Doctoral Thesis

Production of bioplastic in recombinant bacteria from basic research to application

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Production of Bioplastic in Recombinant Bacteria:
From Basic Research to Application

A dissertation submitted to the
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Zürich, 1999
Das Ganze ist mehr als die Summe seiner Teile.

Christian von Ehrenfels

für Birgit
Acknowledgments

After nearly four years of working in science, I finally made it and finished my PhD thesis. Following a rule of our Tuesday morning seminars, I asked myself, what was it good for? Well, mainly I had the opportunity to learn. After learning about bioplastics, bacterial physiology, organizing my research and publishing papers, I learned also a great deal about time. E. g. I had to accept Hofstädters law, that “it always takes longer than you expect, even when you take into account Hofstädters Law”. I also learned that “deadlines might be flexible” and that “playing around” is an integral part of science. For these opportunities, I would like to thank all the people who supported me during the past years, especially:

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Curriculum Vitae
Summary

Polyhydroxyalkanoates (PHAs) are polyesters accumulated in a wide variety of bacteria as carbohydrate reserve material, when grown on carbon substrates in excess and limiting resources of nitrogen. Since medium chain length (mcl) PHAs, a group of PHAs with a monomer length of 6 to 14 carbon atoms, have not only interesting polymer characteristics but are in addition biodegradable and can be produced from renewable resources, these polyesters are being investigated as alternative material to conventional thermoplastics.

Several strategies for the fermentative production of mcl PHAs have been explored. However, the economic breakthrough of bacterially produced biodegradable polyesters on the market place has not yet been achieved. One promising new strategy to produce mcl PHAs from renewable resources at low prices is the development of PHA-accumulating transgenic plants.

The aim of this thesis was to develop methods for mcl PHA production in bacteria, which could be transferred to plants and thereby enable commercial mcl PHA production. To this end, we structured the task into the following approaches: We wanted to develop a method for mcl PHA production in a non-PHA producing bacterial organism (Chapter 2). We aimed at isolating gene(s) which play an important role in mcl PHA biosynthesis in bacteria (Chapter 3) and we tried to increase mcl PHA production from simple substrates in Pseudomonas bacteria (Chapter 4).

Plants and E. coli do not produce PHA. Thus, we chose E. coli to be our bacterial model organism in order to generate a mcl PHA producer by recombinant DNA technology. In Chapter 2 we describe the generation of this recombinant by introduction of genes necessary for mcl PHA production from simple substrates like gluconate. We found that by action of a thioesterase and the PHA synthase an artificial mcl PHA biosynthesis pathway involving both β-oxidation and fatty acid synthesis could be established.
Since the maximum mcl PHA accumulation in transgenic plants is <0.5 % (w/w), we tried to develop methods to increase mcl PHA in bacterial model organisms. One approach was to find important PHA genes, which could be transferred into plants. In Chapter 3, we describe such a gene named *phaD*, which is important for mcl PHA production in *Pseudomonas* bacteria. A knock out of this gene showed negative affects on mcl PHA accumulation which were reversed by genetic complementation. Although we were not able to establish the function of the gene precisely, we found that it affected another PHA gene coding for a granule associated protein. Thus, we propose that the gene investigated codes for a novel type of PHA protein, which interacts indirectly with the granule structure. In Chapter 4 we present another method for increasing mcl PHA accumulation by using a metabolic engineering approach. By reducing the activity of isocitrate dehydrogenase in addition to inactivation of the glyoxylate shunt, we were able to increase mcl PHA formation significantly. According to our hypothesis this is achieved by directing intracellular carbon flow from the citric acid cycle towards the fatty acid synthesis pathway.

Since this thesis was part of the EU research project entitled “Sustainable production of biodegradable polyesters in starch-storing crop plants”, the goal of this project was to develop methods for mcl PHA production in bacterial systems which might be transferable to plants able to accumulate mcl PHA. With respect to knowledge-transfer a first step has already been taken. The genes which we discovered to be necessary for generating a mcl PHA producing bacterium (namely a thioesterase and the PHA synthase) are now being expressed in potatoes in the group of Dr. G. Eggink (ATO-DLO, Wageningen, the Netherlands) and in peas in the group of Dr. T. Wang (John Innes Centre, Norwich, UK).
Zusammenfassung


Es wurden bisher mehrere biotechnologische Fermentationsprozeduren zur großtechnischen Erzeugung dieser Polyester entwickelt. Trotzdem ist der wirtschaftliche Durchbruch der mcl PHA mit Hilfe dieser Technologie nicht gelungen. Eine vielversprechende neue Technik zur kommerziellen Produktion der PHA ist die Entwicklung von mcl PHA-produzierenden transgenen Pflanzen.

Das Ziel dieser Doktorarbeit war die Erarbeitung von Wissen und Methoden, die die kommerzielle Herstellung von mcl PHA in Pflanzen möglich machen sollen. Um dieses Ziel zu erreichen, haben wir folgende Ansätze verfolgt: Wir wollten eine Methode entwickeln, um in einem nicht-PHA produzierenden Bakterium mit Hilfe der rekombinanten Gentechnologie den Polyester herstellen zu können (Kapitel 2).

Wir haben versucht ein Gen zu isolieren welches eine wichtige Rolle in der PHA Biosynthese spielt (Kapitel 3). Wir wollten durch “metabolic engineering” die PHA Ausbeute in Pseudomonas Bakterien erhöhen (Kapitel 4).

Pflanzen, wie auch die E. coli Bakterien, produzieren natürlicherweise keine PHA. Deshalb haben wir E. coli gewählt, um in diesem bakteriellen Modellorganismus die mcl PHA Synthese durch Einbringen von wichtigen Genen zu erreichen. Im Kapitel 2 beschreiben wir die Herstellung dieses mcl PHA-produzierenden E. coli Bakteriums, in welches wir die Gene der Thioesterase und der PHA Synthase transformiert haben. Unsere Hypothese zum Biosyntheseweg des mcl PHA in
diesem rekombinanten Organismus beinhaltet die Stoffwechselwege der β- 
Oxidation von Fettsäuren und der Fettsäuresynthese. 
Da die bisher produzierten transgenen Pflanzen lediglich <0.5 % (w/w) mcl PHA 
produzieren, war eine wichtige Zielrichtung unserer Projekte die Steigerung der mcl 
PHA Ausbeute am Beispiel von bakteriellen Modellorganismen. Im Kapitel 3 haben 
wir deshalb ein neues Gen untersucht, welches offensichtlich eine wichtige Rolle 
für hohe Ausbeuten in der mcl PHA Produktion spielt. Wenn wir das Gen im 
Bakterium inaktivieren, wird die Polyestersynthese negativ beeinträchtigt. Wenn 
wir in dieser Mutante das Gen wieder zufügen, werden die Wildtyp-Charakteristika 
der mcl PHA Produktion wiederhergestellt. Obwohl wir die genaue Funktion dieses 
Gens bisher nicht aufklären konnten, nehmen wir an, daß es indirekt im 
Zusammenhang mit einem wichtigen PHA-Granula-assoziierten Protein wirkt. Im 
Kapitel 4 zeigen wir eine Möglichkeit, wie man die Ausbeute von mcl PHA in 
*Pseudomonas* Bakterien durch Eingriffe am Citratzyklus deutlich erhöhen kann. 
Durch Inaktivierung des Glyoxylatzyklus und Senkung der Aktivität der 
Isocitratdehydrogenase konnten wir intrazelluläre Metabolite vom Citratzyklus zur 
Fettsäuresynthese und damit zur mcl PHA Produktion umleiten. 
Wir hoffen mit unserer Arbeit Wissen bereitgestellt zu haben, welches auf 
pflanzliche Systeme übertragbar ist und somit die Herstellung von transgenen PHA-
produzierenden Pflanzen unterstützen wird. Mit der Expression der Thioesterase 
und der mcl PHA Synthase Gene (Kapitel 2) in Kartoffeln durch die Arbeitsgruppe 
von Dr. G. Eggink (ATO-DLO, Wageningen, Niederlande) und in Erbsen durch die 
Arbeitsgruppe von Dr. T. Wang (John Innes Centre, Norwich, UK) ist ein erster 
Schritt getan, um diesen Wissentransfer zu gewährleisten.
CHAPTER 1

General Introduction
Chapter 1  General Introduction

1.1. Green Chemistry and Biotechnology

1.1.1. A new era of producing chemicals

For chemists, the traditional synthesis paradigm of basing decisions on product yield and the cost of feedstocks with limited concerns for the creation of waste or the toxicity of the product and byproducts is no longer economically or environmentally viable (74). Therefore, pollution prevention has resulted in a new way for scientists to think about chemistry. In addition to the traditional parameters for cost and yield, today’s chemist must also consider the human health and environmental impact of new chemicals and the processes used to make them. This is the essence of green chemistry (4, 74). In the field of green chemistry the new organic synthesis approaches have recently been complemented by biotechnological approaches, in which chemicals are produced by using biological systems. Biocatalysis, the production of a substance de novo by living cells, and biotransformation, the conversion of one substance into another, catalyzed either by whole cells or by isolated enzymes (46), can have several advantages compared to organic synthesis: Biotechnological manufacturing processes are powerful tools for regio- and stereoselective transformations (46). Furthermore, biotechnological processes are often more environmentally friendly compared to organic synthesis based processes (9, 23, 50). Therefore, biotechnology has become an indispensable tool for the manufacture of chemicals, polymers, chiral synthons and pharmaceuticals, and its importance in these fields will increase (46).
1.1.2. Biotechnology: From bioremediation to manufacturing

Environmental biotechnology has the intention of increasing sustainability of production processes by employing biological systems and thereby benefiting the environment (27). In the past, environmental biotechnology has focused mainly on bioremediation strategies to decontaminate polluted soil and groundwater. In a number of cases, it has been shown that bacteria are able to degrade many of these environmental pollutants. However, the persistence of these compounds in the environment suggests that biodegradation does not occur effectively in situ. Therefore, more recent efforts have focused on phytoremediation - the use of green plants for in situ (24, 60). Although the remediation field obtained a boost by novel developments in the field of phytoremediation, more recently a transition of environmental biotechnology from remediation to manufacturing is taking place (20).

One example, illustrating this transition, is the paper-making industry. Deinking recycled paper with solvents, breaking down wood pulp, and bleaching with chlorine all produce environmentally damaging waste streams. In the past, research has focused on detoxifying these wastes biologically. However, recently, work has moved further upstream in the paper-making process, with the aim of producing novel enzymes like xylanases, which break down lignin in trees during the pulping process, and cellulases, which digest wood fibers into pulp, and protocols that avoid the production of toxic substances (20). In addition to cleaning up industries that already rely on biological raw materials, biotechnology is starting to provide renewable alternatives to traditional petrochemicals including biodegradable polymers.

As these examples show, and as industrial researchers have hoped for years, biotechnology can offer important new manufacturing approaches that extend well beyond the pharmaceutical industry. Using nontoxic, renewable resources, genetically engineered enzymes and organisms may soon be producing materials as diverse as paper and plastic (20).
Polymers represent the most abundant class of organic molecules of our biosphere (22). Complex polymers like coal and lignin occur in amounts of approximately $835,000 \times 10^6$ and $700,000 \times 10^6$ tonnes, respectively. Furthermore, approximately $40,000 \times 10^6$ tonnes cellulose and $20,000 \times 10^6$ tonnes lignin are currently synthesized each year (68). In contrast to these numbers, the production of approximately $100 \times 10^6$ tonnes per annum of various plastic materials is rather low (12). However, although only comparably small amounts of plastics are produced, these materials are the source of increasing problems (68). Since most of the plastic material is used for short-term applications, it contributes to the increasing amount of solid waste. Landfill used to be an important route of disposal in Europe but landfill capacity is diminishing nowadays (49). To overcome this problem three methods for the reduction of solid waste have been studied: material recycling, which has the disadvantage of downgrading materials quality constantly, thermal recycling or incineration, which is by now in practice in many countries (67), and composting. Incineration and recycling together divert a substantial fraction of polymer refuse away from landfill. However, some critics argue that municipal solid-waste recycling, as it is practised now, is not a sustainable option (30). Therefore, and for applications where polymer recycling is impractical or impossible, composting might be the option of choice. For composting materials must be biodegradable, which means that the chemical structure of the material is changed by biological activity leading to naturally occurring end products (49). Most of the current large volume polymers are not biodegradable. Thus, biodegradation for waste disposal can only become a reality when new biodegradable polymers and facilities for biodegradation become available. Although biodegradable polymers have fundamental advantages compared to conventional thermoplastics, such as their biodegradability and their production from renewable feedstocks, they also have disadvantages. One major reason which limits their application is their high price (2). Other concerns arise from the lack of evidence of a positive life-cycle-assessment balance (27), the production of greenhouse gases other than carbon
dioxide when composted in landfills (63) and their poor physical and technical properties (68). However, since the evaluation of many of the biodegradable polymer species is still at the very beginning, it is assumed that further studies will probably give hints for overcoming the problems these polymers are facing today (77). The most important biodegradable polymers are either natural polymers and derivatives (starch, cellulose acetate), synthetic polymers (polylactic acid, polyglycolic acid) and microbial polyesters (polyhydroxyalkanoate).

Starch is a food source stored in most plants. It is a polymer of D-glucose, containing amorphous and crystalline regions of linear amylose and branched amylpectin (49). The main material characteristic of starch is its hydrophilicity, hence limiting the useful applications. To reduce sensitivity of starch to water it can be blended with more hydrophobic compounds. Starch polymers are readily biodegradable whereas starch blends showed decreased biodegradation compared to the pure polymer (57). The key applications are in the pharmaceutical and food packaging field (49). Another natural biopolymer, which is also commercially available, is cellulose acetate. Cellulose (D-glucose connected via β-1,4 glycosidic bonds) acetate is a modified polysaccharide produced via the reaction of acetic anhydride with wood pulp. Biodegradation depends on the degree of substitution, which is expressed as the average number of acetyl groups per anhydroglucose unit. Biodegradation is inversely proportional to the degree of substitution and is also reduced by blending (45). Applications include adhesive tape and textiles (49). Polylactic acid (PLA) is a synthetic polymer, being produced via the polymerisation of lactic acid followed by the copolymerisation with hydroxy acids (47). High molecular weight PLAs exhibit the attractive properties of polystyrenes, enabling thermoforming, without displaying the brittleness inherent in styrene polymers. The main application has been in the medical field where the high production costs can be absorbed (49). Polyglycolic acids (PGA) are the simplest linear aliphatic polyesters being produced by polymerisation of glycolic acid. To reduce crystallinity copolymerisation e. g. with lactic acid is carried out (28). Although PLA and PGA are biodegradable it has not been shown whether they decompose easily.
Polyhydroxyalkanoates (PHA), which are microbial polyesters, are the best researched group of biodegradable plastics (49).

