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# Genomic and Transcriptomic Analyses of the Facultative Methanotroph *Methylocystis* sp. Strain SB2 Grown on Methane or Ethanol

Alexey Vorobev,<sup>a</sup> Sheeja Jagadevan,<sup>a</sup> Sunit Jain,<sup>b</sup> Karthik Anantharaman,<sup>b</sup> Gregory J. Dick,<sup>b,c,d</sup> Stéphane Vuilleumier,<sup>e</sup> Jeremy D. Semrau<sup>a</sup>

Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, Michigan, USA<sup>a</sup>; Department of Earth and Environmental Sciences, University of Michigan, Ann Arbor, Michigan, USA<sup>b</sup>; Center for Computational Medicine and Bioinformatics, University of Michigan, Ann Arbor, Michigan, USA<sup>c</sup>; Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, Michigan, USA<sup>d</sup>; Equipe Adaptations et Interactions Microbiennes dans l'Environnement, UMR 7156 Université de Strasbourg-CNRS, Université de Strasbourg, Strasbourg, France<sup>e</sup>

**A minority of methanotrophs are able to utilize multicarbon compounds as growth substrates in addition to methane. The pathways utilized by these microorganisms for assimilation of multicarbon compounds, however, have not been explicitly examined. Here, we report the draft genome of the facultative methanotroph *Methylocystis* sp. strain SB2 and perform a detailed transcriptomic analysis of cultures grown with either methane or ethanol. Evidence for use of the canonical methane oxidation pathway and the serine cycle for carbon assimilation from methane was obtained, as well as for operation of the complete tricarboxylic acid (TCA) cycle and the ethylmalonyl-coenzyme A (EMC) pathway. Experiments with *Methylocystis* sp. strain SB2 grown on methane revealed that genes responsible for the first step of methane oxidation, the conversion of methane to methanol, were expressed at a significantly higher level than those for downstream oxidative transformations, suggesting that this step may be rate limiting for growth of this strain with methane. Further, transcriptomic analyses of *Methylocystis* sp. strain SB2 grown with ethanol compared to methane revealed that on ethanol (i) expression of the pathway of methane oxidation and the serine cycle was significantly reduced, (ii) expression of the TCA cycle dramatically increased, and (iii) expression of the EMC pathway was similar. Based on these data, it appears that *Methylocystis* sp. strain SB2 converts ethanol to acetyl-coenzyme A, which is then funneled into the TCA cycle for energy generation or incorporated into biomass via the EMC pathway. This suggests that some methanotrophs have greater metabolic flexibility than previously thought and that operation of multiple pathways in these microorganisms is highly controlled and integrated.**

Microbial oxidation of methane represents one of the key steps in the global carbon cycle. Although methanotrophic metabolism is well known and was first described over a century ago (1), recent discoveries have expanded our understanding of the physiology and diversity of methanotrophs. For example, these microbes were initially considered to be obligate aerobes that grouped in one of two phyla only, the *Alpha*- and *Gamma*proteobacteria, but thermoacidophilic aerobic methanotrophs belonging to the *Verrucomicrobia* phylum are now known (2–4). Also, anaerobic oxidation of methane has newly been shown to occur via three different microbial processes: by a consortium of *Archaea* and *Bacteria* that oxidizes methane through coupling to sulfate reduction (5), by “*Candidatus Methylopirabilis oxyfera*” of the bacterial NC10 phylum that couples methane oxidation to nitrite reduction (6), and most recently, by the Archaeon “*Candidatus Methanoperedens nitroreducens*,” which couples the anaerobic oxidation of methane via reverse methanogenesis to nitrate reduction (7). In addition, microbial methane oxidation may also be tied to iron and manganese reduction, although corresponding organisms have not yet been isolated (8–10).

Moreover, technological advances have recently enabled the sequencing of many methanotrophic genomes, and such information has shown that these microbes utilize multiple pathways for carbon assimilation. For example, early biochemical analyses had indicated that proteobacterial methanotrophs assimilate carbon from methane at the level of formaldehyde, by using either the ribulose monophosphate (RuMP) pathway or the serine cycle.

Genomic analyses, in contrast, indicated that the verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* SolV possessed a complete Calvin cycle. Subsequent studies showed that this methanotroph uses carbon dioxide as its sole carbon source, i.e., this strain oxidizes methane to carbon dioxide to generate energy and fixes the produced carbon dioxide for biomass production (11, 12).

Despite this vast phylogenetic and physiological diversity of methanotrophs, most such organisms can grow on methane or methanol only, even if some of these strains are also able to utilize other C<sub>1</sub> compounds such as formate and formaldehyde for growth (13). Some methanotrophs, however, are facultative, i.e., they can utilize compounds with carbon-carbon bonds as their sole carbon and energy source (13–17). How facultative methanotrophs utilize multicarbon compounds for growth remains to

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Address correspondence to Jeremy D. Semrau, jsemrau@umich.edu.

A. Vorobev and S. Jagadevan contributed equally to this article.

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be addressed in detail. Several hypotheses can be tested to address this question. For instance, facultative methanotrophy could be rendered possible by the possession of specific genes not found in obligate methanotrophs or by alterations in the regulation of key genes involved in carbon metabolism. In order to explore these hypotheses, we sequenced the genome of *Methylocystis* sp. strain SB2, which can grow on acetate or ethanol in addition to methane (18), and experimentally investigated and compared its transcriptome when grown on methane or on ethanol.

## MATERIALS AND METHODS

**Growth conditions.** *Methylocystis* sp. strain SB2 was grown to late exponential phase on nitrate mineral salts (NMS) medium (19) with 10  $\mu$ M copper added as CuSO<sub>4</sub>. Fifty-milliliter cultures were grown in 250-ml Erlenmeyer flasks at 30°C with rotation at 225 rpm in the presence of 0.1% (vol/vol) ethanol as a carbon source or in a methane-to-air ratio of 1:2. Growth was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>) using a Genesys 20 visible spectrophotometer (Spectronic Unicam, Waltham, MA). All cultures were grown in triplicate for subsequent DNA and RNA extraction and sequencing.

**DNA and RNA extraction.** Late-exponential-phase cells grown on methane were harvested by centrifuging at 4,500  $\times$  g for 10 min at 4°C. Cell pellets were resuspended in 1 ml of extraction buffer (100 mM Tris-HCl [pH 8.0], 1.5 M NaCl, 1% [wt/vol] hexadecyltrimethylammonium bromide [CTAB]), followed by bead beating and three cycles of freeze-thaw. DNA was extracted using phenol-chloroform extraction (20).

