Autotrophic Methanotrophy in Verrucomicrobia: *Methylacidiphilum fumariolicum* SolV Uses the Calvin-Benson-Bassham Cycle for Carbon Dioxide Fixation^{∇†}

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Genome data of the extreme acidophilic verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* strain SolV indicated the ability of autotrophic growth. This was further validated by transcriptome analysis, which showed that all genes required for a functional Calvin-Benson-Bassham (CBB) cycle were transcribed. Experiments with ¹³CH₄ or ¹³CO₂ in batch and chemostat cultures demonstrated that CO₂ is the sole carbon source for growth of strain SolV. In the presence of CH₄, CO₂ concentrations in the headspace below 1% (vol/vol) were growth limiting, and no growth was observed when CO₂ concentrations were below 0.3% (vol/vol). The activity of the key enzyme of the CBB cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), measured with a ¹³C stable-isotope method was about 70 nmol CO₂ fixed · min⁻¹ · mg of protein⁻¹. An immune reaction with antibody against the large subunit of RuBisCO on Western blots was found only in the supernatant fractions of cell extracts. The apparent native mass of the RuBisCO complex in strain SolV was about 482 kDa, probably consisting of 8 large (53-kDa) and 8 small (16-kDa) subunits. Based on phylogenetic analysis of the corresponding RuBisCO gene, we postulate that RuBisCO of the verrucomicrobial methanotrophs represents a new type of form I RuBisCO.

Methanotrophs are a unique group of microorganisms within the methylotrophs that oxidize methane (CH₄) to carbon dioxide (CO₂). Until 2007, the phylogenetic distribution of the aerobic methanotrophs was limited to the α and γ subclasses of the proteobacteria (16). In 2007, novel thermoacidophilic aerobic methanotrophs were discovered in geothermal areas in New Zealand, Russia, and Italy (9, 18, 23). These methanotrophs represented a distinct phylogenetic lineage within the *Verrucomicrobia*, for which the genus name *Methylacidiphilum* was proposed (22).

Recently, methanotrophy was discovered in a member of the NC10 phylum. It was shown that “*Candidatus* Methylomirabilis oxyfera,” enriched under strict anoxic conditions, produces its own oxygen from nitrite (12). This oxygen is then used for CH₄ oxidation in a biochemical pathway comparable to those of aerobic methanotrophs.

During the aerobic oxidation of CH₄ and methanol by proteobacterial methanotrophs, formaldehyde is produced. This central metabolite can be further oxidized to CO₂ or directly assimilated via intermediates of the central metabolism. Based on the pathway used for formaldehyde assimilation, methanotrophs were divided into type I and type II. Type II metha-

notrophs use the serine pathway, in which formaldehyde and CO₂ are utilized in a one-to-one ratio to produce acetyl coenzyme A (acetyl-CoA) for biosynthesis (8), while type I methanotrophs use the ribulose monophosphate pathway for the assimilation of formaldehyde to form glyceraldehyde-3-phosphate as an intermediate of central metabolism (16). In the latter pathway, all cellular carbon is assimilated at the oxidation level of formaldehyde. Genome data of some proteobacterial methanotrophs (*Methylococcus capsulatus* Bath, *Methylocella silvestris* BL2 [7, 31]) and nonproteobacterial aerobic methanotrophs (*Methylacidiphilum infernorum* V4, *Methylacidiphilum fumariolicum* SolV, and “*Candidatus* Methylomirabilis oxyfera” [12, 17, 22]) revealed the presence of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), the key enzyme of the Calvin-Benson-Bassham (CBB) cycle. *M. capsulatus* Bath was found to contain RuBisCO in an active form (27), and genome analysis suggested that a variant of the CBB cycle may operate (19, 31). Although hydrogen seems to support moderate growth with CO₂ on agar plates for *M. capsulatus* Bath and some other methanotrophs (3), autotrophic growth in liquid cultures has not been reported. Marker exchange mutagenesis deleting the genes encoding RuBisCO may give definite answers on the exact role of RuBisCO, but unfortunately, a good genetic system for manipulation of these bacteria is lacking.

Analyses of the complete genome sequence of *M. infernorum* strain V4 (17) and a draft genome of *M. fumariolicum* strain SolV showed that these two verrucomicrobial methanotrophs lack the key enzymes for both the ribulose monophosphate and

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serine pathways (22). However, in this study, we show that a complete set of genes encoding the enzymes of the CBB cycle are present, which suggests that these methanotrophs may be able to fix CO₂, probably using CH₄ mainly as an energy source. The CBB cycle has been associated with a large use of ATP per mole of CO₂ fixed (8) and was thus never considered to be a likely pathway to support growth on CH₄. In the present paper, we show, by applying ¹³CH₄ or ¹³CO₂ in growth experiments, that CO₂ is the only carbon source for *M. fumariolicum* strain SolV during growth on CH₄. With a transcriptome study of strain SolV, we show that all genes necessary for a complete CBB cycle are transcribed. The large and small subunits of RuBisCO turned out to be highly expressed. In addition, we developed a novel ¹³C stable-isotope enzyme assay to demonstrate the activity of RuBisCO.

MATERIALS AND METHODS

Organism and medium composition for growth. The *M. fumariolicum* strain SolV used in this study was originally isolated from the volcanic region Campi Flegrei, near Naples, Italy (23). The composition and preparation of the growth medium were described previously (20).

Transcriptome analysis. The available draft genome of strain SolV (23) was improved by adding data (30×10^6 75-nucleotide reads) from an Illumina sequencing run. Genes encoding the enzymes of the CBB cycle were identified by BLAST searches, and their DNA and protein sequences were used for transcriptome analysis. Exponentially growing cells were harvested by centrifugation, 3.1 mg (dry weight) cells was used for isolation of mRNA, and subsequent synthesis of cDNA (328 ng) was done as described before (12). The cDNA was used for single-end Illumina sequencing (12). Expression analysis was performed with the RNA-Seq analysis tool from the CLC Genomics Workbench software (version 4.0; CLC bio, Aarhus, Denmark).

