In vitro methanol production from methyl coenzyme M using the *Methanosarcina barkeri* MtaABC protein complex

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Abstract

Methanol:coenzyme M methyltransferase is an enzyme complex composed of three subunits, MtaA, MtaB and MtaC, found in methanogenic archaea and is needed for their growth on methanol ultimately producing methane. MtaABC catalyzes the energetically favorable methyl transfer from methanol to coenzyme M to form methyl coenzyme M. Here we demonstrate that this important reaction for possible production of methanol from the anaerobic oxidation of methane can be reversed in vitro. To this effect, we have expressed and purified the Methanosarcina barkeri MtaABC enzyme, and developed an in vitro functional assay that demonstrates MtaABC can catalyze the energetically unfavorable (ΔG^o = 27 kJ/mol) reverse reaction starting from methyl coenzyme M and generating methanol as a product. Demonstration of an in vitro ability of MtaABC to produce methanol may ultimately enable the anaerobic oxidation of methane to produce methanol and from methanol alternative fuel or fuel-precursor molecules.

Keywords: anaerobic methane oxidation; methanol from methane; methanogens; CoM; Zn enzyme; corrinoid.

Introduction

Natural gas, consisting primarily of methane (CH_4), is a large energy resource in the US[1], and production of chemicals that can serve as precursors to liquid fuels and other chemicals is a highly desirable goal[2-4]. Currently, CH_4 is used to produce MeOH[5] at an industrial scale with initial conversion of CH_4 to syngas (CO & H_2), a process which suffers from both high capital costs and low conversion efficiencies. For these reasons, biological conversion of CH_4 to fuels and chemicals is a promising alternative[4].

Anaerobic Methanotrophs (ANME) are close relatives of methanogens, and capable of

the anaerobic oxidation of methane to CO_2 utilizing homologous enzymes to those found in methanogens through a reversal of methanogenesis[6]. The proposed first step of anaerobic methane oxidation is a reversal of the final step in methanogenesis, and involves the binding of methane and a heterodisulfide cofactor to the methyl-coenzyme M reductase followed by release of the methylated coenzyme M (CH_3-CoM) and the free thiol coenzyme B[7]. Expression of a methyl-coenzyme M reductase from a member of the ANME-1 clade in the native methanogen *Methanosarcina acetivorans* resulted in acetate production from methane[8]. Further engineering of this strain resulted in lactate production from methane[9], demonstrating the feasibility of biological production of valuable chemicals from methane. Methylotrophic methanogens catalyze the formation of CH_3-CoM from methanol with the methanol:CoM methyltransferase complex (MtaABC)[10]. A reversal of MtaABC activity could allow methanol production from CH_3-CoM produced during anaerobic CH_4 oxidation, however the reversibility of the MtaABC complex has not been reported.

The MtaABC complex of *Methanosarcina barkeri* is expressed from an operon with the assembled heterodimer complex of MtaB and MtaC tightly bound to a corrinoid prosthetic group[10]. MtaBC catalyzes the methyl transfer from methanol onto the corrinoid group, followed by transfer of the methyl group onto CoM by the monomeric, zinc protein MtaA[10-13]. Previous research expressed MtaA and MtaBC from *M. barkeri* in *E. coli* [14,15], with activities of the MtaA and MtaBC complexes demonstrated in separate assays. Here we report expression of the *M. barkeri* MtaABC subunits in *E. coli*, and demonstrate the forward and reverse methyl transfer reaction of the functionally active MtaABC enzyme complex *in vitro*.

Methods

Gene synthesis and subcloning of *MtaA* and *MtaBC*

The protein sequences of MtaA and MtaBC were from *Methanosarcina barkeri* strain Fusaro (EMBL X91893 and Y08310, respectively), but the cDNA was optimized and synthesized by GenScript for expression in *E. coli* (Supplementary Table 1). MtaA and MtaBC sequences were designed to contain His-tags (Supplementary Fig. 1), and each was cloned into a pET-16B expression vector and transformed into *E. coli* BL21 cells. Plasmid from transformed cells was isolated and the gene inserts were sequenced (GeneWiz, Inc.) to confirm presence of the MtaA and MtaBC sequences.

Expression, purification, and activity assay of recombinant MtaA in *E. coli.* Cells containing the MtaA pET-16B expression construct were grown in Luria-Bertani (LB) medium with 100 µg/mL ampicillin at 30 °C to an OD_{600} of 0.7, then was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 16 °C. The MtaA protein was harvested using a previously published methodology[14,15]. Briefly, cells were resuspended in lysis buffer and lysed by sonication, and the suspension was centrifuged to clarify. The resulting supernatant was applied to a Ni-NTA resin column, and then washed to remove non-specifically bound proteins. The His-MtaA fusion protein was eluted from the resin with 30 mL of 50 mM sodium phosphate, pH 6.0, containing 300 mM NaCl and 500 mM imidazole, and dialyzed overnight at 4 °C against 50 mM 3-(4-morpholino) propane sulfonic acid (MOPS)/KOH, pH 7.2. The dialyzed sample was loaded using FPLC onto a Q-sepharose column, and the MtaA protein was eluted with a gradient of 0-1 M NaCl in 50 mM MOPS/KOH, pH 7.2, over 75 mLs at a flow rate of 1 mL/min. Fractions from the gradient were collected and SDS-PAGE confirmed elution of MtaA between 200 and 300 mM NaCl. The activity of purified MtaA was determined using a modified assay based on previously published work[14,16] which measures the demethylation of methylcobalamin in a CoM-dependent manner by MtaA.

Expression, purification, and activity assay of recombinant MtaBC in *E. coli*

Expression and purification of MtaBC from *E. coli* was performed in an identical manner to expression and purification of MtaA. The MtaBC complex eluted from the Q-sepharose column with 500 mM NaCl. The activity of MtaBC in the forward direction was confirmed using a methylcobalamin assay which monitors the MtaB demethylation of methylcobalamin[15].