RNA was extracted from cultures grown with either methane or ethanol as the sole growth substrate. Cultures were collected in the late exponential phase by centrifugation at 4,500  $\times$  g for 10 min at 4°C. Cell pellets were resuspended in 0.75 ml of RNA extraction buffer (0.2 M NaH<sub>2</sub>PO<sub>4</sub>-Na<sub>2</sub>HPO<sub>4</sub> buffer [pH 7.5]–5% CTAB in 2.4 M NaCl). The resuspended cell pellets were subjected to bead beating (1 min at 4,800 rpm) in 2-ml plastic tubes containing 0.5 g of 0.1-mm zirconia-silica beads (Biospec Products), 35  $\mu$ l of 20% SDS, 35  $\mu$ l of 20% laurylsarcosine, and 750  $\mu$ l of phenol-chloroform-isoamyl alcohol (25:24:1). The samples were then centrifuged at 14,000 rpm for 5 min at 4°C. The aqueous phase was mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 14,000 rpm for 5 min at 4°C. RNA was precipitated by adding MgCl<sub>2</sub> (final concentration, 2.5 mM), 0.1 volume of 3 M sodium acetate, and 0.7 volume of isopropanol and incubating overnight at –80°C. RNA was then recovered by centrifugation at 14,000 rpm for 30 min at 4°C. The DNase treatment was carried out using the RNase-free DNase set (Qiagen) in accordance with the manufacturer's instructions. The RNA samples were further purified using the RNeasy Plus kit (Qiagen) using genomic DNA (gDNA) eliminator columns and RNeasy Mini Spin columns according to the manufacturer's instructions. The rRNA content was reduced using a MICROExpress bacterial mRNA purification kit (Ambion). To check for any DNA contamination, PCR was performed with extracted RNA as the template. RNA was reverse transcribed to obtain cDNA by using Superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions.

**Sequencing.** Genomic DNA of *Methylocystis* sp. strain SB2 was provided to the DNA sequencing core at the University of Michigan (<http://seqcore.brcf.med.umich.edu>) for Illumina sequencing using Illumina HiSeq2000 SE50, which generated 77,914,666 reads passing a quality factor (QF) of >30.

For RNA, sequencing platform-specific chemistry was utilized to produce cDNA and sequencing was carried out using platform-specific protocols, producing pair-ended reads of 50 bp in length. All cDNA samples (triplicate biological replicates) were individually bar-coded and sequenced in the same lane.

**Assembly and annotation.** *De novo* assembly of sequenced reads was performed as follows. (i) Reads that were 100% identical over 100% of their length were removed to leave unique reads (GitHub Geo-omics); (ii) unique reads were trimmed using Sickle (version 1.100; GitHub) with

a quality and minimum-length thresholds of 20; (iii) trimmed reads were assembled using Velvet 1.1.07 (21, 22) at hash lengths of 31, 41, and 45; and (iv) the resulting multiple assemblies were combined using Minimus2 (23). The final contigs were annotated using the Integrated Microbial Genomes system (24).

The number of cDNA sequencing reads generated per sample for transcriptome analysis varied between 11.9 and 18.6 million per experiment. Reads were trimmed using Sickle (version 1.100; GitHub) with default parameters. Reads were then aligned to the draft genome scaffold using the Burrows-Wheeler alignment tool (BWA) version 0.6.2 (25), with default parameters for small genomes. No corrections were applied to raw-data sets prior to this analysis. Sequence alignment/map (SAM) files, obtained from BWA, were converted to binary SAM (BAM) files and subsequently sorted and indexed using SAMtools 0.1.17 (26). These reads were converted into fragments via the protocol described in reference 27. Briefly, properly mapped paired reads were counted as a single fragment. Paired reads that were not properly mapped were discarded, and the remaining unpaired reads were treated as independent fragments. The resulting number of fragments per sample ranged from 9 million to 13.8 million. Sorted and indexed BAM files were analyzed by Cufflinks 2.0.2 (28) to calculate the number of fragments per kilobase of transcript per million mapped reads (FPKM) for all genes and thereby detect differentially expressed genes. Cufflinks output files were analyzed using CummeRbund (29), which was also used to estimate the variance between replicates within both methane- and ethanol-grown cultures. A negative binomial model estimated from the data was used to obtain variance estimates, from which *P* values were computed to determine if differential expression of each individual transcript was statistically significant, as described previously (30). Based on relative expression, genes were grouped in six major categories (omitting rRNA genes): very high (>500 FPKM), high (500 to 200 FPKM), moderate (200 to 50 FPKM), modest (50 to 10 FPKM), low (10 to 2 FPKM), and not expressed (<2 FPKM).

**Genome sequence accession numbers.** Data from this Whole Genome Shotgun project have been deposited at DDBJ/EMBL/GenBank under the accession number [AYNA00000000](https://www.ncbi.nlm.nih.gov/assembly/GCA01243000). The version described in this paper is version [AYNA01000000](https://www.ncbi.nlm.nih.gov/assembly/GCA01243000). The transcriptomes of *Methylocystis* sp. strain SB2 under methane and ethanol growth conditions are available at the NCBI Gene Expression Omnibus database under accession numbers [GSM1243002](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1243002) to [GSM1243007](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1243007).

## RESULTS

**Genome assembly and annotation.** The draft genome of *Methylocystis* sp. strain SB2 comprises 3,653,670 bp of sequence consisting of 150 contigs, with an average GC content of 62.7% and a total of 3,657 predicted proteins. General features of the genome and reconstruction of metabolic pathways from genomic analyses are summarized in Table 1 and Fig. 1.

