

Genetic Tools for the Industrially Promising Methanotroph *Methylobacterium buryatense*

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Aerobic methanotrophs oxidize methane at ambient temperatures and pressures and are therefore attractive systems for methane-based bioconversions. In this work, we developed and validated genetic tools for *Methylobacterium buryatense*, a haloalkaliphilic gammaproteobacterial (type I) methanotroph. *M. buryatense* was isolated directly on natural gas and grows robustly in pure culture with a 3-h doubling time, enabling rapid genetic manipulation compared to many other methanotrophic species. As a proof of concept, we used a sucrose counterselection system to eliminate glycogen production in *M. buryatense* by constructing unmarked deletions in two redundant glycogen synthase genes. We also selected for a more genetically tractable variant strain that can be conjugated with small incompatibility group P (IncP)-based broad-host-range vectors and determined that this capability is due to loss of the native plasmid. These tools make *M. buryatense* a promising model system for studying aerobic methanotroph physiology and enable metabolic engineering in this bacterium for industrial biocatalysis of methane.

Methane, the principal component of natural gas, is a promising feedstock for industrial biotechnology. Domestic natural gas supplies are increasing, and natural gas prices have remained lower than oil prices over the last decade (1, 2). Methane has also been identified as a prime mitigation target for reducing greenhouse gas emissions (3). Aerobic methanotrophs oxidize methane under ambient temperatures and pressures and are genetically tractable, making these bacteria potentially useful biocatalysts for converting natural gas into chemicals and liquid fuels via metabolic engineering (1, 4).

Previous industrial interest in aerobic methanotrophs has included attempts to produce biomass for use as single-cell protein or carotenoids in animal feed (5, 6). However, production of biomass from natural gas is complicated by the fact that nonmethane components of natural gas can be toxic to methanotrophs and can also provide substrates for growth of contaminating nonmethanotrophic bacterial species (5). *Methylobacterium buryatense* is a gammaproteobacterial (type I) methanotroph that was isolated directly on natural gas from a soda lake in the Transbaikalian region of Russia, a harsh environment with fluctuating temperatures, salinity, and pH levels (7). *M. buryatense* is naturally adapted to this environment and moderately haloalkaliphilic and consequently grows robustly in pure culture on natural gas, with increased resistance to contamination. The genome of this species was also recently sequenced and annotated by the Joint Genome Institute (8), enabling genomic manipulation for metabolic engineering. These characteristics make *M. buryatense* a strain of interest for industrial biotechnology.

Genetic manipulation of methanotrophic bacteria is often a slow process with marker exchange mutagenesis typically taking weeks per mutation, making it difficult to construct multiple mutations in a timely manner (9, 10). Counterselection protocols can accelerate the process of creating multiple mutations in a strain by eliminating the need for antibiotic marker recycling and can enable other unmarked genomic manipulations such as point mutations and promoter swaps. While there have been reports of successful unmarked allelic exchange using sucrose counterselection in *Methylobacterium* sp. strain 16a (6) and *Methylobacterium capsu-*

latus (Bath) (11), these methods are not widely used and have not been validated in most methanotroph species.

Replicable plasmids are another valuable genetic tool that can be used to express multiple copies of a gene of interest and to rapidly test different genetic constructs for metabolic engineering. A plasmid-based gene expression system has not yet been validated for *M. buryatense*. Broad-host-range replicons that have been successfully used in other methanotrophs include the RP4/RK2 replicon that defines incompatibility group P (IncP) (12) as well as the RSF1010 (IncQ) (13) and pBBR1 (11) replicons.

We report a set of genetic tools and strain modifications for genetic manipulation of *M. buryatense* strain 5GB1, including a sucrose counterselection system and small replicable vector. We validated these tools by knocking out glycogen production and analyzing relative promoter strengths in this methanotroph. This work provides a platform for metabolic engineering of *M. buryatense* for future biotechnological applications.

