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MINI-REVIEW

Methylobacterium extorquens: methylotrophy and biotechnological applications

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Abstract Methylotrophy is the ability to use reduced one-carbon compounds, such as methanol, as a single source of carbon and energy. Methanol is, due to its availability and potential for production from renewable resources, a valuable feedstock for biotechnology. Nature offers a variety of methylotrophic microorganisms that differ in their metabolism and represent resources for engineering of value-added products from methanol. The most extensively studied methylotroph is the Alphaproteobacterium *Methylobacterium extorquens*. Over the past five decades, the metabolism of *M. extorquens* has been investigated physiologically, biochemically, and more recently, using complementary omics technologies such as transcriptomics, proteomics, metabolomics, and fluxomics. These approaches, together with a genome-scale metabolic model, facilitate system-wide studies and the development of rational strategies for the successful generation of desired products from methanol. This review summarizes the knowledge of methylotrophy in *M. extorquens*, as well as the available tools and biotechnological applications.

Keywords *Methylobacterium extorquens* · Methanol · Methylotrophy · Industrial biotechnology

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Introduction

Methylobacterium is a widespread genus that can be readily isolated from various habitats including wastewater (Kohler-Staub et al. 1986), soil (Doronina and Trotsenko 1996), and the phyllosphere where they are present in large numbers (Delmotte et al. 2009; Vorholt 2012). Its ability to use plant-derived methanol as an energy substrate provides an advantage during plant colonization under competitive conditions (Abanda-Nkpwatt et al. 2006; Fall and Benson 1996; Sy et al. 2005). One of the most intensively studied species of the genus is *Methylobacterium extorquens* and in particular strain AM1 which has been investigated as a model organism for methylotrophy for more than 50 years (Anthony 2011). Detailed information on the methylotrophic metabolism has been uncovered from core enzyme functions to pathways up to a genome-scale metabolic model. In addition, omics approaches have helped to describe methylotrophy on a systems level. The latter approaches initially became possible through the determination of the genome of *M. extorquens* AM1, for which a fully annotated genome sequence is available, as well as a genome-scale metabolic model. Furthermore, a versatile set of genetic tools has been developed and applied for gene deletions and overexpression, as well as random mutagenesis and chromosomal gene integration. Finally, bioprocess engineering led to the development of high cell density fermentation protocols for several *M. extorquens* strains (see below).

The accumulated knowledge about the biology and technical use of *M. extorquens* together with the availability of genetic tools has led to a constant increase in interest in the use of this methylotroph as a microbial host for the production of value-added compounds. Accretive industrialization and growing world population encourage the discussion about alternative raw materials to become more independent of steadily

increasing oil prices but also to avoid direct competition with resources used for food production (Schrader et al. 2009).

Methanol is already an important carbon feedstock for the chemical industry with a worldwide production of approximately 53 million tons in 2011 and an expected annual growth rate in the range of 10–20 % (Bertau et al. 2014), and a methanol-based bioeconomy has been proposed (Olah 2013). In addition to its worldwide availability, methanol production is flexible. The majority of today's methanol is produced from fossil raw materials, such as crude oil, coal, and natural gas, but in the future an increasing share is expected to be produced from renewables or sustainable sources, such as biogas, glycerol, wood, or solid municipal waste (Schrader et al. 2009). For example, a biomass-to-methanol (BtM) plant (that produces methanol starting from forest residues) with a capacity of 100,000 t/a is currently being projected in Sweden (Bertau et al. 2014). Such endeavors illustrate that methanol is producible in various ways, and consequently, industrial biotechnology based on this compound is less dependent on market price fluctuations of raw materials. However, compared to sugars, the market price of which is approximately 0.2$/kg (Peralta-Yahya et al. 2012) corresponding to 0.5$ per kilogram substrate carbon, methanol is still more expensive with a price of approximately 0.45$/kg (Bertau et al. 2014), corresponding to approximately 1.2$ per kilogram substrate carbon. On the other hand, sugar is an agricultural commodity, which is subject to strict price regulations and import limitations in Europe. This impairs free-market conditions which apply for any intermediate in the chemical industry, including methanol. As the price for sugar derived from arable land—the common fermentation substrate in industrial biotechnology—is expected to steadily increase due to its linkage to the food sector, and sugar from lignocellulosics still suffers from severe technical challenges, "bio-methanol" will become an attractive alternative for the establishment of sustainable bioprocesses in the future (Schrader et al. 2009).

Within this review, we summarize the current knowledge of methylotrophy in *Methylobacterium* and its use for methanol conversion to value-added products. Additional applied aspects of *Methylobacterium* strains, such as plant growth-promoting microbials in agriculture (Fedorov et al. 2011; Joe et al. 2013; Tani et al. 2012), are beyond the scope of this review. For a recent review on a thermophilic methylotroph, *Bacillus methanolicus*, the reader is referred to Müller et al. (2014).

Model strains of *M. extorquens*, growth substrates and genomes

The first model methylotrophic bacterium and the most extensively studied to date is *M. extorquens* AM1. It was initially called *Pseudomonas* AM1 and was isolated in Oxford as an airborne contaminant in methylamine medium (Peel and Quayle 1961), hence the name AM1 for airborne methylamine. It grows on the reduced C1 substrates methanol and methylamine but also as a facultative methylotroph on multicarbon substrates such as succinate (C4) and acetate (C2) (Large et al. 1961; Schneider et al. 2012a) (for a more detailed list, see Table S1). The genome sequence of *M. extorquens* AM1 revealed a 6.88-Mb genome consisting of a chromosome, a megaplasmid, and three smaller plasmids (Vuilleumier et al. 2009). An additional number of *Methylobacterium* strains have been sequenced (Marx et al. 2012; Vuilleumier et al. 2009) (Table 1) including *M. extorquens* PA1 that was recently selected as a competitive colonizer of the phyllosphere of *Arabidopsis thaliana* (Knief et al. 2010) (PA1 for phyllosphere of *Arabidopsis*). Further *M. extorquens* strains that have been characterized and sequenced include strains that are able to grow on chloromethane (strain CM4) and dichloromethane (strain DM4). All of the strains harbor a genome of 5.4–5.9 Mb and one or two plasmids (except strain PA1 which has none); as mentioned above, strain AM1 also contains a megaplasmid. While the function of the plasmids of *M. extorquens* AM1, DM4, and BJ001 is unknown, the 380-kb plasmid of strain CM4 was shown to carry genes for chloromethane dehalogenation (Roselli et al. 2013).

This review will mainly focus on the model strain *M. extorquens* AM1 and occasionally refer to strain PA1, which in our opinion represents a valuable alternative to *M. extorquens* AM1 for further research on methylotrophy. This is due to the simpler genome structure, the applicability of genetic screens (Metzger et al. 2013), and the overall conserved methylotrophy features compared to the AM1 strain (Nayak and Marx 2014b). In addition, strain PA1 lacks the long history of domestication, which led to divergence from the initially isolated strain (Carroll et al. 2014).

