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Methenyl-Dephosphotetrahydromethanopterin Is a Regulatory Signal for Acclimation to Changes in Substrate Availability in *Methylobacterium extorquens* AM1

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ABSTRACT

During an environmental perturbation, the survival of a cell and its response to the perturbation depend on both the robustness and functionality of the metabolic network. The regulatory mechanisms that allow the facultative methylotrophic bacterium *Methylobacterium extorquens* AM1 to effect the metabolic transition from succinate to methanol growth are not well understood. Methenyl-dephosphotetrahydromethanopterin (methenyl-dH$_4$MPT), an early intermediate during methanol metabolism, transiently accumulated 7- to 11-fold after addition of methanol to a succinate-limited culture. This accumulation partially inhibited the activity of the methylene-H$_4$MPT dehydrogenase, MtdA, restricting carbon flux to the assimilation cycles. A strain overexpressing the gene (*mch*) encoding the enzyme that consumes methenyl-dH$_4$MPT did not accumulate methenyl-dH$_4$MPT and had a growth rate that was 2.7-fold lower than that of the wild type. This growth defect demonstrates the physiological relevance of this enzymatic regulatory mechanism during the acclimation period. Changes in metabolites and enzymatic activities were analyzed in the strain overexpressing *mch*. Under these conditions, the activity of the enzyme coupling formaldehyde with dH$_4$MPT (Fae) remained constant, with concomitant formaldehyde accumulation. Release of methenyl-dH$_4$MPT regulation did not affect the induction of the serine cycle enzyme activities immediately after methanol addition, but after 1 h, the activity of these enzymes decreased, likely due to the toxicity of formaldehyde accumulation. Our results support the hypothesis that in a changing environment, the transient accumulation of methenyl-dH$_4$MPT and inhibition of MtdA activity are strategies that permit flexibility and acclimation of the metabolic network while preventing the accumulation of the toxic compound formaldehyde.

IMPORTANCE

The identification and characterization of regulatory mechanisms for methylotrophy are in the early stages. We report a nontranscriptional regulatory mechanism that was found to operate as an immediate response for acclimation during changes in substrate availability. Methenyl-dH$_4$MPT, an early intermediate during methanol oxidation, reversibly inhibits the methylene-H$_4$MPT dehydrogenase, MtdA, when *Methylobacterium extorquens* is challenged to switch from succinate to methanol growth. Bypassing this regulatory mechanism causes formaldehyde to accumulate. Fae, the enzyme catalyzing the conversion of formaldehyde to methylene-dH$_4$MPT, was also identified as another potential regulatory target using this strategy. The results herein further our understanding of the complex regulatory network in methylotrophy and will allow us to improve metabolic engineering strategies of methylotrophs for the production of value-added products.

Methylobacterium extorquens AM1 is a facultative methylotroph that is capable of growth on both one-carbon (C_1) compounds, such as methanol and methylamine, and multicarbon compounds, such as succinate. Methanol metabolism is predicted to be limited by reducing power (1–3), requires the cofactor dephosphotetrahydromethanopterin (dH$_4$MPT) (4, 5), uses the serine cycle for assimilation, and involves high carbon flux through toxic intermediates, such as formaldehyde, glyoxylate, and glycine (6). In contrast, multicarbon growth is predicted to be energy limited and involves common heterotrophic pathways, such as the tricarboxylic acid (TCA) cycle and the pentose phosphate pathway (7, 8).

Methanol and methylamine metabolism can be divided into five functional metabolic steps, as follows. (i) The oxidation of methanol or methylamine to formaldehyde is catalyzed by either methanol dehydrogenase (MeDH) or methylamine dehydrogenase, respectively. This NADH-independent reaction occurs in the periplasm, utilizing a *c*-type cytochrome as the initial electron acceptor, which is subsequently oxidized for energy metabolism (9, 10). (ii) Once formaldehyde is incorporated into the cell, it is bound to dH$_4$MPT and oxidized through a series of enzymatic

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steps to produce formate. Formate is an important branch point for the distribution between assimilation and energy production (11). (iii) Part of the formate pool is further oxidized to CO_2 due to the activity of formate dehydrogenases (12, 13). (iv) The remainder of the formate pool undergoes a series of enzymatic steps catalyzing the conversion of formate to methylene-tetrahydrofolate (methylene-H_4F), the intermediate that is incorporated into the assimilation cycles. (v) The main assimilation cycles are the serine cycle, an anapleurotic cycle to regenerate glyoxylate known as the ethylmalonyl coenzyme A (EMC) pathway, and the polyhydroxybutyrate (PHB) pathway, a carbon and energy storage pathway.

