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Introducing a new bioengineered bug

Methylobacterium extorquens tuned as a microbial bioplastic factory

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Key words: *Methylobacterium extorquens*, methanol, unsaturated fatty acids, genetic engineering, funtionalized polyhydroxyalkanoates, reductase-dependent β-oxidation, isomerase-dependent β-oxidation, acyl-CoA desaturase, Scopus

Abbreviations: 2KG, 2-ketoglutarate; 3HA, 3-hydroxyalkanoate; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HHx=, 3-hydroxyhex-5-enoate; 3HO, 3-hydroxyoctanoate; 3HO=, 3-hydroxyoct-7-enoate; 3HP, 3-hydroxypentanoate; 3HV, 3-hydroxyvalerate; AC, acetyl-CoA; C6=, 5-hexenoic acid; C8=, 7-octenoic acid; C11=, 10-undecenoic acid; Cn, carbon number n (length of molecule or position of double bond); GWP, global warming potential; KOH, potassium hydroxide; LCL, long-chain-length; MCL, medium-chain-length; PHA, polyhydroxyalkanoate; PhaC, PHA synthase; PHB, polyhydroxybutyrate; PHBV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PPC, propionyl-CoA; SCL, short-chain-length; SC, succinyl-CoA

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Discussion on and use of methanol as chemical feedstock and as alternative fuel has gained momentum during the past years. Consequently, microorganism and product design based on "methylotrophism" is in vogue as reflected by increasing research and development activities in methanol-related areas. A recent example of microorganism and product development is the use of recombinant *Methylobacterium extorquens* ATCC 55366 strains in the production of second generation biopolyesters. Feeding n-alkenoic acids in addition to methanol yielded functionalized polyhydroxyalkanoates (PHAs) and uncovered how *M. extorquens* copes with fatty acids. While some parts of the degradation pathway remain unclear, possible metabolic routes are suggested that may explain the significant loss of double bonds prior to polymerization of PHA precursors and occurrence of odd-numbered monomers derived from even-numbered n-alkenoic acids. In addition, microbial discoloration upon fatty acid feeding is discussed and future directions for further genetic modification of *M. extorquens* are provided.

The Rationale for Engineering Methanol-Utilizing Bacteria

Methylotrophic bacteria offer obvious potential for transforming environmentally damaging substances (e.g., garbage) into useful materials. Garbage and low value biomasses can be converted into methane and methanol via a thermochemical route, and these two one-carbon and non-food substances become available thereafter as main substrates for methylotrophic microorganisms. Methane is one of the two most important greenhouse gases and has been suggested to have a larger global warming potential (GWP) than carbon dioxide,[1] whereas methanol, its alcohol derivative, is poisonous for many living organisms, including human beings, as it is rapidly metabolized to formic acid via methanal (formaldehyde), causing severe metabolic disturbances.[2] However, it is less their potential usefulness as environmental "cleaning agents" that makes methylotrophs attractive than their potential for utilizing inexpensive and, especially, non-food substrates as raw materials in industrial microbiology. The disadvantages associated with the conversion of food substrates (sugars) into biofuels have recently stimulated the search for alternative ways to obtain biofuels and various bioproducts (chemical feedstocks, bioplastics, other biomaterials, etc.). The preferred sources of methane and methanol would be those derived from non-food biomasses such as garbage, lignocellulosics and low value carbon streams including improper cereals and food wastes. Methane can be easily obtained from biomass via anaerobic digestion.[3] Additionally, the conversion process can be enhanced by recycling carbon dioxide.[4] These obvious advantages have resulted in the recent promotion of a "methanol economy" as an alternative route to deal with the inevitable future

