Exploring Hydrogenotrophic Methanogenesis: A Genome Scale Metabolic Reconstruction of *Methanococcus maripaludis S2*

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# Abstract

Hydrogenotrophic methanogenesis occurs in multiple environments ranging from the rumen of multicellular organisms to marine environments and hot springs. In this work we focus on *Methanococcus maripaludis*, a well-studied hydrogenotrophic marine methanogen.Energy conservation in this organism had long been a mystery; only recently, it was reported that it gains energy for growth via electron bifurcation, thriving despite the lack of sufficient coupling sites. To better understand its metabolism and compare it with other methanogens that utilize a traditional chemiosmotic mode of energy conversation, we have built iMR539, a genome scale metabolic reconstruction that accounts for 539 of its 1722 protein-coding genes. Our reconstructed network uses recent literature to not only portray the central electron bifurcation reaction, but also incorporate vital biosynthesis and assimilation pathways, including unique cofactor and coenzyme syntheses. We show that our distinct methanogenic pathway allows us to accurately replicate experimental growth and gene knockout data. Furthermore, we use our reconstruction to probe into the implications of bifurcation by showing its essentiality in the absence of membrane-bound coupling sites and investigating the effects of ferredoxin specificity on the network. Additionally, we demonstrate a novel method of applying thermodynamic constraints to a metabolic model to quickly estimate overall organism free energy. Finally, we describe a reconstruction-specific computational toolbox we created to improve usability. Together, our results provide a computational network for exploring hydrogenotrophic methanogenesis and confirm the importance of electron bifurcation in this process.

# Importance

Understanding and applying hydrogenotrophic methanogenesis could be key to developing new bio-energy technologies around methane gas. Although a significant portion biological methane is generated through this pathway, existing methanogen models portray more traditional energy conservation mechanisms found in other methanogens. We have constructed a genome-scale metabolic network of *Methanococcus maripaludis* that explicitly shows this environmentally-ubiquitous process, including energy conservation through electron bifurcation. Our reconstruction demonstrates the importance of bifurcation in central metabolism, providing both a window into hydrogenotrophic methanogenesis and a hypothesis-generating platform to fuel metabolic engineering efforts.

# Introduction

Methane plays a critical role in the global carbon cycle and as a greenhouse gas, is 21 times more potent than carbon dioxide (1) in absorbing and emitting energy. Additionally, it is a candidate bridge fuel (2) (ref 1) because it burns comparatively cleaner than traditional fossil fuels. Advancing technology is also enabling this gas to be converted to high energy density liquid fuels with a lower carbon footprint (3). Methane is the second most abundant greenhouse gas after carbon dioxide (ref) and is produced in the environment by biological (refs) and non-biological (refs) sources.. Methanogens are the largest biological contributors of methane, producing about 1 Gt of methane gas per year (4). This group of microorganisms are from the domain Archaea and they grow on carbon dioxide or one or two carbon compounds (5, 6) using enzymes containing unique biological co-factors (Shima ref).

Though phylogenetically and metabolically diverse, methanogens are separated into two groups based on the presence or absence of cytochromes (4). The cytochrome-lacking methanogens have a narrow substrate range of only H2 or formate as electron donors that reduce CO2 to methane (Kyle’s ref); they are sometimes referred to as hydrogenotrophic methanogens. In contrast, cytochrome-containing (or methylotropic) methanogens utilize acetate and methylated compounds for methanogenic growth with a minority also being able to grow hydrogenotrophically. Both groups have similar central pathways of CO2 reduction as well as one universally conserved membrane bound energy conservation site (Mtr), but they also possess additional differing modes of energy conversation.

In the last step of methylotrophic methanogenesis, a traditional membrane-bound electron transport via cytochromes serves as the mechanism of energy conservation. Reducing equivalents are transferred to a membrane bound heterodisulfide complex (HdrDE) via cytochromes or methanophenazine resulting in the generation of an ion gradient for ATP synthesis (4, 7). In hydrogenotrophic methanogenesis however, energy is conserved via flavin-linked electron-bifurcation (ref) in lieu of chemiosmosis. Without cytochromes, these organisms use the energy of an energetically exergonic heterodisulfide reduction to drive the endergonic reduction of the ferredoxin associated with the first step of carbon dioxide reduction (Fwd). Donor electrons from hydrogen or formate oxidation bifurcate at the flavin containing subunit A of the heterodisulfide reductase to the high and low potential paths of heterodisulfide and ferredoxin reduction respectively (ref). Thus, the last exergonic methanogenic step drives the first endergonic reaction step in a cycle (reference to the Wolfe cycle). This type of energy conservation is not membrane associated (ref).