Tools developed and applied for *M. extorquens*

Genetic tools have been used since the early research of *M. extorquens* AM1 to identify essential genes for methylotrophy. Historically, chemical mutagenesis was applied (Heptinstall and Quayle 1970) in combination with complementation analysis to classify mutants into different complementation groups (Nunn and Lidstrom 1986). Subsequently, site-directed knockouts (Chistoserdova and Lidstrom 1992) and transposon mutagenesis (Springer et al. 1995) were established. To date, a broad selection of genetic tools for expression, knockout, and transposon mutagenesis, has been described (Table 2), and the genetic toolbox for *M. extorquens* and other Alphaproteobacteria is constantly

Table 1 List of genome sequenced *M. extorquens* strains

Strain	Isolation	C1 substrates	Genome
AM1	Airborne contaminant in methylamine (Peel and Quayle 1961)	Methanol, methylamine	Chromosome: 5.511 Mb Megaplasmid: 1.26 Mb Plasmids: 44 kb, 38 kb, 25 kb (Vuilleumier et al. 2009)
DM4[a]	Soil from a treatment plant for halogenated hydrocarbon waste (Kohler-Staub et al. 1986)	Methanol, dichloromethane (Kohler-Staub et al. 1986)	Chromosome: 5.94 Mb Plasmids: 140 kb, 39 kb (Vuilleumier et al. 2009)
PA1	*Arabidopsis thaliana* phyllosphere (Knief et al. 2010)	Methanol, methylamine (Knief et al. 2010)	Chromosome: 5.471 Mb (Marx et al. 2012)
CM4[b]	Soil of petrochemical factory (Doronina and Trotsenko 1996)	Methanol, methylamine, chloromethane (Doronina and Trotsenko 1996)	Chromosome: 5.778 Mb Plasmids: 380 kb, 23 kb (Marx et al. 2012)
BJ001[c]	*Populus deltoides* × *nigra* DN34 endophyte (Van Aken et al. 2004)	Methanol, methane[d] methylamine (Van Aken et al. 2004)	Chromosome: 5.8 Mb plasmids: 25 kb, 23 kb (Marx et al. 2012)

[a] Previously *Methylobacterium dichloromethanicum* DM4

[b] Previously *Methylobacterium chloromethanicum* CM4

[c] Previously *Methylobacterium populi* BJ001

[d] Was disputed (Dedysh et al. 2004); genome sequencing later revealed the lack of characteristic genes for methane monooxygenase (*pmoABC* and *mmoXYBZDC*)

expanding. Moreover, a recent study provided a modified medium and a genetically engineered strain, which lacks cellulose synthase, optimized for reproducible high-throughput growth experiments (Delaney et al. 2013).

Table 2 List of available genetic tools for *M. extorquens* AM1

Genetic tools	Description	Reference
Expression systems		
Plasmid-borne		
Constitutive		
P_{mxaF}/P_{lac}	pCM80 (TetR), pCM160 (KanR) IncP *oriV*, *oriT* (RK2-derived)	Marx and Lidstrom (2001)
Methylobricks (P_{mxaF}), P_{fumC}, P_{coxB}, P_{tuf}	Derived from promoter-less pCM80 (TetR) (also KanR available)	Schada von Borzyskowski et al. (2014)
Inducible		
Cumate-inducible P_{mxaF}	Regulator cymR chromosomally integrated	Choi et al. (2006)
Cumate-inducible P_{mxaF}	pHC115, cymR encoded on plasmid, comparatively high background expression	Chou and Marx (2012)
Cumate/anhydrotetracycline-inducible P_R	pLC290, pLC291 (both KanR)	Chubiz et al. (2013)
Cumate-inducible P_{syn2}	pQ2148, tight promoter	Kaczmarczyk et al. (2013)
Insertional		
pCM168/pCM172	Chromosomal insertion of desired gene into *katA* gene *loxP*-flanked resistance marker	Marx and Lidstrom (2004)
Transposons used for genetic screens		
pCM639	Mini Tn-5 derivative IsphoA/hah-Tc	Marx et al. (2003c)
pAlmar3	Mariner transposon (used in strain PA1)	Metzger et al. (2013)
Site-directed knockout		
pAYC61		Chistoserdov et al. (1994)
pCM184	Cre-lox-based system allows unmarked deletions	Marx and Lidstrom (2002)
pCM433	SacB-based bhr vector	Marx (2008)

The fact that *M. extorquens* is a facultative methylotroph represents a useful trait to study methylotrophy. Knockout mutants in essential genes for methylotrophy can be generated on an alternative carbon source and then selected on methanol, thus facilitating the discrimination between true essential genes for methylotrophy and potential technical obstacles to generate a particular gene knockout.

Much of the knowledge generated for *M. extorquens* AM1 is based on classical biochemical studies resulting from enzyme discovery to gene discovery or vice versa. More recently, a number of complementary omics approaches have been applied including transcriptomics, proteomics, and metabolomics to characterize methylotrophy more systematically (Table 3). In these studies, cell analytes detected under methylotrophic conditions were compared to those detected under non-methylotrophic growth conditions, usually succinate, which results in similar growth rates (Peyraud et al. 2012). These studies gave valuable insights into methylotrophic metabolism and represent useful tools for further studies. Comparative proteomics and a microarray study revealed proteins/transcripts that were differentially expressed under methylotrophic conditions compared to non-methylotrophic conditions (Bosch et al. 2008; Laukel et al. 2004; Okubo et al. 2007). These experiments demonstrated the induction of proteins/transcripts known to be involved in methylotrophy, as well as many with unknown functions.

Different methods to study the metabolome have been applied to *M. extorquens*. These methods include nucleotide determination (including ATP and NAD) (Guo and Lidstrom 2006), metabolite profiling (Guo and Lidstrom 2008), and quantitative metabolome analyses (Kiefer et al. 2011; Kiefer et al. 2008). In addition, a method to quantify CoA-esters has been established and was employed to demonstrate the ethylmalonyl-CoA pathway (EMCP) in *M. extorquens* AM1 (Peyraud et al. 2009).

Recently, a genome-scale model of the methylotrophic metabolism of *M. extorquens* was established by integrating all available information including omics, biochemical, and mutation studies (Peyraud et al. 2011). The functionality of the metabolic network was analyzed in silico including elementary flux mode analysis, and the model was tested for network topology and features. In addition, methylotrophy was examined experimentally using ^{13}C-fluxomics via NMR and mass spectrometry (Peyraud et al. 2011). The study provided valuable information about the different pathways utilized during methylotrophic growth and demonstrated the presence of a degree of connectivity of the core metabolism as a mosaic of common and specific pathways. It was also proposed that methylotrophy is under constant selection in nature, which is in line with experimental evolution experiments, where prolonged selection on succinate resulted in a loss of the ability to grow on C1 compounds in a part of the population (Lee et al. 2009).

Methylotrophy in *M. extorquens*

Methylotrophy can be divided into different parts, which altogether allow metabolization of the reduced C1 carbon source for energetic and biosynthetic purposes: (1) oxidation of primary C1 substrate to formaldehyde, (2) oxidation of formaldehyde to CO_2, and (3) assimilation into biomass (Chistoserdova 2011). Different combinations of enzymes and pathways exist, which represent "solutions" found in nature. In the following section, the crucial steps of methylotrophy in *M. extorquens* AM1 are described after a short historical perspective. A graphical overview of methylotrophy in *M. extorquens* AM1 is shown in Fig. 1 and a list of the involved genes including information on their essentiality and regulation is given in Table S2.

M. extorquens AM1 as a model organism for enzyme and pathway discovery

As mentioned above, *M. extorquens* AM1 has been an important model organism for methylotrophy in the past half century. In fact, many enzymes and pathways relevant for C1 metabolism in more general terms have been discovered in this bacterium and are now known to be widespread among methylotrophs (Chistoserdova 2011).

The serine cycle for C1 assimilation was elucidated in *M. extorquens* AM1 already more than five decades ago through the seminal work of Quayle and colleagues (Large et al. 1961, 1962a, b; Large and Quayle 1963). The continuous operation of this pathway requires the regeneration of glyoxylate. Interestingly, *M. extorquens* lacks isocitrate lyase (Dunstan and Anthony 1973), the key enzyme of the known glyoxylate cycle (Kornberg and Krebs 1957), and uses an alternative route for glyoxylate regeneration. The exact sequence of reactions of this pathway, termed the EMCP, was only solved a few years ago (Erb et al. 2007; Peyraud et al. 2009). For a historic overview of the elucidation of the serine cycle and the EMCP, we refer to the recent review by Anthony (2011).