**Methane oxidation.** All steps of methane oxidation to carbon dioxide were found. Specifically, the presence of one complete copy of the *pmo* operon, *pmoCAB* (SB2\_03554 to SB2\_03556), was detected, and the absence of genes encoding sMMO was confirmed. Additional copies of *pmoA* (*pmoA2*, SB2\_02329), *pmoB* (*pmoB2*, SB2\_02328), and *pmoC* (*pmoC2*, SB2\_01179) were found in the genome. Although *pmoA2* and *pmoB2* were contiguous, *pmoC2* was not part of the same operon and was located in a different part of the genome. Interestingly, one copy of the recently discovered *pxm* operon, *pxmABC* (SB2\_02930 to SB2\_02932) was also found, the first identification of this operon in an alphaproteobacterial methanotroph. In contrast to the *pmo* operon found in most methanotrophs, *pxm* genes are organized in the noncanonical form *pxmABC* (31). Phylogenetic analysis using neighbor-joining analysis shows that the *pxmA* sequence of *Methylocystis* sp. strain SB2 clustered most closely with those of

**TABLE 1** General features of *Methylocystis* sp. strain SB2 genome

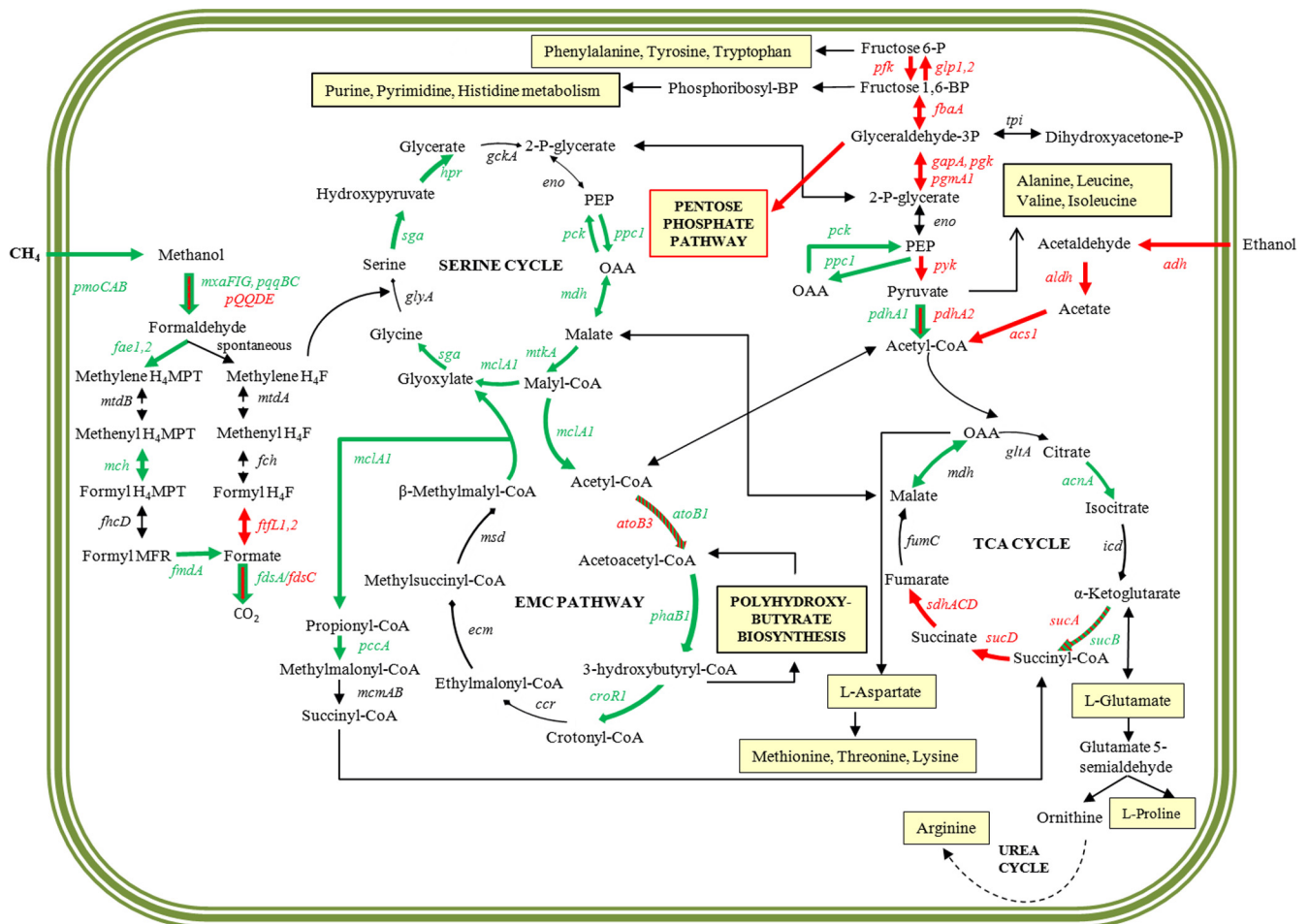
Characteristic (unit)	Value
Complete genome size (bp)	3,653,670
No. of contigs	150
Longest contig (bp)	168,413
G+C %	62.7
Total no. of coding sequences	3,657
Percent coding	98.0
No. of rRNA genes (16S, 23S, and 5S)	14
No. of tRNA genes	46
No. of other RNA genes	14
No. of hypothetical proteins	805
No. of conserved hypothetical proteins	6
No. (%) of protein-coding genes with function prediction	2,698 (74)
No. (%) of protein-coding genes without function prediction	885 (24)
No. (%) of proteins assigned to COGs <sup>a</sup>	2,609 (71)
No. (%) of protein-coding genes coding signal peptides	877 (24)
No. (%) of protein-coding genes connected to transporter classification	312 (8)
No. (%) of protein-coding genes coding transmembrane proteins	853 (23)

<sup>a</sup> COG, cluster of orthologous groups.

*Gammaproteobacteria* such as *Methylomonas* sp. M5 and *Methylobacter marinus* A45 (see Fig. S1 in the supplemental material).

Methanol formed from the oxidation of methane is further oxidized to formaldehyde via the heterotetrameric pyrrolo-quinoline quinone (PQQ)-linked enzyme methanol dehydrogenase (MDH) (32–35, 51). Homologs of *mxoA*, encoding the large subunit of MDH (SB2\_00612), and of *mxoB*, encoding the small subunit of MDH (SB2\_00609), together with genes for cytochrome *c* family protein (SB2\_03431) required for methanol metabolism, were identified. A total of four genes for proteins involved in PQQ biosynthesis (*pqqBCDE*) were found as a single cluster (SB2\_01276 to SB2\_01278). No genes for the small PQQ precursor (*pqqA*) and *pqqFG* were detected in either the genome or the transcriptome of *Methylocystis* sp. strain SB2.

