

Overview of Microbial Metabolomics: A Special Insight to Cyanobacterial Methylotrophy

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Abstract

Metabolomics deals with the identification, qualitative and quantitative measurement of the metabolites acting in the biochemical network. It is widely applied advanced microbiological research to understand the metabolic as well as systems behaviors of industrial important microorganisms. Combining genomics, proteomics and metabolomics data to reveal our molecular hypothesis of selected organisms is an essential interest of this approach. The resulted metabolomic model with internal or external flux balance and metabolic flux coefficient would be served as a platform to set-up our metabolic engineering experiments towards commercial metabolites production. Cyanobacteria are important for their beneficial natural product production, bioremediation and energy applications on which they are highly promising for hydrogen production. Upon considering its applications and available X-omics data in public domain databases, cyanobacterial metabolomics, particularly methylotrophy, have been studied in detail and presented herein. Phylogenetic analysis of this study strongly suggested a strong resemblance between cyanobacteria and facultative methylotrophic bacteria. Accordingly, we have proposed a metabolic pathway for methane and methylamine assimilation in marine cyanobacteria, *Prochlorococcus chlorococcus marinus* MIT9303. Thus, we recommend further research that focuses on the methane and C1 compounds metabolisms involved in living phototrophic prokaryotes to test our hypothesis of methylotrophy.

Keywords: Metabolomics; cyanobacteria; systems biology; methylotrophy; methane; hydrogen; genomics

Introduction

Metabolic engineering is an emerging field of biotechnology which offers tremendous potential for the production of desired metabolites. It is generally referred to as the targeted and purposeful alteration of metabolic pathways found in an organism in order

to better understand and utilize cellular pathways for chemical transformation and energy transduction^{1,2}. It focuses the manipulation of endogenous genes or the introduction of foreign genes into an organism of interest in order to reroute metabolic pathways for the production of specific compounds³. The interest in metabolic engineering is stimulated by potential commercial applications where improved methods for developing strains which can increase production of useful metabolites. The successful development of an engineering process is dependent upon a thorough working knowledge of the genes and metabolites. Computational modeling in metabolic engineering has traditionally been used to guide experimental attempts by anticipating the effect of genetic modifications on metabolism. However, such modeling approaches commonly involve kinetic techniques that require detailed enzyme kinetic information⁴. Metabolic control analysis that requires experiment-based measurements of flux control coefficients. Thus, it involves the prediction of genetic manipulations that would lead to optimized microbial strains, maximizing the production rate of chemicals of interest.

Microbial metabolomics is the now well-established scientific field concerned with the study of naturally occurring, low molecular weight organic metabolites within a microbial cell⁵. The application of metabolomics to the investigation of both free-living organisms obtained directly from the natural environment and of organisms reared under laboratory conditions, where any laboratory experiments specifically serve to mimic scenarios encountered in the natural environment is called environmental metabolomics⁶. The actual functional status of the organism can be mechanistically related to organism phenotype by metabolomic measurements report. Metabolomics can discover unexpected relationships and metabolite responses, which in itself can lead to hypothesis generation. As such, metabolomics is finding an increasing number of applications in the environmental sciences, ranging from understanding



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organismal responses to abiotic stressors^{7,8}. Metabolomics affords also several advantages for studying organism-environment interactions and for assessing organism function and health at the molecular level⁵.

Metabolic flux balance analysis

Metabolic flux analysis is based on a known biochemistry framework. A linearly independent metabolic matrix is constructed based on the law of mass conservation and on the pseudo-steady state hypothesis on the intracellular metabolites. The formulation resulted in a set of linear equations that can be expressed as a stoichiometric matrix A of dimension m by n with vectors for net accumulation, r ($m \times 1$), and metabolic flux, u ($n \times 1$). No kinetic data is required. Dynamic flux balance analysis can be obtained from the same derivation as shown in the following equation with b as the transport term.

$$r = \frac{dX}{dt} = A \cdot v - b$$

Typically, the system that results is under-determined system where $m > n$. However under certain conditions, some pathways are inoperative and can be neglected. The system may become completely determined or over determined and can be solved along with the measurements of external or internal fluxes⁹.

Current scenario of metabolomics

The regulation of metabolic reaction networks is an important task in systems biology and functional genomics. A complete understanding of metabolic regulation requires quantitative information about kinetic laws and the concentrations of metabolites and enzymes. This quantitative knowledge in combination with the known network of metabolic pathways allows the construction of mathematical models that describe the dynamic changes in metabolite concentrations over time. The models are high-dimensional systems of ordinary, non-linear differential equations. The main problems of the approach are the setup of the equations that describe the metabolic pathways in form of kinetic rate equations and the identification of the system parameters. To solve these problems, a variety of metabolome and systems modeling tools have been developed which simplify model construction and

analysis. Most of these tools are able to store and exchange models in the Systems Biology Markup Language and to fit parameters for a given set of experimental data¹⁰.

Metabolomics serves not only as a source of qualitative but also quantitative data of intra-cellular metabolites essential for the model-based description of the metabolic network operating under *in vivo* conditions. To collect reliable metabolome data sets, culture and sampling conditions, as well as the cells' metabolic state, are crucial. Together with the other more established omics technologies, metabolomics will strengthen its claim to contribute to the detailed understanding of the *in vivo* function of gene products, biochemical and regulatory networks and, even more ambitious, the mathematical description and simulation of the whole cell in the systems biology approach. This knowledge will allow the construction of designer organisms for process application using biotransformation and fermentative approaches making effective use of single enzymes, whole microbial and even higher cells¹⁰⁻¹³.

Application of microbial metabolomics

Microbial metabolomics has a potential to benefit from integration of metabolomics into system frameworks. Kinetic mathematical modeling has been described for central metabolism of *Saccharomyces cerevisiae*, *Escherichia coli* and glycolysis in *Lactococcus lactis* but also for biosynthetic pathways leading to shikimate in *Streptococcus pneumonia*, L-phenylalanine and L-threonine in *E. coli*, L-valine/ L-leucine and L-lysine in *Corynebacterium glutamicum* and penicillin in *Penicillium chrysogenum* based on data sets from metabolome measurements¹⁴⁻²⁶. The data modeling process tries to minimize the gap between the measured and modeled data in an iterative approach by parameter fitting and implementation of new model hypotheses about enzyme mechanisms or regulatory elements. Herein, cyanobacterial methylo-trophy is our special interest to be described in detail to understand its metabolic capabilities and cellular behavior in response to environmental methane.

Methane oxidation mechanism

Methanogenesis and methane oxidation are the major biological processes affecting the global cycling of the powerful greenhouse gas methane. Its



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atmospheric concentration has been steadily increasing over the past 300 years. There are two major ways in which methane is removed from the environment: aerobic oxidation by a specialized group of bacteria and anaerobic oxidation by a specialized group of archaea. Methylo-trophic bacteria utilize reduced carbon substrates containing no carbon-carbon bonds (such as methane, methanol, and other methylated compounds) as their sole sources of carbon and energy²⁷. The flux of trace gases between soil and atmosphere is usually the result of simultaneously operating production and consumption processes in soil. Methane is the dominant gaseous product of anaerobic degradation of organic matter and is released into the atmosphere, whereas the other trace gases are only intermediates, which are mostly cycled within the anoxic habitat. A significant percentage of the produced methane is oxidized by methanotrophic bacteria. Nature has cleverly recycled key reactions for the C1 transfers between the oxidation levels of formaldehyde and formate, and these involve analogous enzyme systems and common specialized cofactors, methanopterin and methanofuran²⁸⁻²⁹. The field of methylo-trophy has undergone a significant transformation in terms of discovery of novel types of methylo-trophs, novel modes of methylo-trophy, and novel metabolic pathways²⁷.

