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Rethinking biological activation of methane and conversion to liquid fuels

Chad A Haynes[1] & Ramon Gonzalez[2]*

If methane, the main component of natural gas, can be efficiently converted to liquid fuels, world reserves of methane could satisfy the demand for transportation fuels in addition to use in other sectors. However, the direct activation of strong C-H bonds in methane and conversion to desired products remains a difficult technological challenge. This perspective reveals an opportunity to rethink the logic of biological methane activation and conversion to liquid fuels. We formulate a vision for a new foundation for methane bioconversion and suggest paths to develop technologies for the production of liquid transportation fuels from methane at high carbon yield and high energy efficiency and with low CO_2 emissions. These technologies could support natural gas bioconversion facilities with a low capital cost and at small scales, which in turn could monetize the use of natural gas resources that are frequently flared, vented or emitted.

Meeting the increasing demand for petroleum by the global transportation sector has required the deployment of new extraction technologies, such as enhanced oil recovery and hydraulic fracturing of shale rock as well as exploitation of resources such as tar sands and oil shale[1,2]. This transition to unconventional resources is accompanied by an increase in greenhouse gas (GHG) emissions 0.5–3 times higher than that of conventional resources (**Fig. 1a**)[3].

The use of natural gas, specifically methane, in transportation should be considered a viable option for reducing petroleum dependence and GHG emissions associated with the exploitation of unconventional resources. Globally, natural gas resources that are technically recoverable with new horizontal drilling and efficient extraction technologies are estimated at 7.2×10^3 trillion ft^3 (**Fig. 1b**)[1]. Estimates for the US range between 0.65×10^3 trillion ft^3 and up to 2×10^3 trillion ft^3, a quantity capable of supplying the US with 100 years of natural gas at current usage rates[1]. This increased availability in the US has placed downward pressure on natural gas prices and created a large price spread between natural gas and petroleum on an energy-equivalent basis (**Fig. 1c**).

Methane is not only an energy resource but also a potent GHG, with a greenhouse warming potential 21 times that of carbon dioxide over a 100-year period. When converted to CO_2 equivalents, anthropogenic sources of methane contribute nearly 20% of the world's GHG warming potential each year[4]. In addition to emissions, venting and inefficient flaring of natural gas produced as a byproduct of petroleum extraction is responsible for an estimated 5 trillion ft^3 of GHG released to the atmosphere worldwide[5]. Therefore, technologies to effectively use methane not only from pipeline sources but also from smaller, distributed sources should be pursued as effective means to both produce energy and mitigate GHG warming potential.

Direct use of natural gas as compressed natural gas in the transportation sector is constrained owing to the inherent low volumetric energy density of natural gas and the lack of fueling and end-use infrastructure required for its broader adoption by light duty vehicles[6]. An alternative means to use natural gas as a transportation fuel involves gas-to-liquid (GTL) conversion technologies such as those based on the production of synthesis gas (syngas, a gas mixture predominately composed of carbon monoxide and hydrogen) and subsequent conversion via the Fischer-Tropsch (FT) process (generally approximated as $2(n+1)H_2 + nCO \rightarrow C_nH_{(2n+2)} + nH_2O$). Although attractive, the current GTL-FT approach is a technologically complex, multistep process involving conversion of methane to syngas, catalytic conversion of syngas to long-chain hydrocarbons and subsequent cracking and separation of a broad range of products for market. The process is encumbered by numerous heat and pressure changes, all of which require multiple unit operations that markedly increase technical complexity and capital expenses (CapEx). These process demands result in deployment of exceptionally large-scale facilities to leverage economies of scale that cannot be efficiently scaled down, thus requiring significant CapEx for each facility in upwards of $20 billion (**Fig. 1d**). In addition, production of liquid fuels through GTL-FT processes would lead to GHG emissions approximately 50% higher than that of conventional resources (**Fig. 1a**).

Enabled by recent developments in enzymatic oxidation of methane and synthetic biology, this perspective presents the opportunity to develop industrially relevant biotechnology and bioprocessing for new GTL technologies. Bio-based approaches to GTL are anticipated to be less technologically complex and operate profitably on small scales compared to current GTL-FT. Here we address some of the challenges and technological opportunities for efficient biological activation of methane and synthesis of liquid fuels.

Bioconversion: low CapEx and high efficiency

Biological conversion (bioconversion) processes offer a potential solution to the large-scale, capital-intensive nature of the GTL-FT approach. Corn-grain ethanol fermentation is perhaps the best example of a biological process for fuel production deployed at commercial scale and hence will be contrasted here to the aforementioned GTL-FT process. In ethanol fermentation, sugars are converted to ethanol via a yeast biocatalyst capable of high metabolic and process efficiencies, respectively 97% and 81%, and with ethanol product specificity greater than 90% (ref. 7). The bioprocess operates at mild temperatures and can integrate saccharification and fermentation into a single-unit operation. Taken together, these features result in a process that is less technologically complex than GTL-FT and supports small-scale deployment at significantly lower CapEx (**Fig. 1d**). The lower CapEx of corn-grain ethanol facilities has supported more rapid and widespread deployment in the US than commercial-scale

[1]Booz Allen Hamilton, Washington, DC, USA. [2]Advanced Research Projects Agency–Energy, United States Department of Energy, Washington, DC, USA. *e-mail: ramon.gonzalez@hq.doe.gov or ramon.gonzalez@rice.edu

