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Engineering the heterotrophic carbon sources utilization range of *Ralstonia eutropha* H16 for applications in biotechnology

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Abstract

Ralstonia eutropha H16 is an interesting candidate for the biotechnological production of polyesters consisting of hydroxy- and mercaptoalkanoates, and other compounds. It provides all the necessary characteristics, which are required for a biotechnological production strain. Due to its metabolic versatility it can convert a broad range of renewable heterotrophic resources into diverse valuable compounds. High cell density fermentations of the nonpathogenic *R. eutropha* can be easily performed. Furthermore, this bacterium is accessible to engineering of its metabolism by genetic approaches having available a large repertoire of genetic tools. Since the complete genome sequence of *R. eutropha* H16 has become available, a variety of transcriptome, proteome and metabolome studies provided valuable data elucidating its complex metabolism and allowing a systematic biology approach. However, high production costs for bacterial large-scale production of biomass and biotechnologically valuable products are still an economic challenge. The application of inexpensive raw materials could significantly reduce the expenses. Therefore, the conversion of diverse substrates to PHAs by *R. eutropha* was steadily improved by optimization of cultivation conditions, mutagenesis and metabolic engineering. Industrial by-products and residual compounds like glycerol, and substrates containing high carbon content per weight like palm, soybean, corn oils as well as raw sugar-rich materials like molasses, starch and lignocellulose, are the most promising renewable substrates and were intensively studied.

Introduction

Ralstonia eutropha H16 is a Gram-negative, rod-shaped and facultative chemolithoautotrophic β -proteobacterium isolated from a spring in Germany. Initially described as *Hydrogenomonas eutropha* (121), it has been the subject of frequent taxonomical reclassifications: *Alcaligenes eutrophus*, *Ralstonia eutropha*, *Wautersia eutropha* and finally *Cupriavidus necator* (93). However, the designation *Ralstonia eutropha* remained the most established terminus in the scientific community and will be also used throughout this study.

As a ubiquitous inhabitant of soil and freshwater *R. eutropha* is well adapted to constantly changing environments. Metabolic versatility of this microorganism made it to a model organism in microbiology concerning hydrogen-based chemolithoautotrophy and poly-3-hydroxybutyrate [P(3HB)] synthesis (93). Although aerobic respiration plays the major role in energy generation, *R. eutropha* can utilize nitrate and nitrite as alternative electron acceptors under anoxic conditions (3, 84). During heterotrophic growth, diverse carbohydrates, lipids and organic acids serve as carbon and energy source (Figure 1), whereas in the absence of organic compounds a mixture of H_2 , CO_2 and O_2 enables *R. eutropha* to grow autotrophically. In dependency on the situation in the habitat, *R. eutropha* can easily shift between autotrophy and heterotrophy (50). Mixotrophical growth is observed when *R. eutropha* utilizes concomitantly organic and inorganic substrates as carbon and energy source (33). The Calvin-Benson-Bassham (CBB) cycle of *R. eutropha* is active in the polyhydroxyalkanoate (PHA) synthesis phase even during growth on fructose. On the other side, the complete tricarboxylic acid cycle (TCC) is also active during autotrophic growth (98-99).

During all nutrition modes, *R. eutropha* is able to store P(3HB). For this, two molecules of acetyl-CoA are condensed by a β -ketothiolase (PhaA) to acetoacetyl-CoA. The latter is reduced by the NADPH-dependent acetoacetyl-CoA reductase (PhaB) to (*R*)-3-hydroxybutyryl-CoA, which is further polymerized to P(3HB) by the PHA synthase (PhaC)

(2). These three genes are organized in one operon *phaCAB*, which is constitutively expressed independent from the growth conditions (82). However, intensive synthesis of P(3HB) only starts if a carbon source is available in excess and if one essential nutritional element like N, O, S, Mg, K or P limits cell growth. P(3HB) can be stored up to 90% of the cell dry weight (CDW) by *R. eutropha* H16. Besides 3-hydroxybutyrate (3HB), other short- and medium-chain-length (SCL and MCL) hydroxyalkanoates as well as mercaptoalkanoates are polymerized by PhaC_{Re} (Table S1; 24, 73).

Besides the academic interest, *R. eutropha* has found applications in industry for large-scale production of PHAs (14). PHAs have attracted much interest due to their biodegradability and origin from renewable resources, as well as to thermoplastic/elastomeric properties similar to synthetic polymers (108). With more than 150 different constituents PHAs exhibit a broad spectrum of properties and functionalities (18). Consequently, various technical and medical applications have been developed during the last decades (2, 42, 122).

PHAs have been industrially produced since the 1970s (Imperial Chemical Industries (ICI); Monsanto, Metabolix, Zeneca, etc.). However, the production costs of bioplastics are still 5-10 times higher than costs for the production of conventional plastics from petrochemicals (92). The most critical aspect for industrial production bioproducts is their price competitiveness. Raw materials used for cultivation have an important impact on total production costs (18). Inexpensive raw sugar-rich materials, such as molasses, starch and cellulosic resources, promise to reduce the production costs of microbially synthesized products. Industrial production of plant oils and biodiesel is accompanied with co-production of glycerol-, triacylglyceride- and free fatty acid (FA)-rich residuals as by-products (Figure S1). FA-rich feedstocks are substrates containing high carbon content per weight and can be transformed into biotechnologically relevant products with a very high yield (109). The versatility of *R. eutropha*'s metabolism allows the utilization of a broad range of carbon sources. Further extension of the carbon substrate range as well as optimization of substrate

conversion, are key for accelerating competitiveness of the gained products. Besides, the problem of organic wastes disposal can be partially solved by conversion of residuals to valuable products (18).

Knowledge concerning PHAs and their microbial production is steadily expanding. Novel biopolymers of 3-mercaptoalkanoates and cyanophycin, low-molecular-weight compounds and alcohols recently extended the range of products synthesized by *R. eutropha* (40, 65, 73, 100, 118). New information encouraged reviews every few years (1, 6, 18, 81, 108). Since the genome of *R. eutropha* H16 has been sequenced (85), the energy metabolism of this bacterium has been reviewed (22). The genetic potential of this bacterium as a model organism, the natural and engineered carbon storage pathways for production of PHAs and other bioproducts by *R. eutropha* were analyzed (8, 93-94, 105, 113).

Recently, a variety of studies about the extension of the carbon source utilization range of *R. eutropha* and its optimization became available. To address these items, an understanding of the metabolic network of different renewable substrates concerning genomic and metabolic background of *R. eutropha* is essential. This stimulated to review the recent advances in this field with respect to the application of *R. eutropha* H16 wild type, mutants and metabolically engineered strains in biotechnology. The utilization of substrates that are mainly metabolized *via* the Entner-Doudoroff pathway or are oxidized in β -oxidation cycle and TCC besides lignin derivatives, amino acids, sulfur-compounds will be discussed and their impact on the synthesis of valuable products will be shortly reviewed.

Utilization of gluconate and carbohydrates

Besides gluconate only two carbohydrates, fructose and *N*-acetylglucosamine, are utilized as a carbon source by *R. eutropha* H16 (Figure 1; 53). They are expensive, and their application is therefore economically not feasible for the production of cheap bulk products. Broadening the

substrate range of *R. eutropha* will allow using inexpensive and renewable crop-derived feedstocks. Alternatively, second generation products, like lignocelluloses biomass and other agricultural wastes, provide a good alternative stock of carbon and energy for microbial conversions. Lignocellulose is the most abundant raw material composed of lignin (10-20%) and the carbohydrate polymers, cellulose (30-50%) and hemicelluloses (20-50%) (Figure S1; 52). Cellulose is a polysaccharide of glucose. Hemicellulose is a heteropolymer consisting of glucose, xylose, mannose, galactose, rhamnose, and arabinose. Celluloses and hemicelluloses can be microbially fermented or chemically hydrolyzed into monosaccharides, which can then serve as substrates for biotechnical production of value-added products (66).