Another important discovery was that the catabolic formaldehyde oxidation in *M. extorquens* AM1 depends on tetrahydromethanopterin (H_4MPT), which was previously believed to be unique to methanogenic and sulfate-reducing Archaea (Chistoserdova et al. 1998). In *M. extorquens*, the H_4MPT-dependent pathway functions in the reverse direction compared to the known mode of operation in hydrogenotrophic methanogens and catalyzes the oxidation of formaldehyde to formate. The pathways described above were subsequently discovered in other microorganisms capable of growth on C1 compounds, including aerobic proteobacterial methanotrophs (Chistoserdova et al. 2005b; Vorholt et al. 1999) as well as an anaerobic bacterial methanotroph (Ettwig et al. 2010).

Table 3 Overview of omics studies investigating methylotrophy in *M. extorquens* AM1

Omics study	Reference	Description
Genome	Vuilleumier et al. (2009)	
Genome scale model	Peyraud et al. (2011)	
Proteomics	Laukel et al. (2004), Bosch et al. (2008)	Comparison of methanol and succinate: gel-based approach (2004) and multidimensional protein identifications technology (MudPIT) (2008)
Transcriptomics	Okubo et al. (2007)	Comparison of methanol and succinate microarray
Metabolomics		
Metabolite profiling	Guo and Lidstrom (2008)	Metabolite profiles during growth on methanol and succinate
Quantitative metabolome analysis	Kiefer et al. (2011); Kiefer et al. (2008)	Concentration of central metabolites on methanol and succinate
CoA-esters determination	Peyraud et al. (2009)	Quantification and dynamic labeling of CoA-esters during methylotrophic growth
^{13}C-Fluxomics	Peyraud et al. (2011)	Investigation of metabolic fluxes during methylotrophic growth
Metabolite discovery	(Yang et al. 2013)	Determination of metabolites labeled during growth on ^{13}C-labeled methanol

Methanol oxidation

In *M. extorquens*, methanol oxidation is catalyzed by a periplasmatic pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (MDH) (Anthony 1986). MDH has a hetero-tetrameric $\alpha_2\beta_2$ structure where each α-subunit contains one Ca^{2+} ion and one PQQ as a prosthetic group (reviewed in Anthony and Williams 2003). The large (66 kDa) α-subunit is encoded by *mxaF* and the small (9 kDa) β-subunit by *mxaI*. The electrons taken up by the MDH-bound PQQ during the oxidation of methanol are transferred to the unique cytochrome c_L (*mxaG*), then further via the type I cytochrome c_H to cytochrome *c* oxidase and eventually to oxygen (Anthony 1992). These genes are located in a gene cluster together with genes thought to have auxiliary functions (Morris et al. 1995; Richardson and Anthony 1992). The genes for the biosynthesis of the cofactor PQQ (*pqqABC/DE*, *pqqFG*) are located in two clusters elsewhere in

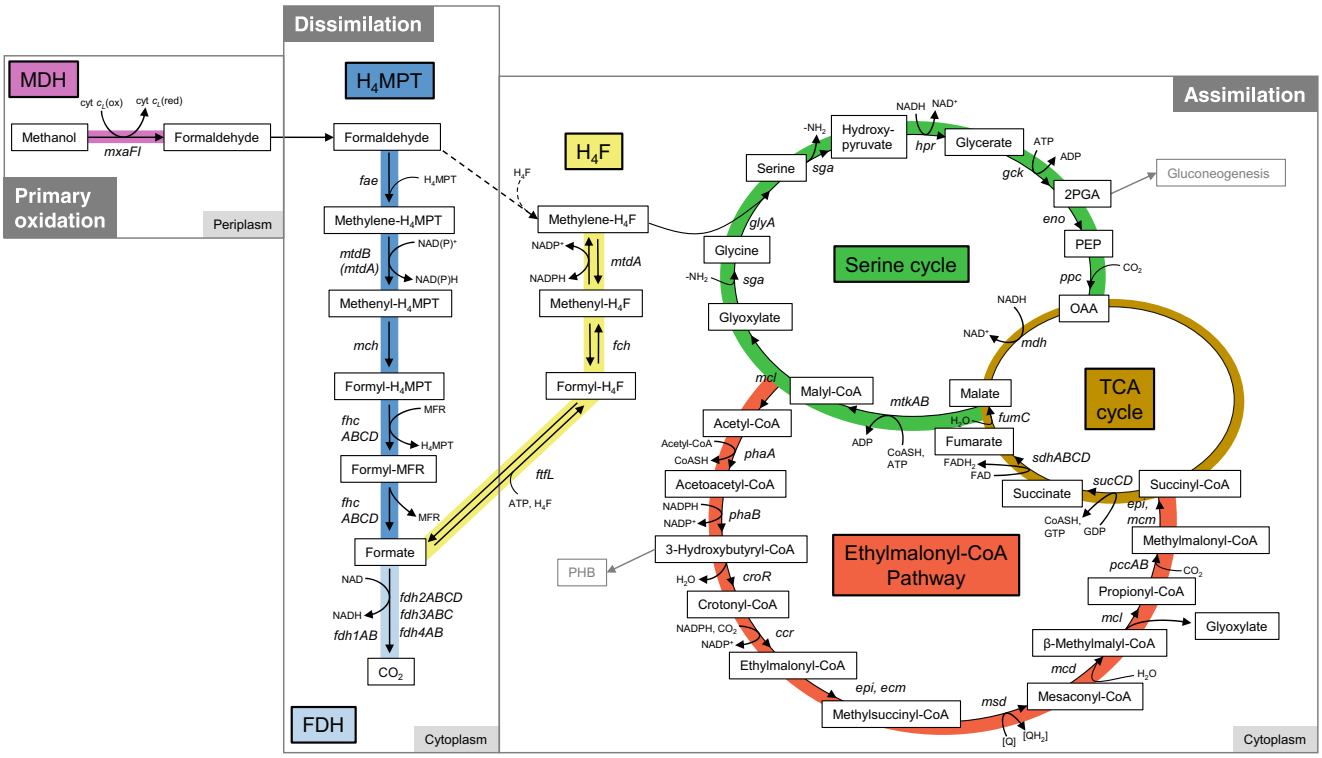

Fig. 1 Overview of methanol metabolism in *M. extorquens* AM1. The coloring marks the different pathways. Genes encoding for the corresponding enzymes are shown in *italics*. For a detailed list of the involved genes, refer to Table S2

the genome (Chistoserdova et al. 2003; Morris et al. 1994; Toyama et al. 1997).

The genome sequence of *M. extorquens* AM1 revealed the presence of several gene-encoding paralogs of the large subunit of methanol dehydrogenase, such as *xoxF1* (Chistoserdova and Lidstrom 1997), which has a sequence identity of approximately 50 %. The enzyme has methanol and formaldehyde oxidation activity (Schmidt et al. 2010) and is able to replace MxaF as a La^{3+}-dependent methanol dehydrogenases under distinct conditions (Nakagawa et al. 2012). In addition to its catalytic role, *xoxF1* is, together with its paralog *xoxF2*, postulated to be involved in regulation (Skovran et al. 2011). Interestingly, *xoxF* is highly expressed in the phyllosphere (Delmotte et al. 2009) and plays a role in plant colonization (Schmidt et al. 2010).