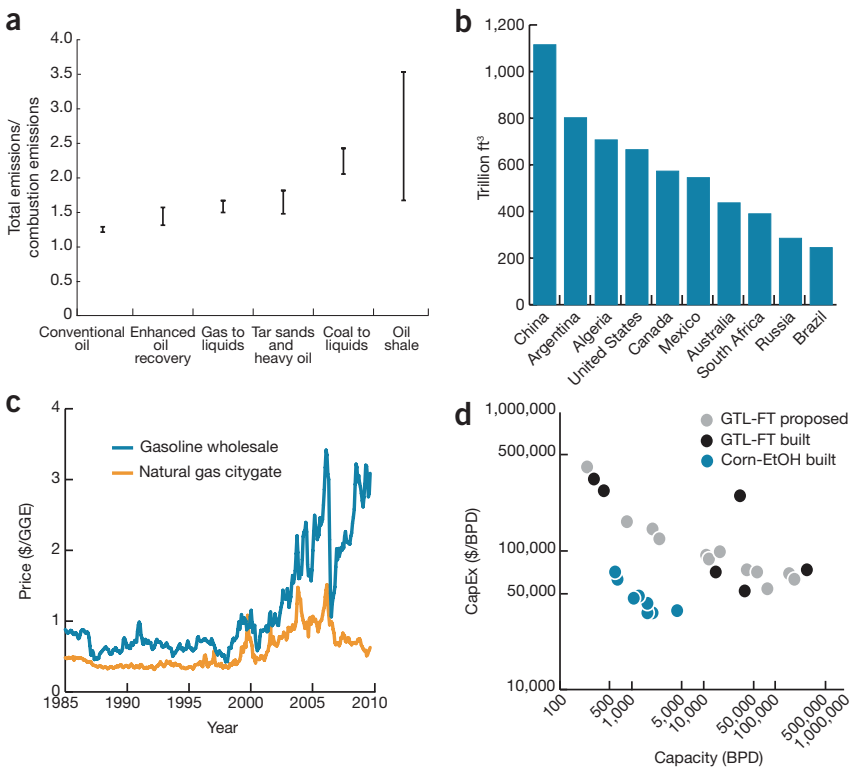

Figure 1 | Macro-level data encourages methane as a liquid fuel feedstock. (**a**) Relative CO_2 emissions associated with the production and use of liquid transportation fuels derived from conventional and unconventional fossil resources. The ratio of total CO_2 emissions to combustion CO_2 emissions is shown[3]. (**b**) Top 10 countries for shale gas extraction. Technically recoverable natural gas resources represent the amount of gas that could potentially be extracted with current technology[1]. (**c**) US natural gas citygate (orange) and gasoline wholesale prices (blue) on an equivalent energy basis. GGE, gallons of gasoline equivalent on an energy basis; 'Natural gas citygate', natural gas that has been transferred from an interstate or intrastate pipeline to a local natural gas utility[57]. (**d**) Comparison of CapEx versus capacity for GTL-FT and corn-ethanol (EtOH) facilities on an equivalent energy basis. GTL-FT data is combination of engineering studies (proposed) as well as built facilities, which includes commercial and demonstration units, whereas corn-ethanol data is entirely from built commercial facilities[58,59]. BPD, barrel of oil equivalent per day.

GTL-FT. A similar widespread adoption has taken place in the case of sugarcane ethanol in countries such as Brazil[8].

Therefore it is reasonable to explore the option of biological technologies as alternatives to state-of-the-art GTL-FT. To leverage the advantages of methane bioconversion, technologies that use biological catalysts (biocatalysts) to selectively activate, functionalize and convert methane to liquid fuels in a consolidated manner are required. Native or engineered organisms capable of methane oxidation as the sole source of energy and carbon, which we refer to here as methanotrophic microorganisms or methanotrophs, can be used for this purpose.

Aerobic methanotrophy for methane bioconversion

Aerobic C-H bond activation, carried out by methanotrophic bacteria such as *Methylococcus* or *Methylomonas* species[9], is an exergonic process catalyzed by methane monooxygenases (MMOs), enzymes capable of converting C-H bonds to C-OH groups[10] and that occur in two forms, either a membrane-bound dicopper particulate MMO or multicomponent diiron soluble MMO (**Fig. 2**)[11,12]. Both enzymes are capable of selective partial oxidization of methane to methanol via the following reaction: $CH_4 + O_2 + 2e^- + 2H^+ \rightarrow CH_3OH + H_2O$ (**Table 1**; reaction 1). The two electrons required by MMOs to activate molecular O_2 and incorporate one oxygen atom into the highly stable C-H bond of methane (dissociation energy 439 kJ mol^{-1}) are derived from the biological cofactors NADH or NADPH, which are generated from the two-electron reduction of their oxidized partners, NAD^+ and $NADP^+$, respectively. These two electrons are provided by the subsequent oxidation of methanol to formaldehyde, catalyzed by the enzyme methanol dehydrogenase (MDH)[13].

The growth of aerobic methanotrophs on methane as the only carbon and energy source requires assimilation of the energy contained in four C-H bonds and the conversion of metabolic intermediates derived from methane activation, such as methanol and formaldehyde, to precursor metabolites used in the synthesis of essential metabolic building blocks such as amino acids, nucleotides and sugar phosphates. This process requires the formation of carbon-carbon (C-C) bonds. Formaldehyde is the native biological intermediate in aerobic methanotrophs, which can be assimilated via the ribulose monophosphate (RuMP) pathway (type I methanotrophs) or the serine pathway (type II methanotrophs)[14,15].

The RuMP pathway combined with glycolysis generates pyruvate, a three-carbon ketoacid, from three formaldehyde molecules with a net generation of one NADH and one ATP[16]. Historically, the major route for carbon assimilation in type I methanotrophs has been reported to follow the Entner-Doudoroff (EDD) variant of the RuMP pathway, which consumes ATP during the production of glyceraldehyde-3-phosphate (a three-carbon intermediate) from formaldehyde[17]. Recently, Kalyuzhnaya *et al.*[18] observed the propensity for *Methylomicrobium alcaliphilum* to use a pyrophosphate-mediated Embden-Meyerhof-Parnas variant of the RuMP pathway as the main route for C1 carbon assimilation under oxygen-limiting conditions. This highly energy-efficient pathway is capable of producing 3 mol glyceraldehyde-3-phosphate as well as 3 mol NADH and 2 mol ATP (via substrate-level phosphorylation) from 9 mol formaldehyde without any additional energy requirement[18]. In an alternative scheme, the serine pathway assimilates formaldehyde via condensation with tetrahydrofolate followed by conversions to acetyl-CoA, which enters the glyoxylate cycle and generates NADH.

Both type I and II methanotrophs are capable of generating ATP via aerobic respiration; however, the energy balance is not completely understood. For example, complete oxidation of one methane will generate one reduced cytochrome c_L via MDH and two NAD(P)H from the oxidation of formaldehyde to formate and formate to carbon dioxide[19]. NAD(P)H and cytochrome c_L can presumably be oxidized to generate proton motive force for ATP production via ATP synthase. However, it is not clear just how much reductant is dedicated to ATP production, considering the need for the as-yet-unidentified physiological MMO reductant. Notably, the use of methane as both a carbon and energy source creates metabolic flux control points, which will need to be leveraged for production of chemicals and fuels.

The only attempts to develop a methane bioconversion process reported in the literature have been the use of aerobic methanotrophs for the production of single-cell protein and carotenoids, neither of which achieved commercial viability[20,21].

Table 1 | Existing and postulated routes to biological activation of methane.

