CHAPTER THIRTEEN

Genetic techniques for studies of methyl-coenzyme M reductase from *Methanosarcina acetivorans* C2A

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Abstract

Methanogenic archaea generate methane as a by-product of anaerobic respiration using CO_2, C_1 compounds (like methanol or methylated amines), or acetate as terminal electron acceptors. Methanogens are an untapped resource for biotechnological advances related to methane production as well as methane consumption. However, key biological features of these organisms remain poorly understood. One such feature

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is the enzyme methyl-coenzyme M reductase (referred to as MCR), which catalyzes the last step in the methanogenic pathway and results in methane formation. Gene essentiality has limited genetic analyses of MCR thus far. Therefore, studies of this important enzyme have been limited to biochemical and biophysical techniques that are especially laborious and often reliant on sophisticated instrumentation that is not commonly available. In this chapter, we outline our recently developed CRISPR–Cas9-based genome editing tools and describe how these tools have been used for the introduction of a tandem affinity purification tag at the chromosomal *mcr* locus in the model methanogen, *Methanosarcina acetivorans* C2A. We also report a protocol for rapid affinity purification of MCR from *M. acetivorans* C2A that will enable high-throughput studies of this enzyme in the future.

1. Introduction

A group of microorganisms within the Archaea, collectively referred to as methanogens, are the predominant source of methane on Earth (Thauer, Kaster, Seedorf, & Buckel, 2008). Methanogens are prevalent in anoxic environments and generate ca. 1 gigaton of methane annually, which accounts for 70%–80% of the annual emissions of this potent greenhouse gas (Schaefer et al., 2016; Thauer et al., 2008). As such, the contribution of methanogens to climate change and the global carbon cycle are apparent. In addition, because methane is a clean burning renewable fuel with high calorific value, methanogens have immense potential to serve as biocatalysts for clean energy-related technological advances (Wood, 2017). However, the absence of tools for high-throughput studies of methanogens has severely crippled efforts toward their use in biotechnological applications. In this chapter, we describe genetic techniques that were recently developed to aid and accelerate studies of MCR, an enzyme of importance for biotechnology and renewable energy, from the methanogenic archaeon *Methanosarcina acetivorans* C2A.

Even though methanogenesis as a metabolic trait is limited to members of the archaeal branch of the tree of life, these organisms are considerably diverse (Liu & Whitman, 2008; Spang & Ettema, 2017; Thauer et al., 2008). Notably, sources of methanogens range from Antarctic lakes where the temperatures are below the freezing point of water (Franzmann, Springer, Ludwig, Conway De Macario, & Rohde, 1992) to "black smokers" at the seabed where temperatures are well above the boiling point of water (Bult et al., 1996). Depending on the strain, the substrate breadth can vary as well; while some methanogens can only grow on $H_2 + CO_2$ or formate, others use methylated

compounds (such as methanol, methylated amines, methylated sulfides) or even acetate as methanogenic substrates (Liu & Whitman, 2008; Thauer et al., 2008). This versatility is an especially desirable trait from a biotechnological standpoint. Despite this apparent diversity, the core methanogenic process is highly conserved, especially the last step that leads to methane formation, which is catalyzed by an enzyme called methyl-coenzyme M reductase (referred to as MCR henceforth).

Since its initial discovery (Gunsalus & Wolfe, 1980), MCR has become the subject of biochemical and biophysical research spanning decades, laboratories, and continents (Ermler, Grabarse, Shima, Goubeaud, & Thauer, 1997; Prakash, Wu, Suh, & Duin, 2014; Thauer, 1998; Wongnate et al., 2016). Structural studies have revealed that MCR is a hexamer comprised of three subunits (α, β, γ) in a $\alpha_2\beta_2\gamma_2$ configuration (Ermler et al., 1997; Grabarse, Mahlert, Shima, Thauer, & Ermler, 2000) (Fig. 1). Each molecule of MCR contains two active sites that lie in a buried pocket within the α subunits (Fig. 1). It has been postulated that the two active sites, which are

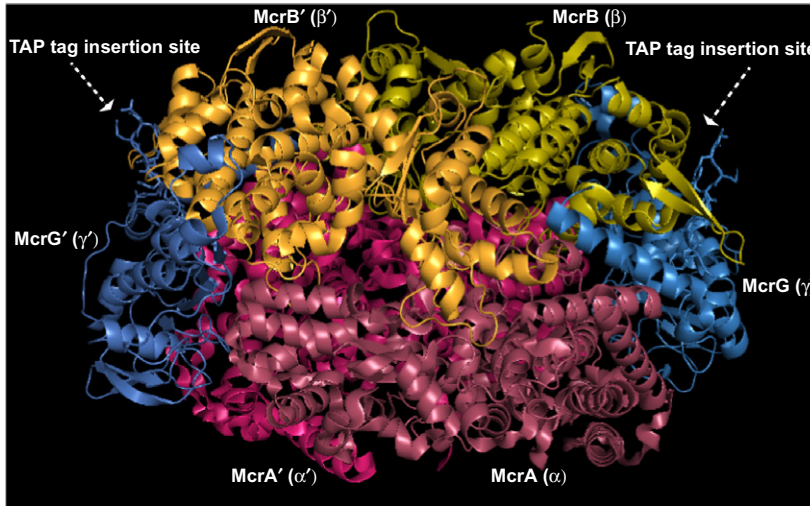

Fig. 1 Structure of methyl coenzyme-M reductase from *Methanosarcina barkeri* (PDB accession number: 1e6y). The α and α′ subunits are colored in shades of pink as indicated; the β and β′ subunits are colored in yellow and orange, respectively; the γ and γ′ subunits are colored in shades of blue as indicated. The N-terminus of the γ and γ′ subunits is highlighted to show the loop where the tandem affinity purification (TAP) tag is inserted. Note: the amino-acid identity of the α, β, and γ subunits between *M. barkeri* and *M. acetivorans* is ≥90%.