Gas and protein analysis. Gas samples (100 µl) were analyzed for CH₄ and CO₂ on an Agilent series 6890 gas chromatograph (GC) equipped with Porapak Q and molecular sieve columns and a thermal conductivity detector (13). To quantify the molecular masses for ¹²CH₄ and ¹³CH₄ (16 and 17 Da) and for ¹²CO₂ and ¹³CO₂ (44 and 45 Da), the same gas chromatograph was coupled with a mass spectrometer (GC-MS) (Agilent 5975C GC MSD; Agilent, Santa Clara, CA) (12). All gas concentrations are given in volume percentages (volume/volume). Protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands).

¹³CO₂ IRMS analysis. The ¹³CO₂ ratio of biomass samples was determined using isotope ratio mass spectrometry (IRMS). The cells were harvested by centrifugation, and the pellet was washed with acidified water (pH 3) and dried overnight in a vacuum oven at 70°C. The dried material (about 0.04 mg) was analyzed on a Thermo Fisher Scientific EA 1110 CHN element analyzer coupled with a Finnigan DeltaPlus mass spectrometer.

Batch cultivation. The effect of CO₂ concentration on the growth of strain SolV was studied in batch cultures using 1-liter serum bottles containing 50 ml of medium and sealed with red butyl rubber stoppers. Incubations were performed in duplicate and at 55°C with shaking at 180 rpm, and mixtures contained 5% CH₄ and air as the only source of O₂ and CO₂. To remove dissolved CO₂ originating from the inoculum, the cell suspension was flushed with sterile air for 10 min before inoculation.

¹³CH₄ experiments were done in 150-ml serum bottles containing 10 ml of culture medium, 40% CO₂, and 5 ml of ¹³CH₄ (99 atom% ¹³C; Sigma-Aldrich). The bottles were inoculated with 0.25 ml of a culture of strain SolV. Initial and final gas concentrations and amounts, as well as mass ratios, were verified by gas chromatographic analysis using a pressure lock syringe. Recovery of ¹³C was calculated from the ¹³C/¹²C ratio of CO₂ and the total amount (moles) of gases in the bottle. For calculating CO₂ in the liquid phase, a partition coefficient of 1.066 (gas/liquid ratio) at 21°C was assumed (32). At the end of the experiment, the biomass was used for the IRMS analysis.

Chemostat cultivation. ¹³CH₄- and ¹³CO₂-labeling experiments were performed in continuous chemostat cultures under N₂-fixing conditions as described before (20). In the first experiment, ¹³CH₄ (99 atom% ¹³C; Sigma-Aldrich) replaced the unlabeled CH₄ in the inlet gas mixture of the chemostat. After a steady state was obtained, the ¹³C-labeling percentage of the gases in the outlet of the chemostat was determined. The ingoing gas now contained 4.7% ¹³CH₄

and 3.5% CO₂ at a flow rate of 10.7 ml · min⁻¹. The outgoing gas (10.3 ml · min⁻¹) contained 2.5% ¹³CH₄, 3.1% ¹²CO₂, and 2.2% ¹³CO₂.

In the second experiment, ¹³CO₂ replaced the unlabeled CO₂. ¹³CO₂ was prepared by pumping a 0.6 M NaH¹³CO₃ (99% ¹³C) solution into a stirred solution (450 ml) of 1.2 M HCl in a closed 500-ml serum bottle. The argon gas supply of the chemostat was led via the headspace of this bottle, and once the ¹³CO₂ concentration was constant, it was supplied into the chemostat. The ingoing gas contained 4.6% CH₄, 0.07% ¹²CO₂, and 3.6% ¹³CO₂ at a flow rate of 10.4 ml · min⁻¹. The outgoing gas, with a flow rate of 10.3 ml · min⁻¹, contained 2.3% CH₄, 1.95% ¹²CO₂, and 3.2% ¹³CO₂. During the experiments, samples of chemostat culture were collected and used for the IRMS analysis of the biomass.

Assuming that carbon in new biomass is labeled according to the label percentage of the CO₂ present (X^{CO_2}) in the chemostat (i.e., gas outlet), the ¹³C label percentage of biomass in the chemostat will develop over time according to the formula: $X^{CO_2} \times (1 - e^{-Dt})$, in which D represents the dilution rate (h⁻¹) of the reactor and t represents time (h).

The "old" biomass in the chemostat at the moment that we started the calculations washed out according to the formula $X^{old} \times (e^{-Dt})$. X^{old} is the ¹³C label percentage of biomass of the first sample taken, at the moment that the ¹³CO₂ gas concentrations in the outlet reached steady state. This value was still close to the natural abundance of ¹³C in the biomass, as only a little ¹³CO₂ was incorporated. The sum of new and old biomass percentages was taken as the predicted average ¹³C label percentage of the chemostat biomass in the course of time during the supply of labeled gases: $X_t = X^{CO_2} \times (1 - e^{-Dt}) + X^{old} \times (e^{-Dt})$.

Recovery calculation of ¹³C from ¹³CH₄ in CO₂ and biomass. The ¹³C recovery in biomass (see Table S1 in the supplemental material) was calculated using the following formula: mole fraction of formaldehyde $\times 0.34 \times 100 +$ mole fraction of CO₂ $\times 0.34 \times 42$. In this formula, the mole fraction refers to the incorporation ratio of formaldehyde and CO₂ into biomass, depending on the carbon assimilation pathway. The values 100 and 42 refer to the labeling percentages of formaldehyde and CO₂, respectively. The value 0.34 refers to the biomass produced according to the observed stoichiometry of CH₄ oxidation (see Results).