Enzyme type	Status	Enzymatic reaction	No. e^-/C-H activation	$\Delta G'^{o}$ (kJ mol^{-1})
O_2 dependent	Existing	1. MMO: $CH_4 + O_2 + 2H^+ + 2e^- \rightarrow CH_3OH + H_2O$	2 e^-	-336
		2. Benzene-DIOX: $C_6H_6 + O_2 + 2H^+ + 2e^- \rightarrow C_6H_8O_2$	1 e^-	-403
	Postulated/proposed	3. CH_4-DIOX: $2CH_4 + O_2 + 2e^- \rightarrow 2CH_3OH$	1 e^-	-378
		4. CH_4-DIOX1: $2CH_4 + O_2 \rightarrow 2CH_3OH$	0 e^-	-298
		5. E-OC: $2CH_4 + O_2 \rightarrow C_2H_4 + 2H_2O$	0 e^-	-341
O_2 independent	Existing	6. MCR: CH_4 + CoM-S-S-CoB $\rightarrow$ CH_3-S-CoM + HS-CoB	0 e^-	30
	Postulated/proposed	7. CH3-succinate synthase: CH_4 + fumarate $\rightarrow$ CH_3-succinate	0 e^-	-15
		8. CH_4-dehydrogenase: $CH_4 + H_2O \rightarrow CH_3OH + H_2$	0 e^-	122
		9. CH_4-carboxylase: $CH_4 + CO_2 \rightarrow C_2H_4O_2$	0 e^-	36
		10. E-syngas: $CH_4 + H_2O \rightarrow CO + 3H_2$	0 e^-	204

Abbreviations: Benzene-DIOX, biphenyl/benzene dioxygenase; CH_4-DIOX, engineered methane dioxygenase; CH_4-DIOX1, engineered methane dioxygenase, E-OC, enzymatic oxidative coupling with oxygen; CH_3-succinate synthase, methylsuccinate synthase; CH_4-dehydrogenase, methane dehydrogenase; CH_4-carboxylase, methane carboxylase; E-syngas, enzymatic syngas.

Anaerobic oxidation of methane: an emerging route

Anaerobic microorganisms carry out fermentation or respiration in the absence of oxygen. Anaerobic metabolism is more energy efficient but is slower than metabolism in aerobic microorganisms. Recently, it has become evident that primary C-H bonds can also be activated through oxygen-independent routes under anaerobic conditions[22]. Anaerobic oxidation of methane (AOM) is carried out by methanotrophic archaea capable of coupling sulfate reduction to methane oxidation to drive favorable thermodynamics, as required by the high degree of reduction of carbon in methane. Strong evidence suggests that the enzyme responsible for the first step of AOM is a homolog of the methyl-coenzyme M reductase (MCR) from anaerobic methanogenic microorganisms[23,24]. Recent structural data indicate that these two MCR homologs share high structural similarity, but the MCR involved in AOM seems to have several different features, including a modification to the F430 cofactor (a tetrapyrrole derivative found only in methanogenic and methanotrophic archaea), altered post-translational modifications and a group of cysteine-rich side chains. Such modifications may be responsible for the enzyme's propensity to operate reversibly; however, a precise understanding of which set of modifications is essential and which is rate enhancing remains to be elucidated (**Fig. 2**)[24]. Furthermore, it should be stressed that no laboratories have been successful at isolating and culturing methanotrophic archaea either in pure culture or in a consortium, which is likely more representative of the microbial ecology of the native environment.

Inefficiencies of aerobic methane bioconversion

We consider a theoretical design for a methanotrophic organism able to convert methane to *n*-butanol (**Fig. 3**). *n*-Butanol, referred to as butanol throughout this perspective, is considered here as a proxy for liquid fuel on the basis of its energy content and compatibility with current liquid fuel infrastructure. One could envision leveraging the latest developments in protein and metabolic engineering and synthetic biology to rewire the metabolism of aerobic methanotrophs to divert carbon from central metabolic pathways to the synthesis of butanol[25]. Alternatively, methane utilization pathways from the aforementioned methanotrophic bacteria can be transplanted to a number of bacteria and yeast that have been engineered for the

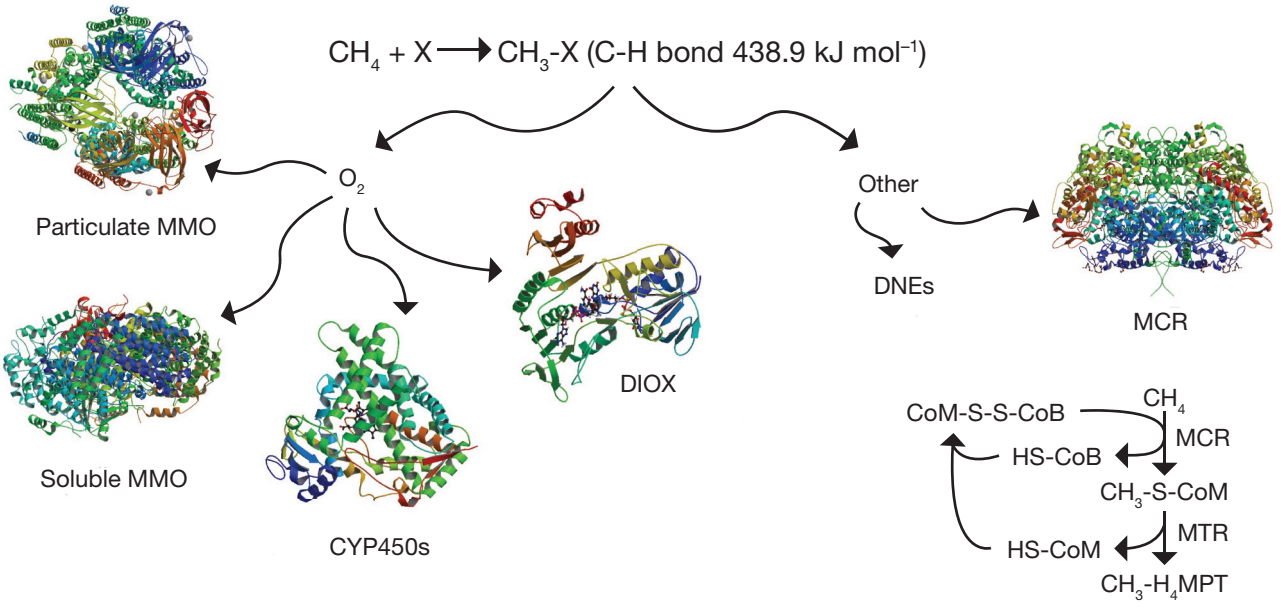

Figure 2 | Native enzymes capable of oxidizing C-H bonds. Ribbons model depictions of known and potential methane activating enzymes: particulate MMO (Protein Data Bank (PDB) code 3RFR)[60], soluble MMO (PDB code 4GAM)[11], CYP450s (PDB code 3KOH)[61], toluene DIOX (PDB code 4EMI)[62], MCR (PDB code 3SQG)[24] and the biochemical reaction pathway catalyzed by MCR and MTR. DNEs, *de novo* enzymes; CoB, coenzyme B; CoM, coenzyme M.