ca. 50 Å apart, might be coupled in a manner similar to a two-stroke engine (Goenrich, Duin, Mahlert, & Thauer, 2005). Within each active site, methyl-coenzyme M (CH_3-S-CoM; methyl-2-sulfanylethanesulfonate) is reduced by coenzyme B (CoB; 7-thioheptanoylthreonine phosphate) to form methane and the mixed heterodisulfide of CoB and CoM (CoM-S-S-CoB) as follows:

$$CH_3 - S - CoM + HS - CoB \rightleftarrows CH_4 + CoM - S - S - CoB$$

The active site of MCR is known as Factor 430 (F_{430}): it contains a porphyrinoid cofactor coordinated to a central Ni atom and the reduced Ni(I) form of F_{430} is essential for catalysis (Moore et al., 2017; Zheng, Ngo, Owens, Yang, & Mansoorabadi, 2016). The low redox potential of the Ni(I)/Ni(II) couple (ca. −650 mV) renders F_{430} especially sensitive to oxidative inactivation, which makes MCR recalcitrant to mechanistic analyses (Goubeaud, Schreiner, & Thauer, 1997). However, recent studies led by Steve Ragsdale's group at the University of Michigan have provided evidence in support of a reaction mechanism that leads to the formation of a methyl radical and a Ni (II)-thiolate intermediate (Wongnate et al., 2016).

Genetic studies of MCR have been scant, only barely keeping pace with other advancements (Bokranz, Baumner, Allmansberger, Ankel-Fuchs, & Klein, 1988; Weil, Cram, Sherf, & Reeve, 1988). We posit two critical hurdles that may have impeded genetic studies of MCR. First, heterologous expression of MCR in a nonnative host such as *Escherichia coli* is likely to be extremely challenging for many reasons: (a) currently unknown electron donors required to maintain F_{430} in the reduced Ni (I) state might be absent, (b) the host might not encode genes for the synthesis of CoM, CoB, or F_{430}, and (c) F_{430} is especially oxygen-labile. Second, all known methanogens are obligate for this metabolic trait (i.e., biomass production and energy conversation are strictly and singularly coupled to methane production) (Spang & Ettema, 2017; Thauer et al., 2008). Thus, not only is MCR universally conserved, but also it is essential for the growth and survival of these organisms. Therefore, gene essentiality is likely to have impeded genetic analyses of MCR even in genetically tractable methanogens. Overall, the dearth of methods for genetic analyses of MCR is a critical hurdle that must be overcome to harness the biotechnological potential of methanogens. To this end, in this chapter we describe how Cas9-based genome editing tools can be used to genetically manipulate the chromosomal *mcr* operon in *M. acetivorans* C2A to introduce a tandem affinity purification (TAP) tag for rapid purification of this important enzyme.

2. CRISPR–Cas9 genome editing in *Methanosarcina* spp.

CRISPR–Cas9-based genome editing has been widely used for genome manipulation in a broad range of organisms, but mostly within the eukaryotic domain (Hsu, Lander, & Zhang, 2014; Li et al., 2015; Peters et al., 2015) (Tables 1 and 2). This technique relies on two components: (a) Cas9 and (b) single guide (sg) RNA (Barrangou & van Pijkeren, 2016). Cas9, originally derived from *Streptococcus pyogenes*, is an RNA-guided DNA endonuclease (Doudna & Charpentier, 2014). The sgRNA has two components: (a) a 20-bp region of homology to a chromosomal locus of interest (also referred to as the target sequence) and (b) an 80-bp scaffold that adopts a secondary structure to enable the sgRNA to bind to Cas9 (Jinek et al., 2012). Upon binding the DNA strand complementary to the target sequence, with a NGG protospacer adjacent motif (PAM) at the 3′ end, the sgRNA triggers Cas9 to generate a double-stranded break (DSB) at the chromosomal locus of interest (Fig. 2). This lethal DSB can be repaired by the native homology-dependent repair mechanism when a homology repair (HR) template to

Table 1 List of plasmids for genome editing in *Methanosarcina* spp.

Plasmid	Features	Source
pAMG40	Vector for fosmid retrofitting that contains pC2A and λattP	Guss et al. (2008)
pJK027A	Vector with P*mcrB*(*tetO1*) promoter fusion to *uidA* that contains ΦC31attB and λattB	Guss et al. (2008)
pDN201	pJK027A-derived plasmid with p*mcrB*(tetO1) promoter fusion to *S. pyogenes cas9*	Nayak and Metcalf (2017)
pDN206	Cointegrate of pDN201 and pAMG40	Nayak and Metcalf (2017)
pDN303	pDN201-derived plasmid with a synthetic fragment containing P*mtaCB1* promoter fusion to a sgRNA targeting *mcrG*	Nayak et al. (2017)
pDN305	pDN303-derived plasmid containing a repair template to introduce a tandem affinity purification tag (containing a 3× FLAG tag and a Twin-Strep tag) at the N-terminus of *mcrG*	Nayak et al. (2017)
pDN307	Cointegrate of pDN303 and pAMG40	Nayak et al. (2017)
pDN309	Cointegrate of pDN305 and pAMG40	Nayak et al. (2017)

Table 2 List of *Methanosarcina acetivorans* strains discussed in this chapter

Strain	Genotype	Construction details	Source
WWM60	Δ*hpt*::*PmcrB-tetR*	—	Guss et al. (2008)
WWM1054	Δ*hpt*::*PmcrB-tetR*, N-terminal TAP tag (3 × FLAG and Twin-Strep tag) upstream of *mcrG*	WWM60 was transformed to Pur^R with pDN247; plasmid-cured strain was isolated by colony purifying Pur^R transformants on solid medium with 8ADP	Nayak et al. (2017)

