



# A methanotrophic bacterium to enable methane removal for climate mitigation

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The rapid increase of the potent greenhouse gas methane in the atmosphere creates great urgency to develop and deploy technologies for methane mitigation. One approach to removing methane is to use bacteria for which methane is their carbon and energy source (methanotrophs). Such bacteria naturally convert methane to CO<sub>2</sub> and biomass, a value-added product and a cobenefit of methane removal. Typically, methanotrophs grow best at around 5,000 to 10,000 ppm methane, but methane in the atmosphere is 1.9 ppm. Air above emission sites such as landfills, anaerobic digester effluents, rice paddy effluents, and oil and gas wells contains elevated methane in the 500 ppm range. If such sites are targeted for methane removal, technology harnessing aerobic methanotroph metabolism has the potential to become economically and environmentally viable. The first step in developing such methane removal technology is to identify methanotrophs with enhanced ability to grow and consume methane at 500 ppm and lower. We report here that some existing methanotrophic strains grow well at 500 ppm methane, and one of them, *Methylobacterium burkatense* 5GB1C, consumes such low methane at enhanced rates compared to previously published values. Analyses of bioreactor-based performance and RNAseq-based transcriptomics suggest that this ability to utilize low methane is based at least in part on extremely low non-growth-associated maintenance energy and on high methane specific affinity. This bacterium is a candidate to develop technology for methane removal at emission sites. If appropriately scaled, such technology has the potential to slow global warming by 2050.

methanotroph | specific affinity | whole-cell K<sub>M</sub> | transcriptomics | climate change

Methane is a potent greenhouse gas and has a global warming potential over 85 times greater than carbon dioxide (CO<sub>2</sub>) on a 20-y timescale (1, 2). Atmospheric methane has been rising quickly in the past 15 y (3) and currently accounts for about 30% of total global warming (4), prompting interest in methane removal technologies (5–8). Methane has an atmospheric lifetime of ~12 y (4), which presents an opportunity for slowing the progress of global warming in the near term if methane removal technologies are promptly deployed (5, 7). Recent projections predict that global warming can be reduced 0.21 to 0.22 °C by removing 0.3 to 1 petagrams methane by 2050 (1, 5). Temperature decreases of this magnitude are predicted to be significant (1, 5), especially when combined with other mitigation strategies (9).

Most proposed methane mitigation solutions are focused on decreasing emissions (5), and these are important goals. However, not all methane emissions are amenable to reduction, and it has been argued that emission reduction strategies must be augmented by methane removal to slow global warming by 2050 (6–8). In addition, any emission reduction strategies that enhance aerobic methanotrophic activity in natural communities may also result in increased nitrous oxide (N<sub>2</sub>O) emission, as demonstrated for rice paddy communities in which N<sub>2</sub>O reduction by denitrifiers is inhibited by stimulation of aerobic methanotrophs due to competition for copper (10). Since N<sub>2</sub>O is ten times more potent as a greenhouse gas than methane (2), any technology for methane mitigation must ensure that it also results in negligible increase in N<sub>2</sub>O emissions.

One potential methane mitigation solution is to employ aerobic methanotrophic bacteria, which are capable of assimilating methane naturally at ambient temperature without producing N<sub>2</sub>O. Two major challenges exist for such methane removal, low concentration and enormous scale. Atmospheric methane is currently at 1.9 ppm (3), a concentration that makes it difficult for aerobic methanotrophs to survive. Although a methanotroph has been described that grows at atmospheric methane, it apparently cometabolizes CO and H<sub>2</sub> to survive (11). The very low rates of methane consumption at 1.9 ppm create significant challenges for reaching the 0.3 to 1 petagram scale noted above. An alternative is to focus on major emission sites where the overlying air is enriched in methane in the 500 ppm range, including landfills, anaerobic digester effluents, rice paddy effluents, and oil and gas production sites (12–15). At these sites, methanotrophic methane removal

## Significance

Atmospheric methane abundance has risen to a historically high value at 1.92 ppm in 2023 and continues to increase rapidly. A key natural sink for methane is attributed to aerobic methanotrophs that can actively oxidize methane and assimilate the carbon into biomass, and thus, they are candidates for methane removal technology. We demonstrate here that an extant gammaproteobacterial methanotroph, *Methylobacterium burkatense* 5GB1C, can grow at low methane concentrations in the range from 200 ppm to 1,000 ppm and exhibits greater methane consumption rates at both low and high methane compared to other methanotroph strains. These features make this strain a promising candidate for methane removal technology at emission sites with enriched methane in air.

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The authors declare no competing interest.

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technology is more likely to be economically feasible at the necessary scale, as demonstrated by studies predicting that sustainable methane removal by methanotrophs is feasible if air contains 500 ppm or more methane (16, 17). A key question then arises regarding which methanotrophs are suitable for such a task, as only a few studies have addressed growth of pure methanotroph cultures fed with methane in the range of 500 ppm (11, 18, 19). We hypothesized that methanotroph strains exist with enhanced abilities to grow on and oxidize methane at 500 ppm.