M. extorquens is a widespread microorganism often associated with the leaf surfaces of plants (14–17). Methane, isoprene, and methanol are the dominant volatile organic compounds (VOCs) in the atmosphere, emitted at 600 Tg/year, 550 Tg/year, and 273 Tg/year, respectively (18–20). Methanol is produced from cell wall metabolism by pectin methyl esterases in the stomata (21, 22) and is released in a transient burst in the early light hours of the diurnal cycle. Therefore, methylotrophs must be able to respond rapidly to such episodic substrate availability. This acclimation process induces the upregulation of the assimilation cycles of the methylotrophic network while preventing the accumulation of toxic intermediates.

Recently, a system-level study addressed the response and metabolic acclimation of *M. extorquens* AM1 after addition of methanol to a succinate-limited chemostat culture. Once methanol was added, there was a transient period of metabolic imbalance, lack of growth, and redistribution of carbon flux to production of CO_2, with metabolic balance and growth resuming after approximately 2 h (23). Evidence was presented that during the period of metabolic imbalance, the methylene-H_4MPT dehydrogenase, MtdA, is a candidate for posttranscriptional restriction of carbon flux to assimilation, due to the 2-h separation of the induction of transcripts from the later increase in activity (23). MtdA has a dual role in methylotrophy: it catalyzes the oxidation of methylene-dH_4MPT to methenyl-dH_4MPT, and it catalyzes the reduction of methenyl-H_4F to methylene-H_4F (24), the entry metabolite in the assimilatory cycles. The role of MtdA during the oxidation of formaldehyde to formate was not completely understood due to an apparent biochemical redundancy of two methylene-H_4MPT dehydrogenases: MtdA and MtdB (25, 26) (Fig. 1). A recent study using an *mtdB* mutant showed that MtdA activity is essential in the oxidation pathway (conversion of formaldehyde to formate) during growth on methanol and methylamine (27).

The current study was undertaken to define the physiological relevance of this posttranscriptional regulatory mechanism by competitive inhibition and its effect in restricting carbon flux to assimilation, using a combination of biochemical, metabolomic, and genetic analyses.

MATERIALS AND METHODS

Bacterial strains and chemostat cultivation. Wild-type *M. extorquens* AM1 carrying pHC61, a plasmid with the *mtac* promoter (28), and *M. extorquens* AM1 overexpressing *mch* from the *mtac* promoter were grown in hypho minimal medium as described by Okubo et al. (29), with the exception that five times more cobalt was added to the medium, to a final concentration of 15 μM (30). The concentration of kanamycin in the medium was 25 μg/ml. Strains were grown in a BioFlo 110 modular fermentor (New Brunswick Scientific) run in chemostat mode, with a 2-liter working volume of minimal medium with a limiting concentration of