*Methanococcus maripaludis* (8) belongs to this group of hydrogenotrophic methanogens. Compared to the larger genome of methylotrophic methanogens, its genome is relatively small and contains for only 1722 protein coding genes (9). However, it grows robustly with a doubling time of 2 hours (8) and is genetically tractable (10), and thus, has been an ideal candidate for studying methanogenesis (ref), unique co-factors and their biosynthesis (Robert white ref), and novel systems of gene regulation (reference or selenocysteine biosynthesis).To avoid environmental fluctuations that can affect gene regulation, a system for continuous culture of *M. maripaludis* (chemostat reference) has been established for steady state transcriptomic (ref), proteomic (ref) studies of *M. maripaludis* strains. A larger system biological approach for predictive studies have also been done by several groups (refs) for this organism. With these tools in place, and the ability for expression of heterologous genes into M. maripaludis (Lie refs and also Biswarup, for eg.), the metabolic engineering of M. maripaludis for various industrial use is the obvious next step.

Genome scale metabolic reconstructions are powerful tools that map and elucidate metabolic pathways. They are organism-specific knowledge bases that can be used for simulating steady-state growth via flux balance analysis (FBA) (11) by generating constraint-based models. Using these models, we can hypothesize different metabolic scenarios that can then be tested experimentally. They have helped guide metabolic engineering efforts for production of industrial biochemicals in multiple organisms (12, 13). Similarly, a genome scale metabolic reconstruction for *M. maripaludis* would not only provide for a better understanding methanogenesis but also for guiding metabolic engineering efforts that could harness the unique metabolism of this hydrogenotrophic methanogen. Other groups have already created metabolic models of *M. maripaludis*; as part of a mutualistic community model with *D. vulgaris* (14)and under axenic conditions (15). In the former case, the model of *M. maripaludis* represented only core metabolism and was used primarily to investigate interactions between the two different species rather than probe the depths of the organism’s metabolism (14). The latter case was the first genome-scale metabolic reconstruction of *M. maripaludis* (15), an important step towards understanding *M. maripaludis* metabolism.

In our model, iMR539, we made important refinements by updating various pathwaysbased on recent literature*.* The most critical was the critical electron bifurcation step that has been described above as it explains the ability for this organism to grow despite the lack of additional membrane related energy coupling sites (ref). This also includes eliminating methanophenazine utilization and synthesis, which is part of the membrane bound electron transport system of the methylotrophic methanogens and absent in hydrogenotrophic methanogens (4). Additional changes include a corrected sulfur assimilation pathway (16), and the addition of various biosynthesis pathways for all of the unique coenzymes involved in methanogenesis (17). We also increased genome coverage and homology by employing likelihood-based gap filling, a technique that fills reaction gaps based on probability rather than on parsimony (18). Our reconstruction is the first manually-curated genome scale reconstruction to employ likelihood based gap filling. Furthermore, we expanded the scope of our reconstruction beyond stoichiometry by creating a new method to approximate overall model free energy. This is an especially salient consideration for methanogenic archaea, which can grow close to the thermodynamic limits that support life (19). A well-established method of applying free energy constraints involves applying the second law of thermodynamics to metabolic models to restrict reaction directionalities in the direction of negative free energy generation (20, 21). Rather than apply thermodynamic constraints to every metabolic reaction, we created a method that predicts overall free energy generated during steady state growth based solely on standard free energies and effective concentrations of external metabolites. In combining these novel thermodynamic considerations with stoichiometric information, iMR539 provides a means to predict energetically feasible strain designs, enhancing our metabolic engineering capabilities with *M. maripaludis.*

# Methods

## Genome Scale Reconstruction Procedure

The process of genome scale metabolic network reconstruction has been reviewed previously (22) and begins with annotating an organism genome using gene-protein-reaction (GPR) relationships stored in a reaction database. Several databases are available for this purpose (23–25); we chose the Department of Energy Systems Biology Knowledgebase (Kbase; www.kbase.us), a suite of tools that includes the Model SEED reaction database (25). We created our first draft reconstruction using the stored Kbase genome for *M. maripaludis S2* (genome id: kb|g.575) and the automated reconstruction method (“Reconstruct Genome-scale Metabolic Model”). For this initial reconstruction, we used the default gram negative biomass composition and filled knowledge gaps using likelihood based gap filling (method currently not available through Kbase Narrative Interface). This yielded the first full draft of the metabolic reconstruction that could be converted to a simulatable model.

We expanded and refined the model by manually adding information from literature sources. In cases where reactions from literature were part of the Model SEED database, we labeled the reactions using SEED identifiers, names, subsystems, and EC numbers. For other cases where we encountered reactions that were not part of the Model SEED, we created unique reaction identifiers and names, then added subsystem information based on our knowledge of the metabolic network. We also adhered to SEED identifiers, names, formulas, and charges for metabolites whenever possible and had very few cases where we specified our own values. Metabolites were compartmentalized using standard tags for cytosol (“c0”) and extracellular (“e0”) compartments. These tags additionally identify *M. maripaludis* as “Organism 0” in the possible future case where we could add other organisms to create a community metabolic reconstruction. Exchange reactions used for introducing metabolites to the extracellular compartment were standardized in “EX\_{metabolite ID}\_e0” format. Comprehensive information on the reactions, metabolites, and genes in our reconstruction can be found in Supplementary Materials.