**Interconversions of C<sub>1</sub> compounds.** Two distinct cofactor-dependent metabolic modules, i.e., tetrahydromethanopterin (H<sub>4</sub>MPT) mediated and tetrahydrofolate (H<sub>4</sub>F) mediated, operate to transfer C<sub>1</sub> units between formaldehyde and formate. The *fol* genes (*folABCEKP*) involved in folate synthesis were identified, with two of these (*folKP*) next to each other (SB2\_02973 and



**FIG 1** Central metabolism of *Methylocystis* sp. strain SB2 grown on methane or ethanol as the sole source of energy and carbon as deduced from genomic and transcriptomic analyses. Genes highlighted in green or red (and corresponding steps) were significantly upregulated on growth on methane and ethanol, respectively. Some steps could be performed via products of multiple genes that were differentially expressed in methane- versus ethanol-grown cultures. These steps are denoted by a single red and green arrow.

SB2\_02974). All genes coding for enzymes involved in H<sub>4</sub>MPT-mediated formaldehyde oxidation were identified in the *Methylocystis* sp. strain SB2 genome. Three different copies for the gene of formaldehyde-activating enzyme (*fae*) involved in the conversion of formaldehyde to methylene-H<sub>4</sub>MPT were found at different locations in the chromosome (SB2\_00144, SB2\_03426, and SB2\_03645). Of these three copies, two showed high (72%) amino acid identity (SB2\_03426 and SB2\_03645) to each other, the third (SB2\_00144) being more distantly related to the two others (<30% amino acid identity). The genes involved in conversion of methylene-H<sub>4</sub>MPT to formyl-H<sub>4</sub>MPT, i.e., methylene-H<sub>4</sub>MPT dehydrogenase (*mtdB*) and methenyl-H<sub>4</sub>MPT cyclohydrolase (*mch*), are located next to each other in the genome (SB2\_01772 and SB2\_01771). The *fhcD* gene (encoding formylmethanofuran H<sub>4</sub>MPT N-formyltransferase), responsible for conversion of formyl-H<sub>4</sub>MPT to formylmethanofuran (SB2\_01313), lies between two formyl-methanofuran dehydrogenase subunits (*fdwAC*, SB2\_01312 and SB2\_01314).

Genome analysis suggests the presence of a complete tetrahydrofolate (H<sub>4</sub>F)-linked pathway in addition to the H<sub>4</sub>MPT-linked formaldehyde oxidation system. It should be noted that formaldehyde is believed to spontaneously (i.e., abiotically) condense with H<sub>4</sub>F to form methylene-H<sub>4</sub>F (34). The enzymes converting methylene-H<sub>4</sub>F to formyl-H<sub>4</sub>F, i.e., methylene H<sub>4</sub>F-dehydrogenase and methenyl H<sub>4</sub>F-cyclohydrolase encoded by genes *mtdA* and *fch*, respectively, were detected next to each other in the genome (SB2\_01771 and SB2\_01772). Two copies of the gene encoding formyl-H<sub>4</sub>F ligase (*ftfL*) were found in two different locations elsewhere in the genome (SB2\_01855 and SB2\_02117). Most subunits of the formate dehydrogenase gene (*fdsABCD*) and *fdhD*, responsible for oxidation of formate to carbon dioxide, were identified adjacent to each other (SB2\_01354 to SB2\_01358) in the genome.

**Carbon assimilation via the serine cycle.** Two pathways for carbon assimilation at the oxidation level of formaldehyde have been characterized in methanotrophs: the ribulose mono-phosphate cycle (RuMP), and the serine cycle. Genes for the two key enzymes of the RuMP pathway, 3-hexulose-6-P synthase and hexulose-P isomerase, were not detected, indicating that *Methylocystis* sp. strain SB2 does not use the RuMP pathway for formaldehyde assimilation. In contrast, all genes involved in the serine cycle, encoding serine-glyoxylate amino-transferase (*sga*), hydroxypruvate reductase (*hpr*), two subunits of malate thiokinase (*mtkAB*), an acetyl-coenzyme A (acetyl-CoA)-independent phosphoenol pyruvate carboxylase (*ppc*), and malyl-CoA lyase (*mcl*), were identified. All these genes are located in close proximity, as two gene clusters: (i) *mcl*, *ppc*, and *mtkAB* (SB2\_01767 to SB2\_01770); and (ii) *hpr* and *sga* (SB2\_01773 and SB2\_01774). The intervening genes between these two clusters were identified as encoding methenyl-tetrahydrofolate (H<sub>4</sub>F) cyclohydrolase (*fch*, SB2\_01771) and methylene-H<sub>4</sub>F dehydrogenase (*mtdA*, SB2\_01772) and are involved in the H<sub>4</sub>F pathway of C1 utilization. Another serine cycle gene, *gck*, encoding glycerate kinase (SB2\_01764), was also located nearby.

**Alternative carbon transformation pathways.** As found previously in the alphaproteobacterial methanotroph *Methylosinus trichosporium* OB3b (36), *Methylocystis* sp. strain SB2 has the ethylmalonyl-CoA (EMC) pathway. However,  $\beta$ -ketothiolase (encoded by *phaA*), which is responsible for the first step in the eth-