Cyanobacterial metabolomics

Cyanobacteria known as blue-green algae, is a phylum of bacteria that obtain their energy through photosynthesis. They are a significant component of the marine nitrogen cycle and an important primary producer in many areas of the ocean, but are also found in habitats other than the marine environment. Cyanobacteria are known to occur in both freshwater, hypersaline inland lakes and in arid areas where they are a major component of biological soil crusts. Stromatolites of fossilized oxygen-producing cyanobacteria have been found from 2.8 billion years ago possibly as old as 3.5 billion years ago³⁰⁻³¹. The ability of cyanobacteria to perform oxygenic photosynthesis is thought to have converted the early reducing atmosphere into an oxidizing one, which dramatically changed the composition of life forms on earth by stimulating biodiversity and leading to the near-extinction of oxygen-intolerant organisms. According to endosymbiotic theory, chloroplasts in

plants and eukaryotic algae have evolved from cyanobacteria via endosymbiosis³².

Phylogeny of cyanobacteria

Phylogenies of cyanobacteria have been reconstructed on the basis of comparing the orders of genes in chromosomes and nucleotide sequences appear to be similar. In the evolution of marine unicellular plankton cyanobacteria, genome rearrangements are fixed with a low rate, whereas in other groups of cyanobacteria the gene order can change several times more rapidly. The gene orders in genomes of cyanobacteria and chloroplasts preserve a considerable degree of similarity³³. The cyanobacterial radiation consists of several lineages of phyletically (morphologically and genetically) related organisms. Several of these organisms show a striking resemblance to fossil counterparts. To investigate the molecular mechanisms responsible for stabilizing or homogenizing cyanobacterial characters, the evolutionary rates and phylogenetic origins of 16S rDNA and the conserved gene *rbcL* are compared³⁴⁻³⁵. Phylogenetic analyses support the hypothesis that cyanobacteria capable of cell differentiation are monophyletic, and the geological record provides both upper and lower bounds on the origin of this clade³⁶.

Cyanobacterial carboxysome

Recent molecular, biochemical and physiological studies have significantly extended current knowledge about the genes and protein components of single-cell CO₂ concentrating mechanism (CCM) and how they operate to elevate CO₂ around RubisCO during photosynthesis³⁵. Cyanobacteria and some chemoautotrophic bacteria are able to grow in environments with limiting CO₂ concentrations by employing a CCM that allows them to accumulate inorganic carbon in their cytoplasm to concentrations several orders of magnitude higher than that on the outside. The final step of this process takes place in polyhedral protein microcompartments known as carboxysomes, which contain the majority of the CO₂-fixing enzyme, RubisCO. The widely accepted models for the role of carboxysomes in the carbon-concentrating mechanism of autotrophic bacteria predict the carboxysomal carbonic anhydrase to be a crucial component. The efficiency of CO₂ fixation by the sequestered RubisCO is enhanced by co-localization with a specialized carbonic anhydrase that



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catalyzes dehydration of the abundant cytosolic bicarbonate and ensures saturation of RubisCO with its substrate CO₂^{37,38}.

Modeling carbonate-silicate cycle

The existence of weathering feedbacks in the carbonate-silicate cycle suggests that atmospheric and oceanic CO₂ concentrations would have been high prior to the presence of a methane greenhouse. With the onset of a methane greenhouse, carbon dioxide concentrations would decrease. Bicarbonate has been proposed as the preferred reductant that preceded water for oxygenic photosynthesis in a bacterial photosynthetic precursor to cyanobacteria; with the drop of carbon dioxide level, Archean cyanobacteria emerged using water as a reductant instead of bicarbonate. A greenhouse transition timescale on the order of 50-100 million years is consistent with results from modeling the carbonate-silicate cycle³¹.

Cyanobacterial hydrogen production

Molecular hydrogen is one of the potential future energy sources as an alternative to the limited fossil fuel resources of today. Cyanobacteria that use two sets of enzymes to generate hydrogen gas: The first one is nitrogenase and it is found in the heterocysts of filamentous cyanobacteria when they grow under nitrogen limiting conditions³⁹. Hydrogen is produced as a byproduct of fixation of nitrogen into ammonia. The other hydrogen-metabolizing/producing enzymes in cyanobacteria are hydrogenases; they occur as two distinct types in different cyanobacterial species. One type of them, uptake hydrogenase has the ability to oxidize hydrogen and the other type of hydrogenase is reversible or bidirectional hydrogenase and it can either take up or produce hydrogen^{39,40}. Uptake of hydrogenase enzymes are found in the thylakoid membrane of heterocysts from filamentous cyanobacteria, where it transfers the electrons from hydrogen for the reduction of oxygen via the respiratory chain in a reaction known as oxyhydrogenation or Knallgas reaction. The hydrogen formed is usually reoxidized by an uptake hydrogenase via a Knallgas reaction and hence there is no net H₂ production in strains with uptake hydrogenases under ambient conditions. Thus it is counterproductive when the goal is to produce hydrogen on a commercial scale⁴¹.

In comparison to the traditional ways of hydrogen production (chemical, photoelectrical), cyanobacterial hydrogen production is commercially viable. Sulfur-deprivation in combination with CH₄ and changes in the pH of the media can be used to further increase the specific capacity of unicellular non-N₂-fixing cyanobacteria to produce H₂ during fermentation⁴². Nitrogenase produces hydrogen as a normal byproduct of the reduction of dinitrogen to ammonia. The Nif₂ nitrogenase in *Anabaena variabilis* is an alternative Mo-nitrogenase and is expressed in vegetative cells grown with fructose under strictly anaerobic conditions⁴⁰. While integrating the existing knowledge and technology, much future improvement and progress is to be done before hydrogen is accepted as a commercial primary energy source⁴¹⁻⁴³.

Mechanism of cyanobacterial methylo-trophy

In our study, a simple text mining approach was carried out to retrieve the information regarding the function of proteins, enzymes, sequences, organisms and other metabolic characteristics from NCBI, iHOP (Information Hyperlinked Over Proteins), MetaCyc, KEGG and UM-BBD (University of Minnesota Biocatalysis/Biodegradation Database). Some of the proteins functions from the sequences of *Prochlorococcus chlorococcus* marinus MIT9303 obtained in text mining were preliminarily annotated by BLASTp search tool of NCBI⁴⁴. The selected sequences were clustered with complete deletion of gaps using ClustalX 2.0 software⁴⁵. Then after, Neighbor Joining trees were searched homogeneous patterns among all lineages using MEGA 4.0 software⁴⁶ with 1000 bootstraps values, JTT model along 0.25 gamma distributions, at uniform rates among sites. Based on the phylogenetic resemblance of proteins involved in reference pathway, a proposed methane metabolic pathway was manually inspected and then verified to reconstruct it with hand curated pathway set derived from the available metabolic information including reactions, Enzyme Commission, biological reliability etc., in KEGG, MetaCyc and UM-BBD databases.

