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The cofactor challenge in synthetic methylotrophy: bioengineering and industrial applications

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Methanol is a promising feedstock for industrial bioproduction: it can be produced renewably and has high solubility and limited microbial toxicity. One of the key challenges for its bio-industrial application is the first enzymatic oxidation step to formaldehyde. This reaction is catalysed by methanol dehydrogenases (MDH) that can use NAD^+, O_2 or pyrroloquinoline quinone (PQQ) as an electron acceptor. While NAD-dependent MDH are simple to express and have the highest energetic efficiency, they exhibit mediocre kinetics and poor thermodynamics at ambient temperatures. O_2-dependent methanol oxidases require high oxygen concentrations, do not conserve energy and thus produce excessive heat as well as toxic H_2O_2. PQQ-dependent MDH provide a good compromise between energy efficiency and good kinetics that support fast growth rates without any drawbacks for process engineering. Therefore, we argue that this enzyme class represents a promising solution for industry and outline engineering strategies for the implementation of these complex systems in heterologous hosts.

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Introduction

In the face of climate breakdown, limiting global warming is essential to avoid triggering a cascade of climate tipping points [1]. This can only be achieved if rapid decarbonisation is implemented across all sectors [2]. Business-as-usual needs to be replaced by drastic policy and societal changes — especially by the Global North [3]. While we should not solely rely on technological innovation to avert a climate catastrophe [4], carbon-negative technologies can play a supporting role for the sustainable production of fuels, high- and low-value chemicals as well as food, feed and others [5,6].

One promising branch of these technologies is the electrochemical reduction of CO_2 to one-carbon (C1) mediator molecules such as methanol (MeOH), formate, carbon monoxide and methane [7]. Although these processes are not yet competitive with petrochemical sources, CO_2-reduction processes are on their way for commercialisation due to catalyst improvements, policy changes and decreasing renewable-energy prices. In addition, some of the gaseous C1 feedstocks are available in industrial off-gases [8,9]. Ultimately, the goal of a circular carbon bio-economy is to convert these C1 molecules and hydrogen by microbial cell factories into a plethora of products [10].

While anaerobic fermentation of gaseous waste C1 feedstocks by acetogens was already demonstrated at industrial scale, the product spectrum is inherently limited by their metabolism. Despite being the most ATP-efficient route for C1 assimilation, the reductive acetyl-CoA pathway requires the generation of products that allow for redox balancing without additional ATP investments. This limits the product range to a few compounds, such as acetate, ethanol or butanol [9].

