Lipid and fatty acid metabolism in Ralstonia eutropha: relevance for the biotechnological production of value-added products

Citation: Riedel, Sebastian L., Jingnan Lu, Ulf Stahl, and Christopher J. Brigham. "Lipid and fatty acid metabolism in Ralstonia eutropha: relevance for the biotechnological production of value-added products." Applied Microbiology and Biotechnology 98:4 (February 2014), pp. 1469–1483.

As Published: http://dx.doi.org/10.1007/s00253-013-5430-8

Publisher: Springer Berlin Heidelberg

Persistent URL: http://hdl.handle.net/1721.1/103371

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Lipid and fatty acid metabolism in *Ralstonia eutropha*: relevance for the biotechnological production of value added products

Sebastian L. Riedel[1,2], Jingnan Lu[3], Ulf Stahl[1], and Christopher J. Brigham[4]*

[1]Department of Applied and Molecular Microbiology, Technische Universität Berlin, Seestr. 13, 13353 Berlin, Germany

[2]Fraunhofer Institute for Production Systems and Design Technology (IPK), Pascalstr. 8-9, 10587 Berlin, Germany

[3]Department of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 USA.

[4]Department of Bioengineering, University of Massachusetts Dartmouth, 285 Old Westport Road, North Dartmouth, MA 02747

*Corresponding author.
Tel: 1-508-999-9149, Fax: 1-508-999-9139, email: cbrigham@umassd.edu

Abstract

Lipid and fatty acid metabolism has been well studied in model microbial organisms like *Escherichia coli* and *Bacillus subtilis*. The major precursor of fatty acid biosynthesis is also the major product of fatty acid degradation (β-oxidation), acetyl-CoA, which is a key metabolite for all organisms. Controlling carbon flux to fatty acid biosynthesis and from β-oxidation allows for the biosynthesis of natural products of biotechnological importance. *Ralstonia eutropha* can utilize acetyl-CoA from fatty acid metabolism to produce intracellular polyhydroxyalkanoate (PHA). *R. eutropha* can also be engineered to utilize fatty acid metabolism intermediates to produce different PHA precursors. Metabolism of lipids and fatty acids can be rerouted to convert carbon into other value added compounds like biofuels. This review discusses the lipid and fatty acid metabolic pathways in *R. eutropha* and how they can be used to construct reagents for the biosynthesis of products of industrial importance. Specifically, how the use of lipids or fatty acids as the sole carbon source in *R. eutropha* cultures adds value to these biotechnological products will be discussed here.

Keywords: *Ralstonia eutropha*, lipid, fatty acid, metabolism, polyhydroxyalkanoate, biofuel

Introduction

Ralstonia eutropha, a Gram-negative betaproteobacterium, is an important organism in biotechnology due to its native ability to produce large quantities of intracellular polyhydroxyalkanoate (PHA) biopolymer. *R. eutropha* is often referred to as the model organism for PHA production and is well-studied in terms of biopolymer homeostasis. In nature, *R. eutropha* accumulates polyhydroxybutyrate (PHB), a type of PHA, up to 90% per cell dry weight (CDW), as a means of carbon and energy storage under stress conditions (Steinbüchel 1991). Because of the bacterium's genetic tractability, many researchers have engineered *R. eutropha* to produce different, and potentially more valuable and versatile, types of PHA (Kahar et al. 2004, Sudesh et al. 2011, Budde et al. 2011b).

Another characteristic that underpins the importance of *R. eutropha* in biotechnology is the bacterium's ability to utilize a multitude of carbon sources for growth and PHA biosynthesis. It has been documented in the scientific literature that sugars (Lutke-Eversloh et al. 2002, Brigham et al. 2012), amino sugars (Holder et al. 2011), carbon dioxide (Ishizaki et al. 2001, Volova et al. 2002, Cramm 2009), short-chain fatty acids (Yang et al. 2010), phenolic compounds (Nickzad et al. 2012), plant oils (Sudesh et al. 2011, Riedel et al. 2012), animal fats (Taniguchi et al. 2003), fatty acids (Brigham et al. 2010) and glycerol (Cavalheiro et al. 2009, Cavalheiro et al. 2012, Tanadchangsaeng and Yu 2012) act as sources of carbon for the production of biomass and of polymer. The use of fatty acids and lipids for microbial production of value-added products has gained popularity, because synthesis of products like PHA is observed to be more efficient when these carbon sources are used, due in part to the high carbon content per mol of these carbon sources (Kahar et al. 2004, Riedel et al. 2012, Tsuge et al. 2013). Also, using engineered strains, PHA incorporating longer chain length monomers (>C6) can be produced using lipids and fatty

acids as a carbon source. *R. eutropha* synthesizes fatty acids by the traditional fatty acid biosynthetic pathway, as discussed below. Examination of the genome sequence of the wild-type strain H16 (Pohlmann et al. 2006) shows that this organism contains all the genes necessary to synthesize fatty acids, starting from acetyl-CoA. *R. eutropha* also degrades extracellular lipids, like those found in plant oils or animal fats, first by action of a lipase that cleaves off the fatty acids from the glycerol backbone (Lu et al. 2013), and then the free fatty acids (FFA) are transported inside the cell for catabolism via the β-oxidation pathway.

Many literature reviews have focused on the PHA production process and the many applications of the polymer. This review will focus on the metabolic pathways necessary for *R. eutropha* to metabolize lipids and fatty acids and how these pathways may be utilized to produce value-added products. Specifically, we examine the metabolic pathway engineering of *R. eutropha* to utilize fatty acid metabolism intermediates for product biosynthesis.

Lipid and fatty acid metabolism in *R. eutropha*

Fatty acid beta-oxidation

In *R. eutropha, E. coli,* and most other Gram-negative bacteria that can utilize fatty acids as sole carbon and energy sources, these fatty acids are metabolized by the β-oxidation pathway. The β-oxidation pathway acts in a cyclic manner to reduce the fatty acyl chain by removing a two carbon acetyl-CoA group every “turn” of the cycle (Fujita et al. 2007). The β-oxidation pathway and the genes and enzymes involved therein are shown in Fig. 1. As fatty acid degradation produces acetyl-CoA, which is a precursor for many different cellular building blocks as well as compounds of potential biotechnological importance, the β-oxidation pathway is often utilized for the microbial biosynthesis of value-added products.

Genome sequencing of *R. eutropha* strain H16 has indicated the presence of several genes that could potentially be involved in specific steps of the process of fatty acid degradation (Pohlmann et al. 2006). More recently, whole genome transcriptome analysis has shown that two putative operons, both containing homologs of each of the β-oxidation pathway genes, are highly expressed when cells are grown using trioleate as the sole carbon source. Deletion of one of these two operons does not affect significantly the growth on lipids and fatty acids, suggesting that activity of gene products expressed from the intact operon compensates for the loss. When both β-oxidation operons are deleted, *R. eutropha* is unable to utilize lipids or fatty acids as carbon sources. Genes encoding FadB homologs, also known as enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase enzymes, are present in each β-oxidation operon (H16_A0461, termed *fadB'* and H16_A1526, termed *fadB1*). A *fadB'* knockout strain exhibited a slight decrease in cell dry mass and PHA production when grown on soybean oil as the sole carbon source. Deletion of both *fadB'* and *fadB1* resulted in a significant decrease of cell dry mass in soybean oil cultures (Insomphun et al. 2013), consistent with the results seen with the double β-oxidation operon deletion strain (Brigham et al. 2010). The main product of the β-oxidation pathway is acetyl-CoA, which is the main building block of PHB biosynthesis in wild-type *R. eutropha* (Peoples and Sinskey 1989a, Peoples and Sinskey 1989b). Propionyl-CoA, which is also a product from β-oxidation of odd chain fatty acids, is a building block for another PHA monomer, 3-hydroxyvaleryl-CoA (3HV-CoA), in wild-type *R. eutropha* (Anderson and Dawes 1990, Yang et al. 2010).

Fatty acid biosynthesis

Fatty acid biosynthesis occurs by condensation of acetyl-CoA to an acyl carrier protein (ACP) in the form of malonyl-ACP (Fig. 2A), and subsequent elongation by addition of two carbon groups

from acetyl-CoA (Fig. 2B) (Fujita et al. 2007). Fatty acid biosynthesis in *R. eutropha* has not been investigated thoroughly, if at all. The presence of fatty acid biosynthesis (*fab*) genes in the genome of *R. eutropha* H16 suggests that it synthesizes fatty acids for cell membrane similar to the *E. coli* pathway (Pohlmann et al. 2006, Fujita et al. 2007). It is unclear what moiety is required for elongation of the nascent fatty acyl-ACP chain during biosynthesis in *R. eutropha*. In *E. coli*, elongation proceeds by the addition of a two-carbon (C2) moiety from the decarboxylation of malonyl-CoA. The precursor for the fatty acid biosynthesis elongation step, malonyl-CoA, is synthesized by the acetyl-CoA carboxylase complex (AccABCD) (Fujita et al. 2007, Broussard et al. 2013). Homologues of *accABCD* genes are present on the genome of *R. eutropha* H16, suggesting that malonyl-CoA is the precursor molecule of fatty acyl-ACP elongation in *R. eutropha.* Expression levels of *accABCD* and *fab* genes in *R. eutropha* H16 were reported to be high during growth, consistent with fatty acid biosynthesis for membrane biogenesis, with a slight decrease in expression during PHB biosynthesis (Shimizu et al. 2013).

Links between *R. eutropha* fatty acid metabolism and other metabolic pathways

It is well known that acetyl-CoA, the main product of fatty acid β-oxidation, is used as a precursor molecule for other pathways, such as amino acid biosynthesis and the citric acid cycle (Fujita et al. 2007, Brigham et al. 2010, Brigham et al. 2012). In *R. eutropha,* intermediates and products of fatty acid metabolism are linked to other key metabolic pathways. Furthermore, both wild-type strains and, to a greater extent recombinant strains of *R. eutropha* can produce PHA precursors, *(R)*-hydroxyacyl-CoA molecules, from fatty acid biosynthesis or β-oxidation (Matsumoto et al. 2001, Mifune et al. 2008, Budde et al. 2011b). To obtain PHA precursors from β-oxidation intermediates, the *phaJ* gene, encoding an *(R)*-specific enoyl coenzyme-A hydratase that catalyzes the conversion of ketoacyl-CoA to the general PHA precursor *(R)*-3-hydroxyacyl-

CoA (Fig. 3), is expressed in *R. eutropha*. To obtain precursors from fatty acid biosynthesis, the *phaG* gene, encoding a 3-hydroxyacyl-ACP: CoA transferase (Fig. 3) can be expressed. The origins of both the *phaJ* and *phaG* genes used in genetic engineering of this type are typically *Pseudomonas* species (Hoffmann et al. 2000, Matsumoto et al. 2001, Davis et al. 2008, Budde et al. 2011b, Sato et al. 2011). However, native *phaJ* genes have been identified and characterized in wild-type *R. eutropha*. Overexpression of either one of these two genes, *phaJ4a* and *phaJ4b*, enhanced 3-hydroxyhexanoate (3HHx) incorporation in PHA synthesized by *R. eutropha* strains grown on soybean oil as the sole carbon source (Kawashima et al. 2012).