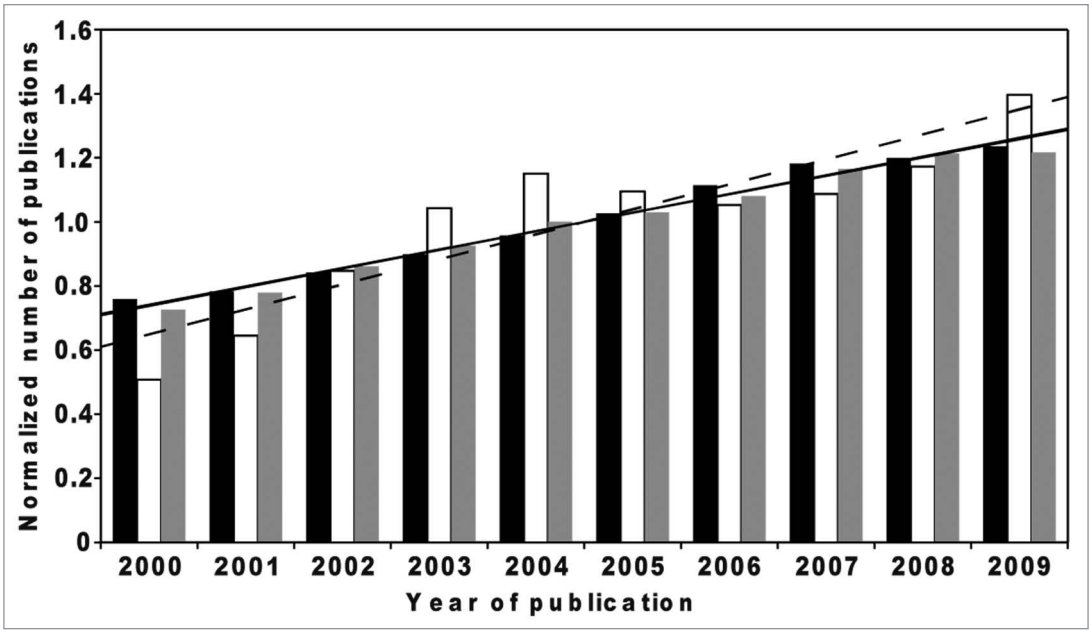

Figure 1. Increase in publications dealing with methanol or glucose (reference carbon substrate). The scientific publication database Scopus (Elsevier, Amsterdam, The Netherlands) was used to analyze documents that were published between 2000 and 2009 and stated methanol or glucose as one of their keywords. Publications from the following research areas were considered: Biochemistry, Genetics and Molecular Biology; Agricultural and Biological Sciences; Environmental Science; Multidisciplinary; Chemical Engineering. For each year, the respective publication number for methanol (white columns), glucose (gray columns) and the number of publications from the entire scientific community (black columns) was divided by the corresponding average number of the entire time period (2000–2009). Trend lines indicate the increase of publications over time. While increases of total publications and those dealing with glucose were nearly identical (overlapping solid lines), the number of documents referring to methanol as one of the keywords showed an above-average increase (dashed line).

shortage of fossil fuels.[5] The rising interest in methanol coincides with increasing activities in corresponding research areas (**Fig. 1**), suggesting that methanol will play an important role in the future, for instance, as a carbon substrate in microbial fermentations.

Starting from methanol, several amino acids, various proteins, the bioplastic polyhydroxybutyrate (PHB) and, very recently, chiral (R)-3-hydroxybutyric acid were successfully produced in methylotrophs.[6,7] Important advances have been made in understanding methylotrophic metabolism.[8] Research into metabolic activities of *Methylobacterium extorquens* regarding C1 compounds and, last but not least, the recent success in sequencing the genome of three *M. extorquens* strains have helped to understand how these microorganisms cope with methanol and what they are capable of producing from it.[9] To extend production capabilities, metabolic engineering approaches have been successfully performed and these have led to product diversification as elegantly reviewed in reference 6.

The Microbial and Enzymatic Technology group of Canada's Biotechnology Research Institute (NRC-BRI) has been carrying out fundamental but mostly applied research on methylotrophic microorganisms since 1988. The Group recognized early on that the World cannot rely only on sugars derived from essentially food sources to support the growth of modern industrial biotechnology. C1 substrates such as methanol and methane offer great potential as carbon sources since they can easily be obtained from various wastes and from low value agricultural streams, with the additional advantage of being non-food sources of carbon. To a large degree, methanol and methane are as advantageous as CO_2 as a carbon source for producing biofuels and various bioproducts. Most of the group's research has focused on the characterization and use of the proprietary *Methylobacterium extorquens* ATCC 55366 strain for producing experimental bioproducts such as PHB, pigments, functionalized PHAs and various recombinant proteins. The Group has also performed significant work on methylotrophic yeasts such as *Hansenula polymorpha* and *Pichia pastoris* and on methane-oxidizing bacteria, in this instance, mostly in an environmental context. The Group possesses also all of the necessary expertise and equipment to scale-up and optimize fermentation processes employing methanol-utilizing microorganisms up to the 1,500 L-scale together with primary recovery and extensive product purification expertise and equipment.

Genomic Potential of *Methylobacterium extorquens* to Function as a Microbial Bioplastic Factory of a Special Kind

We have chosen *M. extorquens* ATCC 55366 to produce second generation, i.e., functionalized biomaterials.[10,11] Selective gene screening gave us reason to believe that this methylotroph expresses the required enzymatic machinery to deal with medium-chain-length fatty acids (MCL-FAs) comprising C-C double bonds

Table 1. Abundance of genes in *M. extorquens* that potentially participate in β-oxidation-driven fatty acid metabolism toward PHA polymerization[A]

Function ID	Name[B]	Gene symbol[C]	Number of genes encoding corresponding enzyme[D]				
			***M ext* AM1**	***M ext* DM4**	***M ext* PA1**	***P put* KT2440**	***R eut* H16**
EC 6.2.1.3	Acyl-CoA synthetase	*fadD*	1	1	2	2	13
EC 1.3.99.3	Acyl-CoA dehydrogenase	*fadE*	1	1	1	2	51
EC 4.2.1.17	Enoyl-CoA hydratase	*fadB/fadJ*[E]	6	5	5	12	52
EC 5.1.2.3	3-hydroxyacyl-CoA epimerase	*fadB/fadJ*[E]	1	1	1	1	0
EC 1.1.1.35	3-hydroxyacyl-CoA dehydrogenase	*fadB/fadJ*[E]	1	1	1	3	10
EC 2.3.1.9	3-ketoacyl-CoA thiolase	*fadA*	2	2	2	7	22
EC 1.1.1.36	3-ketoacyl-CoA reductase	-	1	1	1	0	3
EC 4.2.1.119	Enoyl-CoA hydratase 2	-	0	0	0	0	0
-	PHA synthase	*phaC*	1 (class I)	2 (class I)	1 (class I)	2 (class II)	2 (class I)

M ext, *Methylobacterium extorquens*; *P put*, *Pseudomonas putida*; *R eut*, *Ralstonia eutropha*. [A]Data were obtained from The Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov/). [B]Commonly used name. [C]These gene symbols are commonly used when relating to β-oxidation-driven fatty acid metabolism toward 3-hydroxyacyl-CoA allocation for subsequent polymerization. They are not necessarily related to the function IDs listed above. [D]Including putative enzymes. [E]Putative fatty acid oxidation complex subunit alpha listed as *fadJ* in *M ext* AM1 and DM4 and *fadB* in *P put* KT2440.