Formaldehyde dissimilation

Formaldehyde produced by methanol dehydrogenase in *M. extorquens* AM1 is further oxidized by a H_4MPT-dependent pathway (Chistoserdova et al. 1998) which, in addition to its catabolic role, can also participate in formaldehyde detoxification (Marx et al. 2003a) (Fig. 1). *M. extorquens* AM1 produces a dephosphorylated form of H_4MPT lacking the terminal α-hydroxyglutaryl phosphate unit (dH_4MPT) (Chistoserdova et al. 1998). Several genes involved in the biosynthesis of dH_4MPT in methylotrophs have been identified (Chistoserdova et al. 2005a) and the gene products of two of them, *mptG* (Rasche et al. 2004) and *dmrA* (Caccamo et al. 2004; Marx et al. 2003c)), have been characterized.

The first step in formaldehyde conversion consists of the condensation of formaldehyde with dH_4MPT to methylene-dH_4MPT, a step which occurs spontaneously (Escalante-Semerena et al. 1984). Nevertheless, an enzyme accelerating the condensation of formaldehyde with dH_4MPT, termed formaldehyde-activating enzyme (Fae), could be identified in *M. extorquens* AM1 and is essential for methylotrophy (Acharya et al. 2005; Vorholt et al. 2000). Fae is produced in high amounts under both methylotrophic and non-methylotrophic conditions, whereby the presence of the enzyme on methanol is slightly higher (Bosch et al. 2008). Mutants of the *fae* gene are sensitive to formaldehyde, suggesting that the spontaneous reaction is not efficient enough to detoxify formaldehyde (Vorholt et al. 2000).

The formed methylene-dH_4MPT is oxidized to methenyl-dH_4MPT by methylene-H_4MPT dehydrogenases MtdA and MtdB, which in contrast to their F_{420}-dependent archaeal counterparts (Thauer 1998) represent unique enzymes because they use pyridine nucleotides (NAD and NADP) (Vorholt et al. 1998). The NADP-specific MtdA catalyzes the reaction for methylene-tetrahydrofolate (H_4F) as well as methylene-H_4MPT (Ermler et al. 2002; Hagemeier et al. 2000; Vorholt et al. 1998), while the NAD(P)-dependent MtdB is specific for methylene-dH_4MPT (Hagemeier et al. 2000). They both are essential under methylotrophic growth conditions and play distinct roles in controlling the flux of C1 units (Martinez-Gomez et al. 2013; Marx and Lidstrom 2004).

Methenyl-dH_4MPT is converted to N^5-formyl-dH_4MPT by methenyl-dH_4MPT cyclohydrolase (Mch) (Pomper et al. 1999). The formyl group is then transferred to methanofuran (MFR) by formyltransferase (Ftr). The Ftr-homolog of AM1 (FhcD) forms a complex with three polypeptides that possess sequence identity to formyl-MFR dehydrogenase (FhcABC) (Pomper and Vorholt 2001). Contrary to initial expectations deduced from the analogous enzymatic steps in methanogenic archaea (Vorholt and Thauer 2002), this complex did not catalyze the oxidation of formyl-MFR but instead catalyzed the hydrolysis to formate. The complex was therefore renamed formyltransferase/hydrolase complex (Fhc) (Pomper et al. 2002).

The final oxidation step from formate to CO_2 is catalyzed by several formate dehydrogenases (Fdh), of which only one has been biochemically characterized thus far (Laukel et al. 2003). Subsequently, three more formate dehydrogenases were discovered (Chistoserdova et al. 2007; Chistoserdova et al. 2004). A triple mutant was still able to grow on methanol, but accumulated formate, whereas a quadruple mutant was unable to grow on methanol, which indicates that formate oxidation is essential for growth on methanol (Chistoserdova et al. 2007) and points towards an important role of formate as an intermediate with tightly regulated fluxes and/or yet undiscovered regulatory processes.

Assimilatory pathways

The serine cycle for the assimilation of C1 substrates was first shown in *M. extorquens* AM1 (Large et al. 1961, 1962a, b; Large and Quayle 1963). The C1 unit enters the serine cycle via condensation of methylene-H_4F (see below) with glycine to serine catalyzed by serine hydroxymethyltransferase (GlyA). GlyA is also present in most non-methylotrophic organisms where it provides methylene-H_4F for biosynthesis (Maden 2000). The produced serine is transformed to glyoxylate (to regenerate glycine) and acetyl-CoA. The pathway occurs via intermediates partly shared with gluconeogenesis and the tricarboxylic acid (TCA) cycle and involves a CO_2 fixation step (Anthony 1982) (Fig. 1). None of the genes of the serine cycle are specific for methylotrophic metabolism, but a characteristic feature for methylotrophs is the clustering of the involved genes on the genome (Chistoserdova 2011). In *M. extorquens* AM1, most serine cycle genes are present in one cluster and are transcribed in two operons (Chistoserdova et al. 2003).

The C1-donor methylene-H_4F for the conversion of glycine to serine can principally be produced in two ways: via a direct and indirect route. The direct pathway involves the spontaneous condensation of formaldehyde with H_4F to methylene-H_4F, whereas the indirect route involves the oxidation

of formaldehyde to formate via the H_4MPT-dependent pathway followed by ATP-dependent condensation with H_4F catalyzed by formate tetrahydrofolate ligase (FtfL) (Marx et al. 2003b). The produced formyl-H_4F is converted to methenyl-H_4F (catalyzed by Fch), and subsequently to methylene-H_4F catalyzed by the bifunctional NADP(P)-dependent MtdA enzyme (Vorholt et al. 1998). Experimental data on the operation of the H_4F-dependent pathway in situ is controversial. An earlier study suggested a direct condensation of formaldehyde with H_4F and an indirect route only during a substrate switch (Marx et al. 2005); however, a more recent paper provides evidence for the condensation of formate with H_4F followed by reduction being more important than the direct condensation of formaldehyde with H_4F (Crowther et al. 2008). Future studies using dynamic ^{13}C labeling from methanol will be required to directly demonstrate the carbon flow via cofactor-bound C1 intermediates.

Because metabolic intermediates are withdrawn from the serine cycle, glyoxylate must be regenerated to keep the pathway running. *M. extorquens* AM1 does not use the classical pathway involving isocitrate lyase but instead uses a series of CoA intermediates (Korotkova et al. 2002a; Korotkova et al. 2005). Based on the discovery of the carboxylation activity of crotonyl-CoA reductase (Ccr) (renamed crotonyl-CoA carboxylase/reductase), the EMCP was proposed and shown to operate in *M. extorquens* (Alber et al. 2006; Erb et al. 2007; Meister et al. 2005; Peyraud et al. 2009). The first step of the pathway is the condensation of two acetyl-CoA units, which are then transformed into methylmalyl-CoA over a series of CoA thioester derivatives involving a CO_2 fixation step. Methylmalyl-CoA is cleaved to glyoxylate and propionyl-CoA, which can be converted to C4 compounds, such as succinate, via another carboxylation reaction. This pathway shares many enzymes with other pathways, but unlike the serine cycle, it also possesses unique reactions. Two of these are ethylmalonyl-CoA mutase (Ecm) (Erb et al. 2008) and the above-mentioned crotonyl-CoA reductase/carboxylase (Ccr) (Erb et al. 2007). Unlike other methylotrophy genes, the genes of the EMCP are not localized in large operons but are loosely clustered or not co-localized at all (Hu and Lidstrom 2012).