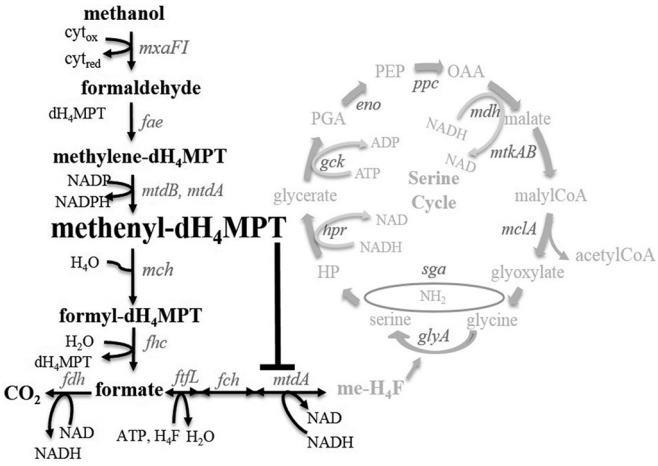

FIG 1 Proposed model for the regulatory role of methenyl-dH_4MPT in methylotrophic metabolism in *M. extorquens* AM1. When methanol availability increases, the carbon flux to the methylotrophic oxidation pathway upshifts and methenyl-dH_4MPT accumulates, inhibiting production of methylene-H_4F and, thus, assimilatory metabolism. Formate utilization shifts to oxidative metabolism, producing more CO_2. Upregulation of the assimilation cycles increases gradually over time. The genes involved in the reactions of the metabolic pathway are indicated next to the arrows. Abbreviations: dH_4MPT, dephosphotetrahydromethanopterin; H_4F, tetrahydrofolate; PGA, phosphoglyceric acid; PEP, phosphoenolpyruvate; OAA, oxaloacetate. The *fae* gene product is formaldehyde-activating enzyme, the *mtdB* and *mtdA* gene products are the methylene-tetrahydromethanopterin dehydrogenases MtdA and MtdB, the *mch* gene product is methenyl-dH_4MPT cyclohydrolase, the *fhc* gene product is the formyltransferase/hydrolase complex, the *fdh* gene products are formate dehydrogenases, the *ftfL* gene product is formate-tetrahydrofolate ligase, and the *fch* gene product is methenyl-H_4F cyclohydrolase. The major assimilation cycle, the serine cycle, is also depicted. The *glyA* gene product is serine hydroxymethyl transferase, the *sga* gene product is serine glyoxylate aminotransferase, the *hpr* gene product is hydroxypyruvate reductase, the *gck* gene product is glycerate kinase, the *eno* gene product is enolase, the *ppc* gene product is PEP carboxylase, the *mdh* gene product is malate dehydrogenase, the *mtkAB* gene products are malate thiokinases A and B, and the *mclA* gene product is malyl-CoA/β-methylmalyl-CoA lyase.

succinate (3.75 mM). When the culture reached an optical density at 600 nm (OD_{600}) of 0.52, medium flow was started, and the dilution rate was maintained at 0.14 h^{-1}. The parameters during the switchover experiment were as previously described (23). Methanol (125 mM) was added, and the OD_{600} was monitored using a Shimadzu (Kyoto, Japan) UV-2401 PC spectrophotometer. Samples for targeted metabolomics and enzyme activity assays were collected over time (before adding methanol, at time zero, and after adding methanol, at 0.3 h, 1 h, 2 h, 4 h, and 7.5 h).

Purification of methenyl-dH_4MPT. Methenyl-dH_4MPT was isolated and quantified as previously described (27) at different times during the transition experiment. Two chemostat runs were pooled per sample, for a total of a 2-liter working volume of culture, and were used for each measurement. Each measurement is reported as an average for two biological replicates. The extraction was done under strictly anoxic conditions and in the dark as previously described (27).

Measurements of formaldehyde and formate in cell extracts. Cell extracts were prepared as follows. Fifty-milliliter aliquots of culture were collected at different times during the transition experiment, subjected to fast filtration, and frozen immediately with liquid nitrogen. The cell pellet was resuspended in 1 ml of 50 mM Tris-HCl, pH 7.5, and cells were broken with a French press (3 passes at 1,000 lb/in^2).

(i) Formate measurements. Formate was measured enzymatically in cell extracts for 3 biological replicates by monitoring Fdh activity (0.3 U/ml) and production of NADH (3 mM) via the increase in absorbance at 340 nm, as previously described (13).

(ii) Formaldehyde measurements. Formaldehyde was measured in cell extracts for 3 biological replicates by the colorimetric Nash method and by monitoring the increase in absorbance at 410 nm (31). For internal concentration estimation, a dry weight of 0.278 g/liter at 1 OD_{600} unit was used, as previously reported (11), with a cell volume of 36 μl/mg dry weight, based on an average cell size of 1 by 3.2 μm (32) and an average of 4×10^8 cells/ml at 1 OD_{600} unit (2).

Sample preparation for metabolite determination. Samples for gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-MS (LC/MS) were collected by fast filtration using 20 ml of cell culture. Cells in the filter were frozen immediately with liquid nitrogen in a precooled 50-ml plastic tube. Samples were lyophilized for 2 h. Hot extraction of the metabolites was achieved as described by Good et al. (33). GC/MS measurements were used to analyze amino acid and organic acid contents as previously described (23). LC/MS was used to identify and determine changes in levels of coenzyme A (CoA) derivatives.

Enzyme assays. Extracts for activity assays were harvested at different time points during the transition from succinate to methanol growth by centrifugation (50 ml) at 4,500 × *g* for 10 min. Supernatants were removed, and pellets were immediately frozen with liquid nitrogen and stored at −80°C. Cells were thawed and resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 7.6. Cells were broken with a French press (3 passes at 1,000 lb/in^2) and cleared by centrifugation (28,078 × *g*, 45 min, 4°C). Fifty-microliter aliquots were used for the following activity assays: methylene-H_4F dehydrogenase (MtdA) activity was measured by monitoring NADPH formation and the increase in absorbance at 340 nm as previously described (23), with the exception that pure methylene-H_4F (Schircks Laboratory, SwitzerlandSUI) was used instead of formaldehyde and H_4F. Hydroxypyruvate reductase (Hpr) activity and serine glyoxylate aminotransferase (Sga) activity were measured as previously described (23).

