1 **A genome-scale metabolic model for *Methylococcus*** 2 ***capsulatus* predicts reduced efficiency uphill electron** 3 **transfer to pMMO.**

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1 **Abstract**

2 Background:

3 Genome-scale metabolic models allow researchers to calculate yields,
 4 to predict consumption and production rates, and to study the effect of
 5 genetic modifications *in silico*, without running resource-intensive
 6 experiments. While these models have become an invaluable tool for
 7 optimizing industrial production hosts like *E. coli* and *S. cerevisiae*, few
 8 such models exist for one-carbon (C1) metabolizers.

9 Results:

10 Here we present a genome-scale metabolic model for *Methylococcus*
 11 *capsulatus*, a well-studied obligate methanotroph, which has been used
 12 as a production strain of single cell protein (SCP). The model was
 13 manually curated, and spans a total of 877 metabolites connected via
 14 898 reactions. The inclusion of 730 genes and comprehensive
 15 annotations, make this model not only a useful tool for modeling
 16 metabolic physiology, but also a centralized knowledge base for *M.*
 17 *capsulatus*. With it, we determined that oxidation of methane by the
 18 particulate methane monooxygenase is most likely driven through uphill
 19 electron transfer operating at reduced efficiency as this scenario
 20 matches best with experimental data from literature.

1 Conclusions:

2 The metabolic model will serve the ongoing fundamental research of C1
3 metabolism, and pave the way for rational strain design strategies
4 towards improved SCP production processes in *M. capsulatus*.

5 **Keywords**

6 COBRA, Genome-scale metabolic reconstruction, C1 Metabolism,
7 Single Cell Protein, Methanotrophy

8 **Availability of Data and Materials**

9 The metabolic model, scripts and corresponding datasets generated
10 and/or analysed during the current study are available in the GitHub
11 repository.

- 12 • Archived: [https://github.com/ChristianLieven/memote-m-](https://github.com/ChristianLieven/memote-m-capsulatus)
13 [capsulatus](https://github.com/ChristianLieven/memote-m-capsulatus)
- 14 • Most Recent: [https://github.com/ChristianLieven/memote-m-](https://github.com/ChristianLieven/memote-m-capsulatus/tree/develop)
15 [capsulatus/tree/develop](https://github.com/ChristianLieven/memote-m-capsulatus/tree/develop)

16
17 The data that support the biomass equation constructed in this study
18 are available from Unibio at

- 19 • <http://www.unibio.dk/end-product/chemical-composition-1>

- 1 • <http://www.unibio.dk/end-product/chemical-composition-2>
- 2 Restrictions may apply to the availability of these data. Data are
- 3 however available from the authors upon reasonable request and with
- 4 permission of Unibio.

5 **Background**

6 The Gram-negative, obligate-aerobe *Methylococcus capsulatus* is a
7 methane oxidizing, gamma-proteobacterium. Since its initial isolation by
8 Foster and Davis [1], the organism has been subject to a wide array of
9 studies. The global role of *M. capsulatus* as a participant in the carbon
10 cycle has been elucidated [2, 3] as well as its effects on human [4] and
11 animal health and disease [5]. Specifically the latter studies have been
12 triggered by a considerable commercial interest in *M. capsulatus* as the
13 primary microbe used for the production of Single Cell Protein (SCP) as
14 animal feed starting in the 70s [6]. Now that hydraulic fracturing has
15 made natural gas a cheap and abundant feedstock [7], the application
16 of *M. capsulatus* for this purpose is being explored again [8, 9]. Another
17 portion of studies, however, has focused on uncovering the biochemical
18 and genetic basis of the organism's unique metabolism [10]. Yet, the
19 greatest interest has been the role and function of the initial enzyme in
20 methanotrophy, methane monooxygenase [11], responsible for
21 oxidation of methane to methanol.

1 *Methylococcus capsulatus* is able to express two distinct types of
2 methane monooxygenases: a soluble form of methane monooxygenase
3 (sMMO) and a particulate, or membrane-bound form (pMMO). The
4 expression of these enzymes is strongly influenced by the extracellular
5 concentration of copper; when *M. capsulatus* is grown in the presence
6 of low levels of copper the sMMO is active, while the pMMO is
7 predominantly active at high levels. Both enzymes require an external
8 electron donor to break a C-H bond in methane. While the electron
9 donor for the sMMO is NADH [12–15], the native reductant to the
10 pMMO has not yet been elucidated due to difficulties to purify the
11 enzyme and assay its activity *in vitro* [11]. Three hypotheses regarding
12 the mode of electron transfer to the pMMO have been suggested
13 previously: 1) formaldehyde oxidation provides the electrons required to
14 oxidize methane in the form of NADH, while the electrons that are
15 released from the oxidation of methanol are passed via a cytochrome
16 ‘redox arm’ to a terminal oxidase, generating ATP. 2) A direct coupling
17 between the pMMO and the methanol dehydrogenase (MDH) allows an
18 efficient exchange of electrons, leading to the latter reaction driving the
19 former. 3) Finally, it is possible that electrons are transferred to the
20 pMMO from MDH through the ubiquinol pool by the action of a reversely
21 operating ubiquinol-cytochrome-c reductase. This mode is referred to as
22 ‘uphill’ electron transfer [17].

1 **Figure 1. Overview of the respiratory chain in *Methylococcus capsulatus***
 2 **as implemented in iMcBath.** Black text in the center of the symbols denotes
 3 the common abbreviation, while the faint grey text below denotes the
 4 corresponding reaction ID in the metabolic model.