## Results and Discussion

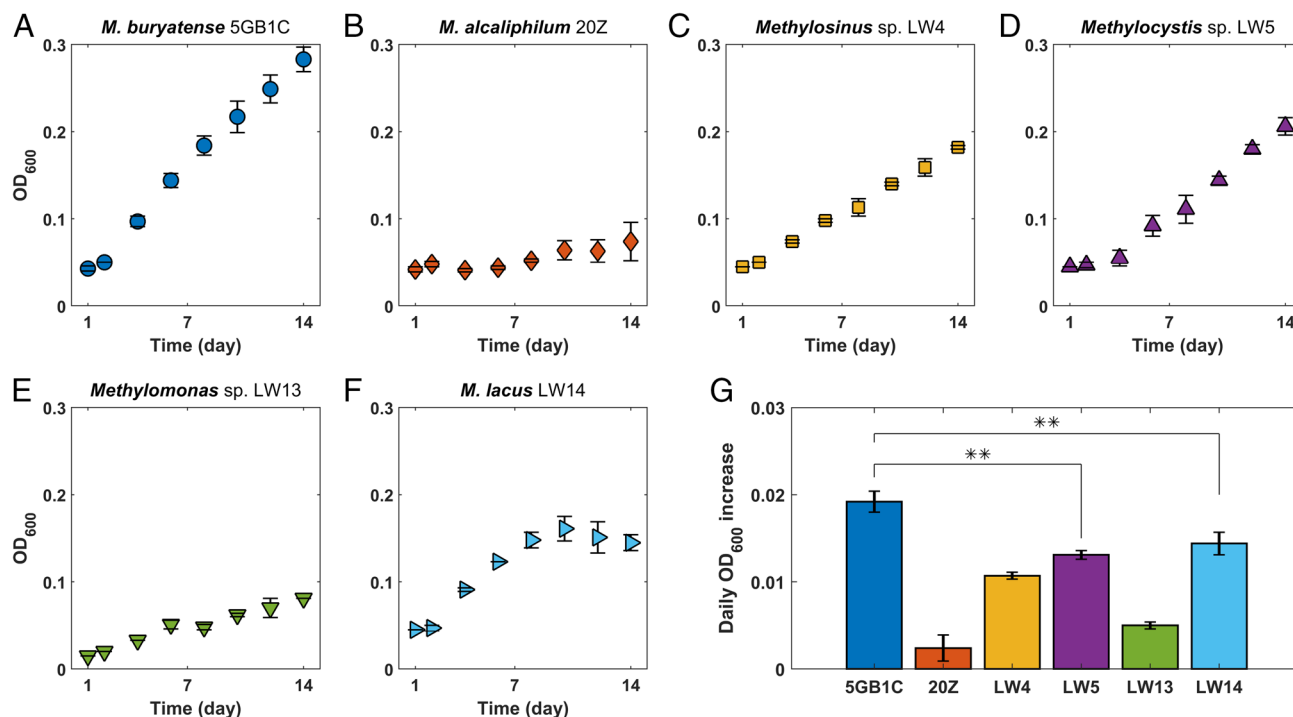
**Screening Aerobic Methanotrophs Capable of Growing at 500 ppm Methane.** We first carried out a screen for methanotrophs showing strong growth at 500 ppm methane, by testing growth in stoppered serum bottles. Six phylogenetically diverse representatives from both alphaproteobacterial and gammaproteobacterial groups were tested from our culture collection, and of these, *M. buryatense* 5GB1C, *Methylobacterium* (previously *Methylosarcina*) *lacus* LW14, *Methylosinus* sp. LW4, and *Methylocystis* sp. LW5 were able to grow at 500 ppm methane. However, *M. lacus* LW14 did not show sustained growth after 10 d at 500 ppm methane. Little or no growth was observed for *Methylobacterium* LW13 or *Methylovivimicrobium alcaliphilum* 20Z incubated for 14 d (Fig. 1). Additionally, *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b, two broadly studied methanotrophs, had previously been shown to not grow or grow poorly at 1,000 ppm or lower concentrations (18), and thus, their growth analyses were not repeated here. In this screen, the four methanotrophs capable of growing at 500 ppm methane show generally linear growth curves (Fig. 1 A, C, D, and F), likely because the headspace in each bottle was refreshed with 500 ppm methane once a day and the daily biomass growth was capped by this restricted supply of methane. Of these four methanotrophs, *M. buryatense* 5GB1C

exhibited the fastest growth and highest optical density values at the end of cultivation at 500 ppm; hence, we focused on this strain in the following analyses. It is worth noting that *M. buryatense* 5GB1C is also a fast-growing methanotroph at high methane concentrations (25% or 250,000 ppm) with a maximum growth rate of 0.22 h<sup>-1</sup> (20).

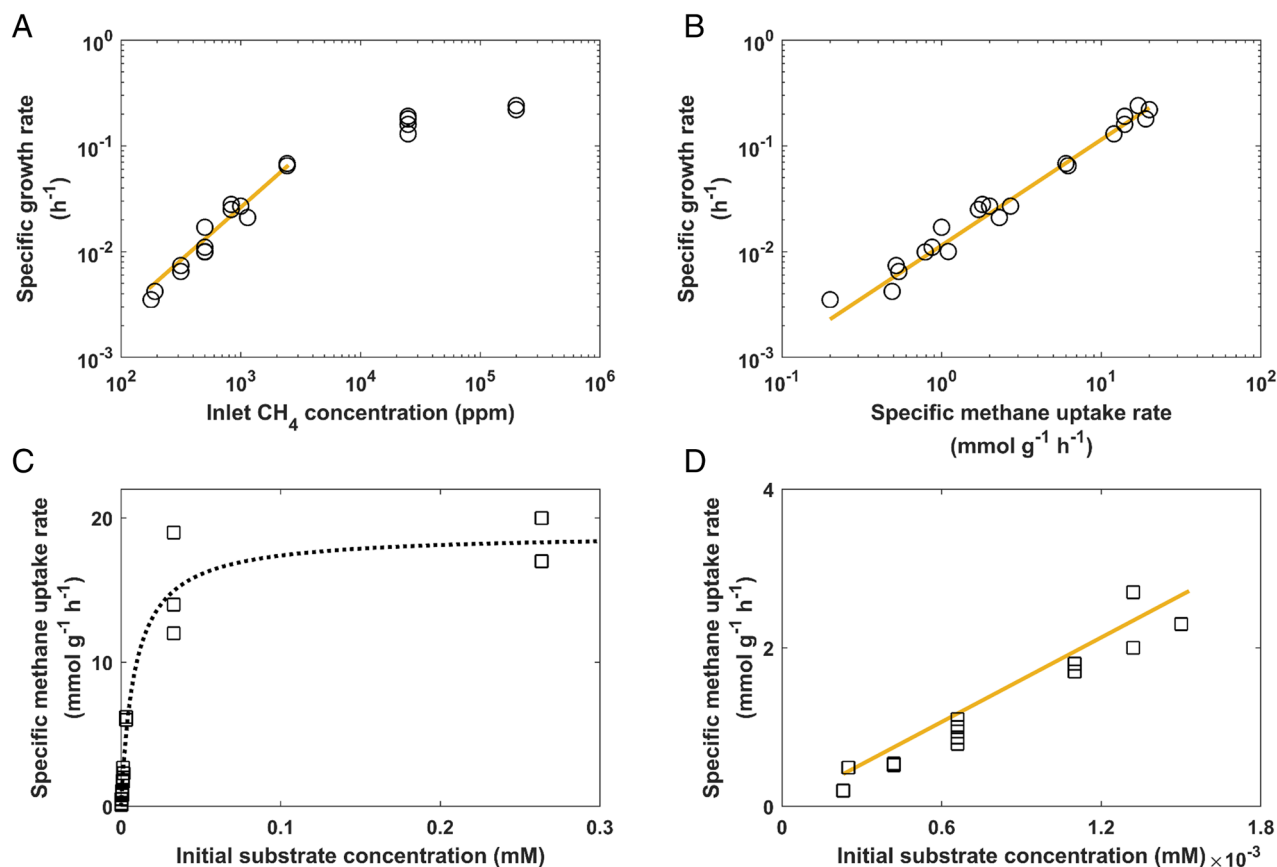
It is reported that lanthanum (La<sup>3+</sup>) addition in medium, which changes the methanol oxidation system induced, can improve the growth rate of *M. buryatense* 5GB1C by 10% in the presence of sufficient methane (21), but the impact on growth at 500 ppm methane was unknown. We tested the addition of lanthanum, but it did not stimulate growth at 500 ppm methane under the conditions tested (SI Appendix, Fig. S1).