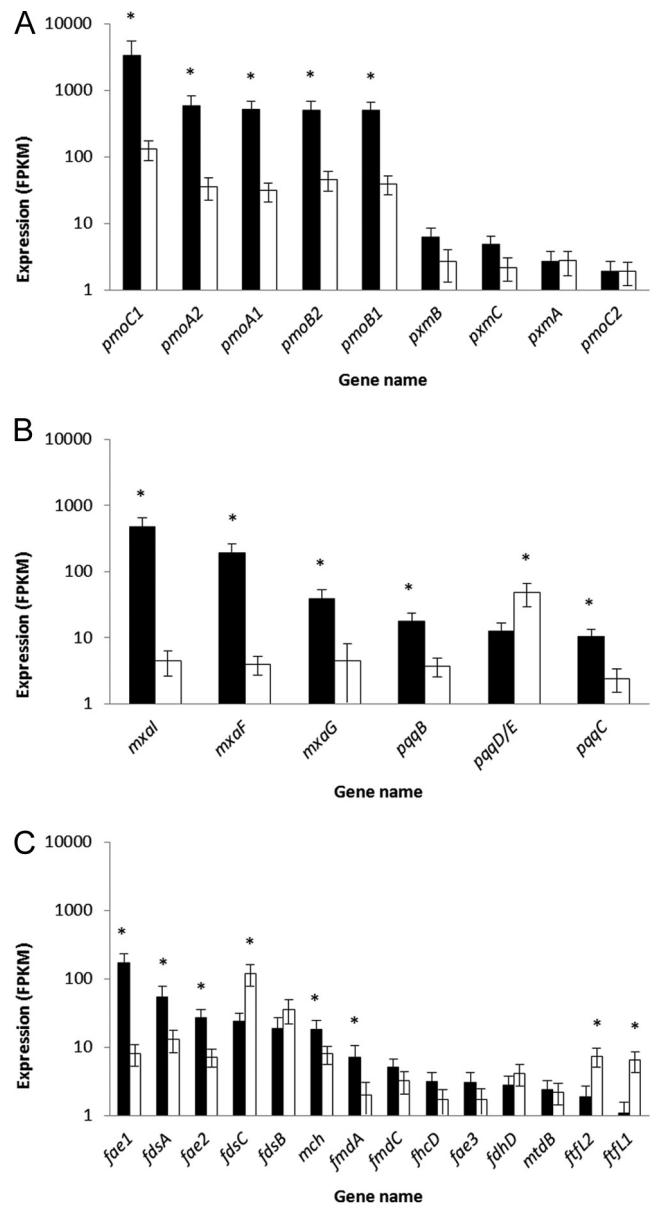


FIG 2 Differential expression of genes involved in methane oxidation (A), methanol oxidation (B), and formaldehyde and formate oxidation (C) in *Methylocystis* sp. strain SB2 grown on methane (black bars) or ethanol (white bars). \*, significantly different expression of genes between methane- and ethanol-grown cultures ( $P < 0.05$ ).

ylmalonyl-CoA pathway, where two acetyl-CoA molecules are combined to form acetoacetyl-CoA, appears to be missing. Three copies of an alternative acetyl-CoA acetyltransferase (encoded by *atoB*), found at different loci of the genome of *Methylocystis* sp. strain SB2, may be involved in the conversion of acetyl-CoA into acetoacetyl-CoA. In addition, genomic evidence for glycolysis, gluconeogenesis, the pentose phosphate pathway, conversion of ethanol to acetyl-CoA, and the complete tricarboxylic acid (TCA) cycle was also found (Fig. 2). No evidence was found for genes encoding key enzymes of methylaspartate and citramalate cycles (17), i.e., glutamate mutase, malate synthase, succinyl-CoA:mesaconate CoA transferase, mesaconyl-CoA hydratase, and

methylaspartate ammonia lyase, involved in the methylaspartate cycle, and citramalate synthase and mesaconate-CoA ligase, involved in the citramalate cycle.

**Comparative transcriptomic analysis of methane- versus ethanol-grown cultures.** Gene expression analysis was carried out on *Methylocystis* sp. strain SB2 grown in NMS medium and either methane or ethanol as the sole growth substrate. On average, 11 million fragments were generated per sample, an amount considered to be largely sufficient to determine differentially expressed genes in bacteria (27). The three biological replicates within both methane- and ethanol-grown cultures were in good agreement with each other, as shown by CummeRbund (29) (see Fig. S2 and S3 in the supplemental material).

Relative expressions of genes as FPKM values are shown in Table S1 in the supplemental material for both methane- and ethanol-grown cultures. Regardless of growth substrate, only a small fraction was either highly or very highly expressed (3.1 and 3.7% for methane- and ethanol grown-cultures, respectively), with the majority of genes expressed either not at all or at a modest or low level (88.6 and 88.1% for methane- and ethanol grown-cultures, respectively).

It is apparent that different central metabolic pathways were upregulated during growth on methane and on ethanol. Not surprisingly, when *Methylocystis* sp. strain SB2 was grown on methane, expression of genes encoding transformation of methane to methanol, transformation of methanol to formaldehyde, and activation and oxidation of formaldehyde to formate were significantly upregulated (Fig. 1 and 2; see Table S2 in the supplemental material). The *pmo* operon was the most highly expressed operon in the transcriptome of methane-grown cultures. Interestingly, expression of *pmoC1* was approximately 6 to 7 times higher than that of other *pmo* genes in the same operon (i.e., *pmoA1* and *pmoB1*), although all genes were highly or very highly expressed. Further, expression of *pmoA2* and *pmoB2* was similar to that of *pmoA1* and *pmoB1*, despite not being part of a canonical *pmo* operon (i.e., *pmoC2* is not contiguous with these genes), and expression of *pmoC2* was quite low. No difference in expression of *pxmABC* was observed between methane- and ethanol-grown cultures. Strikingly, expression of genes involved in methanol, formaldehyde, and formate oxidation for methane-grown cultures was not as high as that as *pmoCAB*, with typically moderate expression. On ethanol, other genes were upregulated, with overall levels of expression moderate at best, e.g., *ftfl1* and *ftfl2* (encoding the reversible conversion of formyl-THF to formate), *fdsC* (encoding the formate dehydrogenase gamma subunit), and *pqqD* and *pqqE* (believed to encode polypeptides that assist in PQQ synthesis in an as-yet-undefined way [37, 38]).

Expression of most genes involved in the serine cycle was higher in methane-grown cultures with the exception of *glyA*, encoding serine hydroxymethyltransferase, *eno*, encoding enolase, and *gckA*, encoding glycerate-2-kinase, which were expressed at similar levels in ethanol-grown cultures (Fig. 1 and 3A; see Table S2 in the supplemental material). Most genes of the serine cycle were expressed at a low or modest level in methane-grown cultures and at a low level in ethanol-grown cultures. Expression of genes identified as part of the EMC pathway varied, with genes encoding the transformation of acetyl-CoA to crotonyl-CoA being expressed at a greater level in methane-grown cultures, although most steps of the EMC pathway were not differentially expressed between methane- and ethanol-grown cultures and