that may be used to produce functionalized biopolyesters, potentially broadening its product repertoire to C4+ compounds. The Integrated Microbial Genomes (IMG) system of the DOE Joint Genome Institute (IGI) (www.jgi.doe.gov/) was used to screen three *M. extorquens* strains (AM1, DM4 and PA1) for their potential to allocate medium-chain-length 3-hydroxyacyl-CoA molecules that may be polymerized to polyhydroxyalkanoates (PHAs). The results were checked against two very prominent bioplastic producers, *Pseudomonas putida* KT2440 and *Ralstonia eutropha* H16, using IMG's genome comparison tools based on enzymatic function ID analysis (**Table 1**). While *R. eutropha* (recently renamed as *Cupriavidus necator*) produces the same type of short-chain-length PHAs (SCL-PHAs, $4 \leq C \leq 5$) that are also found in wild-type *M. extorquens*, pseudomonads process sugars and/or medium-chain-length fatty acids to corresponding medium-chain-length and long-chain-length PHAs (MCL-PHAs, $6 \leq C \leq 14$ and LCL-PHAs, $C > 14$). *R. eutropha* H16 and a genetically modified PHA-deficient mutant strain were reported to incorporate medium-chain-length 3-hydroxyalkanoates (MCL-3HAs) into PHAs,[12] suggesting that *R. eutropha* is also capable of channeling MCL-FAs toward PHA polymerization despite its preference for accumulating SCL-PHAs from sugar. **Table 1** shows that the three sequenced *M. extorquens* strains (AM1, DM4 and PA1) express all enzymes that participate in the conventional β-oxidation cycle associated with CoA-activated fatty acids. Moreover, two enzymes are present (3-hydroxyacyl-CoA epimerase and 3-ketoacyl-CoA reductase) that convert β-oxidation products into PHA precursors (3-hydroxyacyl-CoA) (for a scheme see ref. 13). The assumably minor expression level of the fatty acid degradation machinery, derived from the lower number of the corresponding genes in *M. extorquens*, did not concern us because we were seeking modest levels of MCL-3HA monomeric units within the sought after polyhydroxyalkanoates.

Tuning the Bacterium *Methylobacterium extorquens* for the Production of Functionalized Polyhydroxyalkanoates

Methylobacterium extorquens is a "natural-born" bioplastic factory. It was shown to produce high molecular weight polyhydroxybutyrate and poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) under nitrogen-limiting conditions.[14,15] While these SCL-PHAs can be produced at comparatively low cost, they have been criticized for their poor thermo-mechanical properties which limit their application spectra. The enzymes that polymerize 3-hydroxyacyl-CoA molecules to PHAs are called PHA synthases (or polymerases, PhaC). They are divided into four classes according to their primary structure, substrate specificity and subunit composition.[16] Class I PHA synthases, found in *M. extorquens* and *R. eutropha* (**Table 1**), incorporate SCL-3HAs into polyhydroxyalkanoates. *P. putida* KT2440 possesses two PhaC of type II, which polymerize MCL- and LCL-3-hydroxyacyl-CoA molecules ($C > 14$). Class III and IV PHA synthases have 3-hydroxyacyl-CoA specificities equal to class I PhaC; however, they are part of a dual enzyme complex. Although classified according to the length of their substrates, some PHA synthases show a rather broad substrate specificity, therefore, being able to incorporate SCL, MCL- and LCL-3HAs.

Since the PHA synthases of *M. extorquens* were grouped as type I PHA synthases, we expected that only genetic manipulation would enable our wild-type strain to yield biopolyesters other than SCL-PHAs. The genetic manipulations were performed by inserting a gene encoding for a broad substrate specificity PHA synthase into a recently developed, inducible and tightly regulated gene expression system.[17,18] As indicated above, we were seeking special polyhydroxyalkanoates that are modifiable and easily processable owing to the elongated side chains with terminal double bonds (**Fig. 2**). Such PHAs are called functionalized PHAs. Functionalized PHAs have gained much interest during the past two decades since they may qualify for unique applications and functions, particularly

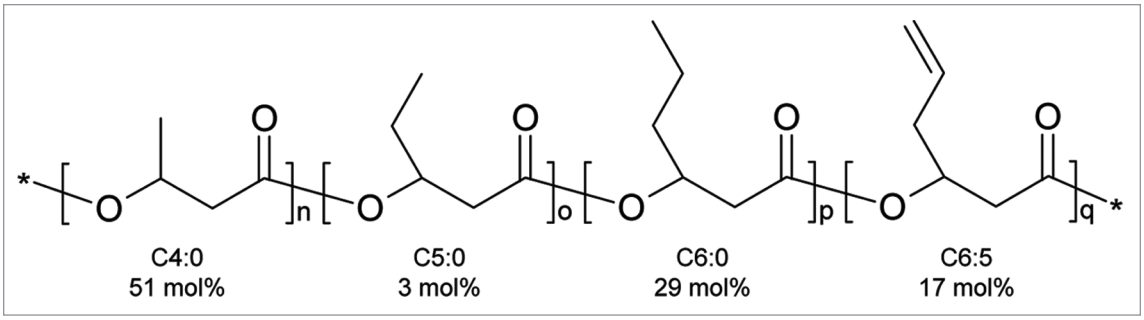

Figure 2. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate-co-3-hydroxyhex-5-enoate) after co-feeding of methanol and 5-hexenoic acid to *M. extorquens* (*M. ex-phaC2* strain) in a bioreactor. This functionalized terpolyester has distinct properties.[11] It is amenable, in theory, to (bio-) chemical modifications and exhibits desirable thermal properties similar to those found in corresponding (saturated) inert analogs. Moreover, it is biodegradable and believed to be biocompatible. The combination of these assets makes this copolymer an interesting candidate for applications in regenerative medicine. For example, it could become the hydrophobic core of a composite material that promotes revascularization. By keeping the medium-chain-length 3-hydroxyalkanoate content below 50 mol%, the melting temperature can be kept above 121°C at which temperatures materials are conveniently autoclaved. Examples for activating functionalized PHAs for some medical applications are reviewed in reference 19.