N-Acetylglucosamine, fructose and gluconate. Fructose, *N*-acetylglucosamine and gluconate are catabolized in the Entner-Doudoroff (ED)-pathway with 2-keto-3-deoxy-6-phosphogluconate aldolase (EDA) as key enzyme, yielding pyruvate (Figure 1). Phosphofructokinase (Pfk) and 6-phosphogluconate dehydrogenase are absent in *R. eutropha* H16; therefore, the Embden–Meyerhof–Parnas (EMP) pathway and the oxidative pentose phosphate pathways are incomplete. However, genes for an anabolically operating EMP-pathway (gluconeogenesis) are present in *R. eutropha* (85). The catabolic EMP-pathway was accomplished in *R. eutropha* by heterologous expression *pfk* from *Escherichia coli* (104). Mutants with a defective ED-pathway serve as a suitable platform for the establishment of fructose utilization *via* the EMP-pathway, because the simultaneous function of EMP- and ED-pathways in the wild type of *R. eutropha* had a negative effect on growth in presence of fructose. Alternatively, the ability of EDA-negative mutants to utilize fructose was restored *via* heterologous expression of the phosphoketolase gene from *Bifidobacterium animalis* (30). These experiments showed that central metabolic pathways can be switched off, while alternative routes can be easily established in *R. eutropha*. Pyruvate formed in the ED-pathway is further decarboxylated by the pyruvate dehydrogenase complex (PDHC) yielding

acetyl coenzyme A (CoA). The latter is used in anabolic pathways for the synthesis of essential compounds, like amino acids and lipids, directed to P(3HB) synthesis and stored as a source of energy and carbon, or directly oxidized in TCC (Figure 1).

The amino sugar and glucose derivative *N*-acetylglucosamine is transported by the phosphoenolpyruvate (PEP)-dependent phosphotransferase transport system (PTS^{Nag}) into cells of *R. eutropha* (Figure 1). The responsible genes are organized in the *nagFECABzwfI* cluster. *N*-Acetylglucosamine enters the cell through a porin (NagC) located in the outer membrane (49). Then, *N*-acetylglucosamine is phosphorylated by two proteins of the PTS^{Nag}, NagF and NagE. Therefore, PEP provides phosphoryl to the soluble NagF, which comprises three PTS domains, EI^{Nag}-HPr^{Nag}-EIIA^{Nag}. Phosphoryl is then transferred from NagF to a cytosolic domain EIIB^{Nag} and a membrane-spanning domain EIIC^{Nag} of NagE. After phosphorylation of *N*-acetylglucosamine by NagE the acetyl-moiety is cleaved off by *N*-acetylglucosamine-6-phosphate deaminase (NagA), and glucosamine is further deaminated by glucosamine-6-phosphate deaminase (NagB) yielding fructose-6-phosphate. Fructose uptake is mediated by a CUT2 family ATP binding cassette (ABC)-type transporter (*frcACB*) (Figure 1; 37, 85). Fructokinase catalyzes the phosphorylation of fructose. Fructose-6-phosphate, which is also obtained from *N*-acetylglucosamine, is then converted to glucose-6-phosphate by phosphoglucose isomerase and is further degraded in ED-pathway with gluconate-6-phosphate as one of the intermediates (Figure 1). If gluconate serves as the substrate, it enters the ED-pathway as gluconate-6-phosphate. The gluconate transport is mediated by a gluconate-H⁺ symporter catalyzed by a gluconate permease (GntP) and a gluconate transporter (GntT) (49).

Although *R. eutropha* H16 harbors only one specific transport system either for fructose or *N*-acetylglucosamine, an additional truncated PTS comprises a variety of PEP-PTS homologous proteins, like PtsN, PtsM, PtsH, and PtsI (85). Recently, it was found that the truncated PTS is indirectly involved in the complex sugar transport system, regulatory

functions of carbon and P(3HB) metabolism (49, 51). Diminished P(3HB) contents and facilitated P(3HB) mobilization were observed in *ptsM*, *ptsH*, and *ptsI* single, double, and triple mutants of *R. eutropha* (49). The lack of PtsI and PtsH impairs the transfer of a phosphoryl group to PtsN, as consequence the non-phosphorylated PtsN leads to a lower P(3HB) content. A deletion of *ptsN* had an opposite effect and led to an increased P(3HB) content in the cells if they were grown on gluconate. Further findings demonstrated that protein-protein interactions of non-phosphorylated PtsN with the key enzyme of a stringent response SpoT1 (GTP diphosphokinase/ppGpp hydrolase) can indirectly alter the global gene expression in the cell (51). Thus, PtsN, in association with SpoT1, was found to participate in the regulation of a stringent response in *R. eutropha* and consequently influences the P(3HB) content.

Glucose. The wild type of *R. eutropha* is unable to utilize glucose as a carbon source. However, glucose-6-phosphate is a common intermediate of the ED-pathway (Figure 1). Crude extracts of fructose-grown cells of strain H16 exhibit hexokinase activity, which phosphorylates besides fructose, also mannose and glucose (37). Furthermore, it was demonstrated, that ^{14}C glucose does not enter the cells. *R. eutropha* H16's behavior was designated as cryptic with regard to glucose, i.e. even cells, which were grown with fructose and equipped with all enzymes necessary for glucose degradation, were unable to metabolize glucose.

Glucose-utilizing strains of *R. eutropha* and other bacteria have been intensively studied for biotechnological production of PHAs. To overcome the incapability of *R. eutropha* H16 to utilize glucose and to extent the substrate range, different attempts were undertaken. Historically, the first approaches were based on the isolation of spontaneous glucose-utilizing mutants (31, 59), later also UV- (96) and chemically (54) induced mutants were obtained. Glucose-utilizing mutants were provided by Schlegel and co-workers to ICI and one of them

(deposited as *R. eutropha* NCIMB 11599) has been intensively exploited for the commercial production of PHA (14, 18). The UV-induced glucose-utilizing mutants were able to transport glucose into the cells and exhibited constitutive glucose-6-phosphate-dehydrogenase activity (96). Multiple independent spontaneous glucose-utilizing mutants were also isolated solely by provision of glucose to the media (31).

Recently, the glucose transport mechanism in G⁺1 mutant of *R. eutropha* H16 was resolved (89-90). The glucose-utilizing G⁺1 mutant was generated in 1965 by Schlegel and Gottschalk by UV-mutagenesis (96). A mutated and derepressed PTS^{Nag} was found to be responsible for the glucose transport (89-90). A point mutation in *nagR* (a negative regulator of *nag* genes) of the G⁺1 mutant introduced a stop codon and caused a truncated repressor protein. Consequently, the derepression of the *nag* operon due to inactive NagR led to a constitutive overexpression of the *nag* operon in mutant G⁺1. Furthermore, a missense Ala153Thr mutation in *nagE* was identified, which positively affected glucose uptake mediated by the mutated PTS^{Nag} in mutant G⁺1. The *R. eutropha* H16 Δ *nagRnagE*Ala153Thr mutant strain showed the same phenotype as strain G⁺1 regarding glucose utilization (90). Another independent study also identified mutations in *nagR* and *nagE* in glucose-utilizing *R. eutropha* NCIMB 11599 (79). The observed single mutation in *nagR* likewise inactivated the regulator, and an additional mutation Gly265Arg in NagE also improved glucose conversion. Although these two glucose-utilizing strains harbor different mutations in the *nagE* genes, they exhibit the same phenotype. The roles of these amino acids have to be studied. A further important finding is that the sugar is transported *via* facilitated diffusion and its transport is uncoupled from phosphorylation by PTS^{Nag}. Intracellular phosphorylation is mediated by glucokinase Glk (90). Another interesting effect of a constitutively expressed and uncoupled PTS^{Nag} transporting sugar by facilitated diffusion is the avoidance of carbon catabolite repression (21), allowing to use media harboring different carbon sources that can be utilized in parallel without diauxic growth (90).

An alternative glucose-utilizing strain of *R. eutropha* was generated by metabolic engineering (101). Heterologous expression of the energy-independent glucose-facilitated diffusion transporter, *glf*, from *Zymomonas mobilis* was used to mediate the glucose transport in the cell. *R. eutropha* H16 harboring *glf* was capable of growing with glucose as sole carbon source after a *lag* phase. Co-expression of *glf* and *glk* (glucokinase from *E. coli*) diminished the *lag* phase. The heterologous Glk compensated a possible metabolic bottleneck, possibly caused by insufficient expression/activity of the wild-type glucokinase. Reconstitution of glucose uptake and phosphorylation in *R. eutropha* via heterologous expression of *glf* and *glk* from *Z. mobilis* was recently confirmed (79). The strains harboring heterologous genes capable of glucose utilization stored comparable amounts of P(3HB) like the wild-type *R. eutropha* grown with gluconate (101).