5 A genome-scale metabolic model (GEM) not only represents a
 6 knowledge base that combines the available physiological, genetic and
 7 biochemical information of a single organism [16], but also provides a
 8 testbed for rapid prototyping of a given hypothesis [17]. Hence, we
 9 present the first manually curated genome-scale metabolic model for
 10 *Methylococcus capsulatus*, with the intent of supplying the basis for
 11 hypothesis-driven metabolic discovery and clearing the way for future
 12 efforts in metabolic engineering [18]. Using the GEM, we investigate the
 13 nature of electron transfer in *M. capsulatus* by comparing the model's
 14 predictions against experimental data from Leak and Dalton [19].
 15 Furthermore, we compare its predictions to those of the model of *M.*
 16 *buryatense* [20] and explain notable differences by considering the
 17 proposed electron transfer modes.

1 **Results and Discussion**

2 **Reconstruction**

3 The presented genome-scale metabolic reconstruction of *M. capsulatus*
4 termed iMcBath was based on BMID000000141026, an automatic draft
5 published as part of the Path2Models project [21]. The whole genome
6 sequence of *Methylococcus capsulatus* (Bath) (GenBank AE017282.2
7 [22]) was used to aid the curation process and to supply annotations
8 (see material & methods). This metabolic reconstruction consists of 840
9 enzymatic reactions that interconvert 783 unique metabolites. The total
10 number of reactions, including exchange, demand and the biomass
11 reactions, is 892. The model attributes reactions and metabolites to
12 three distinct compartments: The cytosol, the periplasm, and the
13 medium that is referred to as extracellular in the model. Gene-Protein-
14 Reaction rules (GPR) associated with 730 unique genes support 85.5%
15 of the included reactions with, leaving 122 reactions without a GPR. The
16 GPRs include representation of 24 enzyme complexes.

17 The model is made available in the community-standard SML format
18 (Level 3 Version 2 with FBC).

19 **Biomass reaction**

20 In stoichiometric models biological growth is represented as the flux
21 through a special demand reaction. The so-called biomass reaction

1 functions as a drain of metabolites, which are either highly reduced non-
 2 polymeric macromolecules such as lipids and steroids or precursors to
 3 typical biopolymers such as nucleic acids, proteins or long-chain
 4 carbohydrates. The stoichiometry of an individual precursor was
 5 calculated from the principal composition of *M. capsulatus* as reported
 6 by Unibio [23]. The monomer composition of individual macromolecules
 7 was calculated from different sources. A detailed account of the
 8 resources is provided in the methods section and an overview of the
 9 biomass reaction is given in Supplement Table 1.

10 The growth-associated maintenance (GAM) and the non-growth
 11 associated maintenance (NGAM) requirements for *M. capsulatus* are
 12 yet to be determined experimentally. Therefore, a GAM value of 23.087
 13 mmol ATP gDW⁻¹ h⁻¹ was estimated according to Thiele et al. [16]
 14 based on the data for *E. coli* published by Neidhardt et al. [24]. The
 15 value for GAM is expected to increase with the growth rate of the cells
 16 [25]. Like de la Torre et al. had done for *M. buryatense* [20], we
 17 assumed the non-growth associated maintenance (NGAM) of *M.*
 18 *capsulatus* to be similar to that of *E. coli* thus setting it to 8.39 mmol
 19 ATP gDW⁻¹ h⁻¹ [26].

1 Metabolism

2 Much of the focus in curating the initial draft model BMID000000141026
3 was put on the central carbon metabolism and respiration of *M.*
4 *capsulatus*.

5 Three possible modes of electron transfer to the pMMO have been
6 proposed.

7 1) In the *redox-arm* mode [27], the methanol dehydrogenase (MDH)
8 passes electrons via cytochrome c555 (cL) [28] and cytochrome c553
9 (cH) [29] to either a CBD- or AA3-type terminal oxidase [30] and thus
10 contributes to building up a proton motive force (PMF) and the synthesis
11 of ATP (Figure 1). The electrons required for the oxidation of methane
12 are provided through ubiquinone (Q8H2), which in turn received
13 electrons from various dehydrogenases involved in the oxidation of
14 formaldehyde to CO₂. Although no binding site has been identified,
15 there is support for pMMO reduction by endogenous quinols [31].

16 2) With the *direct coupling* mode, the MDH is able to directly pass
17 electrons to the pMMO [32–34]. Here, cytochrome c555 is the electron
18 donor to the pMMO instead of ubiquinol. This mode is supported by
19 results from cryoelectron microscopy which indicates that the pMMO
20 and the MDH form a structural enzyme complex [35].

3) The *uphill electron transfer* mode supposes that the electrons from cytochrome c553 can reach the ubiquinol-pool facilitated by the PMF at the level of the ubiquinol-cytochrome-c reductase. This mode was proposed by Leak and Dalton [36] as it could explain the observed reduced efficiency.

To determine which of these modes is most likely active in *M. capsulatus*, Leak and Dalton [36] developed mathematical models for each mode based on previous calculations [37, 38]. However, their models did not reflect the experimental results. De la Torre et al. constructed a genome-scale metabolic model (GEM) for *Methylobacterium buryatense* to investigate growth yields and energy requirements in different conditions [20]. They found that the *redox-arm* mode correlated least with their experimental data for *M. buryatense*.

To study which of the three modes of electron transfer is active in *M. capsulatus*, they were each implemented in the model. The implementation is illustrated in Figure 2. To include the *redox-arm* we implemented the reaction representing the particulate methane monooxygenase, in the model termed as PMMOipp, utilizing Q8H2 as a cofactor. Accordingly, a variant of the pMMO reaction was added to account for a possible direct coupling to the MDH. In this variant reaction, termed PMMODCipp, the cofactor is cytochrome c555, represented as the metabolite focytcc555_p in the model. To enable an

1 *uphill electron transfer*, the reaction representing the ubiquinol-
2 cytochrome-c reductase (CYOR_q8ppi in the model), was constrained
3 to be reversible while keeping PMMOipp active.