In a previous study, chemical inhibition of β-oxidation by acrylic acid in *R. eutropha* H16 was used to produce poly(hydroxybutyrate-*co*-hydroxyhexanoate) [P(HB-*co*-HHx)] copolymers when sodium octanoate was used as the main carbon source. Acrylic acid is an inhibitor of the β-ketothiolase enzyme, FadA, resulting in the diversion of β-oxidation intermediates to PHA biosynthesis (Qi et al. 1998, Antonio et al. 2000, Green et al. 2002). A maximum 3HHx monomer content of 5.7 mol% was observed in PHA produced from *R. eutropha* H16 cultures supplemented with 29.3 mM acrylic acid. In general, this work demonstrated that the greater the concentration of β-oxidation inhibitor used with wild type *R. eutropha* cultures, the less PHA was produced. Using an *R. eutropha* strain expressing a heterologous PHA synthase from *P. aeruginosa*, PHA containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) monomers was produced using sodium decanoate as a carbon source in cultures supplemented with <1 mM acrylic acid, although total PHA accumulated per CDW was low (<5%) (Green et al. 2002). Deletion of the *fadB1* gene has also been shown to alter the monomer composition of PHA in engineered *R. eutropha* strains grown on soybean oil. PHA accumulated in all *fadB1* knockout strains was shown to contain up to 1.4 mol% more 3HHx monomer than the parental

strains. An increase in the mol% of 3HHx monomer was also seen in PHA from *fadB'* gene knockout strains, but this was due primarily to the decrease of mol% of 3HB monomer (Insomphun et al. 2013). These results suggest that the FadB gene products encoded in the different β-oxidation operons in *R. eutropha* play different roles in fatty acid metabolism.

Recently, another link between β-oxidation and PHA homeostasis has been uncovered. Gene expression studies suggested that β-oxidation genes were upregulated during PHB production in wild-type *R. eutropha* grown on fructose, perhaps suggesting a role for this pathway in turnover of acyl-CoA to produce PHB precursors (Shimizu et al. 2013). Also, previous studies shown that a PHA depolymerase enzyme, PhaZ1, in *R. eutropha* H16 performed thiolysis on PHB polymer chains, resulting in the formation of 3-hydroxybutyryl-CoA (3HB-CoA) (Uchino et al. 2007, Uchino et al. 2008). However, in a recent study of *in vitro* PHB degradation by PhaZ1 in the presence of the phasin protein, PhaP1, a second degradation product, crotonyl-CoA, was observed in larger quantities than 3HB-CoA. Crotonyl-CoA is a specific intermediate that is formed from 3HB-CoA by an enoyl-CoA hydratase enzyme in the β-oxidation pathway. In PHB depolymerization, the presence of PhaZ1 and PhaP1 were shown to be necessary to produce crotonyl-CoA (Eggers and Steinbüchel 2013).

In this same study, it was also revealed that the 3HB-CoA that resulted from PHB degradation was mostly of the *(S)* form, and not the *(R)* form that is the precursor for PHB biosynthesis. The 3-hydroxyacyl-CoA intermediates produced by β-oxidation are of the *(S)* form (Fujita et al. 2007, Eggers and Steinbüchel 2013), suggesting a potential link between PHB depolymerization and the fatty acid degradation pathway. It is suggested that the main product of PHB depolymerization in wild-type *R. eutropha* is crotonyl-CoA, which then feeds into the β-oxidation pathway and further metabolized to acetyl-CoA (Fig. 3). The mechanism for

conversion of thiolyzed 3HB-CoA to crotonyl-CoA by PhaZ1 is as of yet unknown, but it is hypothesized that the initial thiolysis product is (*R*)-3HB-CoA, which is not released into the cytoplasm, but converted directly to crotonyl-CoA (Eggers and Steinbüchel 2013).

Ralstonia lipases

Importance of lipase

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) account for 21% of the world's enzyme industry and contribute to an estimated annual sale of US$1.5 billion. Although lipases are ubiquitous among animals, plants, and microorganisms, the industrial lipase biocatalysts primarily come from microbial sources. Many reactions can be catalyzed by lipases owing to their multifaceted chemo-, regio-, and enantioselectivities. Lipases play crucial roles in lipid metabolism by catalyzing the hydrolysis of triacylglycerol (TAG) into diacylglycerol (DAG), monoglycerol (MAG), glycerol, and FFA at the interface of lipid and water. The TAG molecules, together with their cleavage products, form an emulsion within the aqueous media and therefore become bioavailable for cell growth (Lu et al. 2013).

Lipase Gene Organization, Gene Knockout and Overexpression Studies

An extracellular lipase (LipA, locus tag H16_A1322) and its chaperone (LipB, locus tag H16_A1323) were identified and characterized from *R. eutropha* strain H16 (Brigham et al. 2010, Lu et al. 2013). The *lipB* gene is positioned directly downstream of *lipA* on chromosome 1, separated by 56 bps. A transcription initiation site was identified only in the sequence proceeding *lipA* that is potentially shared by *lipB*. Gene expression studies reveal *lipA* and *lipB* are both significantly upregulated in the presence of TAGs (Brigham et al. 2010), further suggesting their co-regulation in an operon.

R. eutropha mutant strains with a Δ*lipA* or Δ*lipB* single deletion mutation completely eradicated the strains' ability to hydrolyze and utilize TAGs from palm oil. Since no extracellular lipase activity was detected in the supernatant of these single deletion strains, LipA was determined to be the only extracellular lipase present from *R. eutropha* H16. Importantly, *lipA* overexpression under a constitutively expressed promoter not only resulted in more secreted LipA enzyme and subsequent TAG hydrolysis, but also shortened the culture lag phase to 5 h as compared to the wild-type (12 h), through faster emulsification of lipids during shake flask experiments (Lu et al. 2013).

Biochemical Studies

LipA belongs to the I.2 subfamily of lipases and shares 48% and 44% sequence identity with lipases from *Ralstonia* sp. M1 and *Pseudomonas* sp., respectively. LipB, on the other hand, has ~45% common sequence with chaperones in the same subfamily. LipA (369 amino acids) contains a putative *N*-terminal signal sequence of 48 residues and is approximately 40 kDa when matured. Various metal-binding residues are present in LipA, even though lipases, including LipA, do not require a metal cofactor. Divalent Ca^{2+}, Mg^{2+}, and Mn^{2+} ions were found to alleviate LipA inhibition caused by chelators. The role of these ions is highly debatable, ranging from association with free fatty acid during product release, lipase structure stabilization, or activation of water during catalysis.

Ser121, Asp292, and His336 make up the active-site catalytic triad of LipA. The catalytic triad of both Ser-Asp-His and Ser-Glu-His appears in this order and is conserved in all serine hydrolases. Although there is no three-dimensional structure available on LipA, multiple X-ray structures of lipases released to date all reveal the presence of a characteristic α/β-hydrolase fold

with distinctive parallel β strands core surrounded by α helices. Although the catalytic triad residues are distant from each other in the primary structure, they reside in close proximity in the folded lipase. Two cysteine residues present in LipA could contribute to the tertiary fold by a disulfide bridge. LipA is highly dynamic and its activity can be greatly increased when encountered with micellar aggregates or emulsion droplets formed by lipid carbon sources, detergents (Triton X-100, SDS), or emulsifiers (Tween 60) (Lu et al. 2013).

Utilization of *R. eutropha* lipid and fatty acid metabolism for synthesis of value-added products

Polyhydroxyalkanoate biosynthesis

As mentioned above, wild-type *R. eutropha* can produce PHB when grown on a wide range of carbon sources. During PHB accumulation, acetyl-CoA produced from fatty acid β-oxidation is channeled primarily into production of PHA via the native biosynthetic pathway (Brigham et al. 2010), and large quantities of intracellular polymer are produced. However, copolymers consisting of short chain length (*scl*, 3-5 carbons) and medium chain length (*mcl*, ≥6 carbons) monomers exhibit thermal and mechanical properties that are closer to those of petroleum based plastics, when compared to PHB homopolymer. These PHA copolymers also exhibit several enhanced properties, such as flexibility and ease of processing, compared to polymers that consist of only *mcl* monomers (Noda et al. 2005b). The *mcl* monomer concentration in a copolymer thus plays an important role, affecting several parameters, *e.g.* melting temperature and crystallinity (Noda et al. 2005a). For example, P(HB-*co*-HHx) co-polymers, containing the *scl* monomer 3-hydroxybutyrate (3HB) and >12 mol% of the *mcl* monomer 3HHx, have been shown to have similar properties as low density polyethylene (Doi et al. 1995).

Engineering *R. eutropha* to produce P(HB-*co*-HHx) copolymer

For *R. eutropha* to produce P(HB-*co*-HHx) from intermediates of fatty acid metabolism, a strain must be engineered containing specific heterologous genes. As the native PHA synthase (PhaC) in *R. eutropha* is specific only for *scl* monomers, it is typically replaced with a PHA synthase gene whose product exhibits broader substrate specificity, with the ability to polymerize both *scl* and *mcl* monomers. For *mcl* monomer (*e.g.*, 3HHx) supply, the cell will typically syphon intermediates from fatty acid metabolism. The concentration of 3HHx-CoA precursor molecules can be increased in *R. eutropha* production strains by expressing one of two genes, *phaJ* or *phaG*, the roles of which are discussed above.

The 3HHx level of the intracellular P(HB-*co*-HHx) produced is affected by the genes present in the *R. eutropha* strain of choice, the type of carbon source used, and the cultivation time. Two recombinant strains, Re2058/pCB113 and Re2160/pCB113 (see genotypes in Table 1), both demonstrated high polymer accumulation per CDW (64-73%) and high 3HHx monomer content (up to 30 mol%) in the resulting PHA when these strains were grown on palm oil as the sole carbon source. These strains contained a broad-specificity PHA synthase, isolated from *Rhodococcus aetheriovarans*, and the *phaJ* gene from *Pseudomonas aeruginosa*, resulting in the increase of intracellular *(R)* 3HHx-CoA concentrations for PHA biosynthesis. In both strains, these genes are present on the PHA production plasmid, pCB113. The orientation of these genes on plasmid pCB113 was based on the native *R. eutropha* PHA biosynthesis operon (Table 1). However, the integration of the PHA operon from pCB113 into the chromosome of Re2058 resulted in significantly less PHA accumulation per CDW (only ~40% PHA per CDW), as compared to Re2058/pCB113 (Budde et al. 2011b). It is likely that a higher gene dosage,

resulting from the location of the operon on the multi-copy plasmid, probably leads to a higher enzyme activity, which therefore allows for a higher accumulation of PHA per CDW.

The classic *R. eutropha* PHB pathway (Fig. 4) was inactivated in strain Re2160 through deletions of the acetoacetyl-CoA reductase genes *phaB1-3*, whose products convert acetoacetyl-CoA to 3HB-CoA, the precursor molecule of the 3HB monomer. Re2160/pCB113 was only able to synthesize 3HB-CoA from the partially metabolized β-oxidation intermediates and therefore produced PHA containing a very high 3HHx content, >50% more 3HHx than is produced in PHA from Re2058/pCB113, where all *phaB* genes are still present on the chromosome (Table 1). However, Re2058/pCB113 had a higher PHA accumulation per CDW, which is likely because the strain still utilizes acetyl-CoA as a precursor for 3HB monomer biosynthesis. This could also provide an explanation for the lower 3HHx monomer concentration in the PHA of Re2058/pCB113 when the strain is grown on lipids. In both strains, decreases in the 3HHx content of the polymer were observed over time in plant oil cultures. Very high levels of 3HHx monomer (40-50 mol%) were measured in intracellular PHA at early time points, but those levels eventually decrease to lower final percentages (around 20-30 mol%). The greatest decrease in the 3HHx concentration occurs at the beginning until the mid-point of the cultivation, but the 3HHx content continues to decline until the end of the cultivation (Riedel et al. 2012).