Although *M. buryatense* 5GB1C is not predicted to generate N<sub>2</sub>O, to confirm the prediction, cultures were tested for N<sub>2</sub>O production after growth at 500 ppm methane for 14 d. N<sub>2</sub>O concentration in the headspace was measured at 0.22 ± 0.10 ppm, comparable to atmospheric N<sub>2</sub>O (0.33 ppm), confirming no N<sub>2</sub>O production under these conditions.

**Characterization of *M. buryatense* 5GB1C Growth and Kinetic Parameters at Low Methane Concentrations.** To more thoroughly evaluate growth performance of *M. buryatense* 5GB1C in response to low methane, we utilized a bioreactor-based system with continuous flow of methane:air mixtures, coupled with gas chromatography (GC) measurement of off-gases. *M. buryatense* 5GB1C was cultivated at seven low methane concentrations (i.e., on 2,500 ppm, 1,000 ppm, 800 ppm, 500 ppm, 300 ppm, 200 ppm, and 100 ppm levels) that were expected to be significantly below the whole-cell K<sub>M</sub> for methane [K<sub>M(app)</sub>], and at 2.5% (v/v, or 25,000 ppm) as a sufficient methane control (Fig. 2 A and B and SI Appendix, Fig. S2 and Table S1). A linear relationship between the methane uptake rate and the specific growth rate was observed for the entire range studied (Fig. 2B). These results



**Fig. 1.** Growth performance of wild-type methanotrophs at 500 ppm methane. (A–G) Growth curves of *M. buryatense* 5GB1C (A), *M. alcaliphilum* 20Z (B), *Methylosinus* sp. LW4 (C), *Methylocystis* sp. LW5 (D), *Methylobacterium* sp. LW13 (E), and *Methylobacterium lacus* LW14 (F) (n = 3). A, B, E, and F = gammaproteobacteria; C and D = alphaproteobacteria. (G) Daily OD<sub>600</sub> increase of the seven methanotrophs during a 14-d growth period (n = 3), which were determined based on the slopes of the linear region of growth curves. \*\*P < 0.01, which was determined by the unpaired t test. Error bars represent SDs.



**Fig. 2.** Characterization of growth and kinetic parameters of *M. buryatense* 5GB1C. (A) Relationship between specific growth rates and the methane concentrations of inlet gas. In the range between 200 ppm and 2,500 ppm methane, the yellow line represents the fitted linear regression curve ( $R^2 = 0.82$ ,  $P = 1.2 \times 10^{-5}$ ). Growth data at 20% (v/v) or 200,000 ppm CH<sub>4</sub> balanced with 5% O<sub>2</sub> and 75% N<sub>2</sub> were based on a previous report (22). (B) A linear relationship between the specific growth rate and the methane uptake rate. The yellow line represents the fitted linear regression curve ( $R^2 = 0.96$ ,  $P = 3.6 \times 10^{-15}$ ). (C) The Michaelis-Menten plot of whole-cell methane uptake rate [mmol methane (gram cell dry weight)<sup>-1</sup> h<sup>-1</sup>] as a function of initial substrate concentration ( $R^2 = 0.96$ ,  $P = 1.6 \times 10^{-17}$ ). The initial substrate concentration was calculated based on Henry's law (Methods and Materials). (D) Linear regression of the linear region of the Michaelis-Menten curve ( $R^2 = 0.93$ ,  $P = 6.6 \times 10^{-8}$ ). Each symbol represents an independent measurement.

also show that *M. buryatense* 5GB1C is able to grow below 500 ppm methane, with growth observed as low as ~200 ppm methane (Fig. 2 A and B and SI Appendix, Tables S1 and S2). No other gammaproteobacterial methanotrophs have been shown to grow at such low methane concentrations (11, 18, 19). At ~100 ppm methane, we observed initial growth of *M. buryatense* 5GB1C for 2 wk; however, biomass barely increased afterward (SI Appendix, Fig. S2C). The culture was allowed to recover for 17 h at ~600 ppm methane and then switched to ~100 ppm methane, but the same behavior was observed (SI Appendix, Fig. S2C). These results indicate that 100 ppm methane may not support long-term growth of *M. buryatense* 5GB1C. Notably, some alphaproteobacterial methanotrophs have been shown to grow at 100 ppm and lower methane, including *Methylocystis* species (18, 19) and *Methylocapsa gorgona* MG08 (11). However, two *Methylocystis* strains for which data are available grew two to threefold more slowly than *M. buryatense* 5GB1C at 1,000 ppm methane and *M. gorgona* MG08 showed sixfold lower methane oxidation rates at 800 ppm methane (SI Appendix, Tables S1 and S2). Assuming a similar linear relationship for the methanotrophs listed in SI Appendix, Table S2, it may be predicted that they would also grow more slowly and consume methane at lower rates than *M. buryatense* 5GB1C at 200 to 500 ppm methane. Finally, our measurements reveal a strong linear correlation for *M. buryatense* 5GB1C between the specific growth rate and the methane concentration of the inlet gas from

200 ppm to 2,500 ppm, corresponding to specific growth rates from 0.004 to 0.07 h<sup>-1</sup> (Fig. 2A).