in the area of regenerative medicine.[19] To date, PHAs of higher value have been mostly obtained from recombinant *Escherichia coli*, *Cupriavidus necator* and wild-type Pseudomonas species, which grow on sugars and/or alkanoic acids. In addition to contributing to the establishment of a new cell factory for specialized bioplastics, we have learned how *M. extorquens* behaves in the presence of MCL-FAs. Knowing that *M. extorquens* is incapable of growing on multicarbon substrates (C > 4), research has focused so far on metabolic behavior and fluxes resulting from C1–C4 components.[8,20,21] In this article, we discuss the metabolic insights we have obtained from exposing wild-type and recombinant *M. extorquens* ATCC 55366 strains to some MCL alkenoic acids (C > 5).[10,11]

The Metabolism of Unsaturated n-Alkenoic Acids in *M. extorquens* Yields an Interesting Array of Intermediates

As expected, only our genetically modified *M. extorquens* ATCC 55366 strains harboring either the *phaC1* or *phaC2* gene of *Pseudomonas fluorescens* GK13 (*M. ex-phaC1* and *M. ex-phaC2*, respectively) were able to produce blends of short-chain-length PHAs (SCL-PHAs, 4 ≤ C ≤ 5) and short-chain-length/medium-chain-length PHAs (SCL/MCL-PHAs, 4 ≤ C ≤ 8) bearing terminal double bonds.[10] Co-feeding methanol + 5-hexenoic acid (C6=) or 7-octenoic acid (C8=) yielded PHA copolymers with less than 10 mol% of MCL monomeric units, which exhibited both saturated and unsaturated side chains. Strain *M. ex-phaC1* proved to be less efficient at incorporating functionality in the form of terminal C-C double bonds in the side chains resulting from the co-substrates. Furthermore, we observed that *M. extorquens* favored C6= and C8= over C11= and that the addition of C8= yielded unexpected C5 monomers within the PHA copolymer. This was surprising because it is commonly accepted that the chemical structure of the substrate is reflected in the monomeric composition of PHA copolymers when fatty acids are fed. In the situation found with our recombinant *M. extorquens* strains, the chemical structure of the fatty acids was only partially reflected in the monomeric composition of the PHAs obtained. Aside from 3-hydroxybutyrate (3HB) originating from methanol assimilation, within the resulting polymers we anticipated to find 3-hydroxyhex-5-enoate (3HHx=) derived from 5-hexenoic acid (C6=), and 3-hydroxyhex-5-enoate (3HHx=) and 3-hydroxyoct-7-enoate (3HO=) derived from 7-octenoic acid (C8=). A significant portion of the n-alkenoic acid derivatives (in some cases more than 50%) had lost their C-C double bonds prior to polymerization, leading to incorporation of both 3-hydroxyalkenoates and their saturated analogs into PHA (**Fig. 2**). Moreover, 3-hydroxyvalerate (3HV= 3-hydroxypentanoate, 3HP) was found in PHAs after feeding either 7-octenoic acid (shake flask experiments),[10] or 5-hexenoic acid (bioreactor studies).[11] This was unexpected because medium-chain-length fatty acids are supposed to undergo a C2 reduction during the β-oxidation cycle, i.e., thereby shortened molecules remain odd- or even-numbered depending on the molecule of origin.

The apparent hydration of C-C double bonds that resulted in a partial loss of functionality and the occurrence of C5 resulting from even-numbered co-substrates prompted us to have a closer look at the multi-carbon fatty acid metabolism in *M. extorquens* ATCC 55366.

Alternative β-Oxidation Pathways for Unsaturated Fatty Acids

Alternative pathways for the degradation of unsaturated long-chain-length fatty acids (LCL-FAs) are known for eukaryotes,[22,23] and for prokaryotes.[24] These pathways are briefly presented below (for a scheme see ref. 25).

Isomerase-Dependent β-Oxidation

When fatty acids possess C-C double bonds at odd-numbered positions, their coenzyme A-activated derivatives run through the β-oxidation cycle until the double bonds hinder further processing. During the final step of the β-oxidation cycle, 3-ketoacyl-CoA is cleaved between C2 and C3 by thiolysis, thereby, shifting the double bonds closer to the coenzyme A tail. Once double bonds reach position

C3, unsaturated acyl-CoA molecules are temporarily diverted from the β-oxidation cycle and isomerized to equivalents bearing a double bond at position C2 in the E (trans) configuration. The isomerization is catalyzed by a Δ^3,Δ^2-enoyl-CoA isomerase, from which this "classical" alternative pathway obtained its name. Resulting 2E-enoyl-CoA molecules subsequently become candidates to the β-oxidation cycle.