Assays for the forward and reverse activity of MtaABC

The formation of CH_3-CoM from the reaction of methanol and CoM was measured by a 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay, which measures free thiol CoM in the solution. The forward MtaABC reaction was used to generate CH_3-CoM for use in the reverse MtaABC activity assay. The CH_3-CoM from the forward MtaABC reaction was purified by HPLC, and the identity of the eluted CH_3-CoM product was confirmed with Electrospray Ionization-MS (ESI-MS). The HPLC-purified CH_3-CoM had a small amount of CoM impurity, so it was reacted with 10 mM of DTNB to deactivate residual free thiol CoM. The CH_3-CoM was again purified by HPLC using the previously described conditions before use in the reverse MtaABC reaction assay. Purified CH_3-CoM was very stable and no hydrolysis was detected. Methanol produced from the reverse MtaABC assay was detected using GC-MS.

Results and Discussion

The biological production of methanol from CH_4 by methanogenic archaea requires three enzymatic steps. The first step is the methylation of CoM catalyzed by methyl-coenzyme M reductase (Mcr) (Fig. 1a). This step has been shown to be reversible in both native methanogens[17] and in a methanogen host expressing a Mcr homolog from an anaerobic

methanotroph of the clade ANME-1[8,9]. The next two steps in methanol production are catalyzed by the MtaABC complex (Fig. 1b and c). Although the individual components of this complex, consisting of MtaA and MtaBC, were previously shown to be reversible[14,15], the reversibility of the entire MtaABC complex had not been demonstrated prior to this work. The Gibb's free energy for the three steps in methyl transfer from methanol to CoM were previously determined[16]. The first step is catalyzed by MtaB and transfers the methyl group from methanol to MtaC, generating CH_3-MtaC and water ($\Delta G^{\circ\prime}$ of -7 kJ/mol). The second step is catalyzed by MtaA and transfers the methyl group from CH_3-MtaC to CoM, generating CH_3-CoM ($\Delta G^{\circ\prime}$ of -20 kJ/mol). Thus, the overall reaction has a $\Delta G^{\circ\prime}$ of -27 kJ/mol and the reversal of this is quite endergonic.

The MtaA and MtaBC proteins of *M. barkeri* were functionally expressed in *E. coli* as shown by SDS-PAGE and *in vitro* assays (Figs. 2-4). Prior research reported that *E. coli* expression of MtaB alone required refolding from inclusion bodies[15,18]. Our approach yielded a functionally folded MtaB without the need to refold from inclusion bodies, as both MtaC and MtaB activities appear in a functionally-active soluble fraction. After *in vitro* assays confirmed functional expression of the MtaA and MtaBC enzymes, we confirmed activity of the full MtaABC complex *in vitro* through DTNB assays (Fig. 5) and mass spectrometry which demonstrated production of CH_3-CoM from methanol in the forward direction (Fig. 6) and production of methanol from CH_3-CoM in the reverse direction (Fig. 7). In order to overcome the ΔG of 27 kJ/mol of the reverse reaction, a large reactant concentration (50 mM methyl CoM) was used, and the free thiol CoM, which are a product of the reverse MtaABC reaction, were continuously consumed in the DTNB assay. The reverse reaction of MtaABC showed a significant increase in absorbance corresponding to a linear velocity of 11-13 μM/min in the first 30 s of the reaction, and was ca. 10-fold greater than the no-substrate control.

Since the MtaABC enzyme complex catalyzes the second and final reaction needed to convert methane to methanol during anaerobic methane oxidation, the demonstration of this reaction *in vitro* represents a significant step toward realizing the potential of converting methane to methanol biologically rather using chemical routes.[20] We envision an engineered strain that combines an effective MCR protein (such as the ANME-1 Mcr) expressed in *M. acetivorans* [8,9] with the MtaABC complex in a suitable host and then provide culture conditions that would promote the reaction from methane to methanol. That would include high methane concentrations under pressure to increase the effective soluble concentration of methane to which the cells would be exposed, combined in a syntrophic co-culture with an organism that uses methanol effectively and with very high affinity (i.e. a very low overall effective K_m value for methanol). The goal of the latter would be to minimize the concentration of methanol in the co-culture system so that the MtaABC reaction is driven in the direction of methanol formation, and this would also drive the reaction from methane to methyl-coenzyme M by minimizing the intracellular concentration of methyl-coenzyme M.

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Supporting Information

Supporting Methods. MtaA and MtaBC expression strategy.

Supplementary Figure 1. MtaBC expression constructs.

Supplementary Table 1. Gene sequences of *MtaA* and *MtaCB*.

AUTHOR INFORMATION

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Author Contributions

LS, WC, ETP and BJB conceived the study. MD, MMK and TDG performed the experiments, and BJB supervised the work. MD and BJB drafted the manuscript, WC, ETP and LS edited the first draft. The final manuscript which was read and approved by all authors.

Notes

The authors declare no competing financial interest.

Figure Captions

Fig. 1 Proposed method for biological production of methanol from methane. (a) Methylotrophic methanogens utilize the MtaABC enzyme protein complex to catalyze the methyl transfer from methanol to CoM to form methyl-CoM, which is energetically favorable in the forward reaction[16]. MtaB catalyzes a methyl transfer from methanol to the corrinoid cofactor of the MtaC subunit. MtaA then catalyzes the transfer of the methyl group to CoM to form methyl-CoM. (b) ΔG values for the three steps of the methyl group transfer from methanol to CoM as calculated by Daas et al[16].(c) MtaABC complex as modeled by Hagemeier *et al*[19]. The structure in blue is MtaB. The structure in green is MtaA. The structure in red is the helical domain of MtaC. The structure in orange is the Rossmann domain of MtaC. The stick structure is the corrinoid bound to the Rossmann domain of MtaC. Reproduced with permission by the National Academy of Sciences (permission to be requested upon publication).