## Model Simulations with Flux Balance Analysis

To make rigorous quantitative growth predictions, a genome scale metabolic reconstruction must be converted to a simulatable model. Reactions and their participating metabolites in the metabolic network are connected via the stoichiometric matrix (S), which contains the stoichiometric coefficients for each metabolite (row) in each reaction (column). The S-matrix is converted to a model via the principles of metabolite mass conservation by recognizing that time-dependent accumulation of metabolites in the system (b) is equivalent to the product of the S-matrix and the vector of reaction fluxes (v)

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| --- | --- |
|  | [1] |

In flux balance analysis (FBA), we further simplify this differential system by assuming our organism is in steady state growth, thus b=0 and the system is linear (26). This assumption bounds our model system to a large solution space that can further be constrained by applying upper and lower bounds to each reaction flux:

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|  | [2] |

To find feasible flux distributions that represent likely physiological states within this solution space, we solved our model by optimizing the biomass objective function, a simulation of maximum cell growth yield (27). We further constrained possible flux distributions by minimizing the squared sum of fluxes, effectively forcing our model to find solutions that minimize the total flux in the system while maximizing growth. All model simulations were performed using the COBRA toolbox 2.0 (28) in MATLAB [7.14.0.739] (The MathWorks Inc., Natick, MA).

To encourage model transparency (29) and assist future users in simulating condition-specific models, we created several functions that create these models, simulate maximum growth with the aforementioned constraints, and print relevant information from the flux distribution (see Supplementary Materials). We also wrote numerous functions to help modify the reaction network, retrieve specific useful pieces of information from model simulations, and diagnose issues that may arise during model use. For several of these functions, we used the Paint4Net toolbox (30) to draw flux maps that show the direction and magnitude of fluxes in a given FBA solution. A limited number of our functions are included here in their current versions (see Supplementary Materials) with the full up-to-date set of tools available on Github (https://github.com/marichards/methanococcus).

## Gene Knockout Phenotype Simulations

Because a model is based around the stoichiometry of reactions contained in the S-matrix, knocking out a gene is akin to knocking out all reactions that depend on the gene. Thus, performing a gene knockout phenotype simulation in a metabolic model requires that model reactions be linked to genes via GPR relationships. We performed gene knockout simulations using our function “simulateKOPanel.m” (see Supplementary Materials), which relies heavily on the “deleteModelGenes.m” function in the COBRA Toolbox 2.0 (28). Our experimental test set included 18 knockout genotypes across 4 different growth conditions, with 30 total wet lab experiments across these conditions (31–36). We simulated growth phenotypes for all 72 combinations of knockout genotypes and growth conditions and then evaluated these growth phenotypes as lethal/non-lethal with a threshold of 10% wild type growth. Predictive accuracy was assessed by comparing predictions on the 30 known phenotypes with wet lab data. We further evaluated our model’s performance using the Matthews correlation coefficient (MCC)(37):

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| --- | --- |
|  | [3] |

## Thermodynamic Calculations

We added standard free energies of formation (1 mM, 25ºC, 1 bar, pH=7, ionic strength = 0.1 M) from the Equilibrator database (38) to all exchanges reactions for which these values could be calculated via the group contribution method (39). To incorporate these values into our reconstruction, we expanded the standard structure to include a “freeEnergy” numerical array with length equal that of the “reactions” array. For calculating overall free energy of a flux distribution, we created an “optimizeThermoModel.m” code (see Supplementary Materials) that is built around running the “optimizeCbModel.m” code in the COBRA Toolbox 2.0 (28). Our code accepts effective concentrations (mM) for specified exchange metabolites, assumes standard concentrations of 1 mM for the remaining metabolites, and uses these values to calculate effective metabolite free energies based on the reconstruction’s stored values for each exchange reaction. Prior to performing FBA, we add these free energies to the exchange reactions, which ordinarily have the form:

We alter these exchanges such that production of a metabolite “creates” free energy equivalent to the metabolite’s free energy of formation:

Here, is the stoichiometric coefficient of a new metabolite “dG” that is used to sum model free energy. Because exchange reactions are model constructs that satisfy mass balance by necessarily entering or exiting the model without creating new metabolites, adding free energies to the model creates an imbalance that we must correct. We restore model balance by allowing “dG” to exit the model via its own exchange reaction:

Measuring the total flux of the exchange reaction gives us an estimation of total free energy being generated in an FBA solution on a per cell mass basis. We have incorporated this thermodynamic calculation into all of our available model simulations (see Supplementary Materials); thus by default, we calculate and print overall model free energy in every flux distribution.