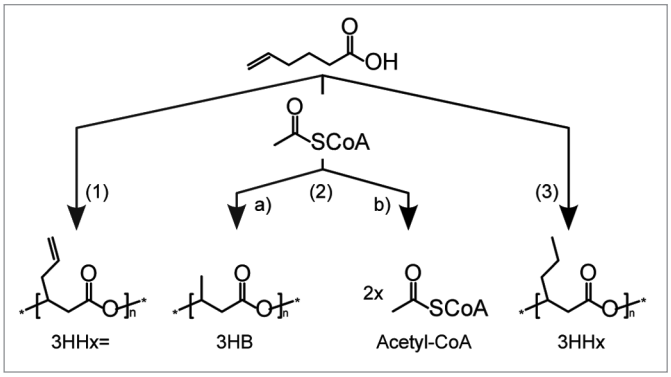

Figure 3. Possible routes for 5-hexenoic acid metabolism: (1) conventional β-oxidation and hydratization to 3-hydroxyhex-5-enoate (3HHx=); (2) thiolysis (resulting in 1x acetyl-CoA) followed by isomerization and (a) hydratization to 3-hydroxybutyrate (3HB) or (b) further β-oxidation cycle reactions to 2x acetyl-CoA; (3) alternative (possibly reductase-dependent) β-oxidation and hydratization to 3-hydroxyhexanote (3HHx) or, more likely, saturation of 5-hexenoyl-CoA followed by conventional β-oxidation and hydratization to 3HHx.

Reductase-Dependent β-Oxidation

Another pathway that seems to be reserved for mitochondrial fatty acid degradation in eukaryotes involves subsequent isomerase actions and NADPH-mediated double bond reduction.[25,26] During this reductase-dependent (also called NADPH-dependent) β-oxidation, 5Z-located double bonds [Z (cis) configuration at position C5] are translocated and finally hydrogenated, leaving β-oxidizable 2E-enoyl-CoA molecules behind. This pathway has been assumed to be present in *Escherichia coli* because 3E,5Z-dienoyl-CoA intermediates that are significant for this pathway were found to accumulate when cells were grown on oleic acid.[27] However, activity of a native $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase, one of the key enzymes in this pathway, was missing.

Question: *Does Methylobacterium extorquens* Possess a Eukaryotic Degradation Machinery for Unsaturated Fatty Acids?

None of the alternative prokaryotic β-oxidation pathways (for a scheme see refs. 24 and 27) seem to be applicable to the overall situation found in *M. extorquens*. Apart from dealing with MCL-FAs with terminal double bonds instead of LCL-FAs with double bonds in E or Z configuration, double bonds at position C5 are ultimately shifted to the C3 position due to thiolase activity between C2 and C3 (isomerase-dependent pathway). This step is necessary to provide the Δ^3, Δ^2-enoyl-CoA isomerase with an intermediate that can be channeled toward the β-oxidation cycle (2E-enoyl-CoA). C8= and C6= molecules would be shortened to 2E-butenoyl-CoA (C4) and, consequently, hydratized to 3-hydroxybutyrate or split into two molecules of acetyl-CoA (route 2 in **Fig. 3**). It is possible that some n-alkenoic acid molecules are metabolized following this route in *M. extorquens*; however, this does not explain the accumulation of saturated 3-hydroxyhexanote. In contrast, the reductase-dependent pathway could explain the apparent saturation of alkanoic PHA precursors, provided that a hypothetical $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase could deal with stereo-unspecific terminal C-C double bonds (**Fig. 4**). This hypothesis is contradicted by the fact that evidence for this pathway is missing in prokaryotes.[27] Indeed, genes encoding for enzymes of this metabolic route (Δ^3, Δ^2-enoyl-CoA isomerase, $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase and 2,4-dienoyl-CoA reductase) have not been reported for *Methylobacterium extorquens* (**Table 2**). Enoyl-CoA isomerase and/or dienoyl-CoA isomerase/reductase functions have been postulated for some prokaryotes (img.jgi.doe.gov/) but it is doubtful that our ATCC 55366 strain possesses all of the enzymes that are required for the reductase-dependent β-oxidation pathway. Nevertheless, a hint toward the NADPH-dependent β-oxidation hypothesis is that double bond elimination reactions seem to be dependent on a vinyl function at position C5. Otherwise, 3-hydroxyoctanoate monomers (originating from 3HO= with vinyl function at position C7) should have been observed to the same extent as 3-hydroxyhexanoate.

A more plausible explanation for the random vinyl hydrogenation, however, may be found in the action of an acyl-CoA desaturase whose gene may be expressed in some *M. extorquens* strains (**Table 2**). This enzyme is also called Δ9-desaturase or stearoyl-CoA 9-desaturase according to the catalyzed reaction, where stearoyl-CoA (C18) is desaturated at the 9th position from the carboxyl end in the presence of two molecules of ferrocytochrome b_5, oxygen and two protons. Interestingly, acyl-CoA desaturases that execute Δ9 and, specifically, Δ5 desaturation of saturated fatty acids were reported to be present in *Lyngbya majuscula*. While desaturation of CoA-activated hexanoic acid to 5-hexenoyl-CoA was found to occur, evidence for dehydrogenation of C8 to 7-octenoyl-CoA was missing.[28] As this desaturation reaction is reversible, it is speculated that the C-C double bond loss of C6= and, to a minor extent of C8=, in *M. extorquens* was performed by the action of an acyl-CoA desaturase, possibly driven by the need of protons for the recycling of NAD(P)H + H^+. **Table 2** summarizes the potential of *M. extorquens, P. putida* KT2440 and *R. eutropha* H16 for alternative alkenoic acid metabolism.

