CHAPTER TWELVE

Recent Advances in the Genetic Manipulation of *Methylosinus trichosporium* OB3b

Soo Y. Ro, Amy C. Rosenzweig[1]
Northwestern University, Evanston, IL, United States
[1]Corresponding author: e-mail address: amyr@northwestern.edu

Contents

Abstract

Methanotrophic bacteria utilize methane as their sole carbon and energy source. Studies of the model Type II methanotroph *Methylosinus trichosporium* OB3b have provided insight into multiple aspects of methanotrophy, including methane assimilation, copper accumulation, and metal-dependent gene expression. Development of genetic tools for chromosomal editing was crucial for advancing these studies. Recent interest in methanotroph metabolic engineering has led to new protocols for genetic manipulation of methanotrophs that are effective and simple to use. We have incorporated these newer molecular tools into existing protocols for *Ms. trichosporium* OB3b. The modifications include additional shuttle and replicative plasmids as well as improved gene delivery and genotyping. The methods described here render gene editing in *Ms. trichosporium* OB3b efficient and accessible.

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1. INTRODUCTION

Methanotrophic bacteria are unique in their ability to utilize methane, a potent greenhouse gas, as their sole carbon source (Hanson & Hanson, 1996). In the first step of their metabolic pathway, methane is oxidized to methanol, which can be utilized as a cheap carbon feedstock for industrial chemical processes (Lawton & Rosenzweig, 2016). For this reason, there have been extensive efforts to understand methanotrophy in multiple species of bacteria. Most aerobic methanotrophs are classified as Type I (Gammaproteobacteria) or Type II (Alphaproteobacteria) based on their use of either the ribulose monophosphate or serine cycles for carbon assimilation, respectively, as well as their phospholipid compositions, cell morphologies, and membrane arrangements (Hanson & Hanson, 1996; Semrau, DiSpirito, & Yoon, 2010). Methane is oxidized to methanol by methane monooxygenase enzymes (MMOs), followed by methanol conversion to formaldeyhde by methanol dehydrogenases (MDHs). There are two types of MMO, a soluble and cytoplasmic iron-containing enzyme (sMMO) and a "particulate" and inner membrane-bound copper-containing enzyme (pMMO) (Ross & Rosenzweig, 2016). With the exception of the genera *Methylocella* and *Methyloferula*, aerobic methanotrophs express pMMO under most conditions (Semrau et al., 2010; Vorobev et al., 2011). Under copper-starved conditions, some Type I and Type II methanotrophs can switch to sMMO expression (Semrau et al., 2010), and some *Methylosinus* and *Methylocystis* species also produce the copper chelator methanobactin (Dassama, Kenney, & Rosenzweig, 2017).

Methylosinus trichosporium OB3b is the best studied Type II methanotroph (Stein et al., 2010). This bacterium can express both sMMO and pMMO, produces methanobactin (Dassama et al., 2017), and can express two distinct MDHs, the calcium-binding MxaF and the lanthanide-binding XoxF (Haque, Kalidass, et al., 2015). Genetic tools developed previously for *Ms. trichosporium* OB3b have been used to mutate or disrupt the genes encoding several important proteins. Recent studies have focused on sMMO (Borodina, Nichol, Dumont, Smith, & Murrell, 2007; Lock, Nichol, Murrell, & Smith, 2017; Semrau et al., 2013; Smith & Murrell, 2011; Smith, Slade, Burton, Murrell, & Dalton, 2002), proteins in the extended *pmo* operon (Gu, Haque, & Semrau, 2017), methanobactin transport and biosynthetic proteins (Dassama, Kenney, Ro, Zielazinski, & Rosenzweig, 2016; Gu,

Baral, DiSpirito, & Semrau, 2017; Gu et al., 2016; Gu, Haque, et al., 2017), and genes related to MDH (Haque, Gu, DiSpirito, & Semrau, 2015).

