enzyme, which is encoded by the genes *fdhA* and *fdhB* enzyme, and which contains a molybdopterin cofactor (Schauer *et al.* 1982). It oxidizes formate to carbon dioxide and, concomitantly, reduces the methanogenesis cofactor F₄₂₀. In addition, the enzyme is only active in the presence of FAD (Schauer *et al.* 1983). FdhC is not well characterized, but the sequence suggests that it is a transmembrane protein, and therefore, the protein could be required for formate transport (Nölling *et al.* 1997). The *fdh* genes also seem to form an operon-like structure with a fourth gene, *orf3*, which is also present in *M. thermautotrophicus* ΔH and annotated as a putative carbonic anhydrase. Because only the *fdhCAB* genes are missing in *M. thermautotrophicus* ΔH, introducing these genes could enable growth on formate, which could be used as a selection marker.

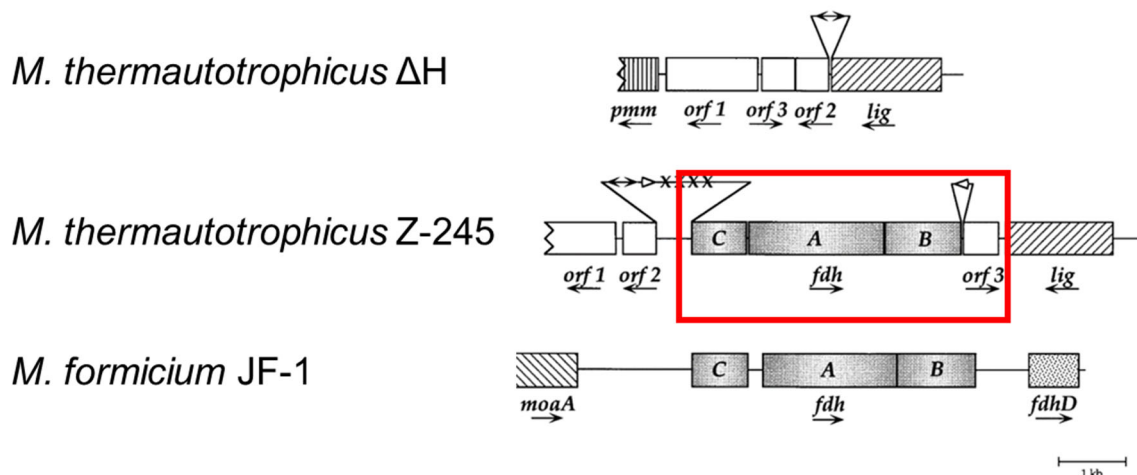


Figure 5. Comparison of the formate dehydrogenase (*fdh*) operon in *M. thermautotrophicus* Z-245 to the *fdh* operon in *M. formicicum* JF-1 and to the same region in *M. thermautotrophicus* ΔH, which lacks the *fdh* operon. Orf, Open reading frame; *fdh*, formate dehydrogenase. Figure adapted from Nölling *et al.* (1997).

4. Alternative transformation methods

While conjugation was successfully established as a transformation method in *M. thermautotrophicus* ΔH, it is a relatively work-intensive method unsuited for high-throughput screening. The genetic toolbox would, therefore, greatly benefit from an alternative transformation strategy. As already mentioned, there were reports that *M. marburgensis* can be transformed by natural competence (Worrell *et al.* 1988) or phage transduction (Meile *et al.* 1990). However, both techniques were used only to transfer genomic DNA between mutants, and neither method had any follow-up

reports. It was also investigated whether *M. thermautotrophicus* Δ H is naturally competent during the establishment of the current conjugation-based genetic system. The addition of plasmid pMVS-V1 to a culture of *M. thermautotrophicus* Δ H did not lead to any transformants (Fink *et al.* 2021), suggesting that at least this strain is not naturally competent. Phage transduction is also unsuitable because it would require time-intensive packaging of foreign DNA into the phages (Karimi *et al.* 2016). The PEG- or liposome-mediated transformation methods commonly used in *Methanosarcina* and *Methanococcus* are also not well suited for *M. thermautotrophicus* Δ H because they require protoplastation. While protoplastation of *M. marburgensis* was possible, the protoplasts could not regenerate their cell wall (Kiener *et al.* 1987). This observation was confirmed in our lab for *M. thermautotrophicus* Δ H (Sebastian Beblawy, unpublished results). While this already rules out many standard transformation methods, electroporation might be a suitable method for the transformation of *M. thermautotrophicus* Δ H.

4.1. Electroporation

Electroporation is a standard transformation method and was already successfully established for *Methanococcus voltae* (Micheletti *et al.* 1991, Patel *et al.* 1994). During electroporation, cells are subjected to short electric pulses that lead to a temporary formation of small pores in the cell membrane, allowing the DNA to enter the cell (Chang *et al.* 1990). Electroporation is an established transformation method for various organisms, from bacteria and archaea to fungi and animal cells (Nickoloff 2008). However, several parameters determine electroporation success and efficiency. The first ones are the electroporation's physical parameters, namely the electric field's strength, the electroporator's capacitance, and the conductivity of the cell suspension. The strength of the electric field is determined by $E = V/d$, with E being the field strength, V being the voltage, and d being the distance between the electrodes (Weaver 1995). In practice, different voltages can be set at the electroporator, and the distance can be changed by using different electroporation cuvettes. The capacitance can be set directly at the electroporator, and the conductivity of the cell suspension is determined mainly by the buffer or medium the cells are in (Weaver 1995). These parameters highly influence the electroporation's

success and need to be determined experimentally for each organism (Kim *et al.* 2005).

In addition, biological parameters can affect transformation efficiency. One factor is the growth phase of the organism, which can severely impact the efficiency of the transformation (Marciset *et al.* 1994). Another major factor is the methylation pattern of the DNA. Bacteria and archaea often have restriction-modification (RM) systems, consisting of a methyltransferase that methylates the microbe's own DNA and a restriction endonuclease that digests incorrectly methylated DNA (Suzuki 2012). The methylation pattern of a transferred plasmid is identical to the *E. coli* strain it was generated in and can affect electroporation efficiency (Molitor *et al.* 2016). For *M. thermautotrophicus* ΔH , it was reported that the genome contains one putative type-I restriction system and that the genomic DNA carries an m6A methylation at the sequence ACANNNNNNNTGG and CCANNNNNNNTGT (Casini *et al.* 2023). In addition, it was reported that incubation with crude extract of *M. thermautotrophicus* ΔH leads to the linearization of circular, non-methylated plasmid DNA. At the same time, linearized DNA does not seem to be affected (Fink 2021). However, the *E. coli* S17-1 strain used for conjugation has standard *E. coli* methylation patterns (Flett *et al.* 1997). While this indicates that the methylation of the DNA does not have a considerable effect on transformation efficiency, it is still an important parameter to evaluate. Electroporation would be a promising candidate for an alternative transformation method, but many parameters must be evaluated.

5. Tools for genome editing

An essential part of any genetic toolbox are tools for genome editing. The classical approach is replacing a target gene with a selection marker *via* homologous recombination in an allele-coupled exchange approach (Sarmiento *et al.* 2011). This was already demonstrated to be feasible in *M. thermautotrophicus* ΔH (Fink *et al.* 2023). The technique has the drawback that the selection marker stays in the genome. Therefore, the number of edits that can be done in one strain is limited to the number of available selection markers. Currently, only neomycin is established as a selection marker for *M. thermautotrophicus* ΔH , which limits the number of possible edits to one. Also, the integration of the selection markers in the genome can lead to polar effects, affecting the transcription, especially of downstream genes (Mateus *et al.* 2021).

Therefore, establishing genome editing techniques that do not leave the selection marker behind would be highly beneficial.

5.1. Markerless mutagenesis

One such technique, markerless mutagenesis or “pop-in-pop-out” recombination, was established in mesophilic methanogens of the genus *Methanococcus* and *Methanosarcina* (Moore *et al.* 2005, Rother *et al.* 2005). The technique is based on homologous recombination and the use of a selection and a counterselection marker (*i.e.*, a gene that confers sensitivity to a compound). If the counterselection marker is a gene already present in the organism, prior deletion is required. In *M. maripaludis*, the hypoxanthine phosphoribosyl transferase-encoding gene (*hpt*) was chosen as a counterselection marker. The *hpt* gene product confers sensitivity to the toxic base analog 8-azahypoxanthine (8-AH). The native *hpt* gene was first deleted in-frame by introducing a suicide plasmid with only the flanking regions of *hpt* and selecting 8-AH-resistant transformants. The resulting Δhpt strain was then used for all further experiments. Markerless mutagenesis was achieved with the suicide plasmid pCRPrNeo, which contains flanking regions upstream and downstream of the target gene, the *hpt* gene, and a neomycin resistance cassette. It is important to note that *hpt* and the neomycin resistance are not in between the flanking regions but elsewhere on the plasmid. After this plasmid was introduced into *M. maripaludis*, clones in which the whole plasmid was integrated *via* single-crossover recombination were selected based on their resistance to neomycin. The resulting clones had a merodiploid phenotype containing both the target site and the whole integrative plasmid (Figure 6). In the next step, 8-AH was added to select for the loss of the *hpt* gene. Because both the up- and downstream flanking regions are present twice, loss of *hpt* can occur in two ways: **1)** if the recombination between the same flanking region as in the first recombination occurs, the wild-type genotype is restored; and **2)** if recombination occurs between the other flanking regions, a clean knock out of the target is achieved (Figure 6). Only one of the two possible recombinations leads to the desired genotype. Therefore, some screening is always required for the second recombination step. With this system, three genes of alanine metabolism were deleted from *M. maripaludis* Δhpt with varying efficiency - between eight of 18 and one of 40 screened colonies had the desired genotype (Moore *et al.* 2005). The system was also used to generate a clean deletion of the uracil phosphoribosyl transferase-encoding gene (*upt*). The *upt* gene

product confers sensitivity to 6-azauracil and can be employed in the same way as the previously mentioned *hpt*. In fact, *upt* is now the primarily used counterselection marker in *Methanococcus* because it was discovered that the deletion of *hpt* has polar effects on hydrogen metabolism, which were not further specified (Sarmiento *et al.* 2011).

The *Methanosarcina* system works on the same principle. The puromycin resistance cassette is used as a selection marker, and the *hpt* gene is used as a counterselection marker. The second recombination event was mediated by site-specific recombination by a recombinase introduced on a non-replicative plasmid (Rother *et al.* 2005).

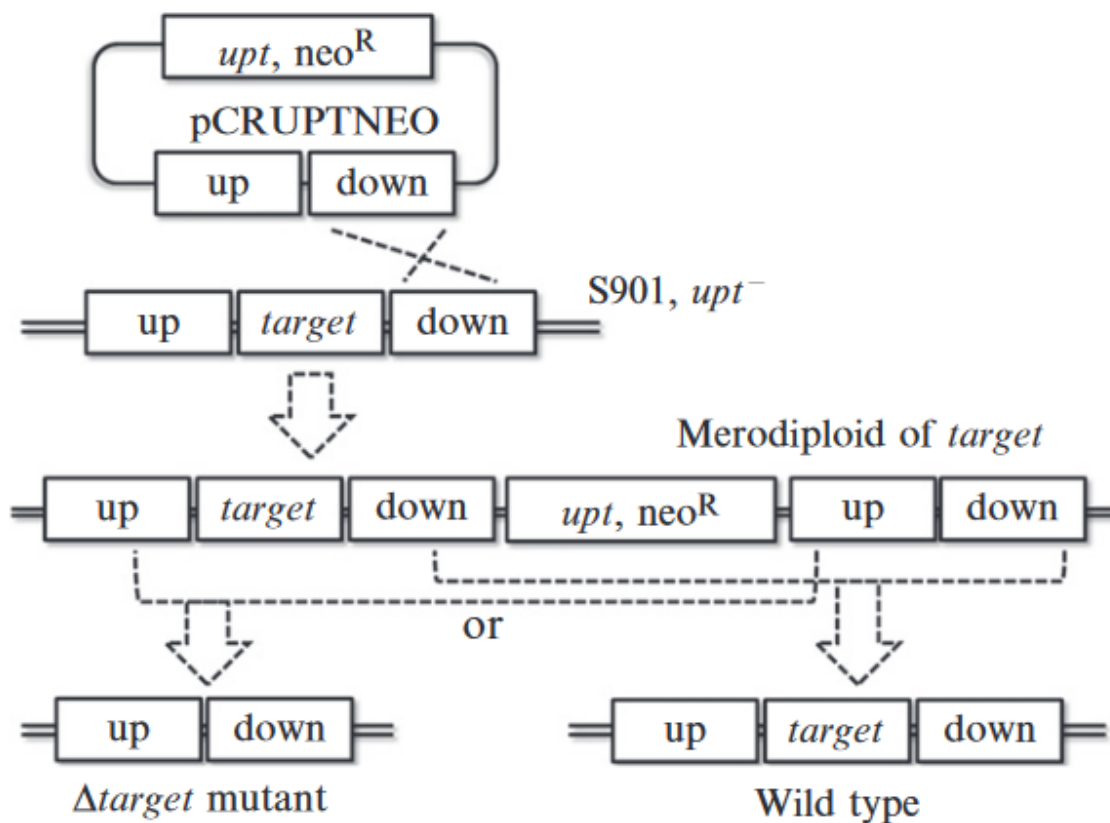


Figure 6. Markerless mutagenesis technique from *M. maripaludis*. An integrative plasmid carrying 1 kb flanking regions up- and downstream of the target gene as well as a selection and counterselection marker (neomycin resistance, *upt*), is introduced to the cell, and successful integration mutants are selected *via* neomycin resistance. The resulting merodiploid state is resolved by excision of the plasmid from the genome, and successful excision mutants are selected by their resistance to 6-azauracil. The resulting state can either be a clean deletion (left) or a restoration of the original genotype (right). Figure from Sarmiento *et al.* (2011).

5.2. CRISPR

Since its first use for genome editing about ten years ago, the **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats - **CRISPR-associated** (CRISPR-Cas) system has become the most common method for genome editing in molecular biology (Adli 2018). CRISPR systems are widespread in bacteria and archaea and play a significant role in adaptive immunity (Barrangou *et al.* 2014). The native CRISPR system of *Streptococcus pyogenes* was adapted as a tool for genome editing (Jinek *et al.* 2012). The system has two core parts: **1)** the Cas endonuclease, with Cas9 being the most commonly used one, which induces double-strand breaks in the DNA; and **2)** the single-guide RNA (sgRNA), which contains a ~20 bp spacer sequence that guides the Cas enzymes to a specific target DNA sequence. While the spacer sequence can be chosen freely, cleavage only occurs when the Cas binds to the DNA at a conserved sequence, the protospacer adjacent motive (PAM), next to the sequence targeted by the spacer. The PAM sequence, which is needed for binding, is highly diverse between different Cas enzymes.

Double-strand breaks in the genomic DNA immediately need to be fixed by the DNA repair mechanism of the cell. Depending on the cell type, this can occur *via* homologous recombination or non-homologous end joining. Non-homologous end joining has rarely been described in archaea. Homologous recombination and so-called “micro homologous end joining,” which requires short homologous sequences for rejoining the DNA ends, seem to be the main repair mechanisms (Pérez-Arnaiz *et al.* 2020).

CRISPR systems were already established for the mesophiles *M. acetivorans* and *M. maripaludis*. For *M. acetivorans*, the Cas9 mentioned above from *S. pyogenes* (SpCas9) was adapted for genome editing. The repair of the DSB was either mediated by HR or by heterologously expressing an NHEJ-machinery from a related *Methanocella* strain (Nayak *et al.* 2017). The SpCas9 was also adapted for *M. maripaludis* (Li *et al.* 2022). However, a Cas12a from *Lachnospiraceae* bacterium ND2006 showed a higher efficiency and a PAM better suited for the AT-rich *Methanococcus* genome (Bao *et al.* 2022). This system was recently transferred to *M. acetivorans* (Zhu *et al.* 2023).

5.2.1. ThermoCas9

All previously mentioned Cas enzymes are from mesophilic microbes, such as the routinely used Cas9 from *S. pyogenes*. While *M. thermautotrophicus* ΔH encodes three CRISPR loci in the genome (Liesegang *et al.* 2010), they have so far not been adapted for use in genome editing. However, CRISPR systems from other thermophiles have successfully been used for genome editing, such as the thermostable Cas9 (ThermoCas9) from the thermophilic bacterium *Geobacillus thermodenitrificans* T12 (Mougiakos *et al.* 2017). The ThermoCas9 was able to cleave target DNA at temperatures up to 65°C *in vitro*, and CRISPR-assisted gene deletion was demonstrated at 55°C for a thermophilic *Bacillus* strain. While the PAM sequence is very simple (5'-NNNNCNAA-3'), the ThermoCas9 tolerates at least two mismatches in the spacer sequence, which needs to be taken into consideration when designing the sgRNA to avoid off-target effects. The ThermoCas9 has also already been successfully used in other thermophilic bacteria, including *Hungateiclostridium thermocellum* (Ganguly *et al.* 2020), *Pseudoclostridium thermosuccinogenes* (Ganguly *et al.* 2022), and *Thermus thermophilus* (Gallo *et al.* 2021).

5.3. Ploidity of *M. thermautotrophicus* ΔH

An important factor for genome editing is the ploidity (*i.e.*, the number of genomes per cell) in the target organism. The ploidity of methanogens vastly differs between different species and even the growth phases of the same species. *M. jannashii* was reported to contain three to 15 genome copies in the exponential growth phase and one to five in the stationary phase (Malandrin *et al.* 1999). For *M. acetivorans*, the genome copy number ranged between three and 17, depending on the substrate, while for *M. maripaludis*, up to 55 genome copies per cell were reported (Hildenbrand *et al.* 2011). The genome copy number was also investigated for *M. thermautotrophicus* ΔH . The strain grows in short filaments, and fluorescence staining revealed that each filament contains two, four, or eight genomes, all spatially separated within the filament. Filament length and genome copy number decreased when cells reached the stationary phase and even further within the stationary phase. However, a filament with less than two genome copies was never observed (Majerník *et al.* 2005). The authors, unfortunately, did not attempt to visualize the cell membrane, so it remains unclear whether the shortest observed filament is one cell with two

spatially separated genomes or a filament with two cells with one genome each. In *M. marburgensis*, enzymatic degradation of the cell wall gave rise to several (3-4) protoplasts per filament. However, the number of genomes in the filaments or the protoplasts was not investigated. While the protoplasts were metabolically active, they failed to regenerate the cell wall (Kiener *et al.* 1987). Also, short filaments with several genome copies might explain why gene deletion attempts *via* homologous recombination in *M. thermautotrophicus* ΔH always lead to a mix of wild-type, single, and double cross-over colony PCR signals initially, which needed several rounds of plating and picking colonies to segregate cells with a clean genotype (Fink 2021, KÜchle 2024).

5.4. Plasmid curing

After a strain was successfully edited with a plasmid-encoded CRISPR system, the plasmid is no longer required. The plasmid hinders further genetic work on the strain because no other plasmids with the same selection marker or *ori* can be transferred into the strain. Therefore, a method to remove the plasmid from the strain, also called plasmid curing, is required. Commonly used methods include treatment with mutagenic chemicals, detergents, or heat-shock treatment (Trevors 1986). Other possible methods include electrocuring (Nickoloff 2008) or several unselective transfers of the plasmid-carrying strain (Volke *et al.* 2021). In general, methods significantly differ and must be determined individually for each organism.

Previous reports showed that the shuttle vectors have high segregational stability in *M. thermautotrophicus* ΔH . After three non-selective liquid transfers and non-selective plating, all 16 analyzed clones still contained the shuttle vector (Fink *et al.* 2021). Also, a plasmid-carrying culture of *M. thermautotrophicus* ΔH was cultivated for eleven days in a continuously fed bioreactor without any selective pressure, and the plasmid was still detectable by colony PCR afterward. However, the authors did not plate the culture afterward, so the possibility remains that a subpopulation of the culture lost the plasmid (Casini *et al.* 2023). Overall, cultivating the strain in unselective conditions might not be a suitable plasmid curing method for *M. thermautotrophicus* ΔH , or at least requires several more transfers.

There was one report of successful curing of *M. marburgensis* from plasmid pME2001 (the *ori* of the shuttle vectors for *M. thermautotrophicus* ΔH) by treating it with

methylnitronitrosoguanidine. However, the author did not give any details on how this was achieved or how they confirmed the absence of the plasmid (Meile *et al.* 1989). Also, methylnitronitrosoguanidine is a mutagen and, therefore, unsuitable for targeted genetic work. Another possible strategy was illustrated for the CRISPR/Cas9 system established in *M. acetivorans*. All genome editing was done in a Δhpt strain, and all CRISPR plasmids included the *hpt* gene. After a successful edit, the cells were directly selected for the plasmid loss by their resistance to 8-ADP (Nayak *et al.* 2017). The same strategy was applied for the CRISPR system in *M. maripaludis* (Li *et al.* 2022).

II. Outline

The relevance of *M. thermautotrophicus* Δ H for methanogen research and the possibility for further industrial applications were the driving forces in establishing a genetic system for *M. thermautotrophicus* Δ H. Based on this success, this dissertation aims to expand the genetic toolbox with the necessary tools, such as additional selection markers, an alternative transformation protocol, and tools for genome editing.

Objective 1: Establishment of additional selection markers for *M. thermautotrophicus* Δ H

- Evaluation of mupirocin sensitivity of *M. thermautotrophicus* Δ H and design of a mupirocin resistance cassette based on the mutagenized target gene
- Evaluation of puromycin sensitivity of *M. thermautotrophicus* Δ H and adaptation of the mesophilic puromycin acetyltransferase (Pac) for its use in the thermophile *M. thermautotrophicus* Δ H
- Creation of a formate-utilizing strain of *M. thermautotrophicus* Δ H by the introduction of the formate dehydrogenase (*fdh*) operon from *M. thermautotrophicus* Z-245 and utilization of the *fdh* operon as a selection marker

Objective 2: Establishment of an alternative transformation protocol

- Transformation of *M. thermautotrophicus* Δ H with an electroporation protocol for *Clostridium ljungdahlii*
- Optimization of electroporation conditions

Objective 3: Establishment of tools for genome editing

- Testing sensitivity of *M. thermautotrophicus* Δ H to counterselection agents 6-methylpurine, 6-azauracil, and 8-azahypoxanthine
- Evaluating possible targets for genome editing
- Establishment of ThermoCas9 as a tool for genome editing in *M. thermautotrophicus* Δ H

III. Materials and Methods

1. Methods

1.1. Microbial cultivation conditions

E. coli strains were cultivated as previously described (Fink *et al.* 2021). *M. thermautotrophicus* strains were cultivated as previously described when grown with hydrogen as the electron donor (Fink *et al.* 2021). For growth with formate as the electron donor, the standard MS medium was aliquoted in serum bottles as usual, but the headspace was then exchanged to 1.52 bar of N₂/CO₂ (80/20 vol-%). The bottles were autoclaved and stored until further use. Before inoculation, each bottle was supplemented with 200 mM sodium formate, 10 µM sodium molybdate, 1 µM sodium selenite, and 0.125 wt% yeast extract from a sterile, anoxic stock solution. For plating with formate as the electron donor, the liquid MS agar was supplemented the same way before pouring the plates. After plating, paper clips were added to the plates for better ventilation. The plates were then incubated in custom-made stainless-steel jars (Fink *et al.* 2021) in an N₂/CO₂ (80/20vol-%) atmosphere at 60°C for the first plating attempts with strain *M. thermautotrophicus* ΔH pMVS1111a:P_{hmtB}-*fdhZ*-245 and in an N₂/CO₂/H₂S (79.9/20/0.1 vol-%) atmosphere at 60°C for all further plating experiments.

1.1.1. Growth measurement

For all growth curves, 1 mL of the respective *M. thermautotrophicus* ΔH culture was taken from the serum bottle with a syringe. The OD₆₀₀ was measured with the undiluted sample in a BioMate™ 160 UV-Vis Spectrophotometer with MS medium as a blank. All growth experiments were done in triplicates.

1.1.2. Cell separation

To achieve better cell separation, a culture of *M. thermautotrophicus* ΔH pMVS1112a:CRISPR6 was treated with a blender before plating according to the protocol described in Küchle (2024). The blender (T10 basic ultra turrax®) was sterilized with 70% ethanol and brought into the anoxic working environment. Inside, 1 mL of an *M. thermautotrophicus* ΔH pMVS1112a:CRISPR6 culture was placed into

a sterile falcon and blended for 50 s at 14,500 rpm (speed level 4) and then for another 20 seconds at 21,100 rpm (speed level 5). Afterward, the cells were spot-plated onto formate-selective plates and treated as described in section III.1.1.

1.2. Genetic manipulation of *E. coli*

1.2.1. PCR

All DNA amplification for cloning purposes was done with the Q5 polymerase (New England Biolabs [NEB]). The following standard mixture was used:

Table 1: Q5 polymerase sample mix

Compound	Volume [μ L]
5 x Q5 reaction buffer	40
dNTPs (10 mM)	4
Primer 1 (10 μ M)	10
Primer 2 (10 μ M)	10
Q5 High-Fidelity polymerase	2
Nuclease free water	133
Template DNA	1
Total volume	200

If the template DNA was gDNA, 5 μ L was used as the template. The mixture was aliquoted into eight tubes with 25 μ L each and placed in the PCR cycler. The cycler was run with the following program:

Table 2: PCR protocol for Q5 polymerase

Step	Temperature [$^{\circ}$ C]	Time	Number of cycles
Initial denaturation	98	2 min	1
Denaturation	98	10 s	32
Annealing	Primer dependent	30 s	
Extension	72	30 per 1000 bp	
Final extension	72	60 s	1
Hold	4	Infinite	1

The annealing temperature was calculated as the average between the two used primers. PCR products were analyzed *via* gel electrophoresis. If the PCR was unsuccessful, it was repeated with the same mixture but as a gradient PCR with an annealing temperature from 52 $^{\circ}$ C to 72 $^{\circ}$ C. If the PCR was still unsuccessful, it was repeated as a gradient PCR but with 5 μ L 5x GC enhancer added to each sample. Successful PCRs were pooled, 2 μ L of DpnI was added, and the mixture was

incubated at 37°C for one hour. Afterward, the PCR product was purified using the PCR purification kit (NEB).

1.2.2. Restriction/ligation cloning

Restriction digest for restriction/ligation cloning was done in the following setup. If several restriction enzymes were used, the water volume was reduced accordingly.

Table 3: Restriction digest sample mix

Compound	Volume [μL]
5x CutSmart® Buffer	5
Template DNA (100-500 ng/μL)	10
Restriction Enzyme	1
Nuclease free water	34
Total volume	50

Usually, one additional enzyme was added to digest the part of the plasmid to be removed further to prevent religation. In addition, 1 μL of calf intestine phosphatase (CIP) or recombinant shrimp alkaline phosphatase (rSAP) was added for the digestion of the backbone to reduce the religation rate further. Digests were incubated for four hours at 37°C, and 5 μL were loaded onto an agarose gel to verify the success of the digest. The rest of the digest was purified using the PCR purification kit (NEB) and eluted in 30 μL elution buffer.

Ligations were done with the following setup.

Table 4: Ligation setup sample mix

Compound	Volume [μL]
T4 DNA Ligase Reaction Buffer	2
Vector DNA	5
Insert DNA	10
T4 Ligase	1
Nuclease free water	2
Total volume	20

The vector and insert DNA concentrations were measured with a microvolume spectrophotometer, and volumes were adapted to reach a three-to-one-molar ratio of vector to insert. Reactions were incubated for 20 min at 20°C in the heating block. 10 μL of the ligation mixture was used to transform *E. coli* NEBstable (section III.1.2.8).

1.2.3. Gibson assembly

Fragments for Gibson assembly were prepared *via* PCR (section III.1.2.1). Gibson assembly was performed with the HiFi DNA assembly kit (NEB) with the following mixture.

Table 5: Gibson assembly sample mix

Compounds	Volume [μ L]
NEBuilder® HiFi Assembly Master Mix	10
Vector DNA	4
Insert DNA	1
Nuclease free water	5
Total volume	20

The vector and insert DNA concentrations were measured with a microvolume spectrophotometer, and volumes were adapted to reach a two-to-one-molar ratio of vector to insert. Reactions were incubated for 15 min at 50°C in the heating block. 10 μ L of the ligation mixture was used to transform *E. coli* NEB stable (section III.1.2.8).

1.2.4. *In-vivo* ligation

In-vivo ligation was applied for promoter and sgRNA spacer exchange and is generally suitable for removing, exchanging, or adding parts of a plasmid up to 100 bp. First, the entire vector of interest except the DNA sequence to be removed was amplified *via* PCR (section III.1.2.1) with a primer that contained around two-thirds of the DNA sequence to be added as overhang, resulting in a linear PCR product with approximately 20 bp – 40 bp of homologous sequences at both ends. The PCR product was purified using the PCR purification kit (NEB) and eluted in 30 μ L of elution buffer, and around 10 μ L was used to transform *E. coli* NEBstable (section III.1.2.8), where the actual ligation occurred.

1.2.5. QuickChange mutagenesis

Quick change mutagenesis was used to introduce the point mutations in the *ileS* gene. The mutagenesis was performed using the QuikChange mutagenesis kit from Agilent. The following mixture was prepared.

Table 6: QuickChange mutagenesis sample mix

Compounds	Volume [μ L]
10X Reaction Buffer	5
Vector DNA (1:10) dilution	1
dNTPs (10 mM)	1
Primer 1 (10 μM)	2
Primer 2 (10 μM)	2
Nuclease free water	38
<i>PfuTurbo</i> DNA Polymerase	1
Total volume	50

The mixture was then loaded into a PCR cyclyer and run with the following program.

Table 7: Quick change PCR protocol

Step	Temperature [$^{\circ}$ C]	Time	Number of cycles
Initial denaturation	95	30 s	1
Denaturation	95	30 s	32
Annealing	Primer dependent	60 s	
Extension	72	7 min	
Hold	4	Indefinite	1

10 μ L of the ligation mixture was used to transform *E. coli* NEBstable (section III.1.2.8).

1.2.6. Error-prone PCR

The error-prone PCR for the mutagenesis of the *pac* gene was done with the error-prone PCR kit from Jena Bioscience according to the following protocol.

Table 8: Error-prone PCR sample mix

Component	Volume [μ L]
10x Reaction Buffer	5
dNTP Error-prone Mix	2
Primer hmtB_mod_pacl.for (10 μM)	1
Primer pac_ascl.rev (10 μM)	1
puc57-<i>P</i>_{snyth}-<i>pac</i> (1:10 dilution)	1
Taq Polymerase	1
PCR-grade Water	34
10x Error-prone Solution	5

Table 9: PCR protocol for error-prone PCR

Step	Temperature [°C]	Time	Number of cycles
Initial denaturation	94	2 min	1
Denaturation	94	30 s	30
Annealing	55	30 s	
Extension	72	1 min	
Hold	4	Indefinite	1

The mutagenized PCR product was purified using the PCR purification kit (NEB) and eluted in 30 µL elution buffer. Afterward, the mutagenized PCR product was digested with *PacI* and *Ascl* and ligated into a digested shuttle vector as described in section III.1.2.2). *E. coli* S17-1 was transformed with 10 µL of the ligation (section III.1.2.8).

1.2.7. Preparation of chemically competent *E. coli*

The chemically competent *E. coli* were prepared with a protocol from Sambrook *et al.* (1989), modified by Christian Klask (2017). The respective *E. coli* strain was inoculated in 5 mL LB medium and incubated overnight (37°C, 150 rpm). For *E. coli* S17-1, 10 µg/mL trimethoprim was added. In the morning, 2 mL of the grown culture was reinoculated in 50 mL LB medium in a 250 mL flask and incubated until the culture reached an OD₆₀₀ of 0.3-0.5. The culture was then harvested by centrifugation in sterilized 50 mL falcons (3600 x g, 15 min, 4°C). Cells were gently resuspended in 25 mL of ice-cold 100 mM CaCl₂ solution and incubated on ice for 5 min. Cells were pelleted again by centrifugation (3600 x g, 10 min, 4°C) and gently resuspended in 4 mL of ice-cold 100 mM CaCl₂ solution with 25 % glycerol. After ten minutes of incubation on ice, 200 µL aliquots of cells were prepared and frozen at -80°C.

1.2.8. Transformation of chemically competent *E. coli*

The chemically competent *E. coli* were transformed according to the protocol developed by Sambrook *et al.* (1989). An aliquot of chemically competent cells was thawed on ice. Once it was thawed, 2-10 µL of the ligation product or 1-2 µL plasmid DNA of diluted plasmid DNA (~ 20 ng) were added and gently mixed by snapping against the tube. Cells were then incubated on ice for 15 more minutes and then incubated at 42°C for 45 s in a heat block. Afterward, cells were quickly cooled on ice, 900 µL of sterile SOC medium was added, and cells were incubated for 60 min (37°C,

150 rpm). Cells were then plated on LB plates with the respective antibiotic(s). Usually, two plates were prepared per transformation, and 100 μ L of the recovery was plated on one and 900 μ L on the other plate.

1.2.9. Plasmid purification

After the transformation, single colonies were picked into 5 mL of liquid LB medium with the respective antibiotics and incubated overnight (37°C, 150 rpm). Plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's description and eluted in 50 μ L elution buffer.

1.2.10. Restriction test digest

Restriction test digests were done in the following setup. If several restriction enzymes were used, the water volume was reduced accordingly.

Table 10: Restriction test digest sample mix

Compound	Volume [μ L]
5x CutSmart® Buffer	2
Template DNA (100-500 ng/μL)	2
Restriction Enzyme	0.5
Nuclease free water	15.5
Total volume	20

Digests were incubated for one hour at 37°C, and 10 μ L were loaded onto an agarose gel.

1.2.11. Agarose gel electrophoresis

A 50x stock of TAE buffer was prepared for gel electrophoresis with the following recipe.

Table 11: TAE buffer composition

Chemical	Amount
Tris base	242.00 g
EDTA	18.61 g
Glacial Acetic acid	57.10 mL

Distilled water was then added to a final volume of one liter. When needed, the stock was diluted 1:50 with distilled water. To prepare an agarose gel, one gram of agarose was added to 100 mL of 1xTAE buffer. The suspension was then repeatedly boiled in the microwave until the agarose was completely dissolved. The liquid gel was cooled

by holding the bottle under a cool water tap for about 30 seconds. Then, 3 μL of MidoriGreen DNA stain was added, the gel was poured into the gel tray, and one or two combs were added. After the gel solidified, it was placed in the running chamber and covered in TAE buffer. Before loading the samples, they were mixed with the appropriate amount of 6x purple loading dye (NEB) unless they already contained a loading dye. The gel was then run at 140 V for 45 minutes. DNA fragments were visualized by UV illumination in the UVP GelStudio PLUS (Analytik Jena AG).

1.2.12. Sanger sequencing

For Sanger sequencing, the following mixture was prepared.

Table 12: Sanger sequencing sample mix

Compounds	Volume [μL]
Plasmid (30-100 ng/ μL)	5.0
Primer (10 μM)	2.5
Nuclease free water	2.5
Total volume	10.0

If the plasmid concentration was higher than 100 ng/ μL , the volume added was reduced, and the volume of nuclease-free water was increased accordingly. The mixture was then sent to GENEWIZ for sequencing. Results were analyzed with Snapgene.

1.2.13. Whole plasmid sequencing

Plasmids pMVS1112a:CRISPR5, pMVS1112a:CRISPR6, pMVS1112a:CRISPR8, and pMVS1112a:CRISPR11 were sequenced with the Plasmid-EZ service of GENEWIZ. For this, 10 μL of a 50 ng/ μL plasmid dilution was sent to GENEWIZ. In case of mismatches between the sequencing result and the expected sequence, the region in question was resequenced by Sanger sequencing.

1.2.14. Storage

E. coli strains confirmed by sequencing were prepared for long-term storage. 1 mL of a fresh overnight culture of the respective *E. coli* strain was resuspended in 0.5 mL of sterile 80 vol% glycerol solution in a sterile 2 mL screw cap vial. The vial was barcoded and stored in our group's aerobic culture collection at -80°C . All strains were stored in duplicates.

1.3. Cloning of Plasmids

1.3.1. Cloning of pMVS1111a:MupR

The *ileS* gene was amplified from the genomic DNA of *M. marburgensis* via PCR with primers Gibson_ileS-puc57.for and Gibson_ileS-puc57.rev. The backbone and promoter of plasmid pLM201 were amplified via PCR with primers Gibson_puc57-ileS.for and Gibson_puc57-ileS.rev, and the two fragments were ligated via Gibson assembly, resulting in plasmid pUC57-P_{synth}-*ileS*. The two point mutations conferring mupirocin resistance were introduced via two successive rounds of QuickChange mutagenesis with primers Mutant-Primer_582.for and Mutant-Primer_582.rev for the first point mutation and Mutant-Primer_589_3.0.for Mutant-Primer_589_3.0.rev for the second one, resulting in plasmid puc57-MupR. The MupR cassette was then cut out from the plasmid by digestion with PaeI and Ascl and ligated into the cargo-module site of shuttle vector pMVS1111a:P_{hmtB}-*fdh*_{Z-245}, resulting in plasmid pMVS1111a:MupR.

1.3.2. Cloning of pMVS1111a:P_{synth}-*pac*

The *pac* gene was already present in our lab on plasmid pCF200 under the control of promoter P_{mcrB} (*M.v.*) (Fink *et al.* 2021). We exchanged the promoter of the *pac* cassette via inverse PCR with primers pac_P_{synth}.for and pac_P_{synth}.rev followed by *in-vivo* ligation, resulting in plasmid puc57-P_{synth}-*pac*. The P_{synth}-*pac* cassette was amplified via PCR with primers hmtB_mod_paeI.for and pac_ascl.rev to add the required restriction sites and ligated into the cargo-module site of vector pMVS1111a:MupR via restriction/ligation with PaeI and Ascl restriction enzymes, resulting in plasmid pMVS1111a:P_{synth}-*pac*.