1.3. Polyhydroxyalkanoates: An overview

1.3.1. History

Polyhydroxyalkanoates are polyesters synthesized and stored in the form of granules by numerous bacteria. The observation of the granules as refractile bodies in bacterial cells under the microscope goes back at least to Beijerinck in 1888 (14). The first determination of the composition of PHA had to wait until 1926 and the work of Lemoigne (43). Although the potential usefulness of the polymers had already been recognized in the 1960s (8), until the mid 1970s the interest in the biopolymers remained directed almost solely at their physiological role (19, 64). In the mid 1970s, following the rise of oil prices, the search for alternative plastic material focussed industrial interest on poly-3-hydroxybutyrate P(3HB). In 1976 Imperial Chemical Industries (ICI) of England started investigating whether P(3HB) could be profitably produced by bacterial fermentation. In 1993 Zeneca Bioproducts took along the polyester fermentation activities initiated by ICI and in 1996 Monsanto bought the production business from Zeneca. The process using Ralstonia eutropha (formerly Alcaligenes eutrophus) produced a copolymer consisting of poly-3-hydroxybutyrate-3-hydroxyvalerate P(3HB/V) and marketed under the tradename BIOPOL. The production volume was 800 tons per year. Monsanto terminated its activities in this area at the end of 1998 and closed the
PHB/V production plant. Other companies which remain active in R & D of polyhydroxyalkanoates in 1999 include Procter & Gamble and Metabolix in the USA.

1.3.2. Storage Material

PHAs are accumulated as discrete granules to levels as high as 90% of the cell dry weight and are generally believed to play a role as sinks for carbon and reducing equivalents. When nutrient supplies are imbalanced, it is advantageous for bacteria to store excess nutrients intracellularly. By polymerizing soluble intermediates into insoluble molecules, the cell does not undergo alterations of its osmotic state and leakage of these valuable compounds out of the cell is prevented (78). Once PHAs are extracted from the bacterial cell, however, these molecules show material properties that are similar to some common plastics such as polypropylene (13). The bacterial origin of the PHAs make these polyesters a natural material, and, indeed, many microorganisms have evolved the ability to degrade these macromolecules. Besides being biodegradable, PHAs are recyclable like the petrochemical thermoplasts.

1.3.3. Chemical and physical structure

Many different PHAs that have been identified to date are linear, composed of 3-hydroxy fatty acid monomers. In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Fig. 1.1.). In all biologically produced PHAs that have been characterized so far, the hydroxyl-substituted carbon atom is of the R configuration. An alkyl group, which can vary from methyl to tridecyl, is positioned at this C-3 or β-position. However, this alkyl side chain is not necessarily saturated: aromatic, unsaturated, halogenated, epoxidized, and branched monomers have been reported as well (72). Substituents in the side chains of PHAs can be modified chemically, for instance by cross-linking of unsaturated bonds (15). The variation in the length and composition of the side chains and the ability to modify their reactive substituents
Chapter I 8 General Introduction

is the basis for the diversity of the PHA polymer family and their vast array of potential applications that are described below. Copolymers of P(3HB/3HV), which is the most studied polymer, can be formed by cofeeding of substrates. Together, polymers containing such monomers form a class of PHAs typically referred to as short-side-chain or short-chain-length PHAs (scl PHAs). In contrast, medium-side-chain or medium-chain-length PHAs (mcl PHAs) are composed of C6 to C16 3-hydroxy fatty acids. These PHAs are synthesized from fatty acids or other aliphatic carbon sources (41). Mcl PHAs are also synthesized from carbohydrates, but the composition of these PHAs is not related to the carbon source (34).

Fig. 1.1.: Chemical structure of PHAs. PHAs are generally composed of (R)-3-hydroxy fatty acids, where the pendant group “R” varies from methyl (C1) to tridecyl (C13). The best known PHAs are P(3HB) (R = methyl), P(3HB-HV) (R = methyl or ethyl), and P(HO/3HH) (R = pentyl or propyl).

The molecular mass of PHAs varies per PHA producer but is generally in the order of 50,000 to 1,000,000 Da. Bacterially produced P(3HB) and other PHAs, however, have a sufficiently high molecular mass to have polymer characteristics that are similar to conventional plastics such as polypropylene. Within the cell, P(3HB) exists in a fluid, amorphous state. However, after extraction from the cell with organic solvents, P(3HB) becomes highly crystalline (18) and in this state it is a stiff and brittle material. Because of its brittleness, P(3HB) is not very stress resistant. Also, the relatively high melting temperature of P(3HB) (around 170°C) is close to the temperature where this polymer decomposes thermally and thus limits the ability to process the homopolymer. Initial biotechnological developments were therefore aimed at making PHAs that were easier to process. The incorporation of 3HV into the P(3HB) resulted in a P(3HB/3HV) copolymer that is less stiff and brittle than
P(3HB). In contrast to P(3HB) and P(3HB/3HV), mcl PHAs have a much lower level of crystallinity and are more elastic (16).

1.3.4. Environmental aspects

Biotechnologists face an enormous challenge in fully exploring the PHA biology to ensure that environmentally friendly polyesters are available for generations to come (36). Besides the typical polymeric properties described above, an important characteristic of PHAs is their biodegradability. In nature, a vast consortium of microorganisms is able to degrade PHAs by using secreted PHA hydrolases and PHA depolymerases (37). The degradation rate of a piece of P(3HB) is typically in the order of a few months (in anaerobic sewage (Fig. 1.2.) to years (in seawater) (10, 37). As important as the biological characteristics and biodegradability of PHAs is the fact that their production is based on renewable resources. Fermentative production of PHAs is based on agricultural products such as sugars and fatty acids as carbon and energy sources. These agricultural feedstocks are derived from CO₂ and water, and after their conversion to biodegradable PHA, the breakdown products are again CO₂ and water. Thus, while for some applications the biodegradability is critical, PHAs receive general attention because they are based on renewable compounds instead of on our diminishing fossil fuel stockpiles (81).
PHAs are natural thermoplastic polyesters, and hence the majority of their applications are as replacements for petrochemical polymers currently in use for packaging (e.g. cheese packages) and coating applications. Initial efforts focused on molding applications, in particular for consumer packaging items such as bottles, cosmetic containers, pens, and golf tees (79). Diaper backsheet materials and other materials for manufacturing biodegradable or compostable personal hygiene articles from P(3HB) copolymers other than P(3HB/3HV) have been described (52). PHAs have also been processed into fibers which then were used to construct materials such as nonwoven fabrics (66). P(3HB) and P(3HB/3HV) have been described as hot-melt adhesives (40). PHAs can be used as a latex, for instance for paper-coating applications (44), or as paint additives. In addition to its range of material properties, PHAs promise to be a new source of chiral synthons.
1.3.6. PHA biosynthesis pathways

1.3.6.1. P(3HB) biosynthesis

Biosynthesis of P(3HB) is carried out via three enzymatic reactions. The first reaction consists of the condensation of two acetyl-CoenzymeA (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl-CoA thiolase encoded by \( p_{ldoA} \). The second reaction is the reduction of acetoacetyl-CoA to \((R)-3\)-hydroxybutyryl-CoA by an NADPH dependent acetoacetyl-CoA dehydrogenase, encoded by \( phhB \). Lastly, \((R)-3\)-hydroxybutyryl-CoA is polymerized by PHB synthase, encoded by \( phbC \) (Fig. 1.3., upper pathway).

P(3HB) is just one type of the many PHAs that are synthesized by numerous different microorganisms, all originating from their own ecological niche and with their own evolutionary history. Among the PHA producing bacteria a great variation of biosynthesis pathways exist (36). A pathway which is similar to that described above but is somewhat extended leads to a copolymer of P(3HB) and polyhydroxyvalerate: P(3HB/3HV). \( R_hodoococcus\ ruber \) accumulate PHAs containing 3HV even in the absence of 3HV precursors in the feed, such as propionate or valerate (6), since the 3HV monomer is derived from acetyl-CoA and propionyl-CoA, where the latter is a product of the methylmalonyl-CoA pathway (80) (Fig. 1.3.).

1.3.6.2. PHA biosynthesis from related substrates

1983 Witholt and coworkers described a novel type of PHA, the mcl PHA, accumulated in \( P_{seudomonas\ oleovorans} \) grown on octane (17).
Fig. 1.3.: Biosynthetic pathway for production of P(3HB/3HV). The upper pathway summarizes biosynthesis of P(3HB). Some microorganisms accumulate P(3HB/3HV) without supplementation of propionate, valerate, or other C_odd fatty acids. Propionyl-CoA in these species is formed through the methylmalonyl-CoA pathway, which originates from succinyl-CoA in the citric acid cycle. Propionyl-CoA and acetyl-CoA are converted to P(3HB/3HV) in the typical PHB synthesizing enzymes: 1 = \( phbA \) coding for \( \beta \)-ketoacyl-CoA thiolase, 2 = \( phbB \) coding for acetoacetyl-CoA dehydrogenase, 3 = \( phbC \) coding for PHB synthase.
Subsequent studies showed that the compositions of the PHAs formed by Pseudomonads of the rRNA homology group I were directly related to the structure of the alkane, alkene or fatty acid carbon sources (11, 35). Thus, these substrates are referred to as related substrates. When the carbon source consists of fatty acids with 6 to 12 carbon atoms, monomers in the PHA are of the same length as the carbon source or have been shortened by 2, 4 or 6 carbon atoms. When the carbon source is a straight chain C13-C18 fatty acid, the composition of the polymer resembles that of C11- and C12-grown bacteria (35). Use of mixtures of hydrocarbons or fatty acids as the carbon source results in the formation of PHAs. The composition of which reflects the ratio of the two carbon sources in the feed. It was shown that during growth on fatty acids PHA biosynthesis in *Pseudomonas* bacteria depends on the fatty acid β-oxidation pathway (33). The substrate specificity of the mcl PHA synthases ranges from C6 to C14 (R)-3-hydroxyalkanoyl-CoA, with a preference for C8, C9 and C10 monomers. However, since the β-oxidation intermediate is (S)-3-hydroxyacyl-CoA, an additional biosynthetic step is required for synthesis of (R)-3-hydroxyacyl-CoA monomer. Whether this is the product of a reaction catalyzed by an enantiospecific hydratase, by an epimerase or by a 3-ketoacyl-CoA reductase is unknown (Fig. 1.4.).

Given the different biosynthetic pathways, it is not surprising that the *pha* loci in the mcl PHA forming *Pseudomonas* are very different from the *pha* loci in the scl-PHA forming bacteria. In *Pseudomonas oleovorans* and *Pseudomonas aruginoasa*, two closely linked genes encoding mcl PHA synthases (PhaCl and PhaC2) are separated by one open reading frame coding for a PHA depolymerase (PhaZ). The two synthases of Pseudomonas are 64% identical in their primary structure. Downstream of the *phaC2* gene an open reading frame of unknown function was detected (*phaD*). Recently, downstream of *phaD*, *phaF* and *phaI* were identified, encoding proteins, associated with PHA granules. Furthermore, it was shown that the PhaF has a function as a negative regulator of *phaC1* expression (Fig. 1.5.).
FIG. 1.4.: Mcl PHA biosynthesis from related substrates. Fluorescent Pseudomonads of the rRNA homology group I can derive monomers for PHA from fatty acid degradation. The pathway involves: 1 = acyl-CoA dehydrogenase, 2 = enoyl-CoA hydratase, 3 = 3-hydroxyacyl-CoA dehydrogenase, 4 = ketoacyl-CoA thiolase and 8 = mcl PHA synthase. Whether an epimerase (7), an enoyl-CoA hydratase (5) or a ketoacyl-CoA reductase (6) is involved in formation of (R)-3-hydroxyacyl-CoA is unknown.
Another example of a microorganism that produces PHA from related substrates is *Aeromonas caviae*. It produces a random copolymer of 3HB and 3-hydroxyhexanoate (3HH), when growing on even-numbered fatty acids or olive oil as sole carbon source. The *pha* locus of *A. caviae* consists of an enoyl-CoA hydratase encoding gene (*phaJ*) and a granule associated protein encoding gene (*phaP*) beside the *phaC* gene, coding for the PHA synthase. PHA production in *A. caviae* proceeds from the β-oxidation pathway intermediate 3-hydroxyenoyl-CoA, which is hydroxylated by the enantiospecific (R)-3-hydroxy-enoyl-CoA hydratase. Other examples of PHA production from related monomers are obtained with strains from *R. eutropha* and *Rhodococcus ruber*. *R. eutropha* accumulated a PHA containing 2-methyl-3-hydroxybutyric acid from tiglic acid (25), while *R. ruber* and other related Gram-positive bacteria synthesized PHAs from 3-hydroxypivalic acid containing 2-dimethyl-3-hydroxypropionic acid (26).

![Diagram](image)

**Fig. 1.5.:** Molecular organisation of the *pha* gene cluster in *Pseudomonas oleovorans*. C1, Z, C2, D, F, I represent the names of the *pha* genes. Pc1 depicts the promoter region upstream of the *phaC1* gene. Loops show the position of putative transcriptional terminators.