In recent years, new genetic manipulation tools have been developed for two Type I haloalkaliphilic methanotrophs, *Methylomicrobium alcaliphilum* 20Z (Kalyuzhnaya et al., 2008; Ojala, Beck, & Kalyuzhnaya, 2011) and *Methylomicrobium buryatense* 5G (Puri et al., 2015; Yan, Chu, Puri, Fu, & Lidstrom, 2016). These protocols have been used to engineer methanotrophs for biofuel production (de la Torre et al., 2015; Henard et al., 2016; Kalyuzhnaya, Puri, & Lidstrom, 2015) and have also enabled studies of lanthanide-dependent methanol oxidation (Chu & Lidstrom, 2016), ectoine biosynthesis (Mustakhimov et al., 2009), and fatty acid biosynthesis (Demidenko, Akberdin, Allemann, Allen, & Kalyuzhnaya, 2017). This chapter describes the incorporation of these newer techniques developed for Type I methanotrophs into the traditional mutagenesis protocol for *Ms. trichosporium* OB3b (Murrell, 1994; Lloyd, Finch, Dalton, & Murrell, 1999; Smith & Murrell, 2011). By simplifying cloning, gene delivery via conjugation or electroporation, and DNA isolation for genotyping, this hybrid method will facilitate future investigations and engineering of *Ms. trichosporium* OB3b.

2. *MS. TRICHOSPORIUM* OB3B GROWTH CONDITIONS

Ms. trichosporium OB3b strains are grown in nitrate minimal salt (NMS) media (Hakemian et al., 2008) (Table 1). Cultures are grown under either low copper (0.1 μM $CuSO_4$) or high copper (10–25 μM $CuSO_4$) conditions in liquid cultures and agar plates. Many genes of interest in methanotrophs are copper regulated, and it may be necessary to alter the copper concentration to ensure viability and to avoid selection against the genetic modifications. Similar considerations may be appropriate for genes regulated by other environmental factors.

Initially, cells at an OD_{600} of 0.1 are inoculated into 50 mL cultures in 250-mL Erlenmeyer flasks sealed with rubber septa. The flasks are then incubated at 30°C with shaking at 200 rpm, and oxygen and methane levels are maintained via daily sparging with a 1:3 methane-to-air ratio at 1 L/min for 5 min. An OD_{600} of ~1.5 is typically reached after 1 week of growth. Agar plates are incubated in GasPak plate incubation chambers (BD) at 30°C, and gas exchange is facilitated by use of a vacuum to create negative pressure inside the chamber followed by gas flow at a 1:1 methane-to-air ratio at

Table 1 *Ms. trichosporium* OB3b Growth Conditions

	Growth	Mating	Selection	Counterselection
NMS medium (1 L)				
$NaNO_3$	0.85 g	0.85 g	0.85 g	0.85 g
K_2SO_4	0.17 g	0.17 g	0.17 g	0.17 g
$MgSO_4{\cdot}7H_2O$	0.037 g	0.037 g	0.037 g	0.037 g
$CaCl_2{\cdot}2H_2O$	0.01 g	0.01 g	0.01 g	0.01 g
40 m*M* $FeSO_4{\cdot}7H_2O$	1 mL	1 mL	1 mL	1 mL
100 m*M* $CuSO_4{\cdot}5H_2O$	Varies	Varies	Varies	Varies
Agar (plates only)	15 g	15 g	15 g	15 g
LB (Difco)	—	1.2 g	—	—
Phosphate buffer solution	10 mL	10 mL	10 mL	10 mL
Trace elements solution	2 mL	2 mL	2 mL	2 mL
50% sucrose	—	—	—	50 mL
Phosphate solution (g/L)				
$Na_2HPO_4{\cdot}7H_2O$	48.06			
KH_2PO_4	23.4			
Trace elements (g/L)				
$ZnSO_4{\cdot}7H_2O$, 0.288; $MnCl_2{\cdot}4H_2O$, 0.16; H_3BO_3, 0.06; $Na_2MoO_4{\cdot}2H_2O$, 0.048; $CoCl_2{\cdot}6H_2O$, 0.048; KI, 0.083				

Table 2 Bacterial Strains

Strain	Growth Medium
Ms. trichosporium OB3b	NMS
E. coli S17-1	LB (Difco)
E. coli CopyCutter EPI400	LB (Difco)

1 L/min for 3 min. Colonies on plates typically appear in 1–2 weeks. Antibiotics such as kanamycin (25 μg/mL) and gentamicin (5 μg/mL) are used to select for mutants, and naladixic acid (10 μg/mL) is used to remove donor *Escherichia coli* strains as described later. The bacterial strains used in this protocol are listed in Table 2.