Cell extracts. Cells of an exponentially growing culture (9 liters, optical density at 600 nm [OD₆₀₀] = 0.56) were collected by centrifugation (4,000 $\times$ g, 4°C, 10 min) and washed two times with 70 ml buffer {25 mM PIPES [piperazine-*N,N'*-bis-(2-ethanesulfonic acid)]-NaOH, pH 7.5}. Finally, the cell pellet (9.6 g [wet weight]) was resuspended in 10 ml of PIPES-NaOH buffer. To this suspension (pH 7), one tablet of protease inhibitor cocktail (Boehringer, Mannheim, Germany) and 1 mg DNase I were added. Cells were broken by passing the cell suspension 3 times through a French press at 20,000 lb/in². Unbroken cells and cell debris were removed from the resulting crude extract by centrifugation at 12,000 $\times$ g for 20 min (4°C, Sorvall SS-34 rotor). This resulted in a turbid supernatant, with increasing turbidity toward the bottom of the tube. The turbid cell extract was centrifuged again at 48,000 $\times$ g for 40 min (4°C). This resulted in clear reddish supernatant and a yellowish pellet. The clarified cell extract obtained was further ultracentrifuged, using a Sorvall Discovery with a 70.1 Ti rotor (150,000 $\times$ g, 4°C, 60 min). This resulted in clear reddish supernatant and a tiny yellow transparent pellet.

Protein gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE were performed using a Mini-Protein 3 cell (Bio-Rad) according to the method of Laemmli (21). Molecular weight standards were obtained from Fermentas and Invitrogen. For SDS-PAGE, protein samples (25 µg) were incubated at 95°C for 10 min in loading buffer (6.5% [vol/vol] glycerol, 0.016 M Tris-HCl [pH 6.8], 0.5% [wt/vol] SDS, 0.003% [wt/vol] bromophenol blue, and 1.3% [vol/vol] β-mercaptoethanol). For native PAGE, loading buffer without SDS and β-mercaptoethanol was used. Proteins in the gel were stained overnight with Coomassie brilliant blue G-250.

Protein bands from native gels were cut out and further analyzed by SDS-PAGE; bands (8.5 mm³) were incubated in 100 µl SDS-PAGE loading buffer at 95°C for 10 min and incubated further overnight at 4°C with gentle shaking. The eluted proteins were 10-fold concentrated and washed several times with Tris-HCl buffer (0.5 M, pH 6.8) using a 3K Vivaspinn column (Sartorius Stedim Biotech) before being loaded on the SDS-PAGE gel.

Molecular mass determination of RuBisCO. For mass determination of RuBisCO, native PAGE (see above) and size exclusion chromatography were applied. Clarified cell extract of strain SolV was passed through a column (16 mm by 130 cm) of Sephacryl S-300 HR (VWR). The column was pre-equilibrated with 20 mM phosphate buffer-100 mM NaCl (pH 7.2). After loading, the column was eluted with the same buffer at 0.5 ml · min⁻¹, and column effluent was monitored at 280 nm. Protein fractions were collected using an automated fraction collector. Peak fractions were then subjected to SDS-PAGE analysis. Calibration proteins obtained from Sigma were albumin egg (45 kDa), albumin bovine (66 kDa),

alcohol dehydrogenase (150 kDa), apoferritin (443 kDa), thyroglobulin (669 kDa), and blue dextran (2,000 kDa).

MALDI-TOF MS analysis. Identification of the protein bands on the SDS-PAGE gels was performed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Gel spots (about 5 mm³) were destained (solutions contained acetonitrile [30, 50, and 100%, vol/vol], diluted with 25 mM ammonium bicarbonate [NH₄HCO₃]), and dried, and the proteins were in-gel digested with 5 µl of trypsin (the solution contained 15 ng trypsin · µl⁻¹ in a mixture of 25 mM NH₄HCO₃ and 5 mM *n*-octyl-β-D-glucopyranoside) on ice for 1 h. The excess trypsin solution was removed, and the gel spot was covered with a solution containing 25 mM NH₄HCO₃ and 5 mM *n*-octyl-β-D-glucopyranoside and was incubated overnight at 37°C. The peptides were extracted by adding 4 µl of a mixture containing (all volume/volume) 50% acetonitrile, 0.5% trifluoroacetic acid (TFA), and 49.5% 5 mM *n*-octyl-β-D-glucopyranoside, followed by 1 h of incubation at room temperature. The liquid was sonicated for 2 min in a water bath and dried in a vacuum centrifuge. The peptides were solved in 4 µl of TFA (0.1%). The dissolved peptides (3 µl) were mixed with 0.3 µl of matrix solution (20 mg α-cyano-4-hydroxycinnamic acid in 0.5 ml 0.1% TFA and 0.5 ml acetonitrile), spotted on a stainless steel target plate, and analyzed on a Biflex III MALDI-TOF mass spectrometer (Bruker, Bremen, Germany) operating in the reflectron mode. Data analysis was performed using X-TOF software (Bruker, Bremen, Germany), and the Mascot search engine (Matrix Science Ltd., Boston, MA) was used to identify the proteins.

Immunoblotting. The proteins from SDS-PAGE and native-PAGE gels were transferred to a nitrocellulose membrane by electroblotting at 100 mA for 45 min. The obtained blots were then prepared for immune reaction as previously described (30). The polyclonal anti-RuBisCO, raised in a rabbit using a synthetic peptide, was used as the primary antibody (product AS03 037; Agrisera, Vännäs, Sweden). This antibody was 3,300-fold diluted in blocking buffer. The monoclonal mouse anti-rabbit IgG alkaline phosphatase conjugate (Sigma catalog no. A2556) was used as the secondary antibody.