4 **Figure 2. The three possible modes of electron transfer to the pMMO**
5 **mapped onto an excerpt of the respiratory chain.** 1) Redox-arm: The
6 methanol dehydrogenase transfers electrons to the terminal oxidase, while
7 the pMMO draws electrons from the quinone pool. 2) Direct coupling:
8 Electrons from the oxidation of methanol are transferred directly to the pMMO.
9 3) Uphill electron transfer: Electrons from the methanol dehydrogenase feed
10 back into the ubiquinol-pool. Black text denotes the common name, while faint
11 grey text denotes the reaction ID in the metabolic model.

12 Following the path of carbon through metabolism downstream from the
13 MDH, the model includes both the reaction for a ubiquinone-dependent
14 formaldehyde dehydrogenase [39], termed FALDDHipp, and an NAD-
15 dependent version, termed ALDD1 (Figure 3). Despite of the initial
16 evidence for the latter reaction [40] having been dispelled by Adeosun
17 et al. [41], it was added to allow further investigation into the presence
18 of a putative enzyme of that function. An additional pathway for
19 formaldehyde oxidation represented in both genome and model is the
20 tetrahydromethanopterin(THMPT)-linked pathway [42].

Figure 3. Three different formaldehyde oxidation pathways are represented in the model. Black text denotes the common name, while faint grey text denotes the reaction ID in the metabolic model.

Formaldehyde assimilation in *M. capsulatus* occurs primarily through the ribulose monophosphate (RuMP)-pathway. As outlined by Anthony [10], the RuMP-pathway has four hypothetical variants. Based on the annotated genome published by Ward et al. [22], we identified not only both C6 cleavage pathways depending either on the 2-keto, 3-deoxy, 6-phosphogluconate (KDPG) aldolase (EDA) or the fructose biphosphate aldolase (FBA), but also the transaldolase (TA) involved in the rearrangement phase that regenerates ribulose 5-phosphate. The alternative to a transaldolase-driven rearrangement step is the use of a sedoheptulose biphosphatase, which was not included in the initial annotation. Strøm et al. could not detect specific activity using cell-free preparations [43]. Yet, we decided to add a corresponding reaction for two reasons. First, the FBA has been characterized to reversibly catalyze sedoheptulose biphosphate cleavage [44], which is reflected by the reaction FBA3 in the model. Second, the pyrophosphate-dependent 6-phosphofructokinase (PFK_3_ppi) was reported to have higher affinity and activity to the reversible phosphorylation of sedoheptulose phosphate than compared to fructose 6-phosphate [45]. Thus, all of the resulting four combinations that make up the RuMP-pathway are represented in this metabolic model (Figure 4).

1 **Figure 4. All four variants of the RuMP pathway are represented in the**
 2 **metabolic model.** If there is a common enzyme name the black text denotes
 3 the common name, while faint grey text denotes the reaction ID in the
 4 metabolic model. Otherwise the black text is also the reaction ID in the model.

5 The genome of *Methylococcus capsulatus* encodes genes for a
 6 complete Calvin Benson Bassham (CBB) cycle [46–48] and a partial
 7 Serine pathway for formaldehyde assimilation [22]. It was argued by
 8 Taylor et al. [49] that *M. capsulatus* can metabolize glycolate (a product
 9 of the oxygenase activity of the ribulose biphosphate carboxylase
 10 (RBPC)) via this pathway. Both Taylor and Ward, further suggested the
 11 presence of unique key enzymes to complete the Serine pathway, such
 12 as hydroxymethyl transferase, hydroxypyruvate reductase and malate-
 13 CoA lyase. However, since the gene annotation did not reflect this and
 14 the RuMP pathway is reportedly the main pathway for formaldehyde
 15 assimilation [50], these putative reactions were not included.

16 All genes of the TCA cycle were identified in the genome sequence and
 17 all associated reactions were included accordingly [22]. Because no
 18 activity of the 2-oxoglutarate dehydrogenase has so far been measured
 19 *in vivo* [50, 51], the associated reactions have been constrained to be
 20 blocked (both lower and upper bounds were set to zero). This way they
 21 can easily be activated if needed. For instance, if a growth condition is
 22 discovered where activity for this enzyme can be detected.

1 Based on reactions already present in BMID000000141026, the
 2 information in the genome annotation, and the measured biomass
 3 composition, we curated the biosynthetic pathways of all proteinogenic
 4 amino acids, nucleotides, fatty acids, phospholipids, panthothenate,
 5 coenzyme A, NAD, FAD, FMN, riboflavin, thiamine, myo-inositol, heme,
 6 folate, cobalamine, glutathione, squalene, lanosterol, peptidoglycan.
 7 Since no corresponding genes could be identified, reactions catalyzing
 8 the biosynthesis of lipopolysaccharide (LPS) were adopted from
 9 iJO1366 [52] under the assumption that the biosynthesis steps among
 10 gram-negative bacteria require amounts of ATP comparable to
 11 *Escherichia coli*.

12 **Figure 5. Oxidation of ammonia to nitrite.**

13 *Methylococcus capsulatus* is able to metabolize the nitrogen sources
 14 ammonium (NH_4) and nitrate (NO_3) in a variety of ways. When the
 15 extracellular concentration of NH_4 is high, alanine dehydrogenase
 16 (ADH) is the primary pathway for nitrogen assimilation into biomass
 17 [53]. In addition, the two monooxygenases are able to oxidize
 18 ammonium to hydroxylamine [15, 54], which is then further oxidized by
 19 specific enzymes first to nitrite [55] (Figure 5), and even to dinitrogen
 20 oxide [56]. NO_3 is reduced to NH_4 via nitrite and ultimately assimilated
 21 via the glutamine synthetase/ glutamine synthase (GS/GOGAT)
 22 pathway. Furthermore, it has been shown that *M. capsulatus* is able to

1 fix atmospheric nitrogen (N_2) [57]. The nitrogenase gene cluster has
2 been identified [58] and annotated accordingly [22], and the
3 corresponding reactions have been included in the model. As the
4 enzyme has not yet been specifically characterized, the nitrogenase
5 reaction was adapted from iAF987 [59]. A schema showing these
6 reactions side-by-side is displayed in Figure 6.