### 1.3.6.3. PHA biosynthesis from unrelated substrates

Substrates which do not structurally resemble the polymer monomers are named unrelated. The synthesis of PHAs others than P(3HB) which are not related to growth substrates occurs relatively seldom in nature (70). It relies on the diversity of central carbon intermediates to CoA thioesters which can serve as PHA precursors and enable the synthesis of different PHAs from renewable resources, such as carbohydrates, lipids or CO₂.
Several fluorescent Pseudomonads of the rRNA homology group I, e.g. *P. putida* KT2442, accumulate a PHA that consists primarily of C10 and C8 monomers, when grown on sugars or gluconate (34, 73). Experiments suggest that these monomers are derived from intermediates of fatty acid biosynthesis and that the composition of the PHAs is probably a reflection of the pool of fatty acid biosynthetic intermediates (Fig. 1.6.). Evidence for the involvement of fatty acid biosynthesis in PHA formation from glucose was obtained by inhibition experiments. When cerulenin (a fatty acid synthesis inhibitor) is added to such cell suspensions, no PHA is formed from glucose whereas PHA is still synthesized from fatty acids (33). These experiments confirmed that PHA formation from glucose is linked to fatty acid biosynthesis. Since fatty acid biosynthesis proceeds via (R)-3-hydroxyacyl-ACP, an enzymatic activity is required that converts this intermediate to (R)-3-hydroxyacyl-CoA. Recently, Rehm et al. determined that the gene product of *phaG* is responsible for this conversion (58).

Other PHA biosynthesis pathways include the citric acid cycle for generation of PHA precursors (Fig. 1.7.). In recombinant *E. coli* a polymer of 4-hydroxybutyrate (4HB) was produced withdrawing succinyl-CoA from the citric acid cycle which is converted to 4HB and introduced in the PHA polymer (75). In addition to intermediates from the fatty acid synthesis pathway and the citric acid cycle, the metabolism of amino acids also provides interesting precursors for PHA biosynthesis (Fig. 1.7.). It has been demonstrated that a mutant of *R. eutropha* that overproduces valine and isoleucine in the presence of sufficient ammonium accumulated P(3HB/3HV) from gluconate and other unrelated substrates under ammonium deficiency (71). Presumably 2-keto acids of the respective amino acids were degraded under these conditions to propionyl-CoA, resulting in incorporation of 3HV (70).
Fig. 1.6.: Biosynthetic pathway for mcl PHA from carbohydrates or gluconate in *Pseudomonas*. Monomers for PHA biosynthesis are derived from fatty acid synthesis including the enzymes: 1 = ketoacyl-ACP synthase, 2 = ketoacyl-ACP reductase, 3 = 3-hydroxyacyl-ACP dehydrase, 4 = enoyl-ACP reductase, 5 = acyl-ACP-CoA transacylase, 6 = PHA synthase.
Other pathways from unrelated substrates include the polyketide synthases (Fig. 1.7.), producing a polymer with poly(3-hydroxy-4-hexenoate) as constituent in transgenic \textit{S. coelicolor} (65). Some \textit{Pseudomonas} spp. can incorporate both scl and mcl PHA monomers in the same polymer chain. Typically, these PHAs are formed when these strains are grown on unrelated carbon sources such as carbohydrates or 1,3-butanediol (1). The PHA synthases synthesizing these scl and mcl PHAs must therefore have a very broad substrate range. This type of mixed PHA is probably exceptional since it has been shown that physical constraints prevent the formation of mixed granules containing both P(3HB) and mcl PHA chains. This was concluded from experiments where a recombinant \textit{P. putida} strain containing both the chromosomal phaC and a copy of the \textit{R. eutropha} phbC on a plasmid was shown to accumulate individual granules composed of either P(3HB) or mcl PHA (56).

Since precursor substrates (e.g. propionic acid, butyric acid) or other related substrates (e.g. fatty acids) are in general more expensive than sugars or molasses and furthermore many of them are toxic (e.g. propionic acid), one major aspect of current research is the synthesis of PHAs from simple carbon sources (70).

\subsection*{1.3.6.4. Mcl PHA production by recombinant organisms}

Mcl PHA biosynthesis proceeds through the action of only one enzyme, which is essentially involved in PHA formation, namely the PHA synthase (59). In addition, a range of other activities affects the amount of PHA that is accumulated, including enzymes that are involved in central metabolism, global metabolic regulation, or control and maintenance of the surface of PHA granules (53, 59).
Fig. 1.7.: PHA synthesis from unrelated substrates. The conversion of carbon sources like carbohydrates, gluconate or CO₂ into PHA other than P(3HB) is shown. Pathways which are utilized include the amino acid biosynthesis, the citric acid cycle (TCA), the polyketide synthesis and the fatty acid synthesis.
Since PHA formation is dependent on the fluxes in central metabolic pathways and the levels of precursors, a detailed knowledge of the molecular physiology of PHA metabolism is critical for successful implementation of transgenic PHA producers (36). Unlike the production of heterologous proteins, which relies mostly on sufficient gene expression, recombinant PHA production involves coordinated expression of heterologous enzymes over a prolonged period and with a concomitant redirection of the metabolism of the host. Only with this knowledge can recombinant processes be successfully developed and lead to what are expected to be the most efficient PHA production processes (36).

1.3.7. PHA granules

PHA granules having a diameter of 350 nm occur frequently in bacteria (5). The core of the granule consists of the hydrophobic polymer matrix surrounded by a phospholipid layer. Three different types of proteins are associated with the phospholipid layer around the PHA granule. The PHA synthase, which couples 3-hydroxyalkanoate monomers to growing polymer chains, the depolymerase, responsible for degradation of the storage polyester, and other granule binding proteins, which might have a structural function (69). The characteristics of these structural proteins are reminiscent of those of oleosins, proteins that associate exclusively with the oil bodies of oil-producing plants. For that reason, these proteins are generally referred to as phasins. It appears that oleosins play a structural role in maintaining the integrity of individual oil bodies by preventing their coalescence (32).

1.3.8. Metabolic engineering

Metabolic engineering is the improvement of industrial organisms using modern genetic tools (7). The ultimate goal is to predict in quantitative terms how to tailor the metabolism of the cell for maximal production of metabolites of industrial interest (21). For more than a decade, metabolic engineers have studied the physiological consequences of particular genetic changes in microorganisms, plants,
and animals, have characterized genome-wide responses of these systems, and have devised mathematical and computational methods to understand connections between genes and cell function. The metabolic engineering goal of identifying genes that confer a particular phenotype is conceptually and methodologically congruent with central issues in drug discovery and functional genomics. Biological research in the past century has established a rich catalog of complicated metabolic pathways, regulatory networks, and other molecular systems in cells. In spite of the complexity in biology, simplifying assumptions have had an important role in a period of scientific and technological progress. Such simplifications are embodied in the terms "rate-limiting step," "drug target," and "function of a gene," all of which are based on the assumption that each single gene product has a significant effect on the elaborate informational and biochemical networks that operate cells. However, there are growing indications that in many cases either single genes do not affect phenotype, or that their influence on phenotype does not arise in a simple, obvious fashion (7). For example, efforts to enhance the ethanol production rate in yeast by overexpression of each enzyme in the glycolysis pathway have produced no significant rate increase (62). From these and other examples, it is nowadays considered a fact, that knowledge of individual enzymes and pathways is insufficient to understand metabolism in its entirety (21). This view is also based on theoretical considerations of the biochemical systems theory. The founding publications of metabolic control analysis called into question the oversimplified assumption that a "rate-limiting step" dictated the rate at which a linear sequence of chemical reactions operates in steady state (29). Instead, this analysis offered a general mathematical proof that activities of multiple steps in the pathway influence the overall rate of the pathway. This theory presages the necessity, now realized experimentally in several metabolic engineering projects, of simultaneous change in several critical genes in order to achieve a significant shift in phenotype (7).
1.3.8.1. Stoichiometric flux analysis

One important technique, which gained popularity in describing the highly complex and nonlinear reaction systems of a cellular metabolic network, is stoichiometric flux analysis (76). The various types of metabolic flux analysis offer a descriptive snap-shot of the physiology of the cell system at different levels of complexity. The simplest of the flux analysis techniques is the flux balancing analysis (FBA). FBA involves the solution of a set of linear algebraic equations relating experimental measurements of substrate inputs and product outputs to the steady-state intracellular flux distribution via a stoichiometric model. The origin of FBA follows from the work of Aiba and Matsuoka (3) who calculated fluxes for citrate production in Candida lypolitica for the purpose of identifying the most experimentally consistent metabolic model. Although widely applicable to a diverse range of systems, strictly speaking FBA is restricted to steady-state situations (although scientists have applied it to analyze fed-batch fermentations of fungi by assuming a pseudo-steady-state (39)) and may require restrictive assumptions regarding metabolically active pathways or cofactor dependence. A complementary approach to the determination of intracellular flux distribution follows from the application of nuclear magnetic resonance (NMR) and/or gas chromatography/mass spectroscopy GC/MS techniques in conjunction with $^{13}$C labeling and traditional FBA (76). This approach augments traditional FBA by providing key intracellular information, such as flux ratios at metabolite branch points, that can function as constraints that, in combination with the stoichiometric model, identify intracellular fluxes (61).

1.3.8.2. Dynamic mathematical models of metabolic networks

While flux analysis provides a descriptive snap-shot of physiology, clearly, its role in the rational design or reengineering of metabolism is limited by its inability to address the dynamic evolution of the system, since stoichiometric models contain no kinetic or regulatory information. Thus, to predict how metabolite or enzyme
levels change with time, the stoichiometric foundation must be augmented by a
description of the systems kinetics and regulatory processes. It is only through this
combination that scientists can arrive at a comprehensive predictive methodology
for the rational design of metabolic networks (76).

1.3.9. Can biotechnology move us toward a sustainable society?

Although conventional, fossil oil-based polymers, such as polyethylene,
polypropylene, and polystyrene offer good material properties at a low price, their
environmental impact and manufacture has traditionally been viewed in a negative
light. As a result, alternative plastic materials like polyhydroxyalkanoates are being
explored that are both biodegradable and produced from renewable resources,
preferably in order to improve the solid-waste management and generate a
sustainable polymer production (27).

Several factors contribute to the environmental impact and the degree of
sustainability of a given product or material. In many instances, however,
environmental impact is the result, not of the product per se, but rather of the
consumption of raw materials and the release of waste products generated during
manufacture. Thus, a "cradle-to-grave" analysis or life-cycle-assessment provides a
good benchmark for assessing environmental impact and sustainability (31).

Recently, Gerngross used life-cycle-assessment to compare the impact of the
production of 1 kg of PHA and 1 kg of polystyrene. Assumptions for PHA
production included a fermentative process with glucose as substrate, a high yield
(> 100 g/L) and a high polymer content (80% PHA w/w) in bacteria. All production
related raw materials and energy consumption were normalized in fossil fuel
equivalents. Gerngross found that the amount of fossil fuel, required to produce 1
kg of PHA (2.39 kg) exceeds that required for the production of 1 kg polystyrene
(2.26 kg) (27).

Thus, the production of PHA using corn as a feedstock with current fermentation
technology is of questionable environmental benefit. Although biological processes
that use renewable resources certainly have the potential to conserve fossil
resources, this case study demonstrates that such an approach can also have the reverse effect. Therefore, future assessments of biological processes must not only incorporate the use of raw materials (which are mostly renewable), but also address the indirect consumption of nonrenewable energy sources required for the process.

1.3.10. PHA production in plants

Taking into account the results of life-cycle-assessment on PHAs produced by fermentation (27), it seems logical to engineer plants to produce polyhydroxyalkanoates. Recently, efforts have been made to produce P(3HB) in plants. Stable expression of the \( phb \) genes has been achieved and the P(3HB) produced is chemically identical to the bacterial products with respect to the thermal properties (melting point, glass transition temperature), while the molecular weight distribution of the polymer was much broader. Still, a significant fraction of the plant P(3HB) had a molecular weight of 1,000,000, which indicated that plants can make P(3HB) of sufficient quality for industrial processing (55).

A first successful attempt, introducing PHA biosynthesis in plants was made using Arabidopsis thaliana, since it is the model organism of choice for heterologous expression studies in plants (54). Because of the presence of endogenous thiolase activity, only the \( phbB \) and \( phbC \) genes from R. eutropha were transfected, resulting in the accumulation of P(3HB) granules in the cytoplasm, vacuole, and nucleus. The expression of the \( phb \) genes had an adverse effect on growth which was possibly due to the depletion of acetyl-CoA from an essential biosynthetic pathway (54). An improved plant production system was subsequently developed by expressing all three \( phb \) genes in the plastid of A. thaliana. The plastid was targeted for P(3HB) production because of the high level of acetyl-CoA in this organelle, which is the site for lipid biosynthesis. A maximum amount of P(3HB) of 14% of the dry weight was accumulated in the leaves (51).

Mcl PHA was produced in recombinant A. thaliana targeting the PhaC1 encoding gene from Pseudomonas aeruginosa to the peroxisomes. The plants produced 0.4% mcl PHA consisting of various monomers (48). The results indicate that the \( \beta- \)
oxidation of plant fatty acids can generate a broad range of \( R-3 \)-hydroxyacyl-CoA intermediates that can be used to synthesize mcl PHAs. Although P(3HB) and mcl PHA synthesis has been achieved in plants, the results obtained so far clearly indicate that there is a long road ahead. In contrast to microorganisms, metabolism in plants is mostly compartmentalized, which complicates the tasks at hand (36). Current and future developments in the molecular biology of plants will undoubtedly find rapid application in the pursuit of PHAs in plant crops. An intriguing development is the potential for transgenic P(3HB) to play a role in engineering new characteristics into existing materials such as cotton (38). Obviously, the limits of transgenic PHA production are unpredictable (36).

### 1.3.11. Scope of this thesis

The work of this thesis was embedded in the international EU-research project “Sustainable production of biodegradable polyesters in starch-storing crop plants”. The overall goal of this project was to produce transgenic plants able to accumulate mcl PHA used for industrial applications. An interdisciplinary group of biological scientists, including experts from microbiology and plant biotechnology, focussed on the identification of bacterial genes and mechanisms important for high-level mcl PHA biosynthesis in order to transfer and express relevant genes in plants to generate mcl PHA producing plants.

Within the EU-project framework the aim of this thesis was to study bacterial synthesis of mcl PHA in order to 1) develop methods for mcl PHA production in recombinant organisms 2) isolate genes which play an important role in mcl PHA biosynthesis in \textit{Pseudomonas} and 3) increase mcl PHA accumulation in bacteria.