## Experimental Measurements

Wild type *M. maripaludis S2* cells were grown in a chemically defined medium (See Supplementary Materials) using a 1-L chemostat under anaerobic conditions as described previously (40). Chemostats were operated in steady state continuous mode under H2-limiting conditions to match model simulation conditions, with gas flows of 10-15 mL/min H2, 40 mL/min CO2, 15 mL/min H2S, and a balance of N2 up to a total 200 mL/min.

We recalculated calibration curves for dry cell weight versus optical density by measuring dry cell weight via cell filtering and optical density via a UV/Vis spectrophotometer {model number?}. Overall chemostat optical density was measured and 20 mL aliquots of cells in media were then sampled directly from chemostat culture by syringe. These aliquots werevacuum filtered through 0.45 µM pore filters to remove all non-cellular components, then dried at room temperature and weighed daily until their weights stabilized.

As described by Thiele and Palsson, the optimal way to obtain accurate ATP maintenance values is to plot ATP production versus growth data from chemostat growth experiments (22). In practice, this required us to measure the growth rate and methane secretion rate of *M. maripaludis* during steady state growth to obtain a set of training data. For each time point, we constrained our model to our measured growth rate and secretion rate and set the model objective to maximize ATP hydrolysis (rxn00062[c0]). We plotted the resulting value of ATP production as a function of growth rate and obtained the growth-associated (slope) and non-growth associated (y-intercept) ATP maintenance values using a linear model. We ensured steady state growth by monitoring dry cell weight via optical density values. Cell growth rate was measured using chemostat dilution rate, which is equivalent to growth rate at steady state {ref?}. Methane evolution rate was quantitatively assessed by measuring total gas outflow using a bubble flow meter and measuring the methane fraction using a {model name/number?} gas chromatograph. The resulting plot can be found in Supplementary Materials.

# Results

## Reconstruction Statistics

The basic statistics for iMR539 are displayed in Table 1. Notably, reactions are categorized as 1) internal reactions, occurring entirely within the cytoplasm; 2) transport reactions, involving translocation of at least one chemical species across the cell membrane; 3) exchange reactions, which supply metabolites to or remove metabolites from the model. Of the 586 internal reactions in our network, over 85% of the internal reactions in our reconstruction are associated with at least one gene. We suspect that a major reason for our increase in gene-associated reactions was our use of likelihood based gap filling, which resulted in the automated addition of 66 genes to our reconstruction before we began to curate it manually. Furthermore, we relied heavily on biochemical knowledge from literature sources, particularly regarding recently-elucidated biosynthesis pathways that were not available in annotation databases. Combined, our use of maximum likelihood gap filling and reliance on published literature sources resulted in more consistent ties to gene homology.

Another salient detail of our reconstruction is that it includes many “dead-end” metabolites and reactions that cannot be synthesized or consumed. Thus, these metabolites and reactions are not part of our simulatable model, but we have included them in our reconstruction because they are all gene-associated; all dead end internal reactions in our reconstruction have at least one gene association. This indicates that we have genetic evidence that each of these reactions and the involved metabolites should be involved in metabolism, but we have not yet elucidated full synthesis or consumption pathways. They represent excellent candidates for further exploration of *M. maripaludis* metabolism, particularly as this reconstruction is updated and expanded in the future.

Conversely, our reconstruction contains 85 internal reactions that lack genes, many of which were added during the automated gap filling phase but some of which were added manually. All of our reactions are annotated with subsystems, allowing us to assess where each reaction, including those without genes, fits into metabolism. Figure 1 shows a breakdown of these reactions lacking genes, where the subsystems have been manually grouped into broader categories (e.g. “Amino Acid Biosynthesis” instead of “Glycine Biosynthesis”). The largest group of these reactions is the “Unique Coenzyme Syntheses”, which includes reactions that synthesize coenzyme M, coenzyme B, tetrahydromethanopterin (H4MPT), methanofuran, coenzyme F420, and coenzyme F430. Although these 24 reactions lack genes, all of them were added manually as hypothetical steps to complete essential biosynthetic pathways and are based on information from biochemical literature. These are distinct from, for example, the 11 reactions encompassed by “Vitamin and Cofactor Synthesis” that were all added to fill biosynthesis gaps but have no supporting literature evidence. In total, 46 of the 85 reactions genes(54%) are gap filling reactions and the remaining 39 (46%) are classified as “hypothetical”, with no known genes currently but with literature evidence pointing to their inclusion in the reconstruction. We expect that as experimental research groups uncover more biochemical phenomena, they will determine genes that tie to the reactions in this latter group. The gap filling reactions, much like dead end reactions and metabolites, point us toward poorly-understood areas of metabolism in our organism and require more investigation into both the reaction pathways and their associated genes.

Our use of likelihood based gap filling not only directs us toward unknown portions of metabolism, but also lets us assign likelihood scores for many of the reactions in the reconstruction. These confidence scores quantify the probability of a given reaction being part of the metabolic reconstruction on a scale of 0-1 and provide a novel metric of evaluating our confidence in the reconstruction. We can then use the scores allow us to quickly hone in on both reactions that lack genes and gene-associated reactions with low gene homology as possible targets for more experimental investigation. They also provide a logical starting point for future users looking to expand upon and improve the existing reconstruction.