Alternative C1 carbon sources

Different *M. extorquens* strains grow on a number of additional C1 compounds, including methylamine, chloromethane, and dichloromethane (Chistoserdova and Lidstrom 2013). For all these different substrates, specific metabolic pathways can be identified (Chistoserdova 2011). Methylamine is oxidized to formaldehyde by a tryptophan tryptophylquinone (TTQ)-dependent methylamine dehydrogenase (encoded by *mau* cluster in strain AM1) with amicyanin as the electron acceptor (Chistoserdov et al. 1994). Strains PA1 and DM4, which both lack the *mau* cluster, were recently shown to use the *N*-methylglutamate pathway for growth on methylamine (Gruffaz et al. 2014; Nayak and Marx 2014b). Chloromethane is dehalogenated by the transfer of the methyl group to H_4F (catalyzed by CmuA and CmuB in the strain CM4) (Studer et al. 2001), which is then further oxidized via the H_4F-dependent pathway using specific enzymes (Studer et al. 2002). Dichloromethane is converted to *S*-chloromethyl glutathione and inorganic chloride by dichloromethane dehydrogenase (DcmA in the strain DM4) (Leisinger et al. 1994). The instable *S*-chloromethyl glutathione decomposes spontaneously to formaldehyde, inorganic chloride, and glutathione (Muller et al. 2011).

Facultative methylotrophy and co-consumption of substrates

As mentioned above, *M. extorquens* is a facultative methylotroph. The alternative non-C1 growth substrates, which have been investigated in detail, are succinate (C4), acetate (C2), and oxalate (C2). Succinate and acetate enter the metabolism on the level of the TCA, and in both cases gluconeogenesis is employed to produce sugars. In the case of growth on acetate, glyoxylate needs to be regenerated via the EMCP (Schneider et al. 2012a). Oxalate is converted to oxalyl-CoA, which is decarboxylated to formyl-CoA and further metabolized via a variant of the serine cycle where glyoxylate is provided by the reduction of oxalyl-CoA (Schneider et al. 2012b). A recent study investigated the activities of all enzymes involved in methanol assimilation under C1, C2, and C4 substrate conditions (Smejkalova et al. 2010).

It can be expected that *M. extorquens* needs to switch carbon sources regularly in its natural environment depending on carbon availability. The adaptation involved in such a switch from multi-carbon (succinate) to single carbon (methanol) was investigated using a systems-level approach (Skovran et al. 2010). This study revealed that immediately after the substrate switch a significant amount of methanol is oxidized to formate and further to CO_2. It was proposed that *M. extorquens* uses a downstream priming approach; i.e. the assimilation cycles (serine cycle and EMCP) are induced before the pathways delivering the corresponding precursors (H_4F-dependent pathway). The study also identified potential control points including the conversion of methenyl- to methylene-H_4F (catalyzed by MtdA) and the conversion of mesaconyl-CoA to β-methylmalyl-CoA (catalyzed by Mcd). The expression of the latter is initially decreased but later increased again, suggesting that in this way the buildup of the toxic intermediate glyoxylate, which is produced in the subsequent step, is blocked. Interestingly, the expression of genes common for both growth substrates is changed greatly

during the switch, but the metabolite pools are relatively constant, suggesting the presence of metabolic set points (Skovran et al. 2010).

In addition to alternative carbon utilization and substrate switches, mixed substrate availability has also been examined. Interestingly, succinate and methanol are co-consumed (Peyraud et al. 2012) if fed simultaneously to a batch culture. This is achieved by partitioning of methanol and succinate to specific metabolic pathways. Methanol is primarily used for energy generation via linear oxidation and dedicated biosynthetic reactions involving C1 precursors such as purine biosynthesis (Peyraud et al. 2012). Succinate is mainly used to provide precursors for biomass production with the concomitant production of reducing equivalents that can be used for ATP generation. The repression of methanol assimilation occurred at the level of the serine cycle, and no operation of the EMCP was observed. It was suggested that co-consumption is a strategy to optimize substrate utilization under environmental conditions (Peyraud et al. 2012).

Taken together, these findings suggest that the metabolism of the facultative methylotroph is optimized to exploit the availability of methanol at any time. This is additionally supported by the fact that MxaF is the most abundant protein not only on C1 but also on multi-carbon substrates (Bosch et al. 2008).

Regulation of methylotrophic pathways

Several transcriptional regulators for methylotrophy have been identified thus far. Each of the gene clusters involved in methanol oxidation (*mxa*, *mxb*, *mxc*) are transcribed in one unit, and the promoter regions for the *mxa* and *mxb* cluster have been identified (showing similarity to the *Escherichia coli* σ^{70} promoter consensus) (Zhang and Lidstrom 2003). In addition, a multiple A-tract regulatory sequence was identified upstream of the promoter regions of five methanol oxidation genes and is essential for normal expression of these genes (Zhang et al. 2005). Five essential genes encoded together with methanol oxidation genes are involved in the regulation of gene expression. The *mxbDM* genes are involved in transcriptional regulation (*mxbM* encodes for a response regulator, *mxbD* for a sensor kinase) of different methanol oxidation genes including *mxaF*, *pqqE*, and *mxaW* (Springer et al. 1997). Another regulator-sensor pair involved in *mxaF* transcription is encoded by *mxcQE* (Springer et al. 1995). One of these couples (*mxcQE*) positively regulates the expression of the other (*mxbDM*) (Springer et al. 1997). The knockout mutants of *mxcQE* and especially *mxbDM* do not express cytochrome c_L but an alternative cytochrome termed c_{553} (Springer et al. 1995), suggesting that c_{553} is negatively regulated by *mxbDM*. Another gene, *mxaB*, shows homology to response regulators (Springer et al. 1998) and is required for the expression of *mxaF* (Morris and Lidstrom 1992). Two additional genes involved in the regulation of methanol oxidation are the *mxaF*-homologs *xoxF1* and *xoxF2*, which appear to fulfill a dual role: a catalytic role (see methanol oxidation chapter) and a regulatory role (Skovran et al. 2011). The double mutant of *xoxF12* is not able to grow on methanol and shows decreased expression of *mxbDM* and *mxcQE*, suggesting that it functions via these regulators (Skovran et al. 2011).

A LysR-type transcriptional regulator, QscR, positively controls the expression of the two serine cycle operons: *qsc1* (*sga*, *hpr*, *mtdA*, *fch*) and *qsc2* (*mtkAB*, *ppc*, *mclA*) and the separate gene *glyA*. The expression of genes that are also involved in central metabolism (*mdh* and *eno*) was not regulated by QscR (Kalyuzhnaya and Lidstrom 2003, 2005). The expression of *qscR* is independent of the growth substrate, but the DNA-binding activity of QscR is increased by formyl-H_4F and decreased by $NADP^+$ and acetyl-CoA (Kalyuzhnaya and Lidstrom 2005). Surprisingly, a recent proteomics study revealed that QscR is strongly down-regulated on methanol (Bosch et al. 2008). Further investigation will be required to understand this finding.

Unlike other methylotrophy genes, the genes of the EMCP are not transcribed in operons (Hu and Lidstrom 2012) and are expressed in multiple patterns during a substrate switch from succinate to methanol and not as a unit (Skovran et al. 2010). Very little is known about their regulation. Recently, a TetR family regulator for the expression of *ccr*, encoding for a key enzyme of the EMCP, was discovered and termed CcrR. Interestingly, this regulator did not affect other genes of the EMCP (Hu and Lidstrom 2012).

M. extorquens in biotechnology

The profound knowledge of the metabolism, proteome, genome, and transcriptome of *M. extorquens*, in addition to the availability of genetic tools and well-established bioprocess regimes, makes this bacterium an attractive candidate for the bioproduction of chemicals from non-sugar substrates (see Fig. 2). Such value-added products might be general substances from precursors common to all organisms such as amino acids, or products based on specific intermediates of the metabolism of *M. extorquens*, including PHB or EMCP intermediates, for the biosynthesis of novel dicarboxylic acids, polyketides, or butanol.