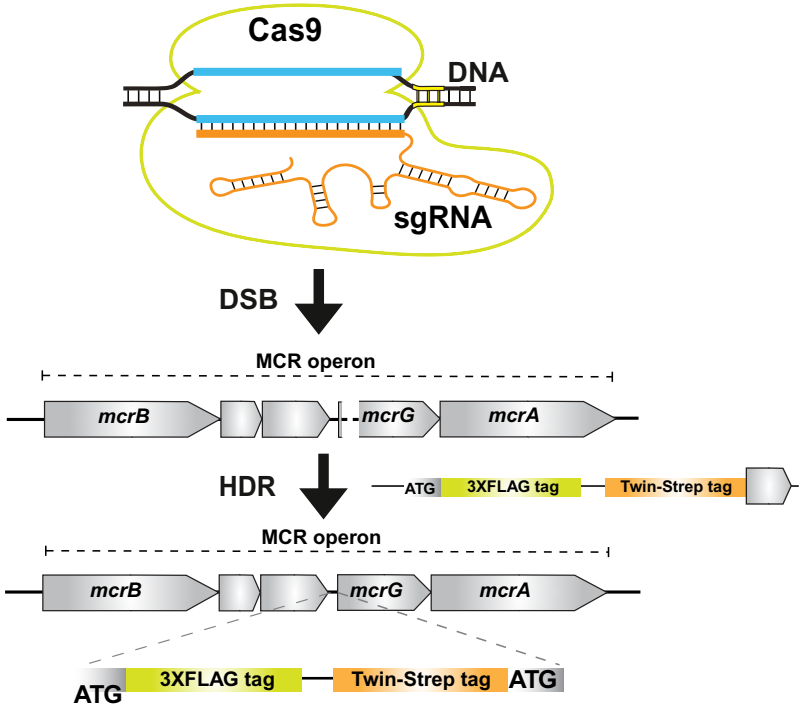

Fig. 2 A schematic overview of Cas9-based genome editing to introduce a tandem affinity purification (TAP) tag at the N-terminus of the *mcrG* locus (locus tag: MA4547) in *M. acetivorans* C2A. Upon binding the target sequence (in *blue*) flanked by an NGG protospacer adjacent motif (PAM; in *yellow*), Cas9 generates a double-strand break at the *mcrG* locus. Homology-dependent repair (HDR) with a repair template containing the TAP tag flanked by regions of the homology surrounding the DSB leads to its introduction at the N-terminus of *mcrG*.

introduce the desired mutation is provided (Fig. 2). Importantly, the introduced mutation must alter or remove the target sequence to prevent additional rounds of cleavage by Cas9.

In a recent study (Nayak & Metcalf, 2017), we described the development of a Cas9-based platform for genetic manipulation of the model archaeon *M. acetivorans* C2A. Since then, we have implemented this platform in another closely related methanogen, *Methanosarcina barkeri* Fusaro as well (unpublished data). The Cas9-based genetic toolbox can be used in conjunction with preexisting genetic tools and provides several advantages over prior techniques:

2.1 Advantages of CRISPR–Cas9 genome editing

- Generation of a single mutant in *M. acetivorans* C2A using this technique takes only ca. 3–4 weeks compared to ca. 8–12 weeks using the previously established genetic technique that requires chromosomal integration/double-crossover of a mutagenic plasmid (Nayak & Metcalf, 2017). Note: The mutant generation time can vary depending on the strain, growth substrate, and the phenotype. The estimate provided above is for the deletion of the *ssuC* locus in *M. acetivorans* C2A using trimethylamine (TMA) as the growth substrate.
- Mutant generation is more efficient. Using the Cas9-based genome editing technique, nearly 100% of transformants contain the desired mutation on the chromosome (Nayak & Metcalf, 2017). In contrast, only ca. 50% of the transformants generated after the double-crossover step using the previous technique typically contain the desired mutation on the chromosome.
- Multiple mutations can be introduced simultaneously. Successful introduction of up to three different mutations without compromising the efficiency and speed of mutant generation has been achieved (Nayak & Metcalf, 2017).
- Gene essentiality can be reliably established. By running appropriate transformation controls for the Cas9-based plasmids, a researcher can establish whether a gene of interest is essential.
- Manipulation of essential genes is feasible. Manipulating essential genes to introduce single nucleotide polymorphisms or affinity tags for purification by the chromosomal integration/double-crossover technique is cumbersome, especially for genes arranged in an operon, due to polar effects or dosage issues induced upon homologous recombination and integration of the mutagenic plasmid. The Cas9-based technique eliminates these hurdles for manipulation of essential genes.

2.2 Features of the Cas9-containing vector (pDN201)

All mutagenic plasmids for Cas9-based genome editing of *Methanosarcina* spp. are derived from a Cas9-containing vector called pDN201 (Nayak & Metcalf, 2017) (Fig. 3). Relevant features of this vector for growth and manipulation in *E. coli* are as follows:

- The vector contains the F origin of replication and the *cat* (chloramphenicol acetyltransferase) cassette that confers chloramphenicol resistance in *E. coli*.
- The vector contains the λ attB attachment site for Gateway cloning.