Activity assay for RuBisCO. (i) Carboxylase activity. As an alternative to the radioisotope method, we developed a stable-isotope method for measuring RuBisCO activity. This was based on the incorporation of ¹³CO₂ into 3-phosphoglycerate (3-PGA) and subsequent destruction of 3-PGA by acid permanganate oxidation. The ¹³CO₂ liberated was quantified by GC-MS as described above. The destruction of 3-PGA to liberate the carboxyl group was performed analogously to the destruction of lactate with permanganate and phosphoric acid (H₃PO₄), as previously described (4), which produces CO₂ and acetate. We used 1% potassium permanganate (KMnO₄) in 0.1 M H₃PO₄ instead of 5% KMnO₄ in 0.07 M H₃PO₄, and the temperature was lowered from 80°C to 50°C. Under such conditions, 15 min of incubation yielded 1 mol of CO₂ per mol of 3-PGA. This was verified by a series of 0.1 to 2 µmol of 3-PGA in 3-ml Exetainer vials (Labco Limited, High Wycombe, United Kingdom). Ice-cold KMnO₄ (500 µl) was added, and the vial was immediately closed with a screw cap with a rubber seal and incubated at 50°C. A linear relation between the amount of 3-PGA and CO₂ produced was measured (GC-MS analysis). Under stronger oxidative conditions, the initial production of CO₂ was more rapid but followed by a continuous slow evolution of CO₂. Apparently, the oxidation product of 3-PGA is prone to further oxidation. The final destruction conditions applied were 0.1% KMnO₄ in 0.1 M H₃PO₄ for 25 min at 50°C.

To limit the background production of CO₂ and increase the sensitivity of the method, we used 20 mM phosphate buffer in the assay mixtures to replace the commonly used organic buffers. The only organic material present in the assay mixture now originated from the RuBisCO-containing samples. Apart from some CO₂ from air, the major part of CO₂ (>80%) present in the Exetainer vial after destruction resulted from the RuBisCO assay samples. As it reflects the sample amount, this CO₂ could act as an internal standard when the ratio of ¹³CO₂ to ¹²CO₂ was taken. This resulted in better time curves than those obtained using the absolute amounts of ¹³CO₂ produced in the destruction vial. Moreover, the GC-MS response for CO₂ was varied over time, while the ratio of ¹³CO₂ to ¹²CO₂ was very constant. The ratio multiplied by the average total amount of CO₂ in the destruction bottles from a time series was used to calculate the amount of ¹³CO₂ produced. Time series were linear for 2 min. The standard RuBisCO assay was performed in a 2-ml vial (VersaVial; Alltech) containing 200 µl of Mg-phosphate buffer (20 mM potassium phosphate, 10 mM MgCl₂, pH 6.9). Cell extracts diluted in 25 mM PIPES-NaOH, pH 7.5, to a total volume of 25 µl and NaH¹³CO₃ (99% ¹³C) (20 µl of a 100 mM stock) were added. The vial was closed immediately with a thin rubber cap (Alltech) to prevent the loss of CO₂. The final pH in this mixture was pH 7.25 (at 50°C), and the CO₂ gas concentration was calculated to be 1.5%. The vial was preincubated for 10 min at 50°C in a water bath, and the RuBisCO activity assay was started by adding 5 µl of stock solution of ribulose-1,5-bisphosphate (25 mM) by a syringe through

the rubber cap. The mixture was vortexed for 2 s and incubated further at 50°C. Samples of 50 µl were taken through the stopper by a syringe every 30 s for 2 min. Samples were mixed with 20 µl of 0.5 M HCl in 3-ml Exetainer tubes and dried under vacuum at 45°C. After the destruction with 0.5 ml permanganate solution as described above and cooling down to room temperature, the vials were stirred on a vortex device to allow CO₂ to equilibrate. The gas phase was measured for ¹³CO₂ and ¹²CO₂ by GC-MS analysis.

(ii) Oxygenase activity. The oxygenase reaction of RuBisCO was assayed in a Strathkelvin RC350 respiration cell at a working volume of 0.7 ml. The assay buffer contained 50 mM HEPES and 10 mM MgCl₂ (pH 7.2) and was air saturated at 50°C. Unlabeled NaHCO₃ was added in the range of 0 to 10 mM, and after the addition of cell extract (12,000 × g supernatant, 1 mg protein), the background O₂ consumption was followed for 10 min before the addition of ribulose 1,5-bisphosphate (0.4 mM). After this addition, consumption rates increased immediately but then slowed down.

pH optimum for RuBisCO assay. The effect of pH on the activity was determined by setting the initial pH value of the Mg-phosphate buffer at values of 6.45, 6.6, 6.85, and 7.05. NaH¹³CO₃ was added at 7, 9, 12, and 16 mM final concentrations, respectively. The final pH was verified at 50°C in an upscaled situation (10-ml tubes) to be 7.0, 7.22, 7.5, and 7.75, respectively. Taking a dissociation constant (pK_a) of 6.1 for carbonic acid (H₂CO₃) at 50°C, the CO₂ percentage in the gas phase was calculated to be between 1.4 and 1.7%.

Phylogenetic analysis. Representative *cbhL* and *cbhS* sequences, encoding the large and small subunits of RuBisCO, were obtained from GenBank and aligned using MUSCLE (10) in MEGA 5.0 (www.megasoftware.net). Phylogenetic trees were calculated using the neighbor-joining method (25) with 1,000 bootstraps (14) to infer the evolutionary relationship. Positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). The Dayhoff matrix-based method was used to compute the evolutionary distances.

Nucleotide sequence accession numbers. The *M. fumariolicum* SolV genes reported in this study were extracted from the draft genome and submitted to GenBank under accession numbers JF706245 to JF706259 and JF714482.

RESULTS

Growth of strain SolV at different CO₂ concentrations. Standard batch incubations of *M. fumariolicum* strain SolV with CO₂ and CH₄, both 5%, resulted in exponential growth within 1 day. However, incubations without CO₂ did not result in any growth on CH₄, even after 40 days of incubation, indicating that CO₂ was necessary for growth. This dependency was further investigated in a series of batch incubations in 1-liter bottles with air as the only source of CO₂ (0.04%) and with 5% CH₄. In all incubations, we observed CO₂ production from CH₄ during the initial period, at a rate directly depending on the inoculum size. As the CH₄ oxidation was not coupled to growth, the CO₂ production rate decreased with time (see Fig. S1A in the supplemental material). Growth resumed only after CO₂ had reached levels above 0.3% (Fig. S1B and S1C). This indicated that the growth of strain SolV is dependent on a minimum concentration of CO₂ in the headspace.