Mannose. In contrast to glucose, no spontaneous mannose-utilizing mutants of *R. eutropha* occurred. Besides the lack of appropriate transporters for mannose, phosphorylation of mannose by the native hexokinase of *R. eutropha* was found to be less efficient than phosphorylation of fructose and glucose (37). Moreover, mannose inhibits the oxidation of fructose in *R. eutropha*. An artificial pathway for the utilization of mannose by *R. eutropha* H16 was established (101). For this, *mak* and *pmi* genes, encoding mannofructokinase and phosphomannose isomerase from *E. coli*, were simultaneously expressed together with *glf*. Glf mediates not only the transport of glucose but also of mannose, while Mak phosphorylates mannose and Pmi converts mannose-6-phosphate to fructose-6-phosphate which is metabolized in the ED-pathway. *R. eutropha* possesses an own gene encoding Pmi. However, moderate growth of the recombinant strains of *R. eutropha*, harboring either *glf* or *glf* together with *mak*, in presence of mannose as sole carbon source, occurred only after prior cultivation on fructose (101). This effect was not observed if the cells were pre-cultivated in presence of gluconate. Consequently, fructose seems to serve as inducer of *pmi* expression in *R. eutropha*.

However, further investigations and corresponding genetic modifications must be achieved in order to exploit the native *pmi* from *R. eutropha*. Although phosphorylation of mannose is possible by a native hexokinase of *R. eutropha* (37), the heterologous Mak improved the growth rate of *R. eutropha* significantly. P(3HB) contents of the recombinant strains grown on gluconate or mannose were similar, showing that mannose might be also utilized for industrial production (101).

Xylose and arabinose. Xylose and arabinose are sugars which can be derived from hemicelluloses (Figure S2). *R. eutropha* H16 is unable to utilize xylose or arabinose probably because it lacks enzymes mediating both, the uptake and the catabolism of these two sugars (85). Uptake of xylose in *E. coli* is achieved by an ABC-transporter. The genome sequence did not reveal any hint for the presence of xylose-specific transporters in *R. eutropha*. In *E. coli*, the catabolism of xylose is initiated by xylose isomerase (XylA), which converts xylose to xylulose (46). Xylulose is then phosphorylated by xylulokinase (XylB) to xylulose-5-phosphate, which is further converted to ribulose-5-phosphate by ribulose-phosphate 3-epimerase (RPE, Table S2). In fungi and yeasts catabolism of xylose is initiated by an NADP-dependent xylose dehydrogenase, which forms xylonolactone from xylose; then lactonase, xylonate dehydratase, 2-keto-3-deoxyxylonate dehydratase and aldehyde dehydrogenase convert the lactone *via* xylonic acid to 2-oxoglutarate (46). *R. eutropha* lacks almost all the necessary genes for xylose uptake and catabolism (85). However, application of a xylose transporter (*xylE*), *xylA* and *xylB* from *E. coli* in addition to a putative *R. eutropha*'s genome encoded RPE was sufficient to establish xylose utilization in *R. eutropha* W50 (70).

Alternatively, *R. eutropha* grows on xylose-containing feedstocks in combination with *Lactococcus lactis* IO-1 by two-stage fermentation method (110). In the first stage, xylose was fermented to lactate and acetate by *L. lactis* and in the second stage PHA was synthesized by *R. eutropha*.

R. eutropha W50 was enabled to utilize arabinose *via* heterologous expression of a set of genes for L-arabinose uptake and metabolism from *E. coli* W3110 (72). The uptake of arabinose was catalyzed by the transporter which is encoded by the *araFGH* operon. In the cells L-arabinose was converted to L-ribulose by heterologous arabinose isomerase (AraA), then ribulose kinase (AraB) phosphorylated L-ribulose to L-ribulose-phosphate, which was finally transformed to D-xylulose-phosphate. The putative RPE of *R. eutropha* might be further involved in the metabolism of this carbohydrate. It was shown that expression of the *araBAD* genes in *R. eutropha* W50 was sufficient for utilization of L-arabinose, however, co-expression of *araFGH* improved the uptake of L-arabinose (72).

Starch. Starch is one of the most abundant carbohydrates contained in plants (potatoes, wheat, corn, rice, etc.), and consequently appears in food wastes and starchy wastewaters (Figure S2; 127). The polysaccharide starch consists of glucose moieties linked by α 1-4 glycosidic bonds. Amylases and glucoamylases hydrolyze this polysaccharide into maltose, maltotriose and glucose. *R. eutropha* lacks genes coding for putative amylases or glucoamylases. Therefore, the utilization of starch by *R. eutropha* is only possible after prior starch hydrolysis. Moreover, since glucose is the end product of starch saccharification, a glucose-utilizing strain of *R. eutropha* must be available (see above). There are at least two different approaches to transform starch into PHA. One method applies a two-step fermentation procedure (127). The starchy wastes are converted by the active sludge containing acidogenic bacteria into volatile FAs (VFAs) such as formic, acetic, propionic and butyric acids. The VFAs could then be converted into bioplastic by PHA producing bacteria like *R. eutropha* during the second fermentation step. Since propionic acid is available after the first step, synthesis of P(3HB-co-3-hydroxyvalerate) is possible by this method. The second approach uses hydrolysis of polysaccharides into sugars by commercially available enzymes (41). Thereby, starchy wastes from a local potato chips factory were successfully converted to

P(3HB) by glucose-utilizing *R. eutropha* NCIMB 11599 after previous enzymatic hydrolysis. However, the energy-consumption during two-step fermentation, down-stream processing and saccharification cause additional costs negatively influencing the competitiveness of PHAs produced by this method. Therefore, the expression of heterologous amylases could be another promising approach.

Lactose and galactose. *R. eutropha* H16 is not able to cleave the disaccharide lactose (Figure S1) or even to utilize galactose. Consequently, growth of the wild type on these sugars as a sole carbon source is not possible. Cleavage of lactose was conferred to the glucose-utilizing *R. eutropha* G⁺1 by heterologous expression of *lacZ* (β -galactosidase), *lacI* (inducer gene) and *lacO* (operator) from *E. coli* in (88). However, utilization of galactose by *R. eutropha* remains unclear. One study showed that *R. eutropha* H16 was able to phosphorylate glucose and mannose, but not galactose, arabinose and sorbose (37). Another study demonstrated that the recombinant glucose-utilizing strain *R. eutropha* G⁺1 excreted galactose into the medium, only if the *lac* genes were applied (88). A concomitant utilization of glucose and galactose was possible only if the *gal* genes were heterologously co-expressed with the *lac* genes. In contrast, recently another study demonstrated that the glucose-utilizing strain of *R. eutropha* DSM 545 (H1G⁺3) was able to grow on galactose, lactose or whey permeate as sole carbon sources harboring only the *E. coli lac* genes (87). The *lac* genes were integrated in the *phaZ* (PHA-depolymerase)-gene to minimize the mobilization of P(3HB). Since strains G⁺1 and H1G⁺3 are UV-induced mutants, they might differ from the wild type and similar spontaneous mutants regarding the galactose metabolism. Therefore, it would be interesting to clarify the mechanisms of galactose uptake and utilization.

Utilization of lignin derivatives

Besides hemicellulose lignin is another abundant waste product derived from lignocelluloses (Figure S1). After chemical, physical or biological pretreatment of lignin, aromatic derivatives as p-coumaric, caffeic, ferulic and sinapinic acid are released (112). These substrates can be further metabolized by some bacteria (*Pseudomonas putida*, *Sphingomonas paucimobilis*) to oxaloacetate and pyruvate with the following intermediates: vanillic, 4-hydroxybenzoic (4-HBA), 2,5-dihydroxybenzoic/gentisic (2,5-DHBA), 3,4-dihydroxybenzoic/protocatechuic (3,4-DHBA), and 3,4,5-trihydroxybenzoic/gallic (3,4,5-THBA) acid. *R. eutropha* H16 was able to utilize 4-HBA, 2,5-DHBA and 3,4-DHBA and the cells stored significant amounts of P(3HB) (112). This can serve as a platform for establishment of the entire lignin degradation pathway in *R. eutropha*. The introduction of heterologous genes responsible for conversion of lignin to 4-HBA, 2,5-DHBA and 3,4-DHBA should compensate the bottleneck in the lignin degradation pathway of *R. eutropha*.