Our findings match results obtained from PHA production studies with *Pseudomonas oleovorans* where the fraction of saturated 3-hydroxyalkanoate

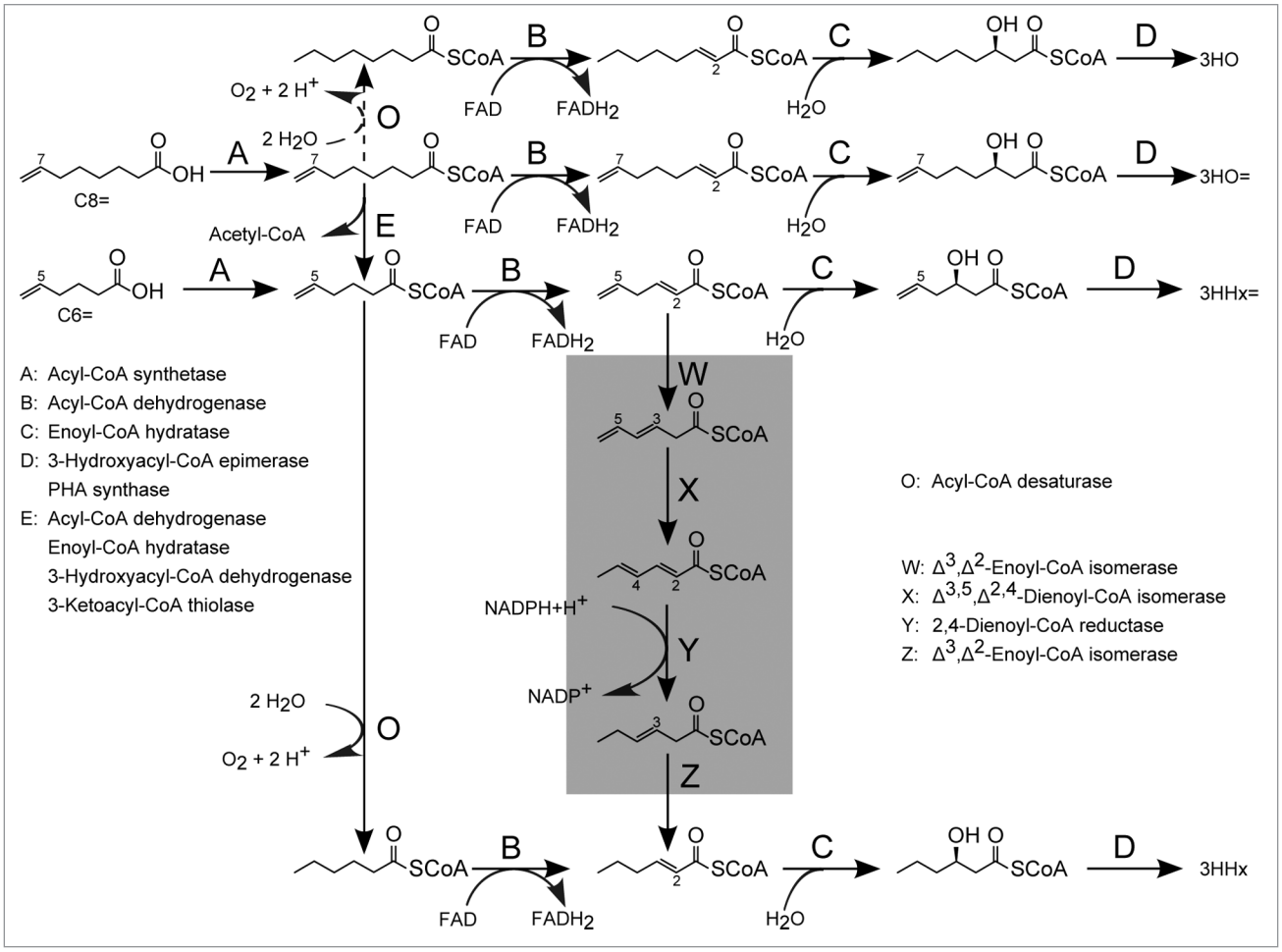

Figure 4. Hypothetical assimilation of 7-octenoic acid (C8=) and 5-hexenoic acid (C6=) to produce medium-chain-length 3-hydroxyalkanoate units in recombinant *M. extorquens*. The highlighted route depicts the reductase-dependent β-oxidation pathway that is found in eukaryotic mitochondria for the degradation of (poly-)unsaturated long-chain-length fatty acids. Genomic screening revealed, however, that the C-C double bond loss is more likely a result of the reverse action of an acyl-CoA desaturase that reduces double bonds preferably at position C5 prior to β-oxidation processing (step O). Conversions catalyzed during steps B, C and E are part of the conventional β-oxidation cycle. 2E-enoyl-CoA (product of conversion B) is most likely hydratized to S-3-hydroxyacyl-CoA and then converted into R-3-hydroxyacyl-CoA prior to PHA polymerization (step D).

units originating from even-numbered and unsaturated 7-octenoic acid was higher than 50 mol%.[29] In contrast, metabolism of 10-undecenoic acid yielded very little amounts of saturated analogs to odd-numbered 3-hydroxyalkenoate monomeric units, which exhibit an even-numbered position of the C-C double bond, thus, not offering equivalent vinyl function at position C5 upon repeated C2 chain reduction. Fermentations using the *M. ex-phaC2* strain and methanol + 5-hexenoic acid revealed that the yield of 3HHx= based on C6= was rather low.[11] The variety of pathways present in *M. extorquens* for dealing with this carboxylic acid may explain this observation (**Fig. 3**).