In order to circumvent endogenous CO₂ production and to test the effect of nongrowth conditions on the viability of strain SolV, a culture was starved for CH₄ for 35 days (at 55°C). When CH₄ was subsequently added, CO₂ production was hardly measurable, and no growth was observed for 7 days. Thereafter, when CO₂ was added, growth resumed within 3 days (Fig. S2).

From various growing cultures of strain SolV, the growth rate was determined at different prevailing CO₂ concentrations. This was done for time intervals of about 6 h, in which CO₂ concentrations in the headspace did not change more than 15%. The results clearly showed the dependency of the growth rate on CO₂ concentration (Fig. 1). The lowest CO₂ concentration at which growth was observed was 0.3% (dou-

FIG. 1. Doubling time of *M. fumariolicum* strain SolV at different CO_2 concentrations (triangles). The growth rates of various growing cultures of strain SolV were determined at different prevailing CO_2 concentrations. This was done for time intervals of about 6 h, in which CO_2 concentrations in the headspace did not change more than 15%.

bling time, about 80 h), and the growth rate increased toward its maximum (doubling time, 10 h) at 1% CO_2 .

^{13}C -labeling experiments in chemostat cultures. Labeling experiments were performed to determine whether CO_2 was the only carbon source in *M. fumariolicum* strain SolV. A CH_4 -limited chemostat culture at a dilution rate (D) of 0.016 h^{-1} was used for this purpose. At steady state, gas concentrations in the inlet and outlet were analyzed, and the CH_4 consumption rate ($\text{ml} \cdot \text{min}^{-1} \pm$ standard error of the mean [SEM]) was calculated to be 0.254 ± 0.009 ($n = 4$). The CO_2 production rate ($\text{ml} \cdot \text{min}^{-1} \pm$ SEM) was 0.167 ± 0.01 ($n = 4$). This results in the following stoichiometry for carbon: $1 \text{ CH}_4 \rightarrow 0.66 \text{ CO}_2 + 0.34 \text{ biomass (CH}_2\text{O)}$. The biomass carbon can be derived either from CH_4 oxidation intermediates or from CO_2 .

In the first experiment, the unlabeled CH_4 supply of the chemostat was changed to $^{13}\text{CH}_4$. The labeling percentages of the gases in the chemostat headspace reached steady state after 1.5 h (42% ^{13}C for CO_2 and $>99\%$ ^{13}C for CH_4), and the $^{13}\text{CH}_4$ consumption and $^{13}\text{CO}_2$ production rates ($\text{ml} \cdot \text{min}^{-1} \pm$ SEM) were calculated (0.254 ± 0.009 and 0.226 ± 0.002 , respectively), thus resulting in a recovery (mean $\pm$ SEM) of $89\% \pm 3.3\%$ of the $^{13}\text{CH}_4$ consumed.

Even when all $^{13}\text{CH}_4$ consumed is converted into $^{13}\text{CO}_2$ and all biomass is derived from CO_2 , a total recovery in $^{13}\text{CO}_2$ is not expected, as part of the $^{13}\text{CO}_2$ produced in the chemostat is incorporated into the biomass. With the above stoichiometry for carbon and a measured 42% ^{13}C -labeling percentage for CO_2 in the chemostat, still 14% (measured ^{13}C -labeling percentage $\times$ moles of biomass) of the label will be incorporated into the biomass, and so only 86% can be recovered as $^{13}\text{CO}_2$ (see Table S1 in the supplemental material).

The measured ^{13}C -labeling percentage of CO_2 in the chemostat was in good agreement with the calculated value of about 40%. This was calculated on the basis of the unlabeled CO_2 in the inlet gas and the amount of $^{13}\text{CH}_4$ converted into $^{13}\text{CO}_2$.

The increase in the ^{13}C percentage of the biomass in the

FIG. 2. (A) Predicted percentage of ^{13}C -labeled biomass, for an *M. fumariolicum* strain SolV chemostat culture supplied with $^{13}\text{CH}_4$ (dashed line) and $^{13}\text{CO}_2$ (solid line). (B) Measured percentages of ^{13}C -labeled biomass in the first 10 h for a SolV culture supplied with $^{13}\text{CH}_4$ (circles) and $^{13}\text{CO}_2$ (squares), combined with the predicted percentages.

chemostat culture over the course of time (Fig. 2A) was predicted under the assumption that all biomass was derived from CO_2 , at the current ^{13}C label percentage of 42%. Before the reactor was supplied with the ^{13}C label, the percentage was 1.1, close to the natural abundance of ^{13}C in CO_2 gas (1.17%). Measured values of the increase of the ^{13}C percentage of the biomass followed exactly the predicted curve (Fig. 2B).

The experiment was repeated with $^{13}\text{CO}_2$ in the inlet gas of the chemostat and unlabeled CH_4 . The labeling percentage of CO_2 in the outlet increased to 62% after 1.5 h (steady state). Unlabeled CO_2 originated from oxidation of the unlabeled CH_4 . Again, the increase of the ^{13}C percentage of the biomass followed the predicted curve (Fig. 2B), with the assumption that CO_2 was the only carbon source.

Within the first hours of the experiment, the increase of the ^{13}C label percentage into biomass was assumed to be linear, and the ^{13}C incorporation rate of the predicted curve for the $^{13}\text{CO}_2$ experiment was calculated to be 1.5 times higher than the predicted curve for the $^{13}\text{CH}_4$ experiment (Fig. 2B), in accordance with the 1.5-times-higher label percentage (62% versus 42%).