Utilization of glycerol

Industrial production of biodiesel and plant oils is still developing; consequently, production of less valuable by-products is also increasing. Transesterification of oils releases waste rich in glycerol and free FAs (Figure S1; 109). As a main by-product of biodiesel industry glycerol is an attractive cheap substrate for enhancing the competitiveness of PHAs and other biobased products (86).

Two glycerol kinases GlyK and two glycerol-3-phosphate dehydrogenases Gly3-DH are located on both chromosomes of *R. eutropha* (Table S2; 85). The genes from the first chromosome were found to be up-regulated, when *R. eutropha* H16 was grown on trioleate (7). Glycerol enters the cells through passive diffusion and is phosphorylated by a kinase

(Figure 1). Glycerol-3-phosphate is dehydrogenated to dihydroxyacetone-phosphate before it is metabolized in the sugar-degrading pathway.

Growth of *R. eutropha* on glycerol occurs at a low growth rate, which is partially due to high activity of hydrogenases overproducing reactive oxygen species, such as H₂O₂. Cells react with a stress response on oxidative stress grown on glycerol. The pattern of metabolic response during glycerol utilization does not differ significantly from autotrophic growth. The activity levels of hydrogenases, ribulose-1,5-bisphosphate-carboxylase/-oxygenase (RuBisCO) and phosphoribulokinase (PRK) are comparable with autotrophic conditions (32, 98).

Several attempts were undertaken to optimize glycerol utilization by *R. eutropha* strains DSM 545 and IPT 026. The glycerol and nitrogen contents in growth media were adjusted for P(3HB) synthesis (15-16). Concentrations of waste glycerol higher as 40 g L⁻¹ were found to inhibit growth, probably due to impurities in the feedstock (16). Beside P(3HB), synthesis of copolymers P(3HB-co-3-hydroxyvalerate) and P(3HB-co-4-hydroxybutyrate-co-3-hydroxyvalerate) has been established from by-products of biodiesel production (17, 36). However, to significantly accelerate the growth rate it was not sufficient to optimize only the growth conditions. A metabolic network of intracellular processes during growth on glycerol was analyzed with elementary flux modes and yield space analysis, and putative links between central metabolism and P(3HB) synthesis were marked (71). A linkage between Gly3-DH's cofactor and NAD(H)/NADP(H) transhydrogenases was indicated. Facilitation of crude glycerol uptake and metabolization in *R. eutropha* might compensate the slow cell growth in order to improve the marketability of the bioproducts. Enhancing the glycerol uptake and improving the function of GlyK and Gly3-DH and their interaction with the anabolic EMP-pathway and its metabolites was suggested (71). Indeed, integration of *glpKEc* alone or together with aquaglyceroporin (*glpF_{Ec}*) in the *R. eutropha* H16 genome compensated the slow growth rate and significantly increased the P(3HB) productivity (35).

Another study demonstrated that presence of glucose in the medium even at low concentrations reduces the consumption of glycerol in *R. eutropha* DSM 545 (103). Probably the high intracellular pool of glycerol-3-phosphate and dihydroxyacetone-phosphate serves as a negative feed-back controller of glycerol consumption. These data are also in accordance with an assumption about inhibition of initial glycerol activation reactions by the products of the EMP-pathway (71).

Another issue of interest is the molecular weight of P(3HB) derived from glycerol. An unspecific incorporation of glycerol by PhaC can lead to a polymer chain termination during prolonged glycerol cultivation (111). Several studies reported on the reduction of the molecular weight of polymers produced from glycerol (16, 111). Since no significant difference in molecular weights of PHAs produced from glycerol or fructose were observed in *R. eutropha* H16 with facilitated glycerol metabolism (*glyKEc* and *glpFEc*), the reduction of intracellular glycerol concentration probably reduces the frequency of chain terminations (35).

Utilization of diverse organic acids

Fatty acids. Since FAs can be transformed into PHAs with a very high yield they are promising substrates for PHA production and have the potential to reduce production costs. In contrast to carbohydrates, a variety of organic acids are taken up by *R. eutropha* H16, so here is no need for substrate range expansions. However, optimization of the conversion of FAs to products has to be achieved. Organic acids, such as acetic, propionic and succinic acids, are fermentation products and common intermediates (in CoA-thioester form) of the central metabolism in *R. eutropha*. Acetic and succinic acid are oxidized in the TCC, while propionyl-CoA is oxidized in the methylcitrate cycle (MCC). Acyl-CoA thioesters of butyric and valeric acid as well as MCL- or long-chain-length FAs first undergo β -oxidation to yield acetyl-CoA (Figure 1).

Since acetyl-CoA can be completely oxidized in TCC, growth on the above mentioned substrates yielding acetyl-CoA is combined with a loss of carbon as CO₂ and a lack of three-carbon (C3) and four-carbon (C4) units in the central metabolism. The anaplerotic glyoxylate pathway, which bypasses the decarboxylation steps of the TCC, mediates the conversion of the acetic residue (C2) to C4-compounds. The glyoxylate pathway comprises cleavage of isocitrate into succinate and glyoxylate; the latter is further condensed with acetyl-CoA by malate synthase to yield malate as intermediate (Figure 1). The concomitant product of the glyoxylate bypass, succinate, is metabolized in the TCC. Another anaplerotic pathway mediates between C3- and C4-compounds during growth on C4-organic acids like succinate or malate (97). The interconversion of C4- and C3-compounds is catalyzed by PEP-carboxykinase to yield PEP from oxaloacetate by decarboxylation (114). Alternatively, decarboxylation of malate/oxaloacetate catalyzed by malic enzyme or oxaloacetate decarboxylase yields pyruvate (Figure 1; 10). Thus, C4-compounds like malate and oxaloacetate are transformed to C3-products like pyruvate and PEP for gluconeogenesis. Decarboxylation of malate to pyruvate is a NADPH-dependent reaction, and thus the C3/C4 metabolism regenerates the main cofactor of P(3HB) synthesis (10, 128).

Acidifying agents commonly occur in the natural environment of *R. eutropha*, and even moderate concentrations of them might lower pH and inhibit cell growth. SCL-FAs, like acetic, propionic, butyric and valeric acid, are often referred to as VFAs. They can freely diffuse through the membrane, acidify the cytoplasm which causes the toxic effect and consequently slows down metabolite production. Dissociated FAs reduce the proton gradient through the membrane, increase osmotic pressure and thus interfere with efficient energy metabolism (119). To overcome the toxic effect of FAs, *R. eutropha* possesses a detoxification mechanism (61-62). Besides the toxicity pure free FAs are relatively expensive. Therefore, the controlled co-feeding at low concentrations in the medium is the prevailing method (105).

(i) Even chain FAs. Even chain FAs provide exclusively 3HB-monomers for PHA synthesis in *R. eutropha* H16. Amongst all acids utilized by *R. eutropha* acetic acid exhibits the second highest dissociation constant and consequently causes severe toxic effects. The detoxification mechanism comprises the rapid metabolization of acetic acid. The tolerance of *R. eutropha* towards acetic acid increases with the cell mass concentration and concomitant increase in utilization rate of acetic acid (126). Therefore, acetate kinase and/or acetyl-CoA synthase activate(s) the acetic-residue with CoA. Acetyl-CoA is further directed to P(3HB) synthesis, TCC and other biosynthetic pathways, such as lipid or amino acid synthesis. Carbon flux analysis showed that the majority of the consumed acetic acid is divided between P(3HB) synthesis and TCC and only a small part is condensed with glyoxylate (128). Growth of *R. eutropha* on acetic acid is associated with the upregulation of such detoxifying enzymes as, for example, catalases, which are involved in a defensive response of *R. eutropha* on the toxic effect of organic acids (62). In a mixture of organic acids acetic acid is consumed after other acids like propionic, lactic or butyric acid, are exhausted (124). Furthermore, two acetic-residues are necessary to produce one molecule of 3HB. If butyric acid is used as sole carbon source, one molecule of butyric acid provides one molecule of 3HB or two molecules acetyl-CoA, which are then oxidized in TCC, resulting in better cell growth and P(3HB) yield. In addition, butyric acid is consumed to a higher rate and less toxic to the cell (124-125). Recently, it was shown that butyric acid serves also as precursor for the production of PHAs with C6-units as described below (48).