1.3.3. Cloning of pMVS1111a:P_{hmtB}-*fdh*_{Z-245}

The *fdh* operon was amplified from plasmid pCF201 via PCR with primers Gibson_fdh.for and Gibson_ileS-puc57.rev. The backbone and promoter of plasmid pCF204 were amplified via PCR with primers Gibson_puc57_fdhv2.for and Gibson_puc57_fdhv.rev, and the two fragments were ligated via Gibson assembly, resulting in plasmid pLM201. We exchanged the promoter of the *fdh* operon to P_{hmtB} via inverse PCR with primers fdh_hmtBNative_puc.for and fdh-HmtBnat.rev followed by *in-vivo* ligation, resulting in plasmid pLM202. The P_{hmtB}-*fdh*_{Z-245} cassette was

amplified *via* PCR with primers hmtB_mod_pacl.for and fdh_spacer_AscI.rev to add the required restriction sites and ligated into the cargo-module site of vector pMVS1111A:P_{synth}-bgaB *via* restriction/ligation with PacI and AscI restriction enzymes, resulting in plasmid pMVS1111a:P_{hmtB}-fdh_{Z-245}.

1.3.4. Cloning of pMVS-V2

The P_{hmtB}-fdh_{Z-245} cassette was cut from pLM202 with FseI and AscI and ligated into the *M. thermautotrophicus* selection marker module of pMVS-V1, resulting in plasmid pMVS-V2.

1.3.5. Cloning of CRISPR plasmids

1.3.5.1. Design of sgRNA spacers

The spacer sequence of the sgRNA was designed in SnapGene. The target gene was searched for sequences matching the PAM of ThermoCas9 (5'-NNNNCNAA-3'), and the 20 bps upstream of the matching sequence were taken as spacer sequence. The spacer sequences were then checked for potential off-targets by an algorithm written by Dr. Isabella Casini adapted for the *M. thermautotrophicus* ΔH genome sequence (Isabella Casini, unpublished results). The algorithm searches the genome of *M. thermautotrophicus* ΔH for sequences that match the spacer with up to three mismatches and that are in front of the PAM sequence. Three mismatches were chosen as the cutoff since this is the highest number of mismatches in the spacer sequence that still resulted in detectable ThermoCas9 activity in the initial testings of the enzyme (Mougiakos *et al.* 2017). The algorithm did not predict any additional targets except the desired ones for all spacer sequences used in this dissertation.

1.3.5.2. Cloning

The first CRISPR module (CRISPR1) was designed *in silico* and completely synthesized (ThermoFischer). It contained the codon-optimized *thermocas9* gene, an *hpt* targeting sgRNA, and the repair template and counterselection marker placeholders. The synthesized module was delivered in a cloning vector, pMA-CRISPR1. Plasmid pMVS1111a:P_{synth}-bgaB was amplified with primers pMESpel.for and Gibson_Pac_pME_AscI to add the required SpeI and AscI restriction sites. The PCR product and pMA-CRISPR1 were digested with SpeI and AscI and ligated together, resulting in plasmid pMVS1111a:CRISPR1. The neomycin resistance cassette of the vector was then replaced with the *fdh* operon from pMVS-V2 *via*

digestion with FseI and AscI and subsequent ligation, resulting in plasmid pMVS1112a:CRISPR1.

The non-targeting sgRNA for module CRISPR2 was synthesized as gBlock. The sgRNA was cloned into pMA-CRISPR1 *via* restriction with PacI and AvrII, resulting in plasmid pMA-CRISPR2. pMVS1112a:CRISPR1 and pMA-CRISPR2 were digested with SpeI and AscI and ligated together, resulting in plasmid pMVS1112a:CRISPR2.

The *upt* repair template for the CRISPR3 module was amplified from plasmid pMVS-*upt_flankonly* with primers *upt_up_PacI* and *upt_down_MluI* to add the required restrictions sites and ligated into the repair template submodule of vector pUC57-CRISPR2 *via* restriction/ligation with MluI and PacI restriction enzymes, resulting in plasmid pUC57-CRISPR3a. The *upt*-targeting sgRNA for module CRISPR3 was synthesized as gBlock. The sgRNA was cloned into pUC57-CRISPR3a *via* restriction with PacI and AvrII, resulting in plasmid pUC57-CRISPR3. pMVS1112a:CRISPR1 and pUC57-CRISPR3 were digested with SpeI and AscI and ligated together, resulting in plasmid pMVS1112a:CRISPR3.

To exchange the promoter of the *upt*-targeting sgRNA from module CRISPR3 was cloned into pMiniT with the NEB PCR cloning kit, resulting in plasmid pMCG_Upt_rev. We exchanged the promoter of the sgRNA *via* inverse PCR with primers *upp_guide_pSynth.for* and *upp_guide_pSynth.rev* followed by *in-vivo* ligation, resulting in plasmid pMCG_Upt_P_{synth}. The sgRNA was cloned into pUC57-CRISPR3 *via* restriction with PacI and AvrII, resulting in plasmid pUC57-CRISPR5. pMVS1112a:CRISPR1 and pUC57-CRISPR5 were digested with SpeI and AscI and ligated together, resulting in plasmid pMVS1112a:CRISPR5.

To increase the repair template size of CRISPR5, the 250 bp upstream of *upt* repair template was amplified from the *M. thermautotrophicus* ΔH genome *via* PCR with primers *uppUP_pUC_CRISPR5.for* and *uppUP_pUC_CRISPR5.rev*. Plasmid pUC57-CRISPR5 was opened just next to the upstream side of the repair template with primers *pUC_CRISPR5_uppUP.for* and *pUC_CRISPR5_uppUP.rev*. The two fragments were ligated together *via* Gibson assembly, resulting in plasmid pUC57-CRISPR6a. The procedure was repeated for the downstream site, with primers *uppDOWN_pUC_CRISPR6a.for* and *uppDOWN_pUC_CRISPR6a.rev* used to amplify the 250 bp downstream from the *M. thermautotrophicus* ΔH genome and

primers pUC_CRISPR6a_uppDOWN.for and pUC_CRISPR6a_uppDOWN.rev used to linearize pUC57-CRISPR6a. The fragments were ligated *via* Gibson assembly, resulting in plasmid pUC57-CRISPR6. pMVS1112a:CRISPR1 and pUC57-CRISPR6 were digested with SpeI and AscI and ligated together, resulting in plasmid pMVS1112a:CRISPR6.

To generate the sgRNA for module CRISPR8, we exchanged the promoter of the sgRNA on plasmid pMCG_Upt_rev *via* inverse PCR with primers mth60_sgRNA.for and mth60_sgRNA.rev followed by *in-vivo* ligation, resulting in plasmid pMCG_mth60. The sgRNA was cloned into pUC57-CRISPR5 *via* restriction with PacI and AvrII, resulting in plasmid pUC57-CRISPR8a. To create the repair template, the neomycin resistance cassette of plasmid pCF702 was removed by inverse PCR with primers pCF702_KanRdel.for and pCF702_KanRdel.rev followed by *in-vivo* ligation. The resulting plasmid, pCF702_kanRdel, has the flanking regions of the *mth60* operon fused together. The fused flanking regions were amplified *via* PCR with primers mth5961_pacI.for and mth58_60_MluI.rev, and the PCR product was digested with AvrII and PacI and ligated into the repair template submodule of pUC57-CRISPR8a, resulting in pUC57-CRISPR8. pMVS1112a:CRISPR5 and pUC57-CRISPR8 were digested with SpeI and AscI, and the fragments were ligated together, resulting in plasmid pMVS1112a:CRISPR8.

The two sgRNAs for module CRISPR11 were synthesized together as one gBlock. The sgRNAs were cloned into pUC57-CRISPR8 *via* restriction with PacI and AvrII, resulting in plasmid pUC57-CRISPR11. pMVS1112a:CRISPR6 and pUC57-CRISPR11 were digested with SpeI and AscI and ligated together, resulting in plasmid pMVS1112a:CRISPR11.

1.4. Genetic manipulations of *M. thermautotrophicus* ΔH

1.4.1. Conjugation

Conjugations with neomycin-selective conditions were done as previously described (Fink *et al.* 2021). The conjugation of pMVS1111a:*P_{hmtB}-fdh_{Z-245}* was performed the same way, except that a formate-selective transfer was also done in addition to the neomycin selective transfer. When grown, 1 mL of the recovery culture was transferred

into formate-selective medium (I.1.1) and plated under formate-selective conditions (I.1.1).

All further conjugations with the *fdh* operon as the selection marker were done using an adapted protocol. *M. thermautotrophicus* Δ H cells were kept in an anoxic environment for all steps. 8.25 ml of an overnight *M. thermautotrophicus* Δ H culture was concentrated to 0.25 ml. A 10 mL overnight culture of *E. coli* S17-1 carrying the plasmid of interest was washed once with fresh LB medium and then resuspended with the concentrated *M. thermautotrophicus* Δ H culture. The mixture was spotted on LB-MS plates in four spots and left to dry. After drying, two paper clips were applied, and the plates were placed in custom-made stainless-steel jars (Fink *et al.* 2021) in an H₂/CO₂/H₂S (79.9/20/0.1 vol-%) atmosphere at 60°C for 24 hours. The next day, spots were washed off the plates with 1 mL of MS medium and transferred into the recovery medium. Two recovery cultures were done per plate. The recovery medium was standard MS medium with formate supplements and 2 bars of H₂/CO₂/H₂S (79.9/20/0.1 vol-%). Recovery cultures were incubated at 60°C with shaking for two days and then transferred into formate-selective medium. The cultures were spot-plated on MS Agar plates with formate supplements when grown. After plating, paper clips were added to the plates for better ventilation. The plates were then incubated in custom-made stainless steel in an N₂/CO₂/H₂S (79.9/20/0.1 vol-%) atmosphere at 60°C. When single colonies became visible, they were picked into liquid formate-selective medium and further analyzed. For the conjugation of pMVS1112a-CRISPR6 and pMVS1112a-CRISPR11, *M. thermautotrophicus* Δ H and *E. coli* mating time was increased to 72 hours.

1.4.2. Electroporation

This describes the standard electroporation protocol for the electroporations with a capacitance of 10 μ F. *M. thermautotrophicus* Δ H cells were kept in an anoxic environment for all steps. 20 mL of a grown overnight culture of *M. thermautotrophicus* Δ H was washed three times with a sterile, anaerobic 10% glycerol solution and concentrated to 200 μ L, and 2 μ g of plasmid DNA was added. The cells were transferred into an electroporation cuvette with a gap width of 2 mm and electroporated with the “Ech2” setting (2.5 kV, 10 μ F, 600 Ω) of the BioRad micropulser electroporator. Cells were immediately transferred into a fresh serum bottle of MS

medium and incubated for 24 hours at 60°C and 150 rpm. Then, 1 mL of the recovery cultures was transferred into a neomycin-selective media bottle with 250 µg/ml neomycin for pMVS-V1 or in formate-selective medium bottle (as described in section III.1.4.1) for pMVS-V2. When grown, cultures were transferred two more times and then analyzed *via* colony PCR.

The BioRad micropulse electroporator has a fixed capacitance. Experiments with a capacitance of 25 µF were, therefore, done with the BioRad GenePulser II at a neighboring laboratory. However, this electroporator was not in an anoxic environment. Therefore, the electroporation protocol had to be adapted. Inside the anaerobic chamber, 20 mL of a grown overnight culture of *M. thermautotrophicus* ΔH was washed three times with a sterile, anaerobic 10% glycerol solution and concentrated to 200 µL, and 2 µg of plasmid DNA was added. The cell suspension was placed in an anoxic 1.5 mL reaction tube and brought to the electroporator. Here, the tube was opened, and the cells were quickly transferred into the electroporation cuvette (2 mm gap width), electroporated (2.5 kV, 25 µF, 600 Ω), and immediately transferred to a fresh, anoxic serum bottle with MS medium. All following steps were completed as described for the lower capacitance.

1.5. Genetic analysis of *M. thermautotrophicus* ΔH

1.5.1. Colony PCR

100 µL of a grown *M. thermautotrophicus* ΔH culture were boiled for twelve minutes at 100 °C in a heating block. Afterward, the following mixture was prepared.

Table 13: Colony PCR sample mix

Compound	Volume [µL]
2x Phire Plant Direct PCR Master Mix	5
Primer 1 (10 µM)	1
Primer 2 (10 µM)	1
Boiled <i>M. thermautotrophicus</i> ΔH sample	1
Nuclease free water	2
Total volume	10

The mixture was placed in a PCR cycler and run with the following program.

Table 14: Colony PCR protocol

Step	Temperature [°C]	Time	Number of cycles
Initial denaturation	98	2 min	1
Denaturation	98	20 s	35
Annealing	52	15 s	
Extension	72	30 per 1000 bp	
Final extension	72	60 s	1
Hold	4	Indefinite	1

Afterward, the sample was loaded onto an agarose gel for analysis (section III.1.2.11).

1.5.2. gDNA extraction

Extraction was performed the same way for *M. thermautotrophicus* ΔH and *M. marburgensis*. 20 mL of a grown overnight culture were harvested *via* centrifugation (3700 g, 15 min). The gDNA was isolated with NucleoSpin® Microbial DNA extraction Kit from MACHEREY-NAGEL according to the manufacturer's description. Cell lysis was performed with the glass beads supplied with the kit in the FastPrep™-24 5G Bead-Beater, which was run with the „wastewater“(6 m/s, 40 s) program. DNA was eluted in 50 µL elution buffer.

1.5.3. Plasmid purification

Plasmid purification was done according to Fink *et al.* (2021). The purification was achieved with the Plasmid Mini Kit (Qiagen) with some adaptations for *M. thermautotrophicus* ΔH. 10 mL of an *M. thermautotrophicus* ΔH culture carrying a shuttle vector was harvested by centrifugation (3700 rpm, 15 min, RT). The pellet was resuspended in P1 buffer with 30 % Sucrose. 2 µL of purified PeiP was added and incubated for 25 min at 60°C in a shaking incubator. 250 µL of buffer P2 was added, and all the following steps were performed according to the kit's manufacturer's specifications. Plasmids were eluted in 30 µL elution buffer. Because the plasmid yields were low (5 - 10 ng/µL, 10 µL of the purified plasmid were retransformed into *E. coli* (section III.1.2.8.). Plasmids were purified from *E. coli* clones (section III.1.2.9) and analyzed *via* restriction digest (section III.1.2.10) and sequencing (section III.1.2.12). The PeiP enzyme was purified from *E. coli* BL21(DE3) pME2508 as previously described (Fink *et al.* 2021).

1.5.4. Storage

Genetically modified *M. thermautotrophicus* Δ H strains confirmed by sequencing were prepared for long-term storage. 1 mL of a fresh overnight culture of the respective *M. thermautotrophicus* Δ H strain was transferred to a serum bottle containing 2 mL of an anoxic and sterile 50 vol% glycerol solution. The serum bottle was then barcoded and stored in our group's anaerobic culture collection at -80°C. All strains were stored in triplicates.

2. Materials

2.1. Strains

2.1.1. *E. coli* strains

Table 15: *E. coli* strains used in this study

Strain	Source
<i>E. coli</i> NEBstable	New England Biolabs
<i>E. coli</i> NEBstable puc57-P _{synth} - <i>fdh</i> _{Z-245}	This study
<i>E. coli</i> NEBstable puc57-P _{synth} - <i>ileS</i>	This study
<i>E. coli</i> NEBstable pUC57-MupR	This study
<i>E. coli</i> NEBstable pMVS1111a:MupR	This study
<i>E. coli</i> NEBstable puc57-P _{synth} - <i>pac</i>	This study
<i>E. coli</i> NEBstable pMVS1111a:P _{synth} - <i>pac</i>	This study
<i>E. coli</i> NEBstable pCF200	Fink <i>et al.</i> (2021)
<i>E. coli</i> NEBstable pCF201	Fink <i>et al.</i> (2021)
<i>E. coli</i> NEBstable pCF204	Fink <i>et al.</i> (2021)
<i>E. coli</i> NEBstable pLM201	Fink <i>et al.</i> (2021)
<i>E. coli</i> NEBstable pLM202	Fink <i>et al.</i> (2021)
<i>E. coli</i> NEBstable pMVS1111a:P _{synth} - <i>bgaB</i>	This study
<i>E. coli</i> NEBstable pMVS1111a:P _{hmtB} - <i>fdh</i> _{Z-245}	Fink <i>et al.</i> (2021)
<i>E. coli</i> NEBstable pMVS-V2	This study
<i>E. coli</i> NEBstable pMA-CRISPR1	This study
<i>E. coli</i> NEBstable pMVS1111a:CRISPR1	This study
<i>E. coli</i> NEBstable pMVS1112a:CRISPR1	This study
<i>E. coli</i> NEBstable pMA-CRISPR2	This study
<i>E. coli</i> NEBstable pMVS1112a:CRISPR2.	This study
<i>E. coli</i> NEBstable pMVS-upt_flankonly	This study
<i>E. coli</i> NEBstable pUC57-CRISPR3a	This study
<i>E. coli</i> NEBstable pUC57-CRISPR3	This study
<i>E. coli</i> NEBstable pMVS1112a:CRISPR3.	This study
<i>E. coli</i> NEBstable pMCG_Upt_rev	This study
<i>E. coli</i> NEBstable pMCG_Upt_P _{synth} .	This study

<i>E. coli</i> NEBstable pUC57-CRISPR5	This study
<i>E. coli</i> NEBstable pMVS1112a:CRISPR5.	This study
<i>E. coli</i> NEBstable pUC57-CRISPR6a	This study
<i>E. coli</i> NEBstable pUC57-CRISPR6	This study
<i>E. coli</i> NEBstable pMVS1112a:CRISPR6	This study
<i>E. coli</i> NEBstable pMCG_mth60	This study
<i>E. coli</i> NEBstable pCF702	This study
<i>E. coli</i> NEBstable pCF702_kanRdel	This study
<i>E. coli</i> NEBstable pUC57-CRISPR8a	This study
<i>E. coli</i> NEBstable pUC57-CRISPR8	This study
<i>E. coli</i> NEBstable pMVS1112a:CRISPR8	This study
<i>E. coli</i> NEBstable pUC57-CRISPR11	This study
<i>E. coli</i> NEBstable pMVS1112a:CRISPR11	This study
<i>E. coli</i> S17-1	(Simon <i>et al.</i> 1983)
<i>E. coli</i> S17-1 pMVS1111a:MupR	This study
<i>E. coli</i> S17-1 pMVS1111a:Psnyth-pac	This study
<i>E. coli</i> S17-1 pMVS1111a:P _{hmtB} - <i>fdh</i> _{Z-245} .	Fink <i>et al.</i> (2021)
<i>E. coli</i> S17-1 pMVS-V2	This study
<i>E. coli</i> S17-1 pMVS1112a:CRISPR1	This study
<i>E. coli</i> S17-1 pMVS1112a:CRISPR2.	This study
<i>E. coli</i> S17-1 pMVS1112a:CRISPR3.	This study
<i>E. coli</i> S17-1 pMVS1112a:CRISPR5.	This study
<i>E. coli</i> S17-1 pMVS1112a:CRISPR6	This study
<i>E. coli</i> S17-1 pMVS1112a:CRISPR8	This study
<i>E. coli</i> S17-1 pMVS1112a:CRISPR11	This study
<i>E. coli</i> OneShot BL21(DE3)	Thermo Fisher Scientific Inc., Massachusetts, US
<i>E. coli</i> JM110 competent cells	Agilent, Santa Clara, US
<i>E. coli</i> Stellar chemically competent cells	Takara Bio Europe, Saint-Germain-en-Laye, France

2.1.2. *Methanothermobacter* strains

Table 16: *Methanothermobacter* strains used in this study

Strain	Source
<i>M. thermautotrophicus</i> ΔH	DSM 1053, DSMZ, Braunschweig, Germany
<i>M. thermautotrophicus</i> Z-245	DSM 3720, DSMZ, Braunschweig, Germany
<i>M. marburgensis</i>	DSM 2133, DSMZ, Braunschweig, Germany
<i>M. thermautotrophicus</i> ΔH pMVS1111a:P _{hmtB} - <i>fdh</i> _{Z-245} .	Fink <i>et al.</i> (2021)
<i>M. thermautotrophicus</i> ΔH pMVS1111a:MupR	This study
<i>M. thermautotrophicus</i> ΔH pMVS1111a:Psnyth-pac	This study
<i>M. thermautotrophicus</i> ΔH pMVS-V2	This study
<i>M. thermautotrophicus</i> ΔH pMVS1112a:CRISPR1	This study

<i>M. thermautotrophicus</i> ΔH pMVS1112a:CRISPR2	This study
<i>M. thermautotrophicus</i> ΔH pMVS1112a:CRISPR6	This study
<i>M. thermautotrophicus</i> ΔH pMVS1112a:CRISPR11	This study

2.2. Primers and gBlocks

Primers were ordered from IDT (Leuven, Belgium) or Sigma Aldrich (Taufkirchen, Germany).

Table 17: Primers used in this study

Primer	Sequence
Gibson_IleS-puc57.rev	TTCGGTTCGGGCGCTGCGGGTCGTGGGGCGGGCGTCACACCTTATCTGAAGGTT - 3'
Gibson_IleS-puc57.for	AGATAATATCACGGTAACCACCGGCTAGCAGGTGATGCATATGCCAATCCAGGAAG
Gibson_puc57-IleS.for	ATAATATCCATAAAACCTTCAGATAAGGTGTGACGCCCCGCCACG
Gibson_puc57-IleS.rev	TCACATGGGGCTTGTAGGATTTTTCGGCTTCCTGGATTGGCATATGCATCACCTGCTAGC
Mutant-Primer_582.for	CTCATGCACGATTTACCCCTT
Mutant-Primer_582.rev	CAAGGGTGAAATCGTGCATGAG
Mutant-Primer_589_3.0.A.for	CATGCACGATTTACCCCTTGATGAAGAGGACAGGAAGATGAGCAAGTCCCTGGGGA
Mutant-Primer_589_3.0.A.rev	CGTTCCCCAGGGACTTGCTCATCTTCCTGTCCTCTTCATCAAGGGTGAAATCGTGCA
Seq_IleS_1.for	CATGAGAAGGAGGACCC
Seq_IleS_1.rev	GGGTGTTGTTGTCCATAC
Seq_IleS_2.for	CAGCATACACGTTGTTGG
Seq_IleS_2.rev	CCTGTGGATGAAGTCCC
Seq_IleS_3.for	CCTACCACAGCCTCTAC
Seq_IleS_3.rev	GGTGCTATGGGTGATAG
pac_pSynth.for	ATATAGTAATAGATAATATCACGGTAACCACCGGCTAGCAGGTGATGCATATGACCGAGTACAAGCCCACC
pac_pSynth.rev	GCTAGCCGGTGGTTACCGTGATATTATCTATTACTATATCCCTATA TAAAGAATTTGAATACTCAAAAAATGGGCGGC
hmtB_mod_pacl.for	GCTTAGCGCATTAATTAACCGCCATTTTTTGTAGTATTC
pac_ascl.rev	GAGCTACGTAGGCGCGCCGTCGTGCGCTCCTTTTCGG
Gibson_fdh.FOR	TTATGATGTATATATCAAAAAAATAGGAGTGATTCTCATGGGATCATCTTTTAAATCGCC
Gibson_fdh.rev	TCCTTTTCGGTTCGGGCGCTGCGGGTCGTGGGGCGGGCGTCATGAATCATTATGCAGGACC
Gibson_puc57_fdhv2.f or	TAACAGCGGCGCTATCAAGGTCCTGCATAATGATTCATGACGCCCCGCCCCACG
Gibson_puc57_fdh.RE V	TTTGCCGTATCTGCAGGCGATTTAAAAGATGATCCCATGAGAATCACTCCTATTTTTTTTG
fdh_hmtBNative_puc.f or	CGATATATTTATATAGGATTATATGAATAGATAATATCACATAAAATGAGGTGGTTAATTATGGGATCATCTTTTAAATCGCCTGCAG
fdh-HmtBnat.rev	ATCCTATATATAAATATATCGCTAATTTTAAGGTTTTTCTGAGCCATCGGTTGGTTCATGGGTAAATTAAGAATACTCAAAAAATGGGCG
Gibson_puc57_fdhv2.F OR	TAACAGCGGCGCTATCAAGGTCCTGCATAATGATTCATGACGCCCCGCCCCACG

Gibson_puc57_fdh.RE V	TTTGCCGTATCTGCAGGCGATTTAAAAGATGATCCCATGAGAATCACTCCTATTTTTTTTG
fdh_spacer_AscI.rev	ATCGGCTTAGGCGCGCCTGCTCATCGTCAATTCTAGTAGAGTCATGAATCATTATGCAGG
pMESpel.for	CATCAGTACACTAGTCCCGCAAAAAAATGCAATAAAATTTGGG
Gibson_Pac_pME_AscI	ACAAGAAAAGGCGCGCCCTGACAGAATTGCCCCC
upt_up_PacI	GATGCTGAGCTTAATTAAGAGGAACTTCTACTGGAGCAC
upt_down_MluI	GCTATACGTAGCTAGCACGCGTGTATCGGTCTCATCCTGC
upp_guide_pSynth.for	AGATAATATCACGGTAACCACGGGCAGCAGGTGATGCATCCTCACGCCATTTAAGGGTGGTCATAGTTCCCCCTG
upp_guide_pSynth.rev	GGTTACCGTGATATTATCTATTACTATATCCCTATATAAAGAGAGTTAATTAACGCATCTCGATCTGATAATAATTAATTAAGACATATAAAGAGAGTTAATTAAGGGTGTCTCTGTTCCAGGG
uppUP_pUC_CRSIPR5.FOR	
uppUP_pUC_CRSIPR5.REV	TGCTCCAGTAGAAGTTCCTCCTGGCAACAAGACCA
pUC_CRSIPR5_uppUP.FOR	TTCATTGGTCTTGTTGCCAGGAGGAACTTCTACTGGAGCACGG
pUC_CRSIPR5_uppUP.REV	GTCCCTGGAACAGGACACCCTTAATTAACCTCTCTTTATATAGGGATATAGTAATAGATAATATCACGG
uppDOWN_pUC_CRSIPR6a.FOR	GGGCAGGATGAGACCGATACCGCCCATCCCAGCTC
uppDOWN_pUC_CRSIPR6a.REV	GCCCCGGAAGTGCCTACGCGTTCAACCATAGTGGGCACCAGG
pUC_CRSIPR6a_uppDOWN.FOR	CTGGTGCCCACTATGGTTGAACGCGTAGGCAGTTCCG
pUC_CRSIPR6a_uppDOWN.REV	TCGAGGAGCTGGGATGGGCGGTATCGGTCTCATCCTGCCCCG
mth60_sgRNA.for	GTGCCGTCCTCTGTGAATGGGGTCATAGTTCCCCTGAGATTATCGCTGTGGTATAATG
mth60_sgRNA.rev	CCATTACAGAGGACGGCACAATGCATCACCTGCTGCCGGTGGTTACC
mth5961_pacl.for	TGCAACTGTTAATTAAGGTACTGTGTGAGGGTCATATTC
mth58_60_MluI.rev	GGCATCATGCAGTACGCGTCCGGGCCATAACACATACC
pCF702_KanR_del.rev	CTTCTTACGGAGAAATCCCAACCTTCATATAATATTGCAAGAACACC
pCF702_KanRdel.for	TTGGGATTTCTCCGTAAGAAGGGAATTGAACCTCCAATC
Seq_Cas1.for	GGGACGATCTGGAGAGG
Seq_Cas1.rev	GATATGTATTTCGTGCTCGAAG
Seq_Cas2.fw	CACACAGGCCAGGAAAG
Seq_Cas2.rev	GTATTGAGACTGGTGATCCG
Seq_Cas3.for	GCCTTCTACCAGAGGAGG
Seq_Cas3.rev	GGAGCTCATCTGCGAAG
Seq_Cas4.FOR	GGATCTTTTCGCCTATTATCAG
Seq_Cas4.rev	CGATTGACCTGAGGGAG
Seq_fdh_pME	GATTTCTGGAATCCGCCATGGG
Seq_FdhA_begin.for	CTAATAGTCGCCGATCCAAG
Seq_FdhA_begin.rev	GGTTCCTGGCTTGAATG
Seq_FdhA_mid.for	GAGAAGCAAAGGATGACTG
Seq_FdhA_mid.rev	CAGCACCCATCTTATTCG
Seq_FdhB_begin.for	GCAGTTAAGAAGGGTTTCG
Seq_FdhB_begin.rev	GGCTCCGTTATAAGGGTTG
Seq_FdhB_end.for	CTGAATGGATCGAGAAAGG

Seq_FdhB_end.rev	CATTCTTTTCGAGATGGAAG
Seq_FdhC_mid.for	CCTATATTCGCATTTCGTGG
Seq_FdhC_mid.rev	ATGTTTGGCCACACTGTG
Seq_pacR_kanaR_RV	CTGACAGAATTGCCCCAGTG
Seq_pME_pUC_713	GGTGGGGTTTTGGTGTGCG
Seq_sgRNA.rev	GGCATACGCGCCTAAG

The CRISPR1 module was ordered from ThermoFisher. All other gBlocks were ordered from IDT.

Table 18: gBlocks used in this study

CRISPR1 module
ACTAGTGATCTATTTATATAGGGATATAGTAATAGATAATATCACGGTAACCACCGGCTAGCAGGTGATGCAT ATGAAGTATAAGATAGGACTCGATATAGGAATCACCTCAATAGGGTGGGCAGTGATAAACCTCGATATACCCA GGATAGAAGACCTGGGTGTCTAGGATATTTCGATAGGGCAGAAAATCCCAAGACAGGTGAATCACTCGCACTCCC CAGGAGGCTCGCCAGGTACGCAAGGAGGAGGCTCAGGAGGAGGAAGCACAGGCTGGAAAGGATAAGGAGGCTT TTCGTGAGGAGGCTTACTGACCAAGGAAGAGCTGAATAAGCTCTTCGAAAAGAACGCAAAATCGACGTGT GGCAGCTCAGGCTTGAGGCCCTTGACAGGAAGCTCAACAACGATGAGCTCGCAAGGATACTGCTCCATCTTGC AAAGAGGAGGGGTTTCAGGTCAAATAGGAAATCAGAAAGGACAAACAAAGAAAATTCACCATGCTCAAGCAC ATAGAGGAAAACAGTCCATCCTGTCTATCCTACAGGACAGTCGCGAGAGATGGTGGTCAAGGATCCCAAATTCCT CACTCCACAAGAGGAATAAGGAGGATAATTACACAAAACACAGTGGCAAGGGACGATCTGGAGAGGGAAATAAA GCTTATCTTCGCCAAGCAGAGGGAGTATGGGAACATAGTCTGTACCGAGGCCTTCGAGCACGAATACATATCC ATATGGGCATCACAGAGGCCCTTCGCATCAAAGGACGATATCGAGAAGAAGGTTGGATTCTGCACCTTCGAGC CCAAGGAAAAAAGGGCCCCCAAAGCAACATACACCTTCCAGTCTTTCACCGTGTGGGAACATATAAACAAAGCT CAGGCTTGTTTTCACAGGTGGGATAAGGGCACTGACAGATGATGAAAGGAGGCTCATCTATAAGCAGGCATTC CATAAAAACAAAATAACATTCCACGACGTTAGGACCTCCTCAACCTGCCCAGCAGACACCAGGTTCAAGGGTC TTCTCTACGACAGGAACACCACACTCAAGGAAAATGAAAAAGTGAGGTTCTTGGAACTGGGTGCATACCACAA AATAAGGAAAGCCATCGACTCCGTCTATGGTAAAGGGGCAGCCAAGTCATTCAGGCCAATAGATTTCGACACA TTTCGGTTACGCACTGACAATGTTCAAGGACGACACAGACATAAGGTCATACCTCAGGAATGAATACGAGCAGA ACGGGAAGAGGATGGAGAACCTTGCCGATAAGGTGTATGACGAAGAGCTCATCGAGGAACCTTCTCAACCTCTC CTTCTCAAAGTTTCGGACACCTCTCCCTCAAGGCACTCAGGAACATACTTCCATACATGGAGCAGGGAGAGGTC TACTCAACCGCATGCGAAAGGGCAGGTTACACATTACAGGACCCAAAAAGAAACAGAAGACCGTTCTGCTTC CCAACATACCACCAATAGCAAACCCCGTTGTTATGAGGGCCCTCACACAGGCCAGGAAAGTTGTTAACGCAAT AATAAGAAATACGGATACCACTCAGTCTCAATACATATAGAGCTGGCCAGGGAGCTCTCCAGTCATTTCGACGAA AGGAGGAAAATGCAGAAAGAGCAGGAAGGTAACAGGAAAAAGAACGAGACAGCAATAAGGCAGCTCGTTGAAT ATGGTCTCACCTTAATCCAACAGGACTCGACATCGTCAAATTCAGCTCTGGTCAGAGCAGAACGGTAAATG CGCATACTCACTTCAGCCAATCGAAATAGAGAGGCTTCTCGAGCCCGGATACACAGAGGTCGACCATGTGATC CCCTACTCAAGGTCCCTTGATGACTCATACACAAATAAAGTGCTGGTCCCTCACAAAGGAAAAACAGGGAGAAGG GGAACAGGACACCCGAGAGTACCTCGGTCTCGGGTCAGAAAGGTGGCAGCAGTTTCGAAACCTTCGTTCTCAC CAACAAACAGTTCTCCAAGAAGAAGAGGGACAGGCTCCTGAGGCTGCATTACGATGAAAACGAGGAGAACGAA TTCAAGAACAGGAATCTGAATGACACAAGGTACATATCCAGGTTCTTCGCCAATTCATCAGGGAACATCTTA AATTTCGAGATTTCAGATGATAAGCAGAAGGTGTACACCGTTAACGGAAGGATAACAGCCCACCTCAGGTCAAG GTGGAATTTCAACAAGAACAGGGAGGAGTCAAATCTCCATCATGCCGTGGATGCCGCAATAGTGGCATGTACA ACACCCTCAGATATCGCCAGGGTTACCGCCTTCTACCAGAGGAGGGAGCAGAACAAAGAGCTTTCAAAAAAGA CAGACCCACAGTTCCCCCAGCCCTGGCCCCACTTCGCAGATGAGCTCCAGGCAAGGCTTTCCAAGAACCCCAA AGAATCAATCAAGGCCCTTAACCTGGGGAACTACGATAACGAAAAGCTTGAGTCACTCCAGCCCGTGTTCGTC TCCAGGATGCCAAAAGGTCCATAACAGGTGCAGCACATCAGGAGACACTTAGGAGGTACATCGGAATCGATG AGAGGTCCGGGAAAATACAGACCGTCGTGAAGAAAAAGCTTTTCAGAGATACAGCTGGACAAGACAGGTCACCT CCCAATGTACGGAAAAGAGTCAGACCCAGGACCTATGAGGCAATAAGGCAGAGGCTCCTTGAGCATAACAAT GACCCCAAGAAGGCCTTCCAGGAGCCCCCTCTACAAGCCAAAGAAAAACGGGGAACTGGGACCCATCATAAGGA CCATCAAGATAATAGATACAACCAACAGGTGATCCCACTGAACGATGGAAAGACCGTGGCCTACAACCTCAA TATAGTCAGGGTCGATGTTTTTCGAGAAGGACGGAAGTACTATTGCGTCCCAATATACACCATAGACATGATG AAGGGTATCCTGCCAAACAAAGCCATAGAGCCCCAACAAACCTACTCAGAGTGGAAGGAAATGCAGGAAGATT ACACATTTCAGGTTCTCACTGTACCCCAATGACCTTATAAGGATAGAGTTCCCCAGGGAGAAAAACCATAAAGAC AGCCGTGGGTGAGGAAATAAAAAATAAGGATCTTTTCGCCTATTATCAGACAAATAGATTCTCAAATGGTGG CTGTCACTTGTTTTCCACGACAACAACCTTCTCCCTCAGGTCAATCGGTTCCAGGACACTGAAAAGGTTTCGAAA AGTACCAGGTTGATGTTCTTGGGAACATATACAAAGTTAGGGGAGAGAAAAGGTTGGGGTCGCCCTCATCATC

ACACTCCAAGGCAGGGGAAACAATAAGGCCCTGTAACTACTGACTTCAGATTAACGAAGGTGCATTGCA
CAAACCTTTTAAAGTGTAAATGCACCTAATTTTTTTTATGATCTACCTAGGGCATAACGCGCTAAGAGTGGGGAA
TGCCCGAAGAAAGCGGGCGATAGGCGATCCCCAACGCCACGGGTCAGTCTGCCTATAGGCAGAAAGCCCTTAT
CATAGTAACCTGAGATCATTGCTGTGGTATAACTTTTCATTATACCACAGCGATAATCTCAGGGGAACATATGA
CGGGGTATGCCATCGGTTACAGGGATGCATCACCTGCTAGCCGGTGGTTACCGTGATATTATCTATTACTATA
TCCCTATATAAAGAGAGTTAATTAACGCATCTCGATTGCTCGGTGCGCTTTTCACACTCCGCGAAAAATTCATAC
CGCTCATTCACTAGGTTGCGAAGCCTACACTGATATATGAATCCAAGCTAGAGCAGGGCTCTTAAAAATTCGGA
GTTGTAGAAGGGTTTGACATCGAGAAACAACGCGTAGGCAGTTCCGGGCTGAAAGTAGCGCCGGGTAAAGGTAA
TCGGGTGGACGGAACGCAGAATTATGGTTACTTTTTGGATACGTGAAACATGTCCCATGGTAGCCCCAAGAC
TTGGGAGTCTATCACAGGGCCCATTTCTGGATATAGGCGCGCCGGTACCTC

non-targeting sgRNA

TTAATTAACCTCTCTTTATATAGGGATATAGTAATAGATAATATCACGGTAACCACCGGCTAGCAGGTGATGCA
TCGGTGAAGAATTCGGGTAAACGGTCATAGTTCCCTGAGATTATCGCTGTGGTATAATGAAAGTTATACCACA
GCAATGATCTCAGGGTTACTATGATAAGGGCTTTCTGCCTATAGGCAGACTGACCCGTGGCGTTGGGGATCGC
CTATCGCCCGCTTTCTTCGGGCATTCCCCACTCTTAGGCGCGTATGCCCTAGGTAGATC

upt-targeting sgRNA

ATCGAGATGCGTTAATTAACCTCTCCCCATGAACCAACCGATGGCTCAGAAAAACCTTAAAAATTAGCGATATAT
TTATATAGGATTATATGAATAGATAATATCACATAAAATGAGGTGGTTAATTCCTCACGCCATTTAAGGGTGG
TCATAGTTCCCTGAGATTATCGCTGTGGTATAATGAAA

mth60-targeting double sgRNA

ATCGAGATGCGTTAATTAACCTCTCTTTATATAGGGATATAGTAATAGATAATATCACGGTAACCACCGGCAGC
AGGTGATGCAATTAAAGTATGGACGTTTCCATGTCATAGTTCCCTGAGATTATCGCTGTGGTATAATGAAAGT
TATACCACAGCAATGATCTCAGGGTTACTATGATAAGGGCTTTCTGCCTATAGGCAGACTGACCCGTGGCGTT
GGGGATCGCCTATCGCCCGCTTTCTTCGGGCATTCCCCACTCTTAGGCGTTTATATAGGGATATAGTAATAGA
TAATATCACGGTAACCACCGGCAGCAGGTGATGCATGGGGAATCTTCACAGCCGCGGTCATAGTTCCCTGAG
ATTATCGCTGTGGTATAATGAAAGTTATACCACAGCAATGATCTCAGGGTTACTATGATAAGGGCTTTCTGCC
TATAGGCAGACTGACCCGTGGCGTTGGGGATCGCCTATCGCCCGCTTTCTTCGGGCATTCCCCACTCTTAGGC
GCGTATGCCCTAGGTAGATCATAA

2.3. Chemicals

Table 19: Chemicals used in this study

Chemical	Manufacturer
6-azauracil	VWR International, Darmstadt, Germany
6-methylpurine	Sigma-Aldrich, Taufkirchen, Germany
8-azahypoxanthine	Thermo Fisher Scientific Inc., US
Agar-Agar Kobe I	Carl Roth, Karlsruhe, Germany
Agarose	Sigma-Aldrich, Taufkirchen, Germany
Aluminium chloride hexahydrate	Chemical store of the university
Ammonium chloride	Carl Roth, Karlsruhe, Germany
Ammonium nickel(II) sulfate hexahydrate	VWR International, Darmstadt, Germany
Ampicillin (sodium salt)	Carl Roth, Karlsruhe, Germany
Boric acid	Fisher Scientific, Schwerte, Germany
Calcium chloride dihydrate	Carl Roth, Karlsruhe, Germany
Carbenicillin	Carl Roth, Karlsruhe, Germany
Chloramphenicol	Carl Roth, Karlsruhe, Germany
Cobalt(II)-chloride hexahydrate	Chemical store of the university
Copper (II) sulfate pentahydrate	Carl Roth, Karlsruhe, Germany
di-Potassium hydrogen phosphate	Sigma-Aldrich, Taufkirchen, Germany
EDTA (Ethylenediamine tetraacetic acid)	Carl Roth, Karlsruhe, Germany
Ethanol	Chemical store of the university

Glycerol	Sigma-Aldrich, Taufkirchen, Germany
Hydrogen chloride / Hydrochloric acid	Sigma-Aldrich, Taufkirchen, Germany
Iron (II) chloride pentahydrate	Merck, Darmstadt, Germany
Iron(III)-chloride	Sigma-Aldrich, Taufkirchen, Germany
L-Cysteine hydrochloride	Carl Roth, Karlsruhe, Germany
Lithium mupirocin	Sigma-Aldrich, Taufkirchen, Germany
Magnesium chloride hexahydrate	Merck, Darmstadt, Germany
Magnesium sulfate heptahydrate	Sigma-Aldrich, Taufkirchen, Germany
Manganese sulfate	Sigma-Aldrich, Taufkirchen, Germany
Neomycin (sulphate salt)	VWR International, Darmstadt, Germany
Nickel (II) chloride hexahydrate	Carl Roth, Karlsruhe, Germany
Peptone	Carl Roth, Karlsruhe, Germany
Potassium dihydrogen phosphate	Sigma-Aldrich, Taufkirchen, Germany
Puromycin dihydrochloride	Thermo Fisher Scientific Inc., Massachusetts, US
Resazurin (sodium salt, 0.025%)	Thermo Fisher Scientific Inc., Massachusetts, US
Sodium bicarbonate	Chemical store of the university
Sodium chloride	Merck, Darmstadt, Germany
Sodium molybdate dihydrate	Carl Roth, Karlsruhe, Germany
Sodium selenate	Thermo Fisher Scientific Inc., Massachusetts, US
Sodium sulfide	Carl Roth, Karlsruhe, Germany
Sodium tungstate dihydrate	Carl Roth, Karlsruhe, Germany
Trimethoprim	Sigma-Aldrich, Taufkirchen, Germany
Tris	Carl Roth, Karlsruhe, Germany
Tryptone	Carl Roth, Karlsruhe, Germany
Vanadium (III) chloride	Sigma-Aldrich, Taufkirchen, Germany
Yeast extract	VWR International, Darmstadt, Germany
Zinc chloride	VWR International, Darmstadt, Germany

2.4. Enzymes and Kits

Table 20: List of enzymes and kits used in this study.