Fig. 2 Expression and purification of MtaA and MtaBC from *M. barkeri* in *E. coli*. (a) SDS-PAGE analysis of MtaA: lane 1, protein marker with band sizes shown in kDa; lane 2, MtaA from Ni-Sepharose column; lane 3, MtaA from Ni-NTA column. (b) SDS PAGE analysis of MtaBC: lane 1, protein marker with band sizes shown in kDa; lane 2, MtaBC from Ni-NTA column, undiluted; lane 3, MtaBC from Ni-NTA column diluted 1:1 in buffer. Expected sizes for MtaA, MtaB, and MtaC are 36 kDa, 49 kDa, and 24 kDa, respectively.

Fig. 3 Methylcobalamin assay of purified MtaA from *M. barkeri* expressed in *E. coli*. The 1 mL assay contained 6 mM methylcobalamin (Sigma-Aldrich) in 50 mM MOPS/KOH, pH 7.2, and 15 µg of purified MtaA with a mineral oil overlay. The reaction was initiated with 10 mM sodium 2-mercaptoethanesulfonate (CoM). The methylation of Mta with methylcobalamin was measured as an increase in absorbance at 310 nm corresponding

to the production of cobalamin (molar attenuation coefficient of 9.24 $mM^{-1}cm^{-1}$). Although the assays were quite noisy, the trends clearly demonstrated the production of colbalamin.

The control assay lacked the MtaA enzyme and showed no production of cobalamin. Assays were repeated twice and both assays are shown with similar results obtained in each (shown as solid and dashed lines).

Fig. 4 Methylcobalamin assay of purified MtaBC from *M. barkeri* expressed in *E. coli*. The 1 mL assay contained 25 μM methylcobalamin in 50 mM MOPS/KOH, pH 7.2, with a mineral oil overlay. The reaction was initiated with 0.1 mg of MtaBC. The methylation of hydroxocobalamin by MtaBC with methylcobalamin was measured as a decrease in absorbance at 528 nm corresponding to the decrease in methylcobalamin (molar attenuation coefficient of 6.3 $mM^{-1}cm^{-1}$). The control assay lacked the MtaBC enzyme and showed no similar decrease in methylcobalamin. Assays were repeated twice and both assays are shown with similar results obtained in each (shown as solid and dashed lines).

Fig. 5 DTNB assays for the forward and reverse MtaABC reactions. (a) The forward reaction measures the MtaABC dependent methylation of 2-mercaptoethanesulfonate (CoM) and subsequent depletion of free thiol CoM. Each 1 mL assay reaction mixture contained 50 mM MOPS/KOH, pH 7.2, with 1 mg of MtaA and 1 mg of MtaBC, 200 μM ATP, 10 mM $MgCl_2$, and 50 mM CoM with a mineral oil overlay at 37 °C. The reaction was initiated with 100 μL of methanol. At various time points, a 10 μL aliquot of the reaction mixture was removed for a 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) assay which contained 1 mL of 100 mM Tris, pH 7.5, and 1 mM DTNB. Absorbance measured at 412 nm (molar attenuation coefficient of 14.15 $mM^{-1}cm^{-1}$) was used for quantitation of

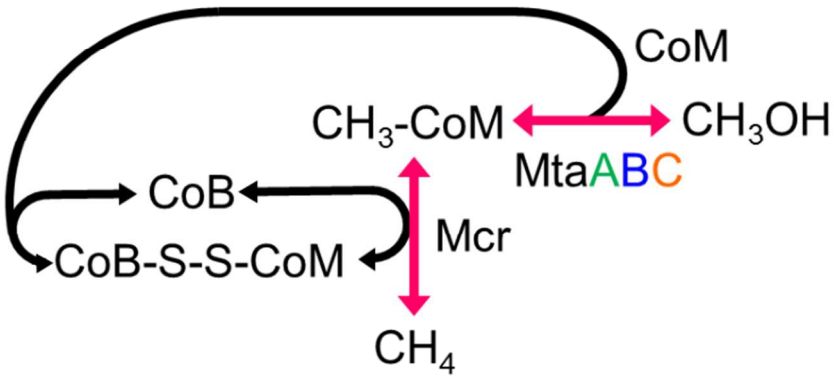
free thiol CoM. Presence of the MtaABC enzyme results in a consumption of free thiol CoM in the assay. A control assay without enzyme showed no decrease in free thiol CoM. (b) The DTNB assay for the reverse reaction measures MtaABC-dependent demethylation of methyl-CoM (CH_3-CoM) to CoM. Each 1 mL assay contained 100 mM Tris, pH 7.5, 200 μM ATP, 10 mM $MgCl_2$, 1 mM DTNB, and 1 mg each of MtaA and MtaBC. The reaction was initiated with 50 mM CH_3-CoM and activity was detected as an increase in absorption at 412 nm, corresponding to an increase in free thiol CoM. A control assay with no enzyme added showed no change in absorbance. A control assay with no CH_3-CoM substrate showed an increase in absorbance from the presence of free sulfhydryls in the MtaABC protein sample, but this increase was smaller than when the CH_3-CoM was present. Assays were performed twice and both assays are shown with similar results obtained in each.

Fig. 6 ESI-MS confirmation of methyl-2-mercaptoethanesulfonate (CH_3-CoM), the product from the MtaABC forward reaction. The solution from the forward MtaABC reaction was heated to 60°C to precipitate out the protein, then loaded onto a C18 reverse phase HPLC column. The CH_3-CoM product was eluted at a flow rate of 1 ml/min with an increasing gradient of methanol on a Shimadzu LCMS 2020. The CH_3-CoM product had a LC retention time of approximately 0.9 min. The identity of the CH_3-CoM peak was confirmed with ESI-MS with a peak at a molecular weight of 178 g/mol for CH_3-CoM plus a sodium ion is indicated by an arrow.