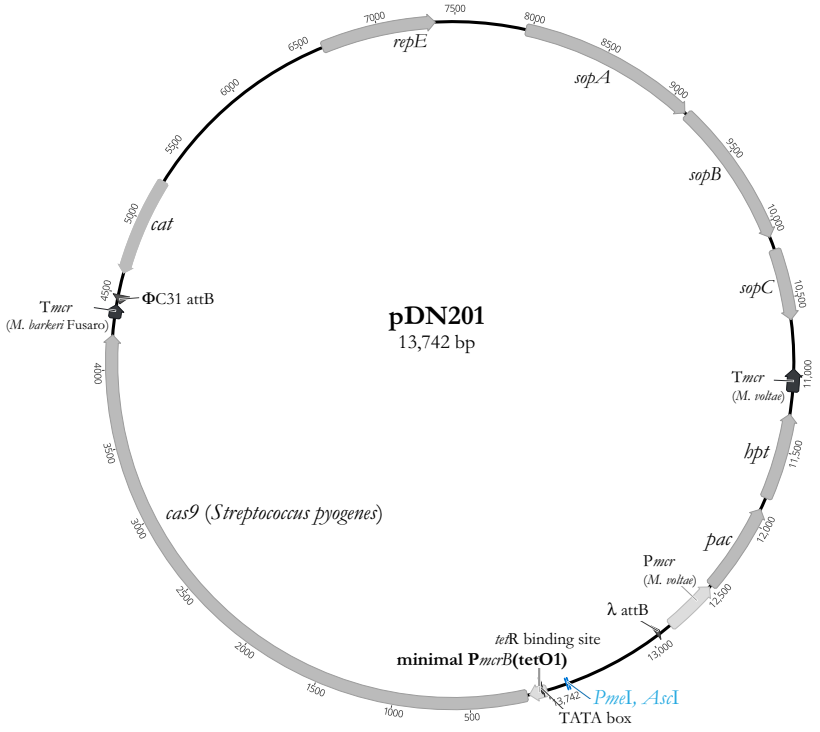

Fig. 3 Plasmid map of the base vector (pDN201) for genome editing in *Methanosarcina* spp. The plasmid contains the *cas9* ORF from *Streptococcus pyogenes* under the control of a tetracycline-inducible promoter P*mcrB*(tetO1). The sgRNA and the homology repair template can be cloned into the *Asc*I and *Pme*I sites, respectively. *cat*, chloramphenicol acetyltransferase; *repE*, replication initiation protein from the *E. coli* F plasmid; *sopA, sopB, sopC*, plasmid partitioning proteins from the *E. coli* F plasmid; T*mcr*, the terminator of the *mcr* operon; *hpt*, hypoxanthine phosphoribosyltransferase; *pac*, puromycin acetyltransferase; *tetR*, tetracycline responsive repressor from Tn10.

The appropriate *E. coli* host is a derivate of DH10B, WM4489, engineered to provide copy-number control through regulation of the *trf33* gene by a rhamnose-inducible promoter (Kim et al., 2012). For plasmid purification, rhamnose should be added to a final concentration of 10–20 m*M* in the outgrowth medium.

Relevant features of this vector for growth and maintenance in *Methanosarcina* spp. (Fig. 3) are as follows:

- The vector contains an operon encoding the *hpt-pac* cassette. The *hpt* (hypoxanthine phosphoribosyltransferase) locus confers sensitivity to 8-aza-2,6-diaminopurine (8-ADP) and can be used to cure the mutagenic plasmid from cells, and the *pac* (puromycin transacetylase) locus confers puromycin-resistance in *Methanosarcina* spp.
- The vector contains the ΦC31 attB attachment site for chromosomal integration in an appropriate host.
- The vector contains the native *cas9* ORF (without any codon optimization) from *S. pyogenes* under the control of a tetracycline-inducible strong promoter P*mcrB*(tetO1) (Guss, Rother, Zhang, Kulkarni, & Metcalf, 2008). Note: As reported in our previous study, addition of tetracycline did not change the efficiency of genome editing (Nayak & Metcalf, 2017), thus the *Methanosarcina* host strain does not need to encode the *tetR* repressor gene.

The appropriate *Methanosarcina* host strain for Cas9-based genome editing should have the following features:

- The native *hpt* locus should be inactivated by a chromosomal deletion. Note: The *hpt* locus can serve as a neutral locus for addition of genes.
- For chromosomal integration of the mutagenic plasmid, the host strain should encode the ΦC31 *int* gene and also contain the ΦC31attB attachment site for site-specific recombination on the chromosome. Note: we do not recommend chromosomal integration of the mutagenic plasmid to avoid inactivation of Cas9 in the host strain.
- For autonomous replication of the mutagenic plasmid, the host strain should *not* encode the ΦC31 *int* gene or contain any ΦC31 attachment site. Note: The base vector pDN201 does not contain genetic elements for autonomous replication in *Methanosarcina* spp. For autonomous replication, the pDN201-derived vector needs to be retrofitted with pAMG40 (containing the pC2A backbone) (Guss et al., 2008) as described in Section 3.5. The vector pDN206, a cointegrate of pDN201 and pAMG40, is routinely used as a positive control in genome editing experiments.

3. Construction of mutagenic plasmids to introduce a TAP tag at the *mcr* locus in *M. acetivorans* C2A

3.1 *In silico* design of sgRNA construct

The sgRNA construct (Fig. 4) has the following components:

- Promoter. We use the promoter of the *mtaCB1* operon, encoding a methanol-specific methyltransferase from *M. acetivorans* C2A to drive the expression of the sgRNA. The promoter elements of this operon were mapped previously in the Metcalf group (Bose & Metcalf, 2008). The transcription start site was mapped to position 537, 295 (G) on the strand of the chromosome and the putative TATA box (TATAT) was mapped to the region between 537, 320–537, 325 on the strand of the chromosome. The *mtaCB1* operon is induced ca. 100-fold in the presence of methanol (Bose & Metcalf, 2008) in the growth medium, and to preserve binding sites for regulatory elements we use the region from 537, 660 to 537, 284 on the strand of the *M. acetivorans* C2A chromosome as the promoter for the sgRNA. Note: As reported in our previous study, addition of methanol does not change the efficiency of genome editing (Nayak & Metcalf, 2017); thus, genome editing can be performed on any growth substrate.
- Target sequence. In general, the following rubric is used for identifying/designing the 20 bp target sequence (or protospacer) based on guidelines provided in previous studies (Cobb, Wang, & Zhao, 2015):
  - 3′ Protospacer adjacent motif should be NGG and should *not* be included in the target sequence. Note: In our experience, including the PAM in the target sequence is the most common error in the design of sgRNAs
  - Last 12 nt (3′ end) of the target sequence (commonly referred to as the seed sequence) + PAM [test all combinations of NGG and NAG, as Cas9 can also use NAG as the PAM (Ran et al., 2013)] should be unique in the genome to prevent off-target matches
  - Preferably, the target sequence should be on the noncoding strand if within the coding sequence of a gene
  - The target sequence(s) should be <500 bp from the HR template

  Note: There are many tools currently available to automate target sequence design for Cas9-based genome editing of a gene/region of interest. We use the "Finding CRISPR sites" tool within the Geneious bioinformatics platform (Kearse et al., 2012) to design the target sequence and identify off-target-binding sites within the genome of the appropriate *Methanosarcina* spp.