γ -Butyrolactone, 4-hydroxybutyric acid (4HB), 4-chlorobutyric acid and 1,4-butanediol are precursors leading to incorporation of 4HB in PHAs. *R. eutropha* synthesizes P(3HB-co-4HB) copolymers with different 4HB-fractions depending on the carbon source (60). However, although these substrates are taken up by the cells, partially degraded and incorporated into the copolymer, *R. eutropha* H16 is not able to grow with 4HB or 1,4-butanediol as sole carbon source (117). γ -Butyrolactone is putatively hydrolyzed, 4-

chlorobutyric acid is dechlorinated and 1,4-butanediol is oxidized to 4-hydroxybutyryl-CoA, and the latter is then incorporated into the copolymer (60). It is assumed that intracellular degradation of 4HB is carried out *via* succinate semialdehyde to succinate (117). P(3HB-*co*-4HB) exhibits biocompatibility, decreased crystallinity and consequently better flexible/elastic properties as P(3HB) and is degraded by lipases and esterases (44).

Optimization of 4HB uptake in *R. eutropha* improves conversion of 4HB to acetyl-CoA and its availability for the central metabolism, while the molar 4HB fraction in the copolymer decreases (107). Synthesis of P(4HB) homopolyester was possible by P(3HB)-leaky mutants of *R. eutropha* JMP222, harboring multiple copies of heterologous PHA biosynthesis genes from *R. eutropha* H16, if 4HB was used as the carbon source (107).

However, the 4HB-precursors are toxic for cells at high concentrations and costly, that is why the two-step batch or controlled co-feeding are the most commonly used procedures. The combination of sugar- or FA-containing cheap substrates as main carbon source with more expensive co-substrates is more beneficial for industrial production. Soybean, spent palm oil or waste glycerol in combination with 4HB-precursor such as γ -butyrolactone were successfully applied for production of P(3HB-*co*-4HB). 4HB fractions of 6–10mol% in P(3HB-*co*-4HB) were achieved by co-feeding soybean oil and 0.5-1% (w/v) γ -butyrolactone (80) or even with up to 15mol% with spent palm oil plus 0.5% (w/v) γ -butyrolactone (91). Successful variation of 4HB-content in P(3HB-*co*-4HB) copolymers synthesized by *R. eutropha* DSM 545 from waste glycerol supplemented with γ -butyrolactone was demonstrated (17). At higher levels of dissolved oxygen, more PHA, though with minor 4HB-fraction, was stored and vice versa. The molar composition and in particular the amount of 4HB in the copolymer, can be also manipulated applying alkanoates as cosubstrates. This effect was observed with propionic, butyric, valeric and hexanoic acids, however with propionic acid the highest molar content of 4HB was detected (55). The presence of propionic acid inhibited the degradation of 4HB, and up to 43mol% of 4HB were incorporated in the

copolymer (17, 55). Besides, the co-utilization of γ -butyrolactone and propionic acid led to synthesis of a new P(3HB-co-4HB-co-3HV) terpolymer. P(4HB) homopolymer could be purified *via* fractionated isolation of PHA from *R. eutropha* grown on mixture of 4HB and propionic acid (55). For further details of 4HB-containing copolymers see below.

After it was shown that PhaC_{Re} is also able to utilize MCL-hydroxyalkanoates (24), the production of copolymers with chain lengths >C5 has been studied intensively in *R. eutropha*. It is known that supply of MCL-precursors is hindered by highly active β -oxidation. Precursors of MCL-PHAs are CoA-thioesters of FAs that are usually completely degraded to acetyl-CoA. Two operons were found to be responsible for FA degradation in *R. eutropha* (Table S2; 7). The FA degradation operons seem to be constitutively expressed on fructose and upregulated in presence of FAs (99). Consequently, high amounts of 3HB-precursors are provided by highly active β -oxidation and abundance of acetyl-CoA. Chemical inhibition of β -oxidation by acrylate resulted, in contrast, in the integration of 3-hydroxyhexanoate (3HHx) and 3-hydroxyoctanoate in copolymers by *R. eutropha* (38). Molecular engineering techniques and appropriate carbon sources optimized P(3HB-co-3HHx) production in *R. eutropha* (12, 77). Therefore, the efflux of β -oxidation intermediates to PHA synthesis was forced *via* overexpression of an (*R*)-enoyl-CoA hydratase or a 3-hydroxyacyl-ACP:CoA transferase genes. Manipulation of the *phaCAB* operon resulted in copolymers with new compositions (76, 105). Two homologues of a (*S*)-specific 2-enoyl-CoA hydratase/(*S*)-3-hydroxyacyl-CoA dehydrogenase from the β -oxidation operons were deleted in order to improve the incorporation of MCL-hydroxyalkanoates (43). Alternatively, P(3HB-co-3HHx) was successfully produced from butyric acid as single carbon source (48). For this, *phaC_{Re}* was replaced by *phaC* from *Rhodococcus aetherivorans* I24 exhibiting broad substrate specificity, and all three genes encoding for acetoacetyl-CoA reductases were deleted in *R. eutropha*. In this mutant, the 3HB-content in the copolymer was reduced. Condensation of an acetic-residue with butyric acid provided as carbon source yielded 2-ketohexanoyl-CoA,

which was thereafter incorporated into the copolymer. The C2- and C4-CoA thioesters are putatively condensed by one of the multiple β -ketothiolases of *R. eutropha*. However, BktB is the most suitable candidate, since it assisted in this reaction in an artificial 1-hexanol pathway (23). Further approaches for MCL-PHA biosynthesis based on FA metabolism are discussed in more detail elsewhere (94).

(ii) Odd chain FAs. Usually odd chain FAs are degraded *via* β -oxidation to acetyl-CoA and propionyl-CoA as end products. Propionyl-CoA contributes to synthesis of a heteropolymer consisting exclusively of 3HB and 3-hydroxyvaleric acid (3HV), i.e. P(3HB-*co*-3HV) (28). Different 3HB/3HV ratios influence physical properties of synthesized copolymers. Generally, the technical applications of P(3HB-*co*-3HV) are more beneficial than P(3HB) due to improved flexibility and reduced crystallinity. Melting temperature of these copolymers decreases with increased 3HV amounts (120).

Once propionyl-CoA is formed from propionic acid, it can be directed to the central metabolism or to PHA metabolism (62). The catabolism of propionic acid in MCC, yields succinate and pyruvate with the latter further decarboxylated to acetyl-CoA (Figure 1, 5). Serving as sole carbon source propionic acid is mainly converted to acetyl-CoA, resulting in a restricted flux of propionyl-CoA to the copolyester synthesis. Interestingly, another β -ketothiolase, BktB, is responsible for P(3HB-*co*-3HV) synthesis. Whereas BktB is able to condense propionyl-CoA and acetyl-CoA, PhaA is not (102). *R. eutropha* lacking *bktB* grows as the wild type on propionic acid, but is impaired in synthesis of 3HV from propionyl-CoA (68). Thus, the copolymer from the *bktB*-lacking mutant contained only up to 3.5mol% of 3HV.

Propionyl-CoA is condensed by BktB with acetyl-CoA, and reduced by PhaB to 3HV-CoA. If the ratio of acetyl-CoA/propionyl-CoA in the cell is high, more 3HB-units are incorporated into the copolymer. In contrast, if the concentration of propionic acid in the

medium is increased, this shifts this ratio towards propionyl-CoA, and higher 3HV-molar fractions in the copolymer (22-45mol%) (27). When acetic and propionic acids are present in the culture medium, acetyl-CoA is obtained from both substrates (27), but it was shown that propionic acid is more favorably consumed as acetic acid (125). Metabolic fluxes show, that only small amounts of propionyl-CoA are converted to 3HV, while MCC consumes the majority of propionyl-CoA (128). Consequently, the acetyl-CoA/propionyl-CoA ratio in the cell is higher only if propionic acid is provided, and the copolymer contained 2-28mol% of 3HV (27). In general, the application of a mixture of acids allows the modulation of PHA synthesis yielding the desired copolymer composition (125).