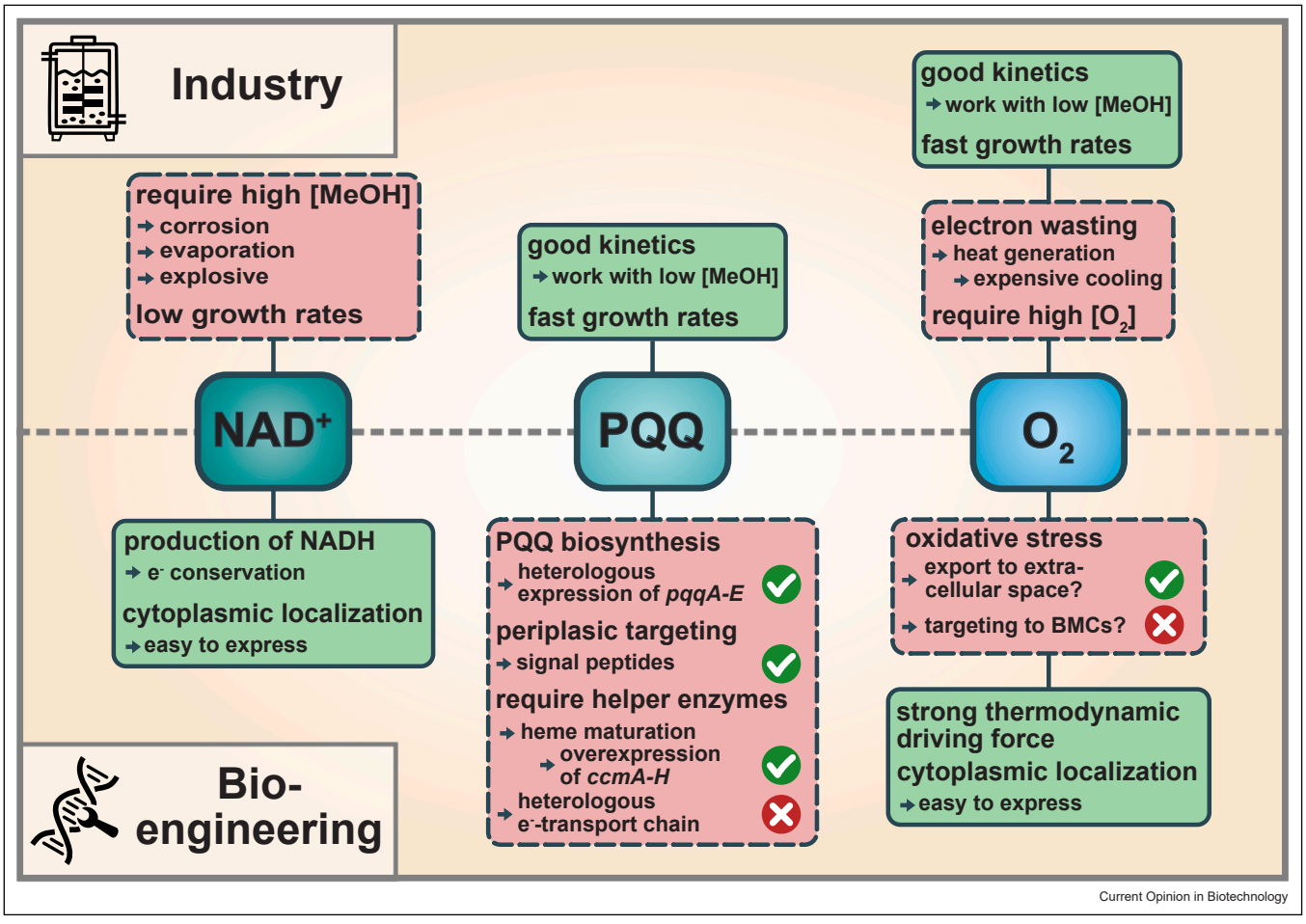
Aerobic growth on reduced C1 compounds, however, can in theory support the production of virtually any bioproduct. Especially MeOH and formate stand out as promising feedstocks. They are, unlike carbon monoxide, methane and CO_2/hydrogen, soluble substrates, which facilitates mass transfer in bioprocesses. In addition, growth on MeOH and formate generally leads to higher energy conversion efficiencies based on combustion energy conversion in the generated biomass. For detailed information and examples, please refer to Ref. [11]. A drawback for the

highly oxidised substrate formate, however, is that during growth, a large fraction of formate is converted to CO_2 for energy generation, whereas the highly reduced substrate MeOH can serve as carbon and energy source without or with very limited CO_2 generation.

Therefore, efficient methylotrophic cell factories are required for the bioconversion of MeOH into products. To tailor native methylotrophs that can efficiently grow on MeOH for industrial needs, we need to extend our understanding of their metabolism and further work on the establishment of routine genetic engineering techniques [12]. In contrast, integration of MeOH assimilation routes into industrially established microbes to generate synthetic methylotrophs circumvents the bottlenecks of natural methylotrophs. Research in synthetic methylotrophy has seen groundbreaking advances with the first fully methylotrophic *E. coli* growing via the reductive glycine pathway (rGlyP) [13] and later via the ribulose monophosphate RuMP cycle [14,15]. Whether native or synthetic methylotrophs are the best option for industry will depend on their productivity (currently limited by growth rate for synthetic methylotrophs) and the desired end product. Hence, the feasibility of both approaches needs to be investigated [16].

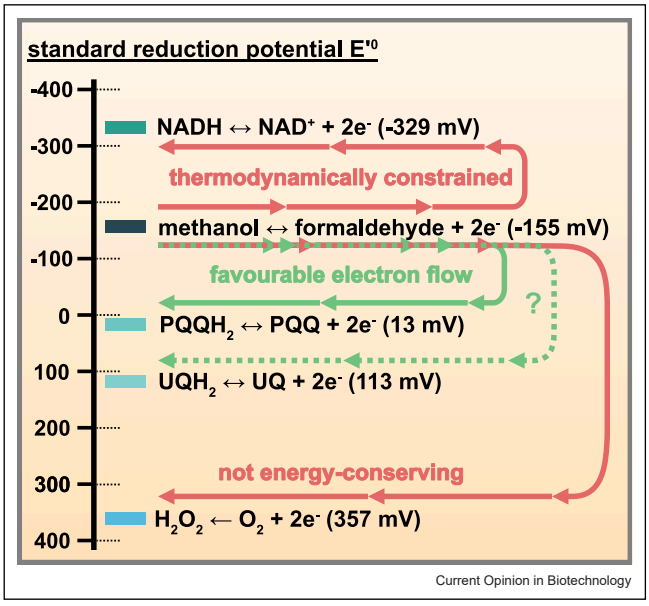
In this review, we focus on the first crucial enzymatic step when designing a synthetic methylotroph: the choice of methanol dehydrogenase (MDH). Most studies about the application of synthetic methylotrophs emphasise the choice of the most efficient assimilation pathway (e.g. RuMP or rGlyP, rather than the serine cycle) as the main objective to optimise titres, rates and yields. While these considerations are essential, they omit the impact of the different types of MDHs on operation costs [7]. Here, we highlight the industrial implications and bioengineering challenges of choosing MDHs with O_2, NAD^+ or