### 1.3.11.1. Chapter 2

In this chapter we describe experiments aimed at the development of a method for mcl PHA production from non-related substrates in recombinant organisms. We chose \textit{E. coli} as a non-PHA-producing model organism. Previously, it was shown
that recombinant *Escherichia coli*, defective in the β-oxidation cycle and harbouring a mcl PHA polymerase encoding gene of *Pseudomonas*, are able to produce mcl PHA from fatty acids but not from sugars or gluconate (42, 59). Since related substrates (e.g. fatty acids) are in general more expensive than unrelated substrates (e.g. carbohydrates such as glucose or gluconate) and many of them are highly toxic (e.g. propionic acid), one major goal of current research is the synthesis of as many PHAs as possible from simple carbon sources (70). Thus, we defined our research goal in enabling *E. coli* to produce mcl PHA from non-related carbon sources such as carbohydrates or gluconate.

By introduction of genes coding for a mcl PHA synthase and the cytosolic thioesteraseI (‘thioesteraseI) into *E. coli* JMU193, we were able to engineer a pathway for synthesis of mcl PHA from gluconate. On the basis of our data we propose a mcl PHA biosynthesis pathway scheme for recombinant *E. coli* JMU193, harbouring PHA synthase and ‘thioesteraseI, when grown on gluconate, which involves both *de novo* fatty acid synthesis and β-oxidation.

1.3.11.2. Chapter 3

In order to identify genes in *Pseudomonas* which contribute to high-level mcl PHA formation, we investigated the role of the *phaD* gene in *Pseudomonas oleovorans*. In *P. oleovorans* major mcl PHA biosynthesis genes are clustered in the PHA locus, which comprises six genes. Five genes have been described and it was shown that they play an important role in mcl PHA biosynthesis. Only for a gene named *phaD* a participation in mcl PHA biosynthesis has yet not been described. In this chapter, we examined whether *phaD* is involved in mcl PHA biosynthesis in *P. oleovorans* by generation of a *phaD* knock out mutant. We found that upon *phaD* inactivation *P. oleovorans* was negatively affected in PHA biosynthesis. However, since we showed that the PhaD protein is not associated to PHA granules, it might belong to a novel class of proteins involved in PHA biosynthesis acting via an indirect mechanism.
1.3.11.3. Chapter 4

In this chapter we present a metabolic engineering approach to increase mcl PHA accumulation in *Pseudomonas putida*.

We have during the past few years made several sets of mutants with the goal to alter the carbon flux towards mcl PHA. While most of these mutants showed clearly reduced mcl PHA levels, a few have shown increased PHA production. Preliminary analysis of one of these mutants (*P. putida* KT217) showed it to be affected in the glyoxylate pathway. This mutation inactivated the glyoxylate shunt and reduced *in vitro* activity of isocitrate dehydrogenase, a rate-limiting enzyme of the citric acid cycle. During growth on gluconate mcl PHA accumulation was significantly higher in the mutant compared to the *P. putida* KT2442 wild type. Stoichiometric flux analysis predicted that the knock out of the glyoxylate pathway in addition to reduced flux through isocitrate dehydrogenase should lead to increased flux into the fatty acid synthesis pathway. Therefore, we assume that enhanced carbon flow towards the fatty acid synthesis pathway increased the amount of mcl PHA that could be accumulated by the mutant.

1.3.11.4. Chapter 5

Chapter 5 is the closing Chapter of the thesis. A summary is given at the beginning describing the motivation for the production of mcl PHA accumulating plants and for carrying out this PhD project. Additionally, the feasibility of generating transgenic mcl PHA plants is assessed and the findings obtained in Chapter 2-4 in this thesis are set in a larger framework. Finally an outlook for the development of sustainable products and processes is given.
References


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CHAPTER 2

Production of Medium Chain Length Poly (3-Hydroxyalkanoates) from Gluconate by Recombinant *Escherichia coli*

STEFAN KLINKE, QUN REN, BERNARD WITHOLT, and BIRGIT KESSLER

Summary

It was shown recently that recombinant *Escherichia coli*, defective in the β-oxidation cycle and harbouring a medium chain length poly(3-hydroxyalkanoate) (mcl PHA) polymerase encoding gene of *Pseudomonas*, are able to produce mcl PHA from fatty acids but not from sugars or gluconate (25, 32). In this study we report formation of mcl PHA in recombinant *E. coli* from gluconate. By introduction of genes coding for a mcl PHA polymerase and the cytosolic thioesterasel (‘thioesterasel) into *E. coli* JMU193, we were able to engineer a pathway for synthesis of mcl PHA from gluconate. We used two expression systems i.e., bad promoter and alk promoter for the ‘thioesterasel and PHA polymerase encoding genes, respectively, which enabled us to modulate their expression independently over a range of inducer concentrations which resulted in a maximum mcl PHA accumulation of 2.3 % of cell dry weight from gluconate. We found that the PHA amount and the ‘thioesterasel activity are directly correlated. Moreover, the polymer accumulated in the recombinant *E. coli* consisted mainly of 3-hydroxyoctanoate monomers. On the basis of our data we propose a mcl PHA biosynthesis pathway scheme for recombinant *E. coli* JMU193, harbouring PHA polymerase and ‘thioesterasel, when grown on gluconate, which involves both *de novo* fatty acid synthesis and β-oxidation.
Introduction

Polyhydroxyalkanoates (PHAs) are polyesters of 3-hydroxyacids produced as intracellular granules by a large variety of bacteria (10, 23, 37). Because of their potential use as biodegradable thermoplastics and as biopolymers being produced from renewable resources, PHAs have been the focus of extensive research of groups from academia and industry (2, 5, 36). Pseudomonads synthesize mainly medium chain length (mcl) PHAs, formed of monomers of 6 to 14 carbons (9, 21, 24). Although PHAs have been commercially developed and marketed (18), the widespread use of these polymers has been hindered by the high cost of production (1, 27). Therefore alternative strategies for PHA production are being investigated. Reduction of PHA production costs can be achieved by several means including utilizing cheap substrates especially carbohydrates such as sugars or molasses (17, 28, 41) or enhancing the product yield e.g. by employing recombinant *Escherichia coli* (27). *E. coli* holds promise as a source for economical PHA production because of its high productivity, the easy purification of PHA and the lack of a depolymerase system degrading the synthesized polymer (13, 15, 27). Moreover transgenic plants are potential candidates for large scale production at relatively low prices, if PHAs can amount to 20 - 40% of the dry weight (29, 30, 39).

Since all wild type *E. coli* and wild type plants are unable to synthesize PHA, these organisms have to be equipped with at least the PHA polymerase encoding gene, which is the key enzyme for PHA accumulation, connecting 3-hydroxyacyl-CoA units. From studies on *P. oleovorans* it is known that two PHA polymerases exist, named PhaC1 and PhaC2 (22). Recently it was found that recombinant *E. coli* deficient in β-oxidation and harbouring a mcl PHA polymerase can produce mcl PHAs when grown on related substrates such as fatty acids; whereas, from carbohydrate substrates such as glucose no PHA was accumulated (25, 32). From studies on *Pseudomonas putida* KT2442 (3), it is known that three main pathways are involved in the synthesis of the 3-hydroxyalkanoate precursors, which are β-
oxidation, *de novo* fatty acid biosynthesis and elongation of 3-hydroxyalkanoates by acetyl-CoA molecules. During growth on fatty acids mainly the β-oxidation pathway is active whereas during growth on carbohydrate or carbohydrate derived substrates such as sugars or gluconate the fatty acid synthesis pathway provides the PHA precursors (20). It could be shown that both metabolic routes, β-oxidation and *de novo* fatty acid biosynthesis, can function simultaneously in the synthesis of PHA (19). Much less is known about the link between the metabolites of the fatty acid metabolic pathways and the PHA precursor. It has been shown that *Pseudomonas* contains a 3-hydroxyacyl acyl carrier protein→coenzymeA (ACP→CoA) transferase (31). However, it is not known whether additional proteins like a 2-trans-enoyle (ACP→CoA) transferase or a thioesterase also link the fatty acid synthesis pathway with PHA precursor formation in *Pseudomonas*. In *E. coli* a well characterized protein which could play a role as link is the ‘thioesteraseI’, whereas occurrence of an 3-hydroxyacyl (ACP→CoA) transferase has not been shown.

In this study we equipped *E. coli* JMU193 (33), deficient in a functional β-oxidation, with a PHA polymerase from *P. oleovorans* and the thioesteraseI from *E. coli*, which was modified by deletion of its leader sequence trapping the periplasmic protein in the cytosol (called ‘thioesteraseI’) (6). This recombinant was able to accumulate mcl PHA from gluconate suggesting a PHA biosynthesis pathway which links *de novo* fatty acid synthesis and β-oxidation (Fig. 2.4.).
Material and Methods

Media and growth conditions

_E. coli_ strains were grown at 37°C in complex Luria-Bertani (LB) medium (34) or in minimal medium E2 (24) supplemented with 1% (w/v) gluconate. The E2 cultures were inoculated from exponentially growing LB precultures. Cells were cultivated in Erlenmeyer flasks and incubated at 225 rpm. Antibiotics were added as needed: 50 μg/ml kanamycin, 100 μg/ml ampicillin, 50 μg/ml streptomycin, 30 μg/ml chloramphenicol. Media were solidified with 1.5% (w/v) agar for plate experiments. Cell densities were measured spectrophotometrically at 450 nm (40). Cultures were harvested by centrifugation and washed with 10 mM MgSO₄ to remove not metabolized substrate. For determination of PHA the cell pellet was lyophilized. For induction of the _bad_ promoter arabinose in concentrations ranging from 0 - 2% (w/v) was added. To induce the _alk_ promoter, dicyclopropylketone (DCPK) was added during the early exponential phase, at OD 450 = 0.4 in the range of 0 - 0.05% (v/v). Incubation continued overnight and samples were taken as described in results. In order to induce the _alk_ promoter pCK01-_alkS_, which contains the _alkS_ regulatory gene was cotransformed with the _alk_ promoter expression plasmid pET702. Strains and plasmids are listed in Table 2.1.

PHA determination

For a qualitative analysis of PHA accumulation, cells were observed by light microscopy after staining with sudan black (12, 35). Heat fixed, stained samples of cell material were observed with a phase contrast light microscope Leitz DMR (Leica, Germany) at a 100 fold magnification. Analysis of PHA accumulation and composition was performed essentially as described by Lageveen et al. (24). Lyophilized cells were subjected to methanalysis (2.5 hrs, 100°C) in an equal volume of chloroform containing methylbenzoate as an internal standard.
### Table 2.1: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> JMU193</td>
<td><em>fadR</em>::Tn10, <em>fad</em>B64</td>
<td>(33)</td>
</tr>
<tr>
<td><em>P. putida</em> KT2442</td>
<td><em>hsd</em>R1, <em>hsd</em>M*, <em>Rf</em>³</td>
<td>(3)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td><strong>Description</strong></td>
<td><strong>Source</strong></td>
</tr>
<tr>
<td>pGEc404</td>
<td>Km⁺, Sm⁺, RSF1010 ori, Mob⁺</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td><em>phaC2</em>, pJRD215</td>
<td></td>
</tr>
<tr>
<td>pBAD22</td>
<td>Ap⁺, pBR322 ori, <em>badp</em></td>
<td>(14)</td>
</tr>
<tr>
<td>pHC122</td>
<td><em>badp-tesA</em>, pBAD22</td>
<td>(6)</td>
</tr>
<tr>
<td>pET702</td>
<td>Sm⁺/Sp⁺, RSF1010 ori, Mob⁺,</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td><em>alkp-phaC1-VSVG-tag</em>, pVLT35</td>
<td></td>
</tr>
<tr>
<td>pCK01-alkS</td>
<td><em>lacZp-alkS</em>, Cm⁺, pSC101 ori</td>
<td>S. Panke (unpublished)</td>
</tr>
<tr>
<td>pCY322</td>
<td>Km⁺, Ap⁺, pBR322 ori, <em>badp</em></td>
<td>(6)</td>
</tr>
<tr>
<td>pCY323</td>
<td><em>badp-tesA</em>, pCY322</td>
<td>(6)</td>
</tr>
</tbody>
</table>

and a mixture of 15% sulfuric acid and 85% methanol. After phase separation and washing twice with water the organic phase was dried with Na₂SO₄ and analyzed by gas chromatography (GC). Analysis of the methylesters was performed on a Fisons HRGC Mega2 gas chromatograph (Fisons, UK) equipped with a 30 m x 0.32 mm Optima-1-0.25 μm column (Machery-Nagel, Germany) operating in split mode (split ratio 25:1) with temperature programming (60°C for 2 min, increments of 5°C/min up to 200°C and increments of 40°C/min up to 280°C, 5 min at 280°C). For peak identification a PHA standard mixture from *P. putida* KT2442 was used. The average results of two independent experiments are shown. Moreover, gas chromatography-mass spectrometry (GC-MS) spectra were obtained using a Fisons HRGC Mega2 gas chromatograph equipped with a 30 m x 0.32 mm Optima-1-0.25 μm column attached by a direct interface to a Fisons MD800 mass spectrometer (Fisons, UK). Spectra were taken as Electron Impact spectra (70 eV). For GC-MS
analysis the trimethylsilyl (TMSi) derivatives of the 3-hydroxyalkanoic acids were analyzed. TMSi derivatization of 3-hydroxyalkanoate methyl esters was accomplished by the addition of 50 µl of N,O-bis(trimethylsilyl) acetamide to a mixture of 200 µl of methanolized sample and 800 µl of chloroform and heating for 15 min at 80°C. Analysis of an aliquot was carried out by GC-MS (26).