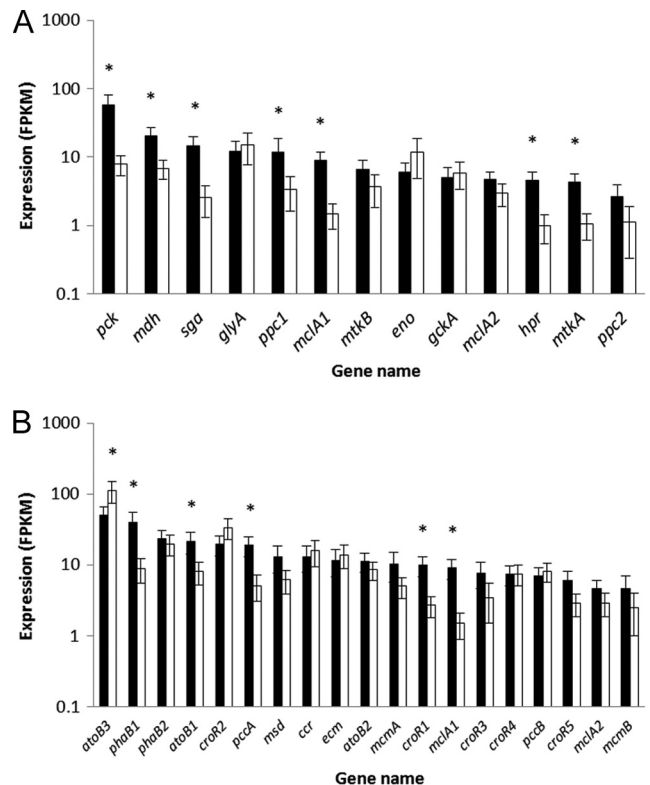


FIG 3 Differential expression of genes involved in the serine cycle (A) and the ethylmalonyl-CoA pathway (B) in *Methylocystis* sp. strain SB2 grown on methane (black bars) or ethanol (white bars). \*, significantly different expression of genes between methane- and ethanol-grown cultures ( $P < 0.05$ ).

were at either low or modest levels (Fig. 1 and 3B; see Table S2 in the supplemental material). Different acetoacetyl-CoA reductases were upregulated on methane- versus ethanol-grown cultures (*atoB1* and *atoB3*, respectively, with *atoB1* modestly expressed with methane as the growth substrate and *atoB3* moderately expressed with ethanol-grown cultures). The expression of a third copy, *atoB2*, was not found to be significantly different and was modestly expressed.

For ethanol-grown cultures, expression of genes likely involved in ethanol oxidation to acetyl-CoA was significantly higher than in methane-grown cultures, with expression varying from low to moderate (Fig. 1 and 4A; see Table S2 in the supplemental material). Genes encoding most steps of glycolysis were also more significantly expressed in ethanol-grown cultures, with expression typically modest (Fig. 1 and 4B; Table S2). Worthy of note, two different pyruvate dehydrogenases were expressed in methane- and in ethanol-grown cultures (*pdhA1* and *pdhA2*, respectively). In addition, differential expression of genes encoding a key step of gluconeogenesis was observed, i.e., two copies of *glp* (*glp1* and *glp2*, encoding fructose-1,6-bisphosphatase) were expressed significantly more in ethanol-grown cultures, although such expression was low. No evidence of pyruvate synthase or pyruvate carboxylase was found in the genome, suggesting that strain SB2 may lack the ability to convert acetyl-CoA to pyruvate or pyruvate to oxaloacetate. Genes encoding portions of the pentose-phosphate pathway displayed higher expression in ethanol-grown cultures, with such expression varying from low to moderate. Specifically,

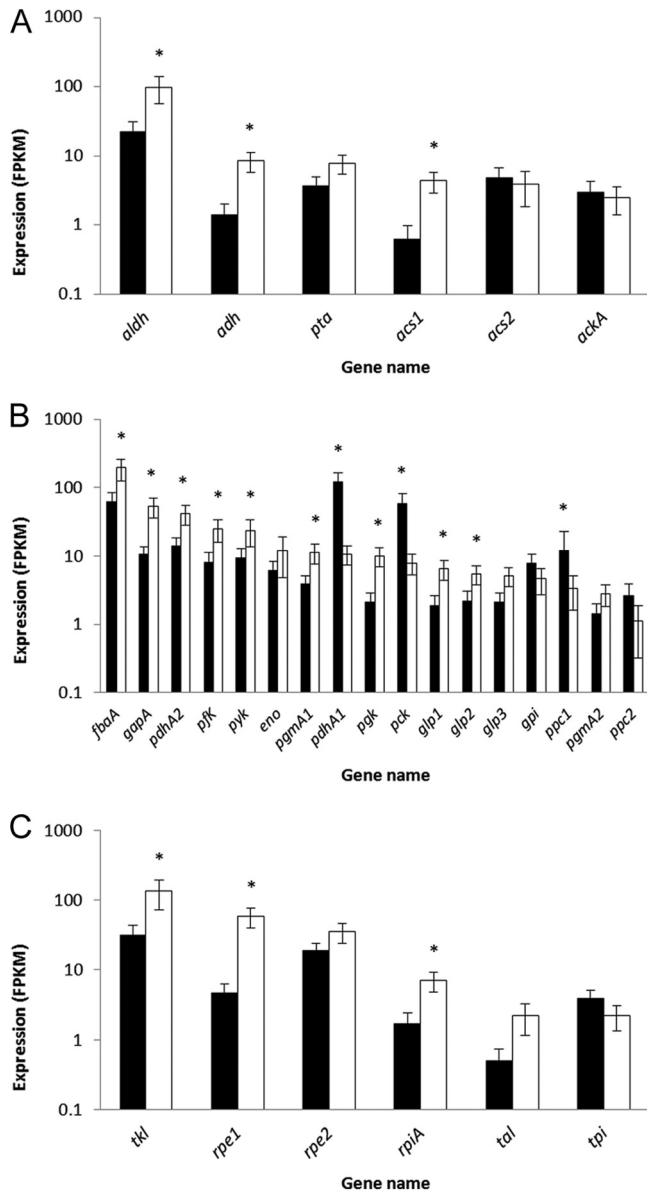


FIG 4 Differential expression of genes involved in ethanol oxidation (A), glycolysis/gluconeogenesis (B), and the pentose phosphate pathway (C) in *Methylocystis* sp. strain SB2 grown on methane (black bars) or ethanol (white bars). \*, significantly different expression of genes between methane- and ethanol-grown cultures ( $P < 0.05$ ).