MATERIALS AND METHODS

Plasmid construction. All plasmids were constructed via Gibson assembly (14) (Table 1). Select plasmids and their sequences have been deposited with the nonprofit plasmid repository Addgene (<http://www.addgene.org/>). The

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i> TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>E. coli</i> S17-1 λpir	Donor strain. Tp ^r Sm ^r <i>recA</i> <i>thi</i> <i>pro</i> <i>hsd</i> (r ⁻ m ⁺)RP4-2-Tc::Mu::Km Tn7 λpir	28
<i>M. buryatense</i> 5GB1	Variant of <i>M. buryatense</i> 5G. Genotype differences are listed in Table S1 in the supplemental material.	This study
<i>M. buryatense</i> 5GB1S	Selected variant of <i>M. buryatense</i> 5GB1 capable of being conjugated with small IncP-based plasmids. Missing native plasmid.	This study
<i>M. buryatense</i> 5GB1C	Variant of <i>M. buryatense</i> 5GB1 intentionally cured of native plasmid by knocking out <i>repA</i> (METBUDRAFT_0076/MBURv2_10076) to <i>repB</i> (METBUDRAFT_0086/MBURv2_10086) native plasmid locus. Capable of being conjugated with small IncP-based plasmids.	This study
<i>M. buryatense</i> Δ <i>glgA1</i>	<i>M. buryatense</i> 5GB1 Δ <i>glgA1</i> (METBUDRAFT_3833/MBURv2_210177)	This study
<i>M. buryatense</i> Δ <i>glgA2</i>	<i>M. buryatense</i> 5GB1 Δ <i>glgA2</i> (METBUDRAFT_3841/MBURv2_210185)	This study
<i>M. buryatense</i> Δ <i>glgA1</i> Δ <i>glgA2</i>	<i>M. buryatense</i> 5GB1 Δ <i>glgA1</i> Δ <i>glgA2</i>	This study
Plasmids		
pCM66	IncP-based broad-host-range plasmid	22
pVK100	IncP-based cosmid	23
pCM433kanT	Minimized, Kan ^r version of pCM433 (19) with <i>bla</i> , <i>tet</i> , and <i>cat</i> antibiotic resistance genes removed.	This study
pAWP45	Kan ^r variant of pCM433 containing flanks to knock out <i>glgA1</i>	This study
pAWP47	Kan ^r variant of pCM433 containing flanks to knock out <i>glgA2</i>	This study
pAWP93	pCM433kanT containing flanks to knock out <i>repA-repB</i> locus of native plasmid	This study
pAWP57	pVK100 derivative used for selection of 5GB1S. Contains RP4/RK2 <i>oriT</i> and <i>oriV</i> as well as the <i>trfA</i> , <i>kor</i> , <i>kfr</i> , and <i>krf</i> operons, but not the <i>trb</i> and <i>tra</i> operons.	This study
pAWP78	Minimized version of pCM66 with <i>bla</i> and <i>tet</i> antibiotic resistance gene fragments removed	This study
pAWP86	pAWP78 containing <i>dTomato</i> with no promoter	This study
pAWP87	pAWP78 containing <i>dTomato</i> driven by <i>M. buryatense</i> <i>mxoF</i> promoter	This study
pAWP88	pAWP78 containing <i>dTomato</i> driven by <i>lac</i> promoter	This study
pAWP89	pAWP78 containing <i>dTomato</i> driven by <i>tac</i> promoter	This study
pAWP92	pAWP78 containing <i>dTomato</i> driven by <i>M. buryatense</i> <i>rpoD</i> promoter	This study

dTomato gene was amplified by PCR from Addgene plasmid 48688. A list of primers used for plasmid backbone amplification and construction of knockout vectors can be found in Table S1 in the supplemental material. A list of promoter sequences for *dTomato* promoter probe vectors can be found in Table S2 in the supplemental material.

***Methylobacterium buryatense* culture and genetic manipulation.**

For these studies, we used a variant of *M. buryatense* 5G that arose during laboratory culture, which we have named strain 5GB1 due to several differences identified by genome sequencing compared to the published draft genome of strain 5G (see Table S3 in the supplemental material) (7, 8).