Product	Manufacturer
JBS Error-Prone Kit	Jena Bioscience, Jena, Germany
NEB HiFI DNA Assembly Mix	New England Biolabs, Frankfurt, Germany
NucleoSpin® Microbial DNA	Macherey-Nagel, Düren, Germany
Phire Plant PCR Master mix	Thermo Fisher Scientific, Massachusetts, US
QIAprep Spin Miniprep Kit	Qiagen, Hilden, Germany
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
QuikChange mutagenesis kit	Agilent, Santa Clara, US
Q5 polymerase	New England Biolabs, Frankfurt, Germany
Restriction enzymes (HF versions were used when available)	New England Biolabs, Frankfurt, Germany
T4 Ligase	New England Biolabs, Frankfurt, Germany

2.5. Devices

Table 21: List of devices used in this study.

Instruments	Model	Manufacturer
Autoclave	Systec VX-95	Systec GmbH, Linden, Germany
Bench-top autoclave	CertoClav EL Autoklav	CertoClav, Leonding, Austria
Blender	T10 basic ultra turrax	IKA-Werke GmbH & Co. KG, Staufen, Germany
Centrifuge (big)	Centrifuge 5920 R	Eppendorf AG, 22331 Hamburg
Centrifuge (glove-box)	mySPIN12	Thermo Fisher Scientific Inc., Massachusetts, US
Centrifuge (small)	Centrifuge 5424	Eppendorf AG, 22331 Hamburg, Germany
Centrifuge (small)	Centrifuge 5427 R	Eppendorf AG, 22331 Hamburg, Germany
Clean-Bench	Sicherheitswerkbank HERAsafe KS 18	Unity Lab Services (Thermo Fisher Scientific Inc., Massachusetts, US)
Dispensette	Dispensette® S	Brand, Wertheim, Germany
Electroporator (low capacitance)	Micro Pulser	Bio-Rad Laboratories GmbH
Electroporator (high capacitance)	Gene Pulser II	Bio-Rad Laboratories GmbH
FastPrep Bead-Beater	FastPrep™-24 5G Bead-Beater	MP Biomedicals, Santa Ana, US
Gas burner	Gasprofi 1 micro	Carl Roth, Karlsruhe, Germany
Gel documentation	UVP GelStudio PLUS	Analytik Jena AG, Jena, Germany
Gel Electrophoresis Power source	EV2310 Electrophoresis Power Supply	Consort, Turnhout, Belgium
Glovebox	Glovebox-System UNIlab Pro SP Schleuse rechts/ MB 20 G	M Braun, Garching, Germany
Heat block (glove box)	H2O3-PRO	Coyote Bioscience Yixing Co, Beijing, China
Heaters and magnetic stirrers	ROTILABO® MH 15	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Heating blocks	Thermo Mixer C	Eppendorf AG, Hamburg, Germany
Hotplate/Stirrer	7X7 ALU HOT/STIR 230V ADV	VWR international, Leuven, Belgium
Incubator (37 °C shaker)	Lab Companion Incubated Shaker ISS-7100R	Jeio Tech, Daejeon, South Korea
Incubator (37 °C)	Incubator IN260	Memmert GmbH & Co. KG, Schwabach, Germany
Incubator (60 °C shaker)	Lab Companion Incubated Shaker ISS-7100R	Jeio Tech, Daejeon, South Korea
Incubators	Universalschrank Model 1060 (37° C)	Memmert GmbH & Co. KG, Schwabach, Germany
Microscope	BX41 Standard Laboratory Microscope with U-PCD2 Phase Contrast Condenser	Olympus, Shinjuku, Japan
Nanodrop	NanoPhotometer® NP80	Implen, München, Germany

PCR Thermo Cycler	Mastercycler® Pro S	Eppendorf AG, Hamburg, Germany
pH Meter	pHennomenal® MU 6100 L	VWR International, Pennsylvania, US
Photometer	BioMate™ 160 UV-Vis Spectrophotometer	Thermo Fisher Scientific Inc., Massachusetts, US
Pipettes	Eppendorf Research® plus	Eppendorf AG, Hamburg, Germany
Precision scale	TA 164i	VWR international, Leuven, Belgium
Scale	Analytical balance TA 164i	VWR International, Pennsylvania, US
Vortex	Vortex-Genie® 2	Scientific Industries Inc, New York
Water purification system	Barnstead™ GenPure™ Pro	Thermo Fisher Scientific Inc., Massachusetts, US

IV. Results

1. Establishment of additional selection markers

M. thermautotrophicus ΔH is an archaeon and a thermophile, which severely limits the number of possible selection markers. Still, there are several promising candidates, namely resistance to mupirocin, puromycin or simvastatin, and formate prototrophy (section 1.3). The resistance of wild-type *M. thermautotrophicus* ΔH against simvastatin was already tested, and while 21.5 $\mu\text{g/mL}$ inhibited growth for at least 48 hours, spontaneously resistant cells emerged afterward (Fink *et al.* 2021). Spontaneously resistant cells emerge even faster than with neomycin, which inhibits growth for at least 60 hours. Because one of the desired criteria for an additional selection marker was the absence of spontaneous wild-type growth, simvastatin was not considered further, and we focused on mupirocin, puromycin, and formate.

1.1. Mupirocin

For the establishment of mupirocin resistance as a selection marker, two conditions need to be met: **1)** The concentration of mupirocin that inhibits the growth of *M. thermautotrophicus* ΔH needs to be determined; and **2)** a suitable resistance marker needs to be generated.

1.1.1. *M. thermautotrophicus* ΔH is sensitive to mupirocin

We first determined the concentration of mupirocin required for sufficient growth inhibition of *M. thermautotrophicus* ΔH . 200 μL of a grown culture of *M. thermautotrophicus* ΔH was transferred into 20 mL of fresh MS medium with mupirocin concentrations between 1 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$. Without mupirocin, *M. thermautotrophicus* ΔH grew in a dense culture overnight. At the same time, all concentrations showed a slower growth than a control without mupirocin, but only the culture with 20 $\mu\text{g/mL}$ of mupirocin did not show visible turbidity after 72 hours. We also assayed the inhibition on plates. Spot-plating 100 μL of a stationary phase culture led to a microbial lawn at 10 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ of mupirocin. Therefore, we increased the concentration to 50 $\mu\text{g/mL}$, which inhibited growth on plates for at least two weeks, after which a small number of colonies became visible. Because higher

concentrations of mupirocin were required to inhibit growth on plates, these concentrations were used to test the putative mupirocin resistance.

1.1.2. Generation of a mupirocin resistance cassette (MupR) based on *ileS*

Mupirocin resistance is not conferred by a traditional resistance gene but by a mutated version of the target gene *ileS* (section 1.3.1). We designed a resistance gene based on resistance-conferring mutations described in literature for the *ileS* of *M. marburgensis* (Jenal *et al.* 1991) and *M. barkeri* (Boccazzi *et al.* 2000). The *ileS* gene is already present in the genome of *M. thermautotrophicus* Δ H. Thus, we decided to use the *ileS* gene from the closely related *M. marburgensis* to avoid the recombination of the resulting shuttle vectors into the genome. We then aligned the amino acid sequences of the *ileS* genes from *M. barkeri* and *M. marburgensis* to check whether the reported resistance-conferring mutations occurred on conserved residues (Figure 7). From the five mutations reported for the gene of *M. barkeri*, only two occur on residues conserved in the *M. marburgensis* gene (G587D and G594E). The other three mutations (A440V, A482T, and G593D) occur on amino acids not conserved between the two proteins. The reported mutation for *M. marburgensis* (G590D) is at the same residue as G594E. It should be noted that while the original report describes the residue as G590, the corresponding residue in the published genome of *M. marburgensis* is G589 (Liesegang *et al.* 2010, Casini *et al.* 2023).

To create a putative mupirocin resistance cassette (MupR), we amplified the *ileS* gene from the genomic DNA of *M. marburgensis*. We then cloned it under the control of the P_{synth} promoter, which is also used for the neomycin resistance cassette on the shuttle vector. The amino acids exchanges G582D and G589D (*M. marburgensis* nomenclature) were introduced *via* quick-change PCR of the entire vector. This resulted in an *ileS* gene leading to two amino acid substitutions and expression under the control of the P_{synth} promoter, which we designated “MupR”, short for mupirocin resistance. The MupR cassette was then cloned into the application module site of the pMVS1111a shuttle vector, resulting in vector pMVS1111a:MupR (Figure 8).

Query 2 IKEITAKYNAEQIEKKITQLWKSDAYRKTREHRKTGKRLFFVDGPPYTTGHIHLGTAWN 61
 I+E Y +IE+K+ W++ D Y + +E R+ R F+DGPPY +G IHLGTAWN
 Sbjct 3 IQEAEKSYRPHRIEEKVQSFEERDIYERVKELREDRPRYSFLDGPYCSGRIHLGTAWN 62

Query 62 KIIKDSILRYYSMNRRNILERPGWDMHGLPIEVKVEGVLFKSKKDIES-FGVENFIEKC 120
 KI+KD+ LR+ SM N+ +PGWD HGLPIE KVEG+LG +SKKDIE G+E F+ KC
 Sbjct 63 KIMKDTYLRFKSMKGFNVRRQPGWDTHTGLPIEHKVEGLLGVRSKKDIEDKIGIEEFVNKC 122

Query 121 KEFAINQKQEMTEQFQRLGVWVWQWEAPYMTLKDDYIEAAWTLKQASEKNLLDVGKRSVN 180
 +EFA+ K MT QFQRLGVWVW W+ PY+T Y+E+ WWTLK+A EK+LL R +
 Sbjct 123 REFAVENKAVMTGQFQRLGVWVWDDPYVTFDPAYMESCWTLKRAHEKDLLVRDLRVIT 182

Query 181 WCPRCETAIADSEVEYAERTDPSIYVKFKIKGEENTFIVITTTTPTWIPANVAVAVHPGF 240
 WCPRCETA+A +E++Y ++ DPSIYVKF + G +T I++WTTTPTWIPAN+AVAVHP F
 Sbjct 183 WCPRCETALALAEIDYHDKEDPSIYVKFPVSG--DTHILVWTTTPTWIPANMAVAVHPDF 240

Query 241 EYSKFRAIRQDGSDEILIAATDLIENVLRQGRYVDYEVLETMLGEDLTKEYESPVGDLV 300
 EY+ R DG E I A L+E VL + + E+L+ + G +L L Y P+ D V
 Sbjct 241 EYA---YARLDG---ETYIMAEALVEKVLGE----EAEILKRVKGTLEGLTYRHPLDDEV 291

Query 301 PVQNEIKHGIYLADYVT-AENTGCVHIAPIHGHGMDDFNVGVKYNLPILCPVGPNGAYTEEA 359
 P+ EI+H + L D+VT E TGCVH APGHG +DF +G KY L ++CPV G +TEEA
 Sbjct 292 PLHREIEHRVILGDHVTLEGTGCVHAPGHGPEDFEIGKKYGLEVICPVDEAGIFTEEA 351

Query 360 GEYAGKNVREANPIVIEDLRKRNRLLAEGTVTHRYGHCWRCKTPIIYLATEQWFLKVTDI 419
 G+Y+G+ V++A+ +I DLR + LL GT++HRYG CWRCKTPIIYLATEQWFLK+T+I
 Sbjct 352 GKYSGRFVKDADEDIADLRSGKLLKAGTISHRYGFCWRCKTPIIYLATEQWFLKITEI 411

Query 420 KDKMLEAIDAVDWPWAGSARFRTWVEGARDWCISRQRYWGMPIPVWCKKCKGKLEVIC 479
 KDKML +D V W P WAG +RFR W+E ARDW ISRQRYWG+PIP+W C++C + V+G
 Sbjct 412 KDKMLRELDRVQWVPSWAGSARFRNWIENARDWTISRQRYWGIPPIWICEECDSIHVVG 471

Query 480 TIRAELEKSGAGSDVE---LHRPYVDKLTIPC-ECGGEKKRVEDVFDVWFDSAVASWATL 535
 + EL E + G ++E +HRP+VD++ + C ECGG KR DV DVW DS VA WA L
 Sbjct 472 SDELRELAVEG-ELEGDFIHRPHVDRIVLECGECGGRMKRTPDVLDVWIDSGVAGWAAL 530

Query 536 KFPQTRQFDEWNPADFITEGHDQTRGWFFYSQLGASMVGFRAPYKSVLMHSTLDAAGK 595
 +P E F EW+P DFITEGHDQTRGWFFYSQLG ++ PY+ VLMHSTLDAAGK
 Sbjct 531 HYPSETELFREWFPPYDFITEGHDQTRGWFFYSQLGCGVIALDEVYRRVLMHSTLDAAGK 590

Query 596 KMSKSLGNVSPIDVIDKYGADTLRAYVLSSAPWDDLKFNQEEVENVHRSINILWNVFR 655
 KMSKSLGNV P DVI+KYGAD LR Y+L + PW+DLKF +E+ NV++ NILWNV+
 Sbjct 591 KMSKSLGNVPEPEDIKYGADVLRFYLLWENKFWEDLKFVWDELNRNVNKMFINILWNVV 650

Query 656 FPLPYMALDNFDPLKVSLSVDKDALREEDRWILSRISQSVVKAVDAMSGYFLHKAVREIL 715
 F YM+LD F P S + + + R+EDRWILSRISV V EA+ H+A REI
 Sbjct 651 FATTYMSLDRFQPGDHSADL--SFRDEDRWILSRINSVALKVTEAIENLHFHRTREIH 708

Query 716 EFTLEDLSRWYIQLIRPRTWTEADDPDKLAAYRVLYEVYVTLTKLISPFMPYLAEMYQN 775
 +F +EDLSRWYI+LIR RTW E DDPDKLAAY LY V TL +SP P++ E++YQN
 Sbjct 709 DFIVEDLSRWYIRLIRSRTWIERDDDPDKLAAYHTLYTVLTKLIVTLSPMAPHVCEDIYQN 768

Query 776 LIRNVDPNALESVHMCWDPKVNAYLDPLEAAMSTARSIVEAASNARQKAGRKLWVPS 835
 L+R +P++ ES+HM DW ++E +D +LEA M R I+EA + AR A KLRWPV
 Sbjct 769 LVGAEPDPSPEIHMLEDW-ILDEGAUVDSQLEADMDIVREIEACARARDTARYKLWVPR 827

Query 836 RIVVSPESEDAACKAVERLRSLVMDQNSKAIVLTPVGESWDELGLEVIPDPSKIGPVFKK 895
 +VV E E KA E L++V+ +Q N+K+I + ++ + P+P+ +GP ++
 Sbjct 828 EMVVVSEDEGLKAAESLKNVIAEQANAKSI---KTSTEFDMKIIARPNPATLGPRLRQ 884

Query 896 DAGKVIPALQKVDGFALKKAFAGEFELSADRTITVTIPGMANFKETLPEGTASAESD 955
 D V+ L+ DG A+K A GEF + D +T F+ LPE SA+ D
 Sbjct 885 DIPLVMRELEGADGSAVKAALDSGDEFTVE-TDGKKFKLTSEDIVFETELPENIVSAQFD 943

Query 956 AGLVYVDANLTPELEAGYAREVIRRLQDMRKELDLVVDENIRVSVRIEDERVLRLVETL 1015
 G V++D LTPE+ +E ARE++RR+QDMRK+LDL V+ +I VSV+ +E L E
 Sbjct 944 GGSVFIDTELTPCIMSEAMARELVRIQDMRKDLDLVEASIEVSVKCEE-FRELTEPQ 1002

Query 1016 KDLIAEVRRAEILNL 1030
 ++ I EVRA L+
 Sbjct 1003 REFIENEVRASLTSF 1017

Figure 7. Comparison of the IleS-amino acid sequence from *Methanosarcina barkeri* (above) and *M. marburgensis* (below). Residues reported to increase resistance to mupirocin in *M. barkeri* are marked in red. Alignment was done in BLAST version 2.15.0 (Morgulis *et al.* 2008).

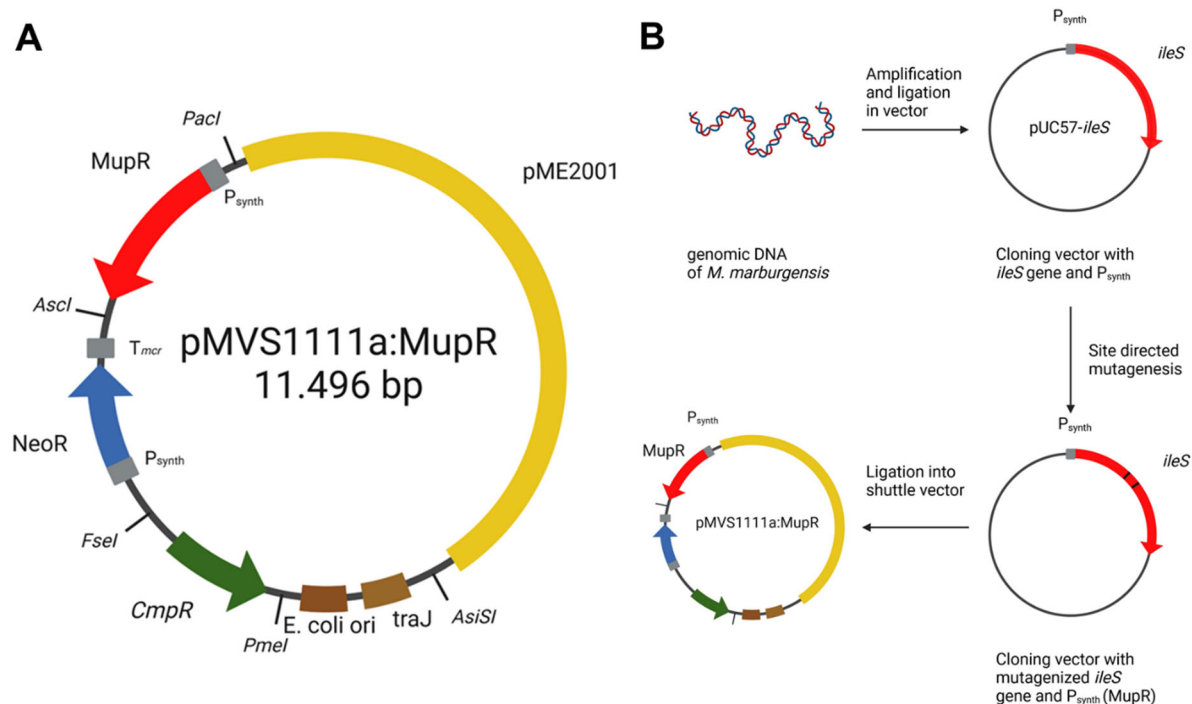


Figure 8. A: Shuttle vector carrying potential mupirocin resistance cassette (MupR) cassette and neomycin (NeoR) resistance cassette. B: Cloning strategy for the generation of vector shown in A. See main text for details. pME2001, cryptic plasmid for *M. marburgensis* (*ori* for *M. thermautotrophicus* Δ H); CmpR, chloramphenicol resistance for *E. coli*; P_{synth}, promoter for *M. thermautotrophicus* Δ H; T_{mcr} Terminator for *M. thermautotrophicus* Δ H; Pacl, Ascl, Fsel, PmeI, AsiSI, recognition sites of restriction enzymes flanking the different modules of the shuttle vector. Figure created with Biorender.com.

1.1.3. Evaluating mupirocin resistance of *M. thermautotrophicus* Δ H carrying pMVS-1111a:MupR

The plasmid pMVS-1111a:MupR carries the putative mupirocin resistance and the already established neomycin resistance cassette. The plasmid was, therefore, first transferred to *M. thermautotrophicus* Δ H via the already established conjugation protocol with neomycin as the selection (Fink *et al.* 2021). After the resulting strain, *M. thermautotrophicus* Δ H pMVS-1111a:MupR, was confirmed to carry the plasmid, the strain's resistance to mupirocin was assessed. Based on the previous findings that 20 μ g/mL of mupirocin was sufficient to inhibit growth for 72 hours in liquid, but 50 μ g/mL was required to inhibit growth on plates, 20, 50, and 100 μ g/mL mupirocin was chosen as testing concentration. We transferred 200 μ L of a grown culture of *M. thermautotrophicus* Δ H pMVS-1111a:MupR and *M. thermautotrophicus* Δ H pMVS-V1 (empty control vector) into 20 mL of fresh MS medium with the respective mupirocin concentrations. *M. thermautotrophicus* Δ H pMVS-1111a:MupR showed visible growth

after 40 hours with 20 µg/mL mupirocin, after 48 hours with 50 µg/mL, and after 64 hours with 100 µg/mL. *M. thermautotrophicus* ΔH pMVS-V1 did not show visible growth after 72 hours for any tested mupirocin concentration. Thus, strain *M. thermautotrophicus* ΔH pMVS-1111a:MupR exhibits an increased resistance to mupirocin, while the control strain, *M. thermautotrophicus* ΔH pMVS-V1, does not, indicating that MupR is a functional mupirocin resistance cassette.

Next, we tested if MupR could also be used as a selection marker (*i.e.*, to select exconjugants directly after conjugation). We repeated the conjugation of pMVS-1111a:MupR and pMVS-V1 (as a negative control for mupirocin selection). However, this time, we transferred the cells from the recovery into three different selective media: the standard selective MS medium with 250 µg/mL neomycin added or mupirocin selective medium with either 20 µg/mL mupirocin or 50 µg/mL added. For both plasmids, growth was visible with neomycin after two days. With 20 µg/mL mupirocin, all cultures showed visible growth after four to five days, including the negative control. However, at 50 µg/mL mupirocin, only the culture transformed with pMVS-1111a:MupR, but not the control, showed visible growth after about eight days. The culture was then spot-plated on plates with 250 µg/mL neomycin and plates with 50 µg/mL mupirocin because cells carrying pMVS-1111a:MupR should be able to grow on both antibiotics, while spontaneous resistant cells would only grow on mupirocin. Single colonies were visible after three days on mupirocin plates, but neomycin plates had no visible growth. A colony from the mupirocin plates was picked into liquid medium with mupirocin. Growth became visible after three days, and the cultures were analyzed *via* colony PCR (Figure S1). While the cultures selected on neomycin showed a PCR signal with plasmid-specific primers, the cultures selected on mupirocin did not show any signal. *M. thermautotrophicus* ΔH pMVS-1111a:MupR also showed growth with 100 µg/mL mupirocin. Therefore, we repeated the conjugation with 250 µg/mL neomycin or 100 µg/mL mupirocin as selective conditions. Again, growth was observed after about a week in the mupirocin-containing medium only for the conjugation with pMVS-1111a:MupR and not for the control, but exconjugants failed to grow when transferred to neomycin-containing medium and did not show any signal in a colony PCR with plasmid specific primers (Figure S2).

In summary, cells transformed with pMVS-1111a:MupR but selected with neomycin grew in MS medium containing 50 µg/mL or 100 µg/mL mupirocin. However, the

cultures directly selected on mupirocin could not grow on neomycin and did not seem to carry the plasmid. Therefore, they were most likely spontaneously resistant cells. Thus, we did not further investigate MupR as a selection marker because selection on mupirocin failed.

1.2. Puromycin

The puromycin-resistance cassette is commonly used as a selection marker in mesophilic methanogens (Enzmann *et al.* 2018). However, the resistance gene, puromycin acetyltransferase, has not yet been used in thermophilic microbes. Thus, the temperature of selective conditions plays an important role.

1.2.1. *M. thermautotrophicus* Δ H is sensitive to puromycin

We first determined the puromycin concentration necessary to inhibit the growth of *M. thermautotrophicus* Δ H at 60°C (the usual growth temperature). Preliminary experiments showed that up to 25 µg/mL of puromycin did not inhibit the growth of *M. thermautotrophicus* Δ H for more than three days. We, therefore, tested the growth inhibition of 50 µg/mL and 100 µg/mL puromycin (Figure 9). While the control cultures without puromycin grew to an OD₆₀₀ of about 0.35±0.004 overnight, the OD₆₀₀ of the cultures with 50 µg/mL or 100 µg/mL of puromycin remained under 0.05 for eleven days, after which the inhibition time was considered sufficiently long, and the measurement was stopped. The bottles from the growth experiment were kept in the incubator for several more weeks, after which there was still no visible growth. Thus, 50 µg/mL puromycin seems to inhibit the growth of *M. thermautotrophicus* Δ H sufficiently, and this concentration was used for all further experiments.

It is unclear whether the puromycin acetyltransferase is functional at 60°C. Therefore, we also tested the growth inhibition of puromycin at lower temperatures. Preliminary experiments showed that *M. thermautotrophicus* Δ H still grows at 42°C, albeit slower (data not shown). Below this temperature, growth drastically slowed down, which is consistent with published reports (Zeikus *et al.* 1972, Wasserfallen *et al.* 2000). Therefore, we also tested the growth inhibition of 50 µg/mL of puromycin at 42°C (Figure 10). At 42°C, the control cultures without puromycin reach their maximum OD₆₀₀ of about 0.18±0.02 after five days. The cultures with puromycin did not surpass an OD₆₀₀ of 0.02±0.02 even after 18 days.

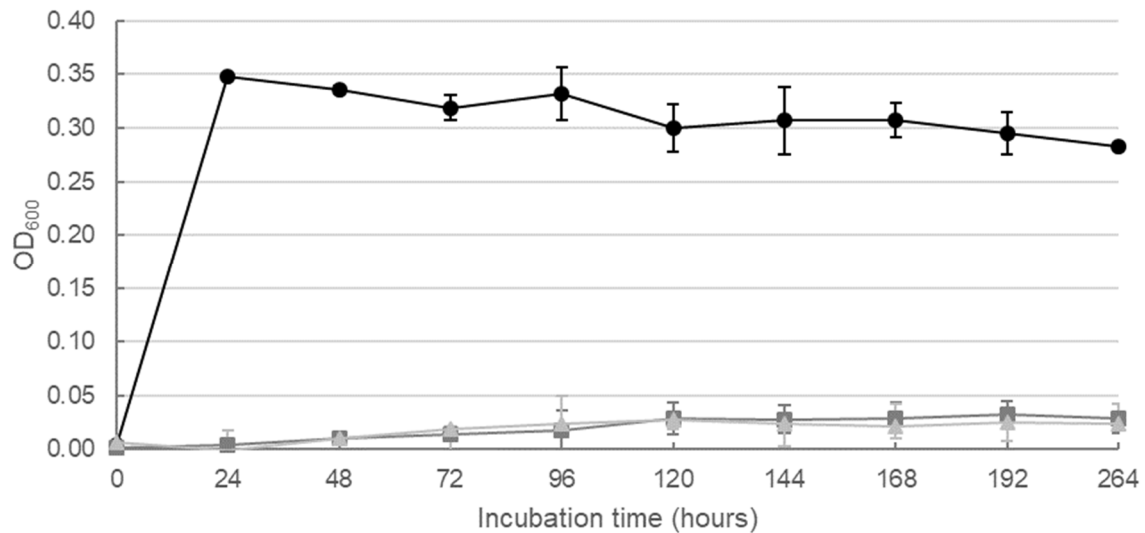


Figure 9. Growth curve of wild-type *M. thermautotrophicus* ΔH in liquid MS medium with 0 μg/mL (circles); 50 μg/mL (squares); and 100 μg/mL (triangles) of puromycin at 60°C. Average (N=3) with error bars indicating standard deviation.

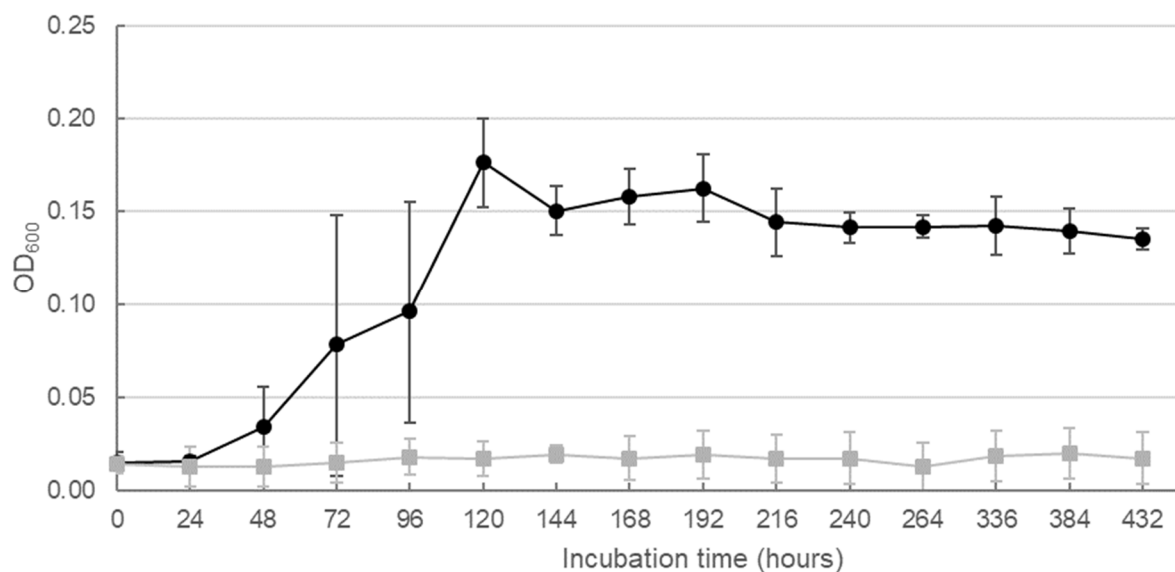


Figure 10. Growth curve of wild-type *M. thermautotrophicus* ΔH in liquid MS medium with 0 μg/mL (circles) and 50 μg/mL (squares) of puromycin at 42°C. Average (N=3) with error bars indicating standard deviation.

1.2.2. Puromycin acetyltransferase does not confer puromycin resistance to *M. thermautotrophicus* ΔH.

After determining that 50 μg/mL puromycin sufficiently inhibited growth, we tested whether the puromycin acetyltransferase could be used as a selection marker for *M. thermautotrophicus* ΔH. For this, we constructed the plasmid pMVS1111a:P_{synth}-*pac*.

Similar to the mupirocin resistance plasmid, this plasmid carries the neomycin resistance in the resistance module and the gene for the puromycin acetyltransferase in the application module, both under the control of the P_{synth} promoter (Figure 11). The plasmid was transferred to *M. thermautotrophicus* ΔH via the established conjugation protocol with neomycin selection, resulting in strain *M. thermautotrophicus* ΔH pMVS1111a: $P_{\text{synth}}\text{-pac}$.

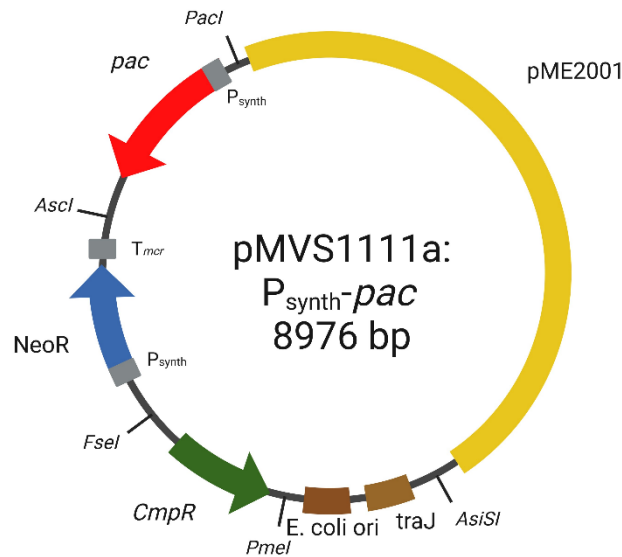


Figure 11. Shuttle vector carrying puromycin acetyltransferase (*pac*) and neomycin resistance (*NeoR*) cassette. pME2001, cryptic plasmid for *M. marburgensis* (*ori* for *M. thermautotrophicus* ΔH); *CmpR*, chloramphenicol resistance for *E. coli*; P_{synth} , promoter for *M. thermautotrophicus* ΔH ; *T_{mcr}*, Terminator for *M. thermautotrophicus* ΔH . *PacI*, *Ascl*, *FseI*, *PmeI*, *AsiSI*, recognition sites of restriction enzymes flanking the different modules of the shuttle vector. Figure created with Biorender.com.