Provision of butyric (128) or oleic (75) additionally to propionic acid improves the total PHA yield. However, only due to an increasing acetyl-CoA/propionyl-CoA ratio and hence higher 3HB supply, while total 3HV content is less affected. An interesting effect on carbon distribution in the cell was observed with the phosphorus limitation strategy during growth of *R. eutropha* DSM 545 with propionic acid and both propionic and butyric acids (39). Unlike nitrogen limitation, phosphorus limitation negatively affected the specific 3HB production rate and supported 3HV production.

Valeric acid (VA) also serves as precursor for PHA. *R. eutropha* H16 synthesizes the copolymer of P(3HB-co-3HV) with up to 75mol% 3HV when grown with VA. If VA is applied as sole carbon source, valeryl-CoA is degraded *via* β -oxidation to acetyl-CoA and propionyl-CoA. Simultaneous application of butyric acid and VA reduces 3HV molar content, due to higher 3HB content (28). BktB plays also a significant role in metabolism of VA. *R. eutropha* lacking *bktB* retains the capability to grow on VA and on propionic acid, but incorporates higher amounts of 3HV (up to 97mol%) into the copolymer (68).

Propionic and valeric acids are not applicable in high concentrations, because of their toxicity and costs. Utilization of diverse cheap feedstocks supplemented with propionic or valeric acid are more suitable for P(3HB-co-3HV) synthesis. Up to 90% PHA of CDW with

7mol% of 3HV was obtained from palm kernel oil with propionic acid as co-substrate or 89% PHA of CDW with 14mol% of 3HV from olive oil supplemented with VA (63). Generally, higher 3HV fractions from VA in comparison to propionic acid are explained due to sufficient provision of acetyl-CoA for the central metabolism from degradation of plant oils. Thus, less VA is cleaved and directed in the central metabolism, but enhanced integration of 3HV into the copolymer occurs.

Levulinic acid (LA) serves as sole carbon and energy source and as precursor for 3- and 4-hydroxyvaleric acid (4HV) for *R. eutropha*. It appears in waste products of wood industry and can be derived from acidic hydrolysis of starch and lignocellulosic biomass (4). LA is the oxidized form of 4HV, and its catabolism starts with activation to levulinyl-CoA by a membrane-bound acyl-CoA synthetase. The acyl-CoA synthetase activity was absent in cells grown on acetate, demonstrating an inducible character of LA conversion (45). Acyl-CoA dehydrogenase and enoyl-CoA hydratase/carnithine racemase were also induced in presence of LA, together with glutathione transferase and a chaperon, which might be involved in a detoxifying response (5). Acyl-CoA dehydrogenase and enoyl-CoA hydratase/carnithine racemase activity support the assumption that LA is degraded *via* β -oxidation. Thus, the expected end products of the catabolism of LA in *R. eutropha* are acetyl-CoA and propionyl-CoA. Since the β -oxidation inhibitor acrylate prevented growth of *R. eutropha* with LA as sole carbon source, thiolytic cleavage of CoA-derivatives of LA was indirectly approved (5). However, no other intermediates, except acetyl-CoA and propionyl-CoA were observed in *in vitro* enzyme assays, supporting the assumption that levulinyl-CoA is directly cleaved into acetyl-CoA and propionyl-CoA (45).

Since the utilization of LA as sole carbon source yields low values of biomass and PHA content, co-feeding of LA with glucose or fructose increases the productivity. When *R. eutropha* was grown under optimized conditions with glucose and LA, cells reached higher cell densities and stored PHA up to 81% of CDW (120). The 3HV monomer composition

varied from 25 to 54mol%. It was shown that at low LA-concentrations, it was utilized at higher rates as glucose and fructose. Concerning PHA synthesis, 3HV-incorporation was less affected by nitrogen limitation, as it is known for the 3HB-monomer. The highest 3HV fraction in copolymers was observed in the transition growth phase, when nitrogen was still available in excess and before significant 3HB synthesis started. Thus, due to the variation of C/N-ratio the desired PHA composition can be achieved (45). During growth on LA, different monomers, such as 3HV and 4HV, can be incorporated into the copolymer (116). Furthermore, recombinant *R. eutropha* was able to synthesize terpolyesters consisting of 3HB, 3HV and minor amounts of 4HV when cultivated with 4HV or 4-valerolactone (115).

Application of 5-chlorovaleric acid alone or in combination with VA as the carbon source resulted in the synthesis of P(3HB-co-3HV-co-5-hydroxyvalerate) (26). Further substrates, such as 5-hydroxyvalerate and ω -pentadecalactone, were successfully used for synthesis of P(3HB-co-3-hydroxypropionate-co-5-hydroxyvalerate) yielding 5-hydroxyvalerate contents up to 10mol% (20). The PHA-negative mutant *R. eutropha* PHB-4 harboring *phaC_{Re}* or mutated copies of *phaC_{Re}*, was cultivated in a two-step fermentation. In the second step different concentrations of 5-hydroxyvalerate or ω -pentadecalacton were tested. The obtained lipase-degradable copolymer exhibited reduced crystallinity, melting temperature and tensile strengths, and increased elongations to break-values in comparison to P(3HB). Alternatively, when other odd chain compounds like 3-hydroxypropionate (3HP), 1,5-pentanediol or 1,7-heptanediol were used as carbon source, synthesis of P(3HB-co-3HP) with a 3HP-fraction up to 7mol% was observed. From these, 1,5-pentanediol led to the highest copolymer content (78). Copolymers containing 3HP up to 2.1 mol% could be also produced from structurally unrelated substrates *via* modified metabolism of *R. eutropha* (34). Heterologous malonyl-CoA reductase and the 3HP-CoA synthetase domain of trifunctional propionyl-CoA synthase from CO₂-fixation pathway of *Chloroflexus aurantiacus* were

introduced in *R. eutropha* and catalyzed 3HP-formation from acetyl-CoA via malonyl-CoA, when the cells were grown with fructose or even chain alkanates.

Amino acids and modifications on amino acid metabolism. *R. eutropha* H16 synthesizes PHAs also from amino acids as sole carbon source although high yield of PHAs were not obtained, since the dissimilation of amino acids is associated with nitrogen supply (56). Moreover, single amino acids are costly. Grown on amino acids, *R. eutropha* accumulates either P(3HB) or P(3HB-co-3HV). 3HV is provided from propionyl-CoA obtained during degradation of leucine, isoleucine, threonine and valine (Figure S2). L-Valine was studied in more detail, since it was more effective regarding P(3HB-co-3HV), yielding the copolymer with 10mol% of 3HV.

Since propionyl-CoA is involved in the metabolism of some amino acids, an auxotrophic mutant of *R. eutropha* H16 with altered anabolism of branched-chain amino acids synthesized P(3HB-co-3HV) from single unrelated substrates such as fructose or gluconate. The mutant excreted valine, leucine and isoleucine into the medium, when a nitrogen source was available in excess, while ammonium limitation yielded P(3HB-co-3HV) in propionic acid-free medium (106). Such intracellular overproduction of the amino acids or related intermediates provides propionyl-CoA from renewable resources.

Recombinant *R. eutropha* PHB-4 harboring *phaC* from *Pseudomonas* sp. 61-3 synthesized P(3HB-co-3-hydroxy-4-methylvalerate) [P(3HB-co-3H4MV)] on fructose medium supplemented with leucine (95). Amino acid co-feeding was chosen as an alternative to supply of costly 4-methylvalerate, since the leucine backbone is identical with 3H4MV (Figure S2). To increase the 3H4MV fraction without leucine co-feeding, the end product feedback inhibition was overcome by mutagenesis, and an increased metabolic flux to leucine biosynthesis was obtained. Consequently, the mutant strain incorporated higher amounts of 3H4MV when grown on fructose.