## Effects of Electron Bifurcation

It has been often assumed that the methanogenic pathway is linear with two main membrane complexes for generating membrane potential. That is the Mtr and the heterodisulfide complex (HdrDE). Without the membrane bound type heterodisulfide reductase of methylotrophic methanogens, *M. maripaludis* lacks one critical energetic coupling site to generate energy for growth. However, it has a cytoplasmic heterodisulfide reductase that conserves energy by an increasingly recognized form of energy conservation in strict anaerobes involving electron bifurcation (refs). As described above, this links the last step of methanogensis to the first step by exergonically driving the endergonic reduction of the 1st step resulting in a circular pathway (ref). This is noteworthy because the assumption of a linear pathway in *M. maripaludis* without accounting for electron bifurcation can affect the downstream predictions in the metabolic model.

Because it is a relatively novel mechanism of energy conservation, this heterodisulfide reduction step was not part of our annotation database and thus was not included in the automated first reconstruction draft. Rather, the default mechanism of energy conservation matched methylotrophic methanogens and utilized methanophenazine, an electron carrier known to be absent from *M. maripaludis* and other hydrogenotrophic methanogens. We replaced the methylotrophic pathway with the correct electron bifurcation pathway, linking heterodisulfide reduction with electrons from H2 to carbon dioxide reduction via reduced ferredoxin. This incident serves as an example of a commonly-encountered reconstruction pitfall, in which information available in annotation databases does not sufficiently represent known biochemical phenomena. Therefore, it is paramount that even as automated reconstruction methods improve, we take the time to carefully evaluate biochemical literature maximize consistency of the reconstruction with experimentally-verified pathways.

To demonstrate the importance of ferredoxin reduction via bifurcation, we altered the existing bifurcating heterodisulfide reductase (Hdr) reaction:

We removed ferredoxin from this reaction, balancing mass and charge to yield:

This scenario represented a case where *M. maripaludis* would not contain a membrane-bound Hdr complex but could not perform electron bifurcation. We optimized our model for growth on CO2 + H2 using this model and were unable to predict *in silico* growth, suggesting that the ferredoxin reduction via electron bifurcation is an essential part of our network. Lack of model growth can be clearly attributed to disruption of the central energy conservation mechanism in *M. maripaludis*, in which electron bifurcation must necessarily reduce ferredoxin for reducing CO2. The only other complex capable of generating large amounts of reduced ferredoxin is the Eha hydrogenase, which utilizes a sodium ion gradient to reduce ferredoxin on a 1:1 basis. Similarly, CO2 reduction to methane requires reduced ferredoxin and pumps out sodium ions on a 1:1 basis. Thus, each cycle of methanogenesis in this scenario effectively produces no sodium ion gradient for synthesizing ATP, the central component necessary for biomass formation. Additionally, methanogenesis also “leaks” small amounts of carbon for biosynthesis; hence, reducing one ferredoxin effectively pumps less than one sodium ion across the cell membrane and creates an overall energy deficit. Overall, this simulation illustrates the essentiality of ferredoxin reduction via electron bifurcation and reinforces the idea that EhA hydrogenase can play only an anaplerotic role in methanogenesis (32).

Interestingly, there is evidence that *M. maripaludis* uses multiple forms of ferredoxin as electron carriers and may link multiple steps, particularly those involved in electron bifurcation, using specific ferredoxins (41). Presently, the full extent of this phenomenon is not well understood and requires more experimental investigation. However, in an effort to represent ferredoxin specificity in our model, we have included a function (see Supplemental Materials) that changes promiscuous ferredoxins to specific ferredoxins for the EhA hydrogenase, heterodisulfide reductase, and formylmethanfuran dehydrogenase (carbon dioxide reduction). Using this function, tightens the coupling between the aforementioned reactions by restricting them all to one pool of electron carriers and allows us to predict how ferredoxin specificity could change possible model flux distributions.

## Other Biochemistry Improvements

A major part of our manual curation was adding biosynthesis pathways for the methanogenic coenzymes, sugars, and lipids. *M. maripaludis* utilizes various unusual coenzymes directly as electron carriers (methanofuran, H4MPT, coenzyme F420, coenzyme B, coenzyme M) and vital pieces of catabolic enzymes (coenzyme F430) during methanogenesis (5). It also synthesizes an archaellin tetrasaccharide as part of N-linked glycosylation (42) and multiple forms of archaeol, an archaeal membrane ether lipid (43). These synthesis pathways, particularly those for the coenzymes, are vital pieces of *M. maripaludis* metabolism, hence we were adamant about including synthesis pathways for these metabolites and adding them to our biomass composition. Although several of these pathways were completely included in the Model SEED database, many reactions were missing and nearly all of the reactions were added manually after automated reconstruction. Including these features in our reaction network and biomass definition distinguishes our model by incorporating multiple pathways that differentiate its metabolism from most other organisms.