Increased β-Oxidation Activity May Stimulate the Production of 3-hydroxyvalerate and Suppress Amino Acid Biosynthesis

The incorporation of 3-hydroxyvalerate (C5) into PHA polymers usually requires the presence of odd-numbered precursors. *M. extorquens* produced PHBV when fed valeric acid in addition to methanol.[14] Co-feeding propanol or propionate (C3) was also shown to yield PHBV,[30] indicating the presence of a C2 chain elongation reaction. This chain elongation may be explained by the condensation of acetyl-CoA and propionyl-CoA to give 3-ketovaleryl-CoA via action of a β-ketothiolase,[31] which is an important enzyme shared between the PHB cycle and the glyoxylate-regenerating ethylmalonyl-CoA pathway that was recently postulated in *M. extorquens*.[32] It is rational to suggest that accumulation of acetyl-CoA molecules, acetyl-CoA being one of the final products of every β-oxidation cycle, leads to coupling of acetyl-CoA with propionyl-CoA provided by the ethylmalonyl-CoA pathway. The fact that 7-octenoic acid yields more acetyl-CoA than 5-hexenoic acid explains why 3HV was more abundant when the longer co-substrate was fed. This observation was made for both the recombinant and the wild-type *M. extorquens* strains. The formation of 3-ketovaleryl-CoA may also explain how fatty acid metabolism may contribute

Table 2. Enzymes that may directly and indirectly participate in reducing C-C double bonds in fatty acids derivatives at position C5[A]

Function ID	Name[B]	Number of genes encoding corresponding enzyme[C]				
		M ext AM1	*M ext* DM4	*M ext* PA1	*P put* KT2440	*R eut* H16
EC 1.14.19.1	Acyl-CoA desaturase	0	1	1	0	1
EC 5.3.3.8	Δ^3, Δ^2-Enoyl-CoA isomerase	0	0	0	1	0
EC 5.3.3.-	$\Delta^{3,5}$, $\Delta^{2,4}$-Dienoyl-CoA isomerase	0	0	0	0	0
EC 1.3.1.34	2,4-Dienoyl-CoA reductase	0	0	0	1	1

M ext, *Methylobacterium extorquens*; *P put*, *Pseudomonas putida*; *R eut*, *Ralstonia eutropha*. [A]Data were obtained from The Integrated Microbial Genomes (IMG) system (http://img.jgi.doe.gov/). [B]Commonly used name. [C]Including putative enzymes.

to hampering microbial growth since a negative relationship between 5-hexenoic acid supply and biomass formation was observed.[11] Under balanced growth conditions on C1 compounds, propionyl-CoA is metabolized to succinyl-CoA via methylmalonyl-CoA which subsequently enters the tricarboxylic acid cycle (TCA cycle). Succinyl-CoA is a direct precursor of succinate which is one of the central metabolites in *M. extorquens* (**Fig. 5**). Reduced TCA cycle activities, owing to decreased levels of succinyl-CoA, would ultimately lead to decreased amino acid formation originating from 2-ketoglutarate. This metabolic imbalance would, consequently, hinder protein biosynthesis which is indispensable for routine cell maintenance and reproduction.

PHBV was also produced when the *M. ex-phaC2* strain was cultivated solely on methanol.[11] Despite the absence of 7-octenoic or 5-hexenoic acid, small amounts of 3-hydroxyvalerate were incorporated into the polymeric chains. To the best of our knowledge, copolymer production based on methanol alone by wild-type *M. extorquens* has not been reported to date. It is suggested that the introduction of the pAll-*phaC2* plasmid triggered a metabolic imbalance, which may also explain elongated lag phases and reduced biomass formation compared to the situation with the wild-type strain. A reduction in growth rate for *M. extorquens* AM1 was recently linked to plasmid-induced cobalt limitation.[33]

Fatty Acid Supply Impacts on Microbial Pigmentation

M. extorquens produces pigments of a strong pink color. We observed an apparent coherence between the length of the n-alkenoic acid present in the growth medium and discoloration of *M. extorquens* in shake flask cultivations. While it is disputable whether carboxylic acids may impact on pigment biosynthesis pathways, extracellular events that result in the loss of pigmentation seem to be more plausible. The solubility of fatty acids in aqueous solutions depends on their length.[24] Accordingly, 10-undecenoic acid (C11=) was the least soluble among the n-alkenoic acids fed to *M. extorquens*. This was visually confirmed by two observations. First, shake flask cultures without manual pH adjustment appeared as oil-in-water emulsions. Second, centrifugation of such cultures led to the formation of a colorless precipitate floating on top of the supernatant fluid. We hypothesize that undissolved fatty acids extracted cell wall-associated carotenoids, which later became discolored due to unknown reactions occurring extracellularly. The absence of pigmentation did not influence the ability of *M. extorquens* to grow, ruling out that biomass levels may have been too low to see the pigmentation. It also confirms past observations to the effect that the pink pigmentation is not vital in this methylotrophic bacterium.[34] *M. extorquens* did not seem to have any use for 10-undecenoic acid; only trace amounts of 3HV were detected from cultivations using the wild-type strain, while the recombinant strains produced only PHB as PHA. Increasing C11= solubility by neutralization with KOH impacted negatively on biomass formation.