Fig. 7 Analysis of reverse MtaABC reaction products on an Agilent 5973 mass spectrometer equipped with EI. (a) Spectrum of a 50 mM methanol standard showing identifying peaks at molecular weights of 31, 30, and 29. (b) Spectrum of methanol detected in the MtaABC reverse reaction assay. (c) Spectrum of control assay, which

lacked the 2-mercaptoethanesulfonate (CoM) substrate, showing no methanol.

Figure 1.



(a)

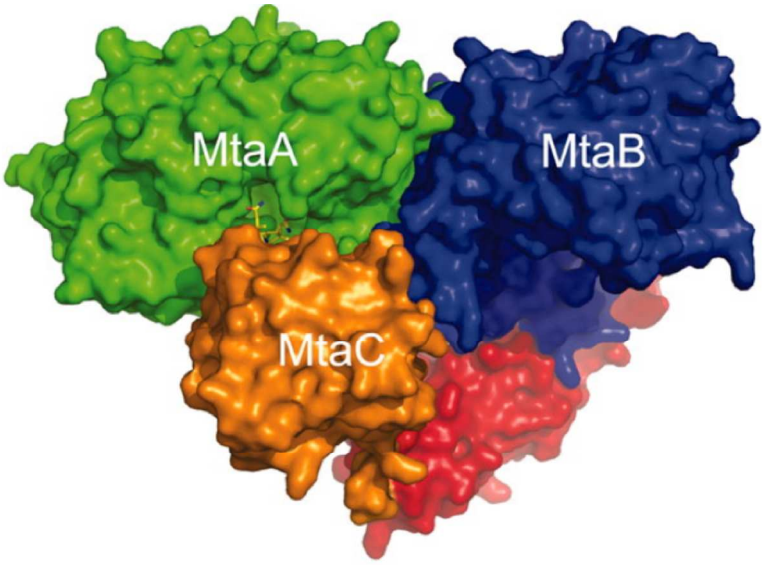
(b)

$$CH_3OH + MtaC \xrightleftharpoons{MtaB} CH_3\text{-}MtaC + H_20 \quad \Delta G^{o'} = -7\ kJ/mol$$

$$CH_3\text{-}MtaC + CoM\text{-}SH \xrightleftharpoons{MtaA} CH_3\text{-}S\text{-}CoM + MtaC \quad \Delta G^{o'} = -20\ kJ/mol$$

$$CH_3OH + CoM\text{-}SH \xrightleftharpoons{MtaABC} CH_3\text{-}S\text{-}CoM + H_20 \quad \Delta G^{o'} = -27\ kJ/mol$$

(c)



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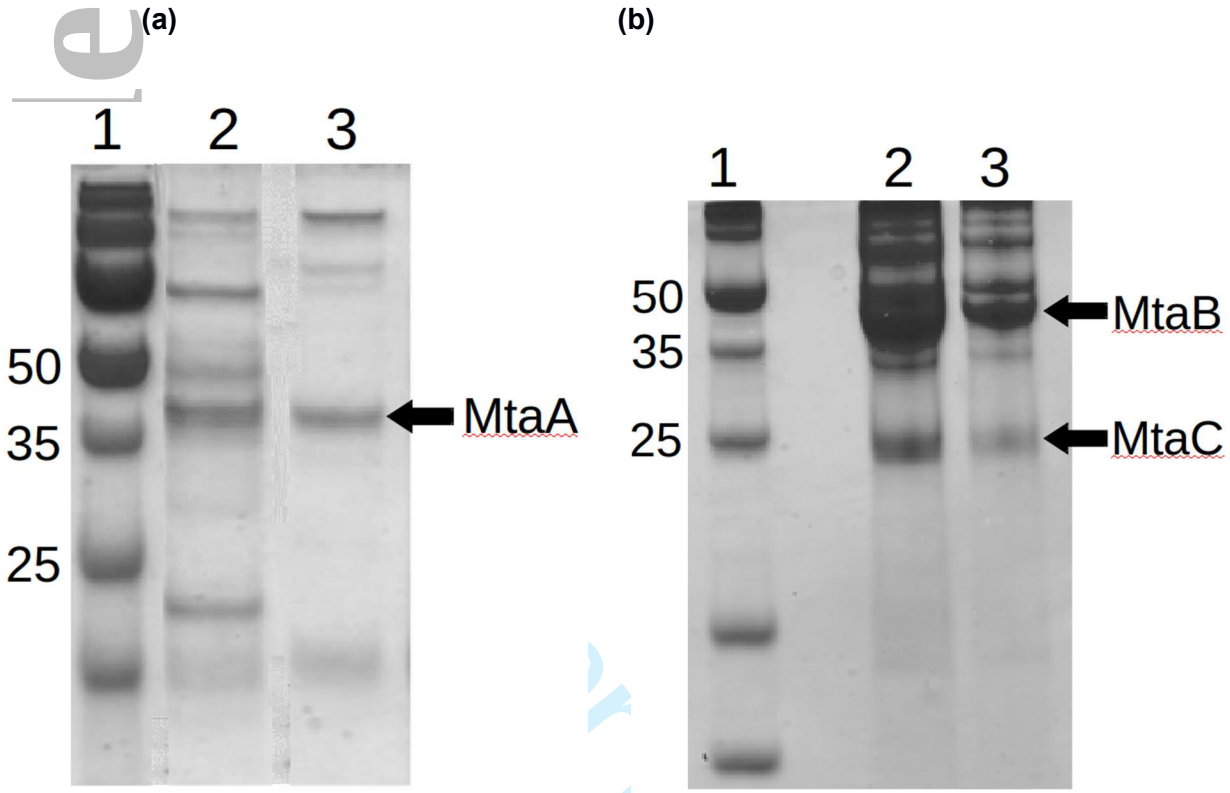