We first tested the strain for its resistance to puromycin at 60°C (Figure 12). While the strain grew to an OD_{600} of 0.29 ± 0.05 overnight with neomycin, cultures with puromycin showed very little growth in general and reached only maximum OD_{600} values of 0.063 ± 0.01 (50 $\mu\text{g/mL}$ puromycin) and 0.034 ± 0.02 (100 $\mu\text{g/mL}$ puromycin). Especially for the cultures grown with 50 $\mu\text{g/mL}$ puromycin, some weak growth was observed between 72 hours and 120 hours, after which the growth seemed to stop again. We already expected that the thermostability of the puromycin acetyltransferase might be an issue. Thus, we also tested the strain for its puromycin resistance at 42°C (Figure 13). While the strain reached a maximal OD_{600} of 0.18 ± 0.02 after three days without puromycin, the OD_{600} only reached 0.025 ± 0.01 after three days with 50 $\mu\text{g/mL}$, which is in a similar range as for the wild-type and did not increase further.

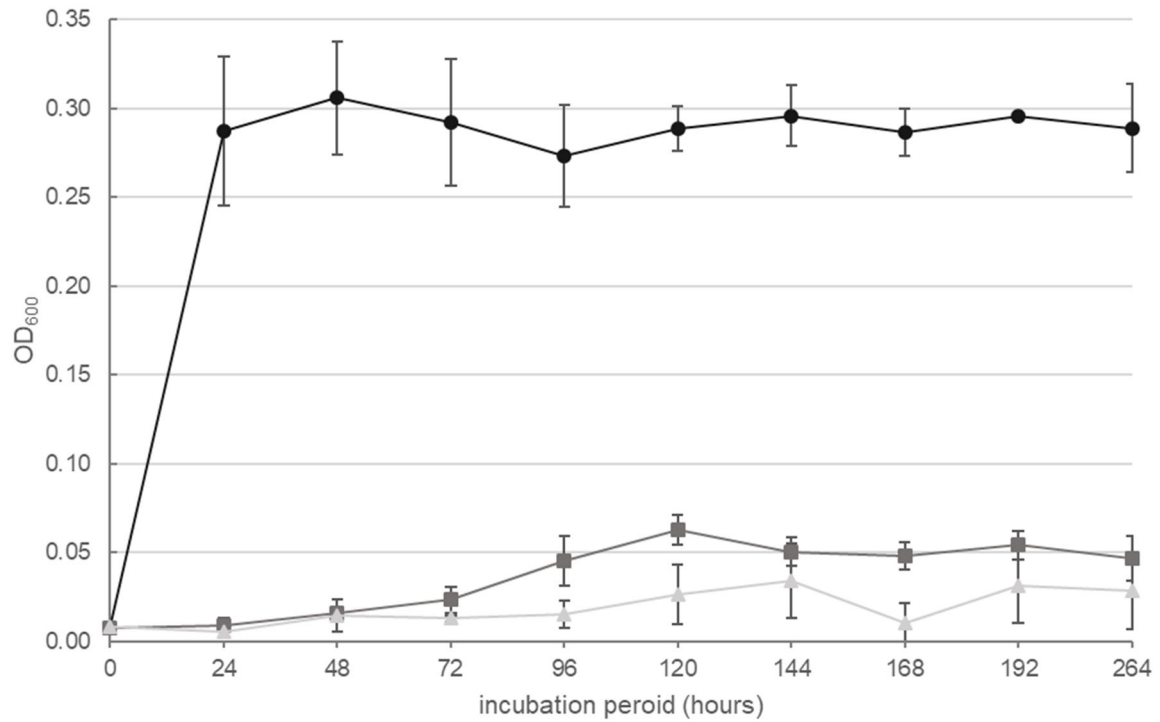


Figure 12. Growth curve of *M. thermautotrophicus* $\Delta H \Delta H$ pMVS1111a:P_{synth}-*pac* in liquid MS medium with 0 $\mu\text{g/mL}$ (circles), 50 $\mu\text{g/mL}$ (squares), and 100 $\mu\text{g/mL}$ (triangles) of puromycin at 60°C. Average (N=3) with error bars indicating standard deviation.

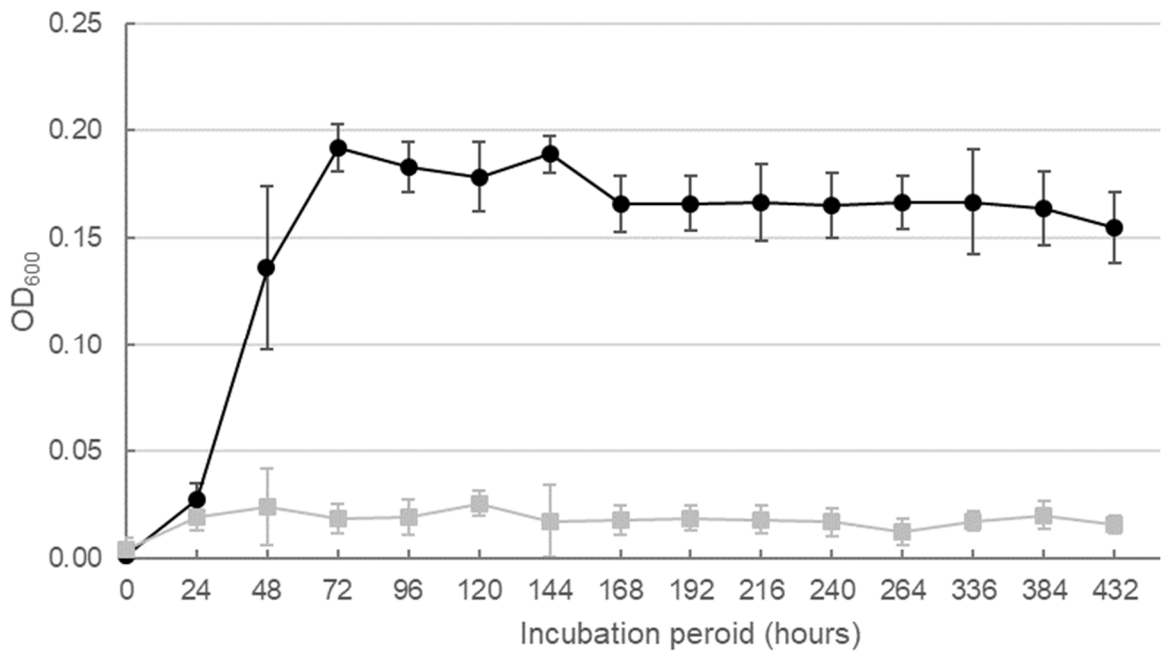


Figure 13. Growth curve of *M. thermautotrophicus* $\Delta H \Delta H$ pMVS1111a:P_{synth}-*pac* in liquid MS medium with 0 $\mu\text{g/mL}$ (circles) and 50 $\mu\text{g/mL}$ (squares) of puromycin at 42°C. Average (N=3) with error bars indicating standard deviation.

Because plasmid pMVS1111a:P_{synth}-*pac* does not seem to confer sufficient puromycin resistance to *M. thermautotrophicus* ΔH, we attempted several optimization strategies. First, we attempted gradual concentration adaptation of the *pac* gene. Because there might have been a slight growth for the strain pMVS1111a:P_{synth}-*pac* at 60°C with 50 µg/mL puromycin, we attempted to grow the strain with 25 µg/mL puromycin.

The strain grew after three days and was transferred into fresh medium with 50 µg/mL of puromycin. To our surprise, the strain now also grew at this puromycin concentration within three days. After three transfers, the strain was plated on MS Agar plates with 50 µg/mL puromycin. Colonies were visible after three days and were then picked into liquid MS medium with 50 µg/mL puromycin. The grown cultures were analyzed *via* colony PCR but did not show any signal with plasmid-specific primers (Figure 14). Attempts to repurify the plasmid also remained unsuccessful, suggesting that the cultures did not carry the plasmid but were spontaneously resistant wild-type.

Next, we attempted to lower the incubation temperature even further. *M. thermautotrophicus* ΔH can also grow at 37°C, but growth only becomes visible after one week. We also tested *M. thermautotrophicus* ΔH pMVS1111a:P_{synth}-*pac* at 37°C because this is the temperature at which the Pac enzyme is active in *Methanococcus* and *Methanosarcina* (Gernhardt *et al.* 1990, Metcalf *et al.* 1997). Growth was only observed after three weeks with 50 µg/mL puromycin, at the same time as the wild-type control. Cultures were, therefore, not further analyzed.

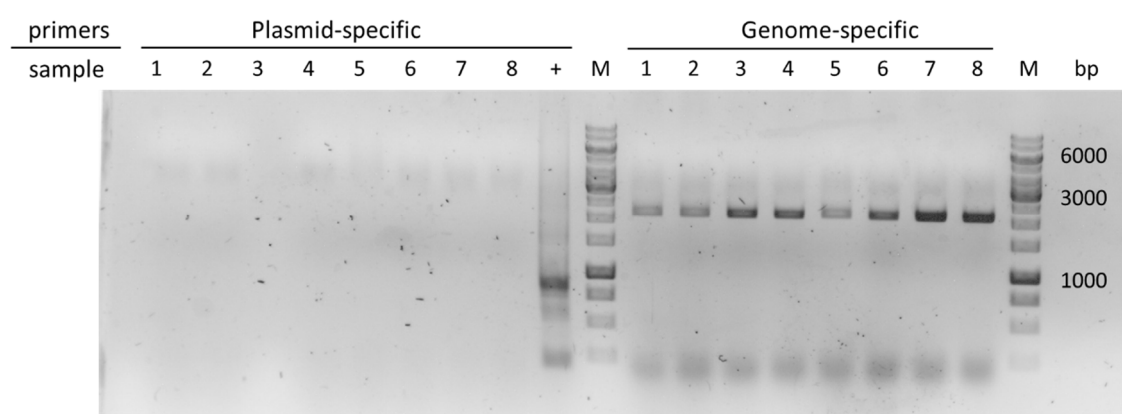


Figure 14. Colony PCR of clones of *M. thermautotrophicus* ΔH pMVS1111a:P_{synth}-*pac* that were adapted on lower concentrations of puromycin. Samples with plasmid-specific and genome-specific primers. Genome-specific primers serve as positive controls for the presence of DNA in the prepared colony PCR sample. 1-8, eight different clones of concentration-adapted *M. thermautotrophicus* ΔH pMVS1111a:P_{synth}-*pac*; +, positive control for colony PCR of plasmid (diluted plasmid sample); M, Marker.

We then focused our efforts on the *pac* gene itself. We hypothesized that the expression level of the *pac* gene might be insufficient. Therefore, we attempted to clone the *pac* gene under the control of our strongest promoter, P_{hmtB} (Fink *et al.* 2021), to create pMVS1111a: P_{hmtB} -*pac*. While the P_{hmtB} -*pac* cassette was successfully cloned in a cloning vector, several attempts to clone the cassette into the shuttle vector failed (Section VII.2). This strategy was, therefore, not further pursued.

One common method to increase the thermostability of antibiotic resistance genes is random mutagenesis, followed by transformation into the target strain and incubation at elevated temperatures. With this method, a thermostable hygromycin resistance cassette (Cannio *et al.* 2001), as well as the thermostable neomycin resistance cassette that is now also used for *M. thermautotrophicus* ΔH (Hoseki *et al.* 1999), were engineered. We also attempted this method for the *pac* gene. We attempted random mutagenesis of only the P_{synth} -*pac* cassette, followed by ligation into the shuttle vector and transformation in *E. coli*. Unfortunately, several attempts failed, with all *E. coli* clones carrying only empty vectors (Figure S 6). In addition, this method requires the transformation of many different *pac* variants, which is not possible to achieve with conjugation. Thus, the establishment of an alternative transformation method would also first be required.

1.3. Formate

Attempts to establish antibiotic markers remained unsuccessful. Next, we attempted to establish formate prototrophy as a selection marker. The closely related strain *M. thermautotrophicus* Z-245 can utilize formate as an alternative electron donor in addition to hydrogen (Nölling *et al.* 1997). To ensure that later experiments do not fail because of general issues with formate utilization, we first examined strain *M. thermautotrophicus* Z-245 for its ability to utilize formate in our hands. For this, cultures of *M. thermautotrophicus* Z-245 pre-adapted to their respective growth conditions (hydrogen or formate) were diluted 1:10 into fresh MS medium and incubated at 60°C (Figure 15). For growth with formate as an electron donor, MS medium was supplemented with 200 mM of sodium formate, as well as 10 µM sodium molybdate, 1 µM sodium selenite, and 0.125 wt% yeast extract to exclude a possible lack of any micronutrients required. For growth with hydrogen as an electron donor, we used standard MS medium. However, the headspace gas phase was adjusted to 1.52 bars of H₂/CO₂ (80/20 vol-%) instead of 2 bars. This should provide an equal number of electrons from either hydrogen or formate in both conditions. The preculturing of *M. thermautotrophicus* Z-245 already brought an interesting observation. While cultures grown with formate as an electron donor would immediately grow again when transferred into medium with hydrogen as an electron donor, cultures grown with hydrogen as an electron donor experienced a lag phase of two to three days when transferred into medium with formate as electron donor before visible growth was observed.

M. thermautotrophicus Z-245 readily grew on both substrates (Figure 15). With hydrogen as an electron donor, cultures grew rapidly to an OD₆₀₀ of 0.16±0.00 in eight hours, after which growth slowed down and reached a maximum OD₆₀₀ of 0.23±0.01 after 26 hours. With formate as an electron donor, a slight lag phase of one hour was observed compared to the cultures with hydrogen. The cultures reached an OD₆₀₀ of 0.13±0.01 in eight hours, after which growth also slowed down, and a maximum OD₆₀₀ of 0.15±0.003 was reached after 14 hours. Thus, we confirmed the growth of *M. thermautotrophicus* Z-245 with formate as the sole electron donor. These results were also published in Fink *et al.* (2021).

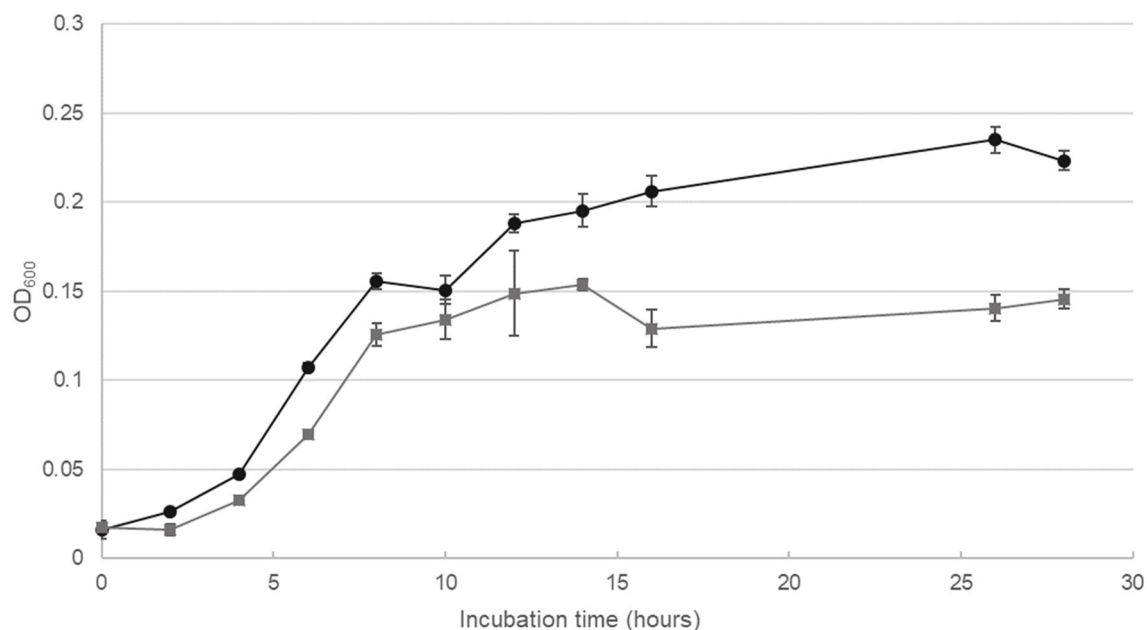


Figure 15. Growth curve of *M. thermautotrophicus* Z-245 in liquid MS medium with 1.52 bars of H₂/CO₂ (circles) or 200 mM of sodium formate (squares). Average (N=3) with error bars indicating standard deviation. Figure adapted from Fink *et al.* (2021).

1.3.1. Expression of the formate dehydrogenase (*fdh*) operon from *M. thermautotrophicus* Z-245 enables *M. thermautotrophicus* Δ H to utilize formate as an electron donor.

Next, we investigated whether the expression of the formate dehydrogenase (*fdh*) operon from *M. thermautotrophicus* Z-245 also enables *M. thermautotrophicus* Δ H to utilize formate as an electron donor. A cloning vector carrying the four genes that seem to form an operon-like structure (*fdhC*, *fdhA*, *fdhB*, and *orf3*) under the native promoter region was already present in our laboratory (pCF201, Fink (2021)). We decided to replace the native promoter with our strongest constitutive promoter (P_{hmtB}) because the native promoter of the *fdh* operon was reported to be repressed by hydrogen in *M. thermautotrophicus* Z-245 (Nölling *et al.* 1997), and this could lead to a lack of expression in the recovery phase during conjugation. We then cloned the cassette P_{hmtB} -*fdh*_{Z-245} into the application module of the shuttle vector, resulting in plasmid pMVS1111a: P_{hmtB} -*fdh*_{Z-245} (Figure 16).

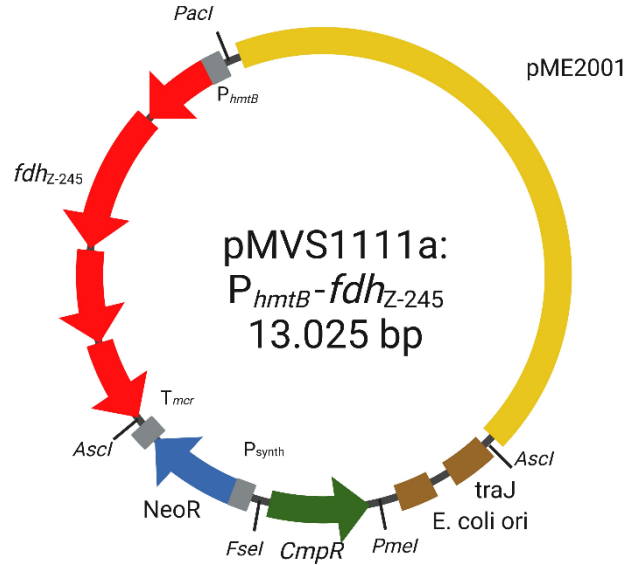


Figure 16. Shuttle vector pMVS1111a:*P_{hmtB}-fdh_{Z-245}* carrying the formate dehydrogenase operon (*fdh*) and neomycin (NeoR) resistance cassette. pME2001, cryptic plasmid for *M. marburgensis* (*ori* for *M. thermautotrophicus* Δ H); *CmpR*, chloramphenicol resistance for *E. coli*; *P_{synth}*, weaker promoter for *M. thermautotrophicus* Δ H; *P_{hmtB}*, stronger promoter for *M. thermautotrophicus* Δ H; *T_{mcr}*, Terminator for *M. thermautotrophicus* Δ H; *PacI*, *AscI*, *FseI*, *PmeI*, *AsiSI*, recognition sites of restriction enzymes flanking the different modules of the shuttle vector. Figure created with Biorender.com.

The plasmid was then transferred into *M. thermautotrophicus* Δ H with the established conjugation protocol with neomycin selection. The resulting strain, *M. thermautotrophicus* Δ H pMVS1111a:*P_{hmtB}-fdh_{Z-245}*, was tested for its ability to grow on formate with the same conditions as strain *M. thermautotrophicus* Z-245 before, except that neomycin was added to the hydrogen and carbon dioxide growth conditions. The growth experiment was also performed with the basic shuttle vector pMVS-V1 as a negative control, and consumption of formate was measured *via* HPLC for selected time points (Figure 17).

Both strains grew quickly with hydrogen as an electron donor, reaching an OD₆₀₀ of around 0.19±0.02 (pMVS-V1) and 0.18±0.01 (pMVS1111a:*P_{hmtB}-fdh_{Z-245}*) after 14 hours. *M. thermautotrophicus* Δ H pMVS1111a:*P_{hmtB}-fdh_{Z-245}* seems to grow slightly slower, but both strains reach the same final OD₆₀₀ of 0.23±0.02. Compared to this, the growth behavior with formate as an electron donor vastly differed between the two strains. Strain pMVS-V1 showed no visible growth, with the final OD₆₀₀ only being 0.03±0.01. Strain pMVS1111a:*P_{hmtB}-fdh_{Z-245}*, on the other hand, showed a prolonged lag phase, but growth became visible on the second day, and the strain reached a

final OD₆₀₀ of 0.20±0.01. Concurrent with growth, the formate concentration of the medium decreased from 200±1 mM to 13±19 mM. In comparison, the formate concentration in the bottles with strain *M. thermautotrophicus* ΔH pMVS-V1 remained at around 200 mM for all measured time points. Thus, we could demonstrate that the plasmid pMVS1111a:P_{hmtB}-*fdhZ*-245 enables *M. thermautotrophicus* ΔH to utilize formate and to grow with formate as the sole electron donor. These results were also published in Fink *et al.* (2021).

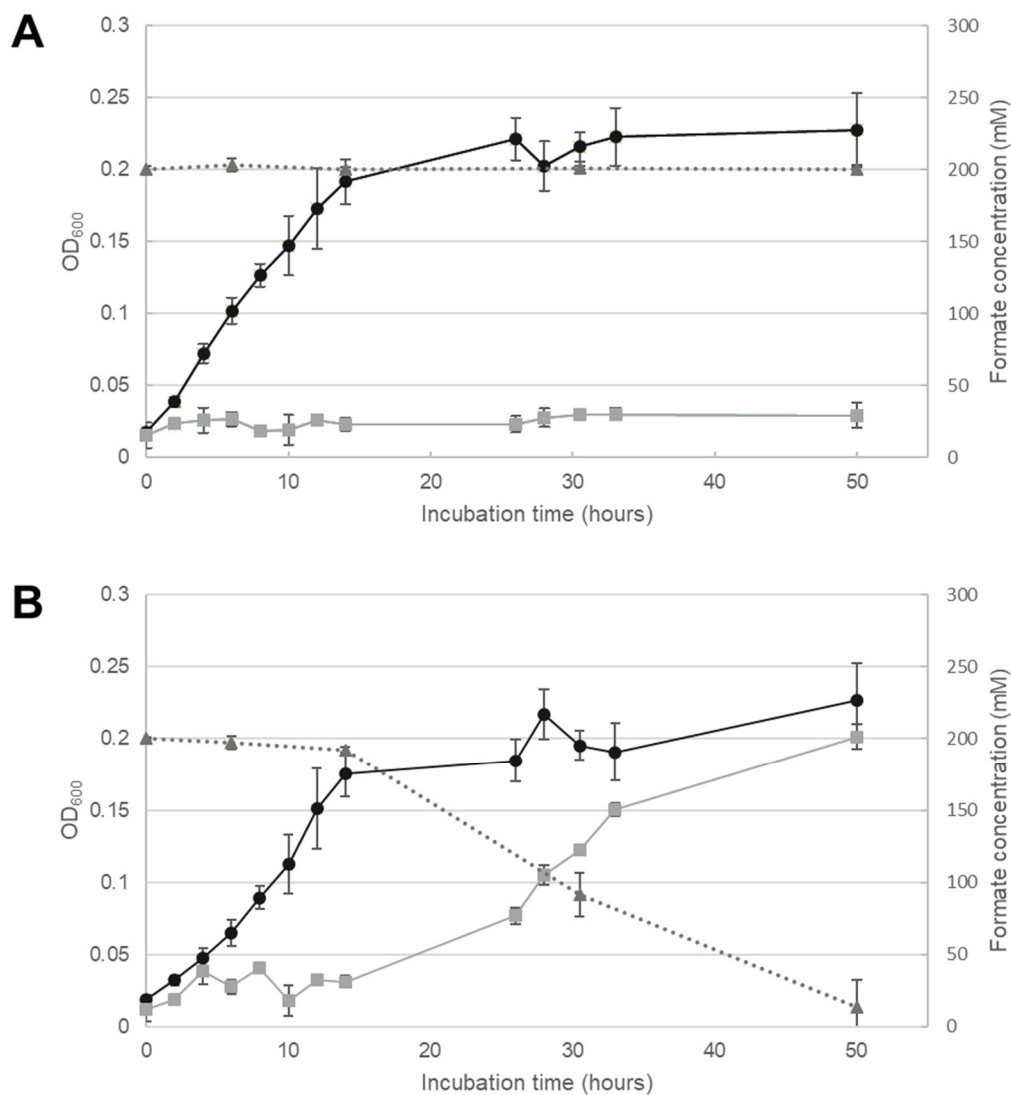


Figure 17. A: Growth curve of *M. thermautotrophicus* ΔH pMVS-V1. B: Growth curve of *M. thermautotrophicus* ΔH pMVS1111a:P_{hmtB}-*fdhZ*-245. Strains were grown in liquid MS medium with 1.52 bars of H₂/CO₂ (80/20 vol-%) with 250 µg/mL of neomycin (circles) or 1.52 bars of N₂/CO₂ (80/20 vol-%) with 200 mM of sodium formate (squares). The formate concentration of selected time points was measured *via* HPLC for the cultures with formate as an electron donor (triangles with dotted line). Average (N=3) with error bars indicating standard deviation. Figure adapted from Fink *et al.* (2021).

1.3.2. The *fdh* operon can be used as a selection marker in *M. thermautotrophicus* Δ H.

After demonstrating that the *fdh* operon from *M. thermautotrophicus* Z-245 enables *M. thermautotrophicus* Δ H to utilize formate as an electron donor, we wanted to test whether the *fdh* operon can also be used as a selection marker in *M. thermautotrophicus* Δ H. We repeated the conjugation of pMVS1111a:*P_{hmtB}-fdh*_{Z-245} with two different selective conditions. The conjugation was performed as usual, and one milliliter of the recovery culture was transferred to the usual neomycin selective conditions (MS medium with 250 μ g/mL of neomycin and H₂/CO₂ [80/20 vol-%] headspace). In addition, another milliliter of the recovery culture was transferred to MS medium that was supplemented as in the previous growth experiment (200 mM of sodium formate, 10 μ M sodium molybdate, 1 μ M sodium selenite, 0.125 wt% yeast extract, and N₂/CO₂ [80/20 vol-%] headspace). There was visible growth after two days for neomycin selection, as usual with this selection marker. For formate selection, growth became visible after around five days. Both strains were spotted on plates supplemented for their respective growth conditions, and colonies were visible after two days on neomycin. Only a very small number of colonies was observed on the plate for formate. Similar plating problems were reported when *M. thermautotrophicus* Z-245 was plated on formate (Fink 2021). However, it was still possible to pick colonies, and colonies from both plates were inoculated into liquid MS medium and analyzed *via* colony PCR (Figure 18).

The culture selected with neomycin and the one selected with formate both gave the expected colony PCR signal with primers specific for the shuttle vector, while wild-type cultures of *M. thermautotrophicus* Δ H and *M. thermautotrophicus* Z-245 did not give any signal (Figure 18A). To further exclude the possibility of contamination with Z-245, we also performed a colony PCR with primers specific for the genomic DNA of *M. thermautotrophicus* Δ H and Z-245 (Figure 18B).

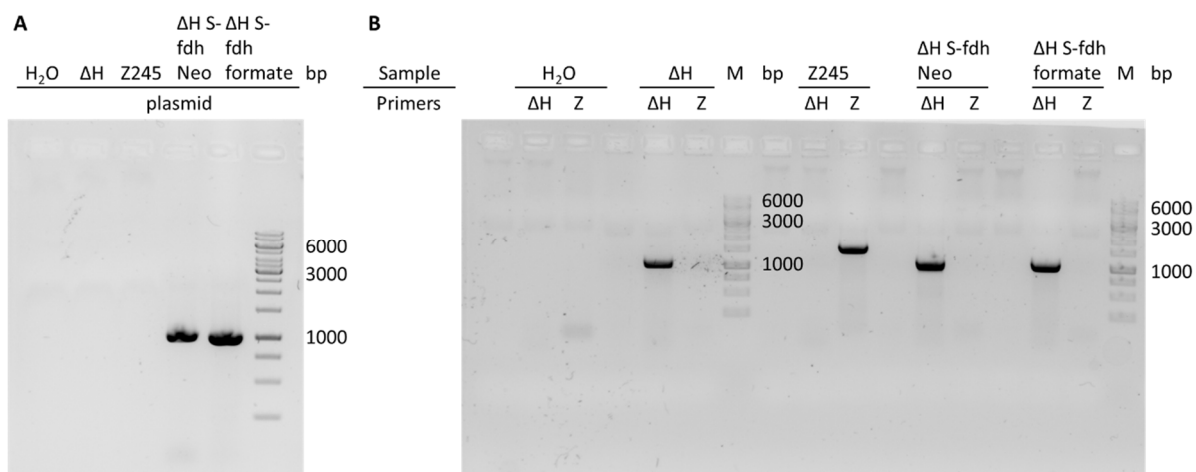


Figure 18. Colony PCR of *M. thermautotrophicus* ΔH pMVS1111a:*P_{hmtB}-fdh_{Z-245}* selected on neomycin or formate, as well as wild-type controls of the strains ΔH and Z-245. A: Colony PCR with plasmid-specific primers. B: Colony PCR with genome-specific primers. Samples: H₂O, negative control containing only water as a sample; ΔH, sample from wild-type *M. thermautotrophicus* ΔH; Z-245, sample from wild-type *M. thermautotrophicus* Z-245; ΔH S-fdh Neo, sample from *M. thermautotrophicus* ΔH pMVS1111a:*P_{hmtB}-fdh_{Z-245}* selected on neomycin; ΔH S-fdh formate, sample from *M. thermautotrophicus* ΔH pMVS1111a:*P_{hmtB}-fdh_{Z-245}* selected on formate. Primers: ΔH, primers specific for the genome of *M. thermautotrophicus* ΔH; Z, primers specific for the native plasmid of *M. thermautotrophicus* Z-245; plasmid, primers specific for the shuttle vector; M, marker.

The samples of *M. thermautotrophicus* ΔH pMVS1111a:*P_{hmtB}-fdh_{Z-245}* under both selective conditions only showed a signal for the primers specific for *M. thermautotrophicus* ΔH, not for the primers specific for *M. thermautotrophicus* Z-245. The presence of plasmid pMVS1111a:*P_{hmtB}-fdh_{Z-245}* was additionally confirmed by isolation of the plasmid from the culture and retransformation into *E. coli*, followed by plasmid preparation and confirmation by sequencing. Thus, we confirmed that the strain growing on formate is *M. thermautotrophicus* ΔH pMVS1111a:*P_{hmtB}-fdh_{Z-245}*, thereby demonstrating that formate prototrophy can be used as a selection marker in *M. thermautotrophicus* ΔH.

Two additional conditions must be met to utilize formate prototrophy fully as a selection marker: **1)** it has to be tested whether spontaneous growth of wild-type *M. thermautotrophicus* ΔH with formate occurs after prolonged incubation; and **2)** the exconjugants must be reliably plateable. To test whether wild-type *M. thermautotrophicus* ΔH grows on formate after prolonged incubations, we diluted a wild-type culture into fresh MS medium prepared the same way as for the formate growth experiments and conjugation and incubated it for several weeks. After four

weeks, there was still no visible growth. The experiment was, therefore, deemed successful and ended.

The plating efficiency of *M. thermautotrophicus* ΔH on hydrogen and carbon dioxide was extensively studied (Fink 2021, Fink *et al.* 2021). Key parameters that increased plating efficiency were the correct plating technique, adding paper clips to the plates for increased ventilation, and adding 0.1 % H₂S to the gas phase. We already added paper clips and used the same technique for plating as with neomycin as the selection agent. However, plates with formate as the selection agent were incubated with only N₂/CO₂ (80/20 vol-%), while plates with neomycin as the selection agent were incubated with H₂/CO₂/H₂S (79.9/20/0.1 vol-%). We, therefore, repeated the spot-plating on formate with N₂/CO₂/H₂S (79.9/20/0.1 vol-%) as the gas phase. The number of colonies drastically increased with these conditions. Interestingly, most cells seemed to grow in a ring-like structure at the outer rim of the spot. Still, several single colonies were obtained in the middle of the spot. Therefore, spot plating with N₂/CO₂/H₂S (79.9/20/0.1 vol-%) as gas phase was deemed a sufficient plating technique for genetically modified *M. thermautotrophicus* ΔH carrying the *fdh* operon.

Overall, we could demonstrate that: **1)** the *fdh* operon enables *M. thermautotrophicus* ΔH to utilize formate as an alternative electron donor; **2)** the *fdh* operon can be used to select genetically modified *M. thermautotrophicus* ΔH cells; **3)** genetically modified *M. thermautotrophicus* ΔH strains carrying the *fdh* operon are well platable when 0.1 % of H₂S is added to the gas phase; and **4)** there is no spontaneous growth of wild-type even after prolonged incubation with formate. This makes the *fdh* operon an ideal selection marker for genome editing.

2. Electroporation as an alternative transformation method

Electroporation as an alternative transformation method would be highly beneficial because it is easier to handle and more versatile than conjugation. Therefore, we set out to establish an electroporation protocol for *M. thermautotrophicus* ΔH. For this, we first reviewed existing protocols. Electroporation had already been established for *M. voltae* (Micheletti *et al.* 1991, Patel *et al.* 1994). However, *Methanococcus* and *Methanothermobacter* species greatly differ in cell-wall composition (Meyer *et al.* 2014). Cell-wall composition plays a significant role in electroporation success and efficiency, and the cell wall of *M. thermautotrophicus* ΔH resembles more closely that of Gram-positive bacteria than that of other methanogens. Thus, we decided to attempt electroporation with a protocol for *Clostridium ljungdahlii* (Molitor *et al.* 2016) already established in our lab.

2.1. *M. thermautotrophicus* ΔH can be transformed with an existing electroporation protocol for *Clostridium ljungdahlii*

We first attempted electroporation with the established protocol for *C. ljungdahlii* with slight adaptations to accommodate *M. thermautotrophicus* ΔH (section III.1.4.2). In brief, for each electroporation, a 20 mL stationary phase overnight culture of *M. thermautotrophicus* ΔH was cooled on ice, concentrated to 200 μL, and washed three times with a sterile, cold, and anoxic 10 vol% glycerol solution in an anaerobic workbench. Afterward, 2 μg of circular pMVS-V1 were added, cells were transferred to a 0.2 mM electroporation cuvette and electroporated with the *Ec2* settings (2.5 kV, 10 μF, 600 Ω) and immediately transferred into the recovery medium (standard MS medium with no antibiotics). After overnight incubation, the serum flasks of the recovery cultures had underpressure, signaling that *M. thermautotrophicus* ΔH consumed most of the gas. 1 mL of the recovery culture was transferred into the standard neomycin selective medium. Previous conjugation experiments showed that the first two selective transfers can give false positive colony PCR results due to DNA carry-over (Fink *et al.* 2021). Therefore, 0.2 mL of the selective enrichment culture was transferred twice into fresh selective medium after growth became visible and then analyzed *via* colony PCR. Each electroporation was done with five replicates plus one

negative control in which no plasmid DNA was added. This negative control was added because we used a higher initial number of cells than with the conjugation protocol, making it difficult to predict when spontaneously neomycin-resistant cells would arise.

After performing the electroporation with these conditions, two of the five replicates showed growth after three days in selective medium and were transferred again. After three selective transfers, both cultures were analyzed *via* colony PCR (Figure 19). Only one of the two cultures showed a band of the expected size, which indicated that this one was a transformant carrying the plasmid, while the other one was spontaneously neomycin-resistant. While it is not possible to calculate a transformation efficiency due to the first selective transfer being in liquid, the first selective transfer only grew after three days, indicating a relatively low number of initial transformants. Thus, *M. thermautotrophicus* Δ H is transformable with an adapted electroporation protocol from *C. ljungdahlii*. However, the electroporation only succeeded in one of five attempts, with a relatively low efficiency. Therefore, we set out to optimize the electroporation conditions.

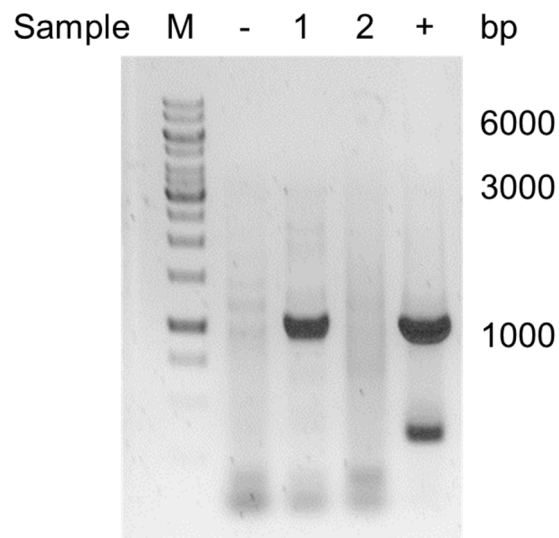


Figure 19. Colony PCR of *M. thermautotrophicus* Δ H cultures after electroporation. Samples: -, negative control containing only water as a sample; 1 and 2, sample from the third selective transfer of *M. thermautotrophicus* Δ H after electroporation; +, diluted pMVS-V1 as sample; M, marker.

2.2. Optimization of electroporation conditions

Several factors can influence electroporation success and efficiency. Therefore, we decided to repeat the basic electroporation procedure with a single alteration, and if

we saw improvement, set this as the new base protocol for further alterations. The need for a liquid enrichment step makes it difficult to calculate transformation efficiency with the neomycin selection marker. It might be possible to plate the cells directly after recovery with formate and *fdh* as the selection marker because there is no spontaneous growth of wild-type cells with the *fdh* operon as a selection marker. Therefore, we attempted electroporation with a plasmid that carries the *fdh* cassette but no neomycin selection marker (pMVS-V2, section IV.4.2.). We first wanted to know whether electroporation also worked with this plasmid and performed the electroporation like for pMVS-V1, except that the recovery culture was treated as usual for formate-selective conditions (two days in MS medium with formate and supplements added, section IV.4.3). No growth was observed in selective medium for any replicates for two weeks, after which the experiment was ended. Based on these results, we decided to use pMVS-V1 for further optimization. All further electroporations were also carried out with five replicates and one negative control, and we used the number of successful replicates as an efficiency indicator.

