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Engineering of Biological Systems for the Synthesis of Tailor-made Polyhydroxyalkanoates, a Class of Versatile Polymers

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Abstract: Medium-chain-length polyhydroxyalkanoates (mcl-PHAs) are bacterial polyesters which are produced in nature by certain *Pseudomonas* strains. These biopolymers are of interest because of their chirality, biodegradability and elastomeric property, while the 3-hydroxy acid monomers are a potential source of chiral synthons. Since wild-type bacteria can only be used to a limited extent for the production of tailor-made functionalized PHAs, various recombinant bacteria have been generated in order to elucidate the PHA synthesis pathway and to control and modify the metabolic carbon flux towards PHA synthesis. Production of PHAs with altered monomer composition and physical properties has already been achieved by pathway engineering. An alternative to the *in vivo* synthesis strategies using whole microorganisms is PHA synthesis *in vitro* with isolated enzymes, which is of interest for incorporation of specific monomers that are not taken up or metabolized by bacterial cells. The various synthesis strategies are discussed in the context of the possible future production of tailor-made functionalized PHAs.

Keywords: Biopolymers · Genetic engineering · *in vitro* synthesis · PHA · Polyhydroxyalkanoates · Tailor-made polymers

Introduction

Bacterial polyhydroxyalkanoates (PHAs) are polyesters of 3-hydroxy acids produced as intracellular granules by a large variety of bacteria. The first example of polyhydroxyalkanoates to be discovered was polyhydroxybutyrate (PHB) [1]. PHB accumulation is found in various microorganisms, representatives of Gram-negative and Gram-positive species (*i.e.* autotrophs, heterotrophs, phototrophs, aerobes, anaerobes) and archaebacteria (as reviewed elsewhere [2–4]). Bacteria synthesize and accumulate PHAs as carbon and energy storage materials under conditions of limiting nutrients in the presence of excess carbon source. When the supply of the limiting nutrient is restored, the PHA can be degraded by intracellular depolymerases and subsequently metabolized as carbon and energy source [5]. The general structure of the molecule is shown in the Figure. The monomer units in these microbial polyesters are all in the *R*-configuration due to the stereospecificity of the biosynthetic enzymes. The molecular weights of the polymers range from 2 x 10^5 to 3 x 10^6, depending on the specific polymer, the microorganism and growth conditions.

The discovery of a polyester consisting mainly of 3-hydroxyoctanoate monomers by de Smet *et al.* [6] was the first example of a new group, so called medium-chain-length (mcl) PHAs which can contain a wide variety of different monomers. To date, more than 120 different monomers have been found in the polymers [7][8]. Among these are 3-hydroxy acids of 6 to 14 carbon atoms with a large variety of saturated, unsaturated, straight

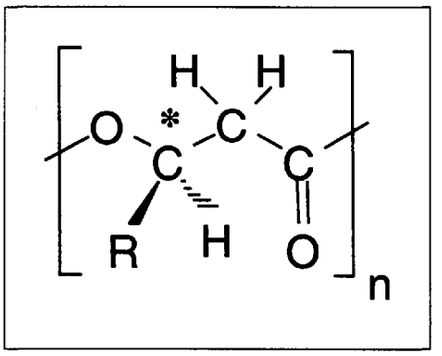

Fig. 1. General structure of polyhydroxyalkanoates. PHAs are classified as short-chain-length PHAs (R=C_{1-2}) which show a high crystallinity or medium-chain-length PHAs (R=C_{3-12}) which are elastomeric materials. If a functional group is incorporated in the side chain, the polymers are often called functionalized PHAs. The number of monomers (n) varies between 1000 and 30 000.

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or branched chains containing aliphatic or aromatic side groups. Furthermore, monomers with various different functional groups in the side chain such as halogen atoms, hydroxy-, epoxy-, cyano-, carboxyl- and esterified carboxyl groups have been introduced into mcl-PHAs (see review [3][8][9]). The mcl-PHAs are of interest for specific uses where chirality and elastomeric property of the polymers are important. In addition, the monomers of PHAs that contain different functional groups in their side chain are receiving more and more attention as sources of chiral synthons.