3. PLASMIDS FOR MUTAGENESIS

For chromosomal site-directed mutagenesis or gene deletion, a broad host range vector pk18*mobsacB* (Schäfer et al., 1994) has been used in several methanotrophs, including *Ms. trichosporium* OB3b (Borodina et al., 2007). However, it is sometimes difficult to insert methanotroph genes, including those in the *pmo* operon, into this plasmid due to leaky expression at high copy number and subsequent toxicity of the product proteins in *E. coli* (Ojala et al., 2011). We have employed the following two strategies to circumvent this issue. First, we use the chemically competent *E. coli* strain CopyCutter EPI400 (Lucigen), designed to control plasmid copy number, to increase the success rate for cloning of methanotroph genes. However, the plasmid must then be incorporated into the *E. coli* donor strain S17-1 (Simon, Priefer, & Pühler, 1983) for subsequent conjugation. Transformation into S17-1 can also fail due to leaky expression. As a second, alternative strategy, we have constructed a modified version of pk18*mobsacB* under p15a copy control (Selzer, Som, Itoh, & Tomizawa, 1983) to generate the plasmid pk18*mobsacB*_p15a (Fig. 1; Table 3). This shuttle vector has improved the efficiency of cloning methanotroph genes, such as those in the *pmo* operon (Ojala et al., 2011).

Replicative plasmids are useful tools for protein overexpression or probing promoters. We tested a replicative plasmid compatible with Type I methanotrophs for function in *Ms. trichosporium* OB3b. The Lidstrom laboratory has constructed a replicative plasmid pAWP89 that can be maintained in *M. buryatense* 5G (Puri et al., 2015). We have determined that

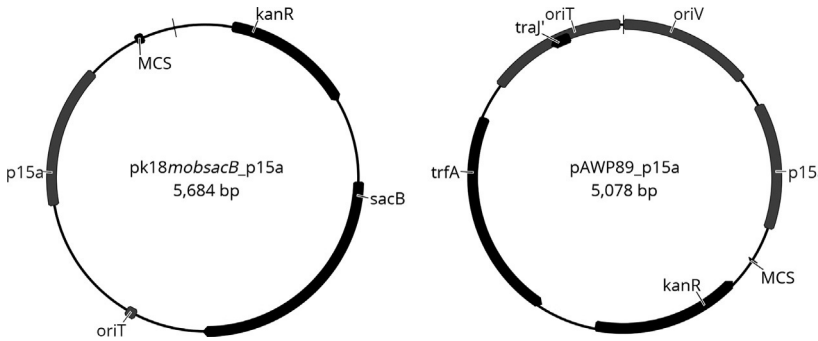

Fig. 1 Plasmid maps of pk18*mobsacB*_p15a and pAWP89_p15a.

Table 3 Plasmids

Plasmid Name	Source/Reference
pk18*mobsacB*	ATCC 87097 (Schäfer et al., 1994)
pk18*mobsacB*_p15a	This study
pAWP89	Addgene 61264 (Puri et al., 2015)
pAWP89_p15a	This study

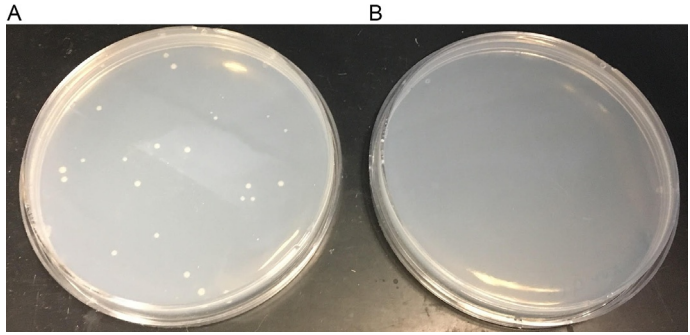

Fig. 2 *Ms. trichosporium* OB3b transformed with pAWP89_p15a (A) compared to wild-type *Ms. trichosporium* OB3b (B) on 25 μg/mL kanamycin NMS agar selection plates.

this plasmid can be maintained in *Ms. trichosporium* OB3b (Fig. 2). For the purposes of overexpressing toxic methanotroph genes, we have replaced the origin of replication in pAWP89 with p15a to create pAWP89_p15a (Fig. 1; Table 3).