Transforming Observations on Cell Metabolism into Genetic Engineering Applications

Our observations with genetically engineered *M. extorquens* strains illuminated metabolic pathways that used to be neglected, arguably because multicarbon metabolism (C > 4) plays a minor role in methylotrophs. Nevertheless, *M. extorquens* possesses a metabolic machinery and the corresponding toolbox that is required for biosynthesis of fine materials beyond PHB and proteins. Certainly, *M. extorquens* as a future microbial bioplastic factory needs further genetic tuning to advance to the "first division" of functionalized PHA producers. The major challenge lies in replacing the two independently working PHA polymerases (synthases) with a single one. Abolishing the native PHA synthase (with its limited substrate specificity) sounds easier to achieve than it is because, in contrast to other facultative PHA accumulators, growth-associated biopolyester catabolism and deregulation is vital in the C1-driven metabolism of *M. extorquens* (**Fig. 5**).[35] Our first attempts to engineer a recombinant $phaC_{wild\text{-}type}$ knock-out mutant expressing the heterologous $phaC2_{P.\ fluorescens}$ gene failed due to poor growth on methanol.[10] The chemically induced recombinant PhaC synthase with a broad substrate specificity appeared to be unable to replace the function of the native PhaC synthase during growth on C1 compounds which is keeping the vital PHB cycle intact. This problem could be tackled by expressing a different PHA synthase with a higher activity or including more heterologous *phaC* copies in the expression vector. Both approaches should result in re-establishing the required flux of metabolites through the PHB cycle.

The second step will be to increase the yield of 3-hydroxyalkenoate monomeric units per mole of alkenoic acid fed. The key to this challenge lies in decoupling of β-oxidation events from C1 metabolism. Cracking the

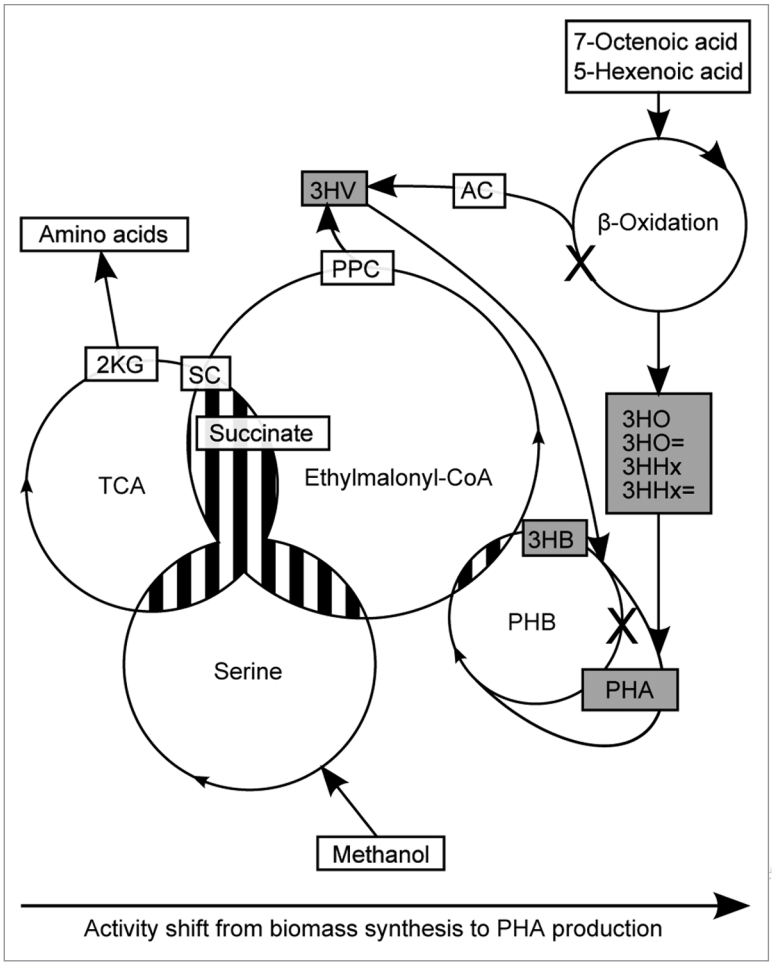

Figure 5. Extended metabolic network in recombinant *M. extorquens*. The central metabolism comprises four cycles (TCA, serine, ethylmalonyl-CoA and PHB) which are interconnected by shared enzymatic activities (the overlapping circle areas are coherent with the number of enzymes shared). C1 from methanol is incorporated into the serine cycle via methylenetetrahydrofolate. In wild-type *M. extorquens*, PHB is constantly produced and degraded through the PHB cycle. The recombinant strains contain a second PHA synthase with broader substrate specificity. Therefore, monomer precursors other than 3HB and 3HV, resulting from β-oxidation of fatty acids, may be used to form PHAs. In the presence of fatty acids, the β-oxidation cycle is assumed to be indirectly linked to the central metabolism by accumulating acetyl-CoA molecules (AC), which subsequently react with propionyl-CoA (PPC) to ultimately form 3-hydroxyvalerate. Thereby reduced propionyl-CoA availability may result in deceleration of the central metabolism, leading to decreased production of some amino acids that originate from 2-ketoglutarate in the TCA cycle. In conclusion, the incorporation of a second PHA synthase seems to induce a metabolic imbalance by shifting activities from biomass synthesis to PHA production. Further genetic engineering approaches to enhance the production of functionalized PHAs include (indicated by X in the flux scheme): knocking out the native PHA synthase to reduce the probability of producing polymer blends and interrupting the β-oxidation cycle to prevent accumulation or overproduction of acetyl-CoA.

conventional β-oxidation cycle would force all of the alkenoic acid derivatives to be channeled toward the production of PHAs (for an example see ref. 13). Furthermore, oversupply of acetyl-CoA would be prevented, which is believed to destabilize the metabolic network of *M. extorquens* (**Fig. 5**).

Finally, elucidation of alternative degradation pathways for unsaturated fatty acids is the third step. This would be less interesting from a material processing point of view as it is not necessary to have a vinyl function on every medium-chain-length 3-hydroxyalkanoate side chain to perform interesting modification reactions; however, it would surely be enlightening to explain the "mysterious" double bond hydrogenation that is reminiscent of the (poly-)unsaturated long-chain-length fatty acid handling observed in mitochondria.

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