7 **Figure 6. Nitrogen assimilation and fixation pathways represented in the**
8 **model.** Black text in the centre of the symbols denotes reaction IDs. Common
9 names are used for metabolites.

10 A metabolic map of all the reactions in the model was built using Escher
11 [60] and is available as Supplement Figure 1.

12 Extension and manual curation

13 Starting reconstruction on the basis of an automated draft required
14 additional effort to be able to use it for calculations. The automated draft
15 used two sets of ID namespaces, BiGG [61] and MetaNetX (MNX) [62].
16 Hence, the first step in the curation efforts consisted of unifying the
17 namespace by mapping all metabolite and reaction identifiers from MNX
18 into the human-readable BiGG namespace. Individual metabolites and
19 reactions with unmappable IDs, that could not be identified in the BiGG
20 database and for which there was little evidence in the form of GPR
21 rules, were removed from the model. Several metabolite formulas

1 contained polymer units, and many reactions lacked EC numbers.
 2 Using the MNX spreadsheet exports 'chem_prop.tsv' and
 3 'reac_prop.tsv' from version 1.0 [63] and 2.0 [64] most of these issues
 4 were resolved. Due to said malformed and missing metabolite formulae,
 5 many reactions were not mass-charge-balanced. We used the
 6 `check\_mass\_balance` function in cobrapy [65] to identify and balance
 7 99.4% of the reactions in the model. The remaining five reactions,
 8 belonging to fatty acid biosynthesis, involve a lumped metabolite that
 9 represents the average fatty acid content of *M. capsulatus*.

10 Another challenge with extending the automated draft was that many
 11 reactions were inverted meaning that reactants and products were
 12 switched, and constrained to be irreversible. Consulting the
 13 corresponding reactions in MetaCyc [66], these instances were
 14 corrected manually. For each precursor in the biomass reaction, the
 15 corresponding biosynthetic pathway was gap-filled and manually
 16 curated. To identify the appropriate pathways, MetaCyc pathway maps
 17 from the *M. capsulatus* Bath specific database were used to compare
 18 the reactions that were present in the draft reconstruction
 19 (<https://biocyc.org/organism-summary?object=MCAP243233>).

20 In order to enable other researchers to integrate iMcBath into their
 21 respective workflows and to simplify model cross-comparisons, we

1 included MIRIAM-compliant annotations for a majority of the model's
2 reactions (96%) and metabolites (98%) [67].

3 As a last step we added transport reactions that were not in the draft
4 reconstruction. Inferring membrane transport reactions from the
5 genome sequence is difficult, as usually the precise 3D structure of
6 transport proteins dictates which metabolite classes can be transported
7 [68]. Even if the substrates are known, the energy requirements of
8 transport are often undefined. Working on protein sequence matches
9 using PsortB 3.0 [69], combined with BLAST [70] matches against
10 TransportDB [71] and the Transporter Classification Database (TCDB)
11 [72], we were able to identify 56 additional transport reactions. We have
12 limited the number of transporters to be added, focusing specifically on
13 transporters with known mechanisms and transport of metabolites
14 already included in the model. A list of putative transport-associated
15 genes that we did not consider is available at
16 <https://github.com/ChristianLieven/memote-m-capsulatus>. Some of
17 these genes were reported by Ward et al. to facilitate the uptake of
18 sugars [22]. Kelly et al. suggest [50] these genes may allow a function
19 similar to *Nitrosomas europaea*, which is able grow on fructose as a
20 carbon-source with energy from ammonia oxidation [73]. This list is a
21 good starting point to study potential alternate carbon source use by *M.*
22 *capsulatus*.

1 Validation of the Model

2 To determine which combination of the three aforementioned electron
3 transfer modes is active in *Methylococcus capsulatus*, we constrained
4 the model based on experiments conducted by Leak and Dalton [19].
5 Since the three modes relate to how the pMMO receives electrons, we
6 focused on the data generated by growing *M. capsulatus* in high-copper
7 medium, which is the condition in which pMMO is predominantly active.
8 We used the average of carbon and oxygen-limited measurements as a
9 reference. Having constrained the model, we compared the Leak and
10 Dalton's measurements for the ratio of oxygen consumption to methane
11 consumption (O_2/CH_4) to the predictions of the model (Figure 7A). We
12 considered the O_2/CH_4 ratio to be a key metric for the respiratory chain
13 in *M. capsulatus*, as it is a function of the mode of electron transfer to
14 the pMMO. The central carbon metabolism was left unconstrained.

15 Under the assumption that the mode of electron transfer to pMMO
16 would be independent of the source of nitrogen, we compared the
17 O_2/CH_4 ratios of the model constrained to employ one of the three
18 modes of electron transfer exclusively to the corresponding reference
19 values of *M. capsulatus* grown on either NO_3 or NH_4 . However, neither
20 of the modes adequately represented the measured O_2/CH_4 ratios of
21 about 1.43 and 1.6, respectively. Although Leak and Dalton had
22 proposed that the *reverse* or *uphill electron transfer* is the most

1 probable mode [36], the model predictions allowing for an unbounded
2 *uphill transfer* did not support this (Figure 7), as the efficiency was
3 almost comparable to predictions using *direct coupling*.