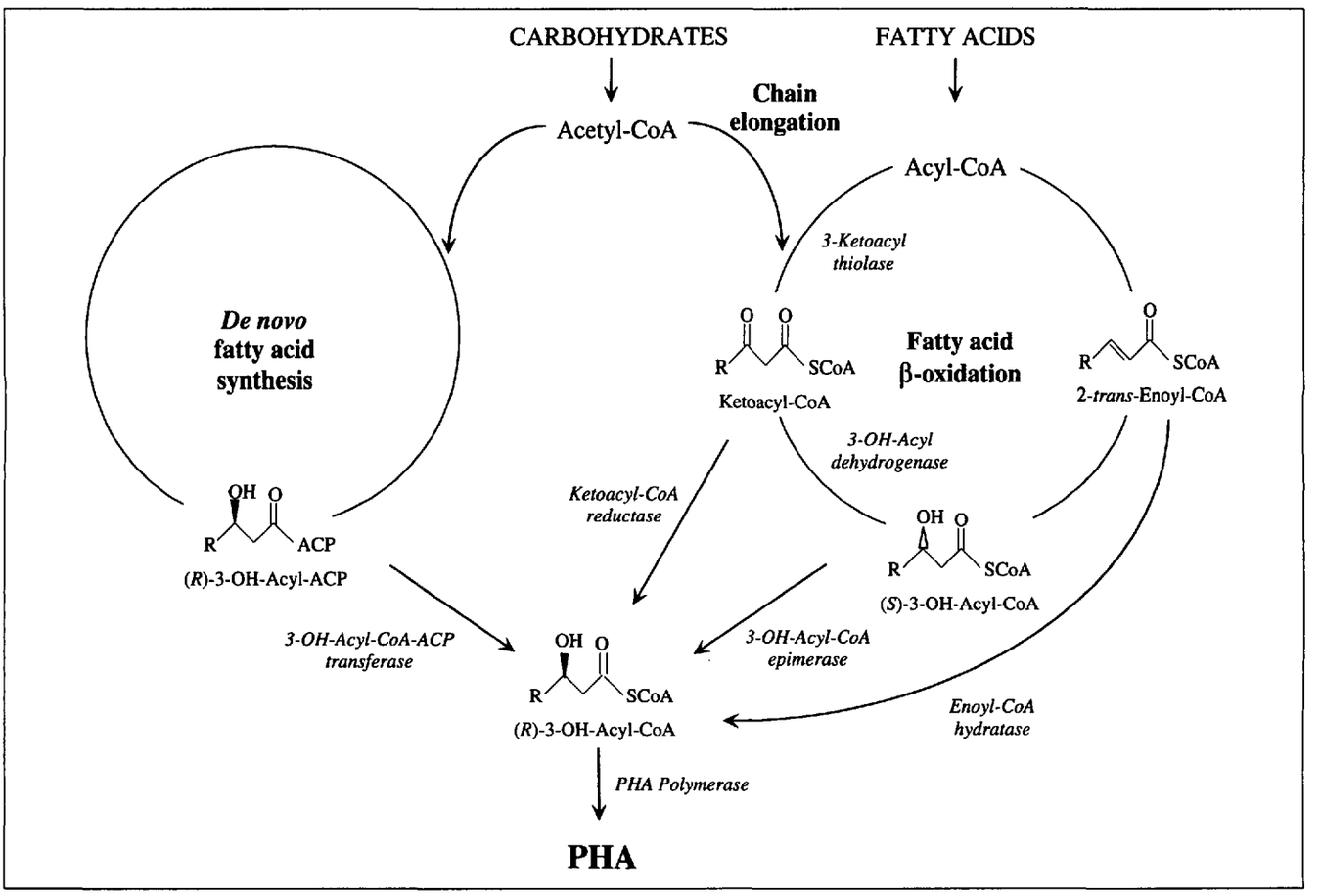
PHA Synthesis in Wild-Type Organisms

Although many species of bacteria are reported to synthesize PHB, including some *Pseudomonas* strains, the ability to accumulate mcl-PHAs is unique to the group of Pseudomonads. The best studied mcl-PHA producers are *P. oleovorans* GPo1, *P. putida* KT2442 and *P. aeruginosa* PAO1. These bacteria contain two PHA polymerases (also called PHA synthases) encoded by *phaC1* and *phaC2* of the *pha* gene cluster [10][11]. It has been shown for *P. oleovorans* that the two PHA polymerases have a small difference in substrate specificity [12]. Moreover, it was demonstrated that both polymerases are functional proteins which are able to catalyze PHA formation independently from each other, *i.e.* only one of the polymerase encoding genes is enough to produce mcl-PHA in heterologous hosts [12–15].