M. buryatense strains were cultured in an atmosphere of 25% methane in air at 30°C. Plates were incubated in sealed jars (Oxoid Limited, Hampshire, United Kingdom), while liquid cultures were grown in 250-ml glass serum bottles (Kimble Chase, Vineland, NJ, USA) or 18- by 150-mm tubes (Bellco Glass, Vineland, NJ, USA) sealed with rubber stoppers and aluminum seals (Wheaton, Millville, NJ, USA). Cultures were grown in modified nitrate mineral salts medium (NMS2), which contains 0.2 g/liter MgSO₄ · 7H₂O, 0.02 g/liter CaCl₂ · 6H₂O, 1 g/liter KNO₃, and 7.5 g/liter NaCl, as well as 1 × trace elements (500 × trace elements contains 1.0 g/liter Na₂-EDTA, 2.0 g/liter FeSO₄ · 7H₂O, 0.8 g/liter ZnSO₄ · 7H₂O, 0.03 g/liter MnCl₂ · 4H₂O, 0.03 g/liter H₃BO₃, 0.2 g/liter CoCl₂ · 6H₂O, 0.6 g/liter CuCl₂ · 2H₂O, 0.02 g/liter NiCl₂ · 6H₂O, and 0.05 g/liter Na₂MoO₄ · 2H₂O). Final concentrations of 50 mM sodium carbonate buffer, pH 9.5, and 2.3 mM phosphate buffer, pH 6.8, were added immediately before use.

All genetic manipulation was performed via conjugation on NMS2 mating plates, which contain less NaCl than the standard medium (2 g/liter) and are supplemented with 15% (vol/vol) nutrient broth. The final concentrations of sodium carbonate buffer and phosphate buffer were

also adjusted to 5 mM and 5.8 mM, respectively. Attempts to introduce vectors via electroporation were unsuccessful. For conjugation, *M. buryatense* strains were spread onto an NMS2 mating plate and grown overnight. An equal volume of *Escherichia coli* S17-1 λpir donor biomass containing the vector of interest was then added to the plate, and the resulting mixture was incubated at 30°C for 2 days. This biomass was then spread onto NMS2 plates containing kanamycin (50 μg/ml) to select for transconjugants, which were subsequently purified from the donor via passaging on NMS2 plates containing rifamycin (50 μg/ml). For sucrose counterselection, double crossovers were selected for by plating kanamycin-resistant transconjugants (single crossovers) on NMS2 containing 2.5% (mass/vol) sucrose and rifamycin. Colonies were then checked for kanamycin sensitivity and genotyped via PCR, using either colony biomass as the PCR template or genomic DNA extracted with DNeasy blood and tissue kit number 69504 (Qiagen, Hilden, Germany).

Genome resequencing. Genomic DNA was prepared via phenol-chloroform extraction. The genome sequencing was performed on an Illumina MiSeq with Illumina's 300-bp paired-end (PE) protocol (2 × 300 bp) by Genewiz (South Plainfield, NJ, USA) with multiplexing. The paired-end reads for each condition were pooled and processed with breseq version 0.24rc6 (15) against the MaGE (16) annotation of *M. buryatense* 5G from 26 November 2013 known as MBURv2. In addition, to identify gene duplications and deletions in an assembly-free approach, we mapped the reads to the 5G draft genome from MaGE using BWA version 0.7.8-r455 (17) using the BWA-MEM algorithm with eight threads and default options, followed by postprocessing of the alignments with SAMtools version 0.1.19-44428cd (18). The reads were attributed to genes with our in-house tool BAM2ORF to compute the reads per kilobase of gene per millions of reads in the sample (RPKM). The RPKM ratios for different conditions were computed and examined manually.

Glycogen quantification. Fifty milliliters of stationary-phase cultures was pelleted for 15 min at $5,000 \times g$. Bacterial pellets were lyophilized overnight, and dry cell weight was recorded for normalization. Cell pellets were then dissolved in 30% KOH and heated for 30 min at 95°C . The mixtures were then cooled on ice, and glycogen was precipitated with 2 volumes ethanol at -20°C overnight. Samples were spun at $21,000 \times g$ for 10 min at room temperature, and the resulting pellets were resuspended in Milli-Q H_2O . Samples were then acidified to pH 3 with 5 N HCl, reprecipitated with 1 volume ethanol at room temperature, and repelleted as before. The pellets were then washed with ethanol and resuspended in Abcam (Cambridge, MA, USA) hydrolysis buffer for glycogen quantification using the Abcam glycogen assay kit (ab65620).