4. GENETIC MANIPULATION VIA CONJUGATION

The most common and efficient method for genetic manipulation in methanotrophs requires conjugation (Lloyd et al., 1999; Murrell, 1994; Smith & Murrell, 2011). Typically after conjugation, the shuttle plasmid is incorporated into the chromosome of the recipient strain, followed by counterselection to ensure double recombination and removal of the vector backbone from the chromosome (Lalioti & Heath, 2001). In *Ms. trichosporium* OB3b, the established conjugation method involves the addition of liquid cultures of both methanotroph and *E. coli* cells onto a 0.2-μm sterile filter paper for mating (Smith & Murrell, 2011). This protocol requires the use and subsequent dismantling of a plastic filter unit or a Pyrex microfiltration glass assembly, followed by cell recovery from filter paper,

which can increase the risk of contamination. A simpler conjugation protocol has been developed for the Type I methanotrophs *M. buryatense* 5G and *M. alcaliphilum* 20Z (Ojala et al., 2011; Puri et al., 2015). We have modified this protocol, which requires only cells grown on agar plates and disposable cell spreaders, for conjugation in *Ms. trichosporium* OB3b (Fig. 3):

1. The vector pk18*mobsacB* or pk18*mobsacB*_p15a (carrying an insert consisting of 5′ and 3′ DNA regions flanking the site of gene insertion, deletion, or mutagenesis, as well as any sequence to be inserted or altered) is transformed into the donor strain *E. coli* S17-1 via either heat shock or electroporation (Simon et al., 1983).
2. One day prior to bacterial mating, the *E. coli* S17-1 cells are streaked onto LB agar plates to obtain a bacterial lawn.

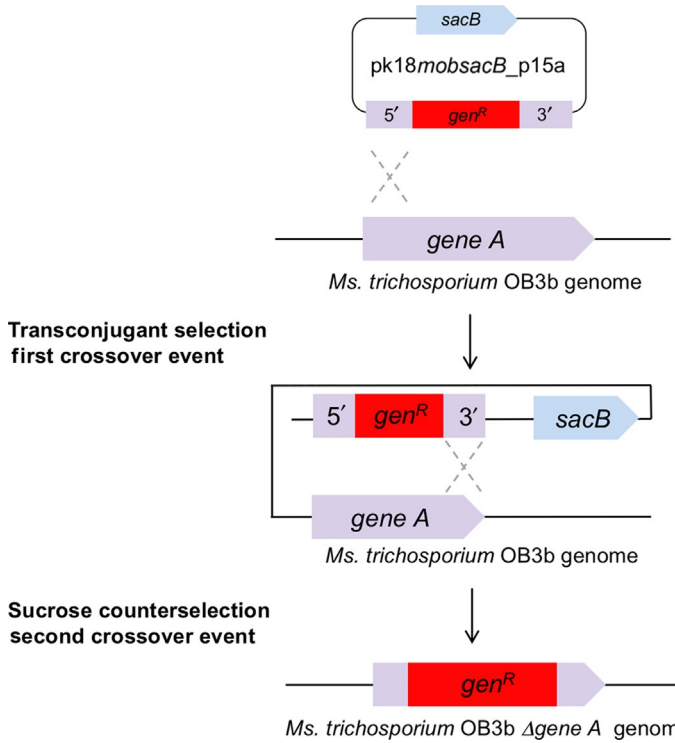

Fig. 3 Gene deletion via conjugation in *Ms. trichosporium* OB3b. Following conjugation, the introduced plasmid recombines into the targeted site in the chromosome during the first crossover. Transconjugant selection followed by sucrose counterselection induces a second crossover event to remove the plasmid backbone from the chromosome and produce the mutant with the gentamicin resistance cassette (*gen*^{R}) disrupting *gene A*.