The presence of nutrient stress-induced DNA-binding protein, starvation induced DNA binding protein, DNA starvation/stationary phase protection protein, nucleoside triphosphate pyrophosphohydrolase, carbon dioxide concentrating



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mechanism protein, RuBisCO expression protein, carbon induced starvation protein, starvation-induced protein involved in peptide utilization during carbon starvation, starvation-inducible outer membrane lipoprotein and starvation sensing protein support our molecular hypothesis for survival of this organism under methane enriched condition (CO₂ deficient environment). It was agreed to earlier reports^{47,48}. RuBisCO large subunit and ferredoxin dependent sulfite reductase are known enzymes reported in this organism, which are required for photorespiration and sulfur metabolism. Using a phylogenetic approach, the functions of carbonic anhydrase, putative ammonia monooxygenase, methanol dehydrogenase regulatory protein, zinc-containing alcohol dehydrogenase (class III), S-(hydroxymethyl)glutathione dehydrogenase, S-formylglutathione hydrolase, bidirectional hydrogenase, carbon-monoxide dehydrogenase, soluble methane monooxygenase, glutathione-independent formaldehyde dehydrogenase, formate dehydrogenase, methylamine dehydrogenase and trimethylamine-N-oxide reductase have been assigned to ensure its metabolic capability for hydrogen production from methane gas and methylamine (Figures 1-11). Accordingly, the proposed methane metabolome of cyanobacteria was constructed as shown in Figure 12 and corresponding biochemical reactions are represented in Table 1. Phylogenetic analysis of every genes involved in this pathway revealed the strong relationships between cyanobacteria and gamma proteobacteria, particularly facultative methylotrophic bacteria that is an indicative of occurring methylotrophic characteristic in this organism. Thus, it suggests that this organism would be served as a potential strain for commercial hydrogen production. It is experimentally supported by earlier reports^{49,50}.



Table 1. Major biochemical reactions of proposed methane metabolism in cyanobacteria

EC	ENZYME	REACTION
4.2.1.1	Carbonic anhydrase	$H_2CO_3 = CO_2 + H_2O$
1.13.12.	Ammonia monooxygenase	$NH_3 + \text{Oxygen} + \text{Ubiquinol} \rightleftharpoons \text{Hydroxylamine} + H_2O + \text{Ubiquinone}$
1.11.1.6	Manganese containing catalase	$2 H_2O_2 = O_2 + 2 H_2O$
3.6.3.	Methanol dehydrogenase regulatory protein	$\text{Methanol} + NAD^+ = \text{formaldehyde} + NADH + H^+$
1.12.1.2	Bidirectional hydrogenase	$H_2 + NAD^+ = H^+ + NADH$
1.2.99.2	Carbon-monoxide dehydrogenase	$CO + H_2O + A = CO_2 + AH_2$
1.14.13.25	Soluble methane monooxygenase	$\text{Methane} + NAD(P)H + H^+ + O_2 = \text{methanol} + NAD(P)^+ + H_2O$
1.2.1.46	Glutathione-independent formaldehyde dehydrogenase	$\text{Formaldehyde} + NAD^+ + H_2O = \text{formate} + NADH + 2 H^+$
1.2.1.2	Formate dehydrogenase	$\text{Formate} + NAD^+ = CO_2 + NADH$
1.4.99.3	Methylamine dehydrogenase	$RCH_2NH_2 + H_2O + \text{acceptor} = RCHO + NH_3 + \text{reduced acceptor}$
1.6.6.9	Trimethylamine-N-oxide reductase	$NADH + H^+ + \text{trimethylamine N-oxide} = NAD^+ + \text{trimethylamine} + H_2O$
1.11.1.7	Peroxidase	$\text{Donor} + H_2O_2 = \text{oxidized donor} + 2 H_2O$
1.1.99.8	Zinc-containing alcohol dehydrogenase (class III)	$\text{A primary alcohol} + \text{acceptor} = \text{an aldehyde} + \text{reduced acceptor}$
1.1.1.284	S-(Hydroxymethyl)glutathione dehydrogenase	$S\text{-}(\text{hydroxymethyl})\text{glutathione} + NAD(P)^+ = S\text{-formylglutathione} + NAD(P)H + H^+$
3.1.2.12	S-Formylglutathione hydrolase	$S\text{-formylglutathione} + H_2O = \text{glutathione} + \text{formate}$
1.5.8.1	Dimethylamine dehydrogenase	$\text{Dimethylamine} + H_2O + \text{electron-transferring flavoprotein} = \text{methylamine} + \text{formaldehyde} + \text{reduced electron-transferring flavoprotein}$
1.5.8.2	Trimethylamine dehydrogenase	$\text{Trimethylamine} + H_2O + \text{electron-transferring flavoprotein} = \text{dimethylamine} + \text{formaldehyde} + \text{reduced electron-transferring flavoprotein}$
1.1.1.244	Methanol dehydrogenase	$\text{Methanol} + NAD^+ = \text{formaldehyde} + NADH + H^+$
2.7.9.3	Selenide, water dikinase	$ATP + \text{selenide} + H_2O = AMP + \text{selenophosphate} + \text{phosphate}$
4.4.1.22	S-(Hydroxymethyl)glutathione synthase	$S\text{-}(\text{hydroxymethyl})\text{glutathione} = \text{glutathione} + \text{formaldehyde}$
4.1.2.32	Trimethylamine N-oxide reductase/demethylase	$\text{Trimethylamine N-oxide} = \text{dimethylamine} + \text{formaldehyde}$
1.14.13.	Oxidoreductases	$\text{Hydantoin-5-propionate} + H_2O_2 \rightleftharpoons 4\text{-Imidazolone-5-propanoate} + \text{Oxygen} + H_2O$
4.1.1.39	Ribulose-bisphosphate carboxylase	$2 \text{ 3-phospho-D-glycerate} + 2 H^+ = \text{D-ribulose 1,5-bisphosphate} + CO_2 + H_2O$
1.8.7.1	Sulfite reductase	$\text{Hydrogen sulfide} + 6 \text{ oxidized ferredoxin} + 3 H_2O = \text{sulfite} + 6 \text{ reduced ferredoxin} + 6 H^+$

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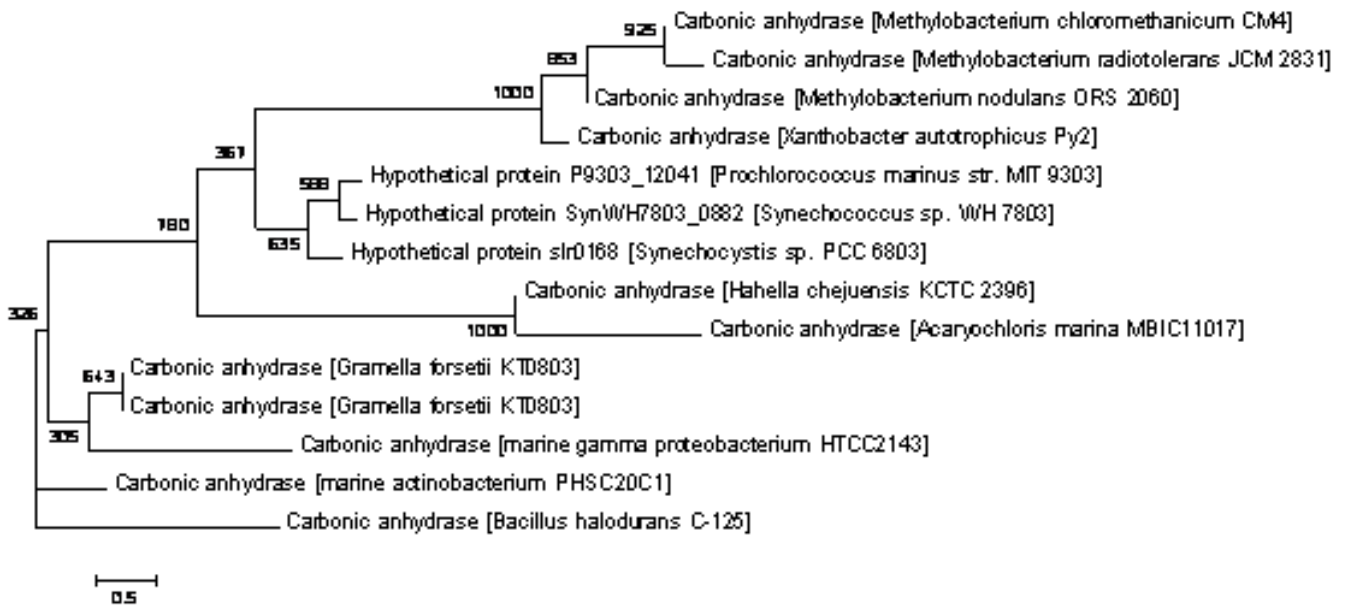