Tomato reporter data. Fifty milliliters of exponential-phase cultures was pelleted for 15 min at $5,000 \times g$ and resuspended in 3 ml 50 mM Tris, pH 7.5. Cell extracts were then created by lysing the samples by French press and clearing them at $15,000 \times g$ for 30 min. Total protein concentrations were measured by bicinchoninic acid (BCA; Pierce, Rockford, IL, USA) for normalization. One hundred microliters of cell extracts was assayed in a 96-well plate (Nunc black optical bottom) after incubation for 4 h at 37°C by measuring fluorescence by plate reader (Tecan Infinite F500) with excitation/emission of 535/590 nm.

Nucleotide sequence accession numbers. The raw sequencing reads from *M. buryatense* 5GB1 and 5GB1S have been deposited with the NCBI as PRJNA265098 and PRJNA265105, respectively.

RESULTS AND DISCUSSION

Development and validation of a counterselection vector for unmarked allelic exchange.

In order to rapidly make multiple deletions in *M. buryatense* 5GB1, we sought to validate a counterselection system for unmarked allelic exchange. We started with the vector pCM433 (19), which contains the *sacB* gene to confer sucrose sensitivity and has been used for allelic exchange in non-methanotrophic methylotrophs. pCM433 contains antibiotic resistance markers for tetracycline, chloramphenicol, and ampicillin; however, tetracycline is less stable at elevated pH, *M. buryatense* is resistant to chloramphenicol, and ampicillin resulted in predominantly false positives following conjugation. We therefore replaced the tetracycline and chloramphenicol resistance markers with one for kanamycin, which can be used for selection of transconjugants in *M. buryatense* 5GB1.

As a proof of concept, we used this vector to knock out glycogen production in *M. buryatense* 5GB1. *Methylobacterium* species have been reported to accumulate very large quantities of intracellular glycogen (upwards of 30% cell dry weight) when grown on methanol or under nitrogen limitation (20, 21). This may significantly impact the carbon conversion efficiency of methane biocatalysis in an industrial process.

We assembled constructs and subsequently knocked out the genes encoding two glycogen synthases in *M. buryatense* 5GB1, *glgA1* and *glgA2*, both individually and in combination. We found that 2.5% sucrose effectively selected for double crossovers during counterselection, while 5% sucrose resulted almost exclusively in false positives (sucrose resistant, kanamycin resistant) (data not shown). Neither the single *glgA* mutants nor the double mutant had a detectable growth defect (Fig. 1A). The single mutants showed intracellular glycogen content similar to that of the wild type in stationary-phase cultures, suggesting that these glycogen synthases may have redundant roles. No glycogen was detectable in the double mutant, confirming the elimination of glycogen synthesis in this strain (Fig. 1B). While *M. buryatense* 5GB1 produces low quantities of glycogen under our batch growth conditions, it is