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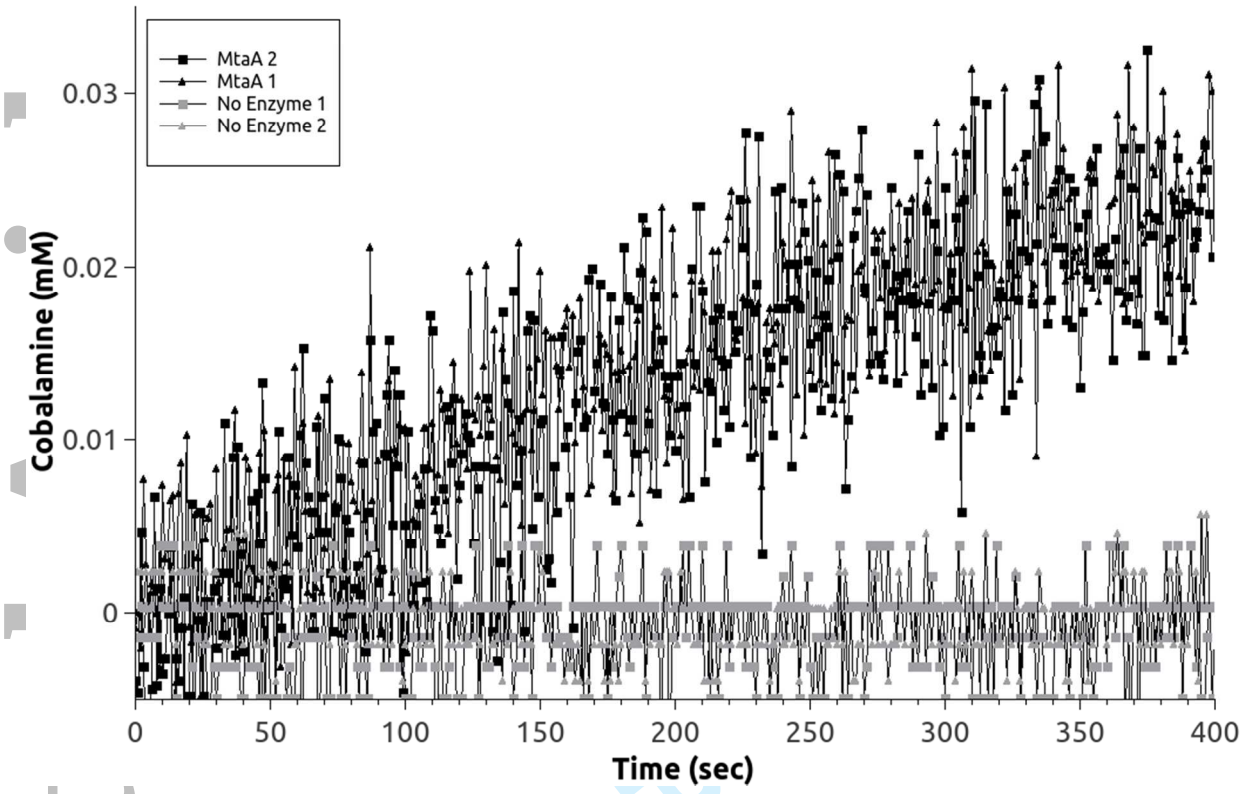