expression of *rpe* (encoding ribulose-5-phosphate-3-epimerase), *tkl* (encoding transketolase), and *rpiA* (encoding ribose-5-phosphate isomerase) was significantly greater for ethanol-grown cultures. Expression of other genes involved in the pentose-phosphate pathway, i.e., *tpi* (encoding triosephosphate isomerase) and *tal* (encoding transaldolase), however, were not differentially expressed (Fig. 1 and 4C; see Table S2 in the supplemental material). Several steps of the TCA cycle, i.e., steps converting  $\alpha$ -ketoglutarate to fumarate (*sucA*, *sucD*, *sdhA*, *sdhC*, and *sdhD*), were also significantly higher for ethanol-grown cultures, and such expression ranged from modest to very high levels (Fig. 1 and 5; see Table S2 in the supplemental material). Most other genes in the TCA cycle were not differentially expressed at a significant level, with

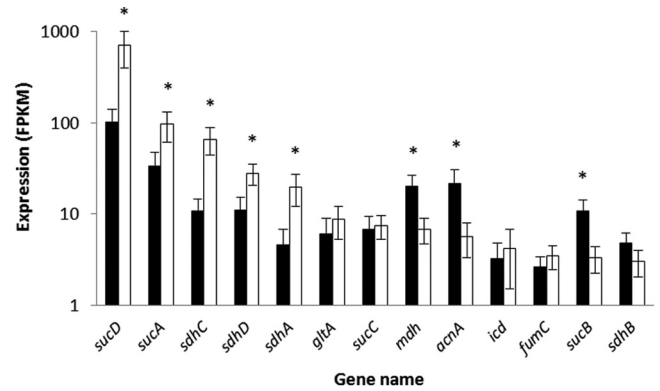


FIG 5 Differential expression of genes involved in the TCA cycle in *Methylocystis* sp. strain SB2 grown on methane (black bars) or ethanol (white bars). \*, significantly different expression of genes between methane- and ethanol-grown cultures ( $P < 0.05$ ).

the exception of *acnA*, encoding aconitate hydratase, and *sucB*, encoding the E2 component of 2-oxoglutarate dehydrogenase, whose expressions were significantly greater in methane-grown cultures but remained modest.

## DISCUSSION

Analysis of the genome of *Methylocystis* sp. strain SB2 confirmed previous findings, i.e., the absence of genes encoding polypeptides of sMMO (18), reduced expression of the *pmo* operon during growth on multicarbon compounds (39), and the EMC pathway integrated with the serine cycle, as previously hypothesized in facultative methanotrophs (17). As found previously (36) for the obligate methanotroph *Methylosinus trichosporium* OB3b, expression of the *pmo* operon in *Methylocystis* sp. strain SB2 was very high for cultures grown on methane. Specifically, the *pmo* operon was the most highly expressed operon in the transcriptome of methane-grown cultures of *Methylocystis* sp. strain SB2, with expression of *pmoC1* approximately 6 to 7 times higher than that of other *pmo* genes in the same operon (i.e., *pmoA1* and *pmoB1*). Expression of *pmoA2* and *pmoB2* was similar to that of *pmoA1* and *pmoB1*, despite not being part of a canonical *pmo* operon (i.e., *pmoC2* is not contiguous with these genes). Expression of *pmoC2* was quite low, suggesting that the products of *pmoA2* and *pmoB2* may also be assembled with that of *pmoC1* to yield a complete and functional pMMO.

Expression of genes involved in methanol, formaldehyde, and formate oxidation was also high in methane-grown cultures, although not as great as that of *pmoCAB*. The exceptionally high expression of pMMO in comparison to other enzymes involved in methane oxidation may reflect the fact that the first step of methane oxidation is relatively slow compared to subsequent steps leading to energy generation or assimilation into biomass. This hypothesis is supported by a previous report that transcripts of *pmoA* are very stable, with a half-life suggested to range from hours to days (40).

It is also noteworthy that the *pxm* operon, encoding a divergent form of the particulate methane monooxygenase, was found in the genome. It is intriguing that *Methylocystis* sp. strain SB2 possesses this operon, since to date, no other alphaproteobacterial methanotroph has been found to possess it. Its function is still unknown, but it has been speculated that it may broaden the range of sub-

strates that a host organism can utilize for growth (31). The transcriptomic analyses of *Methylocystis* sp. strain SB2 reported here, however, indicated very low expression of the *pxm* operon, suggesting that corresponding proteins are not actively used by *Methylocystis* sp. strain SB2 when grown under the conditions considered here.

Methanotrophs that group within *Alphaproteobacteria* utilize the serine cycle for carbon assimilation from methane (16), as confirmed here from genome analysis of *Methylocystis* sp. strain SB2. Interestingly, the serine cycle is differentially expressed with respect to the growth substrate in strain SB2. Expression of the majority of genes of the serine cycle decreased significantly during growth on ethanol, suggesting that this pathway plays a minor role for growth on this substrate. Further, expression of genes encoding the EMC pathway was similar for methane- and ethanol-grown cultures, but expression of many genes involved in the TCA cycle was significantly higher in ethanol-grown cultures. This pattern is reminiscent of that found in *Methylobacterium extorquens* AM1, an alphaproteobacterial facultative methylophil, when grown on acetate. This microbe lacks isocitrate lyase and so cannot generate glyoxylate from isocitrate but rather must generate it via acetyl-CoA shuttled through the EMC pathway. In dynamic <sup>13</sup>C-labeling experiments, most added acetate was oxidized to carbon dioxide via the TCA cycle, with smaller fractions directed into the EMC pathway or combined with glyoxylate to generate malate (41). Under these conditions, phosphoenolpyruvate and pyruvate were synthesized via decarboxylation of malate and oxaloacetate and not from 2-phosphoglycerate, as would be expected if formaldehyde was funneled into the serine cycle.