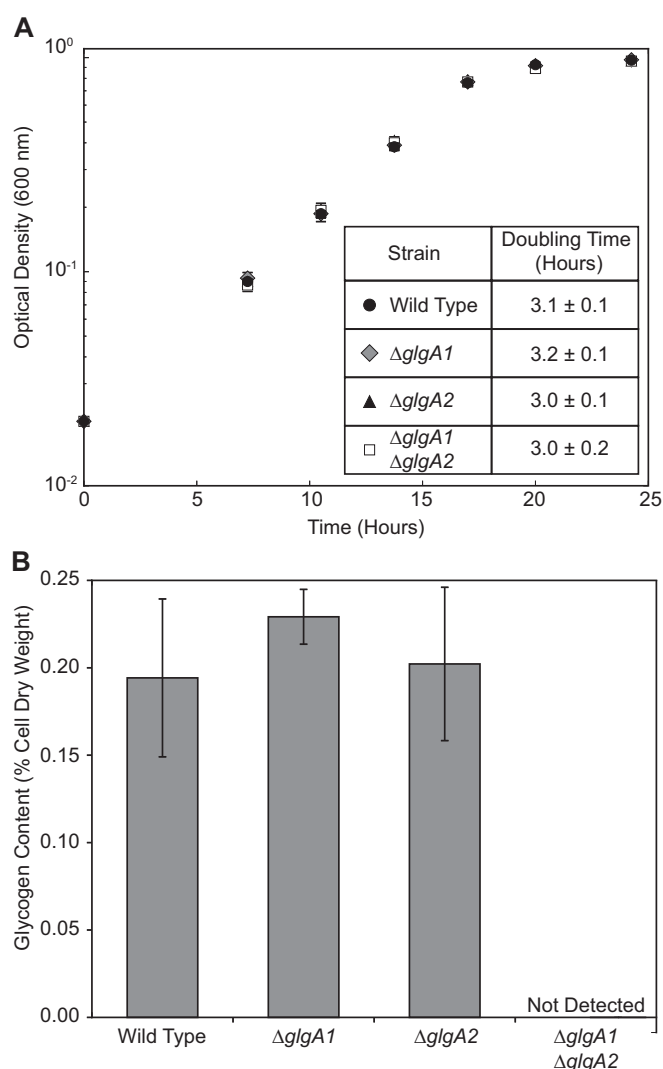


FIG 1 Analysis of *M. buryatense* 5GB1 glycogen synthase (*glgA1*, *glgA2*) mutants. Growth curves and doubling times (A) and intracellular glycogen contents (B) for wild-type and glycogen synthase mutants. Data represent the means from 3 replicates \pm standard deviations.

possible that larger quantities may be produced in an industrial process wherein fluctuations in nutrient availability may occur.

After validating sucrose counterselection in *M. buryatense* using a kanamycin-resistant derivative of pCM433, we further minimized this vector for future use by removing the ampicillin resistance marker. A smaller vector helps facilitate the assembly of complete allelic exchange constructs in one step from PCR-amplified backbone and target flanking regions using Gibson assembly (14). The resulting vector, pCM433kanT (Fig. 2A), has functionality similar to that of the vector successfully used in *Methylobacterium* sp. strain 16a (6). These results demonstrate that sucrose counterselection can be used to make multiple unmarked deletions in *M. buryatense* 5GB1.

Selection of an *M. buryatense* 5GB1 variant that can be conjugated with small IncP-based broad-host-range plasmids. Plasmid-based gene expression systems are useful genetic tools for strain manipulation, as they enable multiple genetic constructs to be rapidly tested. We therefore sought to develop and validate a

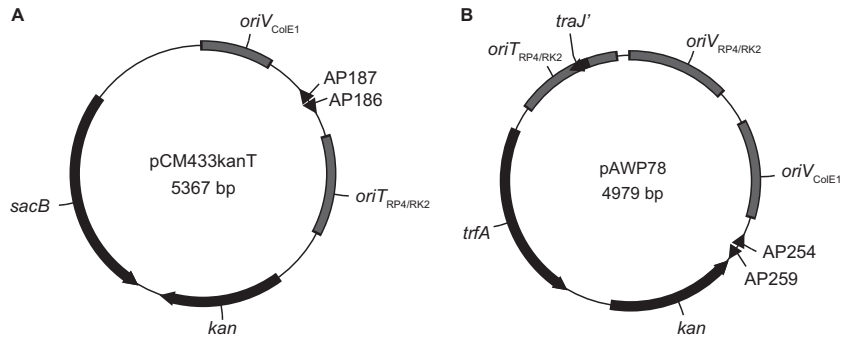


FIG 2 Genetic tools for *M. buryatense*. (A) Sucrose counterselection vector pCM433kanT for unmarked allelic exchange. (B) Minimized IncP-based broad-host-range vector pAWP78 for plasmid-based gene expression. In both cases, the vector backbone was amplified using the annotated primers to add inserts of interest (AP186 and AP187 for pCM433kanT; AP259 and AP254 for pAWP78; see Table S1 in the supplemental material).

plasmid system for *M. buryatense*. The 7.6-kb IncP-based broad-host-range plasmid pCM66 (22) was previously developed for use in nonmethanotrophic methylotrophs, but we were unable to introduce pCM66 into *M. buryatense* 5GB1. We then tested the much larger IncP-based cosmid pVK100 (23 kb) (23), which successfully resulted in kanamycin-resistant transconjugants, demonstrating that the IncP replicon is functional in this strain.