Figure 1



Overview of the industrial challenges and advantages associated with each type of electron acceptor for MeOH oxidation. Industrial challenges are depicted in red boxes with dashed outlines and advantages are depicted in green boxes with solid outlines. Green check marks and red crosses mark challenges for which solutions already exist or not, respectively. Abbreviations: ccm — cytochrome-c maturation, BMCs—bacterial microcompatments.

Figure 2



Redox potential of relevant metabolic half-reactions and the direction of electron flow. All values were calculated with eQuilibrator (pH = 7, pMg = 3, ionic strength = 0.25 M) [22], assuming a concentration of 1 mM for all substrates and products. Note that the reduction potential of bound cofactors (i.e. PQQ and UQ) varies between enzymes depending on cofactor coordination within the enzyme. Abbreviations: PQQ/$PQQH_2$ — pyrroloquinoline quinone/quinol, UQ/UQH_2 — ubiquinone/ubiquinol.

pyrroloquinoline quinone (PQQ) as an electron acceptor (Figure 1). Note that the enzyme oxidizing MeOH with O_2 is generally referred to as methanol oxidase. However, for simplicity, we will refer to them as methanol dehydrogenase (MDH) as a general term in this review.

A key principle behind the different MDH electron acceptors is the difference in thermodynamic driving force, which influences the equilibrium of these reactions. In Figure 2, we exemplify this by depicting the standard reduction potential (E'^0) for the oxidation of MeOH and each electron acceptor. While O_2 and PQQ both have a more positive E'^0 than the oxidation of MeOH to formaldehyde, NAD^+ has a more negative E'^0 and therefore constrains the reaction thermodynamically.

The problem with O_2

Currently, the most common methylotroph established in industry is the native methylotrophic yeast *Pichia pastoris* (renamed to *Komagataella phaffii*), which is commonly used for protein production, usually grown with MeOH as an inducer for protein production and glycerol as a carbon co-substrate. *P. pastoris* was recently engineered to produce malate (2.79 g/L) and fatty acids (23.4 g/L) from MeOH [23–25]. It assimilates MeOH via the xylulose monophosphate cycle and FAD-dependent MeOH oxidases using O_2 as electron acceptor [26]. As O_2 has a very positive E'^0, O_2-dependent methanol dehydrogenase (MOX) benefits from a strong thermodynamic driving force (Figure 2 and Table 1) potentially supporting fast growth and high production rates. Observed growth rates for methylotrophic yeast growing on MeOH, however, are not very high as seen in Table 1, which could be attributed to the generally slower growth rate of yeast compared with fast-growing prokaryotes. This potential advantage comes at a price: using O_2 as an electron acceptor wastes the energy released by the oxidation of MeOH instead of storing it in the form of reduction equivalents. Moreover, this electron wasting results in substantial heat generation, which requires cooling of the bioreactors and increases their operation costs [27]. Furthermore, cells growing via MOX require equimolar amounts of O_2 as a co-substrate, a factor that can become limiting in high-density cell cultures [28]. Finally, the oxidation of MeOH with O_2 generates H_2O_2, which is toxic through the generation of reactive oxygen species [29].

Methylotrophic yeasts oxidise MeOH inside peroxisomes in order to sequester the H_2O_2 generated by the reaction and detoxify it with peroxisomal catalases. Since prokaryotes lack peroxisomes, it is unclear whether heterologous expression of methanol oxidase could support methylotrophic growth. It should be noted, however, that bacteria are known to possess several oxidases, which they use for growth (e.g. [30]). There is at least one catabolic oxidase known in *E. coli*, which is localised in the periplasm and oxidises phenylethylamine [31]. Since this compartment has a different redox state than the cytoplasm and lacks genomic DNA, it is assumed to be less sensitive to H_2O_2 toxicity. Heterologous expression of a functional FAD-dependent oxidase has been shown [32]. However, MOX are large enzymes composed of eight copies of the same subunit, so ensuring proper transport and folding is challenging. Assuming toxicity can be dealt with, for example, via overexpression of a catalase, an oxidase could lead to improved growth rate, while the decrease in yield due to the loss of electrons might be partly counterbalanced by the reduced protein burden and faster growth rate.