294 **Figure 4.**

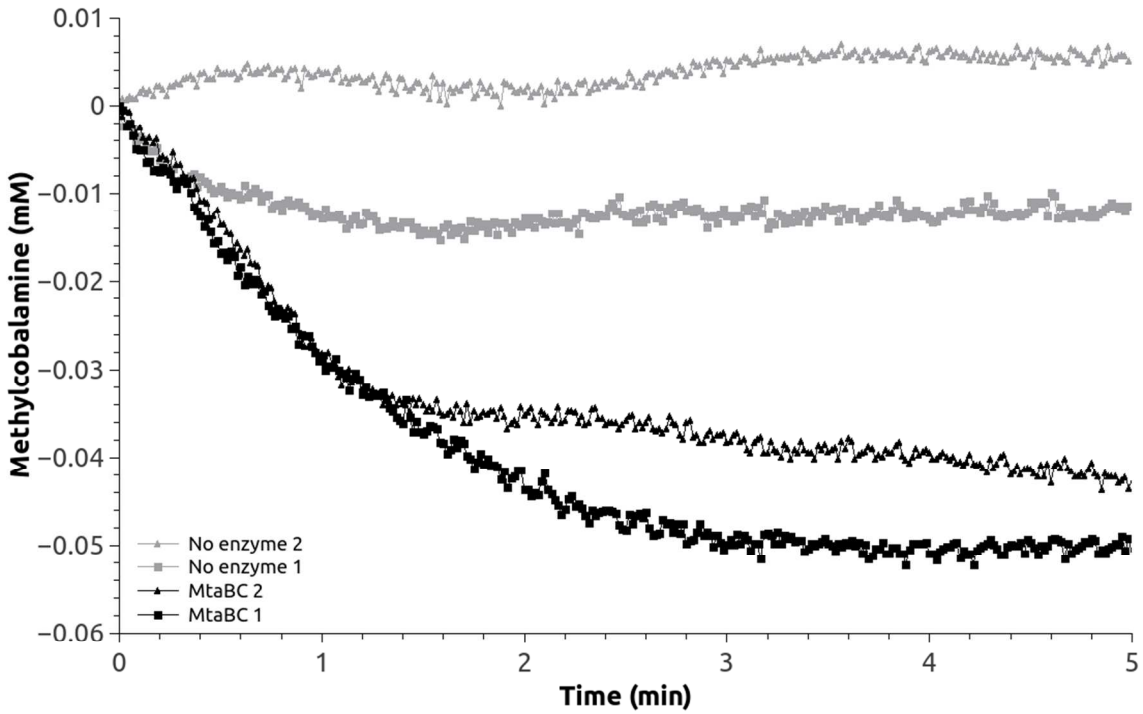

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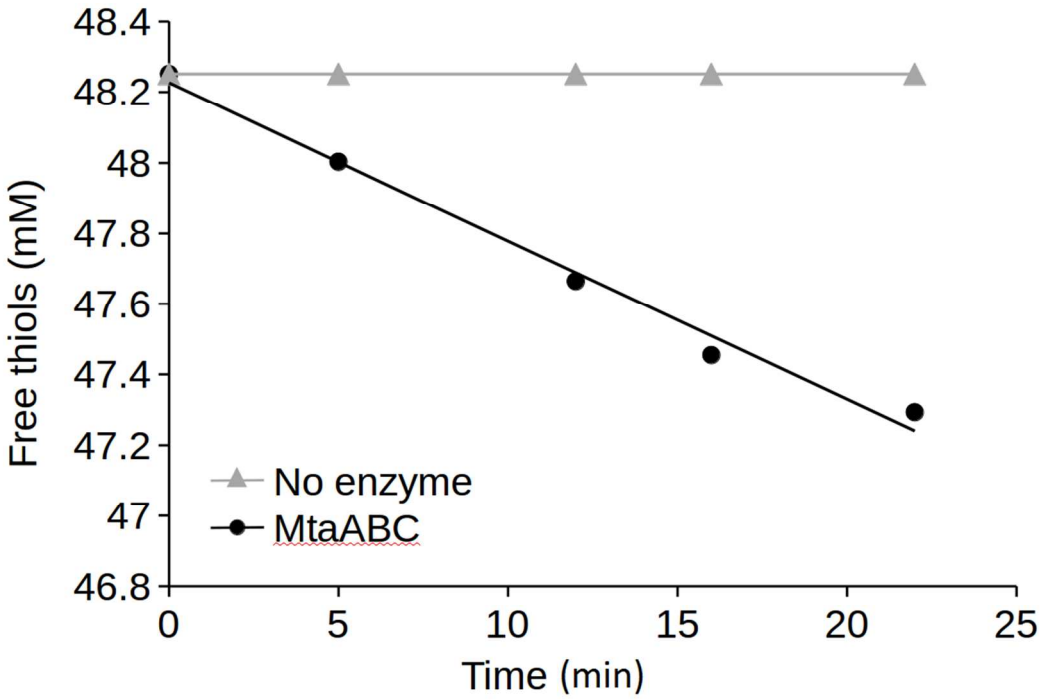
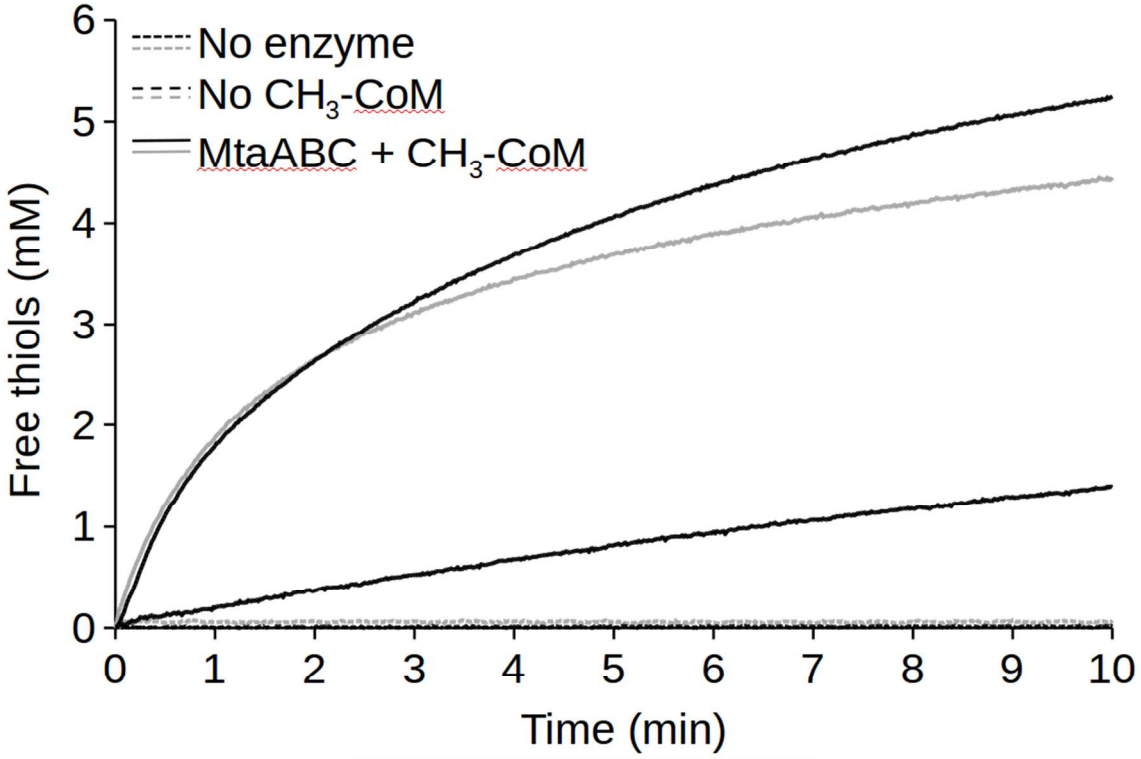
Figure 5.