We took advantage of the fact that *M. buryatense* 5GB1 can be conjugated with pVK100 to select for a more genetically tractable variant strain (Fig. 3A). To do this, we conjugated a smaller derivative of pVK100 (called pAWP57) into *M. buryatense* 5GB1 and then isolated a strain cured of this plasmid by passaging multiple times on medium without kanamycin. We then repeated this mating and curing selection one more time. The resulting strain, 5GB1S, can be conjugated with the smaller IncP plasmid pCM66 with a frequency of roughly 10^{-9} per donor (Fig. 3B).

One potential explanation for this low conjugation frequency is that the plasmid is integrating into the *M. buryatense* chromosome. However, we can reliably reisolate the plasmid and there are no stretches of sequence homology between pCM66 and the published *M. buryatense* 5G genome (8), suggesting that integration is unlikely. Nevertheless, this draft genome sequence remains gapped, so we cannot rule out homology definitively. The genome sequence also contains multiple annotated restriction/modification (R/M) systems (24) that may explain the low mating frequencies. The fact that small plasmids can now be introduced into *M. buryatense* 5GB1S provides a good readout for further improvement of the genetic tractability of this strain, such as by deletion of these R/M systems.

In order to determine why *M. buryatense* 5GB1S can be conjugated with pCM66 but the 5GB1 strain cannot, we sequenced the 5GB1S genome. Notably, we discovered that 5GB1S no longer

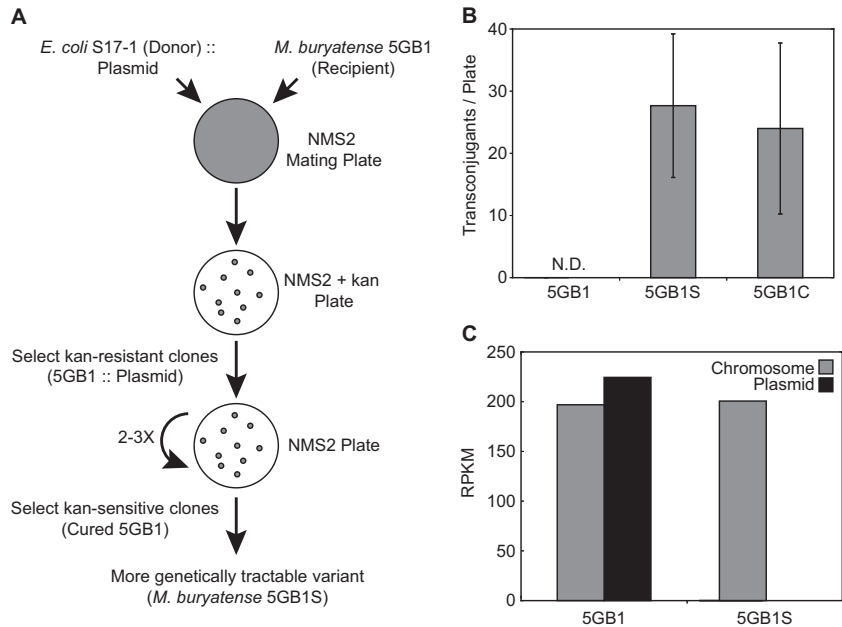


FIG 3 Loss of *M. buryatense* 5GB1 native plasmid results in more genetically tractable variants capable of being conjugated with small IncP-based plasmids. (A) Selection scheme used to obtain the more genetically tractable variant strain 5GB1S. This selection was repeated twice. (B) Number of transconjugants containing the small IncP-based plasmid pCM66 for *M. buryatense* 5GB1, selected strain 5GB1S, and a strain intentionally cured of the native plasmid, 5GB1C. N.D., not detected. (C) Genome sequence reads mapped to chromosome versus native plasmid for strains 5GB1 and 5GB1S. RPKM, reads per kilobase of gene per millions of reads in the sample.