MtdB purification. The *mtdB* gene was amplified and cloned into pET28b as described by Rasche et al. (34). The construct was transformed into *Escherichia coli* BL21-AI. This strain was grown at 37°C in LB medium with kanamycin (50 μg/ml). When the culture reached an OD_{600} of 0.6, IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) and arabinose (0.2%) were added to induce expression of *mtdB*. Cultures were grown for 3 more hours at 30°C, and cells were harvested by centrifugation (4,729 × *g*, 10 min, 4°C). The cell paste (15 g) was resuspended in 7 ml of buffer A (50 mM Tris-HCl, pH 8.0, and 5 mM imidazole), and cells were broken by use of a French press. Cell extracts were cleared by centrifugation (28,078 × *g*, 45 min, 4°C), and the supernatant was applied to a Ni-charged chelating Sepharose column (3 ml) previously equilibrated with buffer A. The column was washed with 5 column volumes of buffer B (50 mM Tris-HCl, pH 8.0, and 200 mM NaCl).

The MtdB-His_6 protein was eluted off the column by using an imidazole gradient of 0 to 500 mM over 25 ml in buffer A. Fractions (6 fractions of 1 ml each) were pooled and desalted using a PD-10 gel filtration column (8.3-ml bed volume, 5-cm bed height) equilibrated with buffer C (100 mM potassium phosphate buffer, pH 7.5). The protein was concentrated using centrifugal filter devices (Amicon-Ultra; 10K, 4,000 × *g*, 15 min, 4°C). The concentrated protein sample (130 μM) was used in the experiments as indicated. Protein concentration was determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL).

Fae activity. Fae activity was monitored by a coupled assay with MtdB. Fae catalyzes the conversion of formaldehyde to methylene-dH_4MPT, which, in addition to $NADP^+$, is used by MtdB to generate methenyl-dH_4MPT and NADPH (24). The production of NADPH can be monitored at 340 nm. To decrease the rate of spontaneous coupling of dH_4MPT and formaldehyde, an alkaline pH and high concentrations of magnesium were used to increase the rate of the enzymatic reaction. The activity assay was performed as described by Vorholt et al. (35), with the following differences: we used 50 mM potassium phosphate buffer, pH 7.8, purified MtdB (130 μM; 50 μl), and the cosubstrate NADP (125 μM). The total volume of the reaction mixture was 0.5 ml. Activity was monitored at 340 nm (i.e., NADPH production) after the addition of formaldehyde (2 mM).

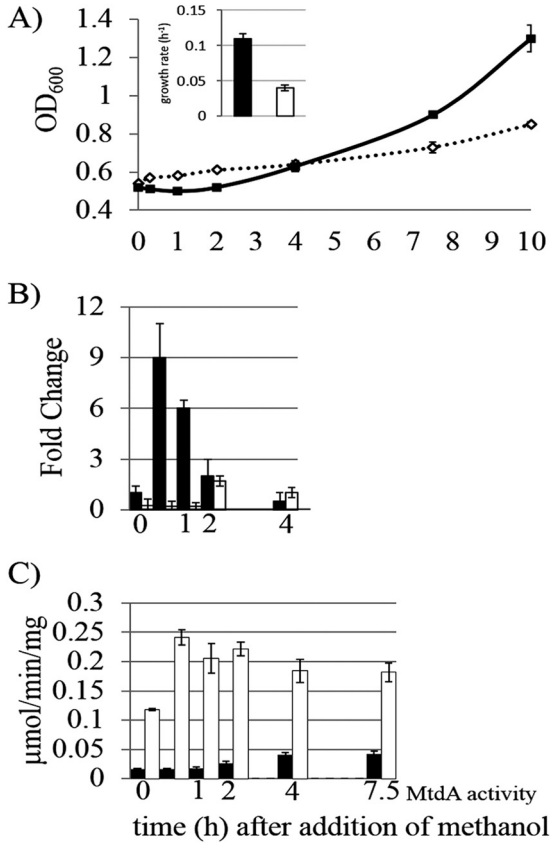

FIG 2 Growth defect and lack of accumulation of methenyl-dH_4MPT as a result of overexpression of *mch* during the transition from succinate to methanol growth. (A) Changes in optical density for a wild-type strain (squares) and a strain overexpressing *mch* (diamonds) after methanol was added to a steady-state succinate-limited culture. Data are means ± standard errors of the means (SEM) ($n = 3$ biological replicates per strain). (B) Fold changes (with respect to succinate growth) of the levels of methenyl-dH_4MPT in the wild type during the transition experiment ($n = 2$ biological replicates). (C) Changes of MtdA activity during the transition experiment ($n = 3$ biological replicates with 2 technical replicates). Filled bars, wild-type strain; open bars, strain overexpressing *mch*.