Thioesterase I assay

Thioesterase I was measured spectrophotometrically in crude extracts according to the modified assay of Barnes and Wakil (4) by monitoring the increase in absorbance at 412 nm assuming a molar extinction coefficient of reduced 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) of 13600 (M⁻¹ cm⁻¹) (11). The assay for thioesterase I contained 50 mM potassium phosphate buffer pH 7.4, 0.1 mg/ml bovine serum albumin, 0.1 mM DTNB, 0.07 mM palmitoyl-CoA and crude extract containing approximately 1 mg of total protein. The total volume of the reaction-mixture was 1 ml and all experiments were performed at 25°C. An enzyme unit was defined as the amount of protein necessary for the hydrolysis of 1 µmol of palmitoyl-CoA per min under these conditions, and activity was expressed as mU per mg of protein. The reaction was started by addition of the substrate. Duplicate samples from two independent experiments were measured for determination of enzymatic activity. The thioesterase I-specific substrate hydrolysis in crude extract was determined by total substrate hydrolysis activity in ‘tesA expressing strains corrected for substrate hydrolysis of non-‘tesA expressing strains (background hydrolysis activity caused by chromosomally encoded thioesterase I and other thioesterases). The latter typically amounted to 15% of the maximum activity of ‘tesA expressing strains. Protein concentrations were measured by using the Bio-Rad Bradford assay (Bio-Rad, Laboratories, Switzerland).
Results

Formation of mcl PHA from gluconate in E. coli.

Initial analysis of mcl PHA production from gluconate was carried out using E. coli JMU193, a fadB mutant, defective in β-oxidation. E. coli JMU193 was equipped with the plasmids pGEc404 and pHC122, harbouring the PHA polymerase encoding gene phaCl from P. oleovorans and the ‘thioesteraseI encoding ‘tesA gene from E. coli, respectively. The phaCl was constitutively expressed, whereas ‘tesA expression was regulated by addition of arabinose. To achieve this we used a construct containing the bad promoter of the arabinose operon and its regulatory gene, araC. The AraC protein is both a positive and negative regulator. In the presence of arabinose, transcription from the bad promoter is turned on. The strain was grown in E2 minimal medium containing gluconate as sole carbon source, appropriate antibiotics and 0.01% (w/v) arabinose for induction of the bad promoter. After monitoring the culture for PHA accumulation by sudan black staining, cells were harvested and assayed for polymer content 45 hours after they reached the stationary phase. In the control strain E. coli JMU193(pGEc404, pBAD), lacking the ‘tesA gene, PHA was detected only in trace amounts (< 0.1%). In contrast E. coli JMU193(pGEc404, pHC122) accumulated 0.6% PHA of cell dry weight (cdw) (Table 2.2.).

In order to investigate PHA polymerase PhaCl of P. oleovorans and due to instability of the above described system (data not shown), we tested other constructs for mcl PHA formation in E. coli. We equipped JMU193 with pCY323 harbouring the ‘tesA gene and carrying a kanamycin resistance gene cassette, pET702 harbouring the PHA polymerase phaCl gene expressed through the inducible alk promoter and containing a sequence encoding the C-terminus of the vesicular stomatitis virus glycoprotein (VSVG-tag) and pCK01-alkS encoding the AlkS regulatory protein of the alk promoter. Strains were grown as described
Table 2.2.: PHA accumulation from gluconate in recombinant *E. coli* JMU193\(^1\) harbouring PHA polymerase and 'thioesterase1.

<table>
<thead>
<tr>
<th>Rec.</th>
<th>Plasmid</th>
<th>Gene</th>
<th>Inducer</th>
<th>PHA(^3) (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pGEC404, pHCl22</td>
<td><em>phaC2</em>, 'tesA'</td>
<td>arabinose</td>
<td>nd(^4) 0.6</td>
</tr>
<tr>
<td>2</td>
<td>pGEC404, pBAD22</td>
<td><em>phaC2</em></td>
<td>arabinose</td>
<td>nd &lt; 0.1</td>
</tr>
<tr>
<td>3</td>
<td>pET702, pCK01-alkS, pCY323</td>
<td><em>phaC1-VSVG, alkS</em>, 'tesA'</td>
<td>DCPK, arabinose</td>
<td>1.2 2.3</td>
</tr>
<tr>
<td>4</td>
<td>pET702, pCK01-alkS, pCY322</td>
<td><em>phaC1-VSVG, alkS</em>, 'tesA'</td>
<td>DCPK, arabinose</td>
<td>0(^5) &lt; 0.1</td>
</tr>
<tr>
<td>5</td>
<td>pCY323</td>
<td>'tesA'</td>
<td>arabinose</td>
<td>0 0</td>
</tr>
</tbody>
</table>

\(^1\) Cells were cultivated in E2 minimal medium with 1% gluconate, antibiotics and inducers (0.01% arabinose, 0.01% DCPK). 25 hours and 45 hours after the cells reached the stationary phase samples were taken and analyzed by GC as indicated in Material and Methods.

\(^2\) recombinant *E. coli* JMU193

\(^3\) The amount of polymer was calculated as the percentage of the cell dry weight.

\(^4\) not determined

\(^5\) below GC detection limit

above except that we added 0.01% (v/v) DCPK to induce *phaC1-VSVG*-tag expression through the *alk* promoter. PHA accumulated to a maximum of 2.3% of cdw 45 hours after the cells reached the stationary phase which is 15% of the amount of mcl PHA accumulated by *P. putida* KT2442 when grown on gluconate, which served as a positive control (data not shown). Only trace amounts of PHA (< 0.1% of cdw) were accumulated in control strain JMU193(pET702, pCK01-alkS, pCY322), lacking 'tesA. In control strain JMU193(pCY323), harbouring 'tesA and lacking *phaC1* no PHA was produced (Table 2.2.).
In order to identify PHA monomers from gluconate grown *E. coli* JMU193 recombinants unequivocally, PHA from lyophilized cells was subjected to methanolysis and GC and GC-MS analysis. Monomers derived from the *E. coli* polymer were compared to monomers derived from a PHA standard, obtained from *P. putida* KT2442 grown on gluconate. Fig. 2.1. shows that *E. coli* JMU193(pET702, pCK01-alkS, pCY323), harbouring thioesteraseI and PHA polymerase, produced PHA with 3-hydroxyhexanoate, 3-hydroxyoctanoate and 3-hydroxydecanoate monomers.

**Effect of alk promoter-phaC1-VSVG-tag inducer DCPK on gene expression and polymer content**

Since application of an inducible expression system for *phaC* gene expression can result in significantly increased PHA accumulation compared to the wild-type expression system in recombinant *E. coli* (32), we employed the inducible *alk* promoter-phaC1-VSVG-tag system to determine the optimal PHA polymerase level for maximum mcl PHA production in JMU193 recombinants. *E. coli* JMU193(pET702, pCK01-alkS, pCY323), containing *alk* promoter-phaC1-VSVG-tag, alkS and *tesA*, was grown in E2 minimal medium with gluconate as carbon source and 0.01% (w/v) arabinose as inducer. Cells were induced in the early exponential phase with DCPK in concentrations ranging from 0 - 0.05% (v/v). PHA content and monomer composition were determined 25 and 44 hours after cells reached the stationary phase. If no DCPK was added to the cultures, only trace amounts of PHA were accumulated (< 0.1%). With increasing DCPK concentration, more PHA was produced. The optimal DCPK concentration for maximum PHA production in JMU193 recombinants was between 0.005 - 0.02% (v/v), resulting in a maximum of 2.0% PHA of cdw 44 hours after cells reached the stationary phase. Further increase of DCPK concentration resulted in a decrease of polymer content to half of this level (Fig. 2.2.). These data are in accordance with previous results showing that PHA polymerase production and PHA accumulation
Fig. 2.1.: Electron ionisation mass spectra of the TMS derivates of standard and *E. coli* derived 3-hydroxyalkanoate methylster.

TMS derive methylsters of 3-hydroxyhexanoate (MW 218) derived from a PHA standard from *P. putida* KT2442 (A) and from *E. coli* JMU193 (pET702, pCK01-alkS, pCY323) (B) grown on gluconate are depicted. Characteristic peaks are: basis peak: M/e 203 (M-15), M/e 145 (M-73), M/e 133 (C5H13SiO2)+, M/e 131 (C5H11SiO2)+, M/e 89 (CH3)3SiO+, M/e 73 (CH3)3Si+. 
Fig. 2.1. - continued:
TMS derivate methylesters of 3-hydroxyoctanoate (MW 246) derived from a PHA standard from P. putida KT2442 (C) and from E. coli JMU193 (pET702, pCK01-alkS, pCY323) (D) grown on gluconate are depicted. Characteristic peaks are: basis peak: M/e 231 (M-15), M/e 175 ((CH3)3SiO=CHCH2CO2CH3), M/e 133 (C5H13SiO2)+, M/e 131 (C5H11SiO2)+, M/e 89 (CH3)3SiO+, M/e 73 (CH3)3Si+.
Fig. 2.1. - continued:
TMS derivate methylesters of 3-hydroxydecanoate (MW 274) derived from a PHA standard from *P. putida* KT2442 (E) and from *E. coli* JM193 (pET702, pCK01-alkS, pCY323) (F) grown on eukaconate are shown. Characteristic peaks are: M/e 259 (M-15), M/e 175 ((CH$_3$)$_3$SiO+=CHCH$_2$CO$_2$CH$_3$), M/e 133 (C$_5$H$_{13}$SiO$_2$)+, M/e 131 (C$_5$H$_{11}$SiO$_2$)+, M/e 89 (CH$_3$)$_3$SiO+, M/e 73 (CH$_3$)$_3$Si+. 
Fig. 2.2.: PHA accumulation by *E. coli* JMU193 (pET702, pCK01- dikS, pCY323) as function of DCPK concentration. Cells were cultivated in E2 minimal medium with 1% (w/v) gluconate, antibiotics, 0.01% (w/v) arabinose and DCPK as indicated. Samples were taken 25 hours (shaded bars) and 44 hours (open bars) after cells reached the stationary phase, lyophilized and analyzed by GC.
are proportional only at low inducer concentrations. At medium and high inducer concentrations PHA polymerase production shows a saturation profile, while in E. coli recombinants PHA accumulation decreased with higher polymerase levels (32). Longer incubation times in the stationary phase (44 hours compared to 25 hours) resulted in higher maximum polymer amounts (2.0 compared to 1.3 % PHA of cdw). Variation of DCPK concentrations had only minor effects on the monomer composition of the PHA produced. Dependent on the DCPK concentration the synthesized polymer consisted of 27 - 33 mol% of 3-hydroxyhexanoate, 62 - 67 mol% of 3-hydroxyoctanoate and 5 - 6 mol% of 3-hydroxydecanoate (Table 2.3.). Interestingly the predominant monomer in the PHA of JMU193 recombinants was 3-hydroxyoctanoate, unlike the 3-hydroxydecanoate found in P. putida (20).

**Effect of bad promoter-tesA inducer arabinose on ‘thioesterase activity and polymer content**

Excessively high levels of ‘thioesteraseI were expected to inhibit flux through the β-oxidation pathway via cleavage of the acyl-CoA thioesters (7). Therefore, we used a ‘tesA expression system which allows fine tuned regulation in order to determine if there is a specific level of ‘thioesteraseI that is optimal for PHA synthesis. We chose a vector in which ‘tesA was cloned downstream of the bad promoter, allowing low-level expression and modulation of gene expression over a wide range of inducer concentrations. The concentrations of arabinose that might permit accumulation of PHA by cleavage of acyl-ACP esters, were chosen on the basis of the data of Guzman and coworkers (14). We cultivated JMU193(pET702, pCK01-alkS, pCY323) on gluconate with addition of appropriate antibiotics, 0.01% (v/v) DCPK and arabinose concentrations ranging from 0 - 2% (w/v). 25 hours after cells reached the stationary phase they were harvested and the PHA content was determined. The polymer content increased with increasing arabinose concentration from trace
Table 2.3.: PHA content and monomer composition of *E. coli* JMU193 (pET702, pCK01-alkS, pCY323).

<table>
<thead>
<tr>
<th>Inducer</th>
<th>PHA (% w/w)</th>
<th>PHA monomer composition$^1$ (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DCPK (% v/v)</td>
<td>C6</td>
</tr>
<tr>
<td>0</td>
<td>&lt;0.1</td>
<td>-</td>
</tr>
<tr>
<td>0.0025</td>
<td>1.2±0.02</td>
<td>29</td>
</tr>
<tr>
<td>0.005</td>
<td>2.0±0.01</td>
<td>27</td>
</tr>
<tr>
<td>0.02</td>
<td>1.9±0.04</td>
<td>30</td>
</tr>
<tr>
<td>0.05</td>
<td>1.1±0.01</td>
<td>33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Arabinose (% w/v)</th>
<th>PHA (% w/w)</th>
<th>C6</th>
<th>C8</th>
<th>C10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.001</td>
<td>0.26±0.006</td>
<td>33</td>
<td>64</td>
<td>3</td>
</tr>
<tr>
<td>0.01</td>
<td>1.24±0.04</td>
<td>23</td>
<td>69</td>
<td>8</td>
</tr>
<tr>
<td>0.1</td>
<td>0.19±0.06</td>
<td>21</td>
<td>71</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$ Cells were cultivated in E2 minimal medium with 1% glucose, antibiotics, DCPK and arabinose at the concentrations indicated. When varying the arabinose concentration 0.01% DCPK was added and when varying the DCPK concentration 0.01% arabinose was added. Cells were harvested 25 hours (arabinose variation) or 46 hours (DCPK variation) after having reached the stationary phase, lyophilized and the PHA composition was measured by GC. C6; 3-hydroxyhexanoate, C8; 3-hydroxyoctanoate, C10; 3-hydroxydecanoate.

$^2$ not analyzed due to the low overall PHA content (<0.1 weight %)

Amounts of polymer (< 0.1 % PHA of cdw), when no arabinose was added, to a maximum of 1.2% PHA of cdw at an arabinose concentration of 0.01% (w/v). Higher concentrations of arabinose resulted in a decreased polymer content (0.1 % PHA of cdw). ThioesteraseI activity measurements in crude extracts from cells harvested at 25 hours after cells reached the stationary phase showed low enzyme activities (20 mU/mg) when no arabinose was added, increasing to 104 mU/mg at 0.01% (w/v) arabinose and decreasing to 27 mU/mg at higher arabinose concentrations. As indicated in Fig. 2.3., the PHA accumulation profile in
recombinant *E. coli* JMU193 correlated with the 'thioesteraseI activity as a function of the arabinose concentration. From this and the fact that 'tesA-negative recombinants did not produce PHA, we conclude that there is a direct involvement of 'thioesteraseI in mcl PHA biosynthesis in *E. coli* JMU193(pET702, pCK01-alkS, pCY323).