4 We altered the efficiency of the three modes to determine whether the
5 fit could be improved. For the *redox arm*, we gradually decreased the
6 mol protons required for the synthesis of 1 mol ATP, thereby improving
7 the efficiency. This did not change the O_2/CH_4 ratio (See Supplement
8 Table 2). We decreased the efficiency of the *direct coupling* mode
9 (PMMODCipp) by forcing a portion of the flux through the regular
10 pMMO reaction (PMMOipp) using ratio constraints. Although this
11 affected the O_2/CH_4 ratio, to achieve a ratio representing the measured
12 value of 1.43 would require a large decrease in efficiency with almost
13 85% of the incoming carbon to be routed through the regular pMMO
14 reaction (See Supplement Table 2). Because of this, we consider a
15 *direct coupling* to be possible, albeit unlikely. Lastly, we iteratively
16 constrained the lower bound of the reaction associated with the
17 ubiquinol-cytochrome-c reductase (CYOR_q8ppi), to reduce the
18 efficiency of the *uphill-electron transfer*.

19 As Figure 7B shows, by constraining the reverse flux through this
20 reaction, it is possible to modulate the ratio of O_2/CH_4 consumption. We
21 decided to fit the lower bound of ubiquinol-cytochrome-c reductase only
22 to the reference O_2/CH_4 ratio of 1.43 with cells grown on NO_3 , in order

1 to avoid an overlap with the effects of NO₂ production. Leak and Dalton
2 pointed out, that the unexpectedly high O₂/CH₄ ratio of 1.6 was the
3 product of latent NH₄ oxidation rather than assimilation [19] leading to
4 elevated levels of NO₂. They were uncertain whether this increase could
5 be attributed to the energetic burden of reducing NH₄ or the uncoupling
6 effect of NO₂.

7 To investigate this effect, we introduced a ratio constraint (see Methods)
8 coupling the uptake of NH₄ to the excretion of NO₂ and explored a
9 number of values for this ratio (Figure 7C). According to the simulations,
10 the energy spent on reducing about 50% of incoming NH₄ to NO₂ is
11 sufficient to account for the observed, high O₂/CH₄ ratio of 1.6. Although
12 this shows that the loss of energy could be significant enough to
13 account for the increased ratio, this does not exclude a potential
14 combined effect because of energy decoupling. Regardless it shows
15 that predictions using the metabolic model can accurately reflect the *in*
16 *vivo* behavior of *M. capsulatus*.

17 **Figure 7 Validation of iMcBath. A:** Using each of the three electron transfer
18 modes exclusively (Redox-Arm, Direct Coupling, Uphill Transfer), the ratios of
19 oxygen to methane consumption (O₂/CH₄) predicted by iMcBath were
20 compared to experimental values from Leak and Dalton [19]. Since none of
21 the three modes matched the reference, the efficiency of the Uphill Transfer
22 was reduced by iteratively constraining the flux through the ubiquinol-
23 cytochrome c reductase (CYOR_q8) reaction as shown in **B**. Using a lower

bound of -2.8 improved the fit to the reference value for cells grown on NO₃ (**A**
 – Uphill Transfer Fit) **C**: To account for the energy loss through NH₃ oxidation,
 several ratios of NH₄ uptake to NO₂ production were considered. The closest
 fit was achieved with a ratio of around 0.5 (**A** – + ½ NO₂ Production)

Comparison with other models

We compared iMcBath, the preceding automated draft reconstruction
 BMID000000141026, and a genome-scale metabolic model of the
 gram-negative, gamma-proteobacterium *Methylobacterium buryatense*
 strain 5G(B1) [20] to illustrate how much the model has progressed
 from the draft through manual curation and how the mode of electron
 transfer affects growth parameters within the group of gamma-
 proteobacteria (See Table 1).

Unsurprisingly, the automated draft generally performs quite poorly in
 comparison with the curated models. It's not possible to produce
 biomass from methane, even in rich media conditions, which is
 indicative of gaps in the pathways leading towards individual biomass
 components. Moreover, ATP can be produced without the consumption
 of substrate, which in turn means that key energy reactions are not
 constrained thermodynamically to have the correct reversibility. In the
 draft, 51% of the reactions are not supported by Gene-Protein-Reaction
 rules (GPR), while in iMb5G(B1) this percentage is only 32% and in
 iMcBath the percentage of these reactions is under 20%. GPRs allow

1 modelers to distinguish between isozymes and enzyme complexes by
 2 connecting gene IDs either through boolean ‘OR’ or ‘AND’ rules. In
 3 iMcBath, 25 complexes in total were curated and formulated as GPR.
 4 Neither the draft model nor iMb5G(B1) make this distinction.

5 In the automated draft, the oxidation of methane was only possible
 6 through a reaction representing the sMMO (MNXR6057). In iMcBath,
 7 this was corrected by also implementing reactions that represent the
 8 pMMO, one with ubiquinone as the electron donor (PMMOipp), and
 9 another that receives electrons from the MDH (PMMODCipp).

10 *Methylococcus capsulatus*, like *Methylobacterium buryatense*,
 11 expresses both the soluble and the particulate monooxygenase
 12 depending on the availability of copper in the medium. In iMb5G(B1)
 13 however only the particulate monooxygenase is represented by the
 14 reaction “pMMO”. The ability of *M. capsulatus* to grow on ammonia,
 15 nitrate and nitrogen has been characterized experimentally [53]. In
 16 addition to that, however, iMcBath also predicts growth on nitrite and
 17 urea. Nitrite is an intermediate in the assimilation of nitrate, yet also
 18 reported to elicit toxic effects [19, 74], hence *in vivo* growth may only be
 19 possible on low concentrations. Growth on urea is possible in the model
 20 since we identified *MCA1662* as an ATP-driven urea transporter and
 21 gap-filled a urease reaction when curating the cobalamine biosynthesis
 22 pathway. As a consequence of this, urea can be taken up into the
 23 cytosol and broken down into ammonia and carbon dioxide, the former

1 of which is then assimilated. Further experimental work is necessary to
 2 verify this *in vivo*. *M. buryatense* 5G(B1) is reported to grow on nitrate,
 3 ammonia and urea, yet without adding the respective transport
 4 reactions iMb5G(B1) only grows on nitrate. While the draft model for *M.*
 5 *capsulatus* contains exchange reactions for all the tested nitrogen
 6 sources except for urea, it couldn't grow at all, which again is likely
 7 because of gaps in the pathways leading to biomass precursor
 8 metabolites.