A further benefit of *M. extorquens* as production host is the standard use of minimal media, which significantly facilitates product recovery during downstream processing compared to conventional bioprocesses, where complex carbon sources, such as molasses or corn steep liquor, are often employed. The toxicity of methanol is another advantage, as most microbes are not able to grow and therefore to contaminate

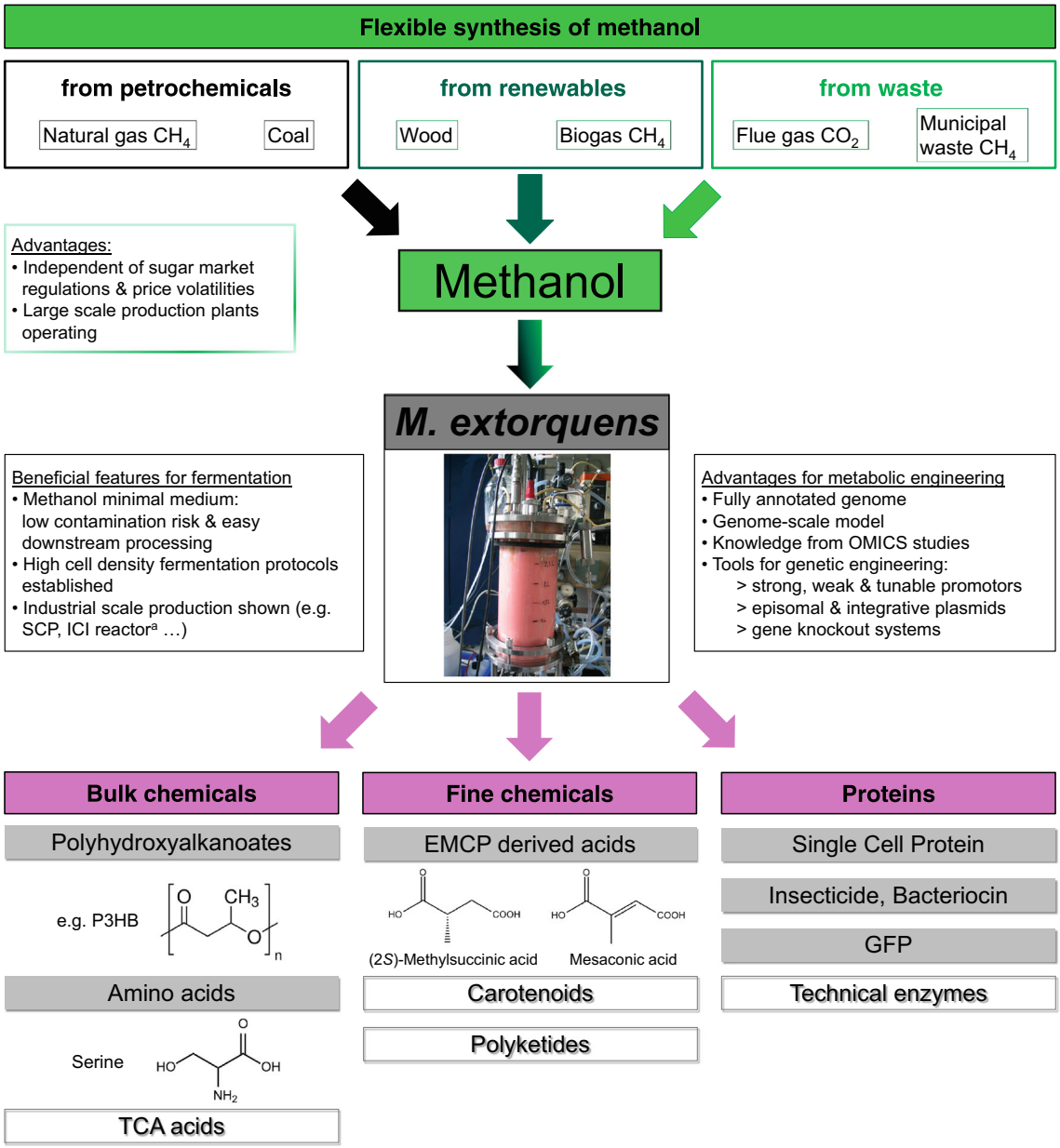

Fig. 2 Use of *M. extorquens* as microbial host for methanol-based product syntheses. Established products are shaded in *gray*, potential products in *white*. Please refer to Table 4 for references of the distinct processes. [a] single cell protein production by Imperial Chemical Industries in 1,500 m^3 air-lift reactor performed in the 1970s and 1980s (Senior and Windass 1980)

methanol minimal media-based fermentation processes. Although *M. extorquens* is able to utilize a wide range of different carbon sources consisting of one-, two-, three-, four-, or five-carbon atoms including alcohols, amines, mono-, and dicarboxylic acids (see Table S1), all the reported biotechnological processes established with *M. extorquens* thus far are based on the substrate methanol. A recent exception is the synthesis of 1-butanol using ethylamine (see Table 4). In addition to methanol, (bio-)ethanol might also become an attractive alternative as a non-sugar-based (and renewable) substrate for the biotechnological production of chemicals by *M. extorquens*. However, no ethanol-based process has been reported to date. In this respect, it should be noted that the maximum growth rates on methanol, which varied from 0.169 to 0.19 h^{-1} (Bélanger et al. 2004; Peyraud et al. 2011), are considerably higher than the 0.069 h^{-1} reported for growth on ethanol (Dunstan et al. 1972). The slow growth of *M. extorquens* AM1 on ethanol is a drawback for competitive industrial applications. In this respect, strain PA1 might represent an attractive alternative candidate with higher growth rates on methanol, ethanol, and multi-carbon substrates (Nayak and Marx 2014a).

Until now, *M. extorquens* has been used for the biotechnological production of various chemicals from methanol, such as polyhydroxyalkanoates (precisely polyhydroxybutyrates),

Table 4 Biotechnological production processes for various compounds established with *M. extorquens* AM1 as a host strain

Substance class	Product	Max. concentration and yield	Description	Reference
Amino acids	L-serine	54.9 g/l (8.3 % from methanol, 39.3 % from glycine	Fermentation of frozen-thawed resting cells at pH 8.2; methanol and glycine as substrates	Sirirote et al. (1986)
		11.3 g/l (4.5 % from methanol, 95.1 % from glycine)	Ca-alginate immobilized resting cells with methanol and glycine as substrates	Sirirote et al. (1988)
PHAs	PHB (600 kDa)	n.s.	Shake flask cultivations	Anderson et al. (1992); Taidi et al. (1994)
	PHB (900–1,800 kDa)	52.9 g/l (0.09–0.12 g/g MeOH; up to 45 % of cdw)	Methanol limited (<0.1 g/l) fed-batch fermentation	Bourque et al. (1995)
	PHB (size n.d.)	149 g/l (0.2 g/g MeOH; 64 % of cdw)	Fed-batch fermentation with defined carbon/nitrogen ratio feed	Suzuki et al. (1986)
		3.3 g/l (33 % of cdw)	Fed-batch fermentation at 1.7 g/l methanol	Bourque et al. (1992)
	PHB-co-3HV	~1 g/l; ratio 0.8–0.2	Addition of 0.5 % (*w*/*v*) valerate to fed-batch fermentation	Bourque et al. (1992)
		0.33 g/l (33 % of cdw); ratio 0.62–0.38	Shake flask cultivation with 0.5 % methanol and 0.05 % *n*-amyl alcohol (both *v*/*v*)	Ueda et al. (1992)
	PHB-co-3HV and PHB-co-3HV-co-3HHx	total 43 % of cdw; ratio of 0.91 to 0.06–0.003	Genomic substitution of native *phaC* by *Aeromonas caviae phaC* + deletion of propionyl-CoA carboxylase *pcc*; growth under cobalt limitation in shake flasks	Orita et al. (2014)
	Functionalized PHB	n.d.; up to 6 % of C5:0, 6:5, 6:0, 8:7, or 8:0 monomers	Overexpression of native or heterologous *phaC*; C5-C11 saturated and unsaturated carboxylic acids as co-substrates	Hofer et al. (2010)
Dicarboxylic acids	Mesaconate	70 mg/l (0.0175 g/g MeOH)	Constitutive overexpression of acyl-CoA thioesterase *yciA*; shake flask cultivation	Sonntag et al. (2014)
	2-Methlysuccinate	60 mg/l (0.015 g/g MeOH)		
Alcohols	1-Butanol	15.2 mg/l	Ethylamine as sole carbon source; co-expression of *adhE*2 from *Clostridium acetobutylicum*, crotonyl-CoA reductase *ter* from *Treponema denticola* and endogenous *croR*	Hu and Lidstrom (2014)
Proteins	Green fluorescent protein (GFP)	0.85–1 mg/g cdw	Construct: pRK310-p*lacZ*-GFP; shake flask cultivation	Figueira et al. (2000)
		1.4 g/l (80 mg/g cdw; 16 % of total protein)	Construct: pCM110$_2$-p*mxaF*-GFP; fed-batch fermentation	Bélanger et al. (2004)
	Haloalkane dehalogenase DhlA	10 % of total protein	pCM80-*dhlA* in ΔphaC strain; shake-flask cultivation	Fitzgerald and Lidstrom (2003)
	Enterocin P (EntP)	150 μg/l	pCM80-*entP*; shake flask cultivation	Gutierrez et al. (2005)
	Insectidical protein Cry1Aa	12.6 mg/l (9 mg/g cdw; 4 % of total protein)	pCM80-*cry1Aa*; shake flask cultivation	Choi et al. (2008)