(a)

(b)



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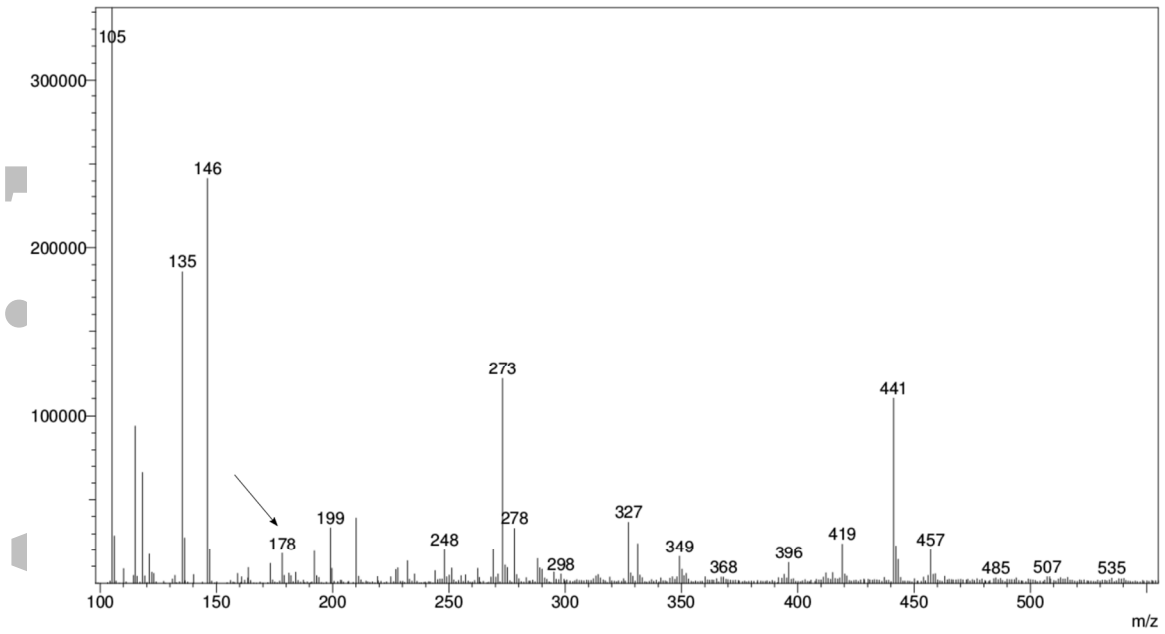
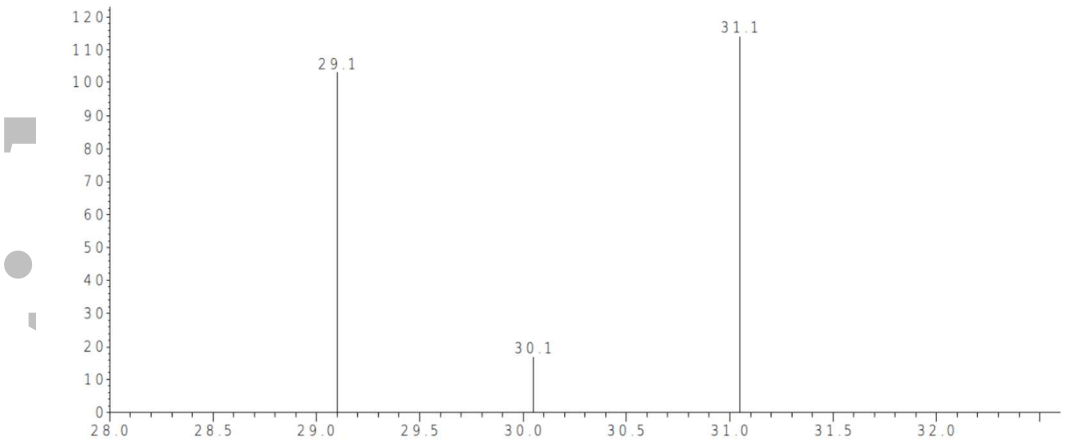


Figure 7.

(a)



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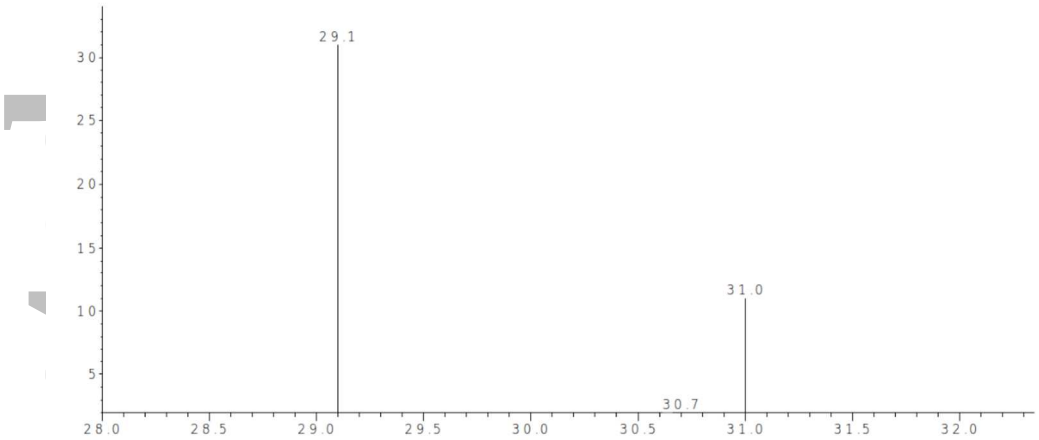