In methanotrophs, a part of the energy produced from methane oxidation is allocated for cell maintenance. For *M. buryatense* 5GB1C grown at methane sufficiency (14% methane; 140,000 ppm), the non-growth-associated ATP maintenance energy (NG-ATPM) is 10 to 15 mmol ATP per gram of dry weight per hour (mmol ATP g<sup>-1</sup> h<sup>-1</sup>) (23). However, *M. buryatense* 5GB1C grown with 200 ppm methane exhibits a methane uptake rate of 0.2 to 0.5 mmol methane g<sup>-1</sup> h<sup>-1</sup> (Fig. 2 A and B and SI Appendix, Table S1) and thus can only yield up to 3.0 mmol ATP g<sup>-1</sup> h<sup>-1</sup> assuming six mole ATP generated per mole of methane consumed (24). Since during active growth, the methane consumed must be partitioned into carbon allocated for biomass generation and carbon for ATP generation, the actual NG-ATPM must be significantly lower than 3.0 mmol ATP g<sup>-1</sup> h<sup>-1</sup>. Indeed, fitting our measurements with the Herbert-Pirt model (25) yielded an NG-ATPM of 0.36 mmol ATP g<sup>-1</sup> h<sup>-1</sup> (SI Appendix, Fig. S3A), comparable to the NG-ATPM (0.6 mmol ATP g<sup>-1</sup> h<sup>-1</sup>) required for retentostat-grown *Saccharomyces cerevisiae* at a growth rate of ~0.001 h<sup>-1</sup> (26). However, the NG-ATPM derived from the linear regression has a high *P* value (0.75) and a wide 95% CI from 0 to 2.8 mmol ATP g<sup>-1</sup> h<sup>-1</sup> (SI Appendix, Fig. S3A). We also used a genome-scale reconstruction (GEM) model (27) to predict growth rates at 1,000 ppm methane or lower. Results show that the NG-ATPM must be ~0.4 mmol ATP g<sup>-1</sup> h<sup>-1</sup> or lower to allow



reasonable growth rate predictions at low methane concentrations (*SI Appendix, Fig. S3 B–D*). These findings suggest that *M. buryatense* 5GB1C is able to decrease the NG-ATPM as a function of decreased substrate availability, as reported previously for *S. cerevisiae* (26). It may be predicted that such a capability could contribute to the relatively strong growth of *M. buryatense* 5GB1C at low methane, since it would enhance the energy from methane oxidation available to support biomass production.

It has been suggested that methanotrophs able to grow at 100 ppm methane and below contain a special high-affinity pMMO (18, 19), although this is apparently not the case in *M. gorgona* MG08 (11). To assess whether *M. buryatense* 5GB1C might show methane oxidation kinetics indicative of a high-affinity pMMO, we carried out whole-cell Michaelis–Menten analysis (Fig. 2C), determining that the whole-cell  $K_m$  [ $K_{M(\text{app})}$ ] and the whole-cell  $V_{\text{max}}$  ( $V_{\text{max}(\text{app})}$ ) for methane are  $8.8 \pm 1.7 \mu\text{M}$  (equivalent to  $6,681 \pm 1,291$  ppm methane in the gas phase at equilibrium) and  $18.9 \pm 0.9 \text{ mmol g}^{-1} \text{ h}^{-1}$ , respectively. Although the  $V_{\text{max}(\text{app})}$  is higher than other known methanotrophs, indicating a rapid maximum methane oxidation rate,  $K_{M(\text{app})}$  is also high compared to other methanotrophs (18). This result does not support the idea that *M. buryatense* 5GB1C possesses a pMMO with higher affinity to methane than other known methanotrophs. It has been well established that  $K_{M(\text{app})}$  is in part dependent on the overall expression of pMMO (18). The specific affinity  $as^\circ$  which denotes the slope of the linear part of the Michaelis–Menten curve, has been suggested to be a more suitable parameter for comparing methane oxidation rates among methanotrophs at low concentrations (18, 19). Through a linear regression (Fig. 2D),  $as^\circ$  for *M. buryatense* 5GB1C is determined to be  $1,101 \pm 87 \times 10^{-12} \text{ L cell}^{-1} \text{ h}^{-1}$  (or  $1,776 \pm 140 \text{ L g}^{-1} \text{ h}^{-1}$ ), more than fivefold larger than the highest reported value and 30 to 100-fold higher than most tested methanotrophs (11, 18, 19) (*SI Appendix, Table S2*), in keeping with the ability of this methanotroph to grow at methane significantly below the  $K_{M(\text{app})}$ . The underlying mechanism for high  $as^\circ$  is unknown. pMMO phylogenetic analysis suggests that the high  $as^\circ$  measured for *M. buryatense* 5GB1C is likely not due to a high-affinity pMMO, since the *M. buryatense* 5GB1C pMMO subunit DNA sequences are closely related to other gammaproteobacterial pMMO sequences, including those of methanotrophs unable to grow at 500 ppm methane (28).

At low growth rates, bacteria may display significant morphological changes (25). We thus quantified biomass dry weight per OD<sub>600</sub> unit, cell sizes, and the coverage of intracytoplasmic membranes (ICMs) that house pMMO. No significant changes were found in any of these parameters between low (500 ppm methane or less) and high (2.5% or more) methane growth conditions (*SI Appendix, Fig. S4*), which indicates robustness of cell morphology. Particularly, we found a moderate 20% reduction of ICM coverage in cells at 500 ppm methane but without statistical significance (*SI Appendix, Fig. S4A*).

**Transcriptional Response of *M. buryatense* 5GB1C to Low Methane.** In some bacteria, strong transcriptional responses accompany growth under nutrient limitation and at low growth rates: Bacteria often decrease expression of the translation and transcription apparatus, up-regulate functions involved in motility and chemotaxis, and up-regulate amino acid synthesis pathways (25, 29–31). To understand how *M. buryatense* 5GB1C responds to low methane at the transcriptional level, we quantified holistic gene expression of cultures grown at 500 ppm and 1,000 ppm at methane-limited steady-state in the bioreactor, with growth rates of  $0.009 \text{ h}^{-1}$  and  $0.02 \text{ h}^{-1}$ , respectively. Differentially expressed genes are defined as those exhibiting an absolute log<sub>2</sub>-fold change