Many bacteria are also known to utilise bacterial microcompartments (BMCs) to sequester toxic metabolites [33]. These are relatively simple protein shells that can self-assemble and to which enzymes can be targeted *in vivo*. Pores in the BMCs can selectively allow certain metabolites to pass, while blocking certain others, usually on the basis of size, polarity or charge. In some bacteria, BMCs are used to support the oxidation of multi-carbon alcohols such as propanediol or ethanolamine, which generate toxic aldehydes as intermediates [34]. If MOX and catalase could be co-localised in a BMC, such a compartment would essentially work in the same way as a peroxisome.

Table 1

Overview of thermodynamic, kinetic and physiological parameters for different classes of MDH. Thermodynamic data were calculated with eQuilibrator (pH = 7, pMg = 3, ionic strength = 0.25 M), assuming a concentration of 1 mM for all substrates and products [22]. μ_{max} denotes the reported maximum growth rates on MeOH and $Y_{X/S}$ denotes the yield expressed in gCDW/mol of MeOH, both based on data from Ref. [11].

Enzyme	$\Delta G'^{m}$ (kJ/mol)	k_{cat} (s^{-1})	K_M (mM)	μ_{max} (h^{-1})	$Y_{X/S}$	References
MOX	-98.9	5.7–60	0.4–2	0.05–0.11	9–13	[17,18]
N-MDH	33.6	0.1–0.2	21–150	0.2–1.0[a] (0.09)[b]	15–18[a]	[15,19]
Q-MDH	-32.6	0.9–35	0.02–6.6	0.2–0.7	11–15	[20,21]

[a] Measured at 55°C.
[b] Fastest reported growth rate of a synthetic methylotroph.

Taking inspiration from nature, we additionally propose a strategy that has thus far not been explored in the literature: many types of fungi can excrete MOX into their extracellular environment. The enzyme then oxidises MeOH and produces H_2O_2, which damages the outer wall of plant cells. This damage is then used by the fungi to infect the plant. Similarly, it should be possible for bacteria to secrete MOX and catalase, using the extracellular space as a 'compartment'. One possible issue with this strategy is of a kinetic nature, as formaldehyde would have to accumulate in the extracellular environment and from there cross the bacterial membrane (both inner and outer in case of Gram-negative bacteria). Additionally, the volatility and reactivity of formaldehyde might prove problematic during cultivation.

However, neither of the bioengineering strategies described above solve the inherent inefficiency of using O_2 as an electron acceptor. Thus, we conclude that MOX do not represent an optimal option for the design of synthetic methylotrophs suited for industrial applications.

The problem with NAD^+

Growing synthetic methylotrophs via NAD-dependent methanol dehydrogenases (N-MDH) seems to solve all the challenges imposed by MOX. Recent efforts in synthetic methylotrophy have therefore focused on N-MDHs as they are rather easy to express heterologously, do not require additional cofactors or translocation out of the cytoplasm and are O_2 independent [13–15]. Most importantly, N-MDHs allow energy conservation through the transfer of electrons to NAD^+ and therefore neither produce excess heat nor toxic H_2O_2.

These advantages, however, come at a price: oxidation of hydroxyl groups to carbonyl groups is thermodynamically unfavourable and the reaction is biochemically fully reversible. This reflects in the rather low k_{cat} and high K_M values (Table 1) that require feeding of MeOH at high concentrations (500–600 mM) to achieve synthetic methylotrophy [13–15]. For aerobic industrial applications, however, it is generally preferable to operate bioreactors with low MeOH concentrations to reduce substrate loss through evaporation, to improve safety through decreased explosion risk and to reduce corrosion of operational machines [35]. Additionally, poor kinetic properties of N-MDHs result in low growth rates (Table 1), which does not allow for continuous feeding of low concentrations of MeOH since this could outdilute the culture. It is important to note that for thermophilic methylotrophs (growing between 45 and 55°C), the N-MDH reaction becomes more thermodynamically favourable [36], which makes them a viable option for the production of some products [12]. The only well-studied methylotrophic thermophile, *Bacillus methanolicus*, grows relatively fast using an N-MDH (doubling time ~1 hour). However, this organism is notorious for being relatively challenging to cultivate and still has some genetic engineering limitations, including no published genome editing tools [37].