In a similar vein, we sought to accurately represent sulfur assimilation, a pathway not yet fully understood in *M. maripaludis*. Sulfate is known not to be the sulfur source for *M. maripaludis* because sulfate reduction produces sulfite, which inhibits methanogenesis (44). However, because sulfate is the default sulfur source for most microorganisms, our initial reconstruction included a sulfate transporter and sulfate reduction pathway. We removed the sulfate transporter and instead added a transporter for sulfide, the primary sulfur source for *M. maripaludis.* Our updated sulfur assimilation pathway includes a pathway for sulfide oxidation to sulfite, which is essential for multiple biosynthetic pathways, via a hypothesized dissimilatory sulfite reductase-like protein (16). Taken together with additional coenzyme syntheses, these modifications demonstrated the need for rigorous manual curation to add known biochemical pathways that were not part of the automated reconstruction and remove pathways that are known not to function in the organism. By employing these methods and by working in a team of experienced metabolic modelers and biochemical experts, we have created a reconstruction that is qualitatively consistent with accumulated biochemical knowledge of our organism.

## Model Validation

Evaluating the metabolic network reconstruction by qualitatively comparing it to known biochemical phenomena is a useful way to gauge how close the network can represent actual biochemistry. To make more quantitative comparisons, we must convert the reconstruction to a metabolic model by imposing flux constraints on the network, enforcing mass balance on all metabolites, and optimizing to an objective function (see Methods). A common way of quantitatively evaluating the resulting model is to simulate maximum cell growth under steady-state conditions and compare growth yield predictions to experimentally-determined values. Due to the narrow range of possible substrates for our hydrogenotrophic system, our comparison was limited to two conditions: H2-limiting and formate-limiting. Previous work yielded YCH4 (grams [cell mass] per mole of CH4 produced) values of 2.86 ± 0.58 for H2 limitation and 2.31 ± 0.58 for formate limitation (45). These experimental yields were based on a previously-reported conversion factor between optical density and dry cell weight with A600 of 1 corresponding to 0.34 mg(dry weight)∙ml-1 (33). We were unsure of the accuracy of this value and to mitigate our concerns, we re-measured this conversion factor (see Methods). We used our newly-measured value of #VALUE to calculate YCH4 values of 2.86 ± 0.58 for H2 limitation and 2.31 ± 0.58 for formate limitation.

Growth yield predictions can also vary considerably in response to model ATP maintenance energies (46). Our default biomass equation obtained through Kbase specified growth associated maintenance (GAM) as 40.11 (mmol per grams [cell mass]) and the first draft model specified no non-growth associated maintenance (NGAM). Though these figures gave what we considered to be reasonable growth yield predictions, we chose to measure GAM and NGAM ourselves with respect to methane production rate (see Methods). Following our growth experiments, we altered these values to #VALUE# and #VALUE# in for GAM and NGAM, respectively.

Using our recalibrated growth yields and ATP maintenance values, we compared our model’s FBA predictions of maximum growth yields to the experimentally-derived measurements as shown in Figure 2. Both of our computational values agreed closely with the experimental values, falling within the uncertainty range in each case. Though growth yield validation is not an absolute measure of model performance, our model’s ability to closely reproduce experimental results without manual overfitting suggested a high propensity for generating viable growth predictions. This result bodes well for our model’s utility as a predictive tool as we look to use it to generate quantitatively feasible growth hypotheses for novel strain designs.

Gene knockout experiments present a different method for validating a metabolic reconstruction based on its model. At its core, a constraint-based model is built around gene-protein-reaction relationships that connect genotype to growth phenotype. Thus, comparing model predictions of gene knockout lethality provide an excellent way to quantitatively measure the qualitative content of the model. This process hinges on the availability of gene knockout data for the organism being modeled, ideally with the abundance of data found for a traditional model organism such as *Escherichia coli* (47). In the case of *M. maripaludis* transposon mutagenesis has been used to calculate an essentiality index of all genes in *M. maripaludis* (48), but there is relatively little data where gene knockout experiments have been systematically carried out *in vivo.* Because much of methanogenesis revolves around the function of different hydrogenases, the bulk of available gene knockout data involves hydrogenase knockouts on different media. For our test set, we were able to assemble a knockout panel of 30 binary growth phenotypes based on previous publications (31–36). Though the breadth of these knockout genotypes is limited, they are all vital pieces of central carbon metabolism and therefore, they give us a good idea of how well our model can predict knockouts in central catabolism. In comparing with these data, as shown in Figure 3, our model achieved 90% prediction accuracy and a Matthew’s correlation coefficient of 0.67. These high values suggested that our model is an excellent predictor of growth phenotype based on genotype changes in central carbon metabolism. This result was particularly encouraging because we avoided training our model on this dataset in the interest of preventing overfitting our model to the validation set.