n.s. not specified, *n.d.* not determined, *PHA* polyhydroxyalkanoates, *PHB* poly-(*R*)-3-hydroxybutyrate, *3HV* (*R*)-3-hydroxyvalerate, *3HHx* (*R*)-3-hydroxyhexanoate, *cdw* cell dry weight

amino acids, dicarboxylic acids, as well as proteins and alcohols from ethylamine (see Table 4). Here, we provide an overview regarding all the reported bioprocesses using *M. extorquens* followed by a short assessment of the future potential of *M. extorquens* as a microbial production host.

One group of bulk chemicals are polymers, such as polyhydroxyalkanoates (PHAs), which have a wide range of applications in packaging, medicine, or as textile and household materials (Keshavarz and Roy 2010). *M. extorquens* is a natural producer of poly-3-hydroxybutyrate (P3HB), whose production from methanol has been investigated for more than 25 years. Suzuki and colleagues reached a remarkable concentration of 149 g/l P3HB (64 % of cell dry weight (cdw)) at a yield of 0.2 g/g methanol in 1986. Similar yields and total amounts were later achieved by Mokhtari-Hosseini et al. (2009). Both groups used accurately adjusted methanol and nitrogen-limited fed-batch fermentations of *M. extorquens*. The total amounts, yield per hour or yield per cdw of produced PHB by *M. extorquens*, are the highest reported compared to other one-carbon- (carbon dioxide, methane, or methanol) based production processes (Khosravi-Darani et al. 2013). Even in comparison to classic production hosts, such as *Cupriavidis necator* (former *Ralstonia eutropha*) or recombinant *E. coli* converting non C1-carbon sources up to 180 g/l PHB, the production outcomes described for *M. extorquens* were in the same range, although space time yields were lower (Chen 2009). Pure P3HB has, however, only limited fields of application due to its stiffness and brittleness caused by its high crystallinity (Verlinden et al. 2007). Investigations of copolymerization with other hydroxyalkanoate co-monomer(s), which overcome the poor mechanical properties of pure P3HB, were therefore carried out and successfully established by the addition of valerate or n-amyl alcohols to the medium (Bourque et al. 1992; Ueda et al. 1992). The production of an even wider range of functionalized PHBs was established using a combination of different native or heterologously expressed PHB synthases and saturated or unsaturated carboxylic acids as co-substrates (Hofer et al. 2010). However, one must be aware that the addition of precursors increases the production costs, which is especially unfavorable for bulk chemicals such as PHBs. A recent study described the de novo production of the copolymer poly[(3-hydroxybutyrate-co-3-hydroxyvalerate] and the terpolymer poly[(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate)] by a metabolically engineered *M. extorquens* strain growing under cobalt-limited conditions (Orita et al. 2014). Cobalt limitation provides limited flux through the EMCP (Chou et al. 2009; Kiefer et al. 2009), resulting in higher pools of propionyl- and butyryl-CoA, which act as precursors for PHB functionalization. Although the PHA content of the cells is still lower than shown for pure P3HB (see Table 4) production processes, newly available tools, e.g., plasmids harboring inducible promoters, (see Table 2) for metabolic engineering might enable higher yields for the production of functionalized PHAs from methanol in the future.

Amino acids are also considered as chemical bulk products with an overall worldwide market volume of more than 3 million tons per year (Sanchez and Demain 2008). Most of the commercially available amino acids are produced by microbial fermentations, such as serine, which is mainly used in pharmaceutical and cosmetic industry and as a building block for the chemical industry (Ikeda 2003). In contrast to other amino acids such as lysine or glutamate, serine is a central metabolic intermediate and precursor for many other metabolites in the classical amino acid producer *Corynebacterium glutamicum*. This has been a drawback for large-scale and high-yield de novo production of serine; thus, despite intense metabolic engineering efforts, the produced serine concentrations of 36.5 g/l (Stolz et al. 2007) are still comparatively low. Methylotrophs harboring the serine cycle, such as *M. extorquens*, offer the possibility to produce serine directly out of cheap methanol and glycine because glycine serves as acceptor molecule for methylene H_4F leading to serine (see above). Sirriote and coworkers used immobilized freeze-thawed resting cells of *M. extorquens* in a controlled fermentation process to produce serine (Sirirote et al. 1988; Sirirote et al. 1986). Freeze-thawing causes porous cell membranes and enables serine export over the cellular membrane. The fermentation of freeze-thawed cells was carried out at methanol concentrations high enough to inhibit growth of *M. extorquens* but below the lethal dose. Thus, biomass formation was prevented and remarkable serine product concentrations of 54.9 g/l were achieved. Today's processes are based on *Methylobacterium* sp. MN43 (Hagishita et al. 1996), which has been reported to produce up to 65 g/l serine. Further increases might be possible by the application of presently available genetic tools, although the challenges of registering food additives produced by genetically modified organisms must be considered.

The heterologous production of (recombinant) proteins is a multi-billion dollar market ranging from pharmaceutical proteins to industrial enzymes (Goodman 2009; Group F 2009). Only approximately 50 % of biopharmaceutical proteins are produced in bacteria (mostly *E. coli*) or yeast (mostly *Saccharomyces cerevisiae*) (Ferrer-Miralles et al. 2009), whereas industrial enzymes are produced by fungi (e.g., *Aspergillus* spp.), yeast (e.g., *S. cerevisiae*, *Pichia pastoris*), or bacteria (e.g., *Bacillus* spp., *E. coli*) (Adrio and Demain 2010). Using *M. extorquens* for the production of enzymes or recombinant proteins from the carbon source methanol would be an interesting alternative to conventional sugar-based production processes as the use of substrates competing with food production could be avoided and product recovery facilitated by the use of minimal medium and the relatively "clean" supernatant of *M. extorquens* cultures.

