Unravelling Formaldehyde Metabolism in Bacteria

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Formaldehyde metabolism is prevalent in all organisms, where the accumulation of formaldehyde can be prevented through the activity of dissimilation pathways. Furthermore, formaldehyde assimilatory pathways play a fundamental role in many methylotrophs, which are microorganisms able to build biomass and obtain energy from single- and multicarbon compounds with no carbon–carbon bonds.

Keywords: formaldehyde ; assimilation ; dissimilation ; methylotrophy

1. Introduction

Formaldehyde is toxic to all living organisms due to its nonspecific reactions with proteins and nucleic acids, giving rise to the widespread development of mechanisms for its detoxification. However, the focus of this entry is formaldehyde metabolism in microorganisms, specifically in bacteria [1]. The primary atmospheric sources of formaldehyde are either anthropogenic (indoor pollution through building materials, vehicle exhaust gases, various combustion sources, and fugitive industrial emissions) or biogenic (live and decaying plants, biomass burning, and seawater) [2][3][4]. Formaldehyde can also be formed as part of bacterial metabolism in biological processes such as the demethylation of lignins or Strecker degradation of glycine with methylglyoxal [5]. High levels of formaldehyde can hamper cell functions due to its cytotoxic effect, which is provoked by its electrophilic reactivity [6]. Formaldehyde forms reactive dihydroxydimethyl peroxides and free radicals in the presence of oxidizing molecules, causing oxidative stress and ultimately cell death [7][8][9][10]. Unspecific binding of formaldehyde to various macromolecules leads to the inactivation of their biological functions [11]. This way, exposure to formaldehyde can damage vital cell components as DNA, RNA, and proteins through processes such as a multistep formation of adducts and cross-links [6][12][13][14][15].

Despite its highly toxic properties, formaldehyde acts as a key intermediate in methylotrophic metabolism directly originating from the oxidation of one-carbon (C1) compounds, excluding rare exceptions [1][6][16][17][18][19][20]. Methylotrophs are microorganisms with the ability to build biomass and obtain energy from either single-carbon compounds such as methane, methanol, or formate, or multicarbon compounds with no carbon –carbon bonds such as dimethyl ether and dimethylamine [20]. All those compounds are commonly known as C1 compounds and will hereafter be referred to as such. Methylotrophs have specifically developed formaldehyde assimilation (i.e., fixation) systems in the course of evolution in order to use formaldehyde for biomass production [1]. Four different cyclic C1 assimilatory pathways have been described for aerobic methylotrophs: formaldehyde fixation can take place via the ribulose monophosphate (RuMP) cycle and the serine cycle in bacteria, while yeasts use the xylulose monophosphate (XuMP) pathway; in a few known methylotrophs, after oxidation of C1 substrates to CO_2, assimilation of the latter takes place via the ribulose bisphosphate (RuBP) pathway as in classic autotrophic microorganisms [20][21][22]. Because the focus of this review is formaldehyde assimilation in bacteria, the XuMP pathway will not be further explored [23]. Likewise, as anaerobic methylotrophy does not involve formaldehyde and the RuBP pathway relies on autotrophic CO_2 assimilation, they will also be excluded from this review [23]. Alongside these assimilation pathways, methylotrophs have also developed formaldehyde dissimilation (i.e., oxidation) mechanisms similar to all living organisms in order to cope with excess formaldehyde and preserve their cellular functions [1]. Remarkably, in methylotrophic *Methylorubrum extorquens* (formerly *Methylobacterium extorquens*) AM1, tolerance to formaldehyde is heterogeneously distributed in bacterial cell populations with two distinct gene expression profiles characteristic for tolerant or susceptible cells. While susceptible cells exhibit global stress response to treatment with formaldehyde, the response of the tolerant subpopulation does not seem to involve the formaldehyde oxidation pathway but features a number of chaperones and electron transport chain factors [24].