3. The wild-type recipient strain *Ms. trichosporium* OB3b is streaked onto an NMS agar medium plate (Table 1) and given 1 week to grow, forming at least a partial bacterial lawn.
4. The donor and recipient strains are harvested from plates and evenly plated and spread onto the same mating agar plate (Table 1) with a cell ratio of 2:1 donor-to-recipient strain. Specifically, a sterile cell spreader is dragged once across the plate containing the recipient methanotroph cells, and then is immediately used to inoculate the mating plate (Table 1). This is repeated for the donor (*E. coli*) strain, and the donor cells are plated directly on top of the recipient cells in the mating plate.
5. These plates are then incubated at a 1:1 methane-to-air ratio for 48 h at 30°C in a GasPak chamber.
6. A fraction of the mated cells, just enough to be visible on the bottom of the sterile cell spreader, are scraped off the mating plate and directly plated onto a selection agar plate (Table 1) containing kanamycin (25 μg/mL). The selection plate is incubated under the same conditions as the mating plate.
7. Single transconjugant colonies that appear are picked with a sterile loop and separately streaked onto selection plates containing kanamycin (25 μg/mL) as well as naladixic acid (10 μg/mL) to eliminate remaining cells from the donor *E. coli* strain.
8. Single transconjugant colonies on selection plates are picked using a sterile loop and streaked onto counterselection agar plates (Table 1) containing 2.5% sucrose and appropriate antibiotics. Single colonies from these counterselection plates are then picked and streaked once more to obtain sufficient biomass for genotype screening as described in Section 6.1.

5. GENE MANIPULATION VIA ELECTROPORATION

In situations where cloning or conjugation fails, electroporation can be employed, as reported for *Methylocella silvestris* BL2, *Methylocystis* sp. strain SC2, and *M. buryatense* 5G (Baani & Liesack, 2008; Crombie & Murrell, 2011; Yan et al., 2016). Linear DNA fragments produced via PCR can be also electroporated into *Ms. trichosporium* OB3b and integrated into the chromosome (Dassama et al., 2016). Thus far, intact plasmids purified from *E. coli* cloning strains have not been successfully incorporated into *Ms. trichosporium* OB3b via this method.

5.1 Construction of Linear DNA Fragment

Homologous recombination of a linear DNA fragment is a technique used for gene disruption, insertion, and modification (Orr-Weaver, Szostak, & Rothstein, 1981; Sharan, Thomason, Kuznetsov, & Court, 2009). One common use of this technique is gene disruption via insertion of a linear DNA fragment containing an antibiotic resistance cassette into the middle of the target gene. Construction of the resulting linear DNA fragment can be performed via several methods, including fusion PCR (Shevchuk et al., 2004). In that method, ~500 bp DNA regions flanking the gene of interest and a gentamicin resistance cassette are amplified by PCR. Primers are designed to add 25 bp regions onto the 5′ and 3′ ends of the gentamicin resistance cassette that overlap with the DNA regions flanking the gene of interest. The three PCR products are then joined together through two rounds of fusion PCR (Fig. 4). PCR products for each round of amplification are isolated and purified through gel extraction. In addition, it is necessary to perform a final cleanup of the PCR product using a PCR Purification Kit (Qiagen) or equivalent technique prior to sequencing and electroporation.

5.2 Electrocompetent Cell Preparation

50 mL cultures of *Ms. trichosporium* OB3b cells are grown in NMS medium (0.1 μ*M* $CuSO_4$) as described in Section 2. Cells are harvested at an OD_{600} of 1.0, centrifuged at 7000 × *g* at 4°C for 10 min, and washed twice with 20 mL of 10% sterile glycerol at 4°C before a final 1 mL resuspension in 10% glycerol at 4°C. Fresh electrocompetent cells are recommended for every electroporation round.