PhaJ will also generate 3HB-CoA, as well as 3HHx-CoA, from β-oxidation intermediates. However, logically if PHA precursors are being produced from β-oxidation intermediates by the action of a PhaJ enzyme, 3HHx-CoA generation will occur one “turn of the cycle” earlier than the 3HB-CoA generation step. Acetyl-CoA competes between cell growth (TCA-cycle) and 3HB-CoA production in Re2058/pCB113. When cells stop growing, an increasing concentration of acetyl-CoA can be directed into the 3HB-CoA pool. It is notable that the strains accumulate

PHA from the beginning and also reach high accumulation levels (up to 60% PHA per CDW) before nitrogen limitation occurs (Riedel et al. 2012), probably through the overexpression of the PHA production genes.

A previous study reports the expression of a heterologous *phaG* gene for the production of P(HB-*co*-HHx) in *R. eutropha* grown on sugars as carbon substrates. The *phaG* and *phaC* genes, encoding a 3-hydroxyacyl ACP:CoA transferase and PHA synthase, respectively, originated from *Pseudomonas* sp. 61-3. With PhaG shunting intermediates from fatty acid biosynthesis for PHA production, an overall *mcl* 3HA monomer content of 3-5 mol% was observed when cells were grown on fructose or gluconate. The *mcl* monomers present ranged from 3HHx (<1.0 mol%) to 3-hydroxydodecanoate (3HDD, ≤1.1 mol%) (Matsumoto et al. 2001). PHA accumulation per CDW could be increased from 26% per CDW to 44 % per CDW by inserting *phaA* and *phaB* from *R. eutropha* (Table 1). It is unclear if other polymer variations can be obtained with *R. eutropha* strains expressing *phaG* using different carbon sources, as this remains to be tested at this writing. One potential reason for the low amounts of *mcl* 3HA monomers in the resulting PHA from the aforementioned study is that the fatty acid biosynthesis pathway, from which PhaG produces PHA precursors, is down-regulated under polymer biosynthesis conditions (Shimizu et al. 2013). Future work could examine PHA produced from strains overexpressing *phaG* and fatty acid biosynthesis genes.

Fatty acids can be defined by carbon chain length into four categories; short-chain fatty acids (SCFA, <6 carbons in chain length), also known as volatile fatty acids (VFA); medium-chain fatty acids (MCFA, 6-12 carbons); long-chain fatty acids (LCFA, 13-21 carbons) and very-long-chain fatty acids (VLCFA, >22 carbons). Using MCFAs as a carbon source, instead of plant oils that contain only LCFAs, allows for more integration of 3HHx monomers into the PHA polymer

produced by recombinant *R. eutropha* strains (Mifune et al. 2008, Mifune et al. 2010, Budde et al. 2011b). This phenomenon was also observed by using crude palm kernel oil (CPKO), which contains large amounts of MCFA, in *R. eutropha* cultures. A maximum of one 3HHx-CoA molecule can be generated from each fatty acid during β-oxidation. Overall, when starting from shorter fatty acids during β-oxidation, fewer acetyl-CoA molecules will be produced, which will decrease the potential intracellular ratio between 3HB-CoA and 3HHx-CoA molecules. Also, the number of cycles and therefore time required for formation of 3HHx-CoA molecules is decreased, with decreasing fatty acid chain length, since breakdown of shorter fatty acids results in fewer rounds of β-oxidation, before a 3HHx-CoA molecule is formed. For example, Re2058/pCB113 produces PHA containing around 20 mol% 3HHx in fermentations with palm oil (containing only LCFA, with the shortest fatty acid C14:0 at around 1%). However, the same strain produced around 30 mol% 3HHx with CPKO (containing a high concentration of MCFA: C12:0, 48%; C10:0, 3%; C8:0, 3%) as the sole carbon source (Riedel et al. unpublished data).

Polyhydroxyalkanoate production from plant oils.

Plant oils have been shown to be excellent carbon sources for PHA production using *R. eutropha* as the biocatalyst. Efficient P(HB-*co*-HHx) accumulation using recombinant *R. eutropha* strains, between 72-87% per CDW, from various plant oils (soybean oil, olive oil, corn oil, jatropha oil, palm oil, and palm kernel oil) was shown by several groups (Fukui and Doi 1998, Tsuge et al. 2004, Loo et al. 2005, Mifune et al. 2008, Ng et al. 2010). The PHA synthases used in these studies are enzymes with broad substrate specificity that can incorporate both *scl* and *mcl* monomers into the final polymer. However, the 3HHx concentration of the stored polymer was low, only 2-5 mol% from these experiments. Mifune et al., (2010) were able to produce this copolymer containing a higher 3HHx level (up to 9.9 mol%) together with a high PHA content

of 79% per CDW from soybean oil, through insertion of a *phaJ* from *Aeromonas caviae* into the PHA biosynthesis operon (MF03, Table 1). Higher 3HHx concentrations could only be reached by feeding MCFA, although this would be undesired due to the cost of these carbon substrates (Table 1). These authors were also able to produce copolymers with high 3HHx levels of up to 26 mol% from soybean oil using a strain containing a *phaB1* deletion (NSDGΔB, Table 1), but at the cost of overall PHA accumulation (max 26% per CDW). It is interesting to compare the PHA accumulation behaviors of the Δ*phaB1* strains during cultivation on octanoate (representing MCFA) or soybean oil (representing LCFA). These strains produce PHA with higher 3HHx content when grown on soybean oil compared to octanoate, but accumulate much less PHA per CDW (Mifune et al. 2010). The 3HHx integration in PHA from this work is the opposite of expected results, based on studies discussed above. In another study, strain Re2160/pCB113, produced P(HB-*co*-HHx) containing extremely high 3HHx levels (up to 62 mol%) in the resulting copolymer when cells were grown on the plant oils mentioned above and additionally with coconut oil, CPO and palm olein as sole carbon source (Wong et al. 2012).

Besides efficient PHA accumulation per CDW as described above, a high total PHA production per liter is desired for an economical PHA production process. Kahar et al., (2004) produced 95.5 g/L P(HB-*co*-HHx) from soybean oil under phosphate limitation (Table 2). The fermentation was initiated with 20 g/L soybean oil and 0.4% ammonium chloride. Soybean oil and ammonium chloride were fed in pulses, shortly before limitation back to initial concentrations. The culture medium included the antibiotic kanamycin to stably maintain the plasmid containing the gene encoding a PHA synthase ($phaC_{Ac}$) with broad substrate specificity from *A. caviae*. High cell density fermentations, using palm oil as the sole carbon source for P(HB-*co*-HHx) biosynthesis, were performed by Riedel at al. (2012). Up to 140 g/L CDW with a

PHA content of 73%, containing 19 mol% 3HHx, was produced in these fermentations. This equals a total PHA production of >100 g/L, and the data suggest that the fermentation process is scalable with a space time yield (STY) greater than 1 g PHA/L/h (Table 2). The molecular weight of PHA produced in these fermentations decreased from 500,000 Da to 300,000 Da over the course of cultivation. The polydispersity index (PDI) increased slightly from 1.9 to 2.1, indicating narrow molecular weight distributions. The decrease of the molecular weight of the PHA could be a result of the metabolism of glycerol, which gets released during TAG utilization in the oil culture (Tsuge et al. 2013). Glycerol functions as a chain transfer agent during PHA polymerization, resulting in polymer with lower molecular weight when glycerol is present in the fermentation broth (Madden et al. 1999). The genes *phaC2*$_{\mathrm{Ra}}$ and *phaJ*$_{Pa}$ were both present on a plasmid in the strain Re2058/pCB113. It is worth noting that the plasmid addiction system of this strain (Budde et al. 2011b) was robust in these high cell density fermentations (Riedel et al. 2012). Therefore an addition of kanamycin to the media for plasmid maintenance was not necessary, which would reduce production costs in an industrial process. Sato et al., (2013) also recently constructed a plasmid for P(HB-*co*-HHx) production, which is stable during high cell density fermentations without the addition of any antibiotic (Table 2).

Using a lipid recovery method from Budde et al. (2011a), Riedel et al (2012) showed the lipid consumption profile and fatty acid distribution of residual lipids over the course of high cell density fed batch palm oil fermentations. The data suggest that TAGs get cleaved first, followed by DAGs and MAGs. The proportions of the residual fatty acids stayed constant during feeding period, suggesting that the different FFAs cleaved from TAGs are consumed at an almost equal rate. However, after the palm oil feeding was stopped, the proportions of some fatty acids in the residual lipids changed. These data suggest that *R. eutropha* prefers unsaturated C18 fatty acids

as accumulation of an increasing proportion of stearic acid (C18:0; 5 fold) was observed in the media, along with respective decreasing concentrations of oleic (C18:1, 1.3 fold) and linoleic acids (C18:2, 4 fold).

R. eutropha PHA production from lipid waste streams

Lipid waste streams have also been a focus for PHA production with *R. eutropha,* because of their low price and their availability in relatively large quantities. Efficient PHB accumulation per CDW using waste frying oil as the sole carbon source in flask cultures of wild-type *R. eutropha* was shown by both Taniguchi et al., (2003) (waste sesame oil, 63% PHB per CDW) and Obruca et al., (2013) (waste rapeseed oil, 62% PHB per CDW). Using random chemical mutagenesis on the wild-type strain, a mutant (strain E01) was isolated that was able to produce 87.9% PHB per CDW from waste rapeseed oil. High production of P(HB-*co*-HV) from waste rapeseed oil, using propanol as the 3HV precursor, was shown by Obruca et al., (2010). A final copolymer concentration of 105 g/L with a 3HV content of 8 mol% was produced. The authors demonstrated a yield of 0.83 g PHA/g oil (Table 2). A P(HB-*co*-HV) content of 80% per CDW, with a low 3HV level (1 mol%), was obtained in flask cultures of wild-type *R. eutropha* when grown using tallow as the sole carbon source (Taniguchi et al. 2003). Tallow has a high melting temperature (40-45°C) and was solid under the cultivation conditions, but it was fully degraded and consumed during cultivation.

The melting temperature of a fat is dependent on the chain lengths of the fatty acids and the portion of unsaturated fatty acids in the individual TAG molecules (*i.e.*, longer chain lengths and fewer double bonds in the requisite fatty acids increases melting temperature). Fats with a higher melting temperature are harder to emulsify for use in *R. eutropha* cultures. This can increase the

lag phase or even lead to a lack of cell growth, depending on the type of cultivation method (shaking flask culture or fermentation).