9 The difference between *M. capsulatus* and *M. buryatense* becomes
 10 apparent when comparing the growth energetics of iMcBath and
 11 iMb5G(B1). iMb5G(B1) produces more $\text{mmol gDW}^{-1} \text{ h}^{-1}$ ATP than
 12 iMcBath. Because of this the hypothetical growth-rates predicted by
 13 iMb5G(B1) are higher than those of iMcBath regardless of the
 14 respective nitrogen source. This difference is likely a direct
 15 consequence of the mode of electron transfer to the monooxygenase
 16 and thus the efficiency of the methane oxidation reaction in total. When
 17 comparing the ratio of the uptake rate of oxygen and the uptake rate of
 18 methane for the two models, we can see that the resulting values in
 19 iMb5G(B1) are lower than in iMcBath. It was recently reported, that
 20 instead of the reverse-electron transfer and redox-arm mode active in
 21 *M. capsulatus*, a mixture of *direct coupling* from pMMO to MDH and
 22 *reverse electron transfer* seems to be the most likely mode in *M.*
 23 *buryatense* 5G(B1) [20].

- 1 **Table 1. Model Comparison.** The presented reconstruction iMcBath, the
- 2 automated draft BMID000000141026 and iMb5G(B1), a genome-scale
- 3 reconstruction of *Methylobacterium buryatense* strain 5G(B1).

	iMcBath	BMID000000141026	iMb5G(B1)
Structure			
	SMMOi,		
Methane Oxidation	PMMOipp,	MNXR6057	pMMO
	PMMODCipp		
Growth on N-Sources	Urea, NO ₂ ,	No Growth	NO ₃
	NO ₃ , NH ₄ , N ₂		
Performance			
ATP Production Closed			
Exchanges	False	True	False
ATP Production Rate - NH ₄	0.775	1000	1
ATP Production Rate - NO ₃	0.365	1000	0.519
Growth Rate - pMMO - NH ₄	0.299	No Growth	0.34
Growth Rate - pMMO - NO ₃	0.205	No Growth	0.27
O ₂ /CH ₄ Ratio - pMMO - NH ₄	1.434	No Growth	1.156
O ₂ /CH ₄ Ratio - pMMO - NO ₃	1.415	No Growth	1.116
Specifications			
Reactions without GPR	174	950	129
Enzyme Complexes	43	0	0
Total # Genes	730	589	313
Total # Metabolites	877	935	403
Total # Reactions	898	1858	402

1 Conclusion

2 IMCBATH is the first, manually curated, genome-scale metabolic model
3 for *Methylococcus capsulatus*. With iMcBath, we combine biochemical
4 knowledge of half a century of research on *Methylococcus capsulatus*
5 into a single powerful resource, providing the basis for targeted
6 metabolic engineering, process design and optimization, omic-data
7 contextualization and comparative analyses. We applied the metabolic
8 model to study the complex electron transfer chain of *M. capsulatus*, by
9 analyzing the three modes of electron transfer that had been proposed
10 previously [36]. We did so by corresponding each mode with the flux
11 through a reaction in the model, and consequently comparing the
12 predicted O₂/CH₄ ratios to experimentally measured values by Leak and
13 Dalton [19]. Simulation and experiment agreed either when the model
14 was constrained to employ the *uphill electron transfer* at reduced
15 efficiency or *direct coupling* at strongly reduced efficiency for *M.*
16 *capsulatus* grown *in silico* on NO₃ as the source of nitrogen. Although
17 an *uphill electron-transfer* seems more likely, further experimental
18 validation is required as neither mode can be ruled out exclusively. The
19 experimentally observed effect of NH₄ oxidation to NO₂ could be
20 replicated by considering the energy burden alone.

21 Future applications of the metabolic model could include hypothesis
22 testing of the regulation of the MMO in other growth conditions [50],

1 studying the effects of the predicted hydrogenases on the energy
2 metabolism of *M. capsulatus* [22], and exploring venues of metabolic
3 engineering for an improved production of metabolites [75]. Another

4 **Methods**

5 **Model Curation**

6 After mapping the reaction and metabolite identifiers from the MetaNetX
7 namespace to the BiGG namespace, we proceeded with the curation
8 efforts as follows: First, we chose a subsystem of interest, then we
9 picked a pathway and using information from either the genome
10 sequence, published articles, the metacyc or uniprot databases, and
11 lastly, we enriched each enzymatic step in said pathway with as much
12 information as possible. Information that was added included for
13 instance: GPR, reaction localization, EC numbers, a confidence score,
14 possible cofactors and inhibitors and cross references to other
15 databases such as KEGG, BiGG and MetaNetX. For each metabolite
16 involved in these reactions, we defined the stoichiometry, charge and
17 elemental formula, either based on the corresponding entries in the
18 BiGG database or on clues from literature.

19 If reactions from a pathway were present in the draft, we checked their
20 constraints and directionality. This was necessary as many of the
21 irreversible reactions in the draft reconstruction seemed to have been

1 'inverted' when compared to the corresponding reactions in the
2 reference databases, which made flux through them impossible in
3 normal growth conditions.