5.3 Electroporation

1. 100–500 ng of the linear DNA fragment is added to 100 μL electrocompetent cells in sterile microcentrifuge tubes kept on ice.
2. The mixture is then transferred into a prechilled sterile electroporation cuvette with a 0.2-cm gap width (Bio-Rad). The cuvette is inserted into an electroporation system (Bio-Rad MicroPulser) and subjected to one pulse at 2.2 kV for 5 ms at 1 Å.
3. After electroporation, the cell mixture is added to 5 mL of NMS liquid medium (without antibiotics) in borosilicate culture tubes sealed with rubber septa.

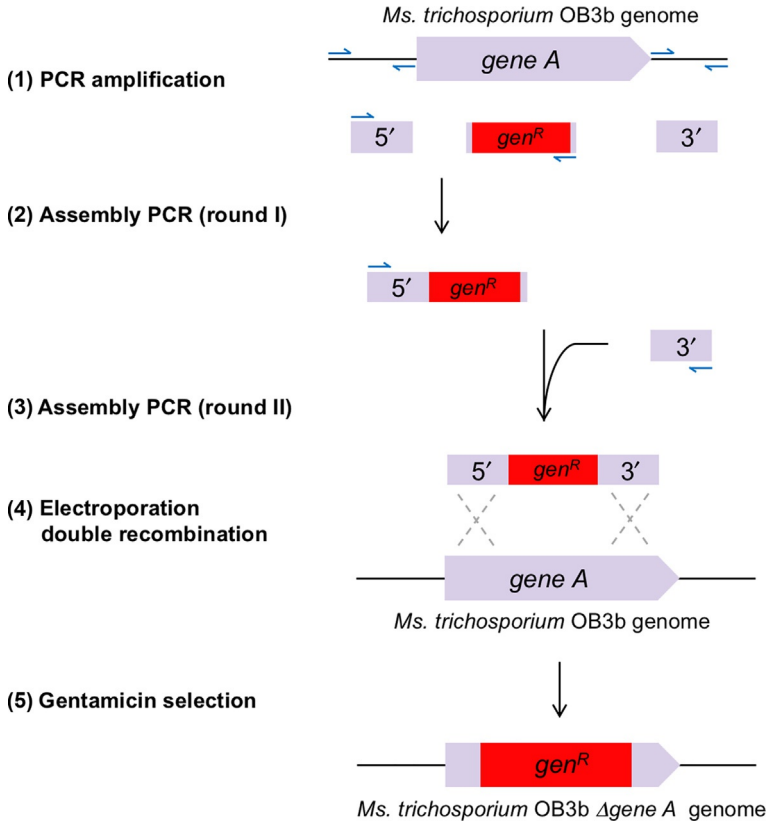

Fig. 4 Construction of a gene-disrupting linear DNA fragment using fusion PCR. (1) PCR amplification of *gen*^{R} and the 5′ and 3′ DNA regions flanking gene targeted for disruption. (2) The 5′ DNA fragment and *gen*^{R} are used as templates for the first round of fusion PCR. (3) The resulting PCR product and the 3′ DNA fragment are used as templates for the second round of PCR to produce the final gene-disrupting linear DNA construct. The linear DNA construct is (4) electroporated into wild-type *Ms. trichosporium* OB3b followed by (5) selection for the *Ms. trichosporium* OB3b Δ*gene A* mutant. Primer binding sites are represented by *half arrows*.

4. The cultures are incubated overnight at 30°C and 200 rpm after gas sparging at a 1:3 methane-to-air ratio.
5. After 24 h of growth, the cells (the outgrowth culture) are centrifuged at 7000 × *g* and 4°C for 10 min, and are then resuspended in 500 μL standard NMS liquid medium.
6. 100–300 μL of the outgrowth culture is then spread onto NMS selection plates containing gentamicin (5 μg/mL).