PHA from short-chain fatty acids

SCFA, or VFA, are an auspicious carbon feedstock for PHA production, since they are inexpensive and widely available in large quantities. VFA are produced via microbial acidogenesis from organic waste streams *e.g.* from anaerobically treated palm oil mill effluent (POME) (Yee et al. 2003, Mumtaz et al. 2008), sludge (Elefsiniotis and Oldham 1993) or food scraps (Digman and Kim 2008). VFA, individually or in mixtures, are suitable as carbon sources for *R. eutropha*. PHA accumulation has been demonstrated, using strain H16, from acetic acid (PHB, (Wang and Yu 2000)), propionic acid (P(HB-*co*-HV), (Kobayashi et al. 2000)), butyric acid (PHB, (Kawaguchi and Doi 1992, Grousseau et al. 2013) and valeric acid (P(HB-*co*-HV), (Khanna and Srivastava 2007, Lindenkamp et al. 2013), or from mixed VFA cultures of acetic, propionic and butyric acid (PHB, P(HB-*co*-HV), (Yu et al. 2002, Yang et al. 2010). Hassan et al. (2002) also showed the direct use of treated POME, which contains three of the abovementioned (acetate, propionate, and butyrate) VFA in a ratio of 3:1:1, respectively (Yee et al. 2003). Lactic acid, which can also be produced during microbial acidogenesis (Zhao et al. 2006) or in large quantities from renewable carbon sources (Datta and Henry 2006), can be used for PHB production (Tsuge et al. 1999). Lindenkamp et al. (2012) was able to produce P(HB-*co*-HV), with an extremely high HV content of 99 mol%, but with low PHA accumulation per CDW (25%), using a mutant *R. eutropha* strain that was missing 9 out of 15 β-ketothiolase gene homologues with valerate as the sole carbon source. The high 3HV levels indicated a reduced 3HB-CoA pool in the mutant strain during PHA accumulation (Lindenkamp et al. 2012).

VFA and lactic acid are inhibitory or toxic for bacterial cell growth in large quantities, depending on culture pH and acid concentration of the feedstock, also because undissociated lipophilic molecules attack the cell membranes, resulting in cell morphology and growth defects (Salmond et al. 1984, Lawford and Rousseau 1993, Roe et al. 1998). These toxic effects occur at very low concentrations of VFA in *R. eutropha* cultures (*e.g.* Wang and Yu 2000). It has been demonstrated that an initial concentration of only >0.3% acetic acid results in significant growth inhibition, and with an initial concentration of up to 0.6%, no cell growth was observed. In order to reach high cell densities using VFA as a carbon source, a sensitive feeding strategy that keeps VFA concentrations at low levels in the culture media is necessary for an effective PHA production process. Cell densities between 64-103 g/L using strain H16 with final PHA contents of 58-73% per CDW have been reached with a pH-controlled two-stage feeding strategy (Tsuge et al. 1999, Tsuge et al. 2001, Kobayashi et al. 2005) (Table 2). The VFA were mixed with ammonium hydroxide solution and potassium phosphate and fed over the base reservoir by keeping the pH at initial values. In this way, VFAs were kept at the low concentrations of ~0.3% (w/v). In the first stage of the culture, VFA was fed in a nitrogen-rich C/N ratio of 10 for the first 12-24 h. In the second stage, the nitrogen content in the feeding solution was decreased, thus increasing the C/N ratio up to 50 for greatest PHA production. The highest PHA accumulation per CDW was reached when the feeding solution was changed during the PHA production phase, where the residual cell dry weight [RCDW = CDW (g/L) – PHA (g/L)] is constant (Table 2). Besides nitrogen, none of these fermentations were knowingly limited for other nutrients.

Biofuels from β-oxidation intermediates and PHA

In recombinant *R. eutropha* strains, short chain alcohol biofuels like *n*-butanol can be produced from PHA precursors like acetoacetyl-CoA and 3-hydroxybutyryl-CoA (Bond-Watts et al. 2011,

Lu et al. 2012), both of which can be produced from intermediates and products of fatty acid metabolism. Recently, a study has been undertaken to produce longer chain fuel molecules using engineered *R. eutropha* strains (Müller et al. 2013). Some organisms produce intracellular lipids that are analogous to PHA in *R. eutropha* (Hillen et al. 1982, Holder et al. 2011), for use as a carbon and energy source during times of carbon starvation. These intracellular lipids can be converted into long chain, energy dense biofuel molecules. Intracellular concentrations of lipids and fatty acids in *R. eutropha* are very low, presumably in part because production of FFA would syphon carbon from the preferred method of carbon storage, PHA. Expression of a truncated thioesterase gene in β-oxidation deficient *R. eutropha* allows the cells to accumulate significant quantities of FFA. An increase of FFA production was observed in β-oxidation deficient and PHB biosynthesis deficient *R. eutropha* strains. This re-routing of carbon metabolism also produced methyl ketones, another important class of biofuel molecules. Expression of a methyl ketone biosynthetic pathway from *Micrococcus luteus* increased production of methyl ketones in *R. eutropha* grown on fructose as the main carbon source. Methyl ketone production was also demonstrated in autotrophic cultures in this study (Müller et al. 2013).

PHA itself can be converted to biofuel by acid-catalyzed hydrolysis to produce hydroxyalkanoate methyl esters or hydroxyalkanoate ethyl esters. Blends of these esters with conventional gasoline were shown to be reasonable fuels, but with lower heats of combustion than unblended gasoline. These PHA-based biofuels do offer a better solution to the “fuel v. food” controversy as compared to corn-based ethanol (Chen 2009, Gao et al. 2011).

Outlook

Outlook for PHA production using VFA, plant oils and waste fats as the sole carbon source

For a successful PHA production using *R. eutropha* as a biocatalyst, the interplay of several factors is needed. First, a strain is needed that accumulates the desired PHA polymer in high levels per CDW (>70%) without the addition of any antibiotics in the culture. Second, a widely available, inexpensive carbon feedstock is needed. Third, feeding strategies that allow for the accumulation of both high cell densities and high levels PHA per CDW in the shortest possible time window are necessary for a maximum space time yield (productivity) during fed batch fermentation.

Efficient laboratory production of *scl* and *mcl* PHA copolymers during fed batch fermentations have been shown with wild type and recombinant strains of *R. eutropha*, respectively, using (waste) plant oils, as sole carbon source, which were liquid at room temperature (Table 2). The next step would be to increase copolymer production to industrial scale. Furthermore, the adaptation of feeding strategies to other TAG-based inexpensive waste streams, such as industrial waste animal rendering fats, is desirable. These fats are more challenging to use in a fed batch fermentation scenario than the oils described above, since most animal fats are solid at room temperature. Using mixtures of VFA, experimentally proven models have been shown to diversify the *scl* monomer composition of P(HB-*co*-HV) (Yu et al. 2002, Yang et al. 2010). The idea is to alternate the composition of the VFA mixture to create tailor-made polymers with desired properties. However, these studies have only been performed in low-density shake flask cultures, which are not suitable for production. These encouraging models need to be expanded and adapted to fed batch fermentations to realize high productivity (*e.g.*, how fixed ratios over the course of the fermentation, changing ratios during different stages of growth and PHA production, and/or alternating concentrations of the feed stream have an influence over polymer composition).

Also, there is still potential to be reached in total PHA production with the discussed fed batch fermentations (Table 2). The type of PHA monomer and the particular concentration of each monomer have great influence on the polymer properties. Recent metabolome studies by Fukui et al., (Fukui et al. 2013) using *R. eutropha* H16 have indicated the presence of greater intracellular 3HB-CoA and 3HHx-CoA pools during PHB production phase when octanoate (representing fatty acid β-oxidation) was used, instead of fructose as the sole carbon source. Varying pool concentrations of PHA precursor molecules could have an influence of the PHA monomer composition (Lindenkamp et al. 2012).

Outlook for *R. eutropha* LipA

LipA has many desired characteristics for utilization in the biotechnology and biomedical industries. Being a secreted extracellular lipase, LipA can be easily overexpressed and isolated. It can be immobilized for high efficiency, low contamination, and continuous usage in large-scale commercial processes. Due to its wide substrate specificity and ability to completely hydrolyze TAG into glycerol and FFA, it can be utilized to quantify the released glycerol or FFA in biosensors and medical diagnostic tools. Its non-specific hydrolysis property also makes LipA a desirable candidate for the production of biodiesel from residual soy, rapeseed, and palm refinery waste oils. LipA from *R. eutropha* exhibits optimum activity in the range of pH 7-8 at 50°C, which could be ideal for the formulation of detergents for stain removal. LipA could also contribute to the food industry in applications ranging from hydrolysis of the lipid membrane of tea shoots for processing black tea to the interesterification of low-cost palm oil into expensive cocoa butter-type TAGs.

Outlook for biofuels produced by *R. eutropha*

Since *R. eutropha* is able to utilize a versatile array of carbon sources and is able to store large quantities of carbon, it can be exploited in biotechnology applications, including the production of biofuels. Since biofuels should be cost-effective to compete with petroleum and also produced in large quantities, the study of *R. eutropha* as a biofuel producer is still in its infancy. Methods of optimizing the carbon flow from feedstock to product must be examined using complex metabolic engineering strategies. Since *R. eutropha* in its natural state makes PHA, the task of optimizing polymer biosynthesis for biotechnology applications coincides with regulation of gene and metabolic pathway expression that is native to the organism. However, since *R. eutropha* does not produce biofuel compounds like alcohols and methyl ketones naturally, metabolic "rewiring" manipulations must be performed at both the pathway and regulatory levels in order to produce abundant biofuel to construct a competitive biosynthetic process.

Fatty acid metabolism in *R. eutropha* can provide the intermediates and products needed to feed into an engineered, de novo metabolic pathway for biofuels. As discussed above, inactivation of β-oxidation provides a platform for the production of long chain carbon compounds. However, one can envision the engineering of the *R. eutropha* native fatty acid biosynthesis pathway to produce fuel molecules. A biosynthesis of this type would likely be tied to nutrient starvation, similar to PHA biosynthesis. The first step in designing a reagent that can produce biofuels from fatty acid biosynthesis pathway intermediates is to alter the expression of pathway genes, since they are down regulated upon entering nitrogen starvation (Shimizu et al. 2013). Further study of the fatty acid biosynthesis pathway in *R. eutropha* is required for this undertaking.