Several different parameters were varied to optimize the protocol. On the physical side, we tested cuvettes with different gap widths, different cell volumes, water as buffer, and different resistance and capacitance settings of the electroporator. On the biological side, we tested plasmids with different methylation patterns and different growth stages of *M. thermotrophicus* ΔH . Most protocol variations either lead to a similar or a smaller number of successful replicates compared to the original protocol (VII.4.) However, we did see an improvement when we used an electroporator with increased capacitance.

Because our laboratory's electroporator has a fixed capacitance and resistance, we switched to an electroporator in a neighboring laboratory for these experiments. While this electroporator had variable settings for resistance and capacitance, it was not in an anoxic environment, which required some changes to the protocol. Cells were still concentrated and washed in our anaerobic workbench but then transferred to a closed Eppendorf tube and brought outside the anaerobic workbench. The anoxic Eppendorf tube was opened at the electroporator, and the cells were quickly transferred to the electroporation cuvette, electroporated, and immediately transferred again to anoxic recovery medium. The total time of oxygen exposure was around 30 seconds. We tested different resistances at an increased capacitance (25 μF). With a capacitance

of 25 μF and a resistance of 600 Ω , we saw a drastic increase in successful electroporations, with four successful electroporations out of five (Table 22).

Table 22. Results of electroporation experiments with different electroporator settings. Electroporation was done in quintuplicates for each experiment. Resistance: Indicates resistance setting of electroporator. Capacitance: Indicates the capacitance setting of the electroporator. Confirmed transformation cultures: Indicates how many of the five electroporations resulted in an *M. thermautotrophicus* ΔH strain carrying pMVS-V1.

Resistance (Ω)	Capacitance (μF)	Confirmed transformant cultures
200	25	0
400	25	2
600	25	4
80	25	0

We reattempted electroporation with pMVS-V2 afterward but without success. We also increased the plasmid amount for each electroporation because pMVS-V2 is considerably larger than pMVS-V1, and 2 μg of pMVS-V2 contains considerably fewer plasmid copies than 2 μg of pMVS-V1. However, even with 10 μg of pMVS-V2, electroporations remained unsuccessful.

In summary, *M. thermautotrophicus* ΔH is transformable by electroporation, but not every electroporation is successful. Also, the transformation of larger plasmids was not possible, and of the attempted optimizations, only increasing the capacitance had a measurable positive effect on the transformation success rate.

3. *M. thermautotrophicus* Δ H promoters show activity in *E. coli*

Throughout the work presented here, we observed severe cloning issues with most of the plasmids (details in section VII.2). We hypothesized that this could be caused by the activity of the *M. thermautotrophicus* Δ H promoters in *E. coli*, leading to the expression of the genes under the control of these promoters, causing stress for the cells.

We decided to test this by transforming plasmid pMVS-V1 into *E. coli* NEBstable and plating the culture on chloramphenicol (normal *E. coli* resistance gene on the vector) or kanamycin. The neomycin resistance employed for *M. thermautotrophicus* Δ H also confers resistance to kanamycin (Hoseki *et al.* 1999). If the promoter is active in *E. coli*, this should cause the cells to be resistant to kanamycin.

The *E. coli* cells transformed with plasmid pMVS-V1 grew densely when plated on LB plates with chloramphenicol. We also saw many colonies when cells were plated on LB plates with kanamycin. There were, however, considerably fewer colonies than on the chloramphenicol plate (Figure 20 A). To exclude that the observed phenotype was caused by some other part of the shuttle vector, we repeated the experiment with the pUC57:P_{synth}-NeoR, which is a cloning vector that only carries the kanamycin/neomycin resistance under the control of the P_{synth} promoter (Figure 20 B). Again, a considerably smaller number of colonies grew on the kanamycin plate than on the control plate. Plating *E. coli* NEBstable on kanamycin without transforming with plasmid DNA did not lead to any colony formation. Because the number of colonies was smaller than when plated on the correct antibiotic, we hypothesized that the selection pressure of the kanamycin might have led to mutations in the promoter region of the kanamycin/neomycin resistance gene that enabled *E. coli* to express the resistance gene. We, therefore, picked ten clones of *E. coli* pMVS-V1 grown on kanamycin and three clones grown on chloramphenicol, isolated the plasmid DNA, and sequenced the promoter region (Figure 20 C). None of the sequenced plasmids contained mutations in the P_{synth} promoter of the kanamycin/neomycin resistance gene.

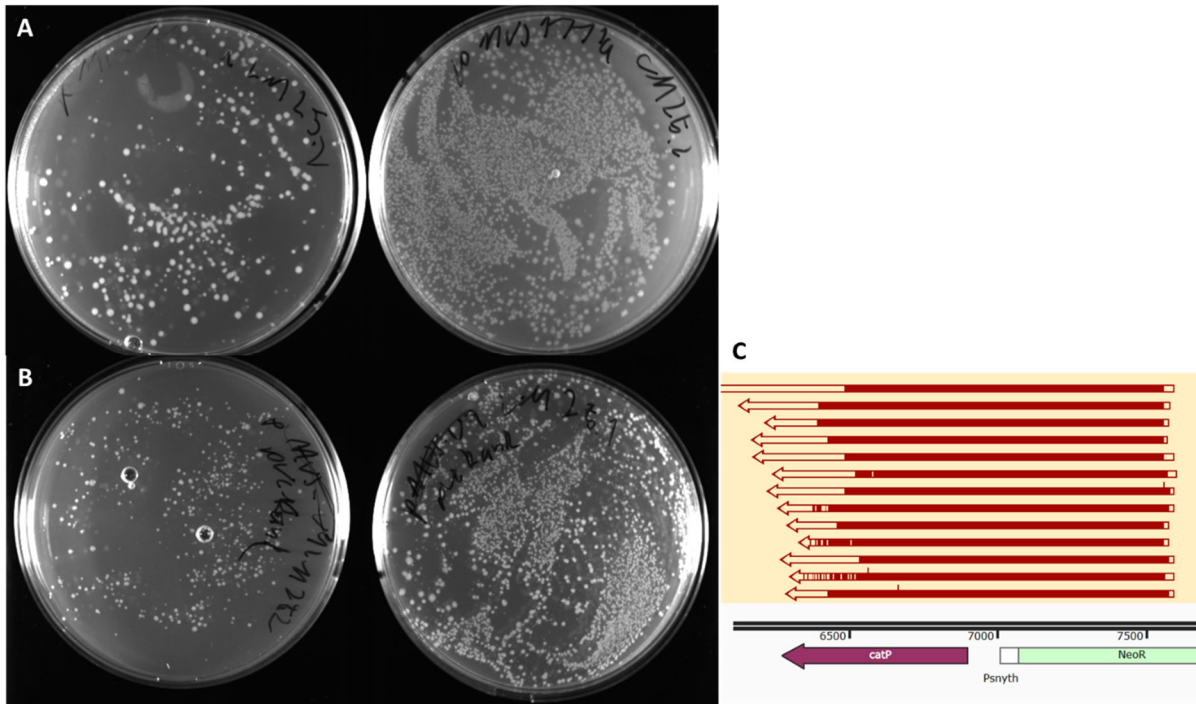


Figure 20. Evaluation of kanamycin resistance of *E. coli* transformed with the NeoR cassette in different vectors. Chemically competent *E. coli* NEB stable cells were transformed with either pMVS-V1 or pUC57- P_{synth} -neoR and plated on the standard selection antibiotic for the plasmid (right) or on kanamycin (left). A: LB plates of *E. coli* transformed with pMVS-V1. B: LB plates of *E. coli* transformed with pUC57: P_{synth} -NeoR. C: Alignment of Sanger sequencing results of ten plasmids prepped from clones of pMVS-V1 grown on kanamycin (top seven) and three plasmids prepped from clones of pMVS-V1 grown on chloramphenicol (bottom three) to sequence of pMVS-V1.

Next, we wanted to investigate whether the observed kanamycin resistance is dependent on the promoter of the kanamycin/neomycin resistance gene. For this, we repeated the transformation experiment with three different plasmids: pMVS-V1, pMVS-V1_ P_{hmtB} , and pMVS-V1_ P_{mcrB} (*M.v.*) (Figure 21). The three plasmids are identical except for the promoter of the kanamycin/neomycin resistance gene (Fink 2021). pMVS-V1_ P_{hmtB} carries the resistance gene under the control of P_{hmtB} instead of P_{synth} . In *M. thermautotrophicus* ΔH , the P_{hmtB} promoter is a constitutive promoter that leads to stronger expression than P_{synth} (Fink *et al.* 2021). pMVS-V1_ P_{mcrB} (*M.v.*) carries the resistance gene under the control of P_{mcrB} (*M.v.*). This construct was shown to not confer neomycin resistance to *M. thermautotrophicus* ΔH (Fink *et al.* 2021). Cells transformed with pMVS-V1_ P_{hmtB} produced a similar number of colonies on LB-kanamycin plates as cells transformed with pMVS-V1 (Figure 21 A and B). However, for cells transformed with pMVS-V1_ P_{mcr} (*M.v.*), only two colonies were visible on the plate with kanamycin (Figure 21 C). We concluded that the kanamycin/neomycin

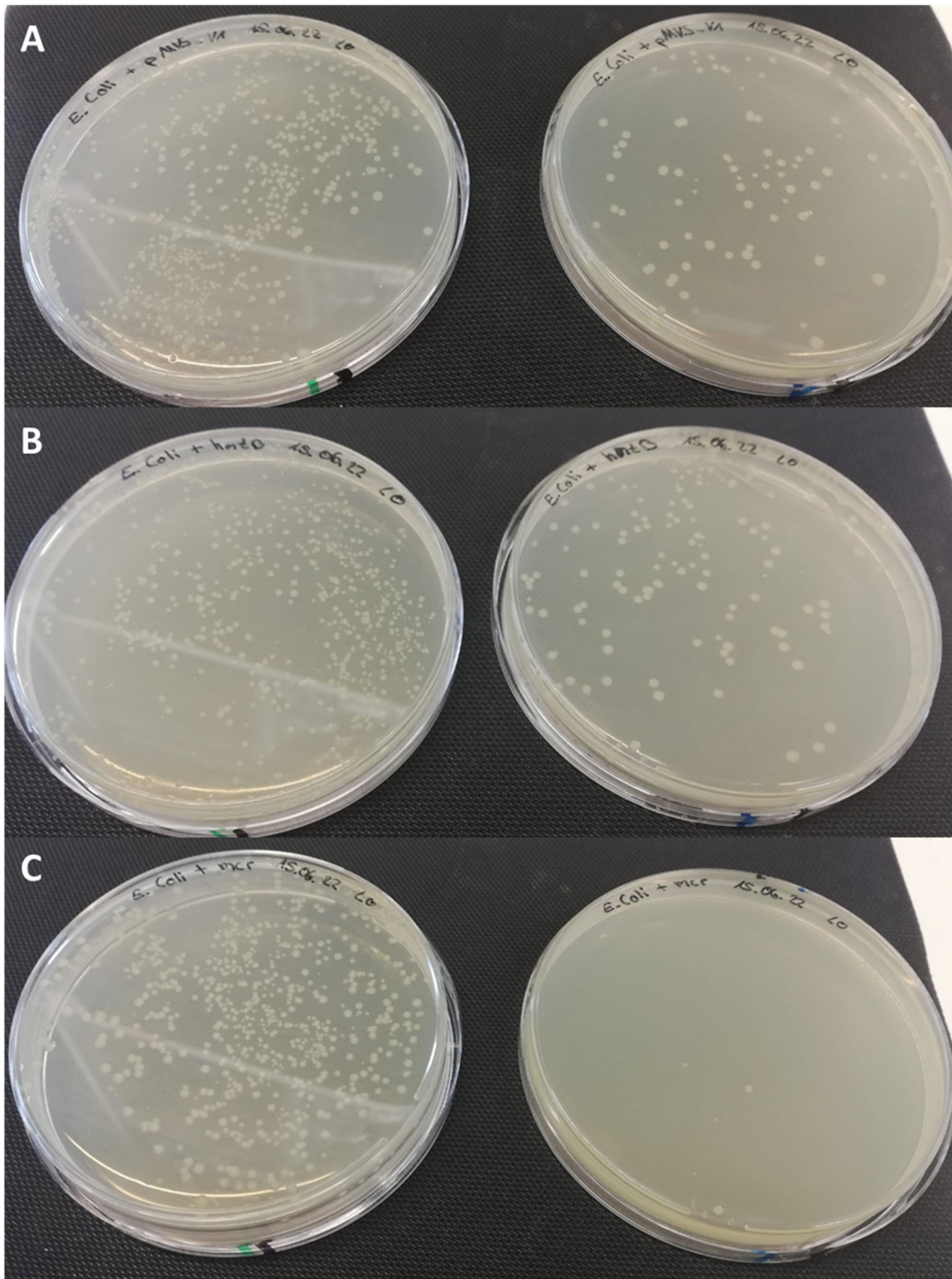


Figure 21. Evaluation of kanamycin resistance of *E. coli* transformed with different shuttle vectors. A: LB plates of *E. coli* transformed with pMVS-V1. B: LB plates of *E. coli* transformed with pMVS-V1_ *P_{hmtB}*. C: LB plates of *E. coli* transformed with pMVS-V1_ *P_{mcrB}* (*M.v.*). Chemically *E. coli* NEB stable were transformed with different vectors and plated on LB medium with either chloramphenicol (left) or kanamycin (right).

resistance under the control of promoter $P_{mcrB (M.v.)}$ does not confer kanamycin resistance to *E. coli*, and that promoter $P_{mcrB (M.v.)}$ is, therefore, most likely not active in *E. coli*.

We, therefore, concluded that the P_{synth} and P_{hmtB} promoters are active in *E. coli* and can drive the expression of the kanamycin/neomycin resistance gene. Because the kanamycin/neomycin resistance cassette's expression seems dependent on the promoter, we also tried to determine putative sequences functioning as *E. coli* promoter elements in P_{synth} or P_{hmtB} bioinformatically. Especially for *E. coli*, a lot of different prediction tools are available. Based on a recently published assessment of different tools (Zhang *et al.* 2022), we picked two promoter prediction tools that scored well in the assessment and are accessible through online web tools, iProEP (Lai *et al.* 2019) and iPromoter-2L 2.0 (Liu *et al.* 2019). Analysis of P_{synth} was not possible because both tools only accept promoters that are at least 81 bp long. While both tools predicted P_{hmtB} to be an active promoter in *E. coli*, they also predicted this for $P_{mcrB (M.v.)}$, which did not exhibit any activity in our experiments (Figure 22). Thus, the reason for promoter activity in *E. coli* remains unclear.

- A** $P_{mcrB(M.v.)}$ AATTATTGTGTTATTAACATCTTATATATAAACTTTTCTATTTAATGTTAATGAAAAAGTG
AATATATATACATAGAGTAATGTTATGATGTATATATCAAAAAAATAGGAGTGATTCTC
- P_{synth} TTTATATAGGGATATAGTAATAGATAATATCACGGTAACCACCGGCAGCAGGTGATG
CAT
- P_{hmtB} CCCATGAACCAACCGATGGCTCAGAAAAACCTTAAATTAGCGATATTTTATATAG
GATTATATGAATAGATAATATCACATAAAATGAGGTGGTTAATT
- B** The length of the query sequence 1 (>PmcrB) is 81 -bp. It has 1 subsequence (length of 81-bp with 1-bp step). The #1 subsequence (1-81) in the query sequence 1 **is predicted as a promoter**. Its probability score of being a promoter is **0.880332**.

The length of the query sequence 2 is less than 81-bp, this sequence has no biological meaning for promoter.

The length of the query sequence 3 (>PhmtB) is 81 -bp. It has 1 subsequence (length of 81-bp with 1-bp step). The #1 subsequence (1-81) in the query sequence 3 **is predicted as a promoter**. Its probability score of being a promoter is **0.922006**.

- C** The length of the query sequence 1(PmcrB) is 81 bp. It has 1 subsequence (length of 81 bp with 1bp step).
The [#1 subsequence \(1-81\)](#) of the query sequence 1 is a σ^{70} -promoter sequence.
- The length of the query sequence 2(PhmtB) is 81 bp. It has 1 subsequence (length of 81 bp with 1bp step).
The [#1 subsequence \(1-81\)](#) of the query sequence 2 is a σ^{70} -promoter sequence.

Figure 22. Analysis of promoters $P_{mcrB(M.v.)}$, P_{synth} and P_{hmtB} . A: Sequences of the three promoters. TATA boxes are in bold, B-recognition elements are in italics, and ribosomal binding sites are in red. Annotation for P_{synth} and P_{hmtB} was taken from Fink *et al.* (2021), and $P_{mcrB(M.v.)}$ annotation was based on *Methanococcus* consensus sequences described in Xu *et al.* (2023). B: Analysis result of promoter prediction tool iProEP (Lai *et al.* 2019). C: Analysis result of promoter prediction tool iPromoter-2L 2.0 (Liu *et al.* 2019). Both promoter prediction tools can only analyze sequences of 81 bp length, so the last 81 bp of $P_{mcrB(M.v.)}$ and P_{hmtB} were used as queries. P_{synth} is shorter than 81 bp and could, therefore, not be analyzed.

4. Establishing a thermostable CRISPR system for traceless genome editing in *M. thermautotrophicus* Δ H

After establishing a selection marker without a background of spontaneously growing wild-type, we were able to focus on establishing genome editing tools. While the integration of foreign DNA in the genome *via* homologous recombination was demonstrated to be feasible in *M. thermautotrophicus* Δ H, most analyzed single colonies seemed to contain a mix of single- and double-recombination events and wild-type DNA (Fink 2021). Two studies from our lab were successful in deleting genes with homologous recombination. For the deletion of the *mth60* operon, a clean deletion strain was achieved by only one additional round of plating and picking colonies (Fink 2021, Fink *et al.* 2023). However, when the deletion of nitrogen-fixation-related genes *nifl*₁ and *nifl*₂ was attempted, a clean deletion was only achieved after several transfers and an additional blender treatment (Küchle 2024). In addition, the deletion of the *mth60* operon was also attempted with the *fdh* operon as a selection marker. While the integration of the suicide vector in the genome was confirmed, all analyzed single colonies seemed to contain a mix of single- and double-recombination events and wild-type DNA. All attempts to resolve the genotype remained unsuccessful (Fink 2021).

The just-described high amounts of transfers required and the failure in utilizing the *fdh* operon in generating clean deletions suggest that markerless mutagenesis would be a cumbersome method for genome editing in *M. thermautotrophicus* Δ H. Markerless mutagenesis enables selection without any additional tools besides a counterselection marker (which usually stems from the host organism). However, it requires two successive rounds of homologous recombination, with the second one theoretically having a 50:50 chance of resulting in the wild-type genotype instead of the desired deletion. Based on the previously described experiences with homologous recombination, markerless mutagenesis would most likely require very tedious passaging and screening of clones. On the other hand, a CRISPR system would put direct selective pressure on the success of the double-homologous recombination because failing to do so would lead to repeated double-strand breaks and, eventually, cell death. Therefore, we focused on establishing a CRISPR system.

4.1. Putative targets for gene editing via CRISPR in *M. thermautotrophicus*: counterselection markers

Because a system for plasmid curing is still missing in *M. thermautotrophicus* Δ H, we focused on putative counterselection markers as the first targets for the CRISPR system. The genes encoding two phosphoribosyl transferases, *hpt* (hypoxanthine phosphoribosyl transferase) and *upt* (uracil phosphoribosyl transferase), are commonly used as counterselection markers in other methanogens (Moore *et al.* 2005, Rother *et al.* 2005). The encoded enzymes confer sensitivity to DNA base analogs, such as 6-azauracil (6-AU) and 8-azahypoxanthine (8-AH). Usually, the genes are present in the microbes. Therefore, a deletion is required before they can be used as counterselection markers. This makes them suitable targets for the CRISPR system. However, the sensitivity of different archaea to the toxic base analogs vastly differs (Bowen *et al.* 1987, Worrell *et al.* 1990). Therefore, we first tested which of the toxic base analogs inhibits the growth of *M. thermautotrophicus* Δ H sufficiently.

4.1.1. Hypoxanthine phosphoribosyl transferase (Hpt)

The *hpt* gene has been reported to confer sensitivity to the toxic base analogs 6-methylpurine (6-MP), 8-aza-2,6-diaminopurine (8-ADP), and 8-azahypoxanthine (8-AH) in other archaea (Moore *et al.* 2005, Rother *et al.* 2005, Santangelo *et al.* 2010). *M. marburgensis* has been reported to be sensitive to 8-ADP and 8-AH (Worrell *et al.* 1990).

We first tested the sensitivity of *M. thermautotrophicus* Δ H to 6-MP. For this, a stationary culture was diluted 1:100 into fresh medium with different concentrations of 6-MP. We tested concentrations of up to 500 μ g/mL 6-MP, which is 50 times the concentration reported to inhibit the growth of *Thermococcus kodakarensis* (Santangelo *et al.* 2010). All cultures grew overnight. We, therefore, concluded that 6-MP does not inhibit the growth of *M. thermautotrophicus* Δ H at these concentrations. We then repeated the experiment with 8-AH. Concentrations of up to 100 μ g/mL 8-AH showed no difference in visible growth compared to a control without 8-AH. A slight growth delay was visible at 200 μ g/mL 8-AH, and with 500 μ g/mL and 1000 μ g/mL 8-AH, growth was only visible after two or three days, respectively. 8-ADP was not

commercially available at the time we performed the experiment and, therefore, was not tested.

4.1.2. Uracil phosphoribosyl transferase (Upt)

The *upt* gene was reported to confer sensitivity to the toxic base analog 6-azauracil (6-AU) in *Methanococcus* (Moore *et al.* 2005). Therefore, we tested the sensitivity of *M. thermautotrophicus* to different concentrations of 6-AU. Due to insolubility at a lower pH, the 6-AU stock solution had to be prepared in 100 mM sodium hydroxide. Because the highest concentration tested was a 1:50 dilution from the stock, we also included a control with 2 mM of sodium hydroxide added to check for any growth defects caused by the addition of base (Figure 23).

The control cultures with no additions reached the stationary phase after 24 hours at an OD₆₀₀ of 0.3±0.02. On the other hand, cultures in which 2 mM sodium hydroxide was added showed a slight delay in growth, with an average OD₆₀₀ of only 0.18±0.07 after 24 hours. However, the growth inhibition was much stronger for cultures where 6-AU was added. For cultures with both 100 µg/mL and 200 µg/mL of 6-AU, OD₆₀₀ stayed below 0.05 for 48 hours. While the cultures with 100 µg/mL of 6-AU already reached an OD₆₀₀ of 0.2±0.07 after 72 hours, growth was delayed

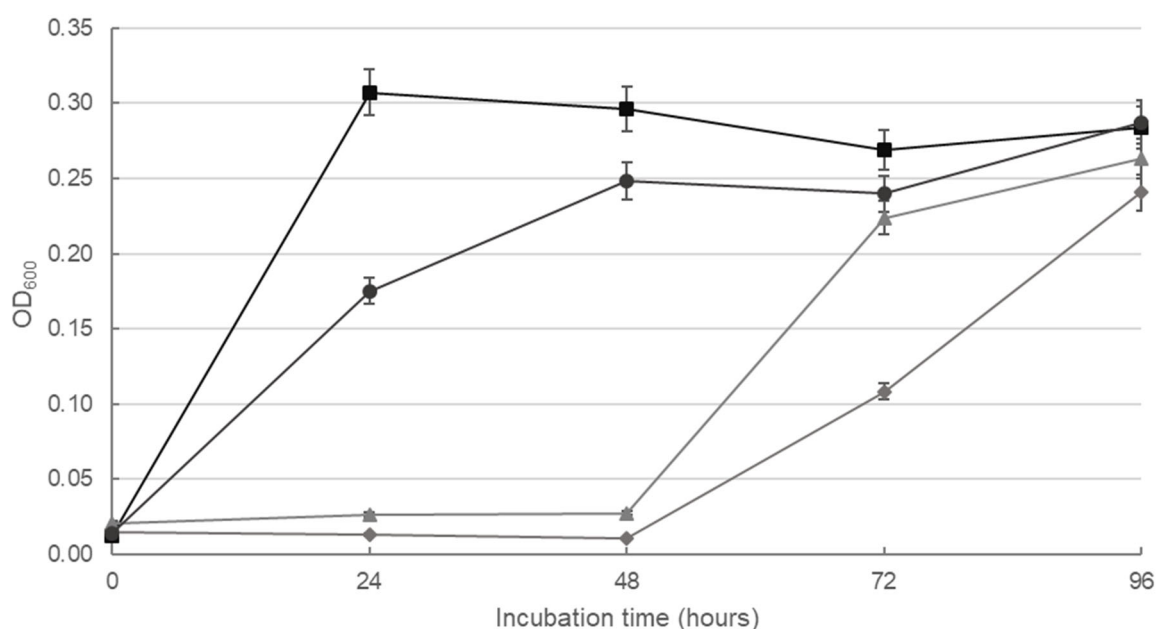


Figure 23. Growth curve of *M. thermautotrophicus* ΔH in MS medium with 0 µg/mL (squares), 100 µg/mL (triangles), and 200 µg/mL (diamonds) 6-AU, or with 2 mM of sodium hydroxide (circles). Average (N=3) with error bars indicating standard deviation.

further for the cultures with 200 µg/mL of 6-AU, which only reached an OD₆₀₀ of about 0.11±0.07 at this time point. After 96 hours, all cultures had reached a similar OD₆₀₀, with an average of 0.26±0.01 for 100 µg/mL of 6-AU and 0.24±0.02 for 200 µg/mL of 6-AU, and the experiment was ended. Thus, we could show that 100 µg/mL of 6-AU inhibits the growth of *M. thermautotrophicus* for at least 48 hours.

4.2. Modular CRISPR module allows quick exchange of parts of the CRISPR system

After we could show that *M. thermautotrophicus* ΔH is sensitive to at least one of the base analogs commonly used for *hpt* and *upt* counterselection, we decided that they are both suitable targets for testing the CRISPR system. We then started cloning the first CRISPR plasmids. To avoid the overgrowth of putative successfully edited strains by spontaneous-resistant wild-type cells, we used the *fdh* operon as the selection marker for all CRISPR plasmids. The only available shuttle vector with the *fdh* operon, pMVS1111a:P_{hmtB}-*fdh*_{Z-245}, had the *fdh* operon in the application module, which we wanted to use for the CRISPR system. Because the *fdh* operon was established as a selection marker, we moved it from the application module site to the site for the selection marker for *M. thermautotrophicus* ΔH. We called this new shuttle vector series with only the *fdh* operon as a selection marker for *M. thermautotrophicus* ΔH pMVS1112a (Figure 24 B). In addition, we also replaced the neomycin resistance in the basic shuttle vector pMVS-V1 with the *fdh* operon, thereby creating the shuttle vector pMVS-V2 (Figure 24 A). This vector contains no additional genetic cargo besides the basic elements required for plasmid maintenance and selection in *E. coli* and *M. thermautotrophicus* ΔH. It can, therefore, be used as a positive control for conjugations with selection by formate prototrophy. This is an important control because not every conjugation attempt with *M. thermautotrophicus* ΔH is successful. Adding a positive control for the conjugation makes it easier to judge whether the conjugation failed because of issues with the conjugation protocol or because of an issue with a specific plasmid.

Building on the shuttle vector's modular design, we employed a modular design within the CRISPR module, with each submodule being flanked by recognition sites of restriction enzymes. As with the modules of the shuttle vector, we chose restriction enzymes with comparatively few recognition sites in the genome of *M.*

thermautotrophicus ΔH (numbers in brackets are the occurrences of recognition sites in the genome of *M. thermautotrophicus* ΔH), namely *Ascl* (6) and *SpeI* (30) for the outsides of the module, and *AvrII* (56), *MluI* (42), *PacI* (34) for within the CRISPR module. This segments the CRISPR module into four submodules.

The first submodule, flanked by *SpeI* and *AvrII* recognition sites, contains the *Cas9* gene. Because the commonly used *Cas9* from *S. pyogenes* is only active up to 40°C, we decided to use a thermostable *Cas9* from *G. thermodenitrificans* T12 (Thermo*Cas9*), which was reported to be active up to 65°C (Mougiakos *et al.* 2017). The *thermoCas9* gene was codon optimized for *M. thermautotrophicus* ΔH and cloned under the control of P_{synth} , the weakest promoter available for *M. thermautotrophicus* ΔH (Fink *et al.* 2021).

The second submodule, flanked by *AvrII* and *PacI* recognition sites, contains the sgRNA of the Thermo*Cas9*. All sgRNAs designed for this project only differ in the spacer sequence from the sgRNA sequence given in Mougiakos *et al.* (2017). The Thermo*Cas9* has a PAM of 5'-NNNNCNAA-3' (Mougiakos *et al.* 2017). Targeting sgRNAs were, therefore, designed by replacing the previous spacer sequence with a 20-bp sequence directly upstream of the PAM sequence in a target gene. Because the Thermo*Cas9* tolerates up to two mismatches in the spacer sequence (Mougiakos *et al.* 2017), the specificity of the spacer was confirmed by an algorithm that would screen the *M. thermautotrophicus* ΔH genome for sequences upstream of PAM sites with up to three mismatches to the spacer sequence (section III.1.3.5.1). The only match found for all tested spacer sequences was the sequence of the desired target.

The third submodule, flanked by *PacI* and *MluI*, contains the repair template. Repair templates used in this work consisted of 750 bp to 1 kb flanking regions up- and downstream of the target gene.

The fourth submodule, flanked by *MluI* and *Ascl*, contains the counterselection marker for plasmid curing. Because a counterselection marker is currently not available for *M. thermautotrophicus* ΔH , the module contains a random 150 bp place-holder sequence.

Unlike the shuttle vector modules, the CRISPR modules are not given a number for each submodule to avoid overcomplicated names for the plasmids. Instead, each CRISPR module is just given a single number, such that the different CRISPR plasmids discussed below are called pMVS1112a:CRISPR"X".

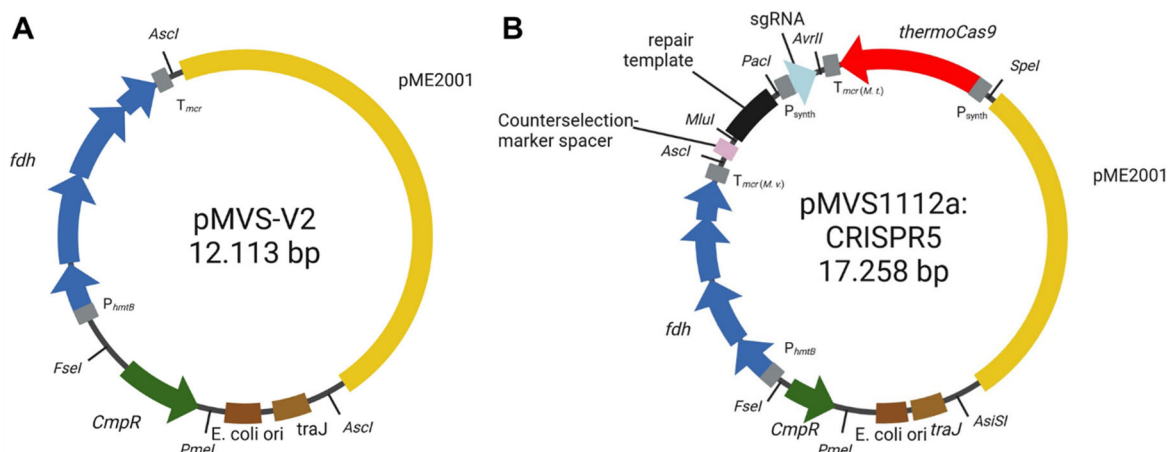


Figure 24. A: Design of the formate-selection positive control vector pMVS-V2. Vector pMVS-V2 was created by replacing the neomycin selection marker from pMVS-V1 with the *fdh* operon and is used as a positive control for conjugations with the *fdh* operon as a selection marker. B: Exemplary design of the pMVS1112a:CRISPR shuttle vectors. The shuttle vector series pMVS1112a carries the *fdh* operon in the *M. thermautotrophicus* Δ H selection marker module. The pMVS1112a:CRISPR shuttle vector series has the CRISPR module in the application module site. The CRISPR module contains four submodules that are each flanked by two rare restriction enzyme recognitions sites: The Cas submodule containing ThermoCas9 under the control of the P_{synth} promoter (flanked by *SpeI* and *AvrII* sites); the sgRNA submodule containing the sgRNA of the CRISPR system (flanked by *AvrII* and *PacI* sites, promoter and spacer of sgRNA differ between different CRISPR plasmids); the repair template submodule (flanked by *PacI* and *MluI* sites, repair template differs between different CRISPR plasmids); and a spacer module reserved for a putative counterselection marker (flanked by *MluI* and *AscI*). pME2001, cryptic plasmid for *M. marburgensis* (ori for *M. thermautotrophicus* Δ H); *E. coli* ori and *traJ*, genes required for plasmid maintenance and conjugational transfer of plasmids in *E. coli*; *CmpR*, chloramphenicol resistance for *E. coli*; *fdh*, *fdh* operon from *M. thermautotrophicus* Z-245; P_{synth} , weaker promoter for *M. thermautotrophicus* Δ H; P_{hmtB} , stronger promoter for *M. thermautotrophicus* Δ H; T_{mcr} (*M.v.*), Terminator region of *mcr* operon from *M. voltae*; T_{mcr} (*M.t.*), Terminator region of *mcr* operon from *M. thermautotrophicus* Δ H. *SpeI*, *AvrII*, *PacI*, *MluI*, *AscI*, *FseI*, *PmeI*, *AsiSI*: recognition sites of restriction enzymes flanking the different modules of the shuttle vector. Figure created with Biorender.com.

4.3. First CRISPR plasmids suggest activity of ThermoCas9 in *M. thermautotrophicus* Δ H

We attempted conjugation of the first CRISPR plasmids and the control plasmid pMVS-V2. First attempts failed due to technical difficulties: An overnight culture of *E. coli* S17-1 carrying the respective plasmid is usually reinoculated into fresh medium in the morning and then used for conjugation after it grew to an OD₆₀₀ of 1.8 – 2. The *E.*

coli S17-1 carrying pMVS1112a:CRISPR1 and other CRISPR plasmids would eventually reach such an OD₆₀₀, but only after twelve hours or more. This was substantially slower than the *E. coli* S17-1 carrying simpler shuttle vectors. This was most likely caused by the size of the CRISPR plasmids (16-18 kbp) and the fact that the *fdh* operon, the *thermoCas9* gene, and the sgRNA are all under the control of promoters that are active in *E. coli* (Section III.3).

Therefore, we modified the conjugation protocol to accommodate the slow growth of *E. coli* S17-1 with large plasmids. Because the *E. coli* grew relatively slowly, we decided to inoculate the main culture used for conjugation the evening before. This led to an *E. coli* culture with the desired OD₆₀₀ the following morning. We also hypothesized that the relatively short recovery time for *M. thermautotrophicus* ΔH of only four hours could become an issue with more complex plasmids. Therefore, we increased the recovery time to two days and directly added the formate plus supplements to the recovery so that recipient cells have all the required nutrients and formate is directly available as soon as the *fdh* operon is expressed (Figure 25).

Before attempting gene deletion with the CRISPR system, we wanted to test whether the ThermoCas9 is active in *M. thermautotrophicus* ΔH. We constructed two plasmids, pMVS1112a:CRISPR1 and pMVS1112a:CRISPR2. The CRISPR module of pMVS1112a:CRISPR1 contains an sgRNA targeting the *hpt* gene under the control of the P_{synth} promoter and no repair template. If the ThermoCas9 is active in *M. thermautotrophicus* ΔH, the nuclease activity of the ThermoCas9 should lead to double-strand breaks at the target site, leading to the death of the recipient cell. Therefore, we did not expect any exconjugants for this plasmid.

Plasmid pMVS1112a:CRISPR2 also contains an sgRNA under the control of the P_{synth} promoter but with a random spacer sequence that has no match in the genome of *M. thermautotrophicus* ΔH. Therefore, plasmid pMVS1112a:CRISPR2 should lead to the expression of a CRISPR complex with no target in the *M. thermautotrophicus* ΔH and, therefore, should be inactive. Thus, the transformation of pMVS1112a:CRISPR2 should not negatively affect the growth of *M. thermautotrophicus* ΔH or conjugation efficiency.