Mch activity. Wild-type *M. extorquens* and *M. extorquens* overexpressing *mch* were grown in succinate liquid medium. At an OD_{600} of 0.8, 50 ml of each culture was centrifuged (4,800 × *g*, 10 min, 4°C), and the cell pellets were immediately frozen with liquid nitrogen and stored at −80°C until needed. Extracts were thawed and resuspended in 1 ml of 50 mM potassium phosphate buffer, pH 8.0. The extracts were broken by use of a French press. Cell extracts were cleared by centrifugation (28,000 × *g*, 45 min, 4°C), and the supernatant was equilibrated with N_2 for 10 min. The activity of Mch was followed photometrically under anoxic conditions by monitoring the decrease in absorbance at 335 nm ($\varepsilon = 21.6\ mM^{-1}\ cm^{-1}$) as described previously (27).

RESULTS

Methenyl-dH_4MPT accumulates during the transition from succinate to methanol growth. The initial response of *M. extorquens* to the switch from succinate to methanol growth is a 2-h growth lag (Fig. 2A, squares), which has been suggested to be the result of a lack of increase of activity of MtdA (23). Since methenyl-dH_4MPT has been shown to inhibit MtdA *in vitro* (27), it is

possible that a similar mechanism affects the activity of MtdA during the switch from succinate to methanol growth. If so, then methenyl-dH$_4$MPT should accumulate during this growth lag. To test this hypothesis, levels of dH$_4$MPT derivatives were measured during the transition from succinate to methanol growth. A succinate-limited chemostat culture of wild-type *M. extorquens* was grown to steady state (OD_{600} of 0.52) at a growth rate of 0.16 h^{-1}. Medium flow was stopped, and samples were taken at time zero. Immediately after samples were taken, 125 mM methanol was added to the culture. Cells were harvested at multiple time points after the addition of the C_1 substrate, for up to 7 1/2 h. dH$_4$MPT species in each sample were extracted, purified, and measured. Of the four species (methylene-, methenyl-, formyl-, and free dH$_4$MPT), only methenyl-dH$_4$MPT accumulated, to a level 7- to 11-fold higher than that in the culture grown on succinate, within 30 min after addition of methanol (Fig. 2B, filled bars). However, the accumulation of this intermediate was transient, and the relative concentration decreased after the initial accumulation. The accumulation and subsequent decrease of methenyl-dH$_4$MPT correlated with the growth lag observed (Fig. 2A and B, filled symbols). The onset of growth occurred at 2 h, which correlated with only a 2- to 3-fold accumulation of methenyl-dH$_4$MPT. By 4 h, the level was below the initial level, correlating with a growth rate of 0.11 h^{-1}. The inhibitory effect of the accumulation of methenyl-dH$_4$MPT on MtdA activity was corroborated by use of cell extracts of samples taken over the time course after the addition of methanol (Fig. 2C, filled bars). No increase in MtdA activity was detected when the levels of methenyl-dH$_4$MPT were 7-fold higher than those seen for succinate growth. As the level of methenyl-dH$_4$MPT dropped, MtdA activity increased (Fig. 2B and C, filled bars). It has been shown previously that *mtdA* transcripts increase immediately after the transition, while MtdA activity does not increase for 2 h (23). Together, these results suggest a mechanism in which methenyl-dH$_4$MPT accumulation inhibits MtdA activity and assimilatory metabolism, contributing to the growth lag observed when a culture is challenged to switch from succinate to methanol growth.