^{13}C -labeling experiments in batch cultures. The labeling study with $^{13}\text{CH}_4$ in the chemostat culture always resulted in high $^{13}\text{CO}_2$ production from $^{13}\text{CH}_4$ oxidation. This could have been circumvented by sparging much larger gas volumes through the reactor. This option was not used, since this is technically not feasible in our chemostat setup; instead, labeling experiments in two batch cultures with a high concentration (40%) of unlabeled $^{12}\text{CO}_2$ and a relatively limited amount (3.2%) of $^{13}\text{CH}_4$ were performed. If all $^{13}\text{CH}_4$ was converted to $^{13}\text{CO}_2$, the CO_2 pool would be labeled for an additional 7.4% only ($3.2\%/40\% \times 100$). Concentrations of the gases and $^{13}\text{C}/^{12}\text{C}$ mass ratios of CO_2 at the start of and after growth, when 80 to 95% of the added CH_4 was converted, were carefully

FIG. 3. $^{13}\text{CO}_2$ incorporation into 3-PGA and its subsequent destruction in closed vials with acid permanganate solution. $^{13}\text{CO}_2$ concentrations quantified in the vial represent *M. fumariolicum* strain SolV RuBisCO activity in turbid cell extract ($12,000 \times g$ supernatant [triangles]) and in the pellet after centrifugation at $48,000 \times g$ (squares).

measured. It could be calculated that in both bottles, at least 96% of the label of $^{13}\text{CH}_4$ was recovered as $^{13}\text{CO}_2$. This pointed to complete oxidation of CH_4 and the sole use of CO_2 as the carbon source.

If we assume that carbon assimilation by fixation of CO_2 and that 0.34 mol CO_2 incorporated into the biomass per mole CH_4 converted, then close to 2% of the total ^{13}C produced would have been incorporated. This makes the total ^{13}C recovery at least 98%. At the end of the experiment, the $^{13}\text{C}/^{12}\text{C}$ ratio of the biomass in both bottles was determined by IRMS. The analysis showed that 4.6% of the biomass carbon was ^{13}C labeled. This percentage was in very good agreement with the average measured ^{13}C percentage of the CO_2 during growth (1.2% at the start of and 7.7% at the end of growth, so an

average of 4.5%) and again confirms that CO_2 is the main carbon source.

RuBisCO in strain SolV. The labeling experiments described above showed that *M. fumariolicum* strain SolV fixes CO_2 , and the draft genome data of strain SolV contained a RuBisCO gene, encoding the key enzyme of the CBB cycle (see below). Therefore, cell extracts of strain SolV were tested for the presence of RuBisCO activity and analyzed by SDS-PAGE (13% gel) and native PAGE (5% gel).

For the activity assay, we developed a method based on the $^{13}\text{CO}_2$ incorporation into 3-PGA and its subsequent destruction in closed vials with acid permanganate solution. $^{13}\text{CO}_2$ concentrations in the vial were quantified on the basis of $^{13}\text{C}/^{12}\text{C}$ ratios as measured by GC-MS.

Activity was proportional to the amount of turbid cell extract added (concentrations of 10 to 250 μg protein per 250 μl assay mixture were tested) and linear for 2 min (Fig. 3). No $^{13}\text{CO}_2$ was incorporated without extract or the substrate ribulose-1,5-biphosphate (0.5 mM). Activity was optimal between pHs 7.2 and 7.5. At pHs 7.7 and 7.0, activity dropped by about 10%. When kept on ice, the activity in extracts was stable for at least a month. The specific activity of the turbid cell extract was 55 to 70 nmol CO_2 fixed $\cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$. The oxygenase activity of RuBisCO measured at an oxygen concentration of around 150 μM was about 4.5 nmol $\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$, and this activity was inhibited by the addition of NaHCO_3 (50% inhibition at 4 mM NaHCO_3 [results not shown]). Virtually all the RuBisCO activity (>90%) appeared to be present in the clarified cell extract ($48,000 \times g$ supernatant). The corresponding pellet contained only very small amounts of RuBisCO activity that could be attributed to a residual soluble fraction (Fig. 3).

When the proteins in the crude extract were analyzed by 13% SDS-PAGE, a consistent pattern of proteins was observed, which did not change upon subsequent centrifuging steps (Fig. 4A). Dominant bands (indicated with arrows) were cut out of the gel and identified by tryptic digestion and MALDI-TOF MS peptide mass analysis. The results showed

FIG. 4. (A) *M. fumariolicum* strain SolV proteins in the crude extract were analyzed by 13% SDS-PAGE after subsequent centrifuging steps. (B) The proteins were then transferred onto a nitrocellulose membrane and tested for the presence of RuBisCO, using a peptide antibody against the large subunit of this enzyme. Lane numbers in both figures are the same. Lane 1, crude extract; lane 2, $12,000 \times g$ supernatant; lane 3, $48,000 \times g$ supernatant; lane 4, $150,000 \times g$ supernatant; lane C, control (crude extract without peptide antibody). The upper and lower bands indicated with arrows correspond to the large and small subunits of RuBisCO, respectively, identified by MALDI-TOF MS analysis.