![Correlation of PHA accumulation and 'thioesterase activity](image)

Fig. 2.3.: Correlation of PHA accumulation and 'thioesterase activity. *E. coli* JMU193 (pET702, pCK01-alkS, pCY323) was cultivated in E2 minimal medium with 1% (w/v) gluconate, antibiotics, 0.01% DCPK (v/v) and arabinose as indicated. The cells were harvested 25 hours after having reached the stationary phase, lyophilized and the PHA content was determined by GC (open circles). 'Thioesterase activity (closed squares) was determined in crude extracts by spectrophotometric measurements and corrected by subtracting the 'thioesterase activity of crude extracts of *E. coli* JMU193 lacking the 'thioesterase encoding gene, from crude extracts of *E. coli* JMU193 expressing the 'thioesterase encoding gene.
Discussion

We have generated recombinant *E. coli* strains capable of producing mcl PHA from gluconate. To this end we equipped an *E. coli* strain blocked in β-oxidation with the PHA polymerase encoded by *phaC1* or *phaC2* gene from *P. oleovorans*, and the cytosolic ‘thioesteraseI’ encoding ‘tesA’ gene from *E. coli*. The thioesterase hydrolyzes acyl-ACPs, therefore producing enhanced intracellular levels of free fatty acids (6) which can then be channelled into the β-oxidation and used by the PHA polymerase as substrates for incorporation into PHA.

Mcl PHA was detected only in recombinant *E. coli* JMU193 harbouring both, the *phaC* and ‘tesA’ encoding plasmids. The involvement of ‘thioesteraseI’ in PHA biosynthesis in JMU193 recombinants is further indicated by the direct correlation between ‘thioesteraseI’ activity and PHA accumulation. Additional indirect evidence for the importance of ‘thioesteraseI’ for PHA production is the fact that PHA accumulation started in the stationary phase. This is in agreement with findings of Cronan and coworkers, who reported that expression of ‘tesA’ results in an increase of total fatty acid synthesis, particularly in the stationary phase, whereas in cultures lacking ‘thioesteraseI’ it is known that the overall rate of lipid synthesis is inhibited (6, 8). Thus, mcl PHA biosynthesis in *E. coli* JMU193(pET702, pCK01-alkS, pCY323) may be assumed to include the following steps (Fig. 2.4.):

‘ThioesteraseI’ producing cells generate free fatty acids, which can accumulate to concentrations that are 15 fold higher than those seen in parallel cultures of strains lacking ‘thioesteraseI’ (6). The free fatty acids are produced by hydrolysis of the thioester bond linking acyl-ACPs generated during *de novo* fatty acid synthesis. Intracellular fatty acids are activated by action of the acyl-CoA synthase (FadD) resulting in acyl-CoAs which can be channelled into the β-oxidation pathway. Because of the deficient β-oxidation cycle in *E. coli* JMU193, 3-hydroxyacyl-CoAs and 2-*trans*-enoyl-CoAs accumulate and are channelled into PHA by involvement of the PHA polymerase.
Fig. 2.4. (page 55): Hypothetical pathway of mcl PHA biosynthesis of PHA polymerase and 'thioesterasel containing E. coli JMU193 grown on gluconate. Gluconate is degraded via the central carbohydrate metabolism (indicated by triple arrows) leading to acetyl-CoA. Via 4 conversions of the fatty acid synthesis pathway, acetyl-CoA is metabolized to acyl-ACP: acetyl-ACP, transferred from acyl-CoA, and malonyl-ACP are coupled to give 3-ketoacyl-ACP by the β-ketoacyl-ACP synthase (1). 3-ketoacyl-ACP is converted to (R)-3-hydroxyacyl-ACP by the β-ketoacyl-ACP reductase (2). The β-hydroxyacyl-ACP dehydrase (3) yields 2-trans-enoyl-ACP which is metabolized by enoyl-ACP reductase (4) to acyl-ACP. Acyl-ACP is hydrolyzed by the 'thioesterasel (5) to result in the corresponding fatty acid which is activated by acyl-CoA synthase (6) to the corresponding acyl-CoA. Acyl-CoA is degraded in the β-oxidation cycle resulting in 2-trans-enoyl-CoA by the acyl-CoA dehydrogenase (7) and to yield (S)-3-hydroxyacyl-CoA by action of the enoyl-CoA hydratase (8). Because of the fadB mutation of E. coli JMU193 (indicated by double sticks), these latter intermediates accumulate and can be transferred by either an isomerase (9) or a (S)-specific hydratase (10) into (R)-3-hydroxyacyl-CoA, which is converted by the PHA polymerase (11) into poly (R)-3-hydroxyalkanoate (PHA). Reactions boxed in ellipsoids are carried out by genes which were introduced into E. coli JMU193 encoding the 'thioesterasel and PHA polymerase C1 or C2 proteins. Question marks indicate uncertainties about the actual pathway according to data from Pseudomonas.

(32). Following our pathway hypothesis we assume that recombinant E. coli JMU193 accumulated mcl PHA via functioning of de novo fatty acid synthesis and certain steps of the β-oxidation cycle linked by the 'thioesterasel. Pseudomonas has also been shown to accumulate mcl PHA by simultaneous functioning of both fatty acid metabolic routes (19).

Both PHA polymerase and 'thioesterasel were produced by fine tuned gene expression systems. For induction of the alk promoter, producing the PHA polymerase, an optimum inducer concentration of 0.01 % DCPK was determined which is in agreement with previous data and reflects the fact that only small amounts of PHA polymerase are sufficient for maximum PHA production (32). For the bad promoter, expressing the 'tesA gene, we found the inducer concentration which resulted in maximum PHA accumulation to be 0.01 % arabinose. As expected, we found that no induction or induction with low inducer concentrations (≤10⁻⁷ % arabinose) yielded in low 'thioesterasel activity. The fact that in the repressed state (zero induction) 'thioesterasel activity could be detected is in accordance with Guzman et al. (14). They found that although the bad promoter is tightly controlled, protein levels in the repressed states are not always
zero. Increase of the inducer concentration led to an increased 'thioesterasel activity (140 mU at 10^2 % arabinose) which correlated with the maximum PHA production. Unexpectedly, with further increase of the inducer concentration the 'thioesterasel activity decreased for which the reason is still unknown. Since we were not able to detect inclusion bodies of the 'thioesterasel protein (data not shown), the activity decrease might well be due to feed back inhibition by free fatty acids. Further experiments have to be carried out to optimize the 'thioesterasel activity in order to increase the PHA amount accumulated in the cell.

We have found that E. coli JMU193 equipped with the PhaC1 polymerase from P. oleovorans accumulates 3-hydroxyoctanoate as the predominant monomer of PHA when grown on gluconate. These observations are in agreement with previous findings showing that E. coli JMU193 harbouring either PhaC1 or PhaC2 polymerase of P. oleovorans accumulated polymer with 3-hydroxyoctanoate as the predominant monomer when grown on fatty acids (32). In contrast it was reported when GPP104, a mcl PHA negative mutant of P. putida KT2442, harbouring either the PhaC1 or PhaC2 polymerase encoding gene from P. oleovorans is cultivated on glucose, the main constituent of the polyester is 3-hydroxydecanoate (20). Furthermore it was shown that many Pseudomonas species including P. putida, P. aeruginosa, P. aureofaciens and P. mendocina accumulate a polymer with 3-hydroxydecanoate as the major constituent when cells are grown on gluconate or carbohydrate substrates (16, 20, 38). Similarly, when E. coli, deficient in β-oxidation, is equipped with the PhaC1 polymerase encoding gene from P. aeruginosa, and PHA is accumulated from fatty acids, 3-hydroxydecanoate is also the predominant monomer (25). The change of the predominant monomer in the mcl PHA using the PhaC polymerases of P. oleovorans in the P. putida mutant GPP104 and in E. coli JMU193 reflects the importance of the varying concentration profiles of 3-hydroxyacyl CoAs for PHA monomer composition in these different strains.

In summary, this is the first report on production of mcl PHA in engineered E. coli grown on gluconate. In comparison to recombinant E. coli strains able to accumulate mcl PHA from fatty acids (25, 32), our strains possess the economic
advantage that they can use inexpensive carbohydrate or carbohydrate derived substrates such as gluconate as sole carbon source for mcl PHA production. Although for biotechnological application the PHA content of engineered *E. coli* has to be increased significantly, this study demonstrates that mcl PHA production from cheap carbon sources in *E. coli*, an important goal for commercial application of these polymers, is basically feasible. Moreover, the presented strategy of mcl PHA production might be relevant with respect to PHA synthesis in plants for which several projects from industry and university related groups are currently under way.

**Acknowledgments**

We thank John E. Cronan, Jr. for providing plasmids pBAD22, pHCl22 and pCY322/3, Sven Panke for plasmid pCK01-*alkS* and Wouter Duetz for helpful suggestions.

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References


CHAPTER 3

Role of phaD in Accumulation of Medium Chain Length Poly(3-Hydroxyalkanoates) in Pseudomonas oleovorans

STEFAN KLINKE, BERNARD WITHOLT and BIRGIT KESSLER

submitted for publication to Applied and Environmental Microbiology
Summary

*Pseudomonas oleovorans* is capable of producing poly (3-hydroxyalkanoates) (PHAs) as intracellular storage material. To analyze the possible involvement of *phaD* in medium chain length (mcl) PHA biosynthesis, we generated a *phaD* knock out mutant by homologous recombination. Upon disruption of the *phaD* gene mcl PHA polymer accumulation was decreased. The PHA granule size was reduced and the number of granules inside the cell was increased. Furthermore, mutant cells appeared to be smaller than wild type cells. Investigation of mcl PHA granules revealed that the pattern of granule associated proteins was changed and that the predominant protein PhaI was missing in the mutant. Complementation of the mutant with a *phaD* harboring plasmid partially restored the wild type characteristics of mcl PHA production and fully restored the granule and cell size. These results indicate that the *phaD* gene encodes a protein which plays an important role in mcl PHA biosynthesis. However, although its main effect seems to be the stabilization of mcl PHA granules, we found that the PhaD protein is not a major granule associated protein and therefore might act by an indirect mechanism involving the PhaI protein.
Introduction

Polyhydroxyalkanoates are storage polymers accumulated by a wide variety of bacteria under nutrient-limitation and carbon excess (25). *Pseudomonas oleovorans* produces medium chain length (mcl) poly(3-hydroxyalkanoates) (PHAs), which are composed of monomers with a chain length varying from 6-14 carbon atoms, when grown on fatty acids (5, 14). PHAs are under investigation because of their potential as biodegradable polymers produced from renewable resources (1, 24).

Three different types of proteins are involved in the synthesis and degradation of PHA, and the assembly or maintenance of intracellular PHA granules (26): PHA synthases, PHA depolymerases and phasins. The group of granule associated proteins named phasins show analogy to oleosins, a class of amphipathic proteins that form a layer at the surface of triacylglycerol inclusions found in seeds and pollen of plants (8, 17).

In *P. oleovorans* GPo1 the major mcl PHA biosynthesis genes are clustered in the PHA locus: Two synthase encoding genes are separated by an open reading frame (ORF) that encodes a depolymerase. Downstream of the second synthase encoding gene there is a fourth ORF (*phaD*) (9), for which a role in PHA biosynthesis has not yet been described. Recently, two additional genes downstream of *phaD* encoding the PhaI and PhaF protein have been identified (20). PhaF and PhaI are major PHA granule binding proteins (20). Moreover, the PhaF protein behaves as a negative regulator of *phaC1* gene expression.

Sequence alignments of PhaD with protein databases revealed no significant homology to PHA synthases or PHA depolymerases. Additionally, no homology between PhaD and other phasins, which themselves do not show significant homology within their group, was detected. However, Valentin et al. reported that deletion of *phaD* in addition to inactivation of *phaI* and *phaF*, encoding the major granule associated proteins of *P. putida*, lead to a significant decrease in mcl PHA accumulation (27).
In this study, we investigated the role of \textit{phaD} in mcl PHA biosynthesis in \textit{P. oleovorans}. A \textit{phaD} knock out mutant was negatively affected in PHA biosynthesis, despite the fact that the PhaD protein was not associated with PHA granules.
**Material and Methods**

**Bacterial strains, plasmids and growth conditions**

*Pseudomonas* strains were grown at 30° C in minimal medium E2 (14) with addition of 0.2% (w/v) citrate for precultures. To stimulate PHA production, cells were grown in minimal medium 0.1 N E2 with addition of 15 mM octanoate. *E. coli* strains were grown at 37° C in complex Luria-Bertani (LB) medium (22). Cells were cultivated in Erlenmeyer flasks and incubated at 225 rpm. Antibiotics were added as needed: 100 μg/ml piperacillin, 12.5 μg/ml tetracycline, 30 μg/ml rifampicin and 100 μg/ml kanamycin. Media were solidified with 1.5% (w/v) agar for plate experiments. Cell densities were measured spectrophotometrically at 450 nm (30). Cultures were harvested by centrifugation and washed with 10 mM MgSO₄. For determination of PHA the cell pellet was lyophilized. The strains and plasmids are listed in Table 3.1.

**DNA manipulation**

Basic recombinant DNA techniques, such as preparation and purification of plasmid DNA, restriction endonuclease digestion, nuclease S1 digestion, dephosphorylation, agarose gel electrophoresis, transformation of *E. coli* and Southern blotting were essentially carried out as described by Sambrook (22).