From the landmark studies on synthetic methylotrophy in recent years [13–15], it is apparent that N-MDHs, while providing a convenient shortcut to establish synthetic methylotrophy, are insufficient for fast and efficient growth. In the first work, showing growth on MeOH via the rGlyP in *E. coli*, a doubling time of 55 hours was measured, compared with 8–10 hours on formate, indicating that the MeOH oxidation step is likely to be the bottleneck. The most recent work, using the RuMP cycle in *E. coli*, showed a much faster doubling time of 8 hours. However, proteomics revealed that N-MDH accounted for roughly 40% of the total proteome [15]. While yield was not reported in any of these works, it is apparent that a slow doubling time and/or the extreme protein burden associated with these strains will limit yields.

Being aware of these kinetic and thermodynamic bottlenecks, several groups have tried to find or engineer N-MDHs with superior characteristics [19,38]. Some of these efforts have uncovered mutations leading to modest improvements in enzyme velocity (3.5-fold improved V_{max}) [38] and good improvements in enzyme affinity (~100-fold improved K_M) [39]. However, these enzymes still present a low activity ($k_{cat} < 1\ s^{-1}$, Table 1).

It is unclear whether further efforts will lead to additional improvements, and how far away current variants of N-MDHs are from the optimum.

It has also been proposed that fusion enzymes between MDH and formaldehyde dehydrogenase could improve kinetics by swiftly removing the formaldehyde as it is generated [40]. Scaffolding alone is unlikely to speed up the enzymatic reaction directly *in vivo*, since diffusion is orders of magnitude faster than enzyme turnover [41], and hence it is unlikely to be the limiting step. A substrate channelling approach, on the other hand, could improve thermodynamics and reduce the rate of backward reaction to improve overall kinetics [42]. Additionally, substrate channelling has been shown to reduce toxicity of intermediates and limit cross-reactivity [43]. Since formaldehyde is highly reactive and readily cross-links proteins and DNA [44], a channelling strategy could be worth pursuing.

Another intriguing possibility is the direct condensation of formaldehyde with the C1 carrier tetrahydrofolate (THF), producing CH_2–THF, which can subsequently be assimilated via, for example, the serine cycle. This reaction is thermodynamically feasible and has been shown to occur *in vivo*, albeit at a very slow rate [45]. To the best of our knowledge, there are no reports of MDHs with this bifunctional activity, however, there are many categories of formaldehyde-producing enzymes known to produce CH_2–THF from a wide array of different substrates [46–50]. The common feature of these enzymes seems to be a THF-binding domain. It is speculated that free formaldehyde is channelled through an internal cavity from the oxidoreductase domain to the THF-binding domain, where the condensation happens, leading to the release of CH_2–THF [51].

The quinone solution?

For the establishment of efficient synthetic methylotrophs in industry, however, neither the electron-wasting MOX nor the thermodynamically unfavourable N-MDHs seem optimal. Instead, using PQQ as a cofactor represents a good compromise. While the use of this cofactor does not conserve reducing power via NADH, PQQ-dependent methanol dehydrogenases (Q-MDH) still conserve some energy, generating a proton gradient that can be used for the synthesis of approximately 1 ATP/MeOH (compared with ~2.5 ATP/MeOH from NADH oxidation). This efficiency compromise potentially allows growth on low MeOH concentrations as well as faster growth and production rates [11].

With the exception of Gram-positive thermophiles, methylotrophic bacteria have evolved to use Q-MDHs [52]. PQQ is a complex quinone cofactor and its biosynthesis requires at least five genes (*pqqABCDE*), whose functions have recently been established [53]. Despite the complexity of its biosynthesis, it has been shown that heterologous expression of these five genes is sufficient for PQQ biosynthesis in organisms such as *E. coli*, which natively lack this capability [54]. Two more genes called *pqqF* and *pqqG* are sometimes present in PQQ-biosynthesis operons and are thought to act as proteases for the cleavage of the *pqqA* peptide after the cross-linking reaction catalysed by the *pqqE* product [55]. However, they do not seem to be essential as they are not required for heterologous expression (at least not in *E. coli*) and they are often missing even in obligate methylotrophs. Indeed, the presence of *pqqBCDE* has been used as a bioinformatic marker for the identification of PQQ-utilising bacteria [56]. Lack of the necessary gene products, however, does not prove that an organism has no PQQ-dependent enzymes, as this cofactor is water-soluble and can be harvested from the environment. *E. coli*, for example, lacks the PQQ biosynthesis genes but harbours two PQQ-dependent dehydrogenases in its periplasmic space [57,58]. It was recently shown that concentrations as low as 1 nM can be actively imported via a TonB–ExbBD-related transporter called PqqU (YncD) [59].