It is also worth noting that all 3 incorrect predictions had regulatory bases. In these cases, knockouts of 5 or 6 hydrogenases are lethal in formate-grown cells with downregulation of the glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR) cycle. When the GAPOR cycle is upregulated (as in Δ6H2asesupp and Δ7H2asesupp), it provides anaplerotic electrons that allow cell growth in formate media, even when missing up to 7 hydrogenases. Upregulation of the GAPOR cycle is a regulatory difference, and we have chosen to leave the cycle upregulated as a default to allow for a richer spectrum of possible flux distributions. Thus, although our default model does not predict knockout growth phenotypes with 100% accuracy, it can easily be tuned to reflect regulatory differences.

## Thermodynamic Calculations

Free energy plays a key role in biochemistry as all biological systems must have a sufficiently low overall free energy to support growth. When simulating optimal growth using a metabolic model we expect the same rules to apply to our system, hence we can apply thermodynamic constraints to the model based on metabolite free energies of formation. In a previous study, free energies of formation were used to constrain reversibility of all internal model reactions based on the second law of thermodynamics (20). This method, while rigorous, is highly dependent on concentration and can be overly restrictive with regard to predicted flux distributions; thus it is most effective when paired with metabolite effective concentration data (21). Lacking extensive effective concentration data for *M. maripaludis,* we chose to represent free energy constraints in a novel approach where we add free energies only to exchange reactions, the set of metabolites that can be taken up or produced by the model. These metabolites effectively represent the overall biochemical “reaction” of a model, therefore it is reasonable to expect this overall reaction must produce a negative overall free energy to support growth. This additional constraint can be added by restricting overall free energy to be negative, the equivalent of imposing the second law of thermodynamics on the organism itself.

We expect that this straightforward calculation (see Methods) will be a useful addition to our model, particularly as we aim to use it as a platform for generating possible strain designs. With regard to free energy, methanogens are particularly notable in that they subsist close to the thermodynamic limit to support growth (19). It follows that for any potential strain design, we must pay particular attention to the overall free energy of our system, lest it dip below this vital threshold. It may also provide a metric for differentiating between multiple feasible strain designs by ranking them in order of thermodynamic feasibility. At the very least, it serves as an additional capability of our model and as a checkpoint to ensure that our overall stoichiometry matches up with overall free energy. We have included example functions for adding metabolite free energies to our model and performing FBA with an additional free energy calculation (see Supplementary Materials).

## Reconstruction and Model Distribution

Reconstructing a metabolic network is an iterative process and therefore, it is paramount that reconstructions be as clear as possible to encourage future updates and expansions (29). We have strived for clarity in both our nomenclature and in our decision making process for including each reaction present in our reconstruction. Reactions and metabolites in our network are based upon identifiers and names found in Kbase, but also include crosslinks to KEGG identifiers (23), enzyme commission numbers, and reaction subsystems where available. Each reaction and gene in the reconstruction is justified and connected to its literature and/or database source (see Supplementary Materials).

Additionally, we have sought to maximize usability of both our reconstruction and the resulting model. The systems biology markup language (SBML) is a standard medium for distributing metabolic reconstructions (49); thus, we have included our reaction network in SBML. In our experience using reconstructions from other groups, we have found a wide range of usability, from those that can easily be imported and simulated to those that are difficult to use and interpret. In the interest of making our simulations and results easy to reproduce, we have included our reconstruction in MATLAB data structure format and all of our codes for simulating model growth on different media and gene knockout phenotypes (see Supplementary Materials). In the interest of transparency and to support the iterative process of reconstructing metabolic networks, we have also made our codes and reconstruction available on Github (https://github.com/marichards/methanococcus).

# Discussion

Metabolic reconstructions provide us with a computational approach to studying the complexity of genome-scale biochemistry. With iMR539, we have created the most comprehensive reconstruction of *M. maripaludis* currently available, the first manually-curated reconstruction built on top of likelihood based gap filling, and a straightforward new method to add thermodynamic constraints to metabolic models.

Our reconstruction process hinged first and foremost on literature sources, which provided high quality biochemical information. Through manual curation from these sources, we created a model with an accurate representation of the Wolfe Cycle, added pathways to synthesize methanogenic coenzymes, included archaellin and archaeol lipid biosyntheses, and corrected the sulfur assimilation pathway. The efficacy of rigorous manual curation is shown not only in the qualitative accuracy of our reconstruction, but also in the formidable quantitative capabilities of the resulting model.

This reconstruction is also, to our knowledge, the first genome scale metabolic network created using likelihood-based gap filling. Favoring this method over a maximum parsimony method helped us to automatically identify 66 more genes, increasing the gene coverage of our reconstruction prior to the start of manual curation. Simultaneously, we were also able to assign reaction likelihood scores for many of the reactions, lending a measure of confidence level for including each reaction in the network.