Figure 1. Phylogenetic inference of carbonic anhydrase sequences and similarity hits obtained from cyanobacteria

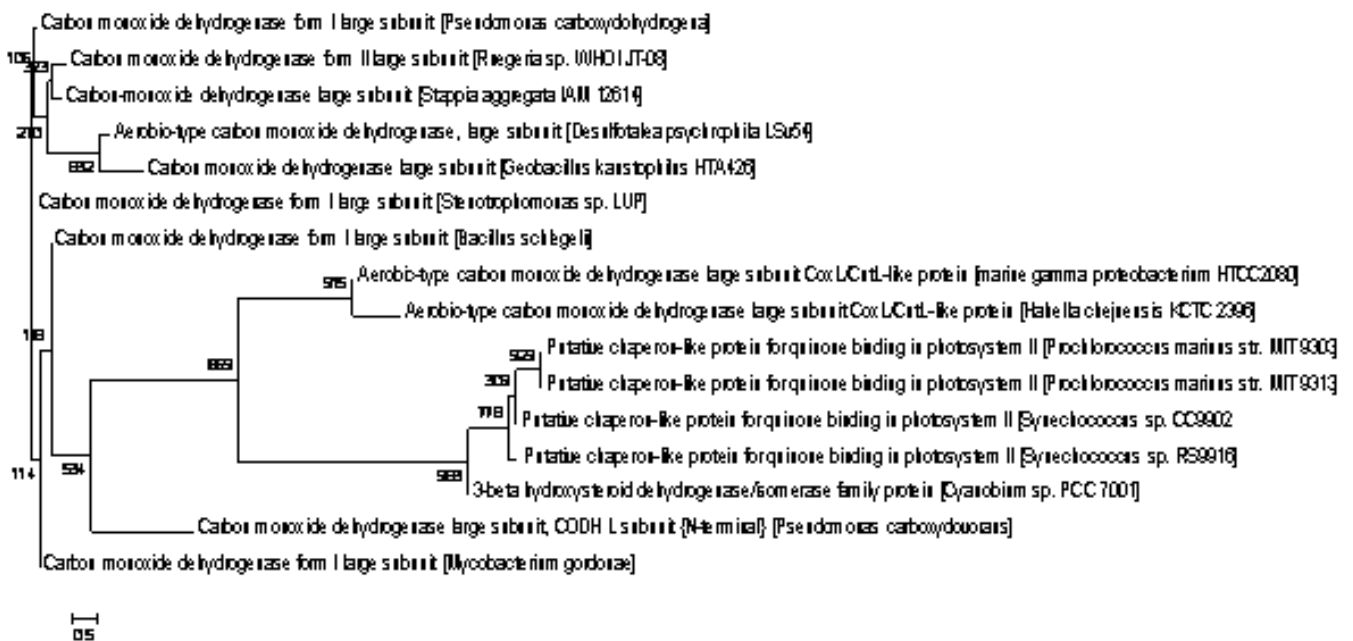


Figure 2. Phylogenetic inference of carbon-monoxide dehydrogenase large subunit sequences and similarity hits obtained from cyanobacteria

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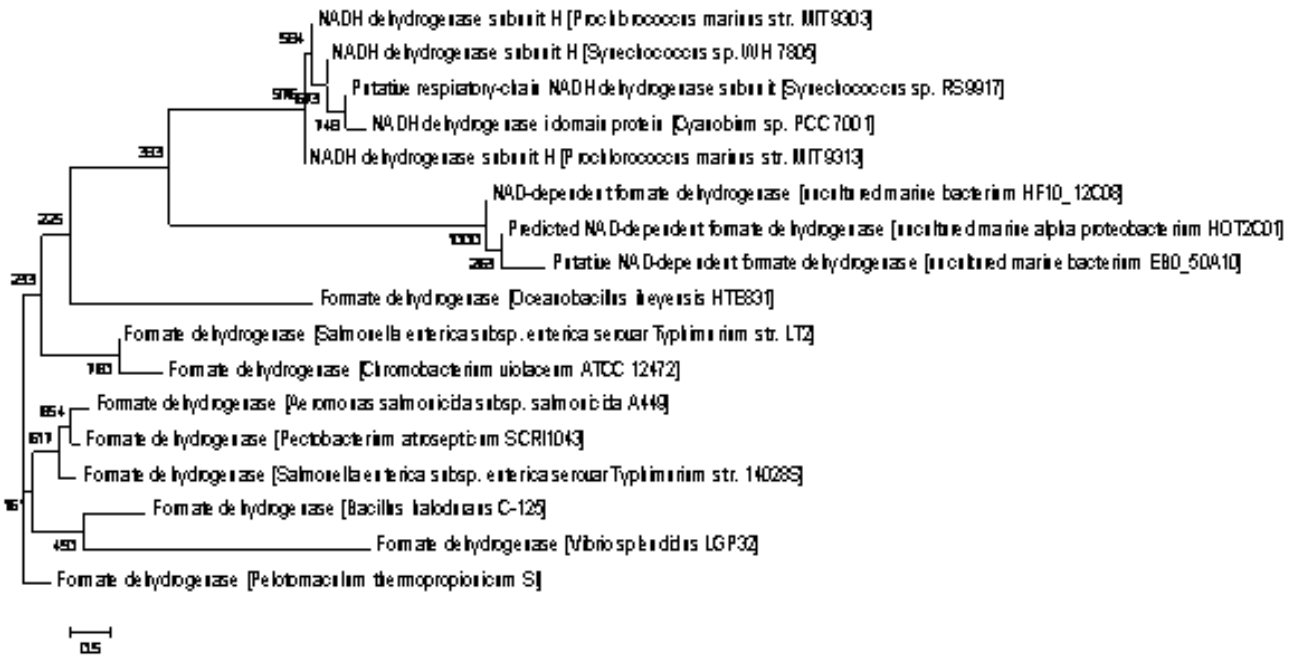


Figure 3. Phylogenetic inference of formate dehydrogenase sequences and similarity hits obtained from cyanobacteria