While PQQ-dependent enzymes were thought to be exclusively present in some prokaryotes, a PQQ-dependent pyranose dehydrogenase was recently discovered in the fungi *Coprinopsis cynerea* and *Trichoderma reesei* [60]. However, fungi do not seem to possess the PQQ-biosynthesis machinery. Additionally, PQQ is widely distributed in plant and animal tissues. However, the role of PQQ in these organisms is not known, and to date, no quinoprotein has been found in plants or animals.

Since PQQ biosynthesis can be engineered, and PQQ-dependent alcohol dehydrogenases (PQQ-ADHs) are highly interesting from the industrial perspective, several groups have tried heterologous expression for the purposes of purification and metabolic engineering [61]. In order to understand why these efforts have generally been unsuccessful, it is necessary to understand the complexity of this category of enzymes and their peculiar electron transport chain. In general, there are three categories of PQQ-ADHs, named type I, II and III, all of which require translocation to the periplasm. This step previously required laborious low-throughput screening [62], but has recently profited from significant technological advances via library-based approaches [63].

Functional PQQ-ADH expression also depends on c-type cytochrome formation (Figure 3). This type of periplasmic heme-containing cytochrome has its own dedicated maturation machinery, which is present in *E. coli* as the *ccmA-H* operon [64]. It is natively only expressed anaerobically but does not contain O_2-sensitive elements and was demonstrated to produce high quantities of c-type cytochromes when overexpressed aerobically [65]. Taken together,

periplasmic export as well as c-type cytochrome formation seem to pose surmountable challenges for functional PQQ-ADH expression in *E. coli* or other heterologous hosts.

In the following section, we provide relevant biochemical background of the different types of PQQ-ADHs and highlight the resulting distinct advantages and challenges.

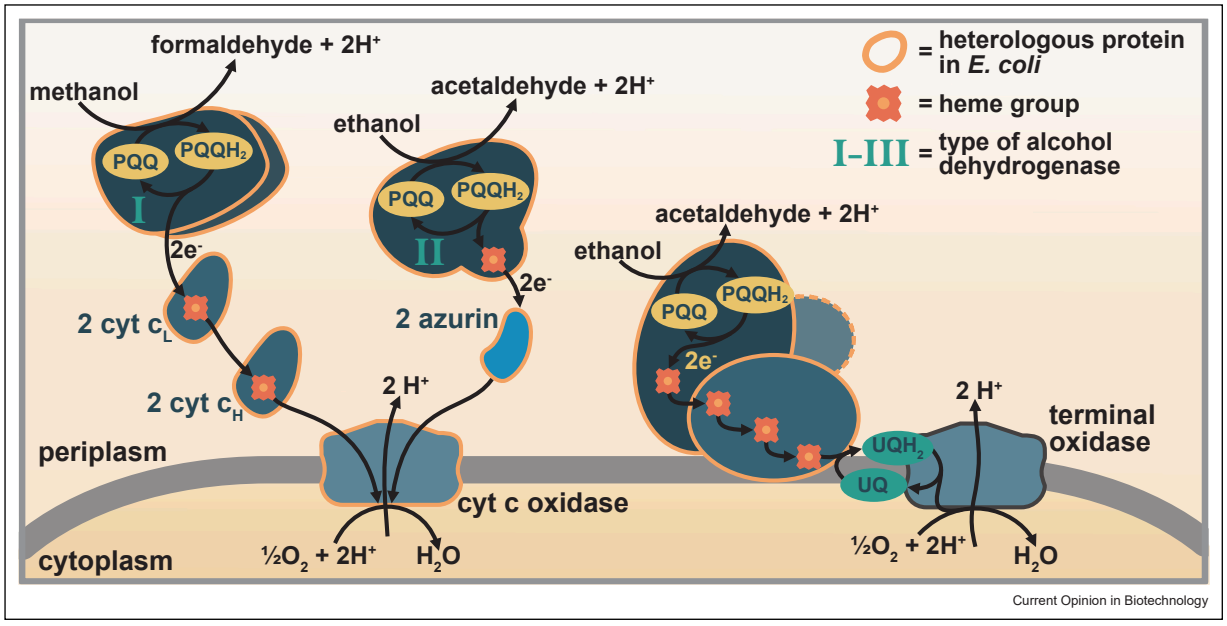
Type I PQQ-dependent alcohol dehydrogenases

Type I PQQ-ADHs are generally heterotetramers ($\alpha_2\beta_2$), though homodimeric (α_2) and even monomeric forms have been reported [19]. While the alpha subunit is responsible for the enzymatic activity, the function of the beta subunit is unknown. The alpha and beta subunits are often encoded in very large operons of 10+ genes, most of which are of unknown function. One hypothesis is that some of these gene products function as chaperones, which are necessary for the proper folding and/or transport of the enzyme. To date, no effort to heterologously express a mxaFI-type quinoprotein has been successful, unless the receiving organism already had its own quinoprotein, and hence most likely the associated machinery.