Southern blot analysis was performed by using either a 2 kb *tet* gene fragment, obtained by *SmaI* digestion of pUT mini-Tn5 Tc, or a 0.6 kb *phaD* internal fragment, amplified by PCR using the primers (A) 5' -CCGCGACACCG-CATCAACAGGCTTAC-3' and (B) 5'-ATGAAGACTCGCGACCCTATCCTC-3'. Both probes were labeled using the DIG labeling and detection kit (Boehringer Mannheim).
Table 3.1.: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. oleovorans</em> GPo12</td>
<td>OCT plasmid, PHA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(11, 23)</td>
</tr>
<tr>
<td><em>P. oleovorans</em> GPo1000</td>
<td>OCT plasmid, PHA&lt;sup&gt;+&lt;/sup&gt;, Rf&lt;sup&gt;+&lt;/sup&gt;</td>
<td>this study</td>
</tr>
<tr>
<td><em>P. oleovorans</em> GPo1001</td>
<td>OCT plasmid, PHA&lt;sup&gt;+&lt;/sup&gt;, Rf&lt;sup&gt;+&lt;/sup&gt;, Tc&lt;sup&gt;-&lt;/sup&gt;, <em>phaD&lt;sup&gt;Q(8b::tet)&lt;/sup&gt;</em></td>
<td>this study</td>
</tr>
<tr>
<td><em>E. coli</em> S17-1 λpir</td>
<td>RP4:2-Tc:Km, Tn7, λ&lt;pir&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, Tp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(16)</td>
</tr>
<tr>
<td><em>E. coli</em> HB101</td>
<td>F-Δ(gpt-proA)62, *leuB&lt;sup&gt;-&lt;/sup&gt;*62, *supE&lt;sup&gt;44&lt;/sup&gt;*44, *ara&lt;sup&gt;-&lt;/sup&gt;-14, *galK&lt;sup&gt;2&lt;/sup&gt;, *lacY&lt;sup&gt;1&lt;/sup&gt;, <em>xyl-5</em>, <em>recA&lt;sup&gt;13&lt;/sup&gt;</em></td>
<td>(22)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>pGEc404</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, RSF1010</td>
<td>(9)</td>
</tr>
<tr>
<td></td>
<td><em>ori</em>, <em>Mob&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>*phaC&lt;sup&gt;2&lt;/sup&gt;, phaD&lt;sup&gt;+&lt;/sup&gt;, pJRD215</td>
<td></td>
</tr>
<tr>
<td>pGEc420</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, Sm&lt;sup&gt;R&lt;/sup&gt;, f1 <em>ori</em>, lacZ, *phaC&lt;sup&gt;2&lt;/sup&gt;, *phaD&lt;sup&gt;+&lt;/sup&gt;, pGEM-7Zf&lt;sup&gt;(+)&lt;/sup&gt;</td>
<td>(9)</td>
</tr>
<tr>
<td>pUT mini-Tn5 Tc</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, Tc&lt;sup&gt;+&lt;/sup&gt;, delivery plasmid for mini Tn5 Tc</td>
<td>(3, 4)</td>
</tr>
<tr>
<td>pRK600</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, ColE1 <em>ori</em>, RK2-Mob&lt;sup&gt;+&lt;/sup&gt;, RK2-Tra&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(4)</td>
</tr>
<tr>
<td>pBCKS</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, ColE1 <em>ori</em>, lacZ, f1 <em>ori</em>, pUC19</td>
<td>Prieto et al. (pers. communication)</td>
</tr>
<tr>
<td>pVLT33</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, RSF1010-<em>lacP/tacP</em></td>
<td>(2)</td>
</tr>
<tr>
<td>pGEc420-tet</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;, *phaD&lt;sup&gt;Q(8b::tet)&lt;/sup&gt;, pGEc420</td>
<td>this study</td>
</tr>
<tr>
<td>pUT-*phaD&lt;sup&gt;Q(8b::tet)&lt;/sup&gt;</td>
<td>Tc&lt;sup&gt;R&lt;/sup&gt;, *phaD&lt;sup&gt;Q(8b::tet)&lt;/sup&gt;, pUT</td>
<td>this study</td>
</tr>
<tr>
<td>pHAD2</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, phaD&lt;sup&gt;+&lt;/sup&gt;, pBCKS</td>
<td>this study</td>
</tr>
<tr>
<td>pHAD5</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, phaD&lt;sup&gt;+&lt;/sup&gt;, pVLT33</td>
<td>this study</td>
</tr>
</tbody>
</table>
**PHA determination**

Analysis of PHA accumulation and composition was performed essentially as described before (10).

**Electron microscopy and analysis of electron microscopy pictures**

Stationary phase cultures (27 hours of growth) were prepared for electron microscopy by high pressure freezing and freeze substitution as described previously (7). The cells were taken up in cellulose capillaries (Type LD OC 02, Microdyn. Wuppertal, Germany) and were subjected to high pressure freezing (HPM 010, Balzers Union, FL). The frozen samples were stored in liquid nitrogen until further use. Freeze substitution was performed in anhydrous acetone containing 2 % osmium tetroxide (28). The samples were plastic embedded in epon/araldite and sectioned on a Reichert Jung Ultracut E microtome equipped with a diamond knife (Diatome AG, Biel, Switzerland), stained with uranyl acetate and lead citrate (21) and examined in a Hitachi H-600 transmission electron microscope (Hitachi, Japan).

To determine the volume of PHA granules from the micrographs, 22 cells of the wild type and 14 cells of the mutant were selected in which the PHA granule radius was measured. 31 granules of the wild-type and 46 of the mutant were analyzed. Only granules with sharp boundaries were selected. Round granules were assumed to have spherical shape (volume = \(\frac{4}{3} \pi r^3\)) whereas oval shaped granules were assumed to be ellipsoidal shaped (volume = \(\frac{4}{3} \pi a^2 b\)). The number of PHA granules per cell was determined only for cells which were fully visible on the EM photographs. 23 wild-type cells and 52 mutant cells were selected. Cell volumes were determined as described above for PHA granule volume determination and the ratio of these volumes was calculated using 23 cells of the wild-type and 14 cells of the mutant.

The granule volumes, the number of granules per cell and the ratio of granule volume to cell volume of wild-type and mutant cells were compared. The data were
analyzed with the software package S-Plus using the two sample t-test and the Wilcoxon rank-sum test.

**Granule isolation and analysis of granule associated proteins**

PHA granules were isolated on a sucrose gradient as previously reported (12). Samples of purified granules were mixed 1:1 (v/v) with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and the bound proteins were separated by SDS-polyacrylamide gels as described before (13). The proteins were electroblotted directly from a gel onto a polyvinylidene difluoride (PVDF) membrane. The amino-terminal sequences were determined by Edman degradation using an automated protein sequencer G 1000 A (Hewlett Packard).
Results

Construction of a \textit{phaD} insertion mutant

In order to determine the role of PhaD in PHA biosynthesis, mutants of \textit{P. oleovorans} disrupted in the \textit{phaD} gene were constructed by a gene replacement procedure. A rifampicin resistant mutant of GPo12, named GPo1000, was generated by growing \textit{P. oleovorans} GPo12 on rifampicin for several cycles. This strain showed an identical PHA accumulation phenotype as the parental strain GPo12 (data not shown). GPo1000 was used for an insertional inactivation of the \textit{phaD} gene by gene replacement using the tetracycline resistance gene cassette (tet).

The \textit{tet} gene was isolated by digesting plasmid pUT mini Tn5 Tc with \textit{SmaI} and cloned into the \textit{NruI} site of pGEc420, which was made blunt with nuclease SI. The resulting vector, called pGEc420-\textit{phaD::tet} (Fig. 3.1.), contains the \textit{tet} gene inserted 8 nucleotides downstream of the start codon of the \textit{phaD} gene. Because of strong terminators up- and downstream of the \textit{tet} gene (4), transcription of the complete \textit{phaD} gene is inhibited in this construct. Plasmid pGEc420-\textit{phaD::tet} was digested with \textit{Clai}, made blunt with nuclease SI and then digested with \textit{EcoRI}. The \textit{EcoRI}-blunt fragment, consisting of \textit{phaC2}, \textit{phaD} with the \textit{tet} insertion and \textit{phaF}, was ligated to the pUT backbone. The plasmid, called pUT-\textit{phaD::tet} (Fig. 3.1.), was used to produce \textit{phaD} insertion mutants of \textit{P. oleovorans}. By mating of donor strain \textit{E. coli} S17-\textit{λpir} pUT-\textit{phaD::tet} and recipient strain \textit{P. oleovorans} GPo1000 the plasmid pUT-\textit{phaD::tet} was transferred into the recipient strain. Selection was done on LB plates with tetracycline and rifampicin to obtain cells in which homologous recombination had occurred. To select only clones with a double recombination event, cells were screened on the β-lactam antibiotic piperacillin, to select for strains which lost their β-lactam antibiotic resistance transferring pUT plasmid. One mutant of \textit{P. oleovorans} GPo1000, named GPo1001, was found to
Fig. 3.1.: phaD gene replacement by homologous recombination in *P. oleovorans* GPo1000. The upper three lines show the construction of the plasmid pUTphaD::tet used for gene replacement of phaD. A double crossover between pUTphaD::tet and homologous DNA in the chromosome of *P. oleovorans* GPo1000 resulted in the phaD::tet - integrated strain GPo1001. The gray box indicates the tet resistance gene. The black bar represents the phaD gene and open arrows depict other genes up- and downstream of the phaD gene. Abbreviations: A, Asp718; C, CiaI; E, EcoRI; N, NruI; S, Stul; Sm, SmaI; X, XmnI.
exhibit the desired phenotype of rifampicin and tetracycline resistance and piperacillin sensitivity. This was presumed to be due to replacement of the wild-type phaD gene in the chromosome by the tet gene disrupted phaD from pUT-phaD::tet, as a result of homologous recombination involving a double cross-over followed by loss of the plasmid (Fig. 3.1.).

**Verification of the P. oleovorans GPo1001 genotype**

Southern blot analysis of the mutant *P. oleovorans* GPo1001 and the parental strain *P. oleovorans* GPo1000 is depicted in Fig. 3.2. When the tet cassette was used to probe an *EcoRI* or *XmnI* digest of chromosomal DNA, a 8.4-kb (*EcoRI*) or 4.8-kb (*XmnI*) fragment was detected in the phaD::tet mutant, providing evidence that this fragment contains phaD::tet. As expected no hybridization of the tet-probe was seen with GPo1000 chromosomal DNA (Fig. 3.2. A). Hybridization of the same digests using phaD as a probe showed a single 6.4-kb or 2.8-kb fragment in GPo1000, respectively. In the phaD::tet mutant a hybridization signal at 8.4-kb or 4.8-kb was detected, consistent with the size expected from insertion of the 2 kb tet resistance cassette into the 6.4-kb or 2.8-kb fragment containing the native phaD gene (Fig. 3.2. B).

**Analysis of P. oleovorans GPo1001 PHA biosynthesis**

To investigate whether the phaD mutation affects the PHA biosynthesis pattern, we determined time dependent PHA accumulation of *P. oleovorans* GPo1001 and its parental strain *P. oleovorans* GPo1000 (Table 3.2.). We found that growth and the polymer accumulation of GPo1001 was significantly lower than that of the parental strain GPo1000; the polymer accumulated by GPo1001 never amounted to more than 20% of that synthesized by GPo1000. Moreover, the difference in PHA accumulation between both strains increased with time varying from 55 to 58 % PHA (w/w) in GPo1000 and from 10 to < 2 % (w/w) in GPo1001 between 23 and
Fig. 3.2.: Southern hybridization of genomic DNA from *P. oleovorans* GPO1000 and GPO1001. Genomic DNA of the parental strain *P. oleovorans* GPO1000 (lane 1,3) and the *phaD*:tet mutant GPO1001 (lane 2,4). DNA was digested with *EcoRI* (lane 1,2) or with *XmnI* (lane 3,4). (A) Hybridization with the *tet* resistance gene cassette sequence. (B) Hybridization with the *phaD* gene sequence. Markers indicate the fragment size in kbp.
Table 3.2.: PHA content and monomer composition of *P. oleovorans* GPo1000 and the *phaD* insertion mutant *P. oleovorans* GPo1001 cultivated on octanoate

<table>
<thead>
<tr>
<th>Organism</th>
<th>Time (hrs)</th>
<th>cdm (g l⁻¹)</th>
<th>PHA (% w/w)</th>
<th>PHA monomer composition (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C6</td>
<td>C8</td>
</tr>
<tr>
<td>GPo1000</td>
<td>23</td>
<td>0.74</td>
<td>54.9</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>0.77</td>
<td>56.1</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>0.86</td>
<td>58.3</td>
<td>7.5</td>
</tr>
<tr>
<td>GPo1001</td>
<td>23</td>
<td>0.44</td>
<td>9.6</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>0.42</td>
<td>8.9</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>0.35</td>
<td>1.1</td>
<td>8.5</td>
</tr>
<tr>
<td>GPo1001 pHAD⁵</td>
<td>23</td>
<td>0.79</td>
<td>38.9</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>0.94</td>
<td>43.8</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>0.94</td>
<td>30.7</td>
<td>3.3</td>
</tr>
</tbody>
</table>

¹ Cells were cultivated in 0.1 NE2 minimal medium with 15 mM octanoate. After 23, 27 and 46 hrs of cultivation cells were harvested, lyophilized and the PHA composition was measured by GC.  
² C6: 3-hydroxyhexanoate, C8: 3-hydroxyoctanoate, C10: 3-hydroxydecanoate.  
³ Cells were grown until they reached the exponential phase (OD₄₅₀ = 0.2) and then induced with 0.5 mM IPTG.

46 hours of growth, indicating a nearly complete intracellular degradation of the PHA within the observed incubation time in GPo1001. The PHA monomer composition was similar in mutant and wild type during the observed period and showed 3-hydroxyoctanoate as predominant monomer in both strains (Table 3.2.).
Analysis of \textit{P. oleovorans} GPo1000 and GPo1001 PHA granule characteristics

Cells and PHA granules of wild type and mutant differed significantly in their appearance as observed by transmission electron microscopy (Fig. 3.3. A,B). To determine whether these differences between mutant and wild type were due to variations of individual cells within one heterogeneous group of bacteria or statistically significant differences between wild type and mutant cells, we analyzed 14 - 52 wild type and mutant cells.