Formaldehyde dissimilation pathways are present not only in methylotrophs but also in nonmethylotrophic microorganisms to prevent the toxic impact of environmental formaldehyde [1]. While for most bacteria, except for methylotrophs, the well-known strategy to maintain intracellular concentrations of formaldehyde at subtoxic levels is its dissimilation, there have recently been attempts at engineering synthetic formaldehyde assimilation pathways into their metabolism using

mainly *Escherichia coli* and *Corynebacterium glutamicum* but also *Pseudomonas putida* and most recently *Bacillus subtilis* as host strains [25][26]. Synthetic methylotrophy is based on implementing methylotrophic pathways into nonmethylotrophic platform microorganisms following synthetic biology approaches with only one successful attempt at autonomous methylotrophy so far without the use of cosubstrate, at growth rates of 0.09 h^{-1} [27]. Although employing natural methylotrophs as bioproduction platforms seems to be a more straightforward approach than creating synthetic methylotrophs, it exhibits limitations due to a narrow understanding of their metabolism and physiology and a restricted synthetic biology toolbox [28]. As an alternative strategy, the transfer of methylotrophy into well-established and biotechnologically relevant hosts offers the possibility of a streamlined implementation of C1-based bioproduction processes.

2. The Understanding of Formaldehyde Metabolism Regulation as a Support to Push Synthetic Methylotrophy

Due to the high toxicity of formaldehyde, its metabolism requires sensitive and fine-tuned regulation systems. Particularly, in methylotrophic microorganisms, formaldehyde metabolism is often regulated through multilevel cascade systems. For example, in the methylotrophic *P. denitrificans*, the formaldehyde metabolism is controlled by a two-component system consisting of FlhR and FlhS [29][30]. The FlhRS system regulates the expression of enzymes that are either involved in production of formaldehyde (Mdh and methylamine dehydrogenase (Madh) or its consumption (GD-Faldh, Fgh) [31][29][30][32]. The FlhRS system is activated, and its expression is induced by the presence of formaldehyde and by the depletion of heterotrophic substrates [29][30]. This regulatory system consists of a signal (FlhS) and a response regulator (FlhR) that binds to DNA [30]. FlhS is a histidine kinase that, when activated through the binding of effector molecules, catalyzes the phosphorylation of FlhR [30]. As soon as FlhR is activated through phosphorylation, it binds to target promoters of C1 metabolism gene clusters, resulting in the expression of genes encoding Fgh and GD-Faldh [29][30]. The upregulation of the expression of these genes takes place during growth on methanol, while they are constitutively expressed at basal levels also during heterotrophic cultivation [30]. Consequently, GD-Faldh and Fgh are synthesized, and thus formaldehyde is converted to CO_2, which prevents the accumulation of its toxic concentrations [30]. This means that initially, only the formaldehyde-consuming enzymes are synthesized. When formaldehyde concentration increases, the activated FlhR binds to promoters of the operons *mxa* (encoding Mdh) and *mau* (encoding Madh), which, however, does not directly lead to their activation [29][30]. The expression of *mxa* and *mau* is induced by binding the additional regulator MxaX or MauR for expression of *mxa* and *mau*, respectively [30]. The regulatory systems derived from *P. denitrificans* were used to create synthetic regulators that can potentially be applied for dynamic gene expression control in synthetic methylotrophs. For example, the sensoring kinase domains of MxaY or FlhS derived from *P. denitrificans* were fused with the cytoplasmic catalytic domain of the osmosensor histidine kinase EnvZ from *E. coli* in order to create a chimeric sensor histidine kinase that responds to the presence of methanol in the environment [29][33]. A similar approach was used to create a methanol sensor by combining the sensing domain of MxcQ derived from *M. organophilum* XX or *M. extorquens* AM1, or *M. extorquens* AM1-derived MxbD with the transmitter domain of EnvZ from *E. coli* [34][35].