4 The energy metabolism and methane oxidation were curated first.
5 Except for the reaction representing the sMMO, all reactions were newly
6 constructed, as they were absent in the draft. Then, in order to achieve
7 sensible FBA solutions for growth on methane, the central carbon
8 metabolism, amino acid and nucleotide biosynthesis pathways were
9 manually curated. Simultaneous to the manual curation a metabolic
10 pathway map was created in Escher, which helped us to maintain a
11 visual checklist of curated pathways.

12 The automated draft contained a rudimentary, generic biomass
13 reaction, which only accounted for the production of proteins, DNA and
14 RNA, but not for the biosynthesis of a cell wall and cell membrane, the
15 maintenance of a cofactor pool, the turnover of trace elements or the
16 energetic costs associated with growth. After calculating a more specific
17 biomass composition for *M. capsulatus*, further pathway curation was
18 necessary to achieve growth *in silico*. This included the biosynthesis
19 pathways of fatty acids (even, odd and cyclopropane varieties),
20 phospholipids, coenzyme A, Ubiquinol, Lanosterol, FAD, FMN,
21 Riboflavin, NAD and NADP, Heme, Glutathione, Cobalamin, Thiamine,
22 Myo-Inositol, and Lipopolysaccharides.

1 To account for the reported differences in ammonia assimilation of *M.*
 2 *capsulatus* when grown in the presence of excess ammonia versus the
 3 growth on either atmospheric nitrogen or nitrate, we curated the
 4 nitrogen metabolism including the oxidation of ammonia to nitrite via
 5 hydroxylamine, the reduction of nitrate and nitrite, the glutamine
 6 synthetase/ glutamate synthase reactions and the alanine
 7 dehydrogenase. Reversible degradation reactions producing ammonia
 8 that would potentially bypass the characterized ammonia assimilation
 9 pathways were constrained to be irreversible accordingly.

10 After we had enriched the annotations already in the draft with
 11 annotations from the metabolic models iJO1366 [52], iRC1080 [76],
 12 iJN678 [77] and iHN637 [78], they were converted into a MIRIAM-
 13 compliant format. As a final step, we manually added transport
 14 reactions to reflect the uptake energetics of cofactors.

15 Throughout the reconstruction process, we iteratively tested and
 16 validated the reconstruction. For instance, we checked the mass and
 17 charge balance of each reaction, attempting to manually balance those
 18 that weren't balanced. In the majority of cases metabolites were missing
 19 formula or charge definitions. In order to remove energy generating
 20 cycles, problematic reactions were manually constrained to be
 21 irreversible. Validation was carried out against growth data [19], which
 22 was also the point of reference for the parameter fitting.

1 Biomass Composition

2 For the principal components of biomass, measurements were made
3 available through the website of our collaborators Unibio [23]. This
4 included specifically the content of RNA (6.7%), DNA (2.3%), crude fat
5 (9.1%), and glucose (4.5%) as a percentage of the cell dry weight. We
6 did not use the percentage values for crude protein (72.9%) and N-free
7 extracts (7.6%) as these measurements are fairly inaccurate relying on
8 very generalized factors. The percentage value of Ash 550 (8.6%)
9 measurements was inconsistent with the sum of its individual
10 components (4.6%) and was hence excluded.

11 On the homepage of UniBio, we were also able to find g/kg
12 measurements of all amino acids except for glutamine and asparagine,
13 trace elements and vitamins, which could directly be converted into
14 mmol/g DW. However, we omitted some of data: The stoichiometries for
15 Selenium, Cadmium, Arsenic, Lead and Mercury were not included in
16 the biomass reaction as their values were negligibly small. Beta-
17 Carotene (Vitamin A) and Gama-Tocopherol (Vitamin E) were omitted
18 because no genes were found supporting their biosynthesis, in addition
19 to both being reportedly below the detection limit [79].

20 For the lack of better measurements, and assuming that *M. buryatense*
21 and *M. capsulatus* are more similar than *M. capsulatus* and *E. coli*, the
22 stoichiometries of glutamine and asparagine, intracellular metabolites

1 such as ribulose-5-phosphate, organic cofactors such as coenzyme A,
2 and cell wall components such as LPS were introduced from de la Torre
3 et al. [20].

4 Using the GC content calculated from the genome sequence [22] and
5 the percentage measurements from Unibio for RNA and DNA, we were
6 able to calculate the stoichiometries of all individual nucleobases.

7 Unibio's measurements that 94% of the crude fat portion were fatty
8 acids conflicted with previously published results, which indicated that in
9 fact phospholipids are likely to be the main lipid component in *M.*
10 *capsulatus* [80, 81]. Thus, we assumed 94% of the crude fat to be
11 phospholipids. This meant that 6% of the crude fat was composed of
12 fatty acids, the distributions of which were again provided by Unibio.
13 However, in order to calculate the stoichiometry of each fatty acid we
14 recalculated the distribution to exclude the unidentified part. Makula had
15 also measured the composition of the phospholipid pool itself [80], from
16 which we calculated the corresponding stoichiometries for
17 phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol
18 and cardiolipin.

19 Bird and colleagues had reported the percentage of squalene and
20 sterols of dry weight [82], which we converted into mmol/g DW without
21 further corrections. Since the genes for hopanoid synthesis were
22 identified we included diplopterol in the biomass reaction [22, 83]. For a

1 lack of more detailed measurements we estimated a similar contribution
2 to the overall composition as squalene. We specifically used lanosterol
3 to represent the general class of sterols in the biomass reaction, since
4 we had only been able to reconstruct the synthesis pathway of
5 lanosterol and since lanosterol is a key precursor metabolite in the
6 biosynthesis of all other sterols.