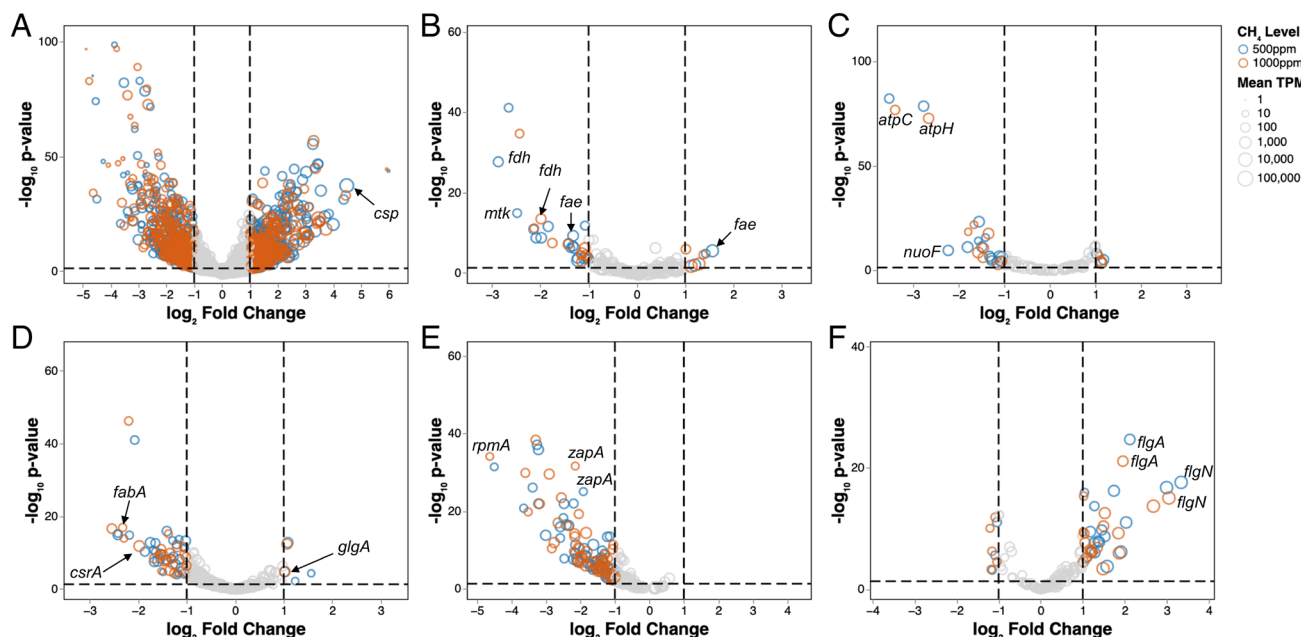
over one and adjusted p value less than 0.05 in comparison to a reference condition, i.e., methane-limited steady-state growth on 2.5% (v/v) methane at a growth rate of  $\sim 0.125 \text{ h}^{-1}$  (22).

Transcription profiles of *M. buryatense* 5GB1C at 500 ppm and 1,000 ppm methane are highly consistent with each other, without any significant variations in gene expression (*SI Appendix, Fig. S5*). When compared to transcriptional profiles under 2.5% methane conditions (32), 725 genes are differentially expressed at both 500 ppm and 1,000 ppm methane (Fig. 3A and *Dataset S1*). Of note, two cold-shock proteins, which are RNA chaperones and in other bacteria are involved in regulation of transcription and translation under stress (33), show strongly changed gene expression but with divergent trends: One (EQU24\_RS15705) is down-regulated by two log<sub>2</sub>-fold, while the other (EQU24\_RS16055) is up-regulated by over three log<sub>2</sub>-fold. Transposases are highly up-regulated in general; however, many but not all of their gene expression levels are low in transcripts per million (TPM < 10) (*Dataset S1*). Cells under stress commonly up-regulate transposase expression (34).

We also analyzed expression of specific genes involved in central metabolism. In the pathway converting methane to CO<sub>2</sub>, genes encoding pMMO (converts methane to methanol) and the MxaF-type methanol dehydrogenase (converts methanol to formaldehyde) are highly expressed but with no significant variations (Fig. 3B). Transcriptional levels of the tetrahydromethanopterin (H<sub>4</sub>MPT) pathway (converts formaldehyde to formate) remain unperturbed except for two genes encoding formaldehyde-activating enzyme (EQU24\_RS13345 and EQU24\_RS14315) displaying significant variations in expression. All six genes encoding two formate dehydrogenases (convert formate to CO<sub>2</sub>) are down-regulated, with greater decreases at 500 ppm methane than at 1,000 ppm methane. These results are accordant with the observation that the excreted formate rate is roughly two times higher at 500 ppm methane ( $0.018 \pm 0.002 \text{ mmol formate g}^{-1} \text{ h}^{-1}$ ) than at 1,000 ppm methane ( $0.009 \pm 0.003 \text{ mmol formate g}^{-1} \text{ h}^{-1}$ ). These values are much lower than corresponding methane uptake rates ( $0.95 \pm 0.08 \text{ mmol CH}_4 \text{ g}^{-1} \text{ h}^{-1}$  at 500 ppm and  $1.5 \pm 0.2 \text{ mmol CH}_4 \text{ g}^{-1} \text{ h}^{-1}$  at 1,000 ppm, *SI Appendix, Table S1*), suggesting that cells growing at low methane tend to reduce carbon loss as formate or CO<sub>2</sub> to allow more carbon assimilation. Gene expression of other central metabolic pathways remains mostly unchanged (Fig. 3B), including glycolysis, the tricarboxylic acid cycle, and the ribulose monophosphate cycle (converts formaldehyde and ribulose 5-phosphate to three-carbon compounds for assimilation). One exception is the incomplete serine cycle (converts formate and CO<sub>2</sub> to acetyl-CoA), where the malate-CoA ligase (EQU24\_RS04635) and the malyl-CoA lyase (EQU24\_RS04630) are down-regulated by two log<sub>2</sub>-fold. Taken together, these results suggest that although the growth rate is decreased by over an order of magnitude, expression of the proteins important for central metabolism pathways is largely unchanged. Such a response is in keeping with a strategy to poise the cells to take advantage of whatever methane is available under these strongly methane-limiting growth conditions.

As for energy metabolism, the NADH-ubiquinone reductase and the F<sub>1</sub>F<sub>0</sub>-type ATP synthase are strongly down-regulated, in keeping with the greatly decreased energy needs at these low growth rates (Fig. 3C).

Gene expression for biosynthesis pathways of fatty acids, amino acids, nucleotides, vitamins, and cofactors remain either stable or down-regulated (Fig. 3D), again, in keeping with the low growth rates and expected decreased fluxes through these pathways. In contrast, genes *glgA* (EQU24\_RS18670) and *glgB* (EQU24\_RS18665) associated with glycogen synthesis are up-regulated by



**Fig. 3.** Transcriptional changes of *M. buryatense* 5GB1C grown at 500 ppm (blue) and 1,000 ppm (orange) methane in comparison to 2.5% (v/v) methane growth conditions. (A–F) Volcano plots of gene expression changes of the entire genome (A), core central carbon metabolism (B), energy metabolism (C), biosynthesis of building blocks and cofactors (D), translation and transcription apparatus (E), and motility and chemotaxis (F). Symbol sizes are correlated with gene expression as shown in the figure. The horizontal dashed line represents  $P = 0.05$ . The two vertical dashed lines represent  $\log_2$ -fold at  $-1$  and  $1$ , respectively. Genes that do not change significantly are colored in gray. Gene abbreviations and gene products: *csp*, cold shock protein; *fae*, formaldehyde activating enzyme; *fdh*, formate dehydrogenase; *mtk*, malate-CoA ligase; *atpC*,  $F_1F_0$  type ATP synthase subunit epsilon; *atpH*,  $F_1F_0$  type ATP synthase subunit delta; *nuoF*, NADH-quinone oxidoreductase subunit NuoF; *fabA*, 3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabA; *csrA*, carbon storage regulator CsrA; *glgA*, glycogen synthase GlgA; *zapA*, cell division protein ZapA; *rpmA*, 50S ribosomal protein L27; *flgA*, flagellar basal body P-ring formation chaperone FlgA; *flgN*, flagellar protein FlgN. An interactive version of this figure is available at <https://erinhwilson.github.io/limited-ch4-tpm-analysis/>.