Successful heterologous expression of homodimeric PQQ-ADH was recently reported: two homodimeric PQQ-ADH (pedE and pedH) were purified from *E. coli* and their activity was demonstrated *in vitro* [66]. However, heterologous expression of the dehydrogenase alone is not sufficient to establish PQQ–MDH activity in a living cell. Reduced PQQ formed during MeOH oxidation needs to be reoxidised. This is accomplished via two additional c-type cytochromes, known as cytochrome c_L and cytochrome c_H, where L and H stand for low and high redox potential (Figure 3). It should be noted that these cytochromes are somewhat uncommon and produced only by methylotrophs and related organisms. Through this multicomponent system, the electrons flow from the reduced PQQ in the active site of the PQQ–MDH via the two soluble c-type cytochromes to a transmembrane cytochrome-c oxidase. This oxidase then donates the electrons to oxygen to form water and generates a proton gradient that can be used for ATP synthesis. In order for the whole system to work, all the components (enzyme, cytochromes and oxidase) must be present and functional, thus posing an exceptional engineering challenge.

Even though type I PQQ-ADH and some cytochromes have been successfully expressed heterologously, there is currently no evidence of successful heterologous expression of cytochrome-c oxidase. The latter is a multisubunit, transmembrane complex, which may prove difficult to transfer between organisms. Note that these issues are not relevant for *in vitro* applications since the enzymes can be easily coupled to artificial electron acceptors such as phenazine ethosulphate/DCPIP, or Wurster's blue [67]. In addition to PQQ, type I enzymes require a metal cofactor, most often Ca^{2+}, although it was recently discovered that

Figure 3



Schematic depiction of PQQ-ADHs type I–III with their associated electron transport chains. The components are not drawn to scale. Type I PQQ–MDH is represented as a homodimer — see main text for further details. In some cases, type III ADH consists of an additional third subunit (dashed outline). Abbreviations: PQQ/$PQQH_2$ — pyrroloquinoline quinone/quinol, UQ/UQH_2 — ubiquinone/ubiquinol cyt - cytochrome.

some enzymes prefer lanthanide ions such as La^{3+} [68], especially among those enzymes that are specific to MeOH. In some organisms, two or more PQQ-ADH are present and differentially regulated in the presence or absence of lanthanides [20,69].

Given the challenges intrinsic to *in vivo* heterologous expression, a different approach might be engineering bacteria that already have type I PQQ-ADHs. This approach requires choosing a genetically tractable bacterium that is either natively methylotrophic or can be engineered to be. One study has shown some initial progress in engineering *Pseudomonas putida* to use the rGlyP for methylotrophic growth using a native type-I PQQ-ADH [70].

Type II PQQ-dependent alcohol dehydrogenases

Alcohol dehydrogenases of type II are monomeric and incorporate a heme-binding site as part of their sequence (Figure 3). While type I PQQ-ADHs have a relatively narrow substrate specificity range, which includes MeOH, type II PQQ-ADHs accept a much wider range of substrates, including primary and secondary alcohols, both aliphatic and aromatic. Activity on MeOH is usually negligible [68]. The electron transport chain also differs from *E. coli* in this class of enzymes: their main electron carrier is thought to be the blue copper protein azurin, from which the electrons are further transferred to a cytochrome c oxidase [71]. Taken together, type II PQQ-ADHs still require the heterologous expression of different components of the electron transport chain but do not accept MeOH as a native substrate. This makes them inferior candidates compared with type I PQQ-ADHs for establishing PQQ-dependent synthetic methylotrophy.

Type III PQQ-dependent alcohol dehydrogenases

Type III PQQ-ADHs are membrane-bound quinohemoproteins found exclusively in acetic acid bacteria, where they catalyse the oxidation of ethanol to acetaldehyde. While type III PQQ-ADHs are reported to have a wide substrate specificity, including primary alcohols up to hexanol, it is frequently stated throughout the literature that they do not accept MeOH as a substrate [68,72–74]. However, there are several examples of type III PQQ-ADHs with up to 37% side activity on MeOH compared with their main substrate ethanol [75].

This type consists of 2 or 3 subunits and transfers electrons via PQQ and 4 heme prosthetic groups to the ubiquinone pool [73] (Figure 3). Subunit I is encoded by *adhA* and binds PQQ and one heme. Subunit II, which is encoded by *adhB*, possesses 3 heme moieties and is responsible for membrane anchoring and ubiquinone reduction [76]. While only AdhA and AdhB were demonstrated to be catalytically active, some candidates require an additional subunit III (AdhS), which is speculated to be a chaperone [77].