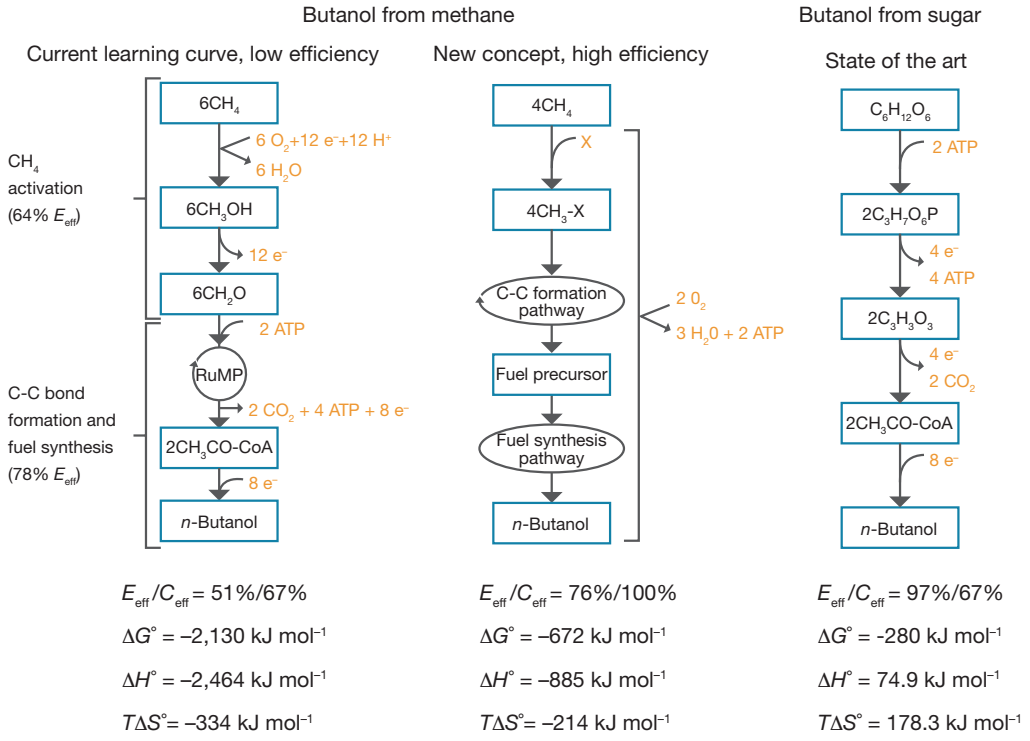

Figure 3 | Pathways for the conversion of methane or glucose to butanol in an engineered microorganism. The left pathway assumes no modification in either methane activation or C-C bond formation pathways, which leads to a low energy efficiency (E_{eff}; based on LHV) and carbon yield. The center pathway shows a hypothetical methanotroph that has been engineered with more efficient pathways for methane activation and C-C bond formation, thus achieving a higher energy efficiency and carbon yield during the conversion of methane to butanol. The right pathway shows the pathways mediating the conversion of glucose to butanol. Energy efficiency, carbon yield/efficiency (C_{eff}) and changes in thermodynamic properties ($\Delta G'^{\circ}$, $\Delta H'^{\circ}$ and $T\Delta S'^{\circ}$) are also shown for each scenario.

efficient production of butanol[26]. Implementation of such strategies would require a number of technological breakthroughs, including the development of effective genetic and metabolic engineering tools for native methanotrophs, the ability to transfer methane utilization pathways to non-native methanotrophs and the improvement of catalytic properties of MMOs. This design represents an example of what can be achieved if one continues on the current learning curve for methane activation and metabolism, which is based on the use of aerobic methanotrophic pathways.

Even if successfully reduced to practice, the aforementioned design suffers from low theoretical energy and carbon efficiencies for aerobic bioconversion, at 51% and 67%, respectively (**Fig. 3**). This would result in a process with large GHG emissions as more than 33% of the carbon in methane would be released as CO_2 during the conversion to butanol, thus exceeding the carbon footprint of GTL-FT fuels from either conventional oil or from natural gas feedstock (**Fig. 1a**).

A more detailed analysis of the overall pathways involved in the conversion of methane to butanol revealed two major sources of inefficiencies (**Fig. 3**). First, methane is activated inefficiently as only 64% of the energy contained in methane is retained in formaldehyde (based on the lower heating value (LHV) of each compound). Second, the conversion of formaldehyde to butanol is also inefficient: 78% energy efficiency based on corresponding LHVs and 67% carbon efficiency. The sources of these inefficiencies are discussed in detail below.

Although MMOs considered in the above design are elegant enzymes capable of exquisite control over the selective and partial oxidation of methane to methanol, they require two electrons to catalyze this oxygen-dependent reaction. This basic requirement necessary to reduce one atom of O_2 to H_2O comes at a cost, as electrons needed for activation must be recovered by the subsequent oxidation of methanol to formaldehyde. The net result is the redox-neutral conversion of methane (CH_4) to formaldehyde (CH_2O), with the concomitant loss of 36% of the energy contained in methane (based on the LHV of each compound). Overall, this two-step process can be considered as the conversion of a fully reduced carbon to simple sugar, given the oxidation state of the carbon atom changes from –4 in methane to 0 in formaldehyde, the latter corresponding to the same oxidation state of carbon in glucose or xylose (the empirical formula of these sugars, CH_2O, is the same as that of formaldehyde).

Synthesis of butanol from methane requires conversion of the one-carbon (C1) intermediate formaldehyde, which is derived from methane activation, to a fuel precursor such as acetyl-CoA via the formation of C-C bonds, followed by the conversion of acetyl-CoA to butanol. Overall, conversion of formaldehyde to butanol is redox balanced and generates 2 mol of ATP per mol of butanol synthesized: $6CH_2O \rightarrow C_4H_{10}O + 2CO_2 + H_2O + 2ATP$ ($\Delta G'^{\circ} = -397.8$ kJ mol^{-1}). At first, this seems to be a metabolically efficient conversion, considering that the ATP yield is on par with that of butanol fermentation from glucose, a very efficient metabolic pathway (**Fig. 3**). However, a more detailed analysis reveals that the energy efficiency of butanol synthesis from formaldehyde is lower (78%) than that from glucose (97%).

The low theoretical energy efficiency of the methane-to-butanol pathway contrasts with the high theoretical energy efficiency of the glucose-to-butanol conversion (51% versus 97%). Notably, the overall free energy change of the conversion pathway is very large in the case of conversion from methane to butanol