about one  $\log_2$ -fold, while other related genes including those for glycogen degradation do not show significant changes in expression. This is consistent with downregulation (1.5 to 2.0  $\log_2$ -fold) of the carbon storage regulator *csrA* (EQU24\_RS07950), which has been shown to negatively mediate glycogen synthesis in *Escherichia coli* (35). It is not clear why the cells would increase carbon storage, but it may reflect a strategy to prepare the cells to accommodate future starvation.

We also observed a strong decline in gene expression of ribosomal proteins, tRNA-ligases, RNA polymerases, and sigma factors (Fig. 3E), suggesting a slowdown of transcription and translation processes. Cell division genes, such as *ftsL* (EQU24\_RS19745), *ftsB* (EQU24\_RS13310), and *zapA* (EQU24\_RS04165), are also significantly down-regulated (Fig. 3E). These changes also reflect decreased need at the low growth rates. By contrast, many genes related to flagellar protein synthesis and chemotaxis are up-regulated (Fig. 3F), as bacteria tend to be more active in searching for nutrients and more favorable environments under stress (29).

All in all, the transcriptional response is in keeping with the low NG-ATPM values, in which the cells down-regulate functions that are not needed at low growth rates, while maintaining or up-regulating those functions that will poise the cells to take advantage of better growth conditions, or alternatively, the onset of complete carbon starvation.

**Global Removal Projections.** In order to assess whether this improved performance of methane removal at 500 ppm could theoretically be feasible for a future methane removal technology, we have carried out projections based on our results compared to literature results. Many examples exist of methanotroph-based biofilter technology for removing methane from waste streams, but the majority of these are carried out at 10,000 ppm (1%) methane

or higher and involve “wild” mixed methanotroph communities (consortia), enriched with high (greater than 1%) methane (36). In the few cases in which methane inlet concentrations below 1% have been reported, elimination capacities (ECs) at 500 ppm methane are estimated to be in the range of 0.5 to 3.2 g  $\text{CH}_4$  removed  $\text{m}^{-3} \text{h}^{-1}$  (37–39) (SI Appendix, Table S3). With the 121  $\text{m}^3$  treatment unit size used in a previous modeling study (16) and assuming 7,200 h (300 d) (16) operation per year, such ECs are projected to result in removal of 0.4 to 2.8 tons methane per year per treatment unit at 500 ppm methane. At the higher end, these ECs are similar to the 5 tons methane per year predicted for pure cultures of methanotrophs (16), strains that are known to not grow significantly at 500 ppm methane (18). These results suggest that the methanotroph strains enriched in published biofilter experiments may not be well-suited for removing such low methane. We have shown the *M. buryatense* 5GB1C specific affinity is more than fivefold higher than the highest reported values and 30 to 100-fold higher than most methanotrophs enriched at high methane. Thus, in theory, the EC for 500 ppm methane with *M. buryatense* 5GB1C should be at least fivefold greater than those in the literature, increasing to 2 to 14 ton per year per treatment unit, and could be significantly higher. The actual EC would depend on how well this strain performs under such conditions compared to general methanotrophs.

Given known biomass yields for *M. buryatense* 5GB1C (22), 0.78 ton biomass dry weight is predicted to be formed per ton methane utilized (Methods and Materials). Methane-derived biomass (single cell protein) has been used for aquaculture feed and is predicted to have a value of ~\$1,600 per ton (40), a cobenefit of methane removal by methanotrophs. If bioreactor systems could be developed that would allow automated biomass harvesting, this cobenefit could substantially add to the attractiveness of a bio-based methane removal system.

Standard biofilters are not designed for use of low methane and alternative bioreactor configurations that focus on enhanced mass transfer could significantly increase ECs at these low methane inlet concentrations. If a combination of strain improvement and bioreactor/bioprocess design could increase ECs 20-fold, treatment units would be projected to remove 40 to 280 tons methane per year. In such a case, 50,000 to 300,000 units deployed worldwide for 20 y at sites with methane enrichment in air averaging 500 ppm would keep 240 million tons methane from entering the atmosphere, an outcome predicted to significantly impact global warming (1, 5). A previous economic analysis (16) suggests that a 20-fold improvement in EC would also become economically feasible, but full environmental life cycle and technoeconomic analyses are needed to more definitively address economic and environmental impacts. The above analysis suggests the use *M. buryatense* 5GB1C either by itself or as part of a consortium as the basis of a methane treatment technology is potentially feasible in the 500 ppm range. More studies are necessary to determine actual feasibility under field conditions.

In summary, *M. buryatense* 5GB1C has emerged from our screening study as a promising candidate for a methane removal technology that does not increase N<sub>2</sub>O emissions, and our results suggest that its ability to grow at low methane relies at least partly on a high specific affinity and a low maintenance energy. The former denotes an inherently rapid methane assimilation that provides the basis for energy production and biomass growth. The latter is in keeping with drastic downregulation of translation and transcription machineries, as both synthesis and maintenance of those components are energy-demanding. They both confer growth advantages in the face of severe substrate limitation, enhancing the energy produced from methane oxidation that can be invested into biomass synthesis. This well-studied bacterium is an excellent candidate to serve as a platform for developing methane removal technology either by itself or as part of a consortium. Strain improvements could be carried out using approaches such as adaptive laboratory evolution and targeted genetic manipulations to improve growth at low methane, and growth improvements could involve testing other medium constituents such as copper concentration. Moving forward, research should also be focused on integration of these methane consumers into deployable and scalable bioreactor systems as well as environmental life cycle and technoeconomic analyses of such a methane removal technology to ensure both economic feasibility and environmental benefit.

## Methods and Materials

**Strains and Bacterial Cultivation.** *Methylosinus* sp. LW4, *Methylocystis* sp. LW5, *Methylomonas* sp. LW13, and *Methylobacterium* (formerly *Methylosarcina*) *lacus* LW14 (41) were grown in nitrate mineral salts (NMS) medium (42). *Methylothermobacterium buryatense* 5GB1C and *Methylothermobacterium alcaliphilum* 20Z<sup>R</sup> were grown in NMS2 medium (20). All methanotrophs were cultivated at 30 °C throughout this study.