L-Serine, L-alanine and L-threonine supplemented in addition to β -oxidation-unrelated substrates (acetic acid, glucose) led to P(3HB-co-4HB) accumulation (57). Supplementation of L-amino acids had a positive effect on CDW with highest values by L-threonine and threefold higher P(3HB-co-4HB) yields, as shown in the absence of amino acids. In presence of L-alanine and L-serine higher 4HB-fractions than with L-threonine occurred. This is putatively due to more favorable utilization of L-threonine for cell growth and 3HB synthesis.

Mercaptoalkanoic acids. Cultivated in presence of sulfur-compounds, such as 3-mercaptopropionic (3MP), 3,3'-thiodipropionic (TDP), 3,3'-dithiopropionic (DTDP), 3-mercaptobutyric (3MB) or 3-mercaptopaleric (3MV) acid, *R. eutropha* incorporates 3MP, 3MB or 3MV into the corresponding copolymers with 3HB (73-74). However, *R. eutropha* cannot utilize these substrates as the sole carbon source. The enzymes mediating uptake of 3MP, TDP and DTDP or cleavage of TDP and DTDP are important for improving the availability of 3MP for polythioester (PTE) synthesis. In contrast, the activity of enzymes metabolizing 3MP to propionyl-CoA are less favorable, since they compete with PTE synthesis for 3MP. PTEs were firstly identified in 2001 (73). The copolymers of 3HB and 3-mercaptoalkanoates exhibit biotechnologically attractive features differing from PHAs in their melting point or glass transition temperature. Moreover, in contrast to PHAs, PTEs are not biodegradable (29).

The metabolism of 3MP, TDP and DTDP in *R. eutropha* is still not completely clear. Genome-wide transcriptome analysis of *R. eutropha* H16 revealed genes which were upregulated and might participate in the utilization of TDP or DTDP (83). Consequently, several deletion mutants were generated to identify genes required for the metabolism of these substrates (25). Single and double deletion mutants lacking ABC-type transporter(s) exhibited slower growth with DTDP (Table S2). Interestingly, only a deletion of the cluster H16_A0357-A0359 led to significantly lower 3MP-fractions in the copolymer and only

during growth with DTDP. In contrast, double deletion $\Delta H16_A0357\text{-}A0359\Delta H16_A3658\text{-}A3660$ resulted in higher 3MP-fractions. Applying DTDP as precursor, less 3MP was also incorporated if *bug* genes encoding putative *Bordetella*-type uptake proteins were deleted. DTDP is probably metabolized by a disulfide reductase to 3MP, while TDP is converted by a sulfide hydrolase to 3MP with concomitant 3HP release. Disulfide interchange proteins DsbA, DsbD, FrnE, rhodanase-related sulfurtransferase and also hydrolase S-adenosylhomocysteinase, might be involved in the cleavage of DTDP since the expression of the corresponding genes was upregulated in presence of sulfur-compounds (83). However, only deletion of *dsbD* led to a significant reduction of 3MP-molar fraction in the copolymer (25).

3MP is further activated to 3MP-CoA and polymerized by PhaC. PhaB3 seems also to be important for P(3HB-*co*-3MP) synthesis, since its expression was two- to fivefold higher than during growth on gluconate (83). On the other hand, the expression of *phaB3* was reduced upon the onset of significant P(3HB) synthesis (11). Genes of FA metabolism were also upregulated during growth with DTDP or TDP suggesting the metabolization of 3MP and 3-hydroxypropionate to propionyl-CoA (Table S2; 83). Other genes, encoding cysteine dioxygenase (CdoA), 2-methylcitrate synthase (PrpC1) and propionate CoA transferase (Pct), of *R. eutropha* were not differentially expressed in the microarray analysis. However, these enzymes have also been studied in terms of PTE synthesis. CdoA converts 3MP to 3-sulfinopropionate (9). 3-Sulfinopropionate can be converted to propionyl-CoA and oxidized in the MCC. The activity of the key MCC-enzyme, PrpC1, was increased in the late stationary phase in presence of 3MP-precursors (83). The lack of *pct* in the corresponding negative mutant of *R. eutropha* did not affect 3MP fraction in the copolymer (69). The gene might be silent but the enzyme was able to activate 3MP with CoA, which suggests that overexpression of *pct* might increase the 3MP fraction in the copolymer.

Since synthesis of P(3MP) homopolythioesters was shown only for *E. coli*, but not for *R. eutropha*, suppression of 3HB-CoA synthesis and deletion of β -ketothiolases was investigated (67). Deletion of 9 of 14 β -ketothiolases in fact resulted in the increased molar 3MP-fraction in the copolymer due to lowered 3HB-contents.

Thus, it was shown that the 3MP-fraction in the copolymer synthesized by *R. eutropha* can be varied. The most important drawbacks like low yield copolymer synthesis and costliness of the precursors need to be addressed. Further improvements by metabolic engineering and establishment of copolymer synthesis from inorganic sulfur as PTE-precursor would be advantageous (123).

Utilization of C1-compounds

Amongst biotechnologically relevant C1-substrates such as CO, CO₂, methanol and formate, only CO₂ and formate are utilized by *R. eutropha* as the sole carbon source (32). CO₂ and formate differ from other substrates discussed in this study, since they are metabolized in a similar manner *via* the CBB cycle.

In the presence of CO₂ as the sole carbon and H₂ as energy source, *R. eutropha* assimilates CO₂ by the key enzyme RuBisCO (Figure 1), while three distinct oxygen-tolerant [NiFe]-hydrogenases deliver energy *via* H₂ oxidation (64). One hydrogenase is membrane-bound and channels electrons into the electron transport chain. The second hydrogenase is soluble and generates reducing equivalents (NADH), which are especially needed in the absence of organic compounds for CO₂-fixation. The third regulatory hydrogenase controls gene expression of the membrane-bound and soluble hydrogenases in response to the availability of H₂ (13, 58).

If formate serves as a substrate, hydrogenases and enzymes of CBB cycle are highly active (32). The activities of two formate dehydrogenases are also increased. These enzymes

oxidize formate to CO₂, which is then fixed in CBB cycle. Because formic acid is very toxic, it is not applicable at high concentrations, as aldehyde it may inhibit enzymes of the central metabolism. In general, it was shown that the cells reduce the amino acid biosynthesis (Shikimate pathway), pyrimidine and purine formation, while the P(3HB) synthesis enzymes demonstrate higher activity in presence of formate (61). Due to its toxicity, low energy potential and low PHA/biomass yield, formate is not suitable as sole carbon source. However, like other VFAs it is a common ingredient of carbohydrate hydrolysates. Moreover, since formic acid can be electrochemically generated from CO₂ and water, it can be further microbially converted to diverse products. Electricity generated from regenerative energies sources can be stored in form of electromicrobial fuels. Recombinant strains of *R. eutropha* were used electromicrobial conversion of CO₂/formate to higher alcohols (47, 65). These auto- and mixotrophical approaches comprise, on one hand, the possibility to store energy and on the other, consume CO₂, which is also beneficial for the environment.

Conclusions

In summary, the metabolic versatility of *R. eutropha* has made this bacterium to a model production strain for PHA metabolism in the past. The understanding of its metabolism based on the availability of the genome sequence and a range of transcriptomic, proteomic and metabolomic data, gene-deletion vectors and expression systems recently broadened the spectrum of applications of *R. eutropha* as a production platform. Thus, the potential for high yield production of diverse tailor-made biopolymers by *R. eutropha* H16 has been demonstrated. The application of metabolically engineered strains of *R. eutropha* revealed a potential of this bacterium as a platform for large-scale biotechnological production of various chemicals and offers an alternative for chemical synthesis. Various ways to extend and optimize the conversion of inexpensive renewable feedstocks have been established. The

autotrophical production of bio-based products is another important separate topic and was only briefly mentioned in this review. However, production of valuable products, derived from the P(3HB) or central metabolism, applying CO₂ as carbon source has been intensively studied. In parallel to chemical synthesis and the application of other production strains, synthesis of additional chemicals like organic acids by *R. eutropha* seems to be promising and should be established in future to replenish the range of synthesized bioproducts. The chemical production of organic acids (acetic, succinic or pyruvic acid) may be replaced by sustainable microbial production. The versatile metabolism of *R. eutropha* can be successfully modulated based on research experience of more than 50 years. Thus, simple, renewable and inexpensive feedstocks can be used by *R. eutropha*. The pathway's bottlenecks can be identified and reconstructed, undesired metabolic fluxes and by-products can be reduced or eliminated based on the advances in genome sequencing, functional genomics, transcriptomics, proteomics and metabolomics.