Besides methanol-sensing regulators, *M. extorquens* AM1 possesses a unique, recently discovered formaldehyde sensor enhanced formaldehyde growth protein A (EfgA), which contributes to formaldehyde detoxification not through enzymatic oxidation but relying on binding with this compound [36]. In response to the transient increase of intracellular formaldehyde concentration, EfgA leads to a rapid halt of protein translation and arrest of cell growth which could limit formaldehyde-induced protein damage [36]. Moreover, the changes in the translation are potentially linked to the global transcriptional response to formaldehyde stress mediated by EfgA [37]. Transcriptional response targets biosynthesis of free formaldehyde, contributes to increased formaldehyde consumption by Fae, mitigates proteotoxicity and genotoxicity, and is reversed when formaldehyde concentration decreases [37]. Expression of *efgA* is regulated by TtmR, a formaldehyde-responsive MarR family transcription factor, and both EfgA and TtmR are required for the optimal transition from multicarbon to C1 growth [38]. It was shown that heterologous expression of *M. extorquens* AM1-derived *efgA* in *E. coli* increases its formaldehyde resistance, indicating the potential application of EfgA in strategies to increase formaldehyde tolerance during the engineering of synthetic methylotrophy [36].

Methylotrophic growth activates formaldehyde assimilation and dissimilation pathways which should actively control intracellular formaldehyde concentrations. In the facultative methylotroph *B. methanolicus* MGA3 some of the genes involved in the RuMP formaldehyde assimilation cycle are upregulated during growth on methanol versus heterotrophic conditions. However, details of the expression regulation are not elucidated, and the regulator is not yet known. *B. methanolicus* MGA3 cells grown on methanol are more sensitive to formaldehyde than MGA3 cells grown on a nonmethylotrophic substrate [39]. The increased formaldehyde sensitivity during methylotrophic growth might be caused by the already high formaldehyde concentration in the cells grown in methanol and thus the saturation of formaldehyde assimilation and dissimilation pathways in this bacterium. For that reason, supplementation with external formaldehyde

can lead to increased formaldehyde toxicity [39]. One of the regulators present in *B. methanolicus* MGA3 is HxlR, which controls the expression of chromosomal genes *hps* and *phi,* upregulated by formaldehyde [40]. It was shown that the introduction of additional copies of *hps* and *phi* through plasmid-based overexpression increases the tolerance of *B. methanolicus* MGA3 cells to high methanol concentrations confirming the central role of that operon in C1 metabolism [40].

The structure of the formaldehyde-responsive transcription factor HxlR was also studied in the nonmethylotroph *B. subtilis*. Similarly to TtmR, HxlR belongs to the MarR family of transcription factors, and it controls the expression of *hxlAB* in *B. subtilis* [41][42]. HxlR recognizes formaldehyde through a protein intra-helical cysteine-lysine cross-linking reaction at its N-terminal α1 helix, which in turn leads to a conformational change and transcriptional activation [42]. The resulting intrahelical methylene bridge is a protein modification with a conformational change that allosterically induces transcriptional activation of HxlR [42]. Another regulator responsible for controlling the expression of genes involved in formaldehyde metabolism in *B. subtills* is AdhR. AdhR regulates the expression of the BSH-dependent formaldehyde dissimilation pathway in *B. subtilis* and belongs to an NmlR clade within the family of MerR repressor-activators [19]. MerR regulators are sensitive to a wide range of molecules such as soft transition metal ions, the superoxide anion, and drug-like compounds, whereas the members of the NmlR clade respond to oxidative and carbonyl stressors [43][44][45][46]. Similar to other formaldehyde sensors, a cysteine residue is conserved within the NmlR clade; for example, Cys52 is conserved in AdhR from *B. subtilis* [43]. Replacement of this residue with alanine leads to the creation of a strain where *adhA* (*adhC*) is not transcribed in a formaldehyde rich environment [43].

Regulation of expression of the formaldehyde metabolic pathway in *C. glutamicum* is not well characterized. The expression pattern of the gene encoding NAD-linked MSH-dependent formaldehyde dehydrogenase is not known, and neither is its regulation [47]. Expression of *ald* encoding acetaldehyde dehydrogenase that catalyzes the oxidation of formaldehyde depends on the carbon source used for the cultivation of *C. glutamicum*. The activity of Ald increases about 10-fold when ethanol is a carbon source as compared to growth with glucose or mixtures of glucose with ethanol [47][48]. This process is regulated by RamA and RamB and putatively by GlxR [47][48]. Thus, due to differential gene expression, the importance of Ald and FadH might vary depending on the physiological conditions [47]. Methanol catabolism is subject to carbon catabolite repression in the presence of glucose and is dependent on the transcriptional regulator RamA, which was previously shown to be essential for the expression of *adhA* and *ald* [49].