To test methanotrophic growth at low methane, colonies of methanotrophs were inoculated into 5 mL fresh medium in glass tubes (18-by-150 mm with an interior volume of ~27.5 mL) sealed with stoppers (20 mm in diameter). Precultures were first grown with 20% (v/v) methane in the headspace and shaken at 200 rpm and then subcultured in 10 mL fresh medium with an initial optical density at 600 nm (OD<sub>600</sub>) between 0.02 and 0.05 in serum bottles (with an interior volume of ~250 mL). Next, 5 mL of certified 2.5% (v/v) methane:air gas (Linde plc) was injected into stoppered bottles, yielding approximately 500 ppm methane in the headspace. The headspace was refreshed daily with the corresponding methane:air gas mixtures. Also, 0.2 mL to 0.3 mL samples were taken and diluted three to five times with sterile media for OD<sub>600</sub> measurements using a Jenway® 7300 Spectrophotometer. Similar experiments were also carried out with glass tubes. Briefly, precultures were subcultured in fresh medium with an

OD<sub>600</sub> between 0.01 and 0.03. Next, 0.5 mL, 1 mL, or 5 mL of certified 2.5% (v/v) methane:air gas (Linde plc) was injected into the stoppered glass tubes, yielding approximately 500 ppm, 1,000 ppm, or 5,000 ppm methane in the headspace, respectively. OD<sub>600</sub> values were measured by a spectrophotometer (Thermo Scientific™ Spectronic™ 20D<sup>+</sup>). The daily OD<sub>600</sub> increase was determined by the slope of growth curves in the linear region. N<sub>2</sub>O concentration in the headspace was measured after 14 d of growth by a gas chromatograph (GC) (Model 8610, SRI Instrument) equipped with an electron capture detector as previously described (43). During La<sup>3+</sup> addition experiments, glass tubes were acid-washed with 1M HCl overnight and then rinsed with ddH<sub>2</sub>O before use.

**Bioreactor Setup and Gas Chromatography Measurements.** The bioreactor was operated as described (22, 44). Briefly, *M. buryatense* 5GB1C growing at the middle to late exponential growth phase was inoculated into 1 L NMS2 medium in the bioreactor (New Brunswick Scientific) connected to the BioFlo® & Celligen® 310 control system (New Brunswick Scientific). Per liter of NMS2 medium, 10 mL 1M carbonate buffer was added. The culture was agitated constantly at 1,000 rpm, and the temperature was maintained at 30 °C. Inlet gas was filtered by a 0.2 µm autoclavable membrane (Whatman plc), and the flow rate was controlled by a mass flow controller (SmartTrak®100, Sierra). The off-gas was automatically sampled every 15 min by a GC (Model GC-2014, Shimadzu Corporation, Japan) equipped with a thermal conductivity detector, as described (31). OD<sub>600</sub> values were measured using a Jenway® 7300 Spectrophotometer.

An empirical calibration curve for methane was obtained by using certified standard methane:air gas mixtures, i.e., 500 ppm, 1,000 ppm, and 2.5% (v/v) methane:air gases. The equation was:  $y = 0.0049x + 0.0003$  ( $R^2 > 0.99$ ), where  $y$  is the methane concentration (%), and  $x$  is the peak area (µV·min) divided by 100. All standard gas tanks were purchased from Linde plc.

In batch conditions, 2.5% methane:air gas was diluted with air at different ratios to create gas mixtures of different methane concentrations. The flow rate of air was constant at 100 cm<sup>3</sup> min<sup>-1</sup>, while the flow rate of 2.5% methane:air gas was set to different rates. Methane concentrations of the resulting mixtures were measured by GC with the calibration curve, and these varied somewhat between experiments (SI Appendix, Table S1).

For steady-state (chemostat) conditions, certified 500 ppm or 1,000 ppm methane:air gas was delivered to *M. buryatense* 5GB1C at a flow rate of 100 cm<sup>3</sup> min<sup>-1</sup>, which underwent a sequence of growth stages: batch growth at 1,000 ppm methane, chemostat (steady-state) growth at 1,000 ppm methane (0.02 h<sup>-1</sup>), chemostat (steady-state) growth at 500 ppm methane (0.009 h<sup>-1</sup>), and batch growth at 500 ppm methane (SI Appendix, Fig. S3). To quantify formate production, 0.8 mL culture was collected from chemostat-grown cultures and pelleted at room temperature. The supernatant was filtered in SpinX® centrifuge tubes (Corning® Costar®) equipped with 0.2 µm membranes at 10,000 ×g for 1 min and stored at -20 °C. Formate concentrations were determined by an ion chromatography system (ICS-5000 ion, Thermo Fisher Scientific) equipped with a Dionex IonPac ICE-AS6 column (9 by 250 mm, Thermo Fisher Scientific) as previously described (27).

**RNA Isolation and RNAseq Analysis.** After growth of three generations under steady-state conditions, ~80 mL culture was harvested in Falcon™ tubes pre-cooled with liquid N<sub>2</sub>, and then, samples were centrifuged at 6,000 ×g for 15 min at 4 °C. The remainder of the procedures for RNA isolation were the same as described previously (21). RNA samples were stored at -80 °C before submission to Azenta/GENEWIZ (USA) for sequencing.

Standard bioinformatics tools were used to process the RNAseq data, assisted by the barrelseq workflow, which is available on Github: <https://github.com/BeckResearchLab/barrelseq> (DOI: 10.5281/zenodo.4323588). Briefly, reads from the fastq field were aligned to the *M. buryatense* 5GB1C genome (NCBI accession NZ\_CP035467.1) using BWA with the BWA-MEM algorithm (BWA version 0.7.17-r1198-dirty, default parameters) (45). SAMTools version 1.9 was used to transform the initial read alignments into sorted BAM files (46). The *htseq-count* tool from the "HTSeq" framework version 2.0.2 was used with modifications (described below) to attribute the reads to ORFs using the "intersection-nonempty" mode, providing estimates of raw read counts (47). Statistical evaluation for differential expression was performed using DeSeq2\_1.2.10 on the raw read counts. Raw read counts were subsequently converted into TPM for each genome feature for use in visualizations (48).