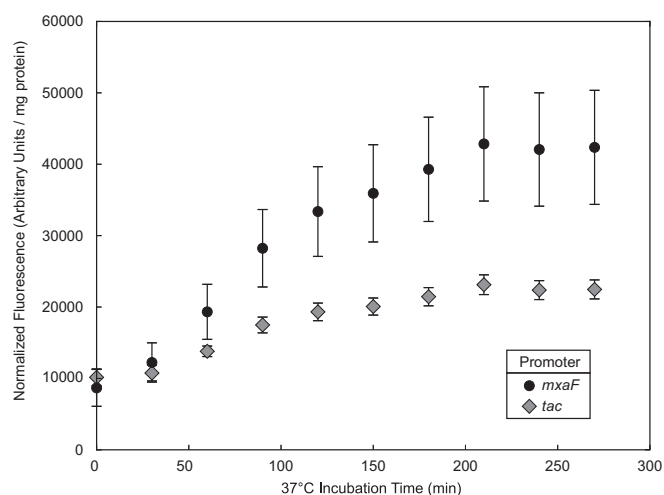


FIG 4 dTomato fluorescence increases over time after cell breakage. Cell extract was incubated at 37°C, and fluorescence (excitation, 535 nm; emission, 590 nm) was measured with a fixed gain at the time points indicated. Data represent the means from 3 replicates \pm standard deviations.

contains the native 80-kb plasmid found in *M. buryatense* 5GB1 (Fig. 3C). Despite this plasmid loss, there is no difference in growth rate under normal laboratory conditions between the 5GB1 and 5GB1S strains (see Fig. S1 in the supplemental material).

To determine if loss of this endogenous plasmid is necessary and sufficient to confer the pCM66 conjugation phenotype, we removed the plasmid from *M. buryatense* 5GB1 by knocking out a locus in the native plasmid containing annotated *repA* and *repB* genes with pCM433kanT using sucrose counterselection. The resulting strain, 5GB1C, no longer contains the endogenous plasmid as assessed by PCR at three separate loci (see Fig. S2 in the supplemental material). Strain 5GB1C can be conjugated with pCM66 at the same frequency and has no change in growth rate compared to strains 5GB1 and 5GB1S (Fig. 3B; see also Fig. S1 in the supplemental material).

These results demonstrate that strains 5GB1S and 5GB1C are able to replicate small IncP-based vectors due to curing of the native 80-kb plasmid found in *M. buryatense* 5GB1 and suggest that there is competition between small IncP-based vectors and the native *M. buryatense* plasmid. The native plasmid does not contain the canonical RP4/RK2 IncP replicon but may share other stability and maintenance functions that result in incompatibility between these plasmids. For example, the native plasmid contains *korB*, which suppresses expression of *trfA*, a gene necessary for replication of IncP vectors (25). The lack of growth phenotype also suggests that the native plasmid does not contain genes essential for growth under our conditions. Loss of this plasmid in *M. buryatense* strains 5GB1S and 5GB1C leads to a more genetically tractable strain capable of being conjugated with smaller vectors that are amenable to modern cloning techniques.

A trimmed IncP-based vector serves as a backbone for promoter probing. After confirming that small IncP-based vectors can be successfully used for gene expression in *M. buryatense* 5GB1S, we sought to improve the vector backbone, pCM66. Smaller vectors are advantageous because they do not contain unnecessary genetic elements that may have unforeseen effects

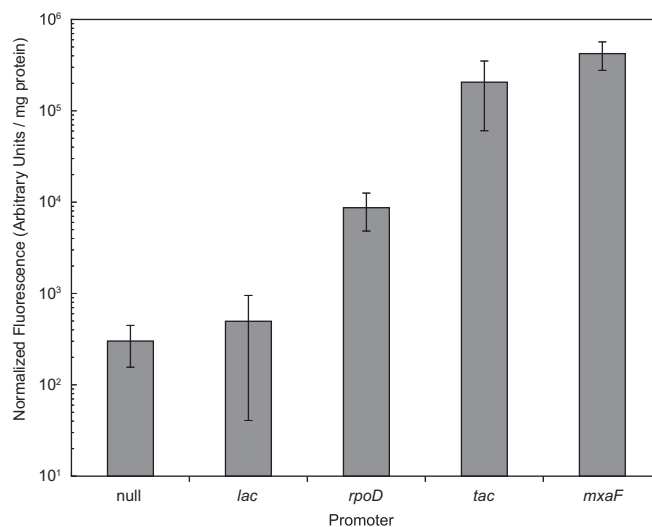


FIG 5 Relative promoter strengths in *M. buryatense*. Fluorescence (excitation, 535 nm; emission, 590 nm) was measured in cell extracts of *M. buryatense* 5GB1S containing probe vectors with promoter-*dTomato* fusions and normalized to protein content. Data represent the mean results for 3 distinct transconjugants \pm standard deviations.

and they enable rapid plasmid construction from PCR products. pCM66 confers only kanamycin resistance but contains fragments of the *bla* and *tetA* genes as well as a complete *tetR* gene left over from restriction-based cloning methods (see Fig. S3 in the supplemental material). We removed these elements using Gibson assembly, resulting in the 5-kb plasmid pAWP78 (Fig. 2B).