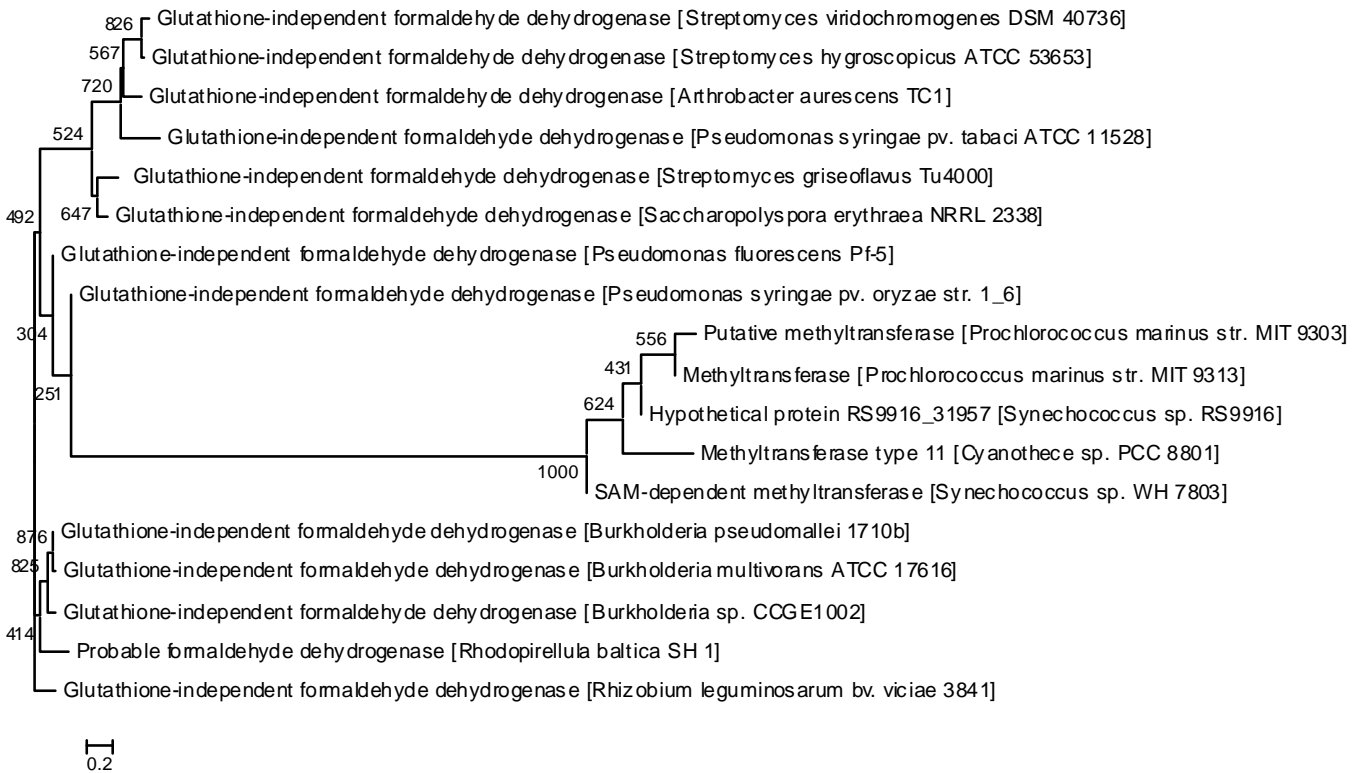


Figure 4. Phylogenetic inference of glutathione-independent formaldehyde dehydrogenase sequences and similarity hits obtained from cyanobacteria

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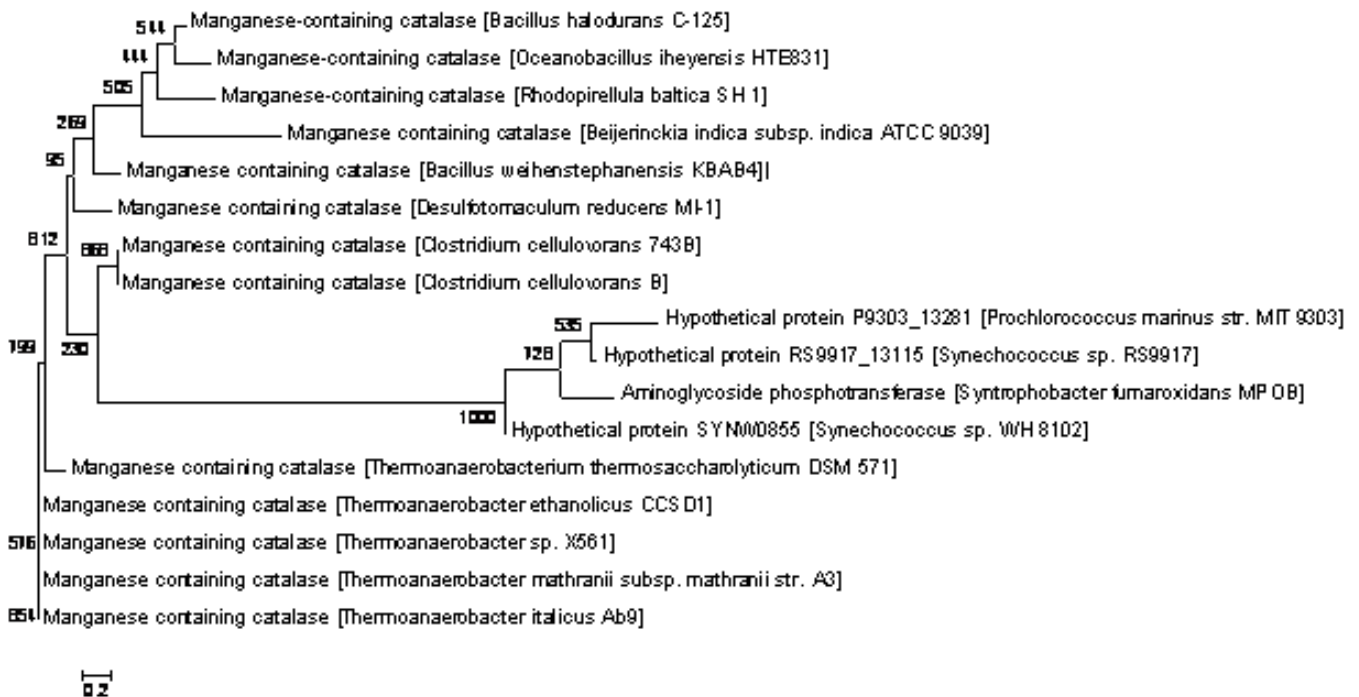


Figure 5. Phylogenetic inference of manganese containing catalase large subunit sequences and similarity hits obtained from cyanobacteria