*htseq-count* was modified to enable the counting of multiply-mapped reads. By default, multiply-mapped reads are assigned an alignment quality of 0 and ignored during counting. In the context of *M. buryatense*, several key genes (*phi*, *hps*, and *tkl*, encoding 6-phospho-3-hexuloseisomerase, 3-hexulose-6-phosphate synthase, and transketolase, respectively) in the RuMP pathway occur in a cluster of 4 tandem repeats (EQU24\_RS22575 - EQU24\_RS21610, EQU24\_RS23130 - EQU24\_RS23165), resulting in most reads for these genes mapping equally well to multiple locations and thus being ignored. In order to include these genes in our expression analyses, *htseq-count* was modified to process these reads by distributing their counts across the locations where they mapped with equal quality. The modified *htseq-count* code to support this mode of counting is available here: [10.5281/zenodo.7434230](https://doi.org/10.5281/zenodo.7434230).

**Measurements of Growth and Kinetic Parameters.** The overall methane consumption rate of the entire culture ( $q$ , in  $\text{mmol h}^{-1}$ ) at concentrations lower than 2,500 ppm methane was determined by the following equation:  $q = \frac{P_i - P_o}{R \cdot T} \cdot Q$ , where  $P_i$  and  $P_o$  are partial pressures of methane in the inlet gas and off-gas, respectively;  $R$  is the ideal gas constant;  $T$  is the temperature; and  $Q$  is the inlet gas flow rate. When the methane concentration was 2.5% (v/v) or above,  $q$  was determined as previously described (44).

In batch conditions, the specific methane uptake rate ( $V$ , in  $\text{mmol g}^{-1} \text{h}^{-1}$ ) was determined by the slope of the overall methane consumption rate ( $q$ ) against the total biomass dry weight (TDW), i.e.,  $V = \frac{dq}{dTDW}$ . The specific growth rate ( $\mu$ ) was determined by the slope of natural log values of TDW against time, i.e.,  $\mu = \frac{d \ln(TDW)}{dt}$ . At least three data points in the exponential growth phase were used for calculating the above parameters. The conversion between the optical density and biomass dry weight was based on previous measurements (22) and reconfirmed in this study for growth at 200 ppm methane. Sample volumes were recorded to correct the culture volume in the bioreactor.

In chemostat conditions,  $V = \frac{q}{TDW}$ , and the specific growth rate ( $\mu$ ) was equal to the dilution rate ( $D$ ):  $\mu = D = \frac{Vol_{out}}{Vol_{SG}}$ , where  $Vol_{out}$  is the volume of culture pumped out of the bioreactor per unit time and  $Vol_{SG}$  is the total *M. buryatense* 5GB1C culture volume (~1 L).

The whole-cell  $K_M$  [ $K_{M(app)}$ ] and the whole-cell maximum methane uptake rate ( $V_{max}$ ) were determined via fitting experimental data with the equation  $V = V_{max(app)} \frac{S}{K_{M(app)} + S}$ , where  $S$  is the initial substrate concentration in the liquid phase. Nonlinear regression and statistics were performed by the built-in function "fitnlm" in MATLAB (Version 2022b).  $S$  was calculated as follows:  $S = H \cdot P_{CH_4}$ , where  $H$  is the Henry's constant for methane at 30 °C ( $1.3 \times 10^{-5} \text{ mol m}^{-3} \text{ Pa}^{-1}$ ) (49), and  $P_{CH_4}$  is the partial pressure of methane. The specific affinity  $a_0$  was calculated by the slope of the linear section of the Michaelis-Menten curve:  $a_0 = \frac{dV}{dS}$ . Linear regression and statistics were performed by using "fitlm" in MATLAB.

**Flux Balance Analysis.** The GEM model of *M. buryatense* 5GB1C was based on a previous study (27). Given different specific methane uptake rates, the GEM

model was used to predict corresponding growth rates with varied NG-ATPM values. Simulation was carried out in both COBRAPy (50) and MATLAB.

**Measurement of Intracytoplasmic Membrane Coverage, Cell Size, and Dry Weight.** *M. buryatense* 5GB1C cells grown at 2.5% and 500 ppm methane were harvested at  $6,000 \times g$  for 10 min at room temperature, and washed with 1 mL of  $1 \times$  phosphate-buffered saline, pH 7.4 (PBS, Invitrogen). Of note, 1 to 5 mL of cells in the exponential phase were harvested to achieve a final  $OD_{600}$  of 1.0 when resuspended in 1 mL of PBS. Cells were pelleted at  $6,000 \times g$  for 10 min and resuspended in 1 mL 4% paraformaldehyde (w/v) (Sigma-Aldrich) in PBS. The cells were incubated on ice for 30 min and then pelleted and washed twice with PBS. The final cell suspensions were stored at 4 °C until staining.

Cells were stained with FM 1-43 (ThermoFisher/Invitrogen, Waltham, MA, USA) to a final concentration of  $5 \mu\text{g mL}^{-1}$  in PBS for 1 h at 25 °C. Then cells were placed on a glass slide coated with 0.01% poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA), topped with a coverslip, and sealed. All images were captured on a Nikon C2si confocal microscope with a  $60 \times$  Plan Apo  $\lambda$  (NA 1.45) oil objective using a pinhole radius at 0.3  $\mu\text{m}$ . The fluorophore excitation wavelength was 488 nm with emission detected with a spectral filter of 490 to 560 nm. All the imaging was performed at room temperature, immediately after staining. Individual cell images were analyzed using NIS-Elements AR Analysis 4.30.02 software (Nikon) to calculate the percent ICM by comparing the total area of the cell (cell size) to the area of internal fluorescence, as previously described (51).

Cell dry weights were measured as described previously (22). Briefly, 225 mL cultures were harvested and centrifuged at  $6,000 \times g$  for 75 min in preweighed tubes. After the supernatant was removed, samples were washed with sterile  $\text{ddH}_2\text{O}$  and centrifuged at  $6,000 \times g$  for 30 min. The supernatant was discarded, and cell pellets were lyophilized overnight (Labconco® FreeZone® 4.5 L – 105 °C Benchtop Freeze Dryer) before weight measurements.

**Calculation of Biomass Efficiency.** *M. buryatense* 5GB1C yields (g C in biomass/g C in  $\text{CH}_4$ ) average 0.5 (22). Assuming the cells are 48% C dry weight (52) and converting to ton  $\text{CH}_4$ , each ton of  $\text{CH}_4$  would generate 0.78 ton biomass.

**Data, Materials, and Software Availability.** The RNA-Seq data have been uploaded to the NCBI Gene Expression Omnibus (GEO) under accession number GSE221011 (53). An interactive version of Fig. 3 is available at <https://erinhwilson.github.io/limited-ch4-tpm-analysis/>.

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