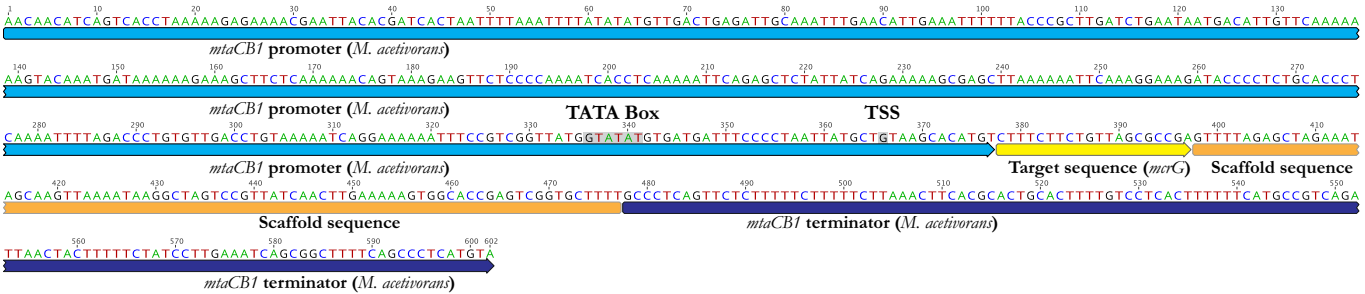

Fig. 4 Design of the synthetic construct for the expression of the single guide (sg) RNA to introduce a tandem affinity purification (TAP) tag at the N-terminus of the *mcrG* locus (locus tag: MA4547) in *M. acetivorans* C2A. The *mtaCB1* promoter (*blue*) and terminator (*purple*) sequences from *M. acetivorans* C2A drive and terminate the expression of the sgRNA, respectively. The sgRNA contains a 20 bp target sequence within the *mcrG* ORF (in *yellow*) and an 80 bp scaffold sequence (in *orange*) that binds to Cas9. *mtaCB1*, genes encoding methanol-specific methyltransferase 1; *TSS*, transcription start site.

- Target sequence to introduce a TAP tag at the *mcr* locus in *M. acetivorans* C2A. We chose to insert the TAP tag at the N-terminus of the *mcrG* locus within the *mcr* operon and used the "Finding CRISPR sites" tool within the Geneious platform to identify candidate target sequence(s) in the 250 bp region after the start codon of *mcrG* (5, 598, 895-5, 599, 144 on the strand of the *M. acetivorans* C2A chromosome). The target sequence was selected as described earlier. Note: An additional constraint for the design of this specific target sequence was to ensure that the seed sequence contains an amino acid with degenerate codons for the design of the HR template.
- Scaffold sequence. The target sequence is fused with 80 bp scaffold region (Fig. 4) derived from Ran et al. (2013) as follows: GTTTT AGAGC TAGAA ATAGC AAGTT AAAAT AAGGC TAGTC CGTTA TCAAC TTGAA AAAGT GGCAC CGAGT CGGTG CTTTT.
- Terminator. We use the putative terminator of the *mtaCB1* operon, encoding a methanol-specific methyltransferase, from *M. acetivorans* C2A to terminate the expression of the sgRNA (Fig. 4). Analysis of reads from RNA-sequencing of methanol-growth *M. acetivorans* C2A indicates that *mta*CB1 transcript terminates at position 534, 768 on the stand of the chromosome (unpublished data). We use the region from 534, 719 to 537, 843 on the strand of the *M. acetivorans* C2A chromosome as the terminator for the sgRNA.

  For each genome editing experiment, the entire sgRNA construct, from the promoter to the terminator, is synthesized as a double-stranded DNA fragment. Appropriate 30-bp overlaps at the 5′ and 3′ end of the synthetic construct are added to aid cloning using the Gibson assembly technique (see Section 3.2). We use the gblocks gene fragments service from Integrated DNA Technologies (IDT, Coralville, IA, USA) to order the synthetic constructs.

3.2 Construction of a pDN201-derived vector with sgRNA

A standardized protocol for construction of a pDN201-derived vector with the sgRNA of interest is outlined below. The sgRNA-containing plasmid is a useful intermediate, as it can serve as a negative control in genome editing experiments (see Section 3.6 for further details)

1. The freezer stock/agar stab of WM7959 (WM4489/pDN201) is streaked out on 1.5% LB agar plates supplemented with chloramphenicol (10 μg/mL) and incubated overnight at 37°C for single colonies.

2. A single colony of WM7989 is inoculated in a test tube containing 5 mL of LB (or SOB) supplemented with chloramphenicol (10 μg/mL) and 10–20 m*M* Rhamnose. Incubation is carried out on a roller/shaker at 37°C overnight.
3. pDN201 plasmid DNA is extracted using any plasmid purification kit. The DNA concentration and quality are checked using a Nanodrop (or another device/technique as available).
4. 1 μg of pDN201 plasmid DNA is linearized with *Asc*I (New England Biolabs, Ipswich, MA, USA) per manufacturer's instructions and gel-purified using any gel extraction kit.
5. The synthetic DNA fragment containing the sgRNA is reconstituted in Tris–EDTA buffer at pH 8.0, per manufacturer's instructions.
6. A Gibson assembly is performed with the linearized pDN201 backbone and synthetic DNA fragment containing the sgRNA using a HiFi DNA assembly master mix (New England Biolabs, Ipswich, MA, USA) per manufacturer's instructions. Note: We recommend performing an *in silico* Gibson assembly reaction to determine the sequence of the overlaps for Gibson assembly. The overlaps for Gibson assembly can be added at the 5′/3′ end of the construct to be synthesized as a double-stranded DNA fragment.
7. ca. 60 μL electrocompetent WM4489 cells is transformed with ca. 1 μL HiFi assembly. A protocol for making electrocompetent cells is available through many resources online for, e.g., the Protocols section of the New England Biolabs website.
8. Appropriate dilutions of the transformation reaction are plated on prewarmed 1.5% LB agar plates supplemented with chloramphenicol (10 μg/mL), and incubated overnight at 37°C.
9. Finally, we screen resulting colonies for insert using primers outside the *Asc*I cloning site, and sequence plasmids from colonies that test positive for insert. A freezer stock of the appropriate *E. coli* strains is generated for future use.