A well-characterized system for the control of formaldehyde metabolism is present in heterotrophic *E. coli*. In this bacterium, *yeiG* encoding Fgh is constitutively expressed, while the expression of Fgh-encoding *frmB,* which belongs to the *frmRAB* operon, increases by 20- to 100-fold over basal levels in the presence of formaldehyde in the environment [50] [51]. FrmR is a member of the CsoR/RcnR family of metal ion-sensing transcriptional repressors, which is responsible for controlling the *frmRAB* operon [52][53][54]. In the absence of formaldehyde, FrmR binds to the promoter of the *frmRAB* operon (P_{frm}), while in its presence FrmR changes its conformation, which leads to the dissociation of the P_{frm}-FrmR complex [54]. The change of protein conformation is caused by the formation of methylene bridges that link adjacent proline (Pro2) and cysteine (Cys35) residues in the FrmR tetramer [54]. The allosteric mechanism of FrmR is triggered directly by formaldehyde *in vitro* [54]. Sensitivity to formaldehyde requires a cysteine (Cys35 in FrmR) conserved in all DUF156 proteins [55].

As highlighted by the limited success in a full transfer of methylotrophy, and the importance of fine-tuning the C1 metabolic landscape, an important approach to synthetic methylotrophy is the implementation of dynamic formaldehyde regulation mechanisms. The formaldehyde-inducible promoter P_{frm} was engineered to obtain variants differing in their basal and induced expression levels [56]. A variant of the formaldehyde-responsive promoter characterized with higher basal and induced expression levels compared with P_{frm} was used for the control of *mdh* and *hxlAB* in a Δ*frmA*Δ*pgi E. coli* genetic background, which led to improved biomass yield in comparison to the strain where the native *E. coli* P_{frm} was used [56]. Furthermore, using the formaldehyde-inducible promoter P_{frm} to drive direct regulation of *rpe* and *tkt* genes involved in the regeneration of Ru5P led to significantly improved methanol assimilation into intracellular metabolites in *E. coli* [57]. Global gene regulation is an additional factor that should be considered in establishing synthetic methylotrophy. The use of the non-native substrate methanol for growth likely triggers the response characteristic for nutrient-limiting conditions in *E. coli* [58]. Such response is characterized by diverting resources away from active growth and division in favour of maintenance and stress resistance leading to inhibition of RNA synthesis [58]. This leads to decreased translation and conservation of amino acids concurrent with the upregulation of many amino acid biosynthetic genes [58]. It was shown that the activation of stringent response via overproduction of guanosine tetraphosphate (ppGpp) or enzymes involved in its biosynthesis (RNA polymerase-binding transcription factor DksA and the stress response sigma factor RpoS) enhances methanol utilization in synthetic methylotroph *E. coli* by enabling the biosynthesis of several limiting amino acids using carbon derived from methanol in comparison to the control strain where such amino acids cannot be synthesized [58].

Altogether, a comprehensive understanding of formaldehyde metabolism and its regulation in native methylotrophs is an invaluable asset in designing strategies for its introduction into nonmethylotrophic species. The hitherto research showcases the importance of finding a balance between oxidation of C1 substrates to formaldehyde, endogenous formaldehyde dissimilation, and introduction of synthetic formaldehyde assimilation pathways in order to properly regulate carbon flux towards assimilation and maintain formaldehyde below toxic levels. As exemplified in this review, formaldehyde dissimilation pathways and their regulation seem to be relatively conserved among different bacterial species regardless of their trophic lifestyle, which means that native pathways on nonmethylotrophs can potentially be used in the engineering efforts for synthetic methylotrophy.

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