Acknowledgements

The authors would like to thank Professors Anthony J. Sinskey and ChoKyun Rha for their guidance and support. We thank Prof. A. Steinbüchel and the editors of *Applied Microbiology and Biotechnology* for giving us the opportunity to publish this review. We thank Mr. John W. Quimby for review of the manuscript and helpful comments and suggestions.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54: 450-72

Antonio RV, Steinbüchel A, Rehm BH (2000) Analysis of in vivo substrate specificity of the PHA synthase from *Ralstonia eutropha*: formation of novel copolyesters in recombinant *Escherichia coli*. FEMS Microbiol Lett 182: 111-117

Bond-Watts BB, Bellerose RJ, Chang MC (2011) Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. Nat Chem Biol 7: 222-227

Brigham CJ, Budde CF, Holder JW, Zeng Q, Mahan AE, Rha C, Sinskey AJ (2010) Elucidation of beta-oxidation pathways in *Ralstonia eutropha* H16 by examination of global gene expression. J Bacteriol 192: 5454-5464

Brigham CJ, Speth DR, Rha C, Sinskey AJ (2012) Whole genome microarray and gene deletion studies reveal regulation of the polyhydroxyalkanoate production cycle by the stringent response in *Ralstonia eutropha* H16. Appl Environ Microbiol 78: 8033-8044

Broussard TC, Price AE, Laborde SM, Waldrop GL (2013) Complex Formation and Regulation of *Escherichia coli* Acetyl-CoA Carboxylase. Biochemistry 52: 3346-3357

Budde CF, Riedel SL, Hübner F, Risch S, Popovic MK, Rha C, Sinskey AJ (2011a) Growth and polyhydroxybutyrate production by *Ralstonia eutropha* in emulsified plant oil medium. Appl Microbiol Biotechnol 89: 1611-1619

Budde CF, Riedel SL, Willis LB, Rha C, Sinskey AJ (2011b) Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from plant oil by engineered *Ralstonia eutropha* strains. Appl Environ Microbiol 77: 2847-2854

Cavalheiro JM, de Almeida MC, Grandfils C, da Fonseca MM (2009) Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. Process Biochem 44: 509-515

Cavalheiro JM, Raposo RS, de Almeida MC, Cesario MT, Sevrin C, Grandfils C, da Fonseca MM (2012) Effect of cultivation parameters on the production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) and poly(3-hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) by *Cupriavidus necator* using waste glycerol. Bioresour Technol 111: 391-397

Chen GQ (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev 38: 2434-2446

Cramm R (2009) Genomic view of energy metabolism in *Ralstonia eutropha* H16. J Mol Microbiol Biotechnol 16: 38-52

Datta R, Henry M (2006) Lactic acid: recent advances in products, processes and technologies — a review. Chem Technol Biotechnol 81: 1119-1129

Davis R, Anilkumar PK, Chandrashekar A, Shamala TR (2008) Biosynthesis of polyhydroxyalkanoates co-polymer in *E. coli* using genes from *Pseudomonas* and *Bacillus*. Antonie Van Leeuwenhoek 94: 207-216

de la Cruz Pradella, J.G., Ienczak JL, Delgado CR, Taciro MK (2012) Carbon source pulsed feeding to attain high yield and high productivity in poly(3-hydroxybutyrate) (PHB) production from soybean oil using *Cupriavidus necator*. Biotechnol Lett 34: 1003-1007

Digman B, Kim DS (2008) Review: Alternative energy from food processing wastes. Environmental Prog 27: 524-525-537

Doi Y, Kitamura S, Abe H (1995) Microbial Synthesis and Characterization of Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules 28: 4822

Eggers J, Steinbüchel A (2013) Poly(3-Hydroxybutyrate) Degradation in *Ralstonia eutropha* H16 Is Mediated Stereoselectively to (*S*)-3-Hydroxybutyryl Coenzyme A (CoA) via Crotonyl-CoA. J Bacteriol 195: 3213-3223

Eggink G, van der Waal H, Huijberts GNM, de Waard P (1993) Oleic acid as a substrate for poly-3-hydroxyalkanoate formation in *Alcaligenes eutrophus* and *Pseudomonas putida*. Ind Crops Prod 1: 157-163

Elefsiniotis P, Oldham WK (1993) Influence of pH on the acid-phase anaerobic digestion of primary sludge. J Chem Technol Biotechnol 60: 89-96

Fujita Y, Matsuoka H, Hirooka K (2007) Regulation of fatty acid metabolism in bacteria. Mol Microbiol 66: 829-839

Fukui T, Doi Y (1998) Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain. Appl Microbiol Biotechnol 49: 333-336

Fukui T, Chou K, Harada K, Orita I, Nakayama Y, Bamba T, Nakamura S, Fukusaki E (2013) Metabolite profiles of polyhydroxyalkanoate-producing *Ralstonia eutropha* H16. Metabolomics DOI: 10.1007/s11306-013-0567-0

Gao X, Chen JC, Wu Q, Chen GQ (2011) Polyhydroxyalkanoates as a source of chemicals, polymers, and biofuels. Curr Opin Biotechnol 22: 1-7

Green PR, Kemper J, Schechtman L, Guo L, Satkowski M, Fiedler S, Steinbüchel A, Rehm BH (2002) Formation of short chain length/medium chain length polyhydroxyalkanoate copolymers by fatty acid beta-oxidation inhibited *Ralstonia eutropha*. Biomacromolecules 3: 208-213

Grousseau E, Blanchet E, Deleris S, Albuquerque MG, Paul E, Uribelarrea JL (2013) Impact of sustaining a controlled residual growth on polyhydroxybutyrate yield and production kinetics in *Cupriavidus necator*. Bioresour Technol DOI: 10.1016/j.biortech.2013.08.120

Hillen LW, Pollard G, Wake LV, White N (1982) Hydrocracking of the oils of *Botryococcus braunii* to transport fuels. Biotechnol Bioeng 24: 193-205

Hoffmann N, Steinbüchel A, Rehm BH (2000) Homologous functional expression of cryptic *phaG* from *Pseudomonas oleovorans* establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. Appl Microbiol Biotechnol 54: 665-70

Holder JW, Ulrich JC, DeBono AC, Godfrey PA, Desjardins CA, Zucker J, Zeng Q, Leach AL, Ghiviriga I, Dancel C, Abeel T, Gevers D, Kodira CD, Desany B, Affourtit JP, Birren BW,

Sinskey AJ (2011) Comparative and functional genomics of *Rhodococcus opacus* PD630 for biofuels development. PLoS Genet 7: e1002219

Insomphun C, Mifune J, Orita I, Numata K, Nakamura S, Fukui T (2013) Modification of beta-oxidation pathway in *Ralstonia eutropha* for production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from soybean oil. J Biosci Bioeng DOI: 10.1016/j.jbiosc.2013.07.016

Ishizaki A, Tanaka K, Taga N (2001) Microbial production of poly-D-3-hydroxybutyrate from CO_2. Appl Microbiol Biotechnol 57: 6-12

Jenkins TC, Wallace RJ, Moate PJ, Mosley EE (2008) Board-invited review: Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J Anim Sci 86: 397-412

Kahar P, Tsuge T, Taguchi K, Doi Y (2004) High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. Polym Degrad Stab 83: 79-86

Kawaguchi Y, Doi Y (1992) Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. Macromolecules 25: 2324-2329

Kawashima Y, Cheng W, Mifune J, Orita I, Nakamura S, Fukui T (2012) Characterization and functional analyses of *R*-specific enoyl coenzyme A hydratases in polyhydroxyalkanoate-producing *Ralstonia eutropha*. Appl Environ Microbiol 78: 493-502

Khanna S, Srivastava AK (2007) Production of poly(3-hydroxybutyric-*co*-3-hydroxyvaleric acid) having a high hydroxyvalerate content with valeric acid feeding. J Ind Microbiol Biotechnol 34: 457-461

Kobayashi G, Tanaka K, Itoh H, Tsuge T, Sonomoto K, Ishizaki A (2000) Fermentative production of P(3HB-*co*-3HV) from propionic acid by *Alcaligenes eutrophus* in fed-batch culture with pH-stat continuous substrate feeding method. Biotechnol Lett 22: 1067-1069

Kobayashi T, Uchino K, Abe T, Yamazaki Y, Saito T (2005) Novel intracellular 3-hydroxybutyrate-oligomer hydrolase in *Wautersia eutropha* H16. J Bacteriol 187: 5129-5135

Lawford HG, Rousseau JD (1993) Effects of pH and acetic acid on glucose and xylose metabolism by a genetically engineered ethanologenic *Escherichia coli*. Appl Biochem Biotechnol 39-40: 301-322

Lee IY, Kim MK, Chang HN, Park YH (1996) Regulation of poly betahydroxybutyrate biosynthesis by nicotinamide nucleotide in *Alcaligenes eutrophus.*. FEMS Microbiol Lett 131: 35-39

Lindenkamp N, Volodina E, Steinbüchel A (2012) Genetically modified strains of *Ralstonia eutropha* H16 with beta-ketothiolase gene deletions for production of copolyesters with defined 3-hydroxyvaleric acid contents. Appl Environ Microbiol 78: 5375-5383

Loo CY, Lee WH, Tsuge T, Doi Y, Sudesh K (2005) Biosynthesis and characterization of poly(3-hydroxybutyrate-*co*-3- hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. Biotechnol Lett 27: 1405-10

Lu J, Brigham CJ, Gai CS, Sinskey AJ (2012) Studies on the production of branched-chain alcohols in engineered *Ralstonia eutropha*. Appl Microbiol Biotechnol 96: 283-97

Lu J, Brigham CJ, Rha C, Sinskey AJ (2013) Characterization of an extracellular lipase and its chaperone from *Ralstonia eutropha* H16. Appl Microbiol Biotechnol 97: 2443-2454

Lutke-Eversloh T, Fischer A, Remminghorst U, Kawada J, Marchessault RH, Bogershausen A, Kalwei M, Eckert H, Reichelt R, Liu SJ, Steinbüchel A (2002) Biosynthesis of novel thermoplastic polythioesters by engineered *Escherichia coli*. Nat Mater 1: 236-240

Madden LA, Anderson AJ, Shah DT, Asrar J (1999) Chain termination in polyhydroxyalkanoate synthesis: involvement of exogenous hydroxy-compounds as chain transfer agents. Int J Biol Macromol 25: 43-53

Matsumoto K, Nakae S, Taguchi K, Matsusaki H, Seki M, Doi Y (2001) Biosynthesis of Poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoates) Copolymer from Sugars by Recombinant *Ralstonia eutropha* Harboring the $phaC1_{Ps}$ and the $phaG_{Ps}$ Genes of *Pseudomonas* sp. 61-3. Biomacromolecules 2: 934-939

Mifune J, Nakamura S, Fukui T (2008) Targeted engineering of *Cupriavidus necator* chromosome for biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from vegetable oil. Can J Chem 86: 621-627

Mifune J, Nakamura S, Fukui T (2010) Engineering of *pha* operon on *Cupriavidus necator* chromosome for efficient biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from vegetable oil. Polym Degrad Stab 95: 1305-1312

Müller J, Maceachran D, Burd H, Sathitsuksanoh N, Bi C, Yeh YC, Lee TS, Hillson NJ, Chhabra SR, Singer SW, Beller HR (2013) Engineering of *Ralstonia eutropha* H16 for Autotrophic and Heterotrophic Production of Methyl Ketones. Appl Environ Microbiol 79: 4433-4439

Mumtaz T, Abd-Aziz S, Rahman NA, Yee PL, Shirai Y, Hassan MA (2008) Pilot-scale recovery of low molecular weight organic acids from anaerobically treated palm oil mill effluent (POME) with energy integrated system. Afr J Biotechnol 7: 3900-3905

Ng KS, Ooi WY, Goh LK, Shenbagarathai R, Sudesh K (2010) Evaluation of jatropha oil to produce poly(3-hydroxybutyrate) by *Cupriavidus necator* H16. Polym Degrad Stab 95: 1365-1369

Nickzad A, Mogharei A, Monazzami A, Jamshidian H, Vahabzadeh F (2012) Biodegradation of phenol by *Ralstonia eutropha* in a Kissiris-immobilized cell bioreactor. Water Environ Res 84: 626-634

Noda I, Bond EB, Green PR, Narasimhan K, Schechtman LA (2005a) Preparation, properties, and utilization of biobased biodegradable nodax copolymers. Polym Biocatal Biomater 900: 280

Noda I, Green PR, Satkowski MM, Schechtman LA (2005b) Preparation and properties of a novel class of polyhydroxyalkanoate copolymers. Biomacromolecules 6: 580-586

Obruca S, Marova I, Snajdar O, Mravcova L, Svoboda Z (2010) Production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Cupriavidus necator* from waste rapeseed oil using propanol as a precursor of 3-hydroxyvalerate. Biotechnol Lett 32: 1925-1932

Obruca S, Snajdar O, Svoboda Z, Marova I (2013) Application of random mutagenesis to enhance the production of polyhydroxyalkanoates by *Cupriavidus necator* H16 on waste frying oil. World J Microbiol Biotechnol DOI 10.1007/s11274-013-1410-5