In *Pseudomonas* three main pathways are involved in the synthesis of mcl-PHA precursors [16][17] (Scheme 1): i) fatty acyl chain elongation, in which acyl-coenzyme A (CoA) is extended with acetyl-CoA; ii) fatty acid degradation by β-oxidation, the main pathway when fatty acids are used as substrate; and iii) *de novo* fatty acid biosynthesis, which is the main route during growth on simple carbon compounds. Based on *in vitro* studies (see below) it is likely that the immediate precursor for polymerization is the (*R*)-form of 3-hydroxyacyl-CoA [18]. Furthermore, the existence of two (*R*)-specific enoyl-CoA hydratases with a substrate specificity towards short- or medium-chain-length substrates in *P. aeruginosa* [19] indicates that the PHA synthesis pathway proceeds *via* a stereospecific hydratase reaction rather than the epimerase activity of the β-oxidation (Scheme 1). Moreover, it could be shown that a 3-hydroxyacyl-CoA-ACP-transferase links the *de novo* fatty acid biosynthesis pathway to PHA synthesis [20] and provides the (*R*)-3-hydroxyacyl-CoA precursor for polymerization (Scheme 1).

Generation of PHA-producing *Escherichia coli* Recombinants

Since wild-type *Escherichia coli* bacteria are not able to produce mcl-PHA and the genetics and physiology of these bacteria are very well studied, *E. coli* has been used to investigate the PHA synthesis pathway and its connection to fatty acid metabolism. If one of the PHA polymerase-encoding *phaC* genes is introduced into commonly used *E. coli* laboratory strains, no PHA accumulation



Scheme 1. Major pathways involved in mcl-PHA synthesis in *Pseudomonas*. Chain elongation, fatty acid β-oxidation and *de novo* fatty acid biosynthesis are involved in mcl-PHA synthesis. Structural formulae are included only for putative mcl-PHA precursor intermediates, such as (*R*)-3-OH-acyl-ACP, ketoacyl-CoA, (*S*)-3-OH-acyl-CoA and 2-*trans*-enoyl-CoA. Enzyme names are written in italics. ACP - acyl carrier protein, CoA - coenzyme A.

can be detected. However, *E. coli* strains blocked in the 3-ketoacyl thiolase (FadA) or 3-hydroxyacyl-CoA dehydrogenase (FadB) enzyme activity of the β-oxidation pathway (Scheme 1) are able to synthesize mcl-PHAs when one of the *phaC* genes of *Pseudomonas* is expressed [13][14][21]. It is assumed that the β-oxidation has to be slowed down in *E. coli* in order to accumulate specific intermediates which can serve as precursors for PHA synthesis. Interestingly, the polymer has a composition similar to that of the polymer synthesized by *Pseudomonas* (Table), although due to the blockage of the β-oxidation, production of a homopolymer had been expected. This indicates that the β-oxidation mutants used are either leaky or other enzymes which can also perform the reaction substitute for the knocked out enzymes. Various *phaC*-expression systems have been used: depending on the carbon source and growth conditions used, PHA amounts up to 33% of cell dry weight have been achieved [13]. The PHA content of these β-oxidation deficient *E. coli* strains could even be further increased to up to 50% of cell dry weight by using acrylic acid, a β-oxidation inhibitor [22].

Recently, it has been shown that mcl-PHA can also be produced in *E. coli* strains containing a functional, non-inhibited β-oxidation pathway. Overexpression of a 3-ketoacyl-CoA reductase encoding gene of *P. aeruginosa* or *E. coli* in addition to one of the PHA polymerase encoding genes resulted in the production of 3 or 8% PHA per cell dry weight, respectively [23][24]. Moreover, *E. coli* recombinants containing, in addition to a PHA polymerase, (*R*)-specific enoyl-CoA hydratases of *P. aeruginosa* with substrate specificities towards either short- or medium-chain-length substrates, produced 29% or 14% PHA per cell dry weight, respectively [19].