6. ANALYSIS OF *MS. TRICHOSPORIUM* OB3B MUTANTS

6.1 DNA Extraction From *Ms. trichosporium* OB3b Cells on Agar Plates

DNA extraction from *Ms. trichosporium* OB3b is less efficient than DNA extraction from many Type I or Type II methanotrophic bacteria. Existing methods use the neutral lysis/CsCl method or a DNeasy Blood Tissue Kit (Qiagen) for DNA extractions from liquid cultures (Gu et al., 2016; Smith & Murrell, 2011). However, growing liquid cultures to genotype multiple colonies is time-consuming. Instead, an improved method has been developed to extract DNA from cells growing on agar plates using the MasterPure Complete DNA Purification Kit (Epicentre, Cat. No. MC85200). This method allows for higher-throughput of genotype screening and provides increased yields of DNA from smaller amounts of cells. The MasterPure Complete DNA Purification Kit protocol has been modified for *Ms. trichosporium* OB3b:

1. Add 20 μL Proteinase K (NEB, 800 U/mL) into 300 μL 2× tissue and cell lysis solution for each sample.
2. Drag a sterile inoculating loop across the agar plate culture until the loop is covered with cells (Fig. 5).

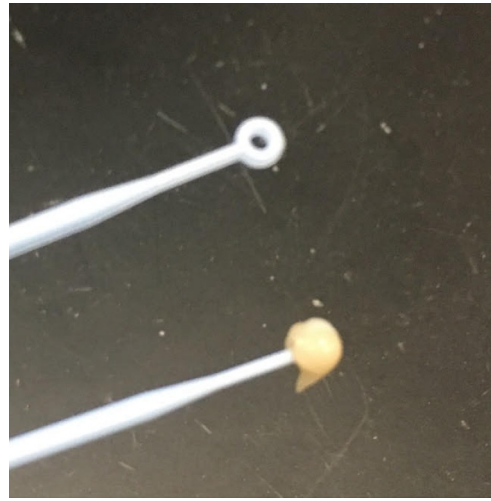

Fig. 5 Amount of *Ms. trichosporium* OB3b cells required for DNA extraction shown on a 1-μL inoculating loop.

3. Swirl the inoculating loop in the lysis solution and resuspend fully by pipetting.
4. Incubate the sample at 65°C overnight at 500 rpm.
5. Add 5 μL RNase A (Epicentre, 5 μg/μL). Incubate for 1 h at 37°C without shaking.
6. Place the samples on ice for 5 min.
7. Add 150 μL MPC protein precipitation reagent and vortex.
8. Centrifuge the sample at 10,000 × *g* at 4°C for 10 min to pellet cell debris.
9. Transfer the supernatant to a new tube and add 500 μL 100% isopropanol on ice. Manually invert the sample 30 times.
10. Centrifuge the sample at 4°C for 30 min at 20,000 × *g* to pellet DNA.
11. Wash the DNA pellet with 750 μL 70% ethanol on ice. Centrifuge at 20,000 × *g* at 4°C for 5 min, remove the ethanol, and repeat the wash step once more.
12. Let the pellet dry at 37°C until residual ethanol is gone.
13. Resuspend the pellet with 50 μL TE buffer and incubate overnight at 4°C.
14. DNA yield can vary from 10 to 60 μg. Additional cleanup steps using silica spin columns (Zymo) may be required for uses other than simple Sanger sequencing.

6.2 Genotyping Mutants

To confirm the genotype of *Ms. trichosporium* OB3b variants, PCR amplification of the altered DNA region is performed. Due to the high genomic GC content of Type II methanotrophs, PCR amplification is more successful under reaction conditions optimized for high-GC samples, such as the 2× iProof GC Master Mix (Bio-Rad). 100 ng of *Ms. trichosporium* OB3b genomic DNA is an appropriate starting point for a 50-μL PCR reaction. Gradient or touchdown PCR at annealing temperatures of 55–65°C can be employed to identify conditions for specific amplification. PCR cleanup (QIAquick PCR Purification Kit, Qiagen) will significantly improve sequencing results.

7. SUMMARY

The modifications in plasmid design, construct delivery, and genotyping described here allow for simpler and more efficient genetic manipulation of *Ms. trichosporium* OB3b, at least until CRISPR or other

gene editing methods are developed (Tian et al., 2017). These advances will expedite metabolic engineering efforts in this methanotrophic strain and enhance studies of proteins involved in the key processes of methanotrophy.

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