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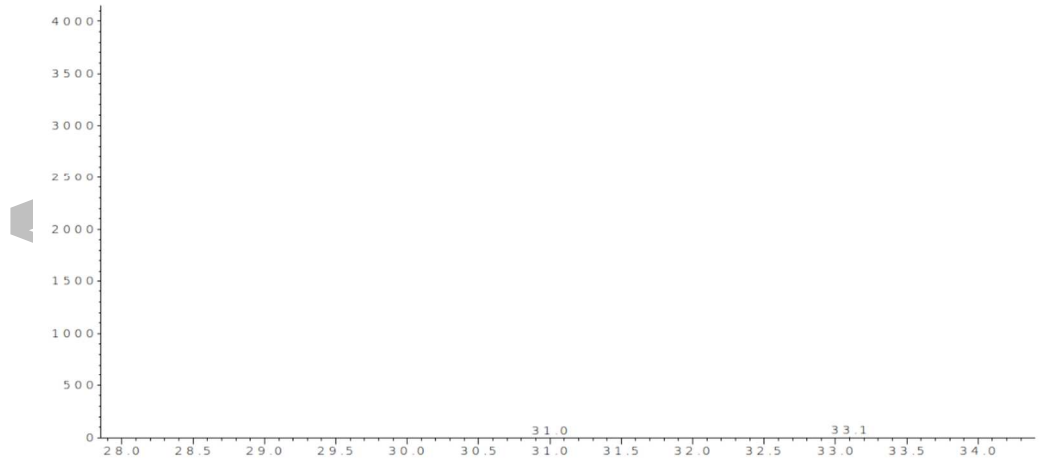

References

1. Blohm A, Peichel J, Smith C, Kougentakis A. The significance of regulation and land use patterns on natural gas resource estimates in the Marcellus shale. *Energy Policy.* 2012;50:358-369.
2. Whitaker WB, Jones JA, Bennett RK, et al. Engineering the biological conversion of methanol to specialty chemicals in *Escherichia coli*. *Metabolic Engineering.* 2017;39:49-59.
3. Whitaker WB, Sandoval NR, Bennett RK, Fast AG, Papoutsakis ET. Synthetic methylotrophy: engineering the production of biofuels and chemicals based on the biology of aerobic methanol utilization. *Current Opinion in Biotechnology.* 2015;33:165-175.
4. Chad A Haynes RG. Rethinking biologocal activation of methane and conversion to liquid fuels *Nature Chemical Biology.* 2014;10:331-339.
5. Fiedler E, Grossmann DB, Keresbohm G, Weiss C. Methanol. *Ullmann's Encyclopaedio of Industrial Chemistry.* Vol 23. Weinheim: Wiley-VCH; 2011:26-48.
6. Hallam SJ, Putnam N, Preston CM, et al. Reverse methanogenesis: Testing the hypothesis with environmental genomics. *Science.* 2004;305(5689):1457-1462.
7. Shima S, Thauer RK. Methyl-coenzyme M reductase and the anaerobic oxidation of methane in methanotrophic Archaea. *Current Opinion in Microbiology.* 2005;8(6):643-648.
8. Soo VWC, McAnulty MJ, Tripathi A, et al. Reversing methanogenesis to capture methane for liquid biofuel precursors. *Microbial Cell Factories.* 2016;15.
9. McAnulty MJ, Poosarla VG, Soo VWC, Zhu FY, Wood TK. Metabolic engineering of *Methanosarcina acetivorans* for lactate production from methane. *Biotechnology and Bioengineering.* 2016.
10. van der Meijden P, Heythuysen HJ, Pouwels A, Houwen F, van der Drift C, Vogels GD. Methyltransferases involved in methanol conversion by *Methanosarcina barkeri*. *Arch Microbiol.* 1983;134(3):238-242.
11. Kruer M, Haumann M, Meyer-Klaucke W, Thauer RK, Dau H. The role of zinc in the methylation of the coenzyme M thiol group in methanol:coenzyme M methyltransferase from *Methanosarcina barkeri*. *Eur J Biochem.* 2002;269(8):2117-2123.
12. van der Meijden P, Heythuysen HJ, Sliepenbeek HT, Houwen FP, van der Drift C, Vogels GD. Activation and inactivation of methanol: 2-mercaptoethanesulfonic acid methyltransferase from *Methanosarcina barkeri*. *J Bacteriol.* 1983;153(1):6-11.
13. Wassenaar RW, Daas PJ, Geerts WJ, Keltjens JT, van der Drift C. Involvement of methyltransferase-activating protein and methyltransferase 2 isoenzyme II in methylamine:coenzyme M methyltransferase reactions in *Methanosarcina barkeri* Fusaro. *J Bacteriol.* 1996;178(23):6937-6944.
14. Harms U, Thauer RK. Methylcobalamin: coenzyme M methyltransferase isoenzymes MtaA and MtbA from *Methanosarcina barkeri*. Cloning, sequencing and differential transcription of the encoding genes, and functional overexpression of the mtaA gene in *Escherichia coli*. *Eur J Biochem.* 1996;235(3):653-659.
15. Sauer K, Harms U, Thauer RK. Methanol:coenzyme M methyltransferase from *Methanosarcina barkeri*. Purification, properties and encoding genes of the corrinoid protein MT1. *Eur J Biochem.* 1997;243(3):670-677.
16. Daas PJH, Gerrits KAA, Keltjens JT, Vanderdrift C, Vogels GD. Involvement of an activation protein in the methanol-2-mercaptoethanesulfonic acid methyltransferase reaction in *Methanosarcina barkeri*. *Journal of Bacteriology.* 1993;175(5):1278-1283.
17. Moran JJ, C. H. House, K. H. Freeman, and J. G. Ferry Trace methane oxidation studied in several Euryarchaeota under diverse conditions. *Archaea.* 2005;1:303– 309.
18. Sauer K, Thauer RK. Methanol:coenzyme M methyltransferase from *Methanosarcina barkeri*. Zinc dependence and thermodynamics of the methanol:cob(I)alamin methyltransferase reaction. *Eur J Biochem.* 1997;249(1):280-285.
19. Hagemeier CH, Krer M, Thauer RK, Warkentin E, Ermler U. Insight into the mechanism of

biological methanol activation based on the crystal structure of the methanol-cobalamin methyltransferase complex. *Proc Natl Acad Sci U S A.* 2006;103(50):18917-18922.

20. Zakariaa, Z, Kamarudin, SK. Direct conversion technologies of methane to methanol: An overview. Renewable and Sustainable Energy Reviews. 2016; 65: 250–261.