For a methanogen living close to the edge of thermodynamic feasibility, we also thought it salient to include some calculation of overall free energy when simulating our model. We have thus introduced a novel method of predicting overall model free energy generation based solely on standard free energies and concentrations of exchange metabolites. Though a relatively trivial calculation, our method gives a quick assessment of whether a predicted flux distribution is thermodynamically possible and could prove a particularly useful tool for guiding future metabolic engineering designs.

We recognize that our reconstruction effort represents only an incremental step toward understanding *M. maripaludis* metabolism and that many other users may follow in our footsteps. With these considerations in mind, we strived for maximum transparency in our metabolic network to make our reconstruction decisions apparent to future users and to make our results easily reproducible. In addition to distributing our model in SBML format, we have also included numerous functions for modifying the reconstruction, simulating growth, and predicting gene knockout lethality. By following this model of reconstruction distribution, we hope to encourage increased transparency and user-friendliness in all metabolic network reconstructions.

Taken together, this work demonstrates both the importance of doing extensive manual curation and the effectiveness of using automated gene homology based methods when reconstructing metabolic networks. Automated methods such as likelihood based gap filling are invaluable in their ability to quickly expand and enrich the reaction network, but literature from experimental studies still serves as the gold standard for adding new pieces to a reconstruction and verifying biochemical pathways. Above all, the goal of a metabolic network reconstruction is to create a realistic representation of organism metabolism that can then be used to push forward biological discovery and fuel innovations in strain design. With this reconstruction, we have contributed a tool to help understand the unique biochemistry of hydrogenotrophic methanogens and unlock their potential as metabolic engineering targets.

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# Tables and Figures

|  |  |  |
| --- | --- | --- |
| ***Methanococcus maripaludis S2* model comparison** | | |
| Model | iMM518 | iMR539 |
| Protein Coding Genes | 518 | 539 |
| % ORF Coverage | 30 | 32 |
| Intra/Extracellular Metabolites | 556/49 | 665/53 |
| Dead End Metabolites | 163 | 265 |
| Internal Reactions | 570 | 645 |
| Exchange Reactions | 49 | 59 |
| Gene-Associated Reactions | 464 | 590 |
| % Reactions Associated with Genes (non-exchange) | 75 | 91 |
|  | | |

Table 1: A comparison between iMR539 and iMM518 indicates that our model covers slightly more of the genome, including over 125 more gene-associated reactions. Our model includes more internal reactions and metabolites, as well as approximately 100 more dead end metabolites. Though these metabolites represent the portion of metabolism that cannot carry flux, all of our model's dead end metabolites are part of gene-associated reactions and thus represent promising avenues for future model expansion

Figure 1: A chart showing broad subsystem groupings of the 85 reactions in iMR539 that are not associated with any genes. Reactions falling underneath the “None” subsystem grouping were present in the Model SEED database but had no subsystems listed there and no obvious membership in another subsystem. Reactions grouped within “Other” were dissimilar both from the other categories and from one another, thus we felt they did not merit creation of multiple additional categories.

Figure 2: Comparing our models’ growth yield predictions on hydrogen and formate, respectively, to experimental data we found that our predictions fell within the experimental error of the measured values. This comparison demonstrates that our models can reproduce these values, suggesting that the models and the reconstruction they were derived from can reliably predict growth yields are quantitatively consistent with *in vivo* results.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Genotype | H2 | Formate | H2 + Formate | Formate + CO |
| ∆hmd | N | N | N | N |
| ∆mtd | N | N | N | N |
| ∆frcA | N | N | N | N |
| ∆fruA | N | N | N | N |
| ∆frcA∆fruA | N | N | N | N |
| ∆vhcAU∆vhuA | N | N | N | N |
| ∆hdrB2 | N | N | N | N |
| ∆fdhA1 | N | N | N | N |
| ∆fdhA2 | N | N | N | N |
| ∆fdhA1∆fdhA2 | N | L | N | L |
| ∆fdhA2∆fdhB2 | N | N | N | N |
| ∆ehbF | N | N | N | N |
| ∆3H2ase | N | N | N | N |
| ∆5H2ase | L | N | N | N |
| ∆6H2ase | L | N | N | N |
| ∆6H2ase∆cdh | L | N | N | N |
| ∆6H2asesupp | L | N | N | N |
| ∆7H2asesupp | L | N | N | N |
| **Total Correct:** | **10 of 10** | **14 of 16** | **2 of 2** | **1 of 2** |

Figure 3: Knockout lethality predictions from running FBA on our models show close agreement with experimental results of hydrogenase knockouts. Green boxes indicate growth phenotypes where our models correctly replicated experimental results; red boxes indicate growth phenotypes where our models were incorrect; white boxes indicate growth phenotypes where we lacked experimental validation data. Across the full spectrum of conditions, our models correctly predicted 27 of 30 conditions (90%) accurately, resulting in a strong Matthews Correlation Coefficient of 0.67. This suggests that our reconstruction produces models that accurately depict the effects of genotype alterations on growth phenotypes.