Overexpression of *mch* releases methenyl-dH$_4$MPT regulation during the transition from succinate to methanol growth. The methenyl-H$_4$MPT cyclohydrolase Mch uses methenyl-dH$_4$MPT as a substrate as part of the oxidation pathway in methanol metabolism (Fig. 1). A correlation between a decrease in Mch activity and an accumulation of methenyl-dH$_4$MPT was found for methylamine-grown cultures of an *mtdB* mutant strain (27). It can be predicted that overexpressing *mch* will remove the methenyl-H$_4$MPT-dependent regulatory mechanism by decreasing the pool of methenyl-dH$_4$MPT. *mch* was constitutively expressed in the wild-type strain, which resulted in a 10-fold increase in Mch activity (from 1.1 ± 0.3 U/mg in the wild-type strain to 10.6 ± 0.8 U/mg in the strain overexpressing *mch*). Growth of the strain overexpressing *mch* during the transition from succinate to methanol growth was significantly different from that of the wild-type strain. Instead of a 2-h lag, as observed for the wild-type strain, steady, slow growth was observed throughout the time course, with a growth rate of 0.04 h^{-1} ($P = 0.002$ by Student's *t* test) (Fig. 2A, diamonds).

Levels of methenyl-dH$_4$MPT were measured in the strain overexpressing *mch* throughout the transition experiment. The strain overexpressing *mch* did not significantly accumulate methenyl-dH$_4$MPT (Fig. 2B, open bars). MtdA activity was measured, and unlike in the wild type, the MtdA activity increased 2-fold immediately after addition of methanol ($P = 0.02$) and then slowly dropped over time (Fig. 2C, open bars). The increased specific activity of MtdA was 10-fold higher than that in the wild type 20 min after the addition of methanol (Fig. 2C). These results demonstrate that overexpression of *mch* removed the methenyl-dH$_4$MPT-dependent inhibition and also affected the growth response.

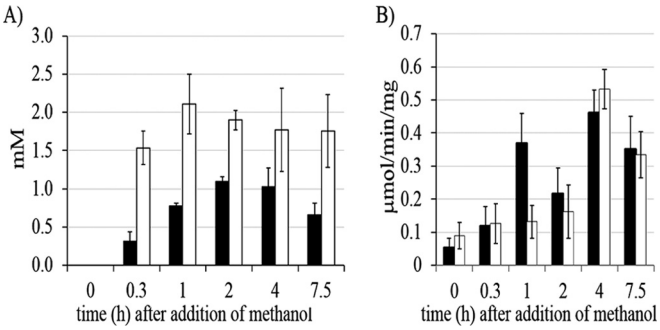

FIG 3 Formaldehyde accumulation during the transition from succinate to methanol growth. (A) Internal concentrations of formaldehyde accumulated during the transition from succinate to methanol growth. Data are means ± SEM ($n = 3$ biological replicates with 2 technical replicates). (B) Fae activity profiles over the transition experiment ($n = 3$ biological replicates with 2 technical replicates). Filled bars, wild type; open bars, strain overexpressing *mch*.

Release of methenyl-dH$_4$MPT regulation results in the accumulation of formaldehyde. A high level of carbon flux through formaldehyde is a signature characteristic of methylotrophy (36). It has been postulated that the methenyl-dH$_4$MPT-mediated regulation of assimilatory metabolism in *M. extorquens* operates to keep formaldehyde from accumulating during the transition from nonmethylotrophic growth to methylotrophic growth (27). In order to test this hypothesis, formaldehyde was measured in cell extracts of wild-type and *mch*-overexpressing strains. When the wild-type control strain was transitioned from succinate to methanol growth, formaldehyde accumulated during the growth lag, to a maximal internal concentration of 1 ± 0.3 mM (2 h after the transition started) (Fig. 3A, filled bars). Once growth resumed, the formaldehyde concentration started to drop, and by 7.5 h, it was at 0.7 ± 0.3 mM.

Formate concentrations had a similar profile over the course of the experiment, with a maximal internal concentration of 0.11 ± 0.04 mM. In contrast, the strain overexpressing *mch* accumulated formaldehyde at a higher rate (Fig. 3A, open bars), starting immediately after the addition of methanol. Twenty minutes into the transition, the strain had accumulated formaldehyde to a concentration of 1.5 ± 0.2 mM, peaking by 1 h, at an internal formaldehyde concentration of 2.1 ± 0.4 mM (versus 0.8 ± 0.1 mM in the wild-type control). By 7.5 h, the formaldehyde concentration remained constant. Formate was not detected during growth on succinate. After the addition of methanol, formate increased immediately, to 0.06 ± 0.02 mM, and remained constant for the first 4 h of the transition experiment.