Although type III PQQ-ADHs also require translocation to the periplasm and heme maturation, they directly transfer electrons to the ubiquinone pool. This makes it easier to wire them with the electron transport chain of *E. coli* since they do not require heterologous expression of helper proteins and especially no heterologous cytochrome-c oxidase. We therefore propose to first establish *in vivo* activity on their native substrate ethanol via whole-cell enzyme assays [78] or in an acetyl-CoA auxotrophic strain [79]. After achieving functional expression, established constructs could be transferred to a formaldehyde sensor strain where catalytic activity on MeOH could be improved via adaptive laboratory evolution or phage-assisted evolution, as demonstrated before for N-MDH [38].

Other approaches

Finally, it could be another approach to change the substrate specificity of PQQ- or ubiquinone (UQ)-dependent dehydrogenases that are native to *E. coli*. This would circumvent the challenges related to the heterologous overexpression of several periplasmic enzymes. Although *E. coli* does not synthesise PQQ, it still expresses two periplasmic PQQ-dependent enzymes: the membrane-bound quinoprotein glucose dehydrogenase (Gcd) and the soluble aldose sugar dehydrogenase (YliI). Both of these catalyse the oxidation of a wide range of sugars [57,58]. Although a homologue of YliI exists in a thermophilic bacterium that accepts MeOH as a substrate [80], it is unclear whether the substrate specificity of both YliI or Gcd could be shifted to accept alcohols and specifically MeOH. Similarly, the approach of engineering *E. coli*'s native UQ-dependent dehydrogenases appears to be far-fetched. Although UQ is thermodynamically suited as an electron acceptor for the oxidation of MeOH, there is no evidence for any UQ-dependent alcohol dehydrogenase in the literature. However, we would like to note that establishing such enzyme activity could become the crown jewel of synthetic methylotrophy, as it would not involve any overexpression of periplasmic enzymes.

Conclusion

Here, we examine the importance of choosing the 'right' electron acceptor for microbial MeOH oxidation from a bioengineering as well as an industrial perspective. We highlight that in spite of a strong thermodynamic driving force and therefore fast kinetics (Table 1), O_2 used by MOX as an electron acceptor does not represent an optimal solution as H_2O_2 production results in cellular stress as well as heat generation, which requires expensive cooling. Furthermore, we argue that the thermodynamic limitations of NAD-dependent MDHs (Table 1 and Figure 2), which have been exclusively used for synthetic MeOH assimilation due to their ease of engineering, not only lead to slow growth rates but

also require feeding of high MeOH concentrations. This is generally undesirable for industrial applications as it results in evaporation, corrosion and safety risks. Based on thermodynamics, kinetics and energy efficiency, Q-MDH therefore present a promising compromise for aerobic oxidation of MeOH in industry. We describe strategies for their functional expression, which requires periplasmic translocation and the concerted activity of a complex enzyme cascade that is heterologous to *E. coli*. However, it is unclear whether this endeavour can be successful. Alternatively, it is important to investigate whether other industrially relevant microorganisms that natively synthesise PQQ and express PQQ-ADHs (e.g. *Pseudomonas putida*, *Gluconobacter oxydans*, *Cupriavidus necator*, etc.) could be more suited for engineering synthetic methylotrophy for industrial applications.

To achieve a better understanding of quinone-dependent enzymes and their relevance for synthetic methylotrophy, many challenges and open questions remain:

1. What is the evolutionary origin of PQQ? Its complex biosynthetic pathway requires several unique enzymes, some of which were only recently characterised. Despite this, it is ubiquitous across all domains of life, and it seems to be beneficial for human health [81].
2. While PQQ seems to be the optimal solution for alcohol oxidation, it is unclear why some solutions based on alternative redox cofactors did not arise. For instance, FAD-dependent dehydrogenases using ubiquinone as electron acceptor exist for a variety of substrates (sugars, alpha-keto acids, saturated hydrocarbons and acyl-CoAs), but to the best of our knowledge, none of them are capable of oxidising alcohols.
3. What determines the electron acceptor (e.g. cytochromes/azurin versus UQ) for PQQ-dependent ADHs and is it possible to use UQ directly as an electron acceptor for alcohol oxidation? Are there examples in nature?
4. What determines the substrate specificity of different types of PQQ-dependent ADH? To what extent can this be engineered?
5. Who will win the race for methylotrophy in industry - native methylotrophs with improved tractability or synthetic methylotrophs with efficient and deeply rewired metabolism?
6. To what extent will methylotrophy become economically and ecologically viable to support industrial decarbonisation?

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare no conflict of interest.

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