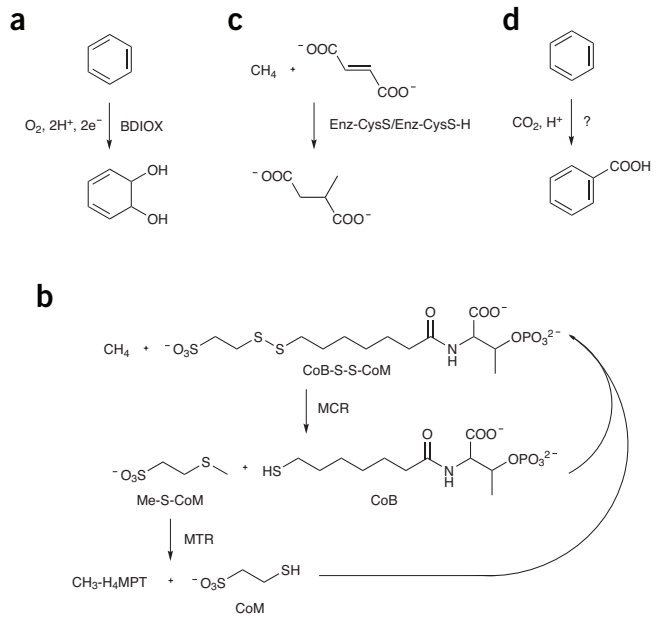

Figure 4 | Selective routes for the activation of C-H bonds in hydrocarbons. (**a**) Aerobic activation of methane catalyzed by biphenyl/benzene dioxygenase (BDIOX). (**b**) Anaerobic activation of methane catalyzed by MRC and MTR. (**c**) Hypothesized activation of methane under anaerobic conditions by addition to fumarate catalyzed by methylsuccinate synthase (represented by the abbreviation 'Enz' for 'enzyme'). (**d**) Anaerobic activation of benzene by direct carboxylation.

($\Delta G'° = -2{,}130$ kJ mol^{-1} butanol), close to ten times the value for the glucose-to-butanol conversion (**Fig. 3**). Most of this energy is lost in the form of heat ($\Delta H'° = -2{,}464$ kJ mol^{-1} butanol), a well-known characteristic of methanotrophic bioprocessing that imposes heat transfer and/or cooling demand[20].

A new concept for methane bioconversion

Guided by the above analysis, we present a new conceptual design for methane bioconversion to butanol that overcomes the aforementioned energy and carbon inefficiencies. **Figure 3** illustrates this concept through an example in which the activation of methane to a metabolic intermediate (for example, methanol) is achieved without the stoichiometric consumption of electrons. Conversion of the activated intermediate (for example, methanol) to the final fuel molecule (butanol) then proceeds through a pathway that retains all of the carbon in the final product. The theoretical energy efficiency for this new design is 77%, and the theoretical carbon yield is 100%. The change in free energy of the overall conversion is –672 kJ mol^{-1} butanol, a value that is ~400 kJ mol^{-1} butanol in excess of the sugar-to-butanol pathway, which operates very efficiently in practice (**Fig. 3**). Overall, the conceptual design relies on efficient pathways for both the activation of methane to a metabolic intermediate as well as the conversion of the activated intermediate to the final fuel product. Details on a number of strategies that can be used to realize this conceptual design are provided below.

Efficient routes for methane activation

Despite numerous challenges, the technology and foundational knowledge exist to develop methane-activating biocatalysts based on MMOs, cytochrome P450s (CYP450s, another class of monooxygenases) or other oxygen-dependent enzymes such as dioxygenases (DIOXs), which are able to activate and functionalize methane more efficiently, with kinetics comparable to native MMOs (**Figs. 2** and **4**). An inspiration for new conceptual frameworks can be found in the enzyme biphenyl dioxygenase, which has been observed to catalyze the dihydroxylation of C-H bonds in benzene with the following stoichiometry (**Fig. 4a**): benzene + NADH + $H^+ + O_2 \rightarrow$ *cis*-1,2-dihydrobenzene-1,2-diol + NAD^+ (**Table 1**; reaction 2)[27]. Notably, the enzyme incorporates both atoms of molecular oxygen into benzene while consuming only one molecule of NADH and hence one electron per each C-H activated. This is half the number of electrons consumed (and hence half of the energy lost) in the case of methane activation by MMOs. On the basis of this reaction, we envision engineering benzene dioxygenase (BDIOX)-like enzymes that simultaneously hydroxylate two molecules of methane with one molecule of oxygen while consuming one NADH (or two electrons; **Table 1**; reaction 3). Despite the favorable thermodynamics of the postulated methane dioxygenase reaction (**Table 1**; reaction 3), engineering an enzyme such as biphenyl dioxygenase attuned to oxidizing an aromatic substrate to one capable of oxidizing two fully saturated C1 compounds will be challenging, considering the role of the aromatic ring and catalytic mechanism. However, this should not be considered impossible. Advancements in computational protein design and the potential to incorporate non-native cofactors and/or amino acids may enable the development of BDIOX-like enzymes capable of activating methane efficiently.

Alternatively, because C-H bonds in benzene (473 kJ mol^{-1}) are stronger than those in methane (439 kJ mol^{-1})[28], it should, in principle, be possible to achieve activation of methane using electrons in a catalytic role as opposed to their consumption in stoichiometric proportions when MMOs, CYP450s and BDIOX-like enzymes are used. Such engineered enzymes could avoid altogether the consumption of electrons and mediate the activation of methane

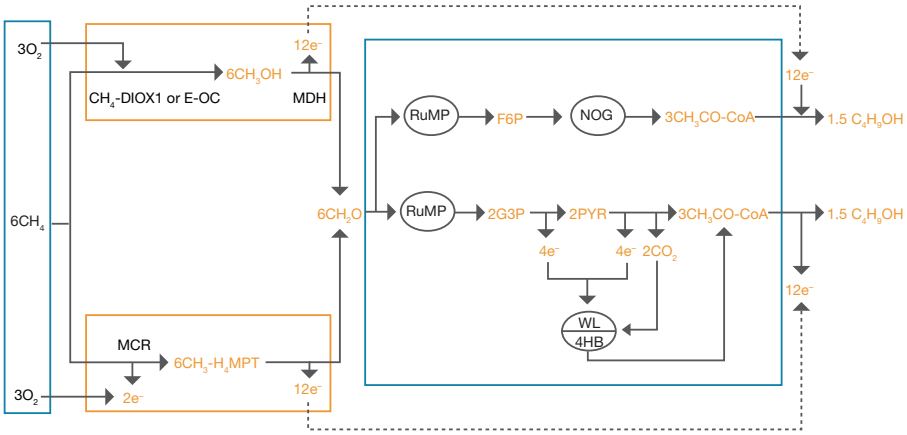

Figure 5 | Metabolic pathways to butanol from metabolic intermediates derived from the efficient activation of methane. Formaldehyde assimilation via the combination of the ribulose monophosphate (RuMP) cycle with nonoxidative glycolysis, the Wood-Ljungdahl (WL) pathway or 3-hydroxypropionic/4-hydroxybutyric (HB) cycle to support efficient butanol synthesis after activation of methane is shown. E-OC, enzymatic oxidative coupling with oxygen; NOG, nonoxidative glycolysis; G3P, glyceraldehyde-3-phosphate; PYR, pyruvate.