3.3 Design of HR Template

General guidelines for the design of a HR template are as follows:

- Genome editing efficiency depends on the size of the homology region provided. The editing efficiency doubled when the size of the upstream and downstream flanks for gene deletion increased from ca. 500 to 1000 bp (Nayak & Metcalf, 2017). Note: we have *not* tested the genome editing efficiency of fragments <500 bp.

- Genome editing efficiency depends on the distance of the HR template from the target sequence. We observed that templates more than 500 bp from the target sequence drastically reduce the efficiency of genome editing (Nayak & Metcalf, 2017). Note: We recommend designing multiple sgRNAs for the generation of deletions >1000 bp in size.

The TAP tag sequence was designed to contain a 3× FLAG tag for quantifying MCR protein levels using western blot with commercially available anti-FLAG antibodies, followed by the Enterokinase cleavage site (DDDDK), and a 2× Strep tag for affinity-purification of MCR (Nayak, Mahanta, Mitchell, & Metcalf, 2017) using the Strep-tactin Superflow Plus resin (QIAGEN, Germantown, MD, USA). In the HR template, the TAP tag sequence is translationally fused to the N-terminus of *mcrG* and the codon for Serine at positions 5, 599, 106 of the *mcrG* coding sequence was changed from TCC to TCA to prevent targeting after the insertion of the TAP tag. A ca. 100 bp region upstream and downstream of the TAP tag is also included in the HR template. The chromosomal regions were amplified by PCR using the genomic DNA from *M. acetivorans* C2A as the template and the TAP tag was synthesized as a double-stranded DNA fragment.

3.4 Cloning the HR template to generate the genome editing plasmid

The protocol for introducing the HR template in the pDN201 derivative(s) containing the desired sgRNA(s) is similar to the protocol described earlier in Section 3.2. Notable differences are as follows:

1. 1 μg of plasmid DNA is linearized with *Pme*I (New England Biolabs, Ipswich, MA, USA) per manufacturer's instructions and the linearized plasmid DNA is purified using any gel extraction kit.
2. A Gibson assembly is performed with the linearized vector backbone and HR template(s) using a HiFi DNA assembly master mix (New England Biolabs, Ipswich, MA, USA) per manufacturer's instructions. Note: We recommend performing an *in silico* Gibson assembly reaction to determine the sequence of the overlaps for Gibson assembly. Overlaps for Gibson assembly are added at the 5′ end of primers if the HR template is to be amplified by PCR, or at the 5′/3′ end of the fragment for synthesis.
3. Resulting colonies are screened for inserts using primers outside the *Pme*I cloning site. Plasmids from colonies that test positive for insert(s) are sequenced. A freezer stock of the appropriate *E. coli* strains is generated for future use.

3.5 Retrofitting plasmids with pAMG40

We recommend retrofitting pDN201-derived plasmids that will be used for transformations with pAMG40. The resulting cointegrate would be able to autonomously replicate in *Methanosarcina* spp. using the pC2A origin of replication and can be readily cured after genome editing. A detailed description of the vector pAMG40 can be found in Guss et al. (2008), but relevant features for this chapter are as follows:

- It contains the entire pC2A plasmid from *M. acetivorans* C2A. A specific region within pC2A that contains the origin of replication remains unknown.
- It contains the λ attP attachment site for Gateway cloning.
- It encodes the *aph* (aminoglycoside phosphotransferase) cassette that confers kanamycin resistance in *E. coli*.

A standardized protocol for retrofitting the pDN201-derived plasmids with pAMG40 using the Gateway cloning technique is as follows:

- Follow steps 1–3 in the protocol outlined in Section 3.2 to purify plasmid DNA. Note: *E. coli* strains with pDN201-derived plasmids should be cultivated in medium supplemented with chloramphenicol (10 μg/mL), whereas WM3357 (WM1788/pAMG40) should be cultivated in medium supplemented with kanamycin (25 μg/mL).
- Perform Gateway cloning with an equimolar ratio of the pDN201-derived plasmid and pAMG40 using the Gateway BP Clonase II Enzyme Mix (Thermo Fisher Scientific, Waltham, MA, USA) per manufacturer's instructions. Note: We typically incubate the BP clonase reaction at room temperature for ca. 1 h and subsequently inactivate BP clonase with 1 μL Proteinase K (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 30 min.
- Transform ca. 60 μL electrocompetent WM4489 cells with ca. 1 μL of the BP clonase reaction.
- Plate appropriate dilutions of the transformation reaction on prewarmed 1.5% LB agar plates supplemented with chloramphenicol (10 μg/mL) and kanamycin (25 μg/mL) and incubate overnight at 37°C.
- Verify cointegrates by analyzing the restriction digest pattern with appropriate restriction enzymes. Note: Cointegrates do not need to be verified by Sanger sequencing as the BP clonase reaction does not contain an error-prone DNA amplification step.
- Generate a freezer stock of the appropriate *E. coli* strains for future use.