Plasmids pMVS1112a:CRISPR1, pMVS1112a:CRISPR2, and the positive control plasmid pMVS-V2 were conjugated with the adapted conjugation protocol. The

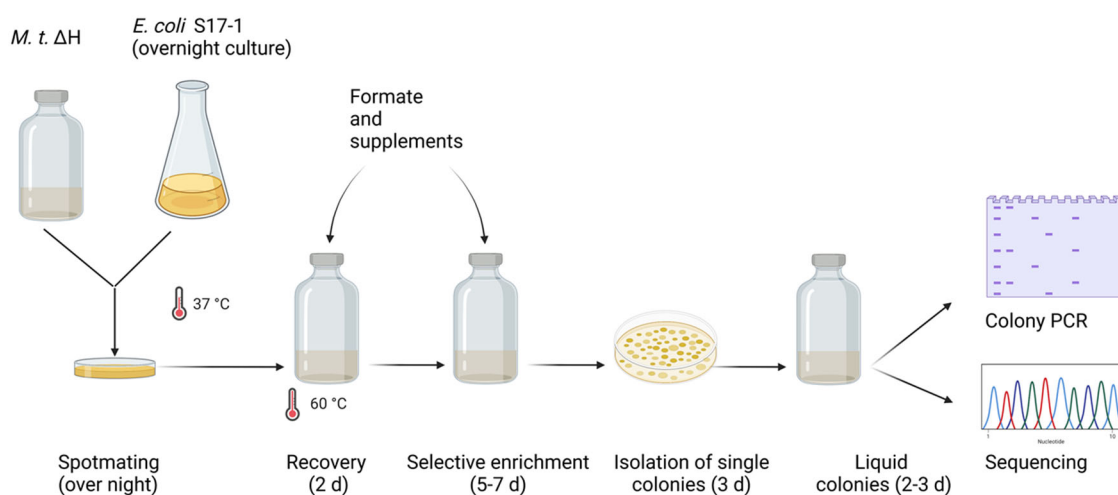


Figure 25. Adaptation of the conjugation protocol for large plasmids and selection on formate. An overnight culture of *E. coli* S17-1 carrying the desired plasmid is mixed with wild-type *M. thermautotrophicus* ΔH and spot-plated on LB-MS plates. Plates are incubated at 37°C overnight. The next day, the spots are washed off and transferred into fresh MS medium with formate and supplements and an (H₂/CO₂) (80/20 vol-%) headspace (recovery with both hydrogen and formate as electron donors). After two days, the culture is transferred into new liquid MS medium with formate and supplements and an N₂/CO₂ (80/20 vol-%) headspace (selective conditions with only formate as an electron donor). Growth in selective conditions usually became visible after five to seven days, at which point the culture was spot-plated on MS agar plates with formate and supplements. Usually, after about three days, single colonies became visible and were transferred into liquid MS medium with formate and supplements. Grown cultures were analyzed *via* colony PCR and plasmid or genomic DNA purification with subsequent sequencing. Figure created with Biorender.com.

selective enrichment culture with pMVS1112a:CRISPR2 and the control plasmid showed visible growth after six days. For the plasmid pMVS1112a:CRISPR1, on the other hand, cells only grew after nine days in one of two technical duplicates, and there was no visible growth for the second culture even after two weeks of incubation. A colony PCR of both strains showed no large-scale deletions in the *hpt* region (Figure S7). These results suggest that the ThermoCas9 is active in *M. thermautotrophicus* ΔH because plasmid pMVS1112a:CRISPR1, which encodes the ThermoCas9 and an *hpt*-targeting sgRNA seemed to inhibit conjugation success and considerably increase the time required for growth under selective conditions (most likely due to escape mutations, section V.4). On the other hand, plasmid pMVS1112a:CRISPR2 expressing the ThermoCas9 and a non-targeting sgRNA did not show a decrease in

conjugation success rate or an increase in the time required for growth under selective conditions.

We then set on to use the ThermoCas9 for actual genome editing. However, we realized that the *hpt* gene might not be a good target for the first attempts at gene deletion. The *hpt* gene seems to form an operon-like structure with the gene *dph2* (Figure 26). *Dph2* encodes an enzyme involved in the posttranslational conversion of a histidine residue into a diphthamide residue in the translation elongation factor Ef-2 (Zhang *et al.* 2010). Loss of diphthamide biosynthesis is either lethal or leads to severe growth defects in eukaryotes and archaea (Narrowe *et al.* 2018). Similar to the reported polar effects of *hpt* deletion on hydrogen metabolism in *Methanococcus* (Sarmiento *et al.* 2011), deletion of *hpt* might have polar effects on *dph2*. In contrast, there is no gene directly downstream of *upt*, making polar effects more unlikely. Therefore, we decided to use *upt* as the target for further CRISPR plasmids.

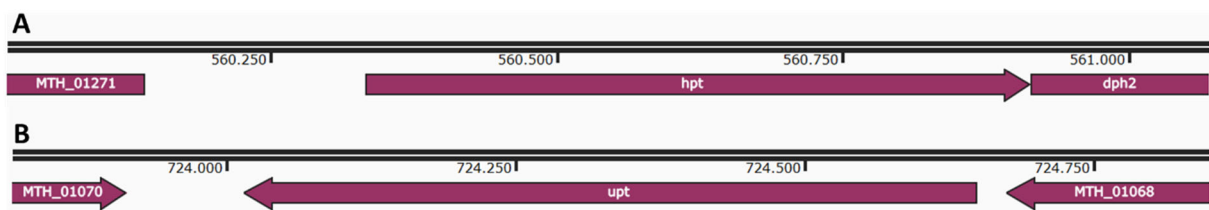


Figure 26. A: *Hpt* gene and surrounding genome region of *M. thermautotrophicus* ΔH. B: *Upt* gene and surrounding genome region of *M. thermautotrophicus* ΔH.

We created plasmid pMVS1112a:CRISPR3, which contained the *thermoCas9* gene, a *upt* targeting sgRNA under the control of the P_{hmtB} promoter, and the 750 bp flanking regions up- and downstream of the *upt* gene fused together as a repair template. We attempted to transfer the plasmid into *M. thermautotrophicus* ΔH. Of the five conjugation attempts, only one was successful, while the conjugation with control plasmid pMVS-V2 was successful three times. The one apparently successful conjugation was further investigated. Bright-field and fluorescence microscopy of the cultures revealed the presence of a second, rod-shaped, non-fluorescent species of microbes in addition to the fluorescent, rod-shaped *M. thermautotrophicus* ΔH cells. Because all methanogens are naturally fluorescent due to the methanogenesis cofactor F₄₂₀ (Lambrecht *et al.* 2017), the observed second cell type is most likely a different non-methanogenic species. While the contamination was not further investigated, we speculated that it could be the thermophilic acetogenic bacterium

Thermoanaerobacter kivui, which is also cultivated in our lab. *T. kivui* grows at 60°C and can also utilize formate (Leigh *et al.* 1981). We attempted to eliminate the contamination by treating the cells with streptomycin. However, while wild-type *M. thermautotrophicus* ΔH was not inhibited by streptomycin concentrations of up to 1000 µg/mL, the putative strain *M. thermautotrophicus* ΔH pMVS1112a:CRISPR3 did not grow at any of the tested concentrations (100 µg/mL - 1000 µg/mL) of streptomycin. Decontamination of strain *M. thermautotrophicus* ΔH pMVS1112a:CRISPR3 was not possible, and conjugation repeatedly failed. Thus, we concluded that there was an issue with this plasmid, potentially caused by the encoded CRISPR system.

4.4. Successful deletion of the *upt* gene with plasmid pMVS1112a-CRISPR6

Due to the issues with pMVS1112a:CRISPR3, we decided to broaden our approach. For *upt*-targeting CRISPR plasmids, we cloned a new construct, pMVS1112a:CRISPR5, which contains the same sgRNA as pMVS1112a:CRISPR3, but under the control of the P_{synth} promoter, which was the one used in pMVS1112a:CRISPR1 and pMVS1112a:CRISPR2, for which we had observed promising results. Because there is also always the possibility that, unlike in other methanogens, *upt* is essential in *M. thermautotrophicus* ΔH, we decided to test a second target in parallel. For this, we picked the *mth60* operon because it was already demonstrated that it could be deleted *via* homologous recombination (Fink *et al.* 2023). Thus, issues with generating an *M. thermautotrophicus* ΔH Δ*mth60* strain with the CRISPR system would mean that the issue lies with the CRISPR system, not with the targeted gene. To facilitate deletion of the *mth60* operon, we cloned pMVS1112a:CRISPR8, which contains an *mth60*-targeting sgRNA under the control of P_{synth} and a repair template consisting of the fused flanking regions, which had been used for *mth60* deletion *via* HR (Fink *et al.* 2023). Conjugation of pMVS1112a:CRISPR5, pMVS1112a:CRISPR8, and control plasmid pMVS-V2 was attempted five times. While three of the five conjugations led to growth under selective conditions with plasmid pMVS-V2, no growth under selective conditions was observed for any of the conjugations with pMVS1112a:CRISPR5 and pMVS1112a:CRISPR8.

Therefore, we decided to revise both plasmids. pMVS1112a:CRISPR5 has a repair template with only 750 bp flanking regions, which is smaller than the 1kb flanking

regions used in the successful deletion of the *mth60* operon (Fink *et al.* 2023). We, therefore, decided to increase the size of the flanking regions to 1 kb for each site, creating plasmid pMVS1112a:CRISPR6. For the *mth60* targeting plasmid, we included a second sgRNA because the to-be-deleted fragment is much larger (2.1 kb) than the one for *upt*, and a second DSB caused by a second sgRNA might increase editing efficiency as evidenced by the design of such systems in other microbes (Nagaraju *et al.* 2016, Hong *et al.* 2018). This resulted in plasmid pMVS1112a:CRISPR11. We also created a variety of control plasmids, which were not yet conjugated until now and are summarized in VII.5.

Conjugations of pMVS1112a:CRISPR6, pMVS1112a CRISPR11, and control plasmid pMVS-V2 were performed with a slightly modified conjugation protocol, where mating time was increased to 72 hours instead of 24 hours to give *E. coli* more time to transfer the large (18 kb) plasmids. Both control cultures with pMVS-V2 showed growth six days after transfer in selective medium. 14 days after transfer into selective medium, there was visible growth in both of the pMVS1112a:CRISPR11 cultures and one of the pMVS1112a:CRISPR6, and the cultures were plated to generate single colonies for further analysis. Both cultures of *M. thermautotrophicus* Δ H pMVS1112a:CRISPR11 did not produce colonies in several plating attempts with all established plating techniques (*i.e.*, spot-plating, spread-plating, pour-plating (Fink *et al.* 2021)).

Spot-plating of *M.thermautotrophicus* Δ H pMVS1112a:CRISPR6 resulted in single colonies, of which five were picked into liquid medium and subsequently analyzed *via* colony PCR. With primers amplifying the *upt* region, all five clones showed two bands, one corresponding to the expected size of the deletion of *upt* (2.3 kb) and one either corresponding to the wild-type (3 kb) or slightly below (Figure 27). Because a detailed genetic analysis is complicated with a mixed signal, we first attempted to resolve the heterozygous genotype of the clones.

Two different strategies were employed to resolve the genotype. In the first strategy, we investigated whether *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 shows increased resistance to 6-AU and whether this resistance can be used to select cells with a clean *upt* deletion. For this, the growth of *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 with formate as electron donor and with 200 μ g/mL or without 6-AU was compared to a control strain (pMVS1111a:P_{hmtB}-*fdhZ*-245). The grown *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 cultures were also analyzed *via* colony

PCR. While the cultures did not exhibit an increased 6-AU resistance compared to the control strain (Figure 28), the subsequent colony PCR did exhibit a clear difference

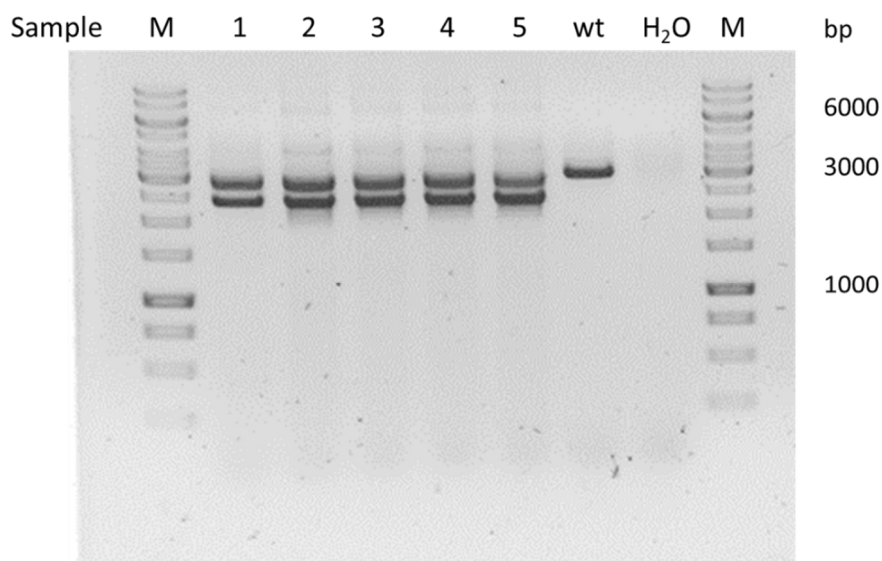


Figure 27. Colony PCR of five clones of *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 with primers amplifying the *upt* region of the *M. thermautotrophicus* Δ H genome. Samples: 1-5, five clones of *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 as the sample; wt, wild-type *M. thermautotrophicus* Δ H as the sample; H₂O, water as the sample; M, Marker.

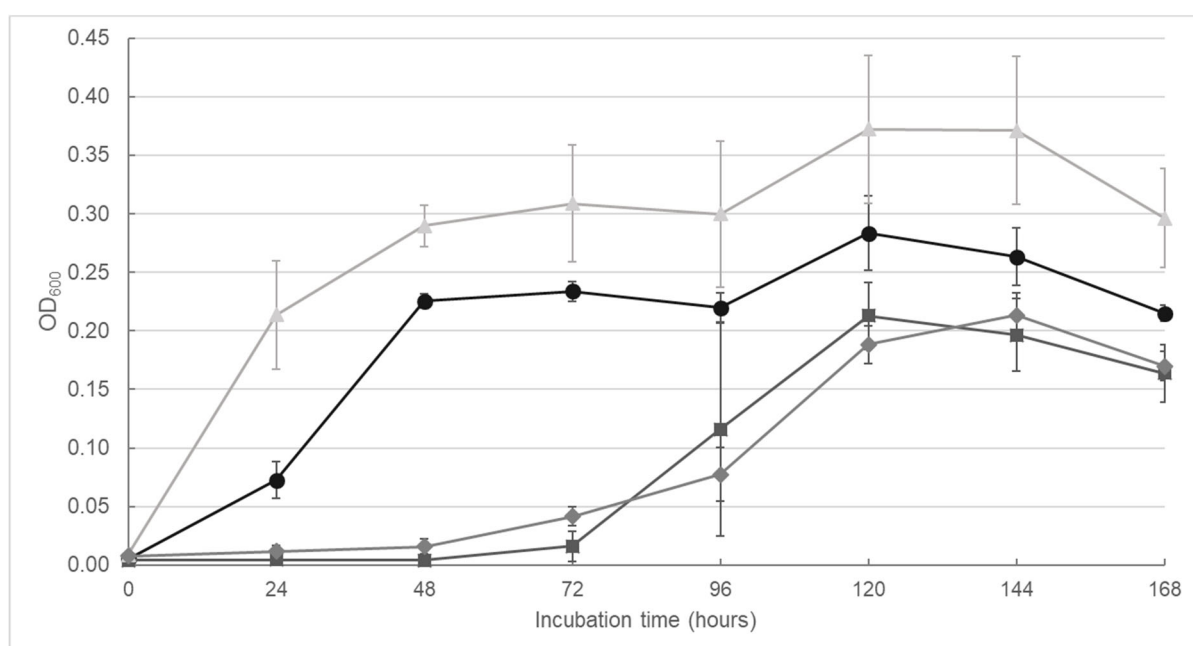


Figure 28. Growth curve of *M. thermautotrophicus* Δ H pMVS-V2 and *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 in MS medium with formate with or without 6-AU. Circles, *M. thermautotrophicus* Δ H pMVS-V2 with 0 μ g/mL 6-AU; squares, *M. thermautotrophicus* Δ H pMVS-V2 with 200 μ g/mL 6-AU; triangles, *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 with 0 μ g/mL 6-AU; diamonds, *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 with 200 μ g/mL 6-AU. Average (N=3) with error bars indicating standard deviation.

between the cultures treated with 6-AU and the untreated cultures (Figure 29). For all three cultures treated with 6-AU, the upper band in the colony PCR was much weaker than the untreated sample, while the lower one (corresponding to the deletion signal) was stronger.

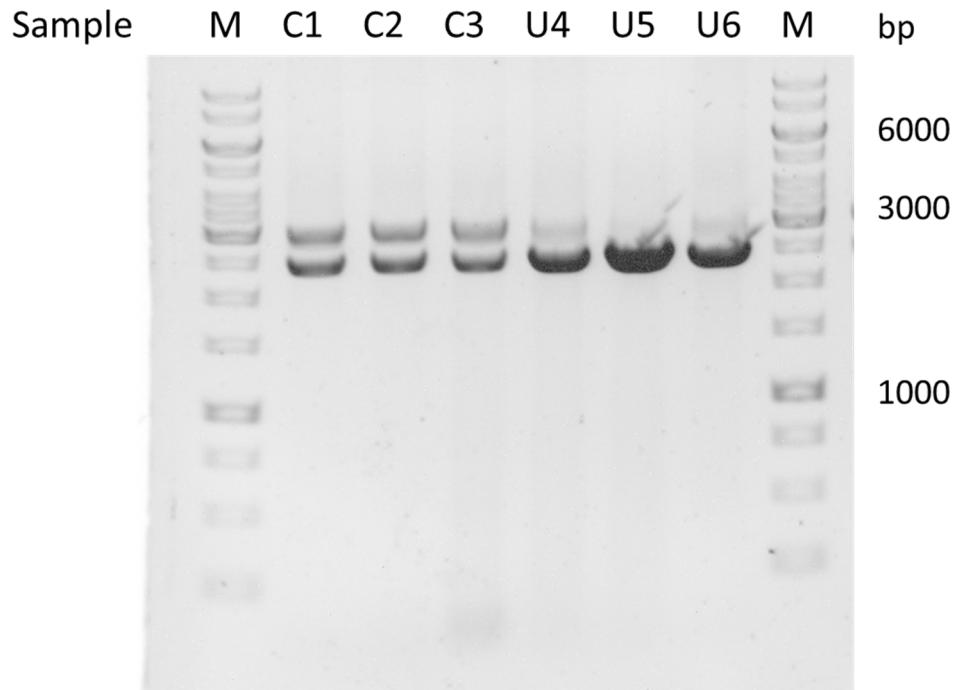


Figure 29. Colony PCR of *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 cultures from the growth curve with (U1-U3) and without (C1-C3) 6-AU with primers amplifying the *upt* region of the *M. thermautotrophicus* Δ H genome. M, Marker.

Parallel to this, we also tested whether the genotype could be resolved without 6-AU counterselection. We hypothesized that the mixed genotype is caused by an insufficient separation of cells while plating, resulting in colonies that originate from a group of cells instead of single cells. *M. thermautotrophicus* Δ H grows in short filaments of several cells (Majerník *et al.* 2005). Those filaments are also attached to other filaments *via* cell appendages (fimbriae) (Fink *et al.* 2023). We first attempted to remove the fimbriae from the cells with an established protocol initially designed for fimbriae purification (Thoma *et al.* 2008, Kühle 2024). For this, a grown culture of *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 was treated with an immersion blender for one minute, washed once with fresh medium to remove the fimbriae, and then spotted on plates. As a control, the same culture was only washed and then spotted on plates. Spots of the blender-treated culture show a clear morphological difference compared to the control (Figure 30 A and B).

While the untreated culture mainly grew in a ring at the outer rim of the spot with a small number of colonies in the middle, the blended culture did not form a ring but instead formed several dozen single colonies in the middle. Several colonies from the blended and the control plate were inoculated into liquid medium. When grown, these colonies were picked into liquid medium and analyzed again *via* colony PCR. Most cultures showed two bands again. However, one of the cultures (blended #4) showed only a faint band in addition to the Δupt signal (Figure 30). This culture was used for the following plating experiments.

Next, we wanted to test whether prolonged incubation aids in resolving the genotype because it was shown that the length and number of genomes per filament decrease during prolonged incubation in the stationary phase (Majerník *et al.* 2005). Therefore, we transferred clone “blended #4” into fresh liquid selective MS medium, incubated the culture for two days until cells reached stationary phase, spread- and spot-plated 100 μ L of culture, incubated the stationary liquid culture for two more days, and then spread and spot-plated again. Colonies were picked from both plates, and the liquid cultures were analyzed *via* colony PCR (Figure 31). All colony PCRs still showed both bands except for colony number three from the plating after two days of incubation (Figure 31A). This culture was, therefore, transferred into fresh medium. However, a second colony PCR showed two bands again (Figure 31C).

In summary, the transformation of *M. thermautotrophicus* ΔH with pMVS1112a:CRISPR6 led to a strain showing the expected band for a *upt* deletion in a colony PCR but a second band in addition. The heterozygous genotype could be resolved with 6-AU but not other tested methods. These results still need to be confirmed by sequencing. However, further attempts to resolve the genotype without 6-AU, sequencing of the 6-AU treated strain showing only a single band in the colony PCR, and further investigation of the not platable *M. thermautotrophicus* ΔH pMVS1112a:CRISPR11 strain could not be conducted before concluding the laboratory work of this dissertation.

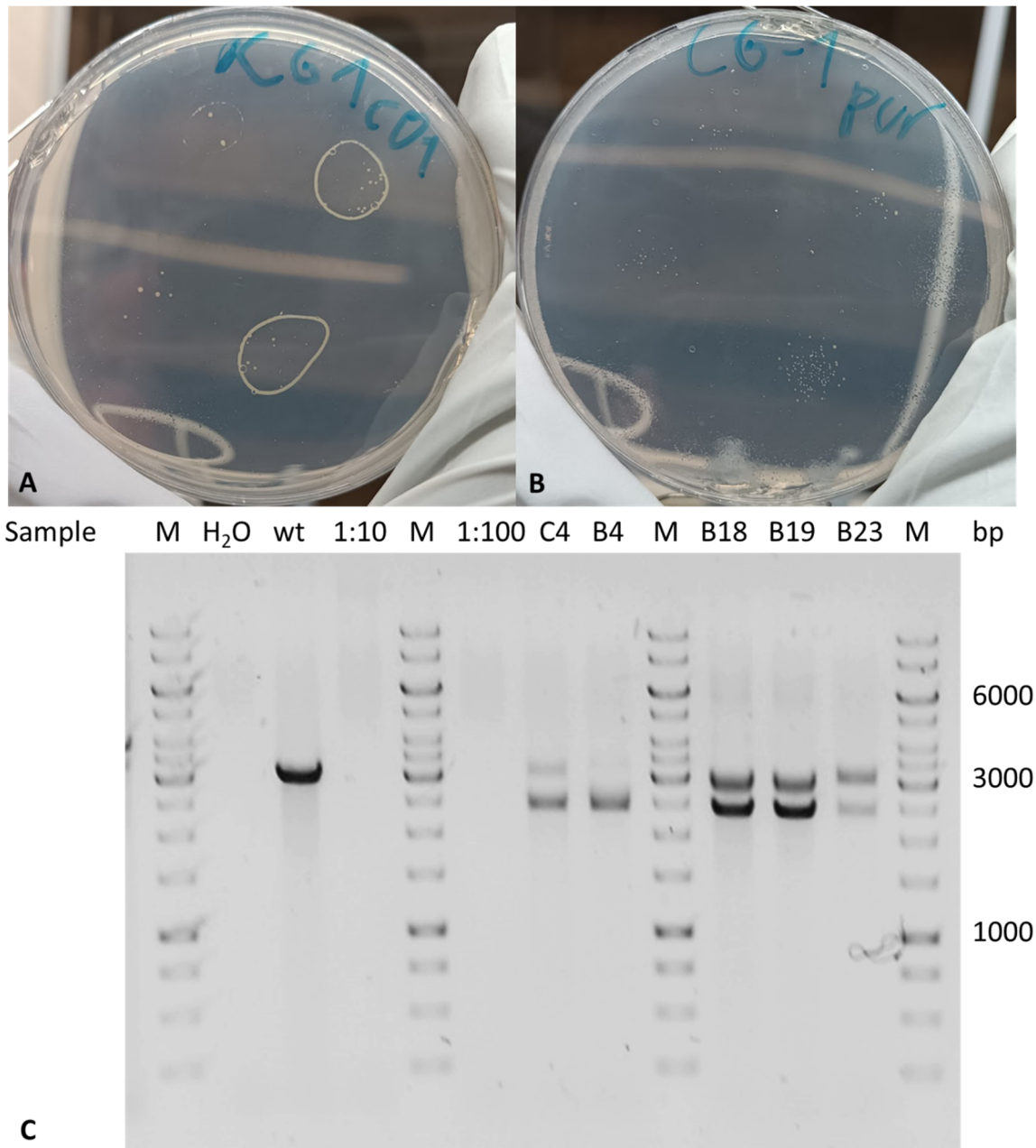


Figure 30. A: Plates of *M. thermautotrophicus* ΔH pMVS1112a:CRISPR6 without blender treatment of the culture before plating. B: Plates of *M. thermautotrophicus* ΔH pMVS1112a:CRISPR6 with blender treatment of the culture before plating. C: Colony PCR of liquid cultures of one colony from plate A and four colonies of plate B. Samples: H₂O, water; wt, *M. thermautotrophicus* ΔH wild-type; 1:10, 1:10 dilution of 100 ng/mL pMVS1112a:CRISPR6 plasmid DNA; 1:100, 1:10 dilution of 100 ng/mL pMVS1112a:CRISPR6 plasmid DNA; C4, liquid culture of colony from a plate without blender pretreatment; B4, B18, B19, B23, four liquid cultures of colonies from a plate with blender pretreatment; M: Marker.

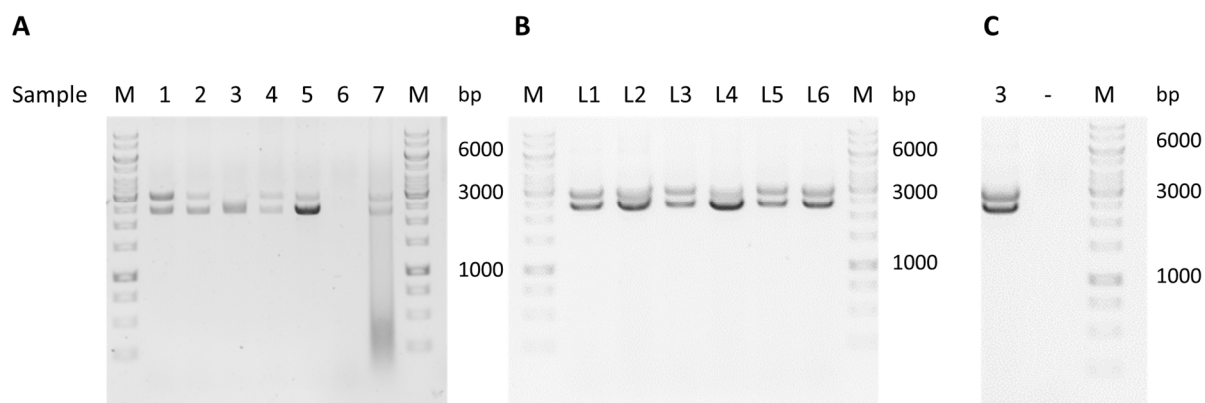


Figure 31: Effects of a prolonged incubation before plating on the genotype of *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6. Colony PCR with primers amplifying the *upt* region of the *M. thermautotrophicus* Δ H genome. A: 1-7, colony PCR of seven cultures incubated for two days before plating. B: L1-L6, colony PCR of six cultures incubated for four days before plating. C: 3: transfer of culture 3 from figure A; -, water control; M, Marker.

V. Discussion

In this dissertation, we present important additions to the genetic toolbox of *M. thermautotrophicus* Δ H. We investigated three putative selection markers, namely a mupirocin resistance cassette, a puromycin resistance cassette, and the *fdh* operon from closely related *M. thermautotrophicus* Z-245, for their usability as selection markers. We could show that the *fdh* operon enables *M. thermautotrophicus* Δ H to utilize formate as an alternative electron donor and can also be used to directly select recombinant cells after conjugation based on their formate prototrophy. Unlike with neomycin resistance as a selection marker (Fink *et al.* 2021), no spontaneous growth of wild-type cells was ever observed, making the *fdh* operon the second selection marker for *M. thermautotrophicus* Δ H, and the first one where the growth of spontaneously resistant wild-type cells is not an issue. This tight selection marker was the basis for the further development of genome editing tools. Here, we designed a modular CRISPR system based on the ThermoCas9 from *G. thermodenitrificans* T12 (Mougiakos *et al.* 2017). We showed that ThermoCas9 is a promising tool for genome editing in *M. thermautotrophicus* Δ H by deleting the gene encoding the uracil phosphoribosyl transferase (*upt*). In addition, we also showed that *M. thermautotrophicus* Δ H is transformable *via* electroporation, which opens new possibilities for the development of further tools.

1. Selection markers

In the first part of this dissertation, we sought to develop additional selection markers for *M. thermautotrophicus* Δ H. So far, only a thermostable neomycin resistance cassette is available as a selection marker in *M. thermautotrophicus* Δ H (Fink *et al.* 2021), and having only a single selection marker severely limits the possibilities for genetic alterations. In addition, the neomycin resistance cassette has the severe drawback that while successful exconjugants usually grow after about two days, prolonged incubation leads to the growth of spontaneously resistant wild-type cells after around five days (Fink 2021). While selection remains possible due to the faster initial growth of exconjugants carrying the neomycin resistance cassette, this could lead to severe issues for any genetic modification that leads to a growth-deficient

phenotype. In this case, successful exconjugants could be overgrown by spontaneously resistant cells.

We first focused on selection markers used in other methanogens, namely simvastatin, mupirocin, and puromycin resistance cassettes. Because it was already previously reported that with simvastatin, the growth of spontaneously resistant cells also occurs within a few days (Fink 2021), simvastatin was not further investigated in this work.

1.1. Mupirocin

We first investigated mupirocin resistance as a selection marker. Firstly, we tested the concentrations of mupirocin required for inhibiting the growth of *M. thermautotrophicus* Δ H. While there was already a delayed growth starting from 1 μ g/mL of mupirocin, 20 μ g/mL was needed to inhibit growth for at least three days in liquid culture, and 50 μ g/mL was required to inhibit growth on plates. This is a higher concentration than reported for *M. marburgensis*, where 10 μ g/mL inhibited growth for at least 48 h (Kiener *et al.* 1986). However, the authors only measured the growth inhibition for 48 h in liquid, which might explain the differences. We used 50 μ g/mL mupirocin for all further experiments because this concentration was required on plates.

Next, we engineered a putative mupirocin resistance cassette. A strain carrying plasmid pMVS1111a:MupR grew with up to 100 μ g/mL mupirocin. In contrast, a control strain carrying the empty shuttle vector did not grow with any of the tested concentrations for 72 h, indicating that the MupR cassette increases the mupirocin resistance of *M. thermautotrophicus* Δ H. Therefore, we investigated whether the MupR cassette could be used as a selection marker in *M. thermautotrophicus* Δ H. The conjugation of pMVS1111a:MupR, but this time, we selected the exconjugants with mupirocin instead of neomycin. Selection was not possible at 20 μ g/mL because the cultures grew at the same time as the negative control. With 50 μ g/mL or 100 μ g/mL mupirocin, there was visible growth only for the cultures from the pMVS1111a:MupR conjugation, not from the control. However, we could not detect the plasmid *via* colony PCR, and cells failed to grow on neomycin, indicating that the cells do not carry pMVS1111a:MupR but are spontaneously resistant to mupirocin. The selection of recombinant *M. thermautotrophicus* Δ H with mupirocin and MupR was considered unsuccessful and not further pursued.

The main issue with mupirocin resistance as a selection marker remains the high rate of spontaneous resistance observed. Under any tested mupirocin concentration, spontaneous resistant cells arose after a week at the latest. While the spontaneous resistant cultures were not further investigated for the cause of their resistance, a likely explanation would be mutations in the genomic *ileS* gene because the designed resistance cassette is just a heterologous *ileS* gene with two point mutations. The high rate of spontaneous resistance can also explain why the selection of mupirocin still failed despite strain *M. thermautotrophicus* Δ H pMVS1111a:MupR showing an increased mupirocin resistance: When the mupirocin resistance of strain *M. thermautotrophicus* Δ H pMVS1111a:MupR was compared to a control strain carrying an empty vector, both cultures were inoculated with a similar number of cells. However, this does not reflect the state in selective conditions after a conjugation. Conjugation efficiency for *M. thermautotrophicus* Δ H is estimated to be $4 \cdot 10^{-9} - 6 \cdot 10^{-6}$ exconjugants per initial recipient cells (Fink *et al.* 2021). This means that for one cell that received plasmid pMVS1111a:MupR, there are a million to a billion wild-type cells, which drastically increases the chances that wild-type cells acquire the mutations required to be mupirocin resistant in the time the exconjugants need to grow from their low initial number. The MupR cassette could be further improved by increasing the expression by exchanging the P_{synth} promoter with the stronger P_{hmtB} or by screening spontaneously resistant cells for additional mutations in the *ileS* and introducing them in the MupR cassette. However, this would not resolve the issue of the high rate of spontaneous resistance observed. Also, unlike resistance markers used for other antibiotics, MupR is not an enzyme that degrades the antibiotic, but a mutated isoleucine tRNA-synthetase (IleS), and therefore, most likely also influences the tRNA and amino acid metabolism of *M. thermautotrophicus* Δ H. These issues make mupirocin resistance unsuitable as a selection marker in *M. thermautotrophicus* Δ H, which is also highlighted by the fact that while it was reported as a selection marker in *Methanosarcina* more than 20 years ago (Boccazzi *et al.* 2000), it has only been used in one follow-up work (Zhang *et al.* 2002).

1.2. Puromycin

Puromycin is the standard antibiotic in the well-genetically tractable genera *Methanosarcina* and *Methanococcus* (Enzmann *et al.* 2018). We first investigated whether puromycin inhibits the growth of *M. thermautotrophicus* Δ H. 50 $\mu\text{g/mL}$ of

puromycin inhibited the growth of *M. thermautotrophicus* ΔH for several weeks at 60°C or 42°C. This is a considerably higher concentration than those reported for other methanogens, where concentrations between 1 µg/mL (Metcalf *et al.* 1997) and 2.5 µg/mL (Tumbula *et al.* 1997) were used.

We then assessed whether the *pac* gene increases the resistance of *M. thermautotrophicus* ΔH to puromycin. *M. thermautotrophicus* ΔH pMVS1111a:P_{synth}-*pac* did not show an increased puromycin resistance compared to the wild-type when incubated at 42°C with 50 µg/mL puromycin. At 60°C, the strain seemed to show a small amount of growth between 72 h and 120 h, at which the growth seemed to stop again at an OD₆₀₀ of only 0.06, which is insufficient for selection on puromycin. Adaptation of the strain by gradually increasing temperature or puromycin concentration was also unsuccessful: At 37°C, there was no difference in growth behavior between *M. thermautotrophicus* ΔH pMVS1111a:P_{synth}-*pac* and the wild-type control. Incubating *M. thermautotrophicus* ΔH pMVS1111a:P_{synth}-*pac* with lower puromycin concentration gave rise to a spontaneous resistant population that also could grow with 50 µg/mL puromycin but did not carry the plasmid anymore.

Our results indicate that the *pac* gene does not confer sufficient puromycin resistance to *M. thermautotrophicus* ΔH. Because this is also the case at 37°C, where the *pac* gene confers puromycin resistance to *Methanococcus* (Gernhardt *et al.* 1990), the thermostability of the Pac enzyme cannot be the main reason for this. One possible reason for that could be the high puromycin concentrations used, which are twentyfold higher than what was used for other methanogens. Therefore, the lack of puromycin resistance of *M. thermautotrophicus* ΔH pMVS1111a:P_{synth}-*pac* could be caused by the fact that the Pac enzyme cannot acetylate these high amounts of puromycin quickly enough. This problem could be solved by increasing the *pac* gene's expression or the Pac enzyme's activity. We attempted to exchange the promoter of *pac* for the stronger P_{hmtB} promoter (Fink *et al.* 2021). Unfortunately, this was not possible due to cloning issues in *E. coli* (section VII.2). We also attempted to increase the efficiency and thermostability of *pac* by random mutagenesis. While transformation with a mutagenized set of plasmids should be possible with the newly established electroporation protocol (section III.2), we could not attempt electroporation of a shuttle vector carrying the randomly mutagenized *pac* gene due to persistent cloning issues

(section VII.3). The observed cloning issues are most likely caused by the activity of the *M. thermautotrophicus* ΔH promoters in *E. coli* (section IV.3).

In summary, puromycin remains an interesting antibiotic because of the four antimicrobial compounds (neomycin, simvastatin, mupirocin, puromycin) tested in this and previous work (Fink *et al.* 2021), it was the only one for which no spontaneous resistance was observed when the strains were incubated with a sufficient antibiotic concentration. On the other hand, the *pac* gene did not confer puromycin resistance to *M. thermautotrophicus* ΔH . Optimization attempts were unsuccessful due to cloning issues. These cloning issues might be circumvented by finding promoters that are not active in *E. coli*. Further attempts to establish puromycin resistance as a selection marker should first focus on finding additional promoters that are active in *M. thermautotrophicus* ΔH but not in *E. coli*.

1.3. Formate

Besides using antibiotic resistances, employing prototrophies as selection markers is a common technique in various microbes. Such selection markers are also commonly employed in archaea, especially hyperthermophiles, where antibiotic selection is challenging due to the growth temperature of the microbes. In *Sulfolobus*, for example, recombinant cells are selected by their uracil (Deng *et al.* 2009) or agmatine (Zhang *et al.* 2013) prototrophy. These selection markers have the drawback of using a native gene of the microbe as the selection marker, which requires prior deletion of the said gene from the genome. This can be circumvented by using heterologous genes that encode enzymes that the wild-type microbe is unable to utilize for growth. There are several examples of such systems: Recombinant *Aspergillus niger* can be selected by their ability to utilize acetamid as a carbon and nitrogen source (Kelly *et al.* 1985), and different recombinant *Lactobacillus* species can be selected by their ability to utilize rare sugars (Peterbauer *et al.* 2011). In this work, we could successfully implement a selection marker of this type for *M. thermautotrophicus* ΔH by establishing formate prototrophy.