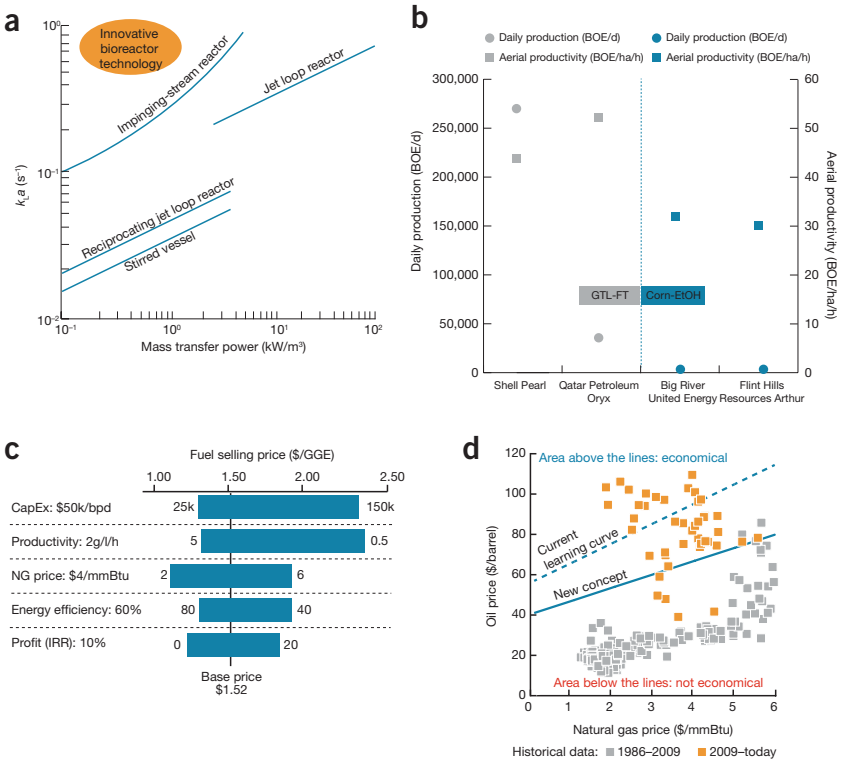

Figure 6 | Bioprocess considerations and techno-economics. (**a**) The k_La versus power chart depicts the power demand required to increase k_La for various bioreactor configurations and identifies an "Innovative bioreactor technology" space with high k_La values and low mass transfer power requirements. Adapted with permission from ref. 50. (**b**) Daily productivities for GTL-FT refineries and corn-ethanol (EtOH) bioprocessing refineries can be compared and normalized to a system-level productivity value, termed aerial productivity, by dividing barrel of oil equivalents (BOEs) produced per day by an estimated total land use coverage (measured in ha) of the respective GTL-FT and corn-ethanol refineries. (**c**) Major cost components of a biological methane-to-liquids process and base values are tabulated to show a sensitivity analysis. The Tornado chart illustrates how the variation in a single parameter influences the overall cost under *n*th plant assumptions. The values to the left and right of the bar are variations on base parameters and show the fuel selling price when all of the other base assumptions are held constant. The underlying process model was parameterized and Aspen-based to enable scenario analysis and couples the biological reactions and chemical unit operations. GGE, gallons of gasoline equivalent; BPD, barrels per day. (**d**) Breakeven analysis for the production of liquid fuels from natural gas. Break-even curves (straight lines) were constructed by comparing the selling price for butanol ($/GGE), based on the analysis shown in **c**, to the selling price of petroleum-derived gasoline[63]. Two bioconversion scenarios discussed in the text and shown in the left (current learning curve) and center (new concept) of **Figure 3** are presented, along with historical data for petroleum and natural gas prices (symbols).

to methanol at high energy efficiencies (**Table 1**; reaction 4).

Although oxygen-dependent activation of hydrocarbons via MMOs, CYP450s and DIOXs has been examined for many years[29], the recently reported enzymes of AOM can have a key role in enabling the new concept for methane bioconversion to liquid fuels (**Fig. 3b**). Specifically, the two-step conversion of methane to methyl-tetrahydromethanopterin (CH_3-H_4MPT), catalyzed by MCR and methyl-H_4MPT:CoM methyltransferase (MTR), proceeds without the stoichiometric consumption of electrons (**Fig. 4b**) and hence has the potential to increase the energy efficiency of methane activation (**Table 1**; reaction 6).

The glycyl radical enzymes benzylsuccinate and alkylsuccinate synthases are well-studied anaerobic enzymes that catalyze the reactions of aromatic hydrocarbons and *n*-alkanes by addition to fumarate, respectively[30,31]. 2-Methylsuccinate, the specific product of methane addition to fumarate, along with a downstream metabolite of its biodegradation (butanoic acid), were only detected in samples from wells that were regularly exposed to methane[32,33]. These findings led to a hypothesis that methane activation by AOM could also proceed via methane addition to fumarate (**Fig. 4c**)[32]. This represents yet another unique opportunity to harness and engineer more efficient activation routes, as no external electrons are required for methane activation (**Table 1**; reaction 7).

Hydroxylation with water is another route to anaerobic activation of C-H bonds in hydrocarbons, which seems to be relevant only for compounds with relatively low C-H bond dissociation energies (<355 kJ mol^{-1}) such as ethylbenzene, *n*-propylbenzene and *p*-cresol. Ethylbenzene dehydrogenase, a molybdenum cofactor-containing enzyme of the dimethylsulfoxide reductase family that catalyzes the anaerobic hydroxylation of ethylbenzene, is the best characterized of the enzymes mediating anaerobic hydroxylation with water[34–37]. Interestingly, such hydroxylation reactions are very energy efficient as they result in the synthesis of the corresponding alcohols and generate reducing equivalents. If applicable to methane activation, such a reaction would result in the following stoichiometry: $CH_4 + H_2O \rightarrow CH_3OH + H_2$ (**Table 1**; reaction 8). However, there are two major challenges. First, the dissociation energy for the C-H bond in methane is nearly 90 kJ mol^{-1} higher than that of typical hydrocarbons activated by water hydroxylation. Second, the overall conversion is significantly endergonic. This point can be made for all of the postulated mechanisms for anaerobic methane activation. Indeed, the thermodynamics of the anaerobic processes are less favorable than aerobic schemes. Anaerobic heterotrophs leverage both substrate and intermediate concentration gradients to overcome thermodynamically challenged reactions; however, in the case of anaerobic methanotrophy, the low solubility of the methane substrate and assumed metabolic flux engineering may attenuate the feasibility of such an approach. In these cases, thermodynamically unfavorable reactions could be overcome by coupling to more favorable reactions, as in the case of native anaerobic methanotrophic archaea[38].