Peoples OP, Sinskey AJ (1989a) Poly-beta-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J Biol Chem 264: 15298-15303

Peoples OP, Sinskey AJ (1989b) Poly-beta-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding beta-ketothiolase and acetoacetyl-CoA reductase. J Biol Chem 264: 15293-15297

Pohlmann A, Fricke WF, Reinecke F, Kusian B, Liesegang H, Cramm R, Eitinger T, Ewering C, Pötter M, Schwartz E, Strittmatter A, Voss I, Gottschalk G, Steinbüchel A, Friedrich B, Bowien B (2006) Genome sequence of the bioplastic-producing "Knallgas" bacterium *Ralstonia eutropha* H16. Nat Biotechnol 24: 1257-1262

Qi Q, Steinbüchel A, Rehm BH (1998) Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid beta-oxidation by acrylic acid. FEMS Microbiol Lett 167: 89-94

Riedel SL, Bader J, Brigham CJ, Budde CF, Yusof ZA, Rha C, Sinskey AJ (2012) Production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by *Ralstonia eutropha* in high cell density palm oil fermentations. Biotechnol Bioeng 109: 74-83

Roe AJ, McLaggan D, Davidson I, O'Byrne C, Booth IR (1998) Perturbation of anion balance during inhibition of growth of *Escherichia coli* by weak acids. J Bacteriol 180: 767-772

Salmond CV, Kroll RG, Booth IR (1984) The effect of food preservatives on pH homeostasis in *Escherichia coli*. J Gen Microbiol 130: 2845-2850

Sato S, Kanazawa H, Tsuge T (2011) Expression and characterization of (*R*)-specific enoyl coenzyme A hydratases making a channeling route to polyhydroxyalkanoate biosynthesis in *Pseudomonas putida*. Appl Microbiol Biotechnol 90: 951-959

Sato S, Fujiki T, Matsumoto K (2013) Construction of a stable plasmid vector for industrial production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) by a recombinant *Cupriavidus necator* H16 strain. J Biosci Bioeng DOI: /10.1016/j.jbiosc.2013.05.026

Schlegel HG, Lafferty R, Krauss I (1970) The isolation of mutants not accumulating poly-beta-hydroxybutyric acid. Arch Mikrobiol 71: 283-294

Shimizu R, Chou K, Orita I, Suzuki Y, Nakamura S, Fukui T (2013) Detection of phase-dependent transcriptomic changes and Rubisco-mediated CO_2 fixation into poly (3-hydroxybutyrate) under heterotrophic condition in *Ralstonia eutropha* H16 based on RNA-seq and gene deletion analyses. BMC Microbiol 13: 169

Steinbüchel A (1991) Polyhydroxyalkanoic Acids. In: Byrom D (ed) *Biomaterials*. MacMillan, London, pp 123-213

Sudesh K, Bhubalan K, Chuah JA, Kek YK, Kamilah H, Sridewi N, Lee YF (2011) Synthesis of polyhydroxyalkanoate from palm oil and some new applications. Appl Microbiol Biotechnol 89: 1373-1386

Tanadchangsaeng N, Yu J (2012) Microbial synthesis of polyhydroxybutyrate from glycerol: gluconeogenesis, molecular weight and material properties of biopolyester. Biotechnol Bioeng 109: 2808-2818

Taniguchi I, Kagotani K, Kimura Y (2003) Microbial production of poly(hydroxyalkanoate)s from waste edible oils. Green Chem 5: 545-546-548

Tsuge T, Tanaka K, Ishizaki A (2001) Development of a novel method for feeding a mixture of L-lactic acid and acetic acid in fed-batch culture of *Ralstonia eutropha* for poly-D-3-hydroxybutyrate production. J Biosci Bioeng 91: 545-550

Tsuge T, Tanaka K, Shimoda M, Ishizaki A (1999) Optimization of L-lactic acid feeding for the production of poly-D-3-hydroxybutyric acid by *Alcaligenes eutrophus* in fed-batch culture. J Biosci Bioeng 88: 404-409

Tsuge T, Ko T, Masako T, Abe H (2013) Effect of Glycerol and its Analogs on Polyhydroxyalkanoate Biosynthesis by Recombinant *Ralstonia eutropha*: A Quantitative Structure-Activity Relationship Study of Chain Transfer Agents. Polym Degrad Stab DOI: 10.1016/j.polymdegradstab.2013.06.026

Tsuge T, Saito Y, Kikkawa Y, Hiraishi T, Doi Y (2004) Biosynthesis and compositional regulation of poly[(3-hydroxybutyrate)-*co*-(3-hydroxyhexanoate)] in recombinant *Ralstonia eutropha* expressing mutated polyhydroxyalkanoate synthase genes. Macromol Biosci 4: 238-242

Uchino K, Saito T, Gebauer B, Jendrossek D (2007) Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. J Bacteriol 189: 8250-8256

Uchino K, Saito T, Jendrossek D (2008) Poly(3-hydroxybutyrate) (PHB) depolymerase PhaZa1 is involved in mobilization of accumulated PHB in *Ralstonia eutropha* H16. Appl Environ Microbiol 74: 1058-1063

Volova TG, Kalacheva GS, Altukhova OV (2002) Autotrophic synthesis of polyhydroxyalkanoates by the bacteria *Ralstonia eutropha* in the presence of carbon monoxide. Appl Microbiol Biotechnol 58: 675-678

Wang J, Yu J (2000) Kinetic analysis on inhibited growth and poly(3-hydroxybutyrate) formation of *Alcaligenes eutrophus* on acetate under nutrient-rich conditions. Process Biochem 36: 201-207

Wong YM, Brigham CJ, Rha C, Sinskey AJ, Sudesh K (2012) Biosynthesis and characterization of polyhydroxyalkanoate containing high 3-hydroxyhexanoate monomer fraction from crude palm kernel oil by recombinant *Cupriavidus necator*. Bioresour Technol 121: 320-327

Yang YH, Brigham CJ, Budde CF, Boccazzi P, Willis LB, Hassan MA, Yusof ZA, Rha C, Sinskey AJ (2010) Optimization of growth media components for polyhydroxyalkanoate (PHA) production from organic acids by *Ralstonia eutropha*. Appl Microbiol Biotechnol 87: 2037-2045

Yee PL, Hassan MA, Shirai Y, Wakisaka M, Karim MIA (2003) Continuous Production of Organic Acids from Palm Oil Mill Effluent with Sludge Recycle by the Freezing-Thawing Method. J Chem Eng Jpn 36: 707-710

Yu J, Si Y, Wong WKR (2002) Kinetics modeling of inhibition and utilization of mixed volatile fatty acids in the formation of polyhydroxyalkanoates by *Ralstonia eutropha*. Process Biochem 37: 731-738

Zhao JH, Zhang B, Cai WM (2006) Influence of temperature on hydrolysis and acidogenesis of kitchen wastes in two-phase anaerobic digestion. Huan Jing Ke Xue 27: 1682-1686

Figure Legends

Fig.1: The fatty acid β-oxidation cycle catabolizes acyl-CoA molecules to produce acetyl-CoA, an essential building block in the cell. One "turn" of the β-oxidation cycle produces one molecule of acetyl-CoA, and shortens the acyl-CoA by the length of two carbons. Enzymes involved in the reactions: (1) Acyl-CoA synthase, (2) acyl-CoA dehydrogenase, (3) enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, (4) 3-ketoacyl-CoA thiolase.

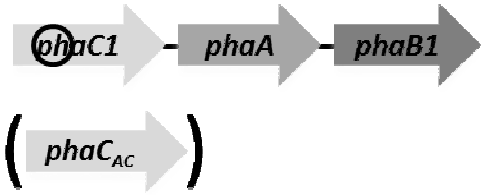
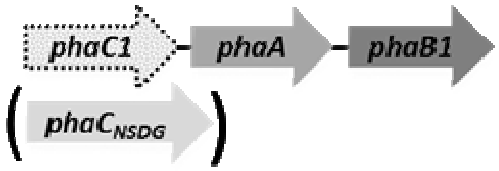
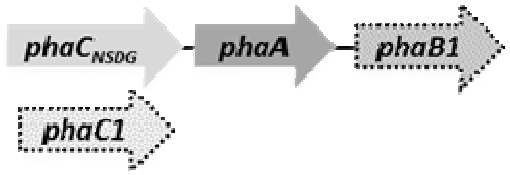
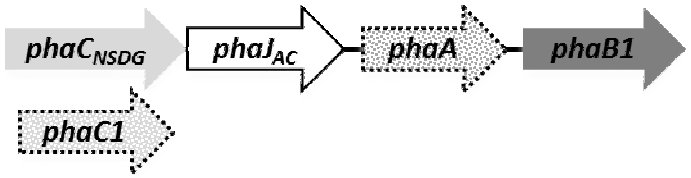
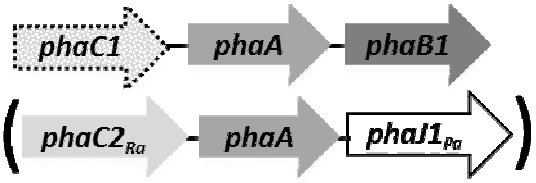
Fig. 2: Fatty acid biosynthesis. The initial condensation of this pathway is the attachment of a malonyl group to ACP and is performed by malonyl-CoA:ACP transacylase (A). The fatty acid biosynthetic cycle produces fatty acyl-ACP (acyl carrier protein) by addition of acetyl-CoA with each "turn" of the cycle (B). The fatty acyl moiety is then used in membrane lipid or lipopolysaccharide biosynthesis. Enzymes involved in these reactions: (5) acetyl-CoA carboxylase complex, (6) malonyl-CoA:ACP transacylase, (7) 3-ketoacyl-ACP synthase, (8) 3-ketoacyl-ACP reductase, (9) 3-hydroxyacyl-ACP dehydratase, (10) enoyl-ACP reductase.

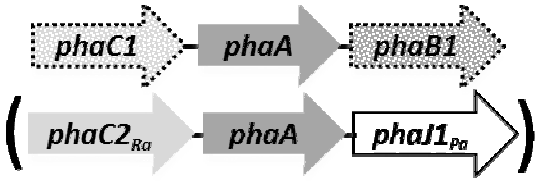
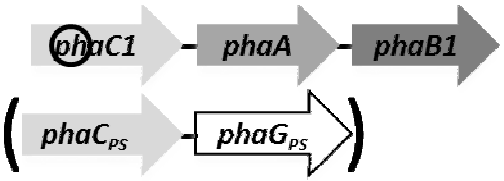
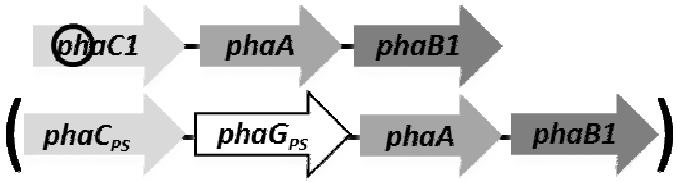
Fig. 3: Relationship of fatty acid metabolic pathways to PHA homeostasis in wild-type and engineered *R. eutropha.* From β-oxidation cycle intermediates, the enzyme PhaJ (reaction #11) can be used to convert enoyl-CoA to *(R)*-3-hydroxybutyryl-CoA, a PHA precursor. *phaJ* genes can be found in wild-type *R. eutropha* strains, as well as expressed heterologously in engineered strains. From fatty acid biosynthesis intermediates, a heterologously expressed enzyme, PhaG (reaction #12) can be used to convert *(S)*-3-hydroxybutyryl-ACP to *(R)*-3-hydroxybutyryl-CoA. These *(R)*-3-hydroxybutyryl-CoA precursors produced via fatty acid metabolism can be incorporated into PHA, using a heterologously expressed PhaC enzyme (reaction #13) to produce polymers with different monomer contents. When PHA (specifically PHB) is degraded by the depolymerase PhaZ1 (reaction #14), crotonyl-CoA (an enoyl-CoA) is produced, which feeds into the β-oxidation cycle (dashed arrow). Enzymes involved in the reactions: (11) *(R)*-specific enoyl coenzyme-A hydratase, (12) 3-hydroxyacyl-ACP:CoA transferase, (13) PHA synthase, (14) PHB depolymerase.