We first demonstrated that the expression of the formate dehydrogenase (*fdh*) operon from *M. thermautotrophicus* Z-245 in *M. thermautotrophicus* ΔH enables *M. thermautotrophicus* ΔH to use formate as an alternative electron donor instead of hydrogen. For this, we constructed strain *M. thermautotrophicus* ΔH

pMVS1111a: P_{hmtB} -*fdh*_{Z-245}, which showed a comparable growth behavior to control strain *M. thermautotrophicus* Δ H pMVS-V1 and *M. thermautotrophicus* Z-245 with hydrogen as the sole electron source. *M. thermautotrophicus* Δ H pMVS1111a: P_{hmtB} -*fdh*_{Z-245} was also able to grow with formate as the sole electron source, unlike control strain *M. thermautotrophicus* Δ H pMVS-V1 or wild-type *M. thermautotrophicus* Δ H. However, growth was considerably slower than the growth of *M. thermautotrophicus* Z-245 despite both strains carrying the same *fdh* operon.

After showing that the *fdh* operon enables *M. thermautotrophicus* Δ H to utilize formate, we could also demonstrate that we can use this characteristic to directly select recombinant cells by their formate prototrophy. For this, we repeated the conjugation with plasmid pMVS1111a: P_{hmtB} -*fdh*_{Z-245} with either neomycin or formate selective conditions. Growth was visible after two days for neomycin selection and five days for formate selection. We confirmed the presence of the shuttle vector in cultures from both selective conditions by colony PCR, plasmid reisolation, and subsequent sequencing. Thus, we could demonstrate that formate prototrophy can be used to directly select recombinant *M. thermautotrophicus* Δ H cells. Lastly, the poor plating efficiency on formate was improved by adding 0.1 % H₂S to the gas-phase of formate-containing plates.

For a selection marker, the *fdh* is relatively large, with a size of 4.5 kb, and it causes severe cloning issues in *E. coli*. In all cloning steps involving plasmids carrying the *fdh* operon, most clones screened somehow seemed to lack the *fdh* from the plasmid backbone, and clones carrying plasmids without *fdh* deletions showed a considerable growth delay (section VII.2). This suggests that the *fdh* operon puts a considerable burden on the *E. coli* cells. In this work, we demonstrated that the promoter P_{hmtB} , which controls the expression of the *fdh* operon, is also active in *E. coli*. Thus, the expression of the *fdh* operon in *E. coli* could cause the observed issues. It is unlikely that this is caused by the activity of the Fdh as an enzyme because Fdh: **1)** requires a molybdopterin cofactor for activity (Baron *et al.* 1989) that is not encoded in the operon; **2)** requires redox cofactor F₄₂₀ as the electron acceptor, which has been described in other archaea and bacteria besides methanogens, but not in *E. coli* (Grinter *et al.* 2021); and **3)** it is unclear whether the enzyme is active at low temperatures at all. Therefore, the observed issues are more likely caused by other factors, such as the

accumulation of misfolded proteins, especially the putative transmembrane protein FdhC.

It is unclear whether all four genes in the operon (*fdhC*, *fdhA*, *fdhB*, *orf3*) are required for growth on formate. The first three genes encode for a putative formate transporter (*fdhC*) and the two subunits of the formate dehydrogenase (*fdhA* and *fdhB*) (Nölling *et al.* 1997). While the *M. thermautotrophicus* Δ H genome encodes an anabolic formate dehydrogenase in the genome, the corresponding enzyme was not found in the proteome, and the genome does not encode a formate transporter (Casini *et al.* 2023). This makes it likely that all three genes are required for growth on formate, although this still needs to be experimentally verified. However, the case is different for *orf3*. While the rest of the *fdh* operon is missing from the genome of *M. thermautotrophicus* Δ H, the strain possesses an *orf3* homolog (Nölling *et al.* 1997). Also, the size of *fdhCAB* transcripts analyzed *via* northern blot (Nölling *et al.* 1997) suggests that *orf3* is not part of the transcript. The recently published genome of *M. thermautotrophicus* Z-245 annotates *orf3* as carbonic anhydrase (Casini *et al.* 2023). It has been suggested that in *M. acetivorans*, a carbonic anhydrase is associated with the acetate transporter to provide the protons required for acetate import (Ferry 2020). Due to the similar chemical characteristics of both compounds, a similar import mechanism would be plausible for the formate transporter FdhC. However, whether the *orf3* has a function for utilization of formate and whether the *orf3* homolog in the genome is already sufficient for this would need to be experimentally investigated.

Growth of *M. thermautotrophicus* Δ H pMVS1111a:*P_{hmtB}-fdh*_{Z-245} with formate as substrate was considerably slower than with hydrogen as substrate and neomycin, especially in the first selective transfer after the conjugation. While growth in neomycin selective medium usually occurs after two days, growth occurs only after five to six days in formate selective medium. Potentially, this might be attributed to a general feature of growth on formate for *M. thermautotrophicus* strains because *M. thermautotrophicus* Z-245 also exhibits a lag phase of one or two days when transferred from medium with hydrogen to medium with formate (Section IV.1.3). However, in *M. thermautotrophicus* Z-245 this observation is likely connected to a regulatory mechanism of not known in detail yet (Nölling *et al.* 1997). Importantly, also in the follow-up transfers, *M. thermautotrophicus* Δ H pMVS1111a:*P_{hmtB}-fdh*_{Z-245} grew considerably slower than on neomycin (Figure 17) and than strain *M.*

thermautotrophicus Z-245 (Figure 15). The lower growth in the later transfers might be explained by the fact that the plasmid-encoded *fdh* operon in *M. thermautotrophicus* Δ H pMVS1111a: P_{hmtB} -*fdh*_{Z-245} is under the control of promoter P_{hmtB} and not the native promoter region from *M. thermautotrophicus* Z-245. While P_{hmtB} is the strongest promoter currently established for genetic work with *M. thermautotrophicus* Δ H, it is unclear how strong the promoter is compared to the native promoter region of the *fdh* operon.

Another important factor to consider is that, unlike the neomycin resistance gene, the *fdh* operon encodes for an enzyme involved in the central metabolism of *M. thermautotrophicus* Δ H. The growth behavior and formate metabolism of the strains *M. thermautotrophicus* Δ H and *M. thermautotrophicus* Z-245 were further investigated in our lab (Casini *et al.* 2023). Both strains were grown in chemostat bioreactors, and growth behavior, methane and biomass production rates, and the transcriptome and proteome of the cells were analyzed. Interestingly, there were significant differences between the two strains despite their close relationship. *M. thermautotrophicus* Δ H showed a significantly higher gas consumption and lower biomass yields than *M. thermautotrophicus* Z-245 (Casini *et al.* 2023). Transcriptome and proteome analysis suggested differences in the formate metabolism of both strains. When growing on hydrogen and carbon dioxide, *Methanothermobacter* species need to synthesize small quantities of formate for purine biosynthesis (Kaster *et al.* 2011). Transcriptome and proteome data suggest that the two strains synthesize formate in different ways. *M. thermautotrophicus* Z-245 synthesizes formate directly from CO₂ via the formate dehydrogenase that was also used in this work, and *M. thermautotrophicus* Δ H synthesizes formate from pyruvate via a pyruvate-formate lyase (Casini *et al.* 2023).

Thus, introducing the *fdh* operon from *M. thermautotrophicus* Z-245 into *M. thermautotrophicus* Δ H could lead to alterations in the formate metabolism. Further results from the same study suggest that this is the case. The *fdh*-operon carrying strain, *M. thermautotrophicus* Δ H pMVS1111a: P_{hmtB} -*fdh*_{Z-245}, was analyzed in chemostat bioreactors when growing with hydrogen and carbon dioxide or with formate as substrates. Interestingly, when grown on hydrogen and carbon dioxide, the gas consumption and biomass production rates of the strain were much more similar to *M. thermautotrophicus* Z-245 than to wild-type *M. thermautotrophicus* Δ H, suggesting that the addition of the *fdh* operon alters the metabolism of *M.*

thermautotrophicus ΔH in a way that it more closely resembles *M. thermautotrophicus* Z-245 (Casini *et al.* 2023). This is important to keep in mind because it can severely impact all metabolism-related genetic studies of *M. thermautotrophicus* ΔH in which the *fdh* operon is used as a selection marker. However, this should not pose an issue when the *fdh* operon does not remain in *M. thermautotrophicus* ΔH , for example, when being used as a selection marker on a CRISPR plasmid, where the plasmid would eventually be cured from the cells after a successful edit.

Growth on formate was comparable between *M. thermautotrophicus* ΔH pMVS1111a: P_{hmtB} -*fdh*_{Z-245} and *M. thermautotrophicus* Z-245 in the chemostat bioreactors (Casini *et al.* 2023). This is different than what we observed in the bottle experiments in this work, where strain *M. thermautotrophicus* ΔH pMVS1111a: P_{hmtB} -*fdh*_{Z-245} showed a slower growth rate. However, the bioreactors were run in a steady state with a dilution rate of 1 d⁻¹, making it likely that both strains were limited by formate availability and could not reach their maximum growth rate.

Despite the cloning issues, slower growth, and effects on the metabolism of *M. thermautotrophicus* ΔH , the outstanding advantage of the *fdh* operon is that with formate as the sole electron source, no spontaneous growth of wild-type cells was observed even after several weeks of incubation, while spontaneously neomycin resistant cells usually arise within a few days. This enables the use of the *fdh* operon for genetic modifications that lead to growth defects or prolonged lag phases. The importance of this was already exemplified in a later part of this work: The strains *M. thermautotrophicus* ΔH pMVS1112a:CRISPR6 and pMVS1112a:CRISPR11 (both carrying the *fdh* operon as a selection marker) only grew after about two weeks in the first selective transfer. These exconjugants would have long been overgrown by spontaneously resistant cells with neomycin selection.

There are several possibilities to improve the applicability of the *fdh* operon as a selection marker. As previously discussed, not all four genes of the operon might be required for growth. It could be tested whether shortened versions of the operon still enable *M. thermautotrophicus* ΔH to utilize formate for growth and whether this impacts the growth rate of the microbes. If vectors based on pMVS1111a: P_{hmtB} -*fdh*_{Z-245} are used for this, one could first select the strains on neomycin, where also the strains with truncated *fdh* operons should be able to grow, which would circumvent the issue that the varying success rate of conjugation makes conjugation success an unreliable

readout. If these experiments show that all four genes are required for formate utilization, the operon could also be split. Either *fdhC* or *fdhAB* could be introduced in the genome of *M. thermautotrophicus* ΔH . If all genes of the operon are required for formate utilization, this strain should not be able to utilize formate. This strain can then serve as the host strain for further genetic experiments, where the remaining genes of the *fdh* operon serve as a selection marker on a plasmid. Splitting *fdhC* and *fdhAB* into two separate transcriptional units should not cause any problems because it has been reported that the genes are transcribed together (*fdhCAB*) and separately (*fdhC* and *fdhAB*) in *M. thermautotrophicus* Z-245 (Nölling *et al.* 1997). Due to its uncertain overall role in formate metabolism, it is unclear where *orf3* would be placed in this scenario. Shortening the *fdh* operon would lead to smaller shuttle vectors and could help resolve the cloning issues in *E. coli*. In addition, just like the *pac* gene, the *fdh* operon would benefit from having a promoter that is not active in *E. coli*.

2. Electroporation as an alternative transformation method

We established electroporation as an alternative transformation method. We first adapted the electroporation protocol from *C. ljungdahlii* (Molitor *et al.* 2016), which was already established in our lab, for *M. thermautotrophicus* ΔH . The initial electroporation attempt succeeded for only one of the five attempts. While it is difficult to calculate the efficiency due to the liquid enrichment step, the first selective transfer only grew after three days, suggesting a low initial number of transformants. The basic shuttle vector pMVS-V2 would be better suited for determining the transformation efficiency because it might allow for direct plating after conjugation. However, electroporation attempts with this vector remained unsuccessful. We, therefore, continued with pMVS-V1 as a vector and used the number of successful electroporations as an indicator to evaluate changes in electroporation conditions.

Electroporation protocols can be altered at many different points, which can all influence electroporation efficiency and success. We tested the effects of a higher capacitance, a smaller cuvette gap width, a higher concentration of cells, a different electroporation buffer (deionized water), different methylation patterns of the plasmid DNA, and different growth phases of *M. thermautotrophicus* ΔH . Most alterations either did not affect the success rate of the electroporation or decreased it. The only

exception was the increase in capacitance, which led to a reproducibly higher success rate (four of five in the first attempt, three of five in two follow-up attempts with the same conditions). However, electroporation with a higher capacitance had to be done in an oxic environment because the electroporator in an anoxic environment in our laboratory had a fixed capacitance of 10 μ F. Also, even the improved conditions did not lead to a successful transformation of pMVS-V2.

In general, the establishment of electroporation as an alternative transformation method was a success. However, the electroporation protocol requires further optimization and testing before it can become an alternative to conjugation. So far, only the basic shuttle vector pMVS-V1 has been transferred. One next step should be to transfer a plasmid that carries genetic cargo because an electroporation protocol that can only be used to transfer the simplest vector available would be of little practical use.

It is unclear why electroporation only seems possible with pMVS-V1 but not pMVS-V2. The posttreatment after electroporation was identical to the conjugation, making issues with the *fdh* operon as a selection marker unlikely as a reason. One possible reason could be the size of the plasmids: pMVS-V2 is 12.1 kbps in size, while pMVS-V1 only has a size of 8.2 kbps. Electroporation efficiency generally decreases with increasing size of the transferred vector, but usually not to a point where no transformants are observed (Marcus *et al.* 1990, Yoshida *et al.* 2009). Whether the observed issues are caused only by the size of the vector could be easily investigated by attempting electroporation with a vector of the same size as pMVS-V2 that carries the neomycin resistance cassette as a selection marker. If this vector is also not transferable by electroporation, the issues are most likely caused by the size of the vector. If it is transferable, the observed issues with pMVS-V2 are most likely caused by the selection marker.

The electroporation protocol itself could also be further optimized. The most obvious next step would be to perform the electroporations anoxic, which was not possible for the higher capacitance because we had to use an electroporator in a different laboratory. We tried to keep oxygen exposure as short as possible, so the cells were kept anaerobic until the actual electroporation and immediately transferred into an anoxic medium again afterward, limiting the oxygen exposure time to around 30 seconds. It has been reported that up to 18 hours of air exposure do not decrease the

viability of *M. marburgensis* (Kiener *et al.* 1983). However, the electroporation also severely stresses the cells, and a considerable fraction likely did not survive the electroporation. This is indicated by the fact that an electroporation recovery culture requires overnight incubation to consume all gas. In contrast, a conjugation recovery culture consumes all the gas in four hours despite a smaller initial amount of cells. Therefore, the already severely stressed cells might be more sensitive to oxygen than usual, and electroporation efficiency might be increased by performing the procedure entirely in an anoxic environment.

The electroporation buffer could also be further optimized. So far, only two buffers have been tested, which were 10% glycerol buffer from the original protocol (Molitor *et al.* 2016) and deionized water, which seemed to decrease electroporation efficiency. The chosen buffers greatly vary between different electroporation protocols. Further buffers commonly used include HEPES (Dennis *et al.* 1995) or sucrose (Baron *et al.* 1995). Especially sucrose might be an interesting candidate for *M. thermautotrophicus* ΔH . A 30 weight% solution of sucrose stabilizes protoplasts of *M. marburgensis* (Kiener *et al.* 1987) and *M. thermautotrophicus* ΔH (Sebastian Beblawy, unpublished results). Thus, it might also aid in stabilizing the transformants after electroporation.

3. Activity of *M. thermautotrophicus* ΔH promoters in *E. coli*

We investigated whether the activity of the P_{hmtB} and P_{synth} promoters in *E. coli* caused the observed cloning issues (section VII.2). We retransformed shuttle- and cloning vectors carrying the kanamycin/neomycin resistance cassette under different promoters into *E. coli* and plated them on the usual selection antibiotic and kanamycin. Transformation of shuttle vectors and cloning vectors that had the kanamycin/neomycin resistance cassette under the control of P_{hmtB} or P_{synth} led to a considerable number of colonies, albeit less than on the usual selection antibiotic. On the other hand, the transformation of a shuttle vector carrying the neomycin resistance cassette under the control of P_{mcr} (*M.v.*) did not lead to a considerable number of colonies. This plasmid was identical to the other shuttle vectors besides the promoter of the kanamycin/neomycin resistance cassette. Therefore, P_{hmtB} and P_{synth} seem to drive the expression of kanamycin/neomycin resistance cassette, thereby conferring kanamycin resistance to *E. coli*.

This was somewhat unexpected because the archaeal transcription factors and promoter elements are much more closely related to the eukaryotic than the bacterial machinery (Wenck *et al.* 2020). Akin to the eukaryotic RNA polymerase II, most archaeal promoters contain two distinct motifs: the TATA-box and the B-responsive element (BRE). They are the binding sites of the TATA-binding protein (TBP) and the transcription factor B, which are required to direct the RNA-polymerase to the transcription initiation site (Gehring *et al.* 2016). P_{hmtB} contains a TATA-box and BRE, while P_{synth} only contains a TATA-box (Figure 22). These elements are distinct from the typical -10 or -35 promoter elements found in bacteria, which serve as binding sites for σ -factors (Paget 2015). We tried to identify putative sequences that could serve as *E. coli* promoter elements using two different promoter prediction tools. While both predicted P_{hmtB} to function as a promoter in *E. coli*, they predicted the same for P_{mcr} (*M.v.*), which did not show activity in *E. coli* in our hands.

This is not the first report of an archaeal promoter being active in *E. coli*. A promoter region from the halophile *Halobacterium halobium* was shown to be a functional promoter in archaea (*Haloferax volcanii*), eukaryotes (*Saccharomyces cerevisiae*), and bacteria (*E. coli*) (Yang *et al.* 2003). However, the promoter region was relatively long (492 bp) compared to P_{hmtB} (101 bp) and P_{synth} (60 bp) and contained the -10 or -35 elements required for bacterial transcription initiation. These sequences are not evident in either P_{hmtB} or P_{synth} . It has also been reported that a β -galactosidase under the control of the *M. voltae* histone promoter (P_{hmvA}) can be used for blue-white screening in *E. coli* (Gardner *et al.* 1999). However, only one vector was tested for this, and the authors did not further investigate whether this was caused by the recognition of the promoter by the *E. coli* transcription machinery or other parts of the vector. The promoter also does not share any homology to P_{hmtB} or P_{synth} .

Thus, the exact sequences that drive transcription in *E. coli* remain to be elucidated. An essential first step would be to implement a different screening assay. Screening by kanamycin resistance applies direct selective pressure on *E. coli*. While there were no mutations in the promoter region of the sequenced plasmids purified from clones selected on kanamycin, the possibility of mutations in the genome of *E. coli* cannot be excluded. Furthermore, it remains unclear why the number of transformants is generally lower with kanamycin selection compared to the selection with the antibiotic for which the resistance gene is on the plasmid. The by far most straightforward assay

to establish would be the thermostable β -galactosidase. We could already demonstrate that the β -galactosidase is active and expressed in *E. coli* by showing that adding X-Gal to an *E. coli* pMVS1111a:P_{synth}-bgaB culture turns the culture blue (data not shown). In addition, *E. coli* strains carrying pMVS1111a:P_{synth}-bgaB and pMVS1111a:P_{hmtB}-bgaB were already prepared in our laboratory, and an assay for the β -galactosidase was also already established for a previous study (Fink *et al.* 2021). Similar to what was done in *Haloferax volcanii* with the *Halobacterium* promoter (Yang *et al.* 2003), the β -galactosidase assay could then be used to screen truncated or mutated versions of the promoters to determine the sequences required for expression in *E. coli*.

As previously discussed, the genetic toolbox would greatly benefit from promoters that are active in *M. thermautotrophicus* Δ H but not in *E. coli*. This could also be screened with the β -galactosidase assay by cloning β -galactosidase in a shuttle vector under the control of the putative promoters, transferring the shuttle vector into *E. coli* and *M. thermautotrophicus* Δ H, and performing the assay with both cell extracts. Initial qualitative screening could already be performed by blue-white screening of *E. coli* on plates to exclude promoters that are active in *E. coli*.

4. Establishing a thermostable CRISPR system for traceless genome editing in *M. thermautotrophicus* Δ H

We investigated whether the thermostable ThermoCas9 could be used for genome editing in *M. thermautotrophicus* Δ H. First, we investigated possible targets, the genes encoding hypoxanthine phosphoribosyl transferase (*hpt*) and uracil phosphoribosyl transferase (*upt*), both employed as counterselection markers in other methanogens. The genes confer sensitivity to toxic base analogs. Therefore, we first tested the sensitivity of *M. thermautotrophicus* Δ H to common base analogs. While one counterselective agent used for *hpt* counterselection (8-AH) and one for *upt* counterselection (6-AU) inhibited the growth of *M. thermautotrophicus* Δ H, the inhibition only lasted for two to three days. However, spontaneous resistant mutants to 8-AH and 6-AU were also reported for *M. voltae* (Bowen *et al.* 1987). Unfortunately, the authors did not give a timeframe for the occurrence of those resistances. Still, 8-AH and 6-AU-based counterselection was later developed for *M. maripaludis*, which shows that the occurrence of spontaneous resistant mutants does not necessarily

prevent the usage of *upt* and *hpt* for genetic purposes. For markerless mutagenesis in *M. maripaludis*, 250 µg/mL of 6-AU or 8-AH (Moore *et al.* 2005) are used. The determined concentrations for *M. thermautotrophicus* ΔH are comparable for 6-AU (100 ug/mL - 200 µg/mL) but somewhat higher for 8-AH (500 µg/mL- 1000 µg/mL). In addition, the *hpt* gene is presumably more difficult to delete because the putatively essential *dph2* gene is located directly downstream, while there is no gene directly downstream of *upt*. We, therefore, decided to target *upt* to avoid issues with polar effects, which have been reported for the deletion of *hpt* in *Methanococcus* (Sarmiento *et al.* 2011).

We used an adapted conjugation protocol for the conjugation of all CRISPR plasmids. Compared to the original protocol, we directly used an overnight *E. coli* S17-1 culture instead of reinoculating it in the morning. This was necessary because the *E. coli* carrying the CRISPR or other shuttle vectors with the *fdh* operon exhibited slower growth than with non-*fdh* shuttle vectors. We also increased the recovery time from four to 48 hours and added formate and supplements already to the recovery culture. We speculated that a prior adaptation with formate would reduce the time for the first selective transfer to grow because growth with formate as the sole electron source also seems to require an adaptation period in *M. thermautotrophicus* strains that are naturally able to do so (section V.1.3). This does not seem to be the case, as the first selective transfer of the basic formate shuttle vector, pMVS-V2, grew after five days, the same amount of time it took for the first selective transfer for the conjugation of pMVS1111a:*P_{hmtB}-fdh_{Z-245}* with the old protocol. While the plasmids are not identical, they only differ in the presence of the neomycin resistance cassette, so their overall behavior should be similar. Still, for better comparability, pMVS-V2 should be conjugated with the old and new protocols in parallel. If there is again no difference between the two protocols, a further possibility would be to incubate the preculture of the conjugation with formate to give the cell a longer adaption time.

We picked ThermoCas9 as an alternative to the non-thermostable SpCas9 most commonly employed in CRISPR/Cas-based genome editing (Mougiakos *et al.* 2017). We then design a CRISPR module for the shuttle vector. The CRISPR module has four submodules for the ThermoCas9, the sgRNA, the repair template, and a spacer for a putative counterselection marker, all separated by rare recognition sites for restriction enzymes. We then decided to test the activity of the CRISPR system *in vivo*

before attempting genome editing. For this, we cloned plasmid pMVS1112a:CRISPR1, with an *hpt* targeting sgRNA, and pMVS1112a:CRISPR2 with a non-targeting sgRNA (pMVS1112a:CRISPR1 still targets the *hpt* gene because the cloning was started before including the considerations about *hpt* discussed above). Because not every conjugation is successful, we also cloned pMVS-V2 as a positive control for the conjugation. Conjugation was successful for all three plasmids, but for pMVS1112a:CRISPR1, only one of the cultures grew in selective medium after nine days, while for pMVS1112a:CRISPR2, both cultures grew after six days, just like for the control plasmid pMVS-V2.

The conjugation's success rate and the first selective transfer's growth were comparable between pMVS-V2 and pMVS1112a:CRISPR2. This suggests that the ThermoCas9 with an sgRNA with no target does not exhibit significant toxicity in *M. thermautotrophicus* ΔH. However, whether there are actually no off-target effects needs to be confirmed *via* whole genome sequencing (WGS). The conjugation of pMVS1112a:CRISPR1 showed a lower success rate and slower growth than the other two plasmids. The plasmid lacks a repair template, and the active *hpt*-targeting CRISPR system should be lethal. However, delayed growth can be expected because one possibility would be that the strain circumvents the CRISPR activity by escape mutations. The slower growth and lower success rate suggested activity of the CRISPR system. Still, point mutations or deletions in the target sequence of the spacer in the genome, the sgRNA, or the *thermoCas9* gene would render the whole system CRISPR inactive and allow the cells to proliferate normally. This would also explain why the culture grew slower in the initial selective transfer but showed comparable growth speed to *M. thermautotrophicus* ΔH pMVS-V2 and pMVS1112a:CRISPR2 in later transfers. First, the cells are inhibited by the activity of the CRISPR system until an escape mutation occurs. The cells with the escape mutation can then proliferate normally because the CRISPR system no longer inhibits them. While a colony PCR did not show any large-scale deletions in the *hpt* region of *M. thermautotrophicus* ΔH pMVS1112a:CRISPR1 (Figure S7), the *hpt* region and the plasmid would still need to be sequenced to investigate potential mutations.

After these promising initial results, we attempted to delete a gene with the CRISPR system. Due to the previously discussed possible issues with the *hpt* gene, we switched our target to the *upt* gene. Also, we included the *mth60* operon as an

alternative target because it was already demonstrated that deletion of the *mth60* operon is possible *via* HR (Fink *et al.* 2023). However, several attempts with two *upt*- (pMVS1112a:CRISPR3 and pMVS1112a:CRISPR5) and one *mth60*-targeting (pMVS1112a:CRISPR8) plasmids did not lead to any exconjugants. However, we got exconjugants for pMVS1112a:CRISPR6 (*upt* targeting) and pMVS1112a:CRISPR11 (*mth60* targeting) in the first attempt. The difference in conjugation success could be caused by differences between the plasmids. pMVS1112a:CRISPR6, pMVS1112a:CRISPR5, and pMVS1112a:CRISPR3 have a smaller repair template (750 bp instead of 1kbp), and pMVS1112a:CRISPR3 has a different promotor for the sgRNA. pMVS1112a:CRISPR11 has two *mth60*-targeting guide RNAs, while pMVS1112a:CRISPR8 only has one sgRNA. However, for the conjugation of pMVS1112a:CRISPR6 and pMVS1112a:CRISPR11, the mating time of *E. coli* S17-1 and *M. thermautotrophicus* Δ H was increased from 24 to 72 hours to give the cells more time to transfer such large plasmids. It is very much possible that the other plasmids are transferable with these conditions, so the conjugations should be repeated with the adapted conditions to verify whether the problem lies with the plasmid or the conjugation conditions.

Growth only became visible in the first selective transfer after two weeks for both successful conjugations. This can most likely be explained by the activity of the CRISPR system, either because the cells need time for the homology-directed repair of the DSB with the repair template or because the initial number of transformants was very low. The latter is supported by the fact that while there were differences in the later colony PCRs of *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6, the colonies exhibited a very uniform genotype in the first colony PCR, which might be because they all stem from one original cell. Unfortunately, the selective enrichment step makes it difficult to calculate how many transformants there were initially.

Several attempts to plate *M. thermautotrophicus* Δ H pMVS1112a:CRISPR11 were unsuccessful with all three plating techniques usually employed for *M. thermautotrophicus* Δ H pMVS1112a:CRISPR11. This issue could be directly related to the *mth60* operon and growth on formate. The operon encodes the fimbriae of *M. thermautotrophicus* Δ H, cell appendages that allow the cells to attach to each other or surfaces (Thoma *et al.* 2008). The operon was successfully deleted *via* homologous recombination with the neomycin resistance cassette as a selection marker (Fink *et*

al. 2023). However, while it was possible to replace the *mth60* with the *fdh* operon in one copy of the genome, it was not possible to achieve a clean knockout with this selection marker (Fink 2021). Because *M. thermautotrophicus* ΔH also seems to exhibit morphological differences when being plated on formate medium (section IV.1.3.2), the fimbriae might play an unknown role in growth on solid medium with formate as the electron donor. This could be further investigated in the existing strain *M. thermautotrophicus* ΔH $\Delta mth58-61::NeoR$ (Fink et al. 2023), in which the *mth60* operon was replaced by the neomycin resistance cassette. The strain carries neither a shuttle vector nor the *fdh* operon and should, therefore, be transformable by pMVS-V2. If transformation with pMVS-V2 repeatedly fails, it would indicate that the fimbriae play an important role for growth on formate. However, in this experiment, the possibility remains that the fimbriae play some role in the conjugation, and the strain is therefore not transformable. A precise determination would only be possible in a clean $\Delta mth58-61$ strain, for example, *M. thermautotrophicus* ΔH pMVS1112a:CRISPR11, after confirmation and plasmid curing. If this strain is not transformable at all, the fimbriae most likely play a role in conjugation. If the strain is only not transformable by plasmids with the *fdh* operon as a selection marker, the fimbriae most likely play a role for the growth on formate.

Because plating on formate was not possible, one alternative to plate *M. thermautotrophicus* ΔH pMVS1112a:CRISPR11 would be to plate the culture with hydrogen as the electron donor. While these conditions would be non-selective, this should not pose a problem when working sterile and possibly after a preselection step with formate in liquid culture. Also, the segregational stability of shuttle vectors was reported to be high in *M. thermautotrophicus* ΔH , with all tested colonies still carrying the plasmid after three unselective transfers (Fink et al. 2021). If there is a direct relation between the fimbriae and growth on formate, this could lead to a successful plating and allow the analysis of single colonies of *M. thermautotrophicus* ΔH pMVS1112a:CRISPR11. When this is also unsuccessful, further strain analysis has to be done with the non-singularized liquid transfers.

M. thermautotrophicus ΔH pMVS1112a:CRISPR6 was platable and further analyzed via colony PCR. While the colony PCR showed bands at the size expected for a *upt* knockout, there was also a second, larger fragment. While this fragment could correspond to the wild-type (expected band size 3.0 kbp), it seems slightly lower

(around 2.8 kbp) on the gel. Because a mixed genotype would be difficult to sequence, we first tried to resolve the genotype with different strategies.

We tested whether the strain with the mixed genotype showed an increased resistance to 6-AU. While the strain did not show a considerable difference in growth behavior (Figure 28), the 6-AU treated samples showed either a much stronger or only the band corresponding to the Δupt genotype in the colony PCR (Figure 29). This suggests that an Δupt strain has an increased resistance to 6-AU. While this was not evident in the growth experiment, the culture at the start of the growth experiment still had a mixed genotype, which probably needed to be resolved before growth in the presence of 6-AU was possible. While the growth experiment should be repeated after the deletion of *upt* was confirmed by sequencing, care has to be taken with a strain that was treated with 6-AU before because spontaneous resistance mutants arise after three days already (section IV.4.1.2). It would, therefore, be challenging to determine whether the *upt* deletion caused the observed resistance. Thus, it is required to reintroduce the *upt* in the Δupt strain, either on a plasmid or in the genome, and demonstrate that this increases the sensitivity to 6-AU again.

Because 6-AU can only be used to select for a *upt* deletion and would not help resolve a heterozygous genotype for other deletion targets, we also attempted different strategies to resolve the genotype in parallel. Mixed colony PCR signals were also observed in previous experiments for gene deletions *via* HR (Fink *et al.* 2023, Küchle 2024). This could be resolved by an additional round of plating and picking for the deletion of the *mth60* operon (Fink *et al.* 2023). We attempted this for *M. thermautotrophicus* ΔH pMVS1112a:CRISPR6. However, several rounds of plating and picking did not resolve the genotype.

Similar results were reported for the deletion of the nitrogenase regulators *nif₁* and *nif₂*. Here, a better cell separation before plating could resolve the mixed genotype. Based on an existing protocol for the purification of the fimbriae, cells were treated with a blender to separate the fimbriae from the cells and achieve a better separation of cells before plating. This treatment resolved the mixed genotype and generated a clean knock-out strain (Küchle 2024). We attempted this treatment for strain *M. thermautotrophicus* ΔH pMVS1112a:CRISPR6. Interestingly, the strain showed a different cell morphology when plated after the blender treatment, suggesting that a better cell separation was achieved (Figure 30). However, all colonies tested still

showed two bands in the colony PCR, albeit the upper band was weak in one of the blended colonies. This culture was then used for the last attempt to resolve the genotype, where we increased the incubation period of the culture before plating because this reduces the filament length (Majerník *et al.* 2005), which was also unsuccessful.

In summary, all attempts to resolve the genotype without 6-AU were unsuccessful. One possible reason might be growth with formate as the electron donor. All methods attempted for cell separation were based on previous findings for *M. thermautotrophicus* Δ H grown with hydrogen as an electron donor. *M. thermautotrophicus* Δ H seems to exhibit a somewhat different growth and plating behavior on formate (section V.1.3). It is, therefore, possible that techniques that can resolve heterozygous genotypes when *M. thermautotrophicus* Δ H grows with hydrogen as the electron donor do not work when *M. thermautotrophicus* Δ H grows with formate as the electron donor. As discussed above for *M. thermautotrophicus* Δ H pMVS1112a:CRISPR11, one way to circumvent this would be to grow and plate the strain with hydrogen as the electron donor. If the heterozygous genotype persists, the different separation techniques could be reattempted with hydrogen as the electron donor.

Further separation techniques could also be attempted. As previously discussed (section I.5.3), *M. thermautotrophicus* Δ H grows in short filaments with several genome copies. If these filaments can be broken down into single, monoploid (or diploid) cells, the heterozygous genotype should be resolved. While protoplastation is not feasible for *M. thermautotrophicus* Δ H because protoplasts do not regenerate the cell wall (section I.4), a more vigorous treatment than the already attempted blending, for example, with a bead beater, might be able to break down the filaments. Observing the cells under a microscope before and after treatment would also be beneficial for observing the effects of different treatments.

We constructed several additional CRISPR plasmids with different promoters, repair templates, and sgRNAs for both *mth60* and *upt* (section VII.5). These plasmids still need to be investigated. They will provide additional valuable insights into the requirements for repair template sizes, sgRNAs, and promoters for the further use of the CRISPR system.

In summary, we could generate promising results that ThermoCas9 can be used for genome editing in *M. thermautotrophicus* ΔH. However, the results still need to be confirmed *via* sequencing, and a reliable method for resolving the heterozygous genotype needs to be established. Future work should first focus on these two issues. If the genotype can only be resolved with hydrogen as an electron donor, future CRISPR plasmids should contain the neomycin resistance cassette and the *fdh* operon. With this, selective conditions can be maintained for both the first selective transfer, where the neomycin resistance is unsuitable because it would be overgrown by spontaneously resistant wild-type cells, and resolving the genotype, where the *fdh* might be unsuitable as a selection marker.

The occurrence of mixed genotypes was already reported in the first publication about the ThermoCas9, where nine out of ten analyzed clones showed a mixed genotype (Mougiakos *et al.* 2017). However, this was done in *Bacillus smithii*, and whether this is a general problem of the ThermoCas9 or a strain-specific problem is unclear. Still, if the heterozygous genotype cannot be resolved, a different Cas enzyme would also be an option. There have been other thermostable Cas9 enzymes described, such as the CaldoCas9 from *Geobacillus stearothermophilus* (Harrington *et al.* 2017), which was successfully used in *Thermanaerobacter ethanolicus* (Le *et al.* 2020) and *Thermus thermophilus* (Adalsteinsson *et al.* 2021).

Another possibility would be the employment of the native CRISPR system of *M. thermautotrophicus* ΔH. The strain possesses three CRISPR loci (Kaster *et al.* 2011). One is a type I-B system, where the PAM of the Cas8 was determined *in vitro* (Cass *et al.* 2015). However, the system has not been evaluated for genome editing, and usually, Cas proteins from class 2 CRISPR systems, such as Cas9 or Cas12, are more commonly used for genome editing (Adli 2018, Moon *et al.* 2019), although intrinsic CRISPR systems have been adopted for genome engineering (Cheng *et al.* 2017, Cañez *et al.* 2019, Xu *et al.* 2021). Alternatively, if *M. thermautotrophicus* ΔH pMVS1112a:CRISPR6 proves to be more resistant to 6-AU than the wild-type, this strain could be used as the basis for a gene deletion system based on markerless mutagenesis. However, this would also require curing the plasmid, which has not yet been established.

VI. Outlook

In this work, we presented significant additions to the genetic toolbox for *M. thermautotrophicus* Δ H. Most importantly, we could establish the *fdh* operon as an alternative selection marker with no wild-type background and electroporation as an alternative transformation method. We demonstrated how the ThermoCas9 could be used for genome editing. This lays the basis for further genetic work with *M. thermautotrophicus* Δ H.

1. Expanding the genetic toolbox for *M. thermautotrophicus* Δ H further

Besides the improvements to the genetic tools established in this dissertation that have already been discussed, there are several additional genetic tools that would greatly benefit the genetic toolbox.

First and foremost, a system for curing plasmids from *M. thermautotrophicus* Δ H is required. This is essential for the further use of the CRISPR system for genome editing because, after a successful edit, the plasmid is not required anymore and needs to be removed before further genetic alterations can be carried out. While some microbes lose their plasmids after a few generations without selection pressure (Volke *et al.* 2021), no clones of *M. thermautotrophicus* Δ H without plasmid were observed even after 20 generations without selective pressure (Fink *et al.* 2021). Other commonly used techniques for plasmid curing are heat shock, mutagenic chemicals, detergents, heat-shock treatment (Trevors 1986), and electrocuring (Nickoloff 2008). There have been previous tests on plasmid curing (Sebastian Beblawy, unpublished results). However, in those experiments, the issue remained that even when a clone without a plasmid was observed, it could not be deduced certainly whether: **1)** it had lost the plasmid; or **2)** it was a wild-type culture that got either dragged along during subculturing or arose from contamination. This problem could now be solved by using strain *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 to test plasmid curing. It contains a plasmid and can be identified by its specific colony PCR signal with primers targeting the *upt* region in the genome. Thus, any cured strain could be easily identified because it would not have a plasmid but still have either the Δ *upt* or a mixed signal in a colony PCR.