3.6 Transformation of *Methanosarcina* spp. with genome editing plasmids

We recommend using WWM60 (Δ *hpt*::p*mcrB-tetR*) (Guss et al., 2008) for genome editing experiments with *M. acetivorans* C2A and setting up appropriate controls as follows:

- Positive control: We recommend using pDN206 (the cointegrate of pDN201 and pAMG40) as a positive control for the transformation reaction.
- Negative control: We recommend using a cointegrate of pAMG40 and the pDN201-derived plasmid with the appropriate sgRNA as a negative control for genome editing as well as a no-DNA negative control for the transformation reaction.

A liposome-mediated transformation reaction as described in detail in Metcalf, Zhang, Apolinario, Sowers, and Wolfe (1997) is used to introduce mutagenic plasmids in *Methanosarcina* spp. For *M. acetivorans* C2A, we use a 10 mL culture volume [mid-late exponential phase culture in high-salt (HS) medium with 50 m*M* TMA as the growth substrate] per transformation reaction. For, *M. barkeri* Fusaro, we use a 30 mL culture volume (mid-late exponential phase culture in HS medium with 125 m*M* methanol as the growth substrate) per transformation reaction. See Wolfe (2011) for details of the HS medium and techniques for anaerobic growth of *Methanosarcina* spp. A standardized protocol for mutant generation using the Cas9-based genome editing technique is as follows:

- Perform liposome-mediated transformation and plate cells on 1.5% HS agar plates supplemented with the appropriate growth substrate and 2 μg/mL puromycin. Incubate plates at 37°C as previously described in Metcalf, Zhang, and Wolfe (1998) for ca. 2 weeks.
- Screen puromycin resistant (Pur^R) transformants for the desired mutation by designing appropriate primers (outside the HR template) and amplifying the chromosome locus by PCR. To screen for the TAP tag at the N-terminus of *mcrG* we designed the following forward (TAC CCA TTC AAT GAC TTC TGC) and reverse (GCAC AGA TTG AAA TGC ACA AG) primers. A 1207-bp band is observed if the TAP tag is present, whereas a 1033-bp band is observed for the WT locus. Note: We typically screen 4–5 Pur^R transformants per mutagenic plasmid and commonly observe that all Pur^R transformants test positive for the desired mutation.
- Streak out 3 Pur^R transformants that test positive for the desired mutation on HS agar plates supplemented with the appropriate growth

substrate and 20 μg/mL 8-ADP. Incubate plates at 37°C as previously described in Metcalf et al. (1998) for ca. 1 week. Screen single colonies for the desired mutation by PCR amplification of the chromosomal locus.

- Pick 2–3 independently derived 8-ADP^{R} isolates that test positive for the desired mutation and inoculate in 10 mL liquid HS medium supplemented with the appropriate growth substrate. Incubate cultures at 37°C.
- When cultures reach mid-late exponential phase, transfer a 100-fold dilution to 10 mL liquid HS medium supplemented with the appropriate growth substrate and 2 μg/mL puromycin to test if the mutagenic plasmid is cured. If cultures are Pur^{S} (i.e., do not grow in medium supplemented with puromycin), the plasmid has been cured. Note: Measure the optical density at 600 nm (OD_{600}) in liquid HS medium with 2 μg/mL puromycin for up to 1 week. If no change in OD_{600} is observed after a week, score the cultures Pur^{S}.
- Confirm that the mutagenic plasmid has been cured by PCR amplification of the *repA* gene from pC2A in 8ADP^{R}, Pur^{S} isolates using the forward primer (CTG CAA TAC ACT TTC TTG TCC) and reverse primer (TCA TCC ACT TTG AAG GGA GAA G). If pC2A^{+}, a 1045-bp band will be observed, whereas no band will be observed if pC2A is absent. It is imperative to set up a positive control (such as pAMG40) for this PCR reaction.
- Promptly generate −80°C freezer stocks of one or two independently derived 8ADP^{R}, Pur^{S} isolates with the desired mutation.

4. Affinity purification of TAP-tagged MCR from *M. acetivorans* C2A

Specific details regarding the cultivation and growth of strictly anaerobic microorganisms are provided in Wolfe (2011). The following protocol describes a technique for aerobic purification of TAP-tagged MCR from *M. acetivorans* C2A. Using this affinity purification technique ca. 1 mg of MCR protein can be reliably purified from 250 mL of culture in 1–2 h (Fig. 5). Aerobically purified MCR is inactive but can be used for several applications such as thermal stability measurements (Nayak et al., 2017) and X-ray crystallography.

A standardized protocol for affinity purification of MCR is described below.

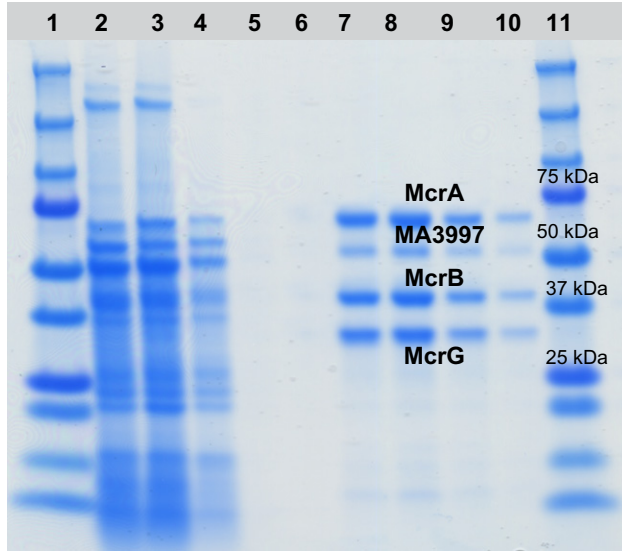

Fig. 5 SDS-PAGE analysis of affinity-purified methyl coenzyme-M reductase (MCR) from *M. acetivorans* C2A. Lanes 1, 11: Protein standard; Lane 2: Crude lysate; Lane 3: Cleared lysate; Lane 4: Flow through; Lane 5: Wash 1; Lane 6: Wash 2; Lane 7: Elution fraction 1; Lane 8: Elution fraction 2; Lane 9: Elution fraction 3; Lane 10: Elution fraction 4. The three subunits of MCR copurified with a fourth band, identified by LC-MS analysis as the hypothetical protein encoded by MA3997.