Another recent development in the anaerobic activation of hydrocarbons relates to degradation of benzene and naphthalene. Although evidence for initial activation of naphthalene via methylation and carboxylation has recently been provided[39], it is the activation of benzene that represents a development of great significance for methane activation, given that C-H bond dissociation energies for benzene are even higher than for methane. Anaerobic degradation of benzene has been reported for pure cultures and consortia with different electron acceptors. After several detailed studies[40,41], it was concluded that activation proceeded primarily via direct carboxylation. (**Fig. 4d**). Although the enzymes and mechanisms of this metabolic process are still unknown, we speculate that methane could be similarly activated by direct carboxylation through a reaction with the following stoichiometry: $CH_4 + CO_2 \rightarrow CH_3COOH$ ($\Delta G'° = 36$ kJ mol^{-1}; **Table 1**; reaction 9). As mentioned earlier for the case of methane hydroxylation by water, this reaction is endergonic and would require thermodynamic coupling to an exergonic reaction to make it feasible.

Although an ATP-dependent phosphorylation is unlikely to mediate direct carboxylation of methane, it is interesting to note that coupling of ATP hydrolysis to AMP ($\Delta G'^{\circ} = -45$ kJ mol^{-1}) with methane carboxylation would make the overall conversion thermodynamically feasible.

Highly efficient C-C bond formation

The engineering of enzymes that consume less than two electrons during methane activation to methanol or equivalent intermediate (as described in the previous section) would create an 'excess' of reducing equivalents when compared to the use of native MMOs (compare **Figs. 3** and **5**). The availability of additional reducing equivalents can be leveraged to recapture the 2 mol of carbon dioxide released during the conversion of 6 mol formaldehyde to 1 mol butanol. **Figure 5** shows two potential pathways that can be used for this purpose: formaldehyde assimilation via the RuMP pathway to fructose-6-phosphate, followed by nonoxidative glycolysis[42] or formaldehyde assimilation via the RuMP pathway, followed by glycolysis to pyruvate, combined with the Wood-Ljungdahl pathway[43] or the 3-hydroxypropionate–4-hydroxybutyrate cycle[44]. A number of other schemes can be envisioned on the basis of known and *de novo* carbon fixation pathways[45–48]. This rewiring of the metabolic network encompasses both a more efficient activation of methane as well as new pathways for the conversion of the activated intermediate to the desired fuel at maximum energy and carbon efficiency. Both schemes would achieve the conversion of methane to butanol at energy efficiency and carbon yield of 76% and 100%, respectively, thus representing a path to realize the conceptual design proposed in **Figure 3**. Yet the technical challenges of incorporating the pathways comprising oxygen-sensitive enzymes, such as the Wood-Ljungdahl pathway, should not be overlooked. Opportunities to operate such integrated schemes in a microaerobic and/or compartmentalized environment need to be investigated.

Figure 5 also depicts a scenario for C-C bond formation and butanol synthesis from metabolites generated by oxygen-independent methane activation pathways discussed in the previous section. **Figure 5** proposes a 'black box' scenario for the anaerobic activation of methane via methyl-CoM reductase to formaldehyde. Alternatively, one may envision a path for the conversion of CH_3-H_4MPT to acetyl-CoA that bypasses methanol. In such a case, it may be conceivable to react carbon monoxide with CH_3-H_4MPT to acetyl-CoA via direct carbonylation by the enzyme carbon monoxide dehydrogenase/acetyl-coenzyme A synthase[49].

Kinetic challenges of the bioconversion of methane

In addition to the need to improve energy and carbon efficiencies, bioconversion of methane to liquid fuels will require overcoming kinetic challenges associated with the low rate of mass transfer of methane to the liquid phase and low volumetric productivities inherent to slow enzyme kinetics from methane activation and fuel synthesis, both of which can markedly increase capital and operating costs.

The kinetic limitations to gas transfer involve both methane and oxygen (if it is used as an oxidant) and are determined by the low solubility of these gases in water (~1 mM at 30 °C and 1 atm) and by gas flammability limits. Methanotrophs, or other biocatalysts, function in the liquid phase; however, gas diffusion across the boundary layer between the methane gas bubble and the liquid phase is the major barrier to mass transfer. For gas-liquid reactors, the gas transfer rate can be evaluated as the product of the volumetric mass transfer coefficient (k_La) and the driving force or difference between the equilibrium concentration (C^*) and the actual concentration (C): i.e., the gas mass transfer rate = $k_La \times (C^* - C)$. k_La is the product of the overall mass transfer coefficient, k_L, and the gas-liquid interfacial area, a, per unit volume. In industrial gas-liquid bioreactors, k_La can be enhanced by manipulating operational parameters (for example, agitation and gas flow rate) and design features (for example, micro-bubble spargers). In addition, one can increase the operational pressure of the bioreactor to increase the delta between the gas equilibrium concentration and actual gas concentration. Regardless of precisely how this is achieved, increasing the gas mass transfer rate eventually requires additional power per unit volume (**Fig. 6a**)[50], increasing both operating and CapEx. For example, increasing pressure requires additional energy to power compressors and robust, mechanically sound vessels, which directly affects CapEx. The techno-economic viability of methane bioconversion depends on high volumetric product synthesis (and methane utilization) rates on scale of grams per liter per hour, which corresponds to a k_La in the range of 700–1,000 h^{-1}. Yet physically achieving high mass transfer alone is insufficient from an economic standpoint. The key feature is the requirement to achieve high mass transfer without incurring costs attributed to increasing energy and/or capital. New bioreactor designs, such as hollow fiber membrane bioreactors and optimization of a number of design and operational parameters, will be necessary to overcome these challenges and increase bioreactor productivity to the levels necessary for techno-economic viability (**Fig. 6a**)[51].

Additionally, the kinetic challenges inherent to a biological process are compounded by the slow rates of methane-activating enzymes in combination with the large molecular weight of the enzyme complex. Overcoming this will require new biocatalysts with high catalytic efficiency, high enzyme concentrations within the cell and high cell densities within the reactor[52].

In light of these kinetic challenges, bioprocess technologies are often regarded as too slow to be profitable operations relative to purely chemical processes. Indeed, the volumetric productivity of a chemical reactor can be 10–100× greater than the volumetric productivity of a bioreactor. However, a direct comparison of the system-level productivity of biological processes such as those involving corn-grain ethanol with chemical processes such as GTL-FT reveals an interesting perspective. By normalizing system-level productivity to barrels of product per day per footprint, measured in area, one observes that the productivity of biological processes is comparable to that of their chemical counterparts (**Fig. 6b**). Despite increased daily productivities in the range of 750–5,700% over corn-grain ethanol refineries, GTL-FT system level productivity, as measured in aerial productivity (barrels of oil equivalent per hectare per hour), is nearly identical to the aerial productivity of corn-grain ethanol bioprocess refineries (**Fig. 6b**)[53]. This observation is important and relates back to the fact that multistep chemical processes such GTL-FT are technologically complex and require area-intensive operations to handle pressure and temperature changes and to operate at a scale large enough to be profitable. GTL-FT does not scale down efficiently, which constrains and limits deployment opportunities to niche situations where feedstock supply is large and relatively inexpensive. The fact that bioprocessing has fewer unit operations enables profitability at smaller scale and opens up deployment opportunities at remote locations such as petroleum-associated gas both on and offshore.