If plasmid curing with typical protocols remains unsuccessful or only works at very low efficiency, the *upt* gene could also be used as a counterselection marker. The *upt* gene could be included on new CRISPR plasmids or any other ones requiring subsequent curing. Strains carrying such plasmids could then be cultivated with 6-AU to select for the loss of plasmids as it was done with the *hpt* gene in *M. acetivorans* (Nayak *et al.* 2017) and *M. maripaludis* (Li *et al.* 2022). However, this would only be possible in a Δ *upt* strain, which requires not only the confirmation of the deletion in *M. thermautotrophicus* Δ H pMVS1112a:CRISPR6 but also that the strain is cured from plasmid pMVS1112a:CRISPR6 by another plasmid curing method.

Another important addition to the genetic toolbox would be the establishment of additional promoters. As previously discussed, promoters not active in *E. coli* would be very beneficial, especially to circumvent cloning issues. Also, having more promoters of different strengths in general would be beneficial. These promoters could be established by testing different native promoters from *M. thermautotrophicus* Δ H genes but also by designing artificial promoters, as recently demonstrated for *M. maripaludis*. The authors identified key promoter elements of *M. maripaludis* and exchanged single bases, generating 81 different promoters with a 10,000-fold difference in strength (Xu *et al.* 2023).

Besides constitutive promoters, inducible promoters are an essential part of genetic toolboxes. Commonly used inducible promoters for other methanogens include promoters inducible by different growth substrates, such as a methanol-inducible promoter for *M. acetivorans* and a methylamine-inducible promoter for *Methanosarcina mazei* (Enzmann *et al.* 2018). However, *Methanosarcina* species can naturally utilize methylamine and methanol as growth substrates (Carr *et al.* 2022). *M. thermautotrophicus* Δ H cannot utilize these substrates. It is, therefore, questionable whether these systems can be transferred to *M. thermautotrophicus* Δ H. Typical bacterial systems, such as the tetracycline-inducible promoter, have also been established for mesophilic methanogens (Enzmann *et al.* 2018). While the system has mainly been employed in mesophilic microbes, it has recently been reported to be functional up to 60°C (Mol *et al.* 2022), which would allow its adaptation for *M. thermautotrophicus* Δ H.

For extensive screening of promoters, a quantification method that can be directly measured and does not require an assay like the β -galactosidase would be beneficial.

One possible alternative would be the fluorescence-activating and absorption-shifting tag (FAST). It is based on a small, non-fluorescent protein tag. Only in combination with fluorogenic chemicals that bind the small protein tag, light of a specific wavelength is emitted upon excitation (Plamont *et al.* 2016). This would allow a faster readout than the β -galactosidase. Also, unlike other fluorescent proteins, such as GFP (Heim *et al.* 1994, Chia *et al.* 2019), the FAST system is active under anaerobic conditions, allowing *in-vivo* experiments in anaerobes (Streett *et al.* 2019). It is also able to overcome the strong autofluorescence of all methanogens caused by the cofactor F₄₂₀ (Lambrecht *et al.* 2017), which was demonstrated by its use in *M. maripaludis* and *M. acetivorans* (Hernandez *et al.* 2022, Fonseca *et al.* 2023). The thermophilic growth conditions should also not be an issue, as the system was recently used in the thermophilic bacterium *T. kiuvi* for the first time (Hocq *et al.* 2023).

2. Applying the genetic toolbox

Besides establishing additional tools, another focus of research should be to apply the established tools for both basic research and industrial applications.

Methanothermobacter strains have been vital for understanding methanogenesis and the enzymes involved, especially one of the key enzymes of methanogenesis, MCR (Thauer 2019). Over the last decades, the focus of protein work has shifted to the more easily genetically tractable mesophilic methanogens. The ability to genetically modify *Methanosarcina* spp. and *Methanococcus* spp. allowed for the introduction of an affinity tag in the genome-encode MCR operon (Nayak *et al.* 2018), deletion of MCR-modifying enzymes (Nayak *et al.* 2017), and investigation of the assembly mechanism by generating chimeric MCRs (Lyu *et al.* 2018). However, *Methanothermobacter* species have always been ideal for protein work due to their fast growth and high final OD₆₀₀ when grown in bioreactors (Thauer 2015, Thauer 2019). The now-established tools allow that future protein research can also be carried out in *M. thermautotrophicus* Δ H.

Another research field that deserves further attention is the formate metabolism of *M. thermautotrophicus* Δ H. While the differences in formate biosynthesis for biomass between different *Methanothermobacter* strains have already been investigated (section V.1.3, (Casini *et al.* 2023)), several points about the utilization of formate as an electron donor remain unclear. Genetically modified *M. thermautotrophicus* Δ H and

wild-type *M. thermautotrophicus* Z-245 both require an adaptation period when switching from hydrogen to formate as an electron donor. While this might be explained by the repression of the *fdh* operon by hydrogen in *M. thermautotrophicus* Z-245 (Nölling *et al.* 1997), the *fdh* operon in *M. thermautotrophicus* Δ H should be constitutively expressed. Thus, the reasons for the observed lag phase must lie elsewhere. A first strategy would be to look for transcriptomic or proteomic differences before and after adaptation to formate. Transcriptomic and proteomic data has already been collected for *M. thermautotrophicus* Δ H MVS1111a: P_{hmtB} -*fdh*_{Z-245} and *M. thermautotrophicus* Z-245 with either hydrogen (Casini *et al.* 2023) or formate (Isabella Casini, unpublished results) as an electron donor. A thorough analysis of the differences in the proteome and transcriptome would be a first step in understanding this issue. Protein biochemistry could also prove helpful for further investigations of formate metabolism: It was shown in *Methanococcus* that during growth on formate, the hydrogenase subunit from the electron-bifurcating hydrogenase–heterodisulfide reductase complex is substituted by the Fdh (Lie *et al.* 2012). In *M. thermophilus*, it was shown that the Fdh was always part of the complex, even when grown with hydrogen as an electron donor (Abdul Halim *et al.* 2021). The latter is likely not the case for *M. thermautotrophicus* Δ H because the wild-type does not have a catabolic Fdh, and the anabolic Fdh was not found in the proteome (Casini *et al.* 2023). Still, the interactions of the Fdh with other enzymes deserve further investigation once methods for expressing tagged proteins are established.

An important industrial application for the genetic tools would be to upgrade the power-to-gas process to power-to-x by generating strains that produce additional compounds besides methane. The main metabolic end product of *M. thermautotrophicus* Δ H will always be methane, and due to its poor energy yield (around 0.5 ATP per methane, Thauer *et al.* (2008)), large quantities are required to generate enough ATP for biomass production and other putative products (Mühling *et al.* 2024). Research should, therefore, focus on high-value products. As previously discussed, terpenoids are a particularly interesting group of compounds because they are high in value, and the precursors should be readily available (section 1.2.1). Production of isoprene (Aldridge *et al.* 2021, Carr *et al.* 2021) and geraniol (Lyu *et al.* 2016) was already demonstrate in mesophilic methanogens. However, only thermophilic methanogens, such as *Methanothermobacter*, are commonly used for power-to-gas processes

(Thema *et al.* 2021). It is, therefore, essential that thermophilic methanogens are also metabolically engineered. Here, *M. thermautotrophicus* ΔH is an ideal candidate. Not only are *Methanothermobacter* strains already used in power-to-gas processes, but the genetic tools available are also more versatile than for the other thermophilic methanogens that became recently genetically tractable (section 1.2.2). Especially once the functionality of the CRISPR/ThermoCas9 is confirmed, genes encoding for metabolic enzymes could not only be introduced on plasmids but also integrated into the genome and maintained without the need for selective pressure. In addition, a genome-scale metabolic model (GSM) was recently published, which allows for the identification of potential bottlenecks and key enzymes to redirect metabolic fluxes (Casini *et al.* 2023). The ThermoCas9 could then also be used to implement the required genetic alterations that were deduced bioinformatically. Overall, with the tools developed here and in previous works (Fink *et al.* 2021, Casini *et al.* 2023, Fink *et al.* 2023), the genetic system is ready to start metabolic engineering in *M. thermautotrophicus* ΔH .

VII. Supplemental information

1. Colony PCR of pMVS-1111a:MupR

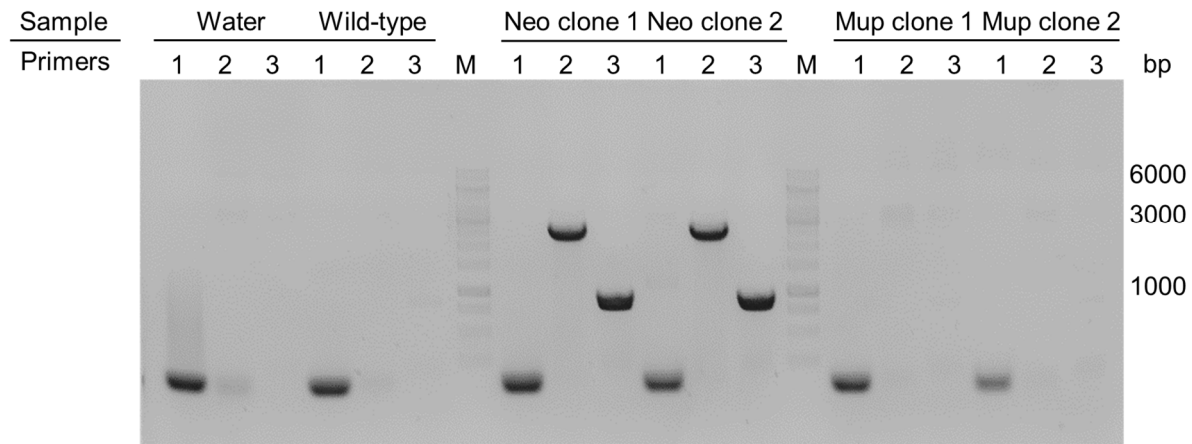


Figure S1: Colony PCR of clones from pMVS-1111a:MupR conjugation. Samples: water, water negative control; wild-type, negative control with wild-type cells of *M. thermautotrophicus* Δ H; Neo clone 1/2, two exconjugants selected with neomycin; Mup clone 1/2, two exconjugants selected with 50 μ g/mL mupirocin; 1, genome-specific primers (PCR was unsuccessful); 2 and 3, two sets of plasmid-specific primers. M, marker.

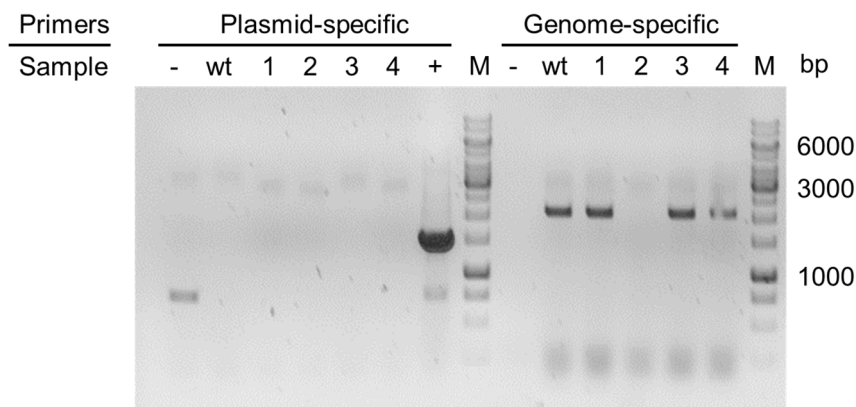


Figure S2: Colony PCR of clones from pMVS-1111a:MupR conjugation selected with 100 μ g/mL mupirocin, Samples: water, water negative control; wild-type, negative control with wild-type cells of *M. thermautotrophicus* Δ H; 1-4, four exconjugants selected with 100 μ g/mL mupirocin; 1, genome-specific primers; 2 and 3, two sets of plasmid-specific primers; M, marker.

2. Cloning issues in *E. coli*

Cloning issues were frequently observed during the cloning of the plasmids used in this dissertation, with a large fraction of *E. coli* clones carrying incorrect plasmids. Issues were especially severe in the cloning step involving the *pac* gene or the *fdh* operon, as illustrated by the following examples.

For cloning steps involving the *fdh* operon, most plasmids prepped from *E. coli* contained incorrect plasmids (Figure S3). For the later cloning work, this issue could be circumvented by a different picking strategy. The LB plates were kept in the incubator for two days instead of one after transformation. On the second day, additional small *E. coli* colonies became visible. These colonies usually carried correct plasmids (Figure S4)

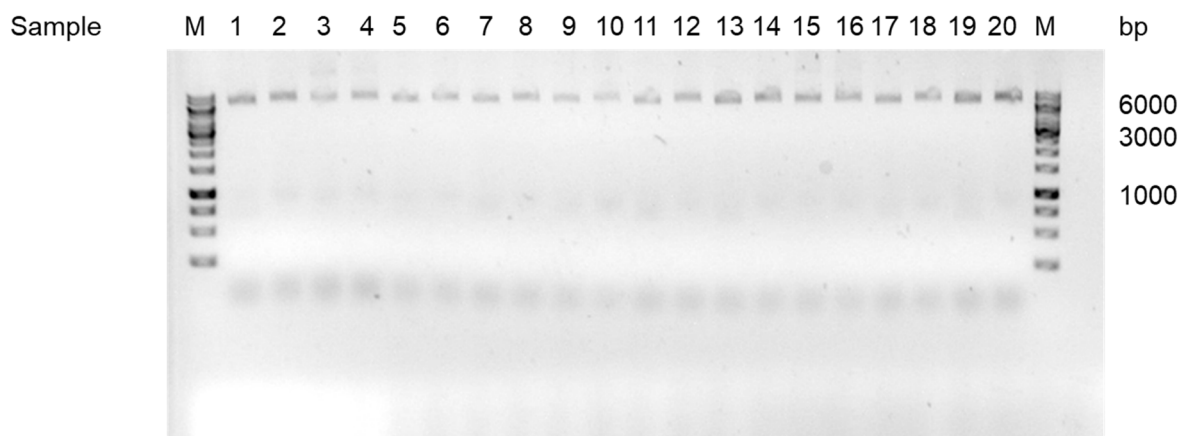


Figure S3: Test digest of plasmids from 20 *E. coli* clones of pMVS1111a: P_{hmtB} -*fdh*_{Z-245}. The P_{hmtB} -*fdh*_{Z-245} fragment was ligated into the shuttle vector *via* restriction ligation with the flanking *PacI* and *Ascl* sites. Plasmids were digested with *HindIII* and *KpnI* for the test digest. Expected band sizes are 9617 bp, 1739 bp, and 1327 bp. M, Marker.

For cloning steps involving the *pac* gene, no growth delay of *E. coli* clones carrying the correct plasmid was observed. Still, the majority of clones carried incorrect plasmids. For example, only one of the eight clones analyzed for the ligation of the P_{synth} -*pac* cassette into the shuttle vector showed the expected band sizes (Figure S5). Cloning issues were even more severe for P_{hmtB} -*pac*. While the cassette was successfully assembled in a cloning vector, several attempts to transfer the cassette into the shuttle vector only led to incorrect plasmids.

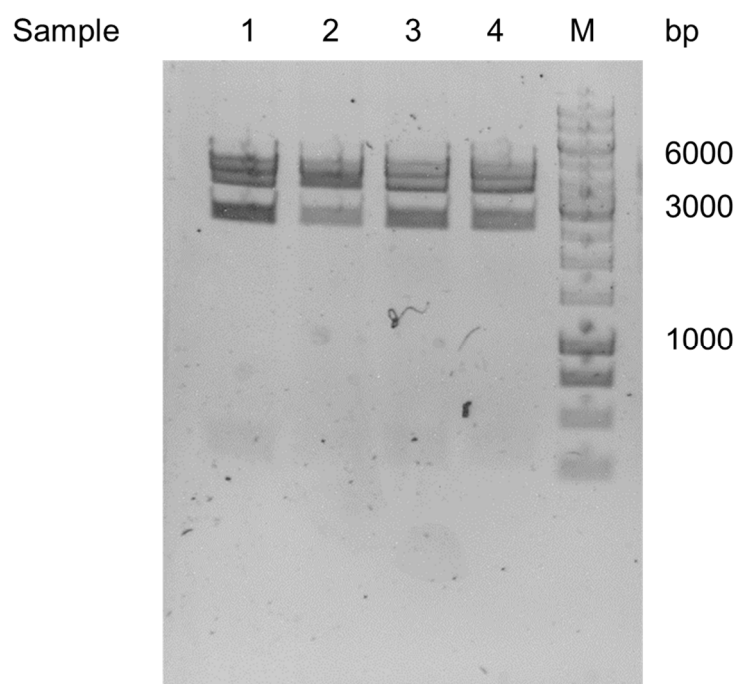


Figure S4: Test digest of plasmids from four *E. coli* clones of pMVS-V2. The P_{hmtB} -*fdhZ*-245 fragment was ligated into the shuttle vector *via* restriction ligation with the flanking *FseI* and *Ascl* sites. Plasmids were digested with *HindIII* and *BamHI* for the test digest. Expected band sizes are 4661 bp, 4000 bp, and 2785 bp. M, marker.

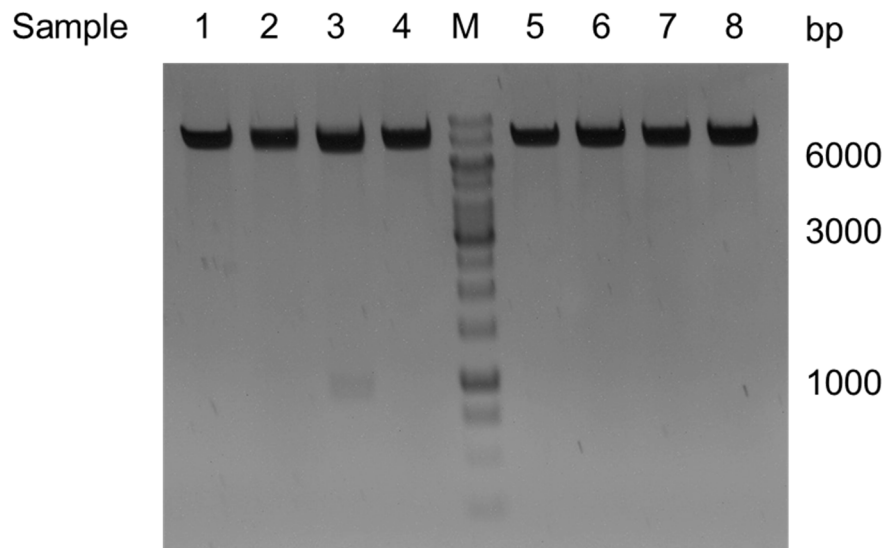


Figure S5: Test digest of plasmids from eight *E. coli* clones of pMVS1111a: P_{synth} -*pac*. The P_{synth} -*pac* fragment was ligated into the shuttle vector *via* restriction ligation with the flanking *PacI* and *Ascl* sites. Plasmids were digested with *SacI* and *KpnI* for the test digest. Expected band sizes are 7706 bp and 928 bp. Correct bands are only visible for clone 3. M, marker.

3. Attempts for random mutagenesis of *pac*

Random mutagenesis of the *pac* gene was attempted by amplifying the $P_{\text{synth-pac}}$ cassette *via* error-prone PCR followed by restriction and subsequent ligation into the shuttle vector. The ligation was used to transform *E. coli* and part of the transformation was plated. Plasmids were prepped for eight single colonies, none of which seemed to contain the correct plasmid. Several reattempts yielded similar results.

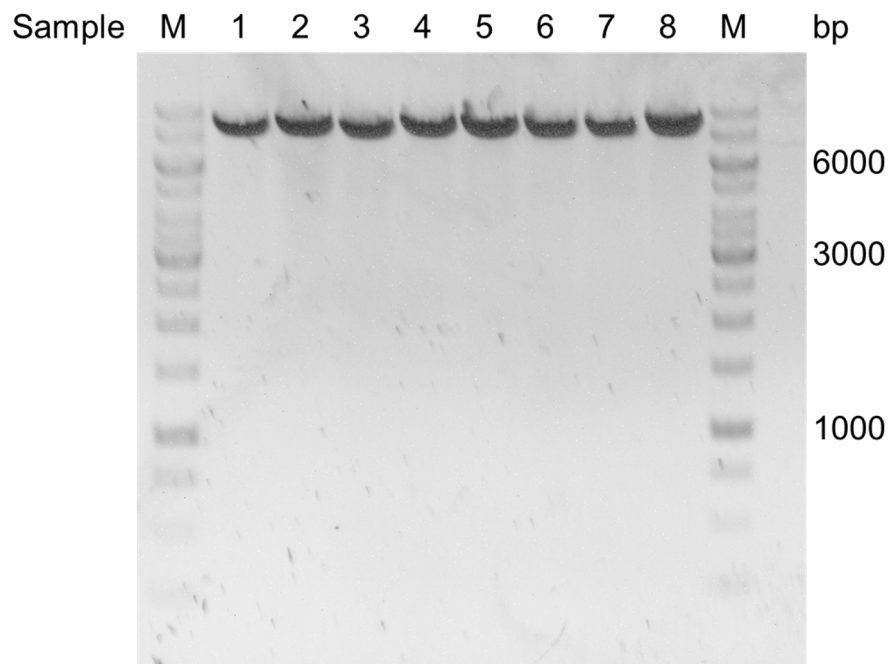


Figure S 6: Analysis of Test digest of plasmids from eight *E. coli* clones of pMVS1111a: $P_{\text{synth-pac}}$ after random mutagenesis of the *pac* cassette. After an error-prone PCR, the $P_{\text{synth-pac}}$ fragment was ligated into the shuttle vector *via* restriction ligation with the flanking *PacI* and *Ascl* sites. Plasmids were digested with *Ascl* and *PacI* for the test digest. Expected band sizes are 8231 and 745 bp.

4. Unsuccessful optimization attempts of the electroporation protocol

Besides the increased capacitance, which increased the electroporation success rate (section IV.2.2), we evaluated several other electroporation parameters. These did not increase the electroporation success rate and are summarized here.

We first investigated whether the methylation pattern of the plasmid DNA affects efficiency because this was also reported for *C. ljungdahlii* (Molitor *et al.* 2016). Prior attempts to express the methyltransferase from *M. thermautotrophicus* Δ H in *E. coli* to create an *E. coli* strain with *M. thermautotrophicus* Δ H-specific DNA methylation were unsuccessful (Christian Fink, personal communication). We, therefore, decided to test the influence of different methylation patterns by purifying the plasmid pMVS-V1 from different *E. coli* strains and attempting electroporation of *M. thermautotrophicus* Δ H (Table S1). All electroporations with different methylation patterns led to no transformants. Therefore, we concluded that using plasmids with different methylation patterns does not increase the success rate of electroporation and might even decrease it. We continued with pMVS-V1 with the original methylation pattern.

Table S1: Results of electroporation experiments with shuttle vector pMVS-V1 that was purified from differently methylating *E. coli* strains. Electroporation was done in quintuplicates for each methylation pattern. Name of *E. coli* strain: The *E. coli* strain from which pMVS-V1 was purified. Strain genotype: Indicates which of the three genes encoding methyltransferases are present or absent. Confirmed transformation cultures: Indicates how many of the five electroporations resulted in an *M. thermautotrophicus* Δ H strain carrying pMVS-V1.

Name of <i>E. coli</i> strain	Strain genotype	Confirmed transformant cultures
(Experiment from above)		1
One Shot BL21(DE3)	<i>dcm-/EcoKI-</i>	0
JM110	<i>dam-/dcm-</i>	0
Stellar	<i>dam-/dcm-/EcoKI-</i>	0

Next, we investigated which conditions of the electroporation itself influence its efficiency. First, we tested using an electroporation cuvette with a smaller gap (0.1 cm instead of 0.2 cm). We concentrated the cells to either the same (200 μ L) or a smaller volume (100 μ L) as before (Table S2). Again, all electroporations led to no transformants. We, therefore, concluded that using electrodes with a smaller gap or a smaller volume of cells does not increase the electroporation efficiency and might even decrease it, and continued with the previous conditions.

Table S2: Results of electroporation experiments: different cell volumes and cuvette widths. Electroporation was done in quintuplicates for each experiment. Cuvette gap width: Indicates the gap width of the electroporation cuvette used. The volume of cells: Indicate the volume to which the culture (20 mL) was concentrated. Confirmed transformation cultures: Indicates how many of the five electroporations resulted in an *M. thermautotrophicus* Δ H strain carrying pMVS-V1.

cuvette gap width (cm)	Volume of cells (μ L)	Confirmed transformant cultures
0.1	200	0
0.1	100	0

Next, we tested whether the electroporation buffer influences the efficiency by repeating the electroporation with the old and new settings (10 μ F and 25 μ F) with deionized water instead of 10% glycerol. All cultures grew well in the recovery, but there were fewer successful transformations than with 10 % glycerol (Table S3). Therefore, we decided to keep glycerol as a buffer.

Table S3: Results of electroporation experiments with a different washing and electroporation buffer. Electroporation was done in quintuplicates for each experiment. Resistance: Indicates resistance setting of electroporator. Capacitance: Indicates the capacitance setting of the electroporator. Washing and electroporation buffer: indicates washing and electroporation buffer used. Confirmed transformation cultures: Indicates how many of the five electroporations resulted in an *M. thermautotrophicus* Δ H strain carrying pMVS-V1.

Resistance (Ω)	Capacitance (μ F)	Washing and electroporation buffer	Confirmed transformant cultures
600	10	deionized H ₂ O	0
600	25	deionized H ₂ O	2

Lastly, we investigated the influences of the growth phase of *M. thermautotrophicus* Δ H on the electroporation success rate. For this, we repeated the electroporation with mid (after six hours) and late (after twelve hours) exponential growth phase cultures and a capacitance of 25 μ F. For each condition, a stationary phase culture was used as positive control. Both experiments with exponential growth phase cultures showed a lower success rate than the ones with stationary cultures (Table S4).

Table S4: Results of electroporation experiments with *M. thermautotrophicus* Δ H cultures in different growth stages. Electroporation was done in quintuplicates for each experiment. Growth time: Indicates the time the *M. thermautotrophicus* Δ H cultures were incubated before electroporation. OD₆₀₀: Final OD₆₀₀ of cultures. Confirmed transformation cultures: Indicates how many of the five electroporations resulted in an *M. thermautotrophicus* Δ H strain carrying pMVS-V1.

Growth time (hours)	OD ₆₀₀	Confirmed transformant cultures
24	0.30	3
6	0.13	0
24	0.31	3
12	0.27	2

5. Overview of CRISPR plasmids

Plasmid	Description
pMVS-1112a_CRISPR1	<i>hpt</i> -targeting sgRNA with P _{synth} promoter, no repair template
pMVS-1112a_CRISPR2	non-targeting sgRNA with P _{synth} promoter, no repair template
pMVS-1112a_CRISPR3	<i>upt</i> -targeting sgRNA with P _{hmtB} promoter, small (750 bp per flanking region) repair template.
pMVS-1112a_CRISPR5	<i>upt</i> -targeting sgRNA with P _{synth} promoter, small (750 bp per flanking region) repair template.
pMVS-1112a_CRISPR6	<i>upt</i> -targeting sgRNA with P _{synth} promoter, larger (1 kb per flanking region) repair template
pMVS-1112a_CRISPR7	<i>upt</i> -targeting sgRNA with P _{synth} promoter, larger (1 kb per flanking region) repair template, <i>thermoCas9</i> with an inducible promoter (Tina Baur, unpublished results)
pMVS-1112a_CRISPR8	<i>meth60</i> targeting sgRNA with P _{synth} promoter,
pMVS-1112a_CRISPR9	<i>meth60</i> targeting sgRNA with P _{synth} promoter, <i>thermoCas9</i> with an inducible promoter (Tina Baur, unpublished results)
pMVS-1112a_CRISPR10	<i>meth60</i> targeting sgRNA with P _{synth} promoter, no repair template
pMVS-1112a_CRISPR11	Two <i>meth60</i> targeting sgRNA with P _{synth} promoter,
pMVS-1112a_CRISPR12	Two <i>meth60</i> targeting sgRNA with P _{synth} promoter, <i>thermoCas9</i> with an inducible promoter (Tina Baur, unpublished results)
pMVS-1112a_CRISPR13	Two <i>meth60</i> targeting sgRNA with P _{synth} promoter, no repair template
pMVS-1112a_CRISPR14	<i>upt</i> -targeting sgRNA with P _{synth} promoter, no repair template
pMVS-1112a_CRISPR15	No Two sgRNA or <i>thermoCas9</i> , only repair template for <i>meth60</i>

6. Colony PCR of first CRISPR experiments

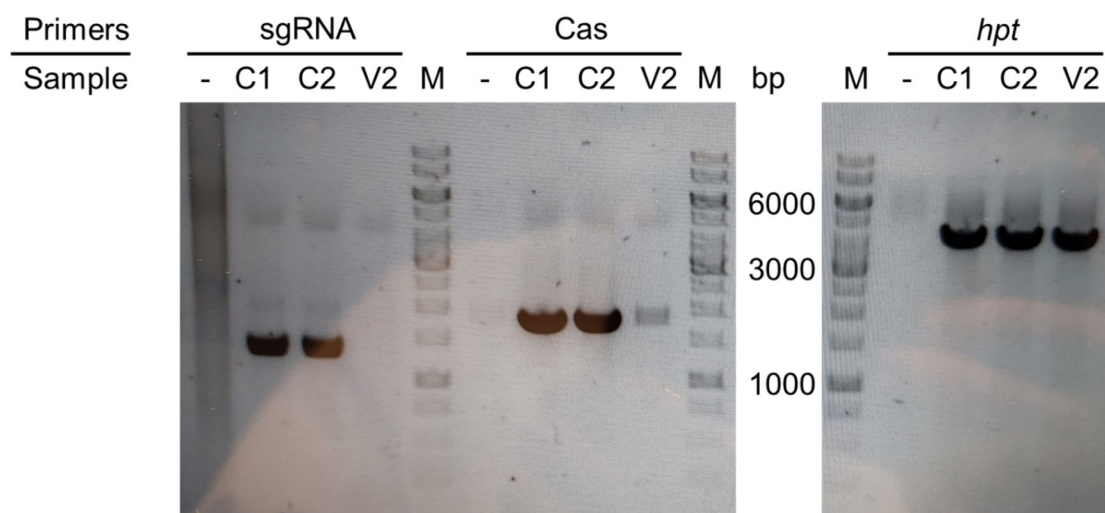


Figure S7: Colony PCR of clones from the conjugation of pMVS1112a:CRISPR1, pMVS1112a:CRISPR2, and pMVS-V2. Samples: -, negative control; C1, *M. thermautotrophicus* Δ H pMVS1112a:CRISPR1; C2, *M. thermautotrophicus* Δ H pMVS1112a:CRISPR2; V2, *M. thermautotrophicus* Δ H pMVS-V2. Primers: sgRNA, primers amplifying the area around the sgRNA on the CRISPR plasmids; Cas, primers amplifying the part of the Cas on the CRISPR plasmids; *hpt*, primers amplifying the *hpt* region in the genome. M, marker

VIII. List of abbreviations

6-AU	6-azauracil
8-ADP	8-aza-2,4-diaminopurine
8-AH	8-azahypoxanthine
Amp	ampicillin
ATP	adenosine triphosphate
<i>bgaB</i>	thermostable beta-galactosidase from <i>Geobacillus stearothermophilus</i>
bp	basepair
BRE	transcription factor B recognition element
Cam	chloramphenicol
Cas	CRISPR-associated
CIP	calf intestine phosphatase
CoA	coenzyme A
CoB	coenzyme B
CODH/ACS	CO dehydrogenase/acetyl-CoA synthase

CoM	coenzyme M
CRISPR	clustered regularly interspaced short palindromic repeats
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
e. g.	<i>exempli gratia</i>
EDTA	ethylenediaminetetraacetic acid
Fdh	formate dehydrogenase
H₄MPT	methyl-tetrahydromethanopterin
Hpt	hypoxanthine phosphoribosyltransferase
HR	homologous recombination
i. e.	<i>id est</i>
IleS	isoleucine tRNA synthetase
kb	kilo basepairs
LB	lysogenic broth (medium for <i>E. coli</i> cultivation)
<i>M.t.</i>	<i>Methanothermobacter thermautotrophicus</i>
<i>M.v.</i>	<i>Methanococcus voltae</i>
MCR	methyl-CoM reductase
MCS	multiple cloning site
MIC	minimal inhibitory concentration
MS	mineral solution (medium for <i>M. thermautotrophicus</i> cultivation)
Mup	mupirocin
MVS	methanothermobacter vector system
NEB	new England Biolabs
Neo	neomycin
OD	optical density
ORF	open reading frame
Ori	origin of replication
P	promoter
Pac	puromycin acetyltransferase
PCR	polymerase chain reaction
PEG	polyethylene glycol
PeiP	pseudomurein endo-isopeptidase P
PhD	doctor of philosophy
pMVS	plasmid Methanothermobacter vector system
Pol	polymerase
ppm	parts per million
R/r	resistance
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SAP	shrimp-alkaline phosphatase
SDS	sodium dodecyl sulfate
sgRNA	single guide RNA

SOC	super optimal broth
spp.	species
Synth	synthetic
T	terminator
TAE	tris-acetate-EDTA
Tet	tetracyclin
TRIS	tris(hydroxymethyl)aminomethane
Upt	uracil phosphoribosyltransferase
Wt	wild-type

IX. Contributions of other researchers

The extensive research work presented in this dissertation would not have been possible without the help of fellow researchers and students. In the course of work presented in this dissertation, I trained and supervised several bachelor and Master students, and parts of their work are also presented here. Tilman Schramm performed parts of the cloning for pMVS1111a:MupR (section IV.1.1.2) for his bachelor's thesis (Schramm 2021). Oskar Geissel cloned plasmid pMVS1111a:P_{synth}-*pac* and performed the puromycin growth experiments (section IV.1.2) during the work for his bachelor's thesis (Geissel 2022). Ferran Agulló i Manobens conducted the growth experiments with 6-AU (section IV.4.1.2) for his bachelor's thesis (Agulló i Manobens 2022). Samuel Farmer worked on optimizing the electroporation conditions (sections IV.2.2 and VII.4) for his master's thesis (Farmer 2024). Franziska Kühle designed the cell separation protocol used in section IV.4.4 for her master's thesis (Kühle 2024).

X. Publications

1. Accepted publications

Fink C, Beblawy S, Enkerlin A M, Mühling L, Angenent L T and Molitor B (2021). A shuttle-vector system allows heterologous gene expression in the thermophilic methanogen *Methanothermobacter thermautotrophicus* ΔH. mBio 12(6): e02766-02721.

2. Submitted publications

Mühling L, Baur T and Molitor B (2024). *Methanothermobacter thermautotrophicus* and alternative methanogens – archaea-based production. Unconventional Organisms in Biotechnology. Holtmann D, Springer Nature Publishing Group, submitted.

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XII. Acknowledgment

This dissertation has been a long but successful journey, and I want to thank everyone who made it possible. First, I want to thank Dr. Bastian Molitor for enabling me to pursue my Ph.D. under his supervision. You guided me through this journey with great scientific and moral support. You always came up with new ideas when things were not working, and I want to especially thank you for your positive and understanding attitude even during difficult times; this really helped me keep going. Also, I want to thank you for the many hours you spent editing manuscripts and this dissertation. I would also like to thank Prof. Dr. Lars Angenent for allowing me to work in his laboratory and being there whenever I needed his expertise.

Second, I would like to thank everyone who helped me in the lab. I want to thank Dr. Christian Fink and Dr. Sebastian Beblawy for training me to work with *M. thermoautotrophicus* and for many helpful tips and discussions in the beginning. I would also like to thank Dr. Tina Baur and Mohamed Balboul for their help with cloning the CRISPR plasmids. In addition, I would also like to thank Dr. Isabella Casini for programming the CRISPR off-target search algorithm and Caroline Schlaiss for many

helpful discussions and suggestions about cloning strategies and conjugation optimization. I would also like to thank once more the students who worked with me during their theses and as HiWis: Tilman Schramm, Oskar Geissel, Ferran Agulló i Manobens, Samuel Farmer, and Franziska Kühle. I had a great time working with you, and your contributions were essential to the success of this dissertation.

I also want to thank my office mates over the years: Sarah Schulz, Patrick Schweizer, Ulrike Biehain, Nils Rohbohm, Han Wang, Antonio Velazquez and Mohammed Balboul. I had a great time in the office with you, and it was always fun. I especially want to thank Gines Martinez-Cano, Dr. Kim Rennhack, Nils Rohbohm, Antonio Velazquez, and Caroline Schlaiss for always being there for me, enjoying the good times together, and cheering me up in difficult ones.

Of course, I also received much support from outside the lab. I want to thank Hannes Maier for many fruitful discussions and support for things inside and outside the lab. I also want to thank my friends from home, Lukas Martin Otto, Joshua Pitz, and Maximilian Bender for our long-lasting friendship and many fun activities during the years. In addition, I would also like to thank my friends from my Bachelor's, Antonia Grauel, Raphael Bader, Michael Ritter, Maria Wahle, Jasemi Loukeri, and Philipp Fink for many fun times in the last years, whether in person or online.

I also want to thank my family, and especially my mother, Linda Mühling. You are always there for me, looking out for me and caring for me, no matter what. I do not think I would have made it this far without you. I also want to thank my father, Alexander Mühling, who unfortunately passed away while I was pursuing my PhD. I miss you.

I especially want to thank my beautiful girlfriend, Ryoko Hiyama. Despite the great distance, you were always there for me and supported me however you could. I am really grateful for that, and I cannot wait to spend more time with you in the future.

Lastly, I want to thank everyone again, including those I might have forgotten. This dissertation would not have been possible without all of you.