Fae is the enzyme that catalyzes the conversion of formaldehyde and dH$_4$MPT to methylene-dH$_4$MPT, using formaldehyde as a substrate. If formaldehyde accumulates, the most likely reason is a decreased Fae activity. For the first 2 h after methanol addition,

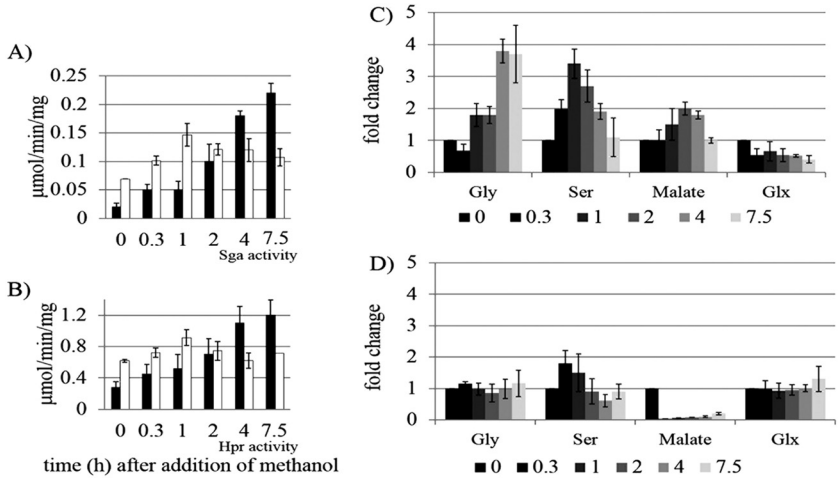

FIG 4 System response of the serine cycle in the strain overexpressing *mch*. (A) Changes in serine-glyoxylate aminotransferase (SgaA) activity during the transition experiment. Data are means ± SEM ($n = 3$ biological replicates with 2 technical replicates). Filled bars, wild type; open bars, strain overexpressing *mch*. (B) Changes in hydroxypyruvate reductase (Hpr) activity during the transition experiment ($n = 3$ biological replicates with 2 technical replicates). Filled bars, wild type; open bars, strain overexpressing *mch*. (C) Targeted metabolomic profiles (fold changes compared to time zero) of four serine cycle intermediates from the control strain (wild-type strain with an empty vector containing the *mtac* promoter) ($n = 3$ biological replicates with 2 technical replicates). (D) Targeted metabolomic profiles (fold changes compared to time zero) of four serine cycle intermediates from the strain overexpressing *mch* under the control of the *mtac* promoter ($n = 3$ biological replicates with 2 technical replicates).

Fae activity did not increase significantly when *mch* was overexpressed ($P = 0.1$ by Student's *t* test) (Fig. 3B, open bars). This activity profile is different from the wild-type control profile, in which there is a 7-fold increase in Fae activity by 1 h (Fig. 3B, filled bars) and a 4-fold increase in Fae activity by 2 h ($P = 0.02$ by Student's *t* test). These results suggest that overexpression of *mch* results in decreased Fae activity, which in turn contributes to formaldehyde accumulation.

Release of methenyl-dH$_4$MPT regulation affects serine cycle enzyme activities. Another possibility for poor growth of the *mch* overexpression strain was a decreased serine cycle enzyme activity. Two enzymes, Sga (serine-glyoxylate aminotransferase), and Hpr (hydroxypyruvate reductase), were tested. In the wild-type control during the succinate-to-methanol transition, the activities of these enzymes started to increase immediately after methanol addition, similar to results reported previously (23). However, the *mch* overexpression strain showed a more rapid increase in enzyme activity for the first hour, and then the activity dropped slowly over time (Fig. 4A and B). Cellular metabolite pool studies were also carried out. The wild-type control strain metabolic profile had similar trends, with pools remaining constant (<2-fold change compared to succinate levels) (Fig. 4C) after the addition of methanol and until growth resumed. Once the culture started growing, glycine accumulated approximately 4-fold (Fig. 4C). Serine levels accumulated 3-fold by 1 h after methanol addition and slowly decreased once growth resumed (at 2 h) (Fig. 4C). In contrast, the pools of intermediates of the serine cycle from the *mch*-overexpressing strain, such as glycine, serine, and glyoxylate, remained constant, while pools of malate decreased significantly compared to succinate levels (Fig. 4D). Together, these data suggest an immediate upregulation of these serine cycle enzymes, with a detrimental effect, an hour into the transition from succinate to methanol growth, at the time when formaldehyde accumulation peaks.