Declaration of Interest

The authors report no declarations of interest.

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Figure 1 Scheme of the central carbon metabolism of *R. eutropha* illustrating the linkage of KDPG pathway, Calvin-cycle, TCC, C3/C4-metabolism and FA β -oxidation (5, 85, 97).

Abbreviations of enzymes: PTS_{Nag}, phospho transferase system; NAGPase, *N*-acetylglucosamine-6-phosphate deacetylase; GPDA, glucosamine-6-phosphate deaminase; PGI, phosphoglucose isomerase; G6PD, glucose-6-phosphate dehydrogenase; 6PGL, 6-phosphogluconolactonase; EDD, gluconate-6-phosphate dehydratase; EDA, 2-keto-3-deoxygluconate-6-phosphate aldolase; TPI, triosephosphate isomerase; FBPA, fructosebisphosphate aldolase; FBPP, fructose bisphosphatase; G3PD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; PPS, phosphoenolpyruvate synthetase; PRK, phosphoribulokinase; RuBisCo, ribulosebisphosphate carboxylase; PDHC, pyruvate dehydrogenase complex; ME, malic enzyme; OD, oxaloacetate decarboxylase; PCK, PEP carboxykinase; PC, PEP carboxylase; PEP, phosphoenolpyruvate; GlyK, glycerol kinase; Gly3P-DH, glycerol-3-phosphate dehydrogenase; FadD, acyl-CoA synthetase; FadE, acyl-CoA dehydrogenase; FadB, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; FadA, β -ketothiolase.

**BBTN-2014-0124.R1 spell checked
and with corrections accepted**

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Figure S2 Schematic representations of amino acid metabolism as basis for artificial pathways for the production of bio-based products. Squares present the natural metabolites and ellipses indicate the products of interest. Synthesis of such PHA precursors like 4-hydroxybutyrate, 3-hydroxy-4-methylvalerate and propionate; alcohols like isobutanol and 3-methyl-1-butanol was already successfully established (black ellipses) based on native and altered degradation of amino acids (56-57, 95, 106). 1-Propanol, 1-butanol, 2-methyl-1-butanol (grey ellipses) can presumably be derived from the amino acid pathways applying heterologous KIVD, ketoisovalerate decarboxylase, and ADH, alcohol dehydrogenase, as it was shown for *E. coli* (19).

Figure S1

Figure S2

Table S1. Overview of monomers which can be polymerized by the wild type and recombinant strains of *R. eutropha* H16

Monomer	Structure	Precursor substrate
3-Hydroxypropionate (3HP)		3-Hydroxypropionic acid, 1,5-pentanediol, 1,7-pentanediol
3-Hydroxybutyrate (3HB)		Any carbon source
4-Hydroxybutyrate (4HB)		4-Hydroxybutyric acid, γ -butyrolactone, 4-chlorobutyric acid, 1,4-butanediol, valine, isoleucine, threonine
3-Hydroxyvalerate (3HV)		Propionic, valeric, levulinic acid, valine, isoleucine, threonine
4-Hydroxyvalerate (4HV)		4-Hydroxyvaleric acid, 4-valerolactone
5-Hydroxyvalerate (5HV)		5-Hydroxyvaleric acid, 5-chlorovaleric acid, ω -penta-decalactone
3-Hydroxyhexanoate (3HHx)		FAs with carbon chain $\geq C4$
3-Hydroxy-4-methylvalerate (3H4MV)		3H4MV, leucine

3-Hydroxyalkanoates with medium chain length		Medium- and long chain length fatty acids
3-Mercaptopropionate (3MP)		3MP, DTDP, TDP
3-Mercaptobutyrate (3MB)		3MB
3-Mercaptovalerate (3MV)		3MV

Commented [L1]: Image exchanged

Commented [L2]: Image exchanged

Table S2. Overview on genes, which products are responsible for substrate utilization

Utilization of carbohydrates		
<i>N</i> -Acetylglucosamine	<i>nagR</i> , H16_A0310	GntR family transcriptional regulator
	<i>nagF</i> , H16_A0311	Protein-N(pi)-phosphohistidine-sugar phosphotransferase II ABC: <ul style="list-style-type: none"> · EI^{Nag} domain · HPr^{Nag} domain · EIIA^{Nag} domain
	<i>nagE</i> , H16_A0312	Protein-N(pi)-phosphohistidine-sugar phosphotransferase II ABC: <ul style="list-style-type: none"> · cytosolic domain EIIB^{Nag} · membrane-spanning domain EIIC^{Nag}
	<i>nagC</i> , H16_A0313	membrane protein (porin)
	<i>nagA</i> , H16_A0314	<i>N</i> -Acetylglucosamine-6-phosphate deaminase (NAGPase)
	<i>nagB</i> , H16_A0315	Glucosamine-6-phosphate deaminase (GPDA)
	<i>zwfI</i> , H16_A0316	Glucose-6-phosphate 1-dehydrogenase
Fructose	<i>frcA</i> , H16_B1498	ABC-type transporter ATPase
	<i>frcC</i> , H16_B1499	ABC-type transporter permease
	<i>frcB</i> , H16_B1500	ABC-type transporter periplasmic protein
	<i>zwf2</i> , H16_B1501	Glucose-6-phosphate dehydrogenase (G6PD)
	<i>pgi</i> , H16_B1502	Phosphoglucose isomerase (PGI)
	<i>frcK</i> , H16_B1503	Fructokinase
Gluconate	<i>eddI</i> , H16_A1178	Gluconate-6-phosphate dehydrogenase
	<i>gntK</i> , H16_A1179	Gluconate kinase
	<i>eda</i> , H16_B1213	2-Keto-3-deoxy-6-phosphogluconate aldolase
	<i>gntP</i> , H16_A3011	Gluconate permease
	<i>gntT</i> , H16_B1207	Gluconate transporter
PEP-PTS homologous proteins	<i>ptsM</i> , H16_A0324	Phosphotransferase system, fructose-specific component IIA
	<i>ptsH</i> , H16_A0325	Phosphocarrier protein (HPr)
	<i>ptsI</i> , H16_A0326	Phosphoenolpyruvate-protein kinase (PTS system EI component)
	<i>ptsN</i> , H16_A0384	Phosphotransferase system mannitol/fructose-specific IIA domain-containing protein
Mannose	<i>pmi</i> , H16_B1152	Phosphomannose isomerase (PMI)
Xylose	H16_A3317	Ribulose-phosphate 3-epimerase (RPE)
Glycerol	<i>glyK</i> , H16_A2507, H16_B1199	Glycerol kinase GlyK
	H16_A2508, H16_B1198	Glycerol-3-phosphate dehydrogenase (Gly3-DH)
Utilization of organic acids		
Long chain length FA	H16_A0459-A0464	Fatty acid degradation operon
	H16_A1526-A1529	Fatty acid degradation operon
Levulinic acid	H16_A1090	Glutathione transferase
	H16_A1091	Acyl-CoA dehydrogenase
	H16_B1346	Enoyl-CoA hydratase/carnithine racemase
	H16_B1347	Chaperone
Mercaptoalkanoic acids	H16_A0357-A0359	ABC-type transporter
	H16_A3658-A3660	
	<i>ahcY</i> , H16_A0244	Hydrolase S-adenosylhomocysteinase
	<i>dsbA</i> , H16_A0161; <i>dsbD</i> , H16_A3455;	Thiol:disulfide interchange proteins

	<i>frnE</i> , H16_B2322	Dithiol-disulfide isomerase
	H16_A2779, H16_A0337, H16_A3718- H16_A3720	Bug-like substrate binding proteins
	H16_B1102	Acetyl-CoA synthase
	H16_A0234 H16_B1192	Acyl-CoA dehydrogenase
	H16_A0179	Enoyl-CoA hydratase
	H16_B0782	Acyltransferase
	<i>cdoA</i>	Cysteine dioxygenase
	<i>prpC1</i> , H16_A1906	2-Methylcitrate synthase
	<i>pct</i> , H16_A2718	Propionate CoA-transferase