4.1 Prepare buffers for aerobic purification of MCR

Buffer NP (1 L)—Will be used as the lysis buffer and wash buffer

- Weigh 6.9 g $NaH_2PO_4 \cdot H_2O$ (final concentration = 50 m*M* NaH_2PO_4)
- Weight 17.53 g NaCl (final concentration = 300 m*M* NaCl)
- Dissolve in ca. 500 mL ultrapure H_2O
- Adjust pH to 8.0 using a 5 *M* NaOH stock solution
- Raise volume to 1 L with ultrapure H_2O
- Filter-sterilize and store at 4°C

Buffer NPD (100 mL)—Will be used as the elution buffer

- Weigh 53.5 mg of desthiobiotin (final concentration = 2.5 m*M* desthiobiotin)
- Dissolve in 100 mL Buffer NP
- Filter-sterilize and store at 4°C

4.2 Cell culture and lysis

- Thaw a cryopreserved stock of WWM1054 (Genotype: TAP tag fused to the N-terminus of *mcrG* in WWM60) (Nayak et al., 2017) at room

temperature and inoculate 2 mL in a Balch tube (Chemglass Life Sciences, Vineland, NJ, USA) containing 10 mL HS medium supplemented with 50 m*M* TMA as the sole growth substrate.

- Incubate Balch tube (Chemglass Life Sciences, Vineland, NJ, USA) at 37 °C. Acclimate WWM1054 to HS medium supplemented with 50 m*M* TMA for 10–15 generations prior to cultivation for protein purification.
- Transfer a 100-fold dilution of a late-exponential phase culture of WWM1054 to an anaerobic culture bottle (such as an "Och" bottle) (Chemglass Life Sciences, Vineland, NJ, USA) containing 250 mL HS medium supplemented with 50 m*M* TMA as the sole growth substrate. Monitor growth by measuring OD_{600} at regular intervals. Note: It is important to "vent" the cultures during the growth phase to depressurize the anaerobic culture bottles.
- Harvest cultures in mid-exponential phase and collect the cell pellet by centrifugation ($3000 \times g$) for 15 min at 4°C.
- Discard supernatant and resuspend cell pellet in 5 mL buffer NP for lysis.
- Sonicate for three bursts of 10 s with 30-s intervals. Keep cells on ice during sonication. Add a small amount of DNase and mix gently to reduce viscosity of the lysate. Store 20 µL of crude lysate at −20°C for SDS-PAGE analysis.
- Clear the crude lysate by centrifugation ($17{,}500 \times g$) for 30 min at 4°C. Store 20 µL of cleared lysate at −20°C for SDS-PAGE analysis and use the remainder for protein purification.
- Equilibrate the cleared lysate to room temperature prior to purification.

4.3 Protein purification

- Equilibrate a gravity column containing 2 mL Strep-tactin Superflow Plus slurry (a 50% suspension) (QIAGEN, Germantown, MD, USA) with 4 mL buffer NP at room temperature. Note: Vortex the Strep-tactin Super Flow Plus slurry for 10 s before transferring to the gravity column.
- Load the cleared lysate on the column and collect 20 µL of the flow-through for SDS-PAGE analysis.
- Wash the column twice with 4 mL of buffer NP two times. Collect 20 µL of each wash for SDS-PAGE analysis. Note: After the second wash, the resin should have a bright yellow color due to the oxidized Ni(II) form of F_{430} in MCR.

- Elute the protein in four fractions with 500 μL buffer NPD per fraction. Use 20 μL of each fraction for SDS-PAGE analysis. Note: The highest concentration of the protein is typically observed in the second or third fraction.
- Measure the protein concentration using a Coomassie Plus (Bradford) assay kit (Pierce Biotechnology, Thermo-Scientific, Rockford, IL, USA) with BSA (bovine serum albumin) as the standard per the manufacturer's instructions.

4.4 Buffers for SDS-PAGE analysis

20% SDS (w/v)
- Weigh out 50 g SDS
- Dissolve in ultrapure H_2O and raise volume to 250 mL
- Store at room temperature

10 × SDS running buffer
- Weigh out 30.25 g Tris Base (final concentration will be 25 m*M*)
- Weigh out 144.1 g glycine (final concentration will be 192 m*M*)
- Dissolve in ultrapure H_2O and raise volume to 1 L
- Store at room temperature

1 × SDS running buffer (prepare fresh on the day of and discard after single use)
- 100 mL 10 × SDS running buffer
- 5 mL 20% SDS
- Fill to 1 L with ultrapure water

Sample buffer (prepare fresh on the day of and discard after single use)
- Add 20 μL β-mercaptoethanol (BME) to 380 μL Laemmli sample buffer (Bio-Rad, Hercules, CA). Note: Handle BME in a fumehood
- Vortex to mix

4.5 SDS-PAGE analysis of affinity-purified MCR

- Add 10 μL 2 × sample buffer to an equal volume of each sample and boil for 10 min
- Load 10 μL of boiled sample in wells of a 4%–20% Mini-Protean TGX denaturing SDS-PAGE gel (Bio-Rad, Hercules, CA)
- Run gel at 70 V until the dye front reaches the bottom of the gel
- Wash gel with ultrapure H_2O for 15 min on a gently shaking platform. Repeat the wash step for a total of three washes.

- Stain with Gel code Blue Stain Reagent (Thermo Fisher Scientific, Waltham, MA) and destain with ultrapure H_2O per manufacturer's instructions.

In ongoing experiments, we are adapting this protocol for affinity purification of MCR under anaerobic conditions to retrieve active protein for kinetic and mechanistic analysis.

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