Fig. 4: Short chain length (*scl*) polyhydroxyalkanoate (PHA) biosynthesis in *R. eutropha*. PHA biosynthesis in wild-type *R. eutropha* proceeds by the formation of acetoacetyl-CoA from two molecules of acetyl-CoA, and subsequent reduction to *(R)*-3-hydroxybutyryl-CoA. The *(R)*-3-

hydroxybutyryl-CoA is polymerized by a PHA synthase (PhaC, reaction #13) to produce polyhydrxoybutyrate (PHB), a PHA homopolymer. The acetyl-CoA can be produced from fatty acid β-oxidation, as discussed in the text. If *R. eutropha* cells are grown in the presence of propionate or odd-carbon chain length fatty acids, propionyl-CoA can be synthesized, and ketovaleryl-CoA can be formed from one molecule each of acetyl-CoA and propionyl-CoA using the β-ketothiolase enzyme, BktB (reaction #17). *(R)*-3-hydroxybutyryl-CoA and *(R)*-3-hydroxyvaleryl-CoA can then be co-polymerized (by PhaC, reaction #13) to form poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-co-HV)], a PHA copolymer. Enzymes involved in the reactions: (13) PHA synthase, (15) β-ketothiolase PhaA, (16) acetoacetyl-CoA (ketovaleryl-CoA) reductase, (17) β-ketothiolase BktB.

Table 1: PHA related genotypes *of Ralstonia eutropha* PHA production strains and type of produced PHA polymer. Dashed arrows indicate deleted genes, circle arrow indicates the presence of a point mutation, HA_x = type of hydroxyalkyl monomer, Hx = HAs with 10 or 12 carbons, wt = wild-type, tr = trace, Fruc = Fructose

Strain (Plasmid)	**PHA Operon of Strain (PHA Genes on Plasmid)**	**Produced PHA (C-source , mol% HA_x)**
H16 [wt]		**PHB** (Fruc, 100 HB)[a] (Palm oil, 100 HB)[a]
PHB-4* (pJRDEE32d13)		**P(HB-*co*-HHx)** (Palm oil, 4 HHx)[b] (Corn oil , 5 HHx)[b] (Oleic acid, 4 HHx)[b]
CNPCN (pCUP3NSDG)		**P(HB-*co*-HHx)** (Palm kernel oil, 3 HHx)[c]
NSDGΔB		**P(HB-*co*-HHx)** (Fruc, tr HHx)[d] (Octanoate, 24 HHx)[d] (Soybean oil, 26 HHx)[d]
MF03		**P(HB-*co*-HHx)** (Fruc, 0 HHx)[d] (Octanoate, 23 HHx)[d] (Soybean oil, 10 HHx)[d]
Re2058 (pCB113)		**P(HB-*co*-HHx)** (Fruc, 0 HHx)[e] (Palm oil, 20 HHx, tr HO)[a] (CPKO, 30 HHx)[f]

Re2160** (pCB113)		**P(HB-*co*-HHx)** (Fruc, 0 HHx)[e] (Palm oil, 30 HHx, tr HO)[a] (CPKO, 40 HHx)[g]
PHB-4 (pRKmHS32)		**P(HB-*co*-HHx-*co*-HO-*co*-Hx)** (Fruc: 0.5 HHx, 0.9 HO, 2.0 Hx)[h] 26% PHA/CDW
PHB-4 (pRKmHS59)		**P(HB-*co*-HHx-*co*-HO-*co*-Hx)** (Fruc: 0.5 HHx, 0.9 HO, 1.5 Hx)[h] 44% PHA/CDW

* point mutation of PHB-4 (DSM 541, (Schlegel et al. 1970)) was characterized by Mifune et al, (2008).

**phaB2 and phaB3 are also deleted from the genome

AC = *Aeromonas caviae*, NSDG = double moutant of PHA synthase derivated from *A. caviae*, Pa = Pseudomonas aeruginosa, Ps = *Pseudomonas sp*. 61-3, Ra = *Rhodococcus aetherivorans* I24,

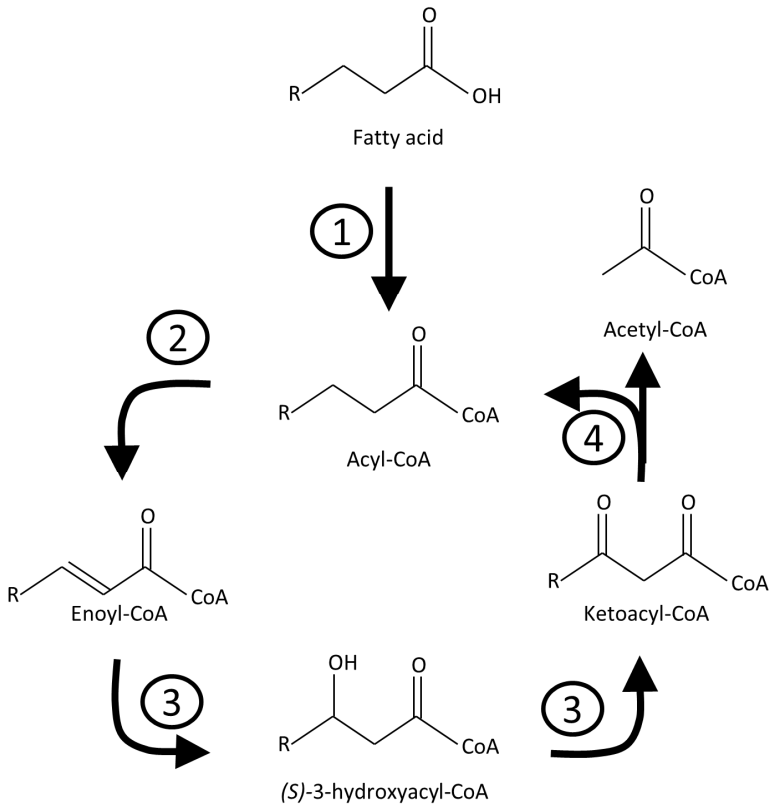
[a] = Budde et al., 2011b, [b] = Fukui and Doi, 1998, [c] = Sato et al, 2013, [d] =Mifune et al., 2010, [e] = Budde et al., unpublished data, [f] = Riedel et al., unpublished data, [g] = Wong et al., 2012, [h] = Matsumoto et al., 2001.

Table 2: **PHA production with *Ralstonia eutropha* H16 (DSM 428, https://www.dsmz.de/catalogues/details/culture/dsm-428.html) or its recombinant strains (m) from different Carbon (C) sources, from shaking flask (SF), batch (b), extended batch (eb) or fed batch (fb) fermentations (F). Palm oil (PO), palm kernel oil (PKO), soy bean oil (SBO) or waste rapeseed oil (WRO) with propanol (PrOH) as HV precursor where used as TAG feedstocks. As VFA were used: Acetic acid (AA), propionic acid (PA), and butric acid (BA). Lactic acid (LA), Glycerol (Glyc) and waste glycerol (wGlyc), where also used for PHA production. *n* shows numbers of replications. Error bars (±), indicating standard divisions for $n \geq 3$, for $n = 2$ minimum and maximum are given. PHA production was triggered with nitrogen (N) or phosphate (PO_4) limitation. n.a. indicates that data were not available.**

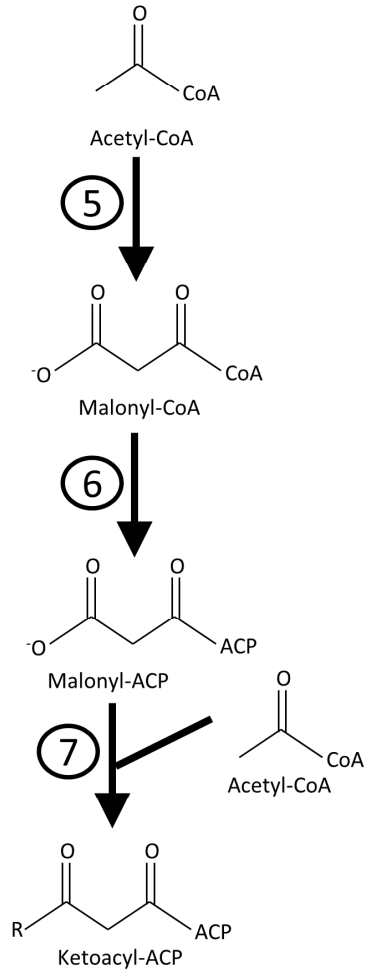
Carbon Source	Strain	Scale (kind)	*n*	Limi-tation	Total N or PO_4 (mM)	Total C (%)	PHA Production				PHA Composition (mol%)			Ref.
							per CDW (%)	total (g/L)	STY (g/L/h)	yield (g/g C)	HB	HV	HHx	
PO	H16	F (b)	6	N^h	19	2	79 ± 2	8 ± 1	0.1	0.61 (0.84, 16-72 h)	100			$m^{\#}$
	m^a	SF	1	N^h	9	1	81± 0	3			96		4	n
	$m^{b\bullet}$	SF	3	N^h	9	1	73 ± 0	3 ± 0			87 ± 0		13 ± 0	o
	$m^{b\bullet}$	F (b)	3	N^h	75	4	71 ± 2	18 ± 1	0.2		83 ± 1		17 ± 1	o
	$m^{c\bullet}$	F (b)	3	N^h	75	4	66 ± 2	11 ± 2	0.1		70 ± 2		30 ± 2	o
	$m^{b\bullet}$	F (eb)	2	N^i	150	6	72 ± 1	33 ± 2	0.3	0.52 (0.77, 48-96 h)	83 ± 1		18 ± 1	p
	$m^{b\bullet}$	F (fb)	2	N^j	480	17	70 ± 0	69 ± 1	0.7		76 ± 1		24 ± 1	p
	$m^{b\bullet}$	F (fb)	3	N^i	480	17	74 ± 2	102 ± 8	1.1	0.63 (0.78, 63-96 h)	81 ± 0		19	p
PKO	m^d	SF	n.a.	n.a.	n.a.	0.5	87 ± 2	4			95 ± 0		5	q
	m^d	SF	n.a.	n.a.	n.a.	0.5	70 ± 2	2			95 ± 0		5	q
	$m^{e\bullet}$	F (fb)	2	N^k	n.a.	n.a.	76 ± 0	126 ± 1	1.9	n.a.	98 ± 0		3 ± 0	r
	H16	F (fb)	2	N^k	n.a.	n.a.	72 ± 1	118 ± 1	1.8	n.a.	100			r
SBO	H16	F (fb)	2	PO_4	80	n.a.	74 ± 2	90 ± 5	0.9	0.74	100			s
	m^d	F (fb)	2	PO_4	80	n.a.	73 ± 2	97 ± 6	1.0	0.73	95 ± 0		5	s