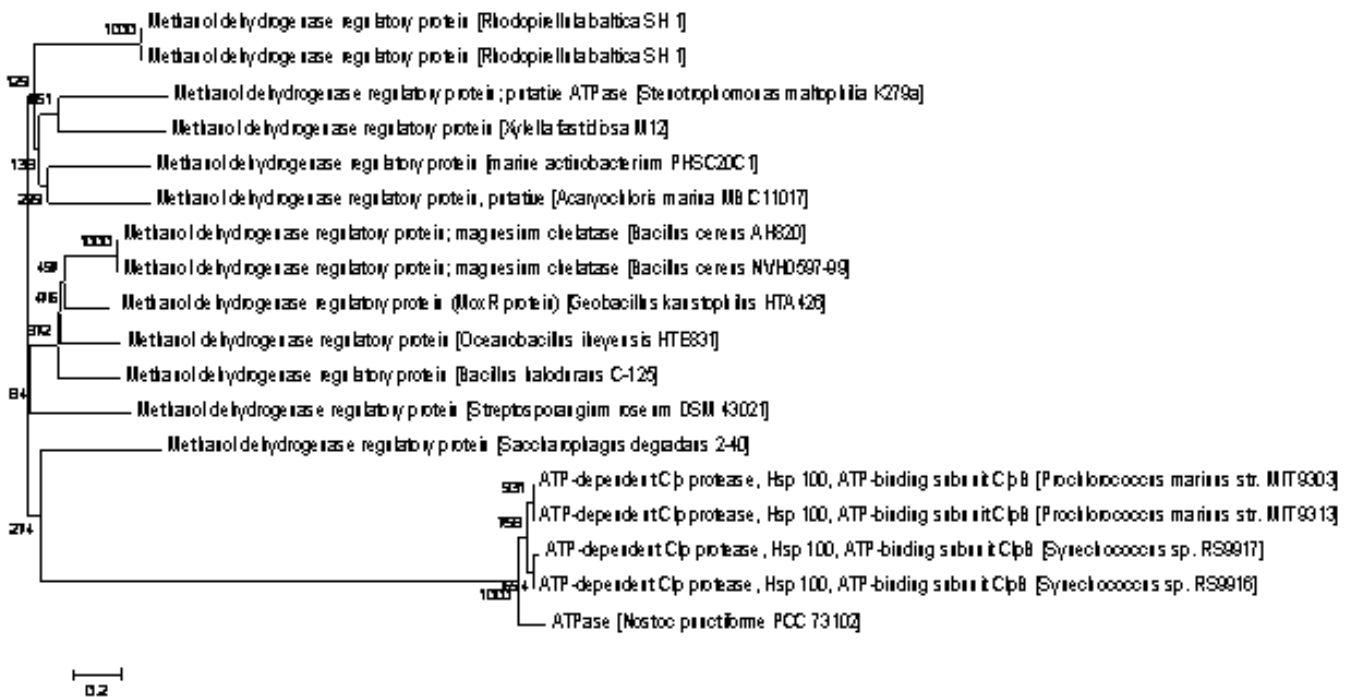


Figure 6. Phylogenetic inference of methanol dehydrogenase regulatory protein sequences and similarity hits obtained from cyanobacteria

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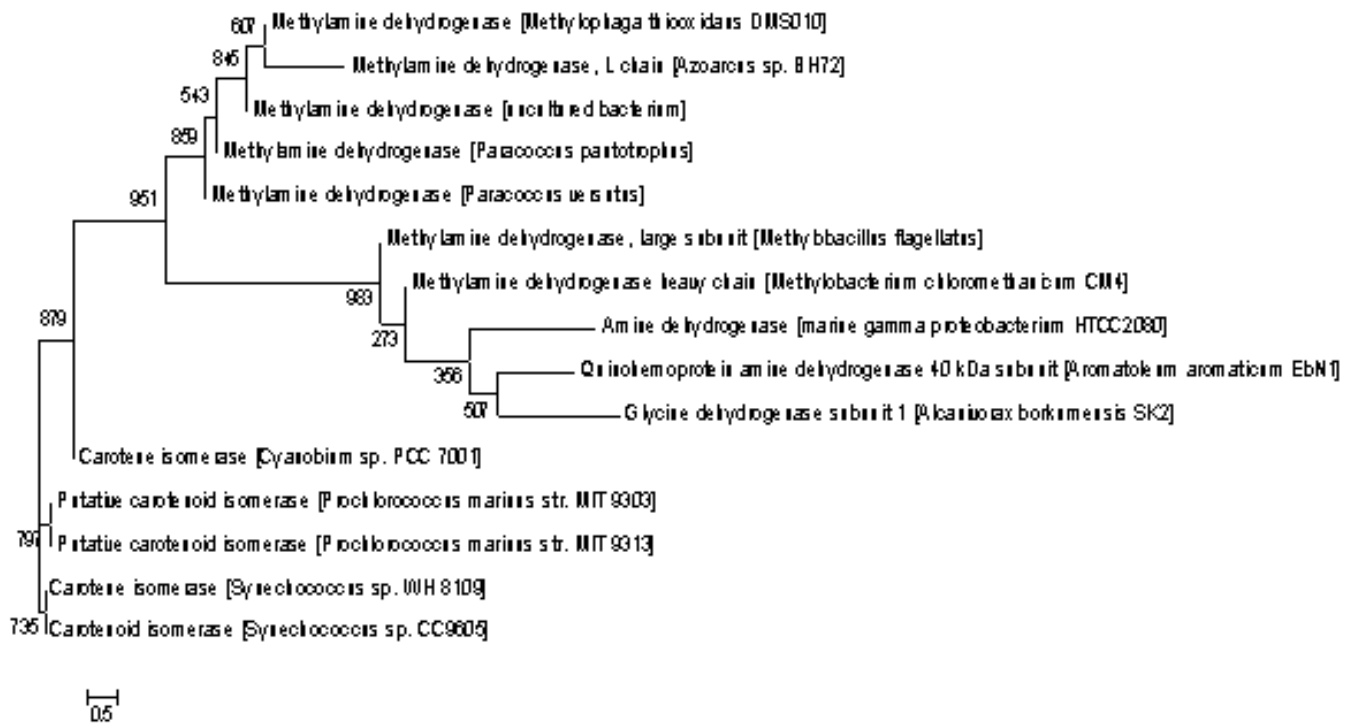


Figure 7. Phylogenetic inference of methylamine dehydrogenase sequences and similarity hits obtained from cyanobacteria