FIG. 5. (A) *M. fumariolicum* strain SolV proteins in the crude extract were analyzed by 5% native PAGE after subsequent centrifuging steps. (B) The proteins were then transferred onto a nitrocellulose membrane and tested for the presence of RuBisCO, using the peptide antibody against the large subunit of this enzyme. Lane numbers in both figures are identical. Lane 1, crude extract; lane 2, $12,000 \times g$ supernatant; lane 3, $48,000 \times g$ supernatant; lane 4, $150,000 \times g$ supernatant. (C) The native band corresponding to the RuBisCO complex (indicated with an arrow in panel B) was analyzed on a 13% SDS-PAGE gel (lane 1).

that the upper band corresponded to the large subunit of RuBisCO, with a predicted mass of 53,930 Da, and the lower band corresponded to the small subunit of RuBisCO, with a predicted mass of 16,550 Da (Fig. 4A). The pellets obtained after the different centrifugation steps did not contain these bands (data not shown). In addition to the MALDI-TOF MS identification, immunoblotting with an antibody against the RuBisCO large subunit was done, and only the 53,930-Da band specifically reacted with this antibody (Fig. 4B, lanes 1 to 4). After being washed extensively with PIPES-NaOH buffer, pellets of the $12,000 \times g$ and $48,000 \times g$ centrifugation steps (yielding intact cells and membranes) showed only a weak reaction with this antibody. In contrast, after a washing of the $150,000 \times g$ pellet, a clear immune response band remained visible (data not shown), suggesting that RuBisCO is present as a high-molecular-mass complex that partly sediments at this high centrifugation speed. This was verified by analyzing all supernatants on a 5% native-PAGE gel (Fig. 5A). The prominent band at about 536 kDa (Fig. 5A) showed an immunoreaction with the RuBisCO antibody (Fig. 5B). The corresponding protein band from an unstained native gel part was cut out of the gel. Proteins from the gel pieces were denatured, eluted, and loaded on a 13% SDS-PAGE gel. This resulted in only 2 protein bands with molecular weights corresponding to the large and small subunits of RuBisCO (Fig. 5C, lane 1). This was confirmed by the MALDI-TOF analysis and antibody reaction (data not shown). The native mass of RuBisCO was also determined by size exclusion chromatography, which showed a somewhat apparent lower molecular mass of about 482 kDa. Most likely, the native RuBisCO protein complex is composed of 8 small and 8 large subunits, with a calculated molecular mass of 563,840 Da.

Transcriptomics. Analyses of the draft genome of *M. fumariolicum* strain SolV revealed that the key genes needed for an operational ribulose monophosphate pathway, the hexulose-6-P synthase and hexulose-6-P isomerase genes, were absent (23). In addition, the crucial genes encoding key enzymes of the serine pathway, the malyl-coenzyme A lyase and glycerate kinase genes, were not found. However, the genes needed for

a full operational CBB cycle were present. All these genes were also transcribed (Table 1), as was verified by transcriptome analysis of exponentially growing cells, especially the genes encoding the large and small subunits of RuBisCO, which were highly transcribed. These results, together with the physiological and biochemical experiments described above, fully support the conclusion that strain SolV makes use of the CBB cycle to fix CO_2 for carbon assimilation.

Phylogenetic analysis of RuBisCO. RuBisCO enzymes can be grouped according to their structures, catalytic properties, and gene arrangements into types I (A, B, C, and D), II, III, and IV (RuBisCO-like) (2). A neighbor-joining tree (Fig. 6) was constructed using representative *cbbL* sequences of each group. Also, for this analysis, the sequences of three (uncharacterized) mesophilic verrucomicrobial methanotrophs available from GenBank (accession numbers JF706245, JF706246, and JF706247) were added. The verrucomicrobial RuBisCO enzymes formed a distinct group in the phylogenetic tree. The RuBisCO operons in *M. fumariolicum* strain SolV and *M. infernorum* strain V4 are arranged in the gene order *cbbL cbbS cbbX* (see Fig. S3 in the supplemental material), which is typical for type IC (2). In both strains, no genes involved in carboxysome formation were detected. The remaining genes of the CBB cycle (Table 1) are spread through the genome of strain V4, sometimes with two or three CBB cycle genes clustered. BLASTP searches with the *M. fumariolicum* SolV RuBisCO large subunit in environmental data sets did not result in sequences fitting into the distinct verrucomicrobial group.

DISCUSSION

Carbon fixation by methanotrophs has been a topic of research for decades, and the assimilation via the CBB cycle by proteobacterial methanotrophs is unlikely, because CBB is associated with high ATP requirements (8). Although *M. capsulatus* Bath possesses CBB cycle genes (31), physiological evidence for an active CBB cycle is lacking thus far. However,

TABLE 1. Genes involved in the Calvin-Benson-Bassham cycle in *M. fumariolicum* strain SolV^a

Enzyme	Gene name	EC no.	GenBank accession no.	Relative expression mRNA
Ribulose 1,5-bisphosphate carboxylase, large subunit	<i>cbbL</i>	EC 4.1.1.39	JF706253	21.6
Ribulose 1,5-bisphosphate carboxylase, small subunit	<i>cbbS</i>	EC 4.1.1.39	JF706253	14.4
RuBisCO accessory protein CbbX, AAA ATPase	<i>cbbX</i>		JF706253	14.3
Ribulose-phosphate 3-epimerase	<i>rpe</i>	EC 5.1.3.1	JF706259	1.1
Phosphoribulokinase	<i>prkB</i>	EC 2.7.1.19	JF706255	1.8
			JF706253	3.8
Ribose 5-phosphate isomerase B	<i>rpiB</i>	EC 5.3.1.6	JF706256	1.5
Triosephosphate isomerase	<i>tpiA</i>	EC 5.3.1.1	JF714482	1.2
Putative phosphoketolase			JF706257	0.7
Transketolase	<i>tktA</i>	EC 2.2.1.1	JF706253	4.3
Fructose-1,6-bisphosphatase II/sedoheptulose 1,7-bisphosphatase	<i>glpX</i> -SEBP gene	EC 3.1.3.11	JF706249	1.3
		EC 3.1.3.37		
Fructose-1,6-bisphosphatase	<i>fbp</i>	EC 3.1.3.11	JF706254	2.5
Fructose-bisphosphate aldolase, class I	<i>fbaA</i>	EC 4.1.2.13	JF706258	0.5
Fructose-bisphosphate aldolase, class II	<i>fba2</i>	EC 4.1.2.13	JF706254	4.4
Glyceraldehyde-3-phosphate dehydrogenase	<i>gapA</i>	EC 1.2.1.59	JF714482	5.1
Phosphoglycerate kinase	<i>pgk</i>	EC 2.7.2.3	JF714482	1.8
6-Phosphogluconolactonase	<i>nagB</i>	EC 3.1.1.31	JF706252	0.3
6-Phosphogluconate dehydrogenase	<i>gnd</i>	EC 1.1.1.44	JF706256	1.2
Glucose-6-phosphate 1-dehydrogenase	<i>zwf</i>	EC 1.1.1.49	JF706252	1.0
Glucose-6-phosphate isomerase	<i>pgi</i>	EC 5.3.1.9	JF706248	0.4
Fructose-6-phosphate aldolase 1	<i>mipB</i>	EC 4.1.2.-	JF706251	0.8

^a The relative levels of transcription of the genes were obtained by a transcriptome analysis of an exponentially growing liquid culture (see Materials and Methods).

available genome data from verrucomicrobial methanotrophs (17, 23) point to an autotrophic lifestyle for these microbes.