Knowledge of relative promoter strengths is important for expressing genes in a given organism. To probe promoter strengths in *M. buryatense* 5GB1S, we constructed a series of vectors using the red fluorescent protein dTomato (26) as a reporter in the pAWP78 backbone. Fluorescent protein expression in methanotrophs is generally assayed in cell extracts (12). We found that cell extracts are also necessary to obtain maximal fluorescence from dTomato expression in *M. buryatense* 5GB1S, as can be seen by the large increase in fluorescence observed over a 200-min incubation at 37°C following cell disruption (Fig. 4). The ratio of fluorescent signals from different promoter probes also changes during incubation, making it crucial to allow the fluorescent signal to mature before comparing different promoter strengths. We were also able to use catechol 2,3-dioxygenase (encoded by *xylE*) successfully as a reporter in cell extracts but not beta-galactosidase (encoded by *lacZ*) (data not shown).

In order to assay a wide range of gene expression levels in *M. buryatense*, we tested the commonly used *lac* and *tac* promoters, as well as endogenous promoters that drive expression of the *M. buryatense* methanol dehydrogenase gene *mx*aF and sigma factor gene *rpoD* (Fig. 5). To assess the heterogeneity of plasmid-based gene expression, we selected three different transconjugants after mating each promoter probe into *M. buryatense* 5GB1S. While we observed substantial heterogeneity between transconjugants harboring the same promoter probe, overall the four selected promoters provided distinct levels of expression that spanned 3 orders of magnitude.

We also tested the relative strengths of these promoters in *E. coli* whole cells (see Fig. S4 in the supplemental material). We did

not detect any activity for the endogenous *mxoF* promoter in *E. coli*, although this was the strongest of the promoters tested in *M. buryatense*. This promoter therefore provides a potentially useful tool for cloning genes that are toxic to *E. coli* for expression in *M. buryatense*, such as the genes that encode the particulate methane monooxygenase (27), the primary methane oxidation system in most aerobic methanotrophs.

These plasmids were conjugated into *M. buryatense* 5GB1S via the commonly used *E. coli* donor strain S17-1 (28). S17-1 contains a chromosomally integrated copy of the IncP plasmid RP4 and has been reported to modify transferred plasmids by inserting DNA from the S17-1 chromosome (29). To determine if our vectors are modified after conjugation into *M. buryatense*, we reisolated plasmids from the 15 transconjugants used to compare promoter strengths. We detected insertions of RP4 DNA downstream of the promoter probe plasmid origin of transfer in 10 cases (data not shown). These modifications presumably arose via recombination between the promoter probe and RP4 DNA cotransferred from the S17-1 genome. However, the presence or absence of these insertions did not correlate with differences in expression between transconjugants.

Together, our results show that genes can be reproducibly expressed at distinct levels using the plasmid system and different promoters (Fig. 5). Plasmids also enable genes to be transiently expressed in *M. buryatense*, as a plasmid can later be removed by curing. Transient gene expression is useful for developing additional genetic tools for metabolic engineering in this methanotroph, including expressing the Cre recombinase for unmarking of transposon mutants via the *cre-lox* system (30) or the Cas9 endonuclease for CRISPR/Cas genome editing (31).

The tools described here enable genetic modification of *M. buryatense*, an aerobic methanotroph with robust growth characteristics in pure culture. *M. buryatense* has the potential to serve as a promising system for studying aerobic methanotroph physiology and for industrial biocatalysis of methane via metabolic engineering. These streamlined plasmids may also serve as useful tools for genetic manipulation of additional methanotroph species as well as other Gram-negative bacteria.

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