Pathway Engineering for Synthesis of PHAs with Altered Physical Properties

In addition to the elucidation of the mcl-PHA synthesis pathway, *E. coli* is an useful candidate to control and modify the carbon flux towards PHA. This is a requisite for the production of polymers with desired monomer composition and physical properties.

Experiments with different substrates (*i.e.* fatty acids with different chain lengths) or different *E. coli* hosts failed to produce PHAs with altered physical properties [25]. However, it appeared that the monomer composition of the PHA produced by different *E. coli* recombinants is determined by the substrate specificity of the introduced enoyl-CoA hydratase or 3-ketoacyl-CoA reductase [19][25]. Thus, specific *E. coli* recombinants can be engineered in order to produce polymers with desired monomer compositions. In addition, it could be shown that pathway engineering can be used to synthesize mcl-PHAs with altered physical properties (Table). Introduction of the acetoacetyl-CoA reductase of *Ralstonia eutropha* and blockage of the ketoacyl-CoA degradation step of the β-oxidation not only caused significant changes in the monomer composition but also caused an increase of the molecular weight and loss of the melting point [25]. A high-molecular weight peak of around 1 000 000 Da was observed that could be caused by the higher C6 monomer content of the polymer and which might alter the ratio of chain elongation to chain termination events, resulting in longer PHA chains. Another possibility is that the high-molecular weight peak is due to the presence of C6 monomer stretches which facilitate strong non-covalent interactions among PHA chains and thus result in the formation of microgels [25].

Table. Monomer composition and physical properties of mcl-PHA isolated from different bacteria

Strain	Monomer composition [mol%] C6	C8	C10	Mw [Da]	Mw/Mn	Tg [°C]	Tm [°C]
P. putida	11	89	<1	187 000	2.4	–31.0	56
E. coli JMU193 (*phaC*⁺)	11	76	13	59 800	2.1	–33.8	55.1
E. coli JMU194 (*phaC*⁺)	20	57	23	71 300	3.0	–32.7	53.9
E. coli JMU194 (*phaC*⁺, *phbB*⁺)	42	38	20	97 000 & 1 000 000	1.8	–30.1	n.d.

C6 = 3-hydroxyhexanoate, C8 = 3-hydroxyoctanoate, C10 = 3-hydroxydecanoate, Mw = molecular weight, Mw/Mn = polydispersity, Tg = glass transition temperature, Tm = melting temperature, JMU193 = *fadB* β-oxidation mutant, JMU194 = *fadA* β-oxidation mutant, *phaC* = gene encoding PHA polymerase, *phbB* = gene encoding acetoacetyl-CoA reductase, n.d. = not detectable.

Stable Recombinants for PHA Production Purposes

In summary, it is possible to produce mcl-PHA polymers in recombinant *E. coli*. However, the lack of stability of the recombinants is often a major drawback for the production of sufficient amounts of PHA [13]. In addition, a major problem in general in applying plasmid containing recombinants in large scale fermentations is plasmid maintenance and stability. The classical approach to maintain the phenotype of the recombinant strain is to add antibiotics to the culture medium. This can have a considerable effect on the reproducibility of the results and the final cost of the product. An attractive alternative using minitransposons for stable, regulated and inexpensive *phaC* gene expression in recombinant bacteria has been developed by Prieto *et al.* and the stability of the system to culture mcl-PHA producing recombinant *E. coli* in a bioreactor operated in batch or continuous cultivation mode in the absence of a selection marker has been exploited [26]. The phenotype was 100% stable throughout the fermentation process.