DISCUSSION

Methanol emission rates from plants are dynamic, with a sharp transient increase in early light hours followed by an exponential decrease (21, 22). As a result, methylotrophs must respond to sudden changes in substrate availability. A recent study showed that when wild-type *M. extorquens* is challenged to switch from succinate to methanol growth, a transient restriction of carbon flux into the assimilation pathways occurs, resulting in a growth lag until the 24 assimilatory enzymes are completely induced (23). The results of the current study suggest a model for this carbon flux restriction that involves reversible inhibition of assimilatory flux by the small molecule methenyl-dH$_4$MPT (Fig. 1). This type of regulatory mechanism facilitates an immediate response to the availability of methanol, while allowing a slower induction of assimilatory capacity and an effective transition to active growth of the strain. This study has confirmed that MtdA is an important metabolic control point regulated by a mechanism dependent on the accumulation of the small molecule methenyl-dH$_4$MPT.

The physiological relevance of this strategy was demonstrated by the accumulation of methenyl-dH$_4$MPT being prevented by overexpression of *mch*. The strain in which the methenyl-dH$_4$MPT regulatory mechanism was bypassed accumulated formaldehyde transiently, with an initial concentration that was 5 times greater than that in the wild type, which in itself may account for the growth defect of the *mch*-overexpressing strain. Although the toxic level of internal formaldehyde is not known, once the formaldehyde concentration rose above 1.5 mM (at 1 h), Sga and Hpr activities decreased. These results support the idea that at early time points during the transition, the activities of serine cycle enzymes increase similarly to what has been observed for the wild-type strain; however, at later time points correlating with high formaldehyde levels, serine cycle enzymes are affected, contributing to the observed growth defect.

Formaldehyde accumulation suggests an inhibitory effect on

formaldehyde-activating enzyme (Fae). The activity of Fae did not increase considerably until after 2 h into the transition from succinate to methanol growth, and based on results from the previous study (23), it can be predicted that by 1 h after the transition, the methanol uptake rate is on the order of 400 nmol min^{-1} mg $protein^{-1}$. Since all the methanol consumed is converted to formaldehyde, and assuming that the *in vitro* Fae activity measured is similar to the *in vivo* activity, the methanol uptake rate would be expected to overwhelm Fae activity at early points in the *mch* overexpression strain but not in the wild-type control strain. The regulation of Fae has not been studied, so the mechanism of this inhibition is not known. It may be at the transcriptional or posttranscriptional level, or both. No effectors have been described for this reaction, but it is possible that an intermediate or cosubstrate in the oxidation pathway is out of balance when *mch* is overexpressed and that this results in an inhibition of Fae activity. At later points, the accumulation of formaldehyde might be indicative of increased MeDH activity and increased methanol uptake.

It is important that the activities of Sga, Hpr, and MtdA from the strain overexpressing *mch* during succinate growth (time zero) were significantly higher than those from the wild-type strain (3-fold, 2-fold, and 7-fold, respectively; $P \leq 0.001$ by Student's *t* test). Sga, Hpr, and MtdA are transcriptionally regulated by QscR (37, 38), a Lys-type regulator known to be coinduced by at least one H_4F derivative. It is possible that constitutive expression of *mch* affects H_4F derivative pools during succinate growth, with effects on the transcription levels of QscR-regulated genes. Regardless, the initial response for both strains resumed, with gradual increases of both Sga and Hpr activities, once methanol was added. An exception was seen for MtdA, as the accumulation of methenyl-dH_4MPT in the wild-type strain, acting as an inhibitor, was also observed.

The results of this study support the hypothesis that the immediate and primary response by *M. extorquens* after a change in substrate availability involves a nontranscriptional regulatory mechanism mediated by the small molecule methenyl-dH_4MPT. This immediate response leads to a major redistribution of carbon flux between assimilation and energy production (23), and the results presented here suggest that this response is at least partially responsible for preventing the accumulation of formaldehyde during the transition. Nontranscriptional regulatory mechanisms have been suggested to play an important role in controlling metabolic fluxes (39), and this is another example in which such a mechanism contributes to an effective acclimation after a metabolic perturbation (27).

Expanding studies into regulatory mechanisms, specifically nontranscriptional regulatory mechanisms, will contribute to understanding the fine-tuning of carbon redistribution in methylotrophy. These results can be translated to successfully overcome associated metabolic challenges resulting from pathway modifications. This ability is particularly important in manipulating or rewiring metabolic networks to drive carbon distribution to specific intermediates that are precursors of value-added products for the pharmaceutical industry and for biofuel production.

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