	H16	F (eb)	1	PO_4 [**]	n.a.	8	81	67	2.5	0.85	100			t
WRO +PrOH	H16	F (fb)	1	N[l]	n.a.	n.a.	76	138	1.5	0.83	92	8		u
LA[*]	H16	F (fb)	1	low N[k]	n.a.	n.a.	58	59	1.2	0.17	100			v
PA[*]	H16	F (fb)	1	low N[k]	n.a.	n.a.	58	37	0.7	n.a.	n.a.	n.a.		w
AA/LA[*] (1:1)	H16	F (fb)	1	low N[k]	n.a.	n.a.	73	55	1.3	0	100			x
BA	m[g]	F (fb)	1	PO4	n.a	6.4	82	38.4	0.57	0.61	100			y
Glyc	m[f]	F (fb)	1	N[k]	n.a.	26.5	71	53	0.9	0.2	100			z
Glyc	m[g]	F (fb)	1	N[k]	n.a.	24.9	62	51	1.5	0.2	100			za
wGlyc	m[g]	F (fb)	1	N[k]	n.a.	n.a.	50	38	1.1	n.a.	100			za

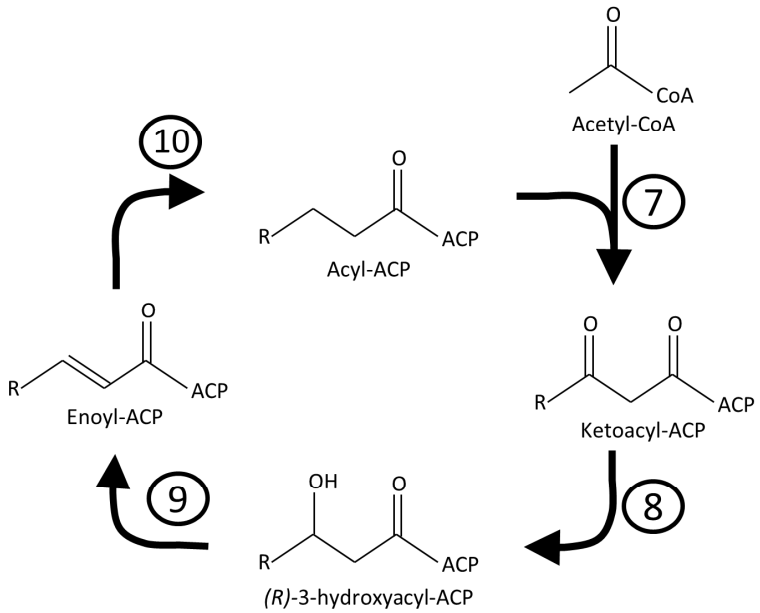
[a] = PHB-4/PJRDEE32d13, [b] = Re2058/pCB113, [c] = Re2160/pCB113, [d] = PHB-4/pBBREE32d13, [e] = CNPCN, [f] = laboratory mutant of H16, [g] = DSM 545 (http://www.dsmz.de/catalogues/details/culture/DSM-529.html), [h] = NH_4Cl, [i] = urea, [j] = NH_4Cl/NH_4OH - pH controlled, [k] = $(NH_4)_2SO_4/NH_4OH$ - pH controlled, [l] = $(NH_4)_2SO_4$, m[#] = Budde et al, 2011, n = Fukui and Doi, 1998, o = Budde et al, 2011, p = Riedel et al, 2012, q = Loo et al, 2005, r = Sato et al, 2013, s = Kahar et al, 2004, t = (de la Cruz Pradella, J.G. et al. 2012) , u = Obruca et al, 2010, v = Tsuge et al, 1999, w = Kobayashi et al, 2000, x = Tsuge et al, 2001, y = Grousseau et al, 2013 z = Tanadchangsaeng and Yu, 2012, z[a] = Cavalheiro et al, 2009, [*] = C-source mixed with ammonium hydroxide solution and fed over base control in two stage fed batch fermentation: 1[st] stage C/N = 10, 2[nd] stage C/N ratio = 23[v] or 50[w,x], resulting in low N levels. Acid concentration controlled at around 2 - 3 g/L. [**] = may also be Cu-, Ca-, and/or Fe-limited. • = no antibiotic additions were necessary for maintenance of plasmid stability.

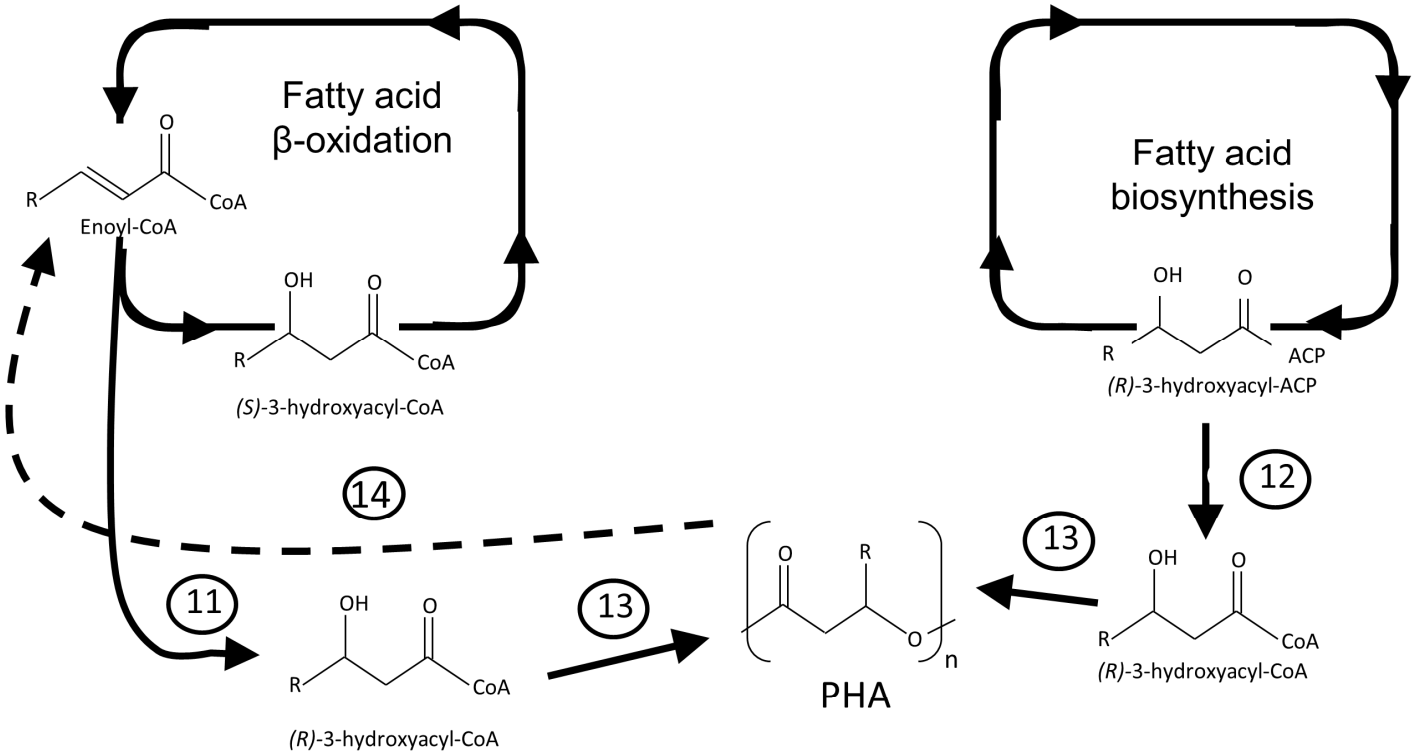

O
R
OH
Fatty acid
1
O
CoA
Acetyl-CoA
2
O
R
CoA
Acyl-CoA
4
O
R
CoA
Enoyl-CoA
O
O
R
CoA
Ketoacyl-CoA
3
OH
O
R
CoA
(S)-3-hydroxyacyl-CoA
3

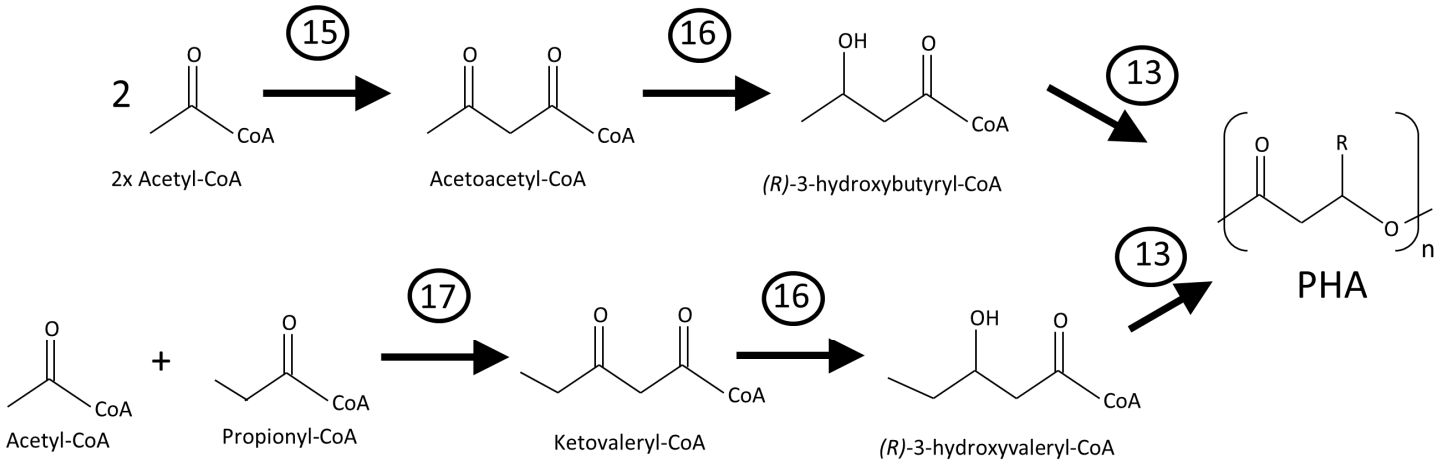
A.


Acetyl-CoA
5
Malonyl-CoA
6
Malonyl-ACP
7
Acetyl-CoA
Ketoacyl-ACP

B.


Acetyl-CoA
7
Ketoacyl-ACP
8
(R)-3-hydroxyacyl-ACP
9
Enoyl-ACP
10
Acyl-ACP


Fatty acid
β-oxidation
Enoyl-CoA
(S)-3-hydroxyacyl-CoA
Fatty acid
biosynthesis
(R)-3-hydroxyacyl-ACP
12
13
14
11
13
PHA
(R)-3-hydroxyacyl-CoA
(R)-3-hydroxyacyl-CoA


2
O
CoA
2x Acetyl-CoA
15
O
O
CoA
Acetoacetyl-CoA
16
OH
O
CoA
(R)-3-hydroxybutyryl-CoA
13
O
R
O
n
PHA
Acetyl-CoA
+
Propionyl-CoA
17
Ketovaleryl-CoA
16
(R)-3-hydroxyvaleryl-CoA
13