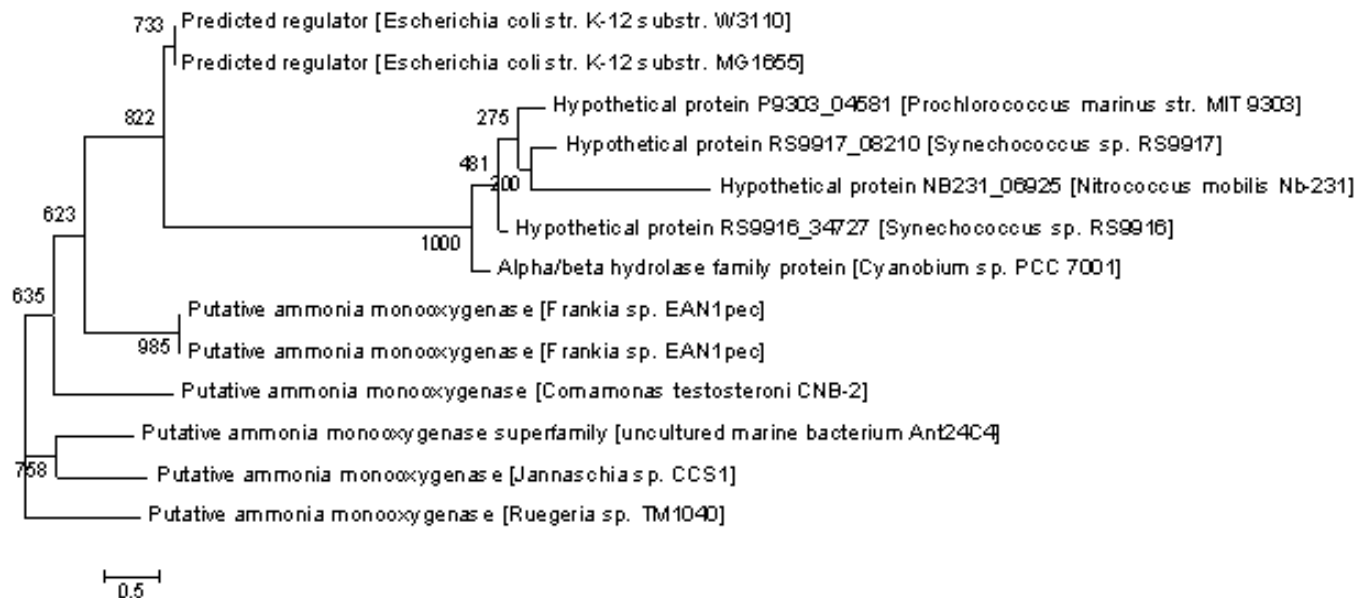


Figure 8. Phylogenetic inference of putative ammonia monooxygenase sequences and similarity hits obtained from cyanobacteria

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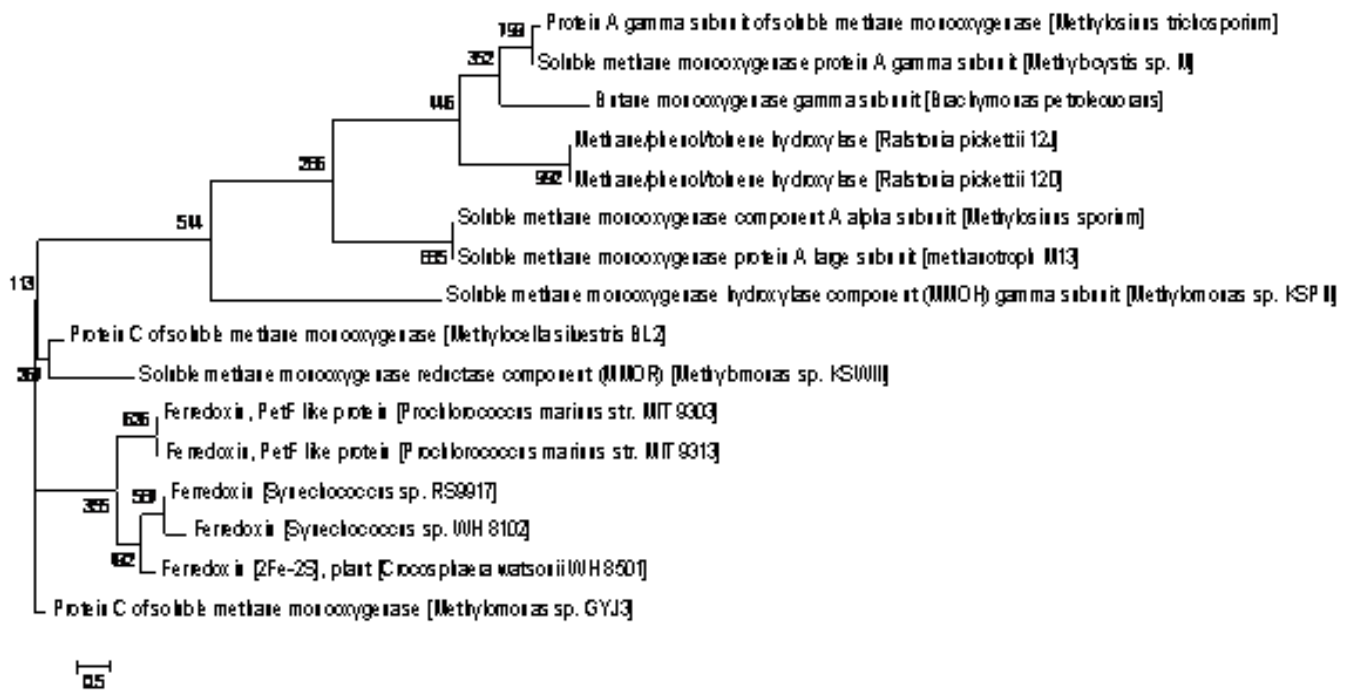


Figure 9. Phylogenetic inference of soluble methane monooxygenase sequences and similarity hits obtained from cyanobacteria

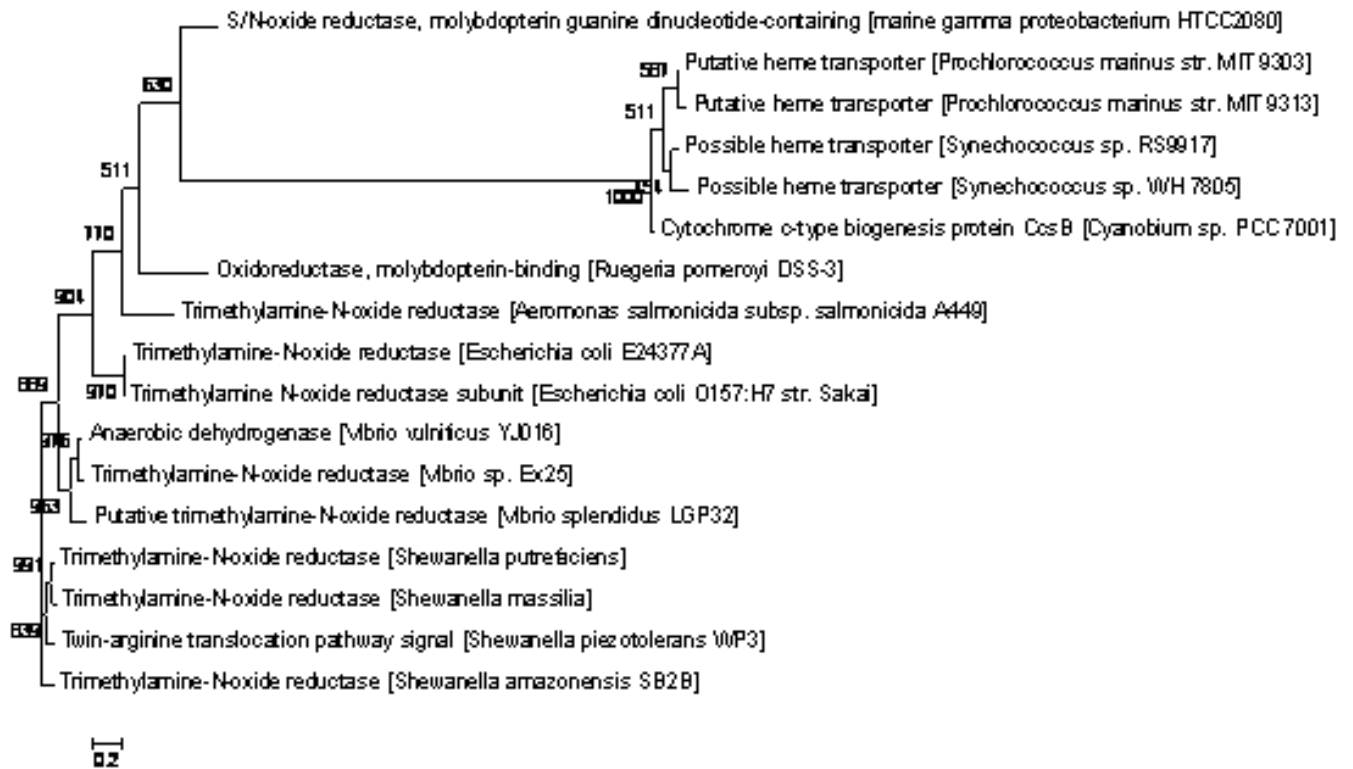


Figure 10. Phylogenetic inference of trimethylamine-N-oxide reductase sequences and similarity hits obtained from cyanobacteria