Economics of natural gas bioconversion to liquid fuels

To evaluate whether methane bioconversion would support cost-effective production of liquid fuels, we conducted a preliminary techno-economic analysis of the key components of the process and determined the sensitivity of fuel selling price to each component. This analysis suggests that the fuel selling price is sensitive to a number of variables including CapEx, volumetric productivity, natural gas price and the energy efficiency of conversion (**Fig. 6c**). As the major cost drivers are interlinked, a system demonstrating low CapEx will require high productivity as well as both high metabolic and process efficiency. To achieve this combination, major breakthroughs are expected in three areas addressed in previous sections of this perspective: (i) high-efficiency biological methane

activation, (ii) high-efficiency biological synthesis of liquid fuel from an intermediate derived from methane activation and (iii) process intensification to address kinetic limitations related to gas transfer and bioconversion rates. If these challenges are addressed, production of liquid fuels from methane at a biorefinery gate selling price of $1.52 per gasoline gallon equivalent (GGE) can be realized (**Fig. 6c**).

A break-even analysis clearly illustrates that the economic viability of the bioconversion of methane to liquid fuels is coupled to the economics of liquid fuel production from petroleum (**Fig. 6d**). Assuming the vision outlined in this perspective is realized, technologies resulting from the reconceptualization of methane bioconversion can be profitable over a wide range of prices of gasoline and natural gas (for example, most scenarios from 2009 to the present day; **Fig. 6d**). More importantly, the viability of the bioconversion of natural gas to liquid fuels increases for scenarios in which high energy prices are considered. For example, with natural gas at $1 per mmBtu, an oil price of at least $45 per barrel is required for methane bioconversion to be economically viable (i.e., a 45:1 price spread). However, with natural gas at $6 per mmBtu, the required price spread is just 13:1 (equivalent to an oil price of at least $80 per barrel).

Also of relevance is that technologies derived via the reconceptualization of methane bioconversion proposed here are more competitive than those resulting from continuing the current research and development trajectory of engineering fuel synthesis pathways into native methanotrophy (**Fig. 6d**) or steam methane reforming to syngas followed by fermentation. In the latter case, methane can be reformed with steam to CO and H_2 ($CH_{4(g)} + H_2O_{(g)} \rightarrow CO_{(g)} + H_{2(g)}$ ($\Delta G'^\circ = +204$ kJ mol^{-1})), which can be assimilated by anaerobic microorganisms such as *Clostridia*. However, compared to an integrated methane bioconversion process, steam methane reforming followed by fermentation requires two distinct unit operations, which will add technical complexity and cost. Ultimately, such a scheme would appear more akin to GTL-FT and would increase the final fuel price.

Concluding remarks

As described here, we present a case for highly energy- and carbon-efficient conversion of methane to liquid fuels via biological processes. In all cases, engineering biocatalysts at both the enzyme and cell level will be necessary and very challenging. Yet it is our expectation that these challenges can be met. The exponential growth in genomic information, coupled with rapid and affordable nucleic acid sequencing and gene synthesis, continues to accelerate the pace of innovation in the fields of synthetic biology and metabolic engineering. Despite this opportunity, it will be critical to consider the potential advantages and challenges to engineering new biological systems. Extensive metabolic engineering of host microorganisms can generally take one of two paths: either engineering native methanotrophs for more efficient methane oxidation and fuel synthesis or engineering robust, genetically tractable microbes such as *Escherichia coli* to be capable of C1 metabolism. Although the latter may be a major challenge, examples from the literature indicate that there is potential to convert obligate heterotrophs to C1 autotrophs through transfer and heterologous expression of C1 assimilation pathways[54].

A number of platforms are currently being explored for the production of liquid transportation fuels from renewable feedstock, presenting opportunities for synergies with methane bioconversion. For example, biomass is primarily composed of lignocellulosic material (multiple C-C bonds and high oxygen content), which is carbon rich but energy limited. Methane, on the other hand, contains no C-C bonds or oxygen, and hence it is energy rich but carbon limited. Given these characteristics, one could envision combining both types of feedstock in a bioconversion that leverages their corresponding advantages and minimizes their limitations, as represented in the following stoichiometric equation (using glucose as a proxy for biomass): $2CH_4 + 0.33\ C_6H_{12}O_6 \rightarrow C_4H_{10}O + H_2O$ ($\Delta G'^\circ = +7.8$ kJ mol^{-1} butanol). Either increasing the pressure of methane to approximately 25 bars or keeping butanol at concentrations below 1 M can help overcome the slightly endergonic nature of this bioconversion.

Photosynthetic systems, which are also used for the production of renewable transportation fuels directly from CO_2 and solar energy, provide another synergistic opportunity. For example, light-dependent reactions of photosynthetic organisms can be harnessed to generate the energy (reducing equivalents and ATP) and oxygen necessary to activate methane aerobically to methanol via native MMO.

In addition, new biological methane activation routes can, in principle, be designed by mimicking existing routes for chemical activation of methane. Two examples are provided in **Table 1**: the enzymatic equivalent of oxidative coupling of methane, using either oxygen (or sulfur) as oxidant[55] (reaction 5) and the enzymatic generation of synthesis gas (reaction 10). This chemistry-inspired approach could be seen as equivalent to the chemical and biological versions of the water gas-shift reaction ($CO_{(g)} + H_2O_{(g)} \rightarrow CO_{2(g)} + H_{2(g)}$, $\Delta G'^\circ = -28$ kJ mol^{-1}), which was first applied industrially for the production of hydrogen gas in the late nineteenth century, and its use continues today. Nearly 100 years later, a biological equivalent was discovered in numerous aerobic and anaerobic bacteria where CO oxidation with water to CO_2, $2e^-$ and $2H^+$ is coupled to respiratory pathways such as methanogenesis, desulfurization and acetogenesis[56].

Methanotrophy has been considered as a means to convert gaseous methane into higher-value products such as energy-dense, liquid hydrocarbons that can be cost-effectively handled, stored and used for transportation. Yet to date, all of the commercial attempts to develop a profitable methane bioconversion process have failed owing to multiple incompatibilities, such as high natural gas prices, poor energy efficiency and slow process kinetics. However, the very recent convergence of both market and technical opportunities has recasted biology's role as an economically viable process for GTL. Relative to state-of-the-art GTL-FT, methane bioconversion processes should be able to profitably operate at a smaller scale with both high energy and carbon efficiency. If such a solution could be deployed, numerous remote sources of methane could be monetized in low-carbon-emissions processes. Further, combining methane bioconversion with renewables, such as biomass, would enable complete energy and carbon conversion of both fossil and renewable feedstock to liquid fuels. As described herein, the challenges for methane bioconversion are many but both intellectually and technically tractable. Through spotlighting these critical challenges, we hope to encourage the broader scientific community to pursue solutions and develop transformational bioconversion processes for more distributed, efficient and profitable use of methane.

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