In the present study, the transcriptome analysis of *M. fumariolicum* strain SolV showed that all of the genes for the enzymes necessary for a CBB cycle are expressed, most prominently the gene encoding RuBisCO. The *cbbR* gene (encoding the RuBisCO operon transcriptional regulator) is present, but its expression is low (only 0.1). On the V4 genome, this gene seems to be coupled to nitrate transport and reduction (17). Whether this gene is involved in the regulation of the RuBisCO operon needs to be resolved.

On SDS-PAGE gels, RuBisCO was identified as one of the most dominant proteins in the cell extracts of strain SolV. Using a novel ¹³CO₂ incorporation assay, we showed the high specific activity of RuBisCO (70 nmol CO₂ fixed · min⁻¹ · mg of protein⁻¹). This activity is similar to that reported for other autotrophic bacteria (5) and corresponds well with the maximum specific growth rate of 0.07 h⁻¹ (doubling time, 10 h), assuming that dry cells contain 50% carbon and 70% protein.

As RuBisCO was found only in the clarified cell extract, we conclude that RuBisCO is not densely packed into polyhedral inclusion bodies (carboxysomes), which generally sediment upon centrifugation at 10,000 × *g* and 40,000 × *g* (15, 24). In addition, the genome data did not show any proteins that could encode carboxysomal shell proteins. Carboxysomes are encountered in cyanobacteria and in a limited number of chemolithotrophs like sulfur- or sulfide-oxidizing and nitrifying bacteria (33). These compartments are thought to enhance the concentration of CO₂ for RuBisCO, which has a low affinity for CO₂. Microorganisms, which contain carboxysomes, are able to grow at an ambient CO₂ concentration; however, our batch incubations have demonstrated that strain SolV needs a high CO₂ concentration for growth (above 0.3%), in agreement with a free form of RuBisCO. Furthermore, the volcanic regions from which the verrucomicrobial methanotrophs were

isolated exhibit high CO₂ concentrations (6). In these environments, there is apparently no need to sequester CO₂.

Based on the apparent molecular masses of the native RuBisCO protein and the two subunits, we predict that it forms an octomeric complex (L₈S₈). This form of RuBisCO is typical for all form I RuBisCOs, which are composed of four large-subunit dimers (L₂) with small subunits (S) decorating the top and bottom of the L₈ octomeric core (28).

Phylogenetic analysis and the gene arrangement of the RuBisCOs from two thermophilic *Methylophilum* strains positions them in a separate cluster within form I RuBisCOs, for which we propose the name type IE. Based on gene arrangement, the verrucomicrobial RuBisCO is most closely related to RuBisCO type IC (2). The *cbbX cbbS cbbL* operon arrangement is typical for type IC and type IE and not typical for other form I enzymes. The IC form has medium to low affinity for CO₂, indicating an adaptation to environments with medium to high CO₂ but with O₂ present, and microorganisms having type IC RuBisCO do not possess carboxysomes (2). This verrucomicrobial RuBisCO type has not been detected before by molecular approaches. This can be explained by the mismatches we observed when comparing the *Methylophilum*-type *cbbL* sequence with all the available RuBisCO primers sets (1, 11, 26, 29).

The ¹³C label percentage in the biomasses of both the batch and the chemostat experiments was in complete accordance with the ¹³C label percentage of CO₂ in the cultures and confirms that biomass carbon was derived exclusively from CO₂. If CO₂ is the only carbon source in *M. fumariolicum* strain SolV, it can be anticipated that CH₄ is completely oxidized into CO₂. This was demonstrated by the recovery study of ¹³CH₄ (see Results). If intermediates of CH₄ oxidation (which are fully ¹³C labeled) were incorporated, the recovery of label into CO₂ would have been much lower: about 66% for

FIG. 6. Phylogenetic analysis of the RuBisCO large subunit (CbbL). Representative amino acid sequences were obtained from GenBank. Bootstrap values (1,000 replicates) are shown at the branches. The bar represents 20% sequence divergence. The neighbor-joining tree is drawn to scale; the evolutionary distances were computed using the Dayhoff matrix-based method, and units are the numbers of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 539 positions in the final data set.

the ribulose monophosphate pathway and 79% or 76% for the serine pathway (see Table S1 in the supplemental material).

The autotrophic nature of verrucomicrobial methanotrophs has large consequences for their detection in the environment. The presence of active methanotrophs has been assessed by isotope-based techniques, like stable isotope probing and phospholipid fatty acid labeling, which rely on the incorporation of labeled CH_4 into DNA/RNA or lipids, respectively. Such methods overlook the involvement of these autotrophic methanotrophs, especially in environments with high CO_2 concentrations.

Conclusions. *M. fumariolicum* strain SolV is an autotrophic methanotroph. It fixes CO_2 via the CBB cycle, with CH_4 as the energy source. It uses a non-carboxysome-associated RuBisCO, in agreement with a high requirement for CO_2 . RuBisCOs in verrucomicrobial methanotrophs form a new group most closely related to type IC. The ultimate proof would require knockout and complementation studies, but this has to await the development of a genetic system.

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