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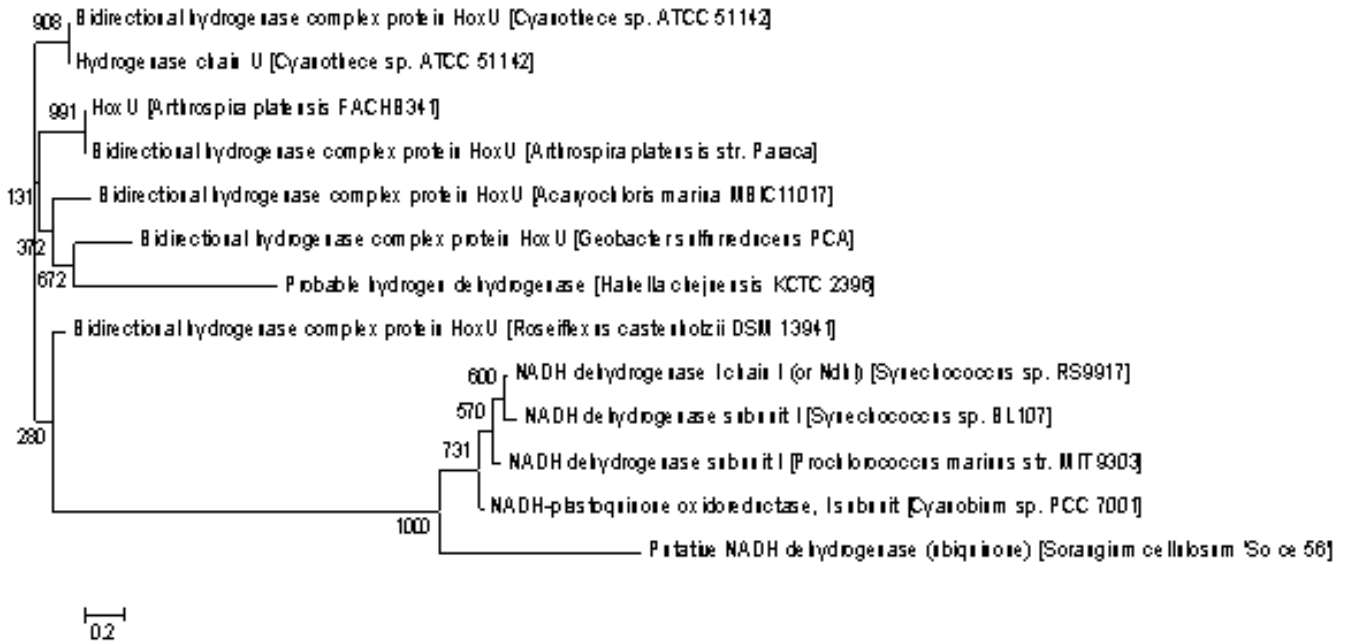


Figure 11. Phylogenetic inference of bidirectional hydrogenase sequences and similarity hits obtained from cyanobacteria

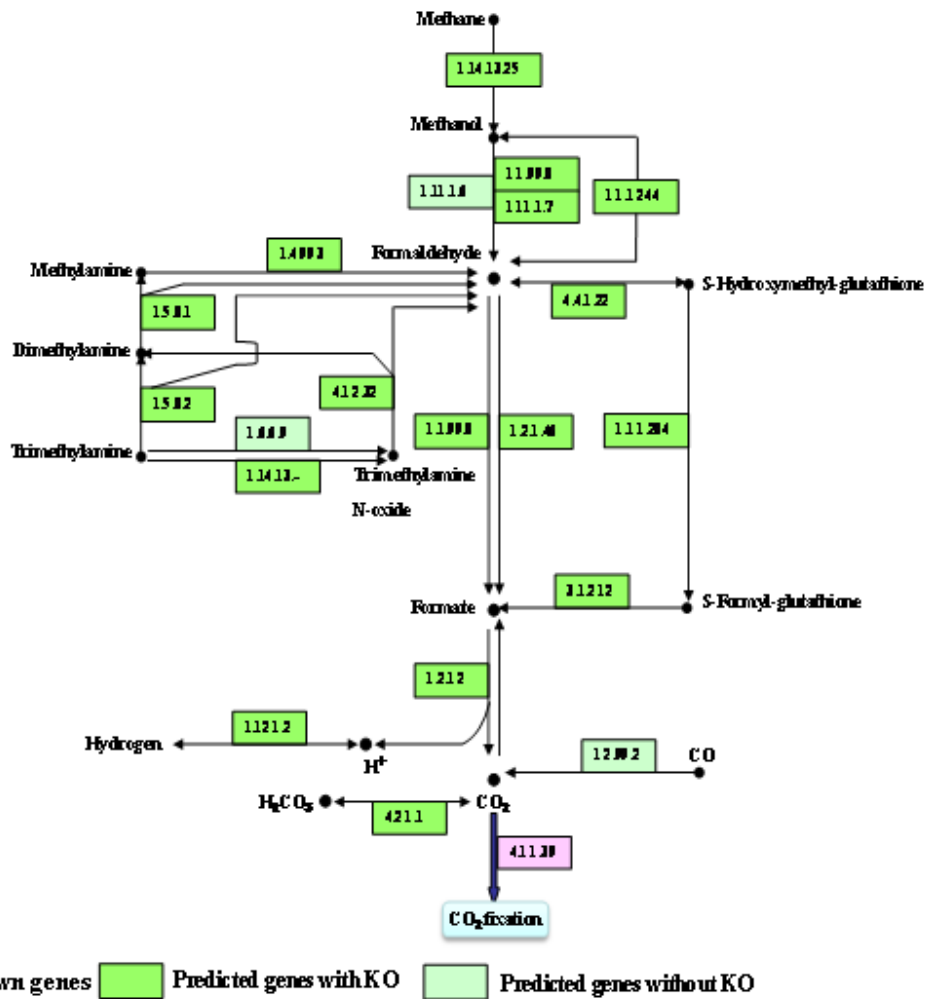


Figure 12. Proposed pathway for methane metabolism in *Prochlorococcus* genomes (KO KEGG orthology)

Conclusion

Overall, we conclude that elucidating the molecular and biochemical mechanisms of methylo-trophy in fresh and marine water cyanobacteria by comparing their genomes functional homologies and metabolomes with Type I, II and X methylo-trophs using systems biology approach will provide an insight to understand their methane assimilation capacities in the ecosystem. It is emphasis the genomes of cyanobacteria can have the metabolic capabilities not only for methane, but also other C1 compounds assimilation by finding the genes involved in their metabolomes. The genes with unknown functions are predicted and complied with known functional genes for reconstruction of methane pathway using bioinformatics resources and thereafter, metabolic model of this pathway is developed with experimental as well as hypothetical data using systems biology approach. The resulted exposure will perhaps coincide with experimental data so as to exploit cyanobacteria for producing hydrogen from C1 compounds and for bioremediation purpose.

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