7 The growth associated maintenance energy requirements were
8 calculated in accordance with protocol procedures [16].

9 Transport Reactions

10 The identification of transport reactions involved the two databases for
11 transport proteins, the Transporter Classification Database (TCDB) [84]
12 and the Transport Database (TransportDB) [71], and two computational
13 tools, PSORTb v3.0 [69] and BLASTp [70]. We employed the following
14 semi-automatic way of inferring the putative function of transport
15 proteins in *M. capsulatus*.

16 Using the protein sequences in AE017282.2_protein.faa [22], we
17 determined the subcellular location of each protein using PSORTb v3.0.
18 We filtered the results and focused only on proteins with a final score
19 larger than 7.5, which the authors of PSORTb consider to be
20 meaningful. We combined this list with the *M. capsulatus* specific
21 entries from the TransportDB, which allowed us to use the PSORT-

1 scores as an additional measure of confidence. At this point, 242
 2 putative transport proteins were identified. From this list we then
 3 selected all proteins which were predicted to transport metabolites and
 4 were already included in the model, which reduced the number to 133.
 5 Since for many of the entries, the exact mechanism of transport is
 6 unknown, we combined the previously selected transporters with the
 7 results from running BLAST against the TCDB. The e-value and
 8 bitscore from BLAST provided yet another measure to confidently
 9 assess the correctness of the automatic TransportDB predictions, and
 10 the Transporter Classification-Numbers (TC-numbers) allowed us to
 11 gather information on the mechanism of transport. This led to a list of 97
 12 transport proteins with high confidence, which was filtered once more as
 13 follows.

14 We checked the general description for a given specific TC-number,
 15 and then considered the BLAST result to read about a given transporter
 16 in more detail, especially with regards to finding the specific substrates.
 17 When we were able to identify the corresponding transport reaction in
 18 the BiGG database, we incorporated only the simplest, smallest set of
 19 reactions. In cases of conflicts between our own BLAST results and the
 20 automatic TransportDB transporter annotation, we preferentially trusted
 21 the BLAST results. Thus we ultimately added 75 transporter-encoding
 22 genes connected via GPR to 56 distinct transport reactions.

1 Ratio Constraint

2 To identify which mode of electron transfer is active in *M. capsulatus*,
 3 we fit the solutions of FBA using iMcBath to measured values [19]. The
 4 authors had experimentally determined the O₂/CH₄ ratio and the growth
 5 yield of *M. capsulatus* in several conditions. They varied the nitrogen
 6 source using KNO₃, NH₄CL, and both simultaneously; the concentration
 7 of copper in the medium, which directly affects the activity of either
 8 sMMO or pMMO; and whether the culture was oxygen or carbon limited.

9 Since each electron transfer mode respectively is represented by the
 10 flux through one specific reaction in the model (PMMOipp,
 11 PMMODCipp, CYOR_q8ppi), we were able to investigate each simply
 12 by constraining the corresponding reaction.

13 Secondly, we accounted for the differential expression of ADH in the
 14 presence of excess NH₄ in the medium versus the GS/GOGAT in the
 15 presence of NO₄ or N₂ by blocking the corresponding reactions
 16 accordingly [53].

17 Several studies have shown that NH₄ is a co-metabolic substrate to the
 18 methane monooxygenases in *M. capsulatus* leading to the production of
 19 hydroxylamine first and nitrite later [54, 74, 85]. Hence, when simulating
 20 the growth on NH₄ we assumed that varying ratios *r* of the nitrogen
 21 taken up would eventually be converted into nitrite (Figure 7).

$$(1) \quad v_{EX_nh4_e} + r v_{EX_no2_e} = 0$$

2 Stoichiometric Modeling

3 The reactome of an organism can be represented mathematically as a
 4 stoichiometric matrix S . Each row of S corresponds to a unique
 5 compound, while each column corresponds to a metabolic reaction.
 6 Hence the structure of the matrix for an organism with m compounds
 7 and n reactions equals $m \times n$. The values in each row denote the
 8 stoichiometric coefficients of that metabolite in all reactions. The
 9 coefficients are either negative for compounds that are educts, positive
 10 for those that are products, or zero for those that are not involved in a
 11 given metabolic reaction.

12 The vector v of length n contains as values the turnover rates of each
 13 reaction. These rates are commonly referred to as fluxes and are given
 14 the unit $\text{mmol gDW}^{-1} \text{ h}^{-1}$. Vector v is also referred to as the flux vector,

15 Declarations

16 Ethics approval and consent to participate:

17 “Not applicable”

1 Consent for publication:

2 “Not applicable”

3 Availability of Data and Materials:

4 The metabolic model, scripts and corresponding datasets generated
5 and/or analysed during the current study are available in the GitHub
6 repository.

7 • Archived:

8 <https://github.com/ChristianLieven/memote-m-capsulatus>

9 • Most Recent:

10

11 The data that support the biomass equation constructed in this study
12 are available from Unibio at

13 • <http://www.unibio.dk/end-product/chemical-composition-1>

14 • <http://www.unibio.dk/end-product/chemical-composition-2>

15 Restrictions may apply to the availability of these data. Data are
16 however available from the authors upon reasonable request and with
17 permission of Unibio.

18 Competing interests:

19 Unibio is a collaborator in the “Environmentally Friendly Protein
20 Production (EFPro2)” project. CL, MJH, and NS are publicly funded

1 through the Novo Nordisk Foundation and the Innovation Fund
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3 interest.

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7 Production (EFPro2)”)

8 Authors’ contributions:

9 CL collected and analysed the data, reconstructed the metabolic model,
10 drafted and revised the manuscript. LP and SBJ provided feedback on
11 the metabolic behaviour of *M. capsulatus*, discussed improvements to
12 the model and revised the manuscript. KVG, MJH and NS conceived
13 and supervised the study and revised the manuscript critically. All
14 authors read and approved the manuscript.

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20

Net equation for each combination in the model:

A **B** **C**