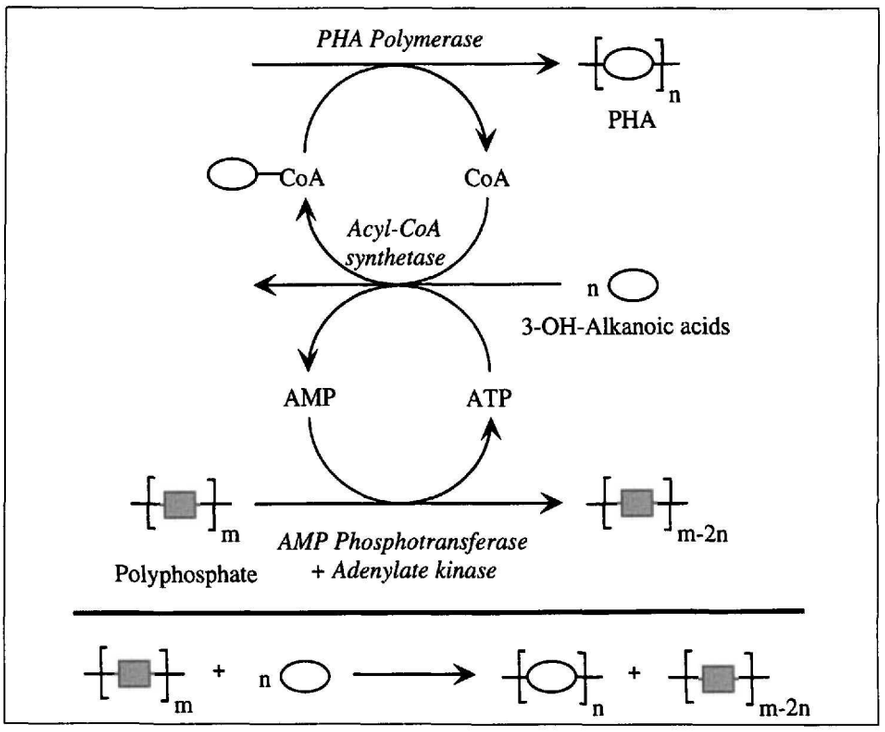
PHA Synthesis with Isolated Enzymes

An alternative approach to the described production of PHA *in vivo* using whole microorganisms is the *in vitro* synthesis of PHA using isolated enzymes. This is of interest if specific functional groups which are either toxic to the cells or are removed during the synthesis process are to be incorporated into PHA.

In vitro synthesis of PHA can be performed in two steps. First, (*R*)-3-hydroxyalkanoyl-CoA, the substrate of the PHA polymerase, can be synthesized enzymatically by coupling free CoA to (*R*)-3-hydroxyalkanoate using an acyl-CoA synthetase. This reaction is energy-de-

pendent and requires ATP. Second, an ester linkage is formed between the (*R*)-3-hydroxyalkanoyl-CoA substrates by the PHA polymerase and CoA is released again [18][27]. Since the co-substrate CoA is very expensive and inhibits the PHA polymerase at high concentrations, the two reactions have been coupled in order to recycle CoA [28]. Furthermore, it is desirable to regenerate ATP, for example by an AMP phosphotransferase/adenylate kinase system [29], in order to develop a cost effective process. Thus, the optimal *in vitro* PHA synthesis system is a multi-enzyme system (Scheme 2) which is currently being developed (de Roo, unpublished results).

Although PHA polymerases have been purified to homogeneity and no primer has been added to the *in vitro* PHA synthesis reaction mixture, a primer might be attached to and co-purified with the PHA polymerase [28][30]. Thus far, it is not known how the initiation of a polymer chain happens and what factors are involved in the chain termination.



Scheme 2. Multi-enzyme *in vitro* PHA synthesis system. A multi-enzyme system consisting of acyl-CoA-synthetase, PHA polymerase, AMP phosphotransferase and adenylate kinase is required for cost efficient synthesis of PHA *in vitro* using 3-hydroxyalkanoic acids and polyphosphate as substrates. CoA and ATP are added only in catalytic amounts and are regenerated by the system.

Conclusions

In addition to wild-type mcl-PHA producers which have been used in the past, we are now able to produce mcl-PHA with various engineered biological systems. Taking together all the information obtained thus far for PHA producing *E. coli* recombinants and considering that fermentation and downstream process technologies are well established for *E. coli*, *E. coli* is clearly an interesting candidate for the production of specific designed PHA polymers. *In vitro* synthesis of mcl-PHA is less likely to become competitive in the foreseeable future. However, the basis has been established and the system may become useful for incorporation into PHAs of specific monomers which are not taken up or are metabolized by cells, or for the synthesis of high molecular weight polymers. In any case, it is not likely that mcl-PHA can ever compete with petroplastics for commodity applications. Instead, mcl-PHAs will find applications as specialties such as hydrophobic coatings, medical implants, functionalized polymers for chromatography, microgranules to be used as binders in paints or in blends that incorporate latexes, and as sources of chiral monomers. In this sense, engineering and investigation of various biological systems for the synthesis of tailor-made mcl-PHAs is of major interest.

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