A similar situation may apply to *Methylocystis* sp. strain SB2 growing on ethanol (Fig. 2). Based on genomic studies, as found for other methanotrophs and *M. extorquens* AM1 (36, 41), strain SB2 lacks isocitrate lyase and thus appears to generate acetyl-CoA from ethanol that is shuttled through the EMC pathway to form glyoxylate. It is interesting that only one enzyme of the EMC pathway was upregulated in ethanol-grown cultures, the conversion of acetyl-CoA to acetoacetyl-CoA. It may be that this is the overall rate-limiting step of the EMC pathway or that comparatively little carbon from ethanol is shuttled through the EMC pathway compared to the TCA cycle when strain SB2 is grown on ethanol. Based on transcriptional analysis, the TCA cycle is expressed and used for generation of ATP and reducing equivalents under these conditions. It is reasonable to assume that a major proportion of ethanol is converted to carbon dioxide, as suggested by lower growth yields than with methane (18). Nevertheless, operation of the TCA cycle, the serine cycle, and the EMC pathway has to be controlled, in a still-unknown way, to allow for both sufficient energy generation and assimilation of carbon for biomass production. Given the increased expression of genes involved in both glycolysis and gluconeogenesis, one intriguing possibility would be that malate and/or oxaloacetate, generated via the TCA cycle, is first decarboxylated by phosphoenolpyruvate carboxykinase to form phosphoenolpyruvate, which is then converted to 2-phosphoglycerate by enolase, as suggested for growth of *M. extorquens* AM1 on acetate (41). 2-Phosphoglycerate could then be converted to either pyruvate via phosphoenolpyruvate (for amino acid production), glyceraldehyde-3-phosphate (for transfer into the pentose phosphate pathway), or to 6-carbon sugars (for formation of purines and pyrimidines). Such a pathway would allow strain SB2

to overcome its apparent inability to form pyruvate directly from acetyl-CoA produced from ethanol oxidation.

In addition, the expression of genes associated with the pentose phosphate pathway also increased in ethanol-grown cultures, suggesting that this pathway plays a key role in the growth of *Methylocystis* sp. strain SB2 with multicarbon compounds. In any event, given the genes and pathways detected and expressed in *Methylocystis* sp. strain SB2, the required mechanisms of metabolic control appear to be quite complex, and further work, particularly involving metabolomics, will be required to completely unravel the pathways by which multicarbon compounds are assimilated and their regulation.

Tight regulation of metabolic pathways is further suggested by the increased expression of several genes in ethanol-grown cultures that code for proteins involved in the reversible conversion of formyl-H<sub>4</sub>F to formate (*fffL1* and *fffL2*), conversion of formate to carbon dioxide (*fdsC*), and PQQ synthesis (*pqqD* and *pqqE*). It is well known that methylophilic organisms employing the serine cycle, as well as the alphaproteobacterial methanotroph *Methylosinus trichosporium* OB3b, have both the tetrahydromethanopterin and the tetrahydrofolate-linked formaldehyde oxidation pathways (36, 42), and it has been previously suggested that formate (and not formaldehyde) may be the entry point for carbon into the serine cycle after reduction to methylene-H<sub>4</sub>F (42). It may be that some carbon from ethanol flows in this direction, but the finding that some genes involved in formate conversion are upregulated in ethanol-grown cultures of *Methylocystis* sp. strain SB2 is unusual and cannot be definitively explained at this time. The upregulation of some genes involved in PQQ synthesis is also surprising, although it has been shown that some forms of ethanol dehydrogenase have a PQQ cofactor (43, 44). One might speculate that *Methylocystis* sp. strain SB2 employs a similar mechanism for the conversion of ethanol to acetate.

A detailed functional genomics comparison of *Methylocystis* sp. strain SB2 to other closely related *Methylocystis* strains not found to be facultative will be of interest to investigate whether mechanisms for assimilation of multicarbon compound assimilation exist in obligately methanotrophic *Methylocystis* strains. A preliminary comparative genomic analysis of the available genomes of *Methylocystis* sp. strain SB2 and of the obligate methanotrophs *Methylocystis rosea* SV97<sup>T</sup>, *Methylocystis* sp. strain SC2, and *Methylocystis* sp. strain Rockwell (ATCC 49242) (45–47), using the suite of bioinformatic tools of the MicroScope online platform (48), indicates that the TCA cycle and EMC pathway are complete in all of these strains (data not shown). We also investigated the complement of genes that were common to *Methylocystis* sp. strain SB2 and strains of *Methylobacterium extorquens* able to utilize multicarbon compounds (AM1 and PA1), but not found in other obligately methanotrophic *Methylocystis* strains, to evaluate whether any such differences could help explain the facultative nature of *Methylocystis* sp. strain SB2. Only nine such genes were found, of which five encode proteins of unknown function and four have putative functions, i.e., a presumed RNase, an HAD-superfamily hydrolase, and two genes that appear to be similar to genes in a circadian clock gene cluster (data not shown). These findings suggest that gene content is not the main issue for facultative methanotrophy. Rather, it may be that alternative pathways for carbon assimilation are either poorly or not expressed in some methanotrophs, thus not allowing the use of multicarbon compounds to support growth in these strains. Alterna-

tively, it may be that such methanotrophs can utilize multicarbon compounds for growth but that appropriate growth conditions that sufficiently enhance expression of required pathways have yet to be identified. For example, it has recently been shown that expression of the high-affinity form of the pMMO, but not the low affinity form, was downregulated in *Methylocystis* strain SC2 in the presence of elevated concentrations of ammonium (49). It may be that the presence of ammonium, or more generally the availability of nitrogen, also affects the expression of alternative carbon assimilation pathways. Such data will be especially interesting to collect in the future. As a recent example, some alphaproteobacterial methanotrophs members of the alpha upland soil cluster, known to have high affinities for methane, can assimilate acetate using an as-yet-unknown pathway (50). It may be that some methanotrophs benefit from oxidizing multicarbon compounds, even if these compounds cannot serve as sole growth substrates, via the limited expression of pathways such as the TCA cycle and EMC pathway.

In summary, we have presented here the annotated genome of the facultative methanotroph *Methylocystis* sp. strain SB2 and also reported for the first time the transcriptome of a facultative methanotroph grown on a multicarbon compound. It appears from these genomic and transcriptomic analyses that the EMC pathway is integrated with the TCA cycle and the glycolysis/glucosoneogenesis pathway to enable facultative growth of *Methylocystis* sp. strain SB2. An effective coordination of these pathways likely involves an unusual regulatory network that now requires to be elucidated. Preliminary analyses suggest that obligate versus facultative methanotrophy does not appear to be due to lack of genes for an alternative carbon assimilation pathway. Closer examination of the mechanisms by which *Methylocystis* sp. strain SB2 and other facultative methanotrophs utilize multicarbon substrates and the means by which these microbes control the expression of such pathways will enhance our understanding of how methanotrophs survive *in situ*, where substrate and nutrient conditions can fluctuate significantly. Such studies will clarify the role that these intriguing microorganisms play in the global carbon cycle, particularly how methanotrophs may respond to changing climatic conditions that are likely to alter the availability of substrates and nutrients.

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