To date, the heterologous production of four different proteins by *M. extorquens* (GFP, insecticidal protein Cry1Aa, a haloalkane dehalogenase, and Enterocin P) has been reported (see Table 4). These studies provide a proof-of-concept for the applicability of *M. extorquens* as a production host for different heterologous proteins. However, protein yields and final concentrations vary highly from microgram per liter scale for Enterocin P to 1.4 g/l (16 % of total protein) reported for GFP by Bélanger et al. (2004). Commercial production processes would require considerably higher yields because yields up to 50 % of cdw and product concentrations of more than 5 g/l protein were achieved for *E. coli* (Panda 2003) and methylotrophic yeast *P. pastoris* (Macauley-Patrick et al. 2005), respectively. It must also be considered that the use of *M. extorquens* as a bacterial host has several disadvantages compared to already established methylotrophic yeast *P. pastoris*, such as a lacking posttranscriptional modification machinery. In particular, more research concerning the protein secretion machinery of *M. extorquens* would be needed to adjust its capabilities to the purpose of high-efficiency protein production. Nevertheless, *M. extorquens* might be very suitable for the production of industrial enzymes due to its relatively "clean" supernatant.

In addition to the possibility of producing long-known bulk chemicals from the alternative carbon source methanol, the C1 metabolism of *M. extorquens* itself provides potential precursors for the production of special chemical compounds. One rather unusual pathway is the EMCP (see above), which comprises of several branched, saturated or unsaturated, and in some cases, chiral C4- and C5-acyl-CoA esters (Erb et al. 2007; Peyraud et al. 2009). Their corresponding dicarboxylic acids, e.g., ethylmalonic acid, (2*S*)-methylsuccinic acid, mesaconic acid, or (2*R*/3*S*)-methylmalic acid, represent new and interesting building blocks for the chemical industry (Alber 2011). Recently, a bacterial thioesterase YciA was identified that is capable of cleaving CoA from mesaconyl- and (2S)-methylsuccinyl-CoA, resulting in de novo production of mesaconic and 2-methylsuccinic acid from methanol (Sonntag et al. 2014). Although still in the milligram per liter range, EMCP-derived dicarboxylic acid production offers the possibility of the sustainable production of novel monomers. The research to increase process efficiency is currently ongoing in our laboratories.

Beside the dicarboxylic acid derivatives, the EMCP intermediates can also be used as precursors for the biosynthesis of next generation fuels. Hu and Lidstrom (2014) used a combined overexpression of a crotonoyl-CoA reductase and alcohol dehydrogenase for the production of 1-butanol out of crotonyl-CoA. Interestingly, these approach required ethylamine as carbon source instead of methanol to synthesize detectable butanol concentrations. Further optimization might not only increase the product concentrations which are in low miligram per liter range but also enable the use of methanol as carbon source.

Taken together, *M. extorquens* is an efficient bacterial host for the production of various chemical compounds starting from methanol as shown by the synthesis of (functionalized) PHB, amino acid serine, proteins, and uncommon dicarboxylic acids. Final product concentrations and yields are often already competitive, and the increasing interest in the use of alternative carbon sources will most likely focus efforts on establishing application processes with the methylotroph *M. extorquens*.

Future potential of *M. extorquens* as production host

Proof of concepts for the production of PHAs, amino acids, proteins, and dicarboxylic acids from methanol by *M. extorquens* have been delivered to date. In these laboratory processes, product concentrations and yields for PHB and serine synthesis have already reached high values. Nevertheless, new available genetic tools and metabolic insights might provide possibilities to maximize the efficiency of these processes.

The natural PHB synthesis pathway in *M. extorquens* will likely play a key role for the further optimization of de novo production processes, as PHB is in most cases an unwanted by-product. This is particularly true for the production of EMCP-derived dicarboxylic acids due to an overlap of the PHB cycle and EMCP (see Fig. 1). Knockout of the PHB synthase gene *phaC* was already described by Korotkova and Lidstrom (2001), but this mutant suffered from a strong growth defect and was highly instable because it produced second-site suppressor mutants at a high frequency. A deeper insight into PHB synthesis regulation showed that regulator protein PhaR might act as a central flux regulator for acetyl-CoA adjusting its distribution either to the EMCP and therefore also to PHB or to the TCA cycle (Korotkova et al. 2002b). The knockout strain Δ*phaR* is as a stable mutant unable to produce PHB, but metabolomics revealed increased flux through the TCA (Van Dien et al. 2003), which is likely disadvantageous for increasing EMCP-related product formation. A deeper understanding of the mechanism of PhaR may not only provide a way to establish a stable PHB-synthase knockout strain without drastic growth defect, but may also be useful for the controlled increase of TCA fluxes. The latter may open opportunities for TCA cycle-derived dicarboxylic acid or amino acid production from methanol by *M. extorquens*.

In addition to mesaconic acid and (2*S*)-methylsuccinic acid, other EMCP-derived dicarboxylic acids, such as ethylmalonic acid, methylmalonic acid, or the chiral (2*R*/3*S*)-methylmalic acid (Alber 2011), are interesting chemical building blocks unavailable in bulk quantities to date. Their production would require another thioesterase that ideally has

specific activity with one of the related CoA-esters. As in vitro screens are hindered by the fact that many (EMCP) CoA-esters are expensive or commercially unavailable, in vivo screening of potential thioesterases might be an option. McMahon and Prather (2014) recently published a screen for thioesterases showing increased activity in comparison to broad-range thioesterase TesB. They expressed different thioesterases in an engineered *E. coli* strain harboring a heterologous pathway with several short-chain acyl-CoA intermediates followed by supernatant analysis for an altered spectrum of released acids or alcohols as the cleave-off products of the acyl-CoAs. This method is most likely also applicable for screening thioesterases active on CoA-ester intermediates of the EMCP in *M. extorquens*. However, lethal effects resulting from unspecific cleavage of essential CoA-esters and insufficient CoA-ester pools for measurable acid concentrations must be taken into consideration when applying this approach.

Along with the function as precursors for the production of novel dicarboxylic acids, CoA-esters of the EMCP, especially ethyl- and methylmalonyl-CoA, could act as monomers for heterologous polyketide synthesis, as they are the most important building blocks for polyketide synthases (Wilson and Moore 2012). Sufficient supply of heterologous polyketide synthesis in *E. coli* or other hosts often suffers from the low amount of available precursors (e.g., methylmalonyl-CoA) or their complete inexistence (e.g., ethylmalonyl-CoA in *E. coli*). Therefore, the expensive addition of necessary substrates or the high effort of establishing additional pathways for polyketide precursor synthesis is required (Gao et al. 2010; Pfeifer and Khosla 2001). This could be avoided by using *M. extorquens* as a host for polyketide biosynthesis, as most of the known precursors such as acetyl-, methylmalonyl-, ethylmalonyl-, malonyl-, and propionyl- are part of the primary metabolism with high metabolic fluxes and likely comparatively high pool sizes. However, the functional expression of a heterologous polyketide synthases in *M. extorquens* has not been proven to date.

M. extorquens is a pink-pigmented methylotroph (Peel and Quayle 1961), and the color is a result of natural carotenoid production, which are likely C30 carotenoids instead of common C40 carotenoids (Sandmann, unpublished). As a native carotenoid producer, *M. extorquens* might be suitable for the production of other commercially interesting carotenoids such as β-carotene. However, exact information on the natural carotenoid biosynthesis, which most likely overlaps with hopane biosynthesis (Bradley et al. 2010), would be necessary for efficient pathway manipulation.

In summary, there is tremendous potential for *M. extorquens* to be applied as a microbial cell factory for the synthesis of various compounds from methanol, in addition to the already established processes (see above). In addition to other bulk chemicals such as TCA- or EMCP-derived dicarboxylic acid, *M. extorquens* metabolism also offers an opportunity for the production of fine chemicals such as polyketides, carotenoids, or terpenoids.

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