Table 3.3. shows that the median of the PHA granule volume decreased by three orders of magnitude in the \textit{P. oleovorans} mutant GPo1001 compared to the wild type \textit{P. oleovorans} GPo1000. The mutant GPo1001 had a higher number of PHA granules per cell (median = 5) compared to the wild type GPo1000 (median = 1). As a result, the ratio of granule volume to cell volume for GPo1001 cells decreased significantly (median = 2\%) compared to that of GPo1000 cells (median = 49\%). The difference found for the granule volume, the granule numbers and the ratio of granule volume to cell volume were statistically significant according to the t-test and the Wilcoxon rank-sum test. Moreover, we found a reasonable agreement between the PHA amount (%w/w) of mutant and wild type derived from electron micrographs and determined by GC measurements (Table 3.3.).

Influence of \textit{phaD} gene disruption on granule associated proteins

SDS-PAGE analysis of isolated granules showed that PhaD is not a major granule associated protein. Interestingly, we found that the pattern of granule associated proteins of GPo1000 and GPo1001 differed significantly. The most obvious difference was a missing protein band in granule preparations of the \textit{phaD} insertion mutant GPo1001 at 18 kDa (Fig. 3.4.). The N-terminus of this 18 kDa protein in GPo1000, \texttt{XKVT VK DDAPGT LG EVR GYARK IML-AGIG AYARV GQEG}, is except for two amino acids, identical to that of PhaI, one of the major granule associated proteins in \textit{P. oleovorans} (20, 27).
Fig. 3.3.: Morphology of *P. oleovorans* *phaD* mutant. Cells were cultivated in 0.1NE2 minimal medium containing 15 mM octanoate and were harvested in the stationary growth phase. Electron micrographs were obtained as described in Material and Methods and depict representative cells. (A) *P. oleovorans* GPO1000. (B) *P. oleovorans* GPO1001. (C) *P. oleovorans* GPO1001 pHAD5. CY, cytoplasm; IB, PHA granule (Inclusion Body); bar, 0.5 μm.
Table 3.3.: Descriptive statistics of PHA granules of *P. oleovorans* GPo1000 and GPo1001 by analysis of electron micrographs¹

<table>
<thead>
<tr>
<th>strain</th>
<th>cells² (nr)</th>
<th>gn³ (nr)</th>
<th>(a) (μm)</th>
<th>(b) (μm)</th>
<th>(v_{ga}) ((\mu m^3))⁴</th>
<th>min⁸</th>
<th>max⁹</th>
<th>mean</th>
<th>median</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPo1000</td>
<td>22</td>
<td>31</td>
<td>0.1-</td>
<td>0.4</td>
<td>0.09-0.55</td>
<td>0.003</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPo1001</td>
<td>14</td>
<td>46</td>
<td>0.02-</td>
<td>0.19</td>
<td>0.02-0.19</td>
<td>4x10⁻⁵</td>
<td>0.03</td>
<td>0.003</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>280</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>46</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

¹ Cultures of *P. oleovorans* GPo1000 and GPo1001 were grown on 0.1 N E2 minimal medium containing 15 mM octanoate. Cells from stationary phase cultures were prepared for electron microscopy as described in Material and Methods. For each sample 13 independent electron micrographs, each showing one or more bacterial cells, were chosen for analysis.

² number of cells (only sharply and completely depicted cells were chosen)

³ number of PHA granules (only sharply and completely depicted granules were chosen)

⁴ radius of PHA granules in μm, calculated by multiplying the measured diameter with the electron microscopy magnification factor

⁵ volume average per PHA granule

⁶ number of PHA granules per cell

⁷ volume of PHA granules (volume average per PHA granule times number of granules per cell) per cell volume (percent)

⁸ minimum value

⁹ maximum value

¹⁰ PHA amount (% w/w) as calculated from micrographs

¹¹ PHA amount (% w/w) as determined by GC measurements

¹² not determined since PHA granules were spherical (assumption: a=b)
Fig. 3.4.: PHA granule associated proteins of *P. oleovorans* GPo1000 and GPo1001. Cells were cultivated in 0.1NE2 minimal medium containing 15 mM octanoate and were harvested in the early stationary phase. PHA granules were isolated and analyzed by SDS-PAGE. Granule associated proteins of *P. oleovorans* GPo1000 (lane A) and GPo1001 (lane B) are shown. Lane C: Molecular mass standard proteins with the masses indicated on the right are depicted. PhaI and PhaF proteins are indicated.
Complementation of the phaD mutant GPo1001

To test if the GPo1001 dependent phenotype described above was attributable to disruption of the phaD gene or was due to another unrecognized mutation or to a polar effect, we prepared a broad host range phaD expression construct to complement the mutant GPo1001. We digested plasmid pGEc404 with StuI and Asp718, generating a 1 kb fragment containing phaD which was cloned into vector pBCKS digested with EcoRV and Asp718. The resulting construct, named pHAD2, was sequenced in order to verify the phaD gene sequence. Subsequently, pHAD2 was digested with EcoRI-Asp718. Prior to ligation the ends of this fragment were dephosphorylated with arctic shrimp alkaline phosphatase (SAP) followed by cloning into EcoRI-Asp718 digested pVLT33, a broad host range vector. This construct, containing the phaD ORF downstream of a tac promoter, was named pHAD5. By triparental mating, using E. coli HB101 pRK600 as a helper strain, pHAD5 was transferred into GPo1001. Clones were selected on citrate containing minimal medium plates with kanamycin. PHA accumulation of GPo1001 pHAD5 was tested in minimal medium 0.1NE2 with 15 mM octanoate and kanamycin. The cells were induced in the early exponential phase (OD450 = 0.2) with 0.5 mM IPTG. As shown in Table 3.2., pHAD5 was able to partly restore the PHA accumulation characteristics of the mutant GPo1001 and was able to restore the growth characteristics fully leading to a cell dry mass (cdm) accumulation which was slightly higher than that in the wild type.

Furthermore, we did transmission electron microscopy studies with the cells of the complemented mutant. As depicted in Fig. 3.3. C, which shows a representative cell of GPo1001 harbouring pHAD5, the cell size and the number and size of granules was similar to that found for wild type cells (Fig. 3.3. A).
Discussion

In this study it is shown for the first time that the \textit{phaD} gene of \textit{P. oleovorans} plays an important role in mcl PHA biosynthesis in this organism. To study the effects of \textit{phaD} we generated the \textit{P. oleovorans} mutant GPo1001, carrying a \textit{tet} gene cassette insertion in the \textit{phaD} gene. The knock out resulted in a decreased mcl PHA accumulation, in a changed granule architecture and in a reduced cell size.

Upon insertion of the \textit{tet} gene cassette in the \textit{phaD} gene, we observed several effects on polymer accumulation in \textit{P. oleovorans}. Firstly, mcl PHA production in the \textit{phaD} mutant was less than 20\% of that of the wild type. The same effect was observed by Wieczorek et al. when the \textit{phaP} gene in \textit{Ralstonia eutropha}, encoding a phasin protein, was inactivated (29). Also in other species, such as \textit{Rhodococcus ruber} and \textit{P. putida}, reduction of PHA accumulation by disruption of genes coding for phasins has been described (19, 27).

To verify the \textit{phaD} knock out in GPo1001, Southern blotting (Fig. 3.2.) and complementation of the mutant GPo1001 by introducing a \textit{phaD} containing plasmid were done. Upon introduction of the \textit{phaD} containing construct mcl PHA levels were partially restored (Table 3.1.). Differences in terms of the mcl PHA content between the complemented mutant and the wild type might be due to a changed expression level of the extrachromosomal \textit{phaD} gene or to a possible disruption of \textit{phaC2} in GPo1001, caused by homologous recombination. Thus, we could demonstrate that the effects on mcl PHA accumulation in the mutant GPo1001 reported here were due to \textit{phaD} inactivation rather than to a polar effect on downstream genes.

We initially concluded from the observations on reduced PHA production by \textit{phaD} inactivation that \textit{phaD} might code for a phasin protein. However, the data on PHA granule number, size and shape in the \textit{phaD} mutant were not in agreement with characteristics of other phasins studied by Wieczorek and Pieper-Fürst (18, 29). It has been shown for \textit{R. eutropha}, that upon inactivation of \textit{phaP} only one large PHA granule was built inside the cell (29), whereas \textit{phaP} overexpression in the
\textit{phaP} mutant led to an increase in granule numbers compared to the wild type (29). As shown in Fig. 3.3. and Table 3.3., knock out of \textit{phaD} increased the number of PHA granules whereas complementation of the mutant by a \textit{phaD} harbouring plasmid restored the number of granules to wild type level.

Moreover, in contrast to phasins described earlier (18-20, 27, 29), PhaD was not bound to PHA granules. As shown in Fig. 3.4., in the wild type we detected two major protein bands, representing the phasins PhaI and PhaF at 18 and 36 kDa, but no protein band of the expected size of the PhaD protein (23 kDa), in accord with previous studies which showed PhaI and PhaF but not PhaD to be major granule associated proteins (20, 27). Moreover, the analysis of granule associated proteins of the \textit{phaD} mutant revealed that the protein pattern of PHA granules was changed and that the PhaI protein was missing (Fig. 3.4.). Preventing major granule associated proteins, like PhaI, to bind to PHA granules has been shown to result in unspecific binding of cytoplasmic proteins to the granules and can therefore result in detrimental effects on metabolism or structural integrity of the cells (15, 26). This unspecific cytoplasmic protein binding to granules and removal from the cytoplasm might explain the change in the pattern of granule associated proteins and might account for the smaller size of the \textit{phaD} mutant cells (Fig. 3.3.). In agreement with this hypothesis, we found that complementation of GPo1001 with a \textit{phaD} harbouring construct led to restoration of the cell size to wild type level (Fig. 3.3.).

In Table 3.2. we report that biodegradation of the PHA polymer in the mutant is significant, reducing the intracellular polymer content after 46 hours of incubation to 1 % (w/w) while biodegradation of PHA in wild type cells is not detectable. It is known that during PHA synthesis the depolymerase is active to a certain extent (31). Doi et al. reported that the rate of PHA accumulation in batch fermentation of \textit{R. eutropha} was about 10 times higher than that of PHA degradation (6). Thus, an overall decrease of PHA in the mutant GPo1001 could be caused by a decreased rate of PHA biosynthesis while the rate of depolymerisation remained constant.
In summary, we conclude that phaD is an important constituent of PHA biosynthesis but does not code for a granule associated protein. Thus, we propose that it acts via an indirect mechanism, preventing expression or binding of major granule proteins, like Phal, leading to the observed effects on PHA accumulation. Therefore, Phal absence might account for the PHA leaky phenotype, which was also proposed by Valentin et al., who deleted the Phal encoding gene (called GA1 in that report) in _P. putida_ and thereby decreased PHA production in batch cultivation by more than 65% (27).

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Inactivation of Isocitrate Lyase Leads to Increased Production of Medium Chain Length Poly(3-Hydroxyalkanaotes) in *Pseudomonas putida*

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Abstract

Medium chain length poly(3-hydroxyalkanoates) (mcl PHAs) are storage polymers, accumulated from various substrates in Pseudomonas bacteria of the rRNA homology group I. In experiments aimed at increasing the PHA production in Pseudomonas, we generated a mcl PHA overproducing mutant of Pseudomonas putida KT2442 by transposon mutagenesis, in which the aceA gene was knocked out. This mutation inactivated the glyoxylate shunt and reduced in vitro activity of isocitrate dehydrogenase, a rate-limiting enzyme of the citric acid cycle. The genotype of the mutant was confirmed by DNA sequencing and the phenotype was supported by biochemical experiments. The aceA mutant was not able to grow on acetate as sole carbon source due to disruption of the glyoxylate bypass and showed 2-5 fold reduction of isocitrate dehydrogenase activity. During growth on gluconate the difference between the mean PHA accumulation in mutant and wild type strain was 52%, leading to a significant increase of mcl PHA accumulation at the end of the exponential phase in the mutant Pseudomonas putida KT217. Stoichiometric flux analysis predicted that the knock out of the glyoxylate pathway in addition to reduced flux through isocitrate dehydrogenase should lead to increased flux into the fatty acid synthesis pathway. Therefore, enhanced carbon flow towards the fatty acid synthesis pathway increased the amount of mcl PHA that could be accumulated by the mutant.
Introduction

Many bacteria are able to accumulate poly(3-hydroxyalkanoates) (PHAs) as carbon and energy reserve. Because of their potential use as biodegradable thermoplastics and as biopolymers that can be produced from renewable resources, PHAs have been extensively studied by academic and industrial groups (2, 6, 30). Pseudomonads synthesize mainly medium chain length (mcl) PHAs, formed of monomers of 6 to 14 carbons (8, 14, 31). Although a few PHAs have been commercially developed and marketed (6, 11), the widespread use of these polymers has been hindered by the high production cost (1, 19). Reduction of these costs could be achieved by several means including enhancing the product yield (19) or using transgenic plants for PHA production, provided that PHA levels can be brought to 20 - 40% of the plant dry weight (23, 24, 32).

We have during the past few years made several sets of mutants of Pseudomonas putida KT2442 with the goal to alter the carbon flux towards mcl PHA. While most of these mutants showed clearly reduced mcl PHA levels, a few have shown increased PHA production. Preliminary analysis of one of these mutants (P. putida KT217) showed it to be affected in the glyoxylate pathway.

From studies on P. putida KT2442, it is known that PHA precursors can be produced via three main pathways: ß-oxidation, de novo fatty acid biosynthesis and elongation of 3-hydroxyalkanoates by acetyl-CoA molecules (12, 32). The ß-oxidation pathway is active during growth on fatty acids, whereas during growth on carbohydrate or carbohydrate derived substrates such as sugars or gluconate, PHA precursors are generated via the fatty acid synthesis pathway (13). Therefore, when cells are grown on gluconate, acetyl-CoA is a key intermediate of the PHA biosynthesis pathway, and since acetyl-CoA plays an essential role in replenishing both the citric acid cycle and the fatty acid synthesis pathway, PHA synthesis competes with the citric acid cycle for acetyl-CoA. To decrease the flux of acetyl-CoA into the citric acid cycle, either the expression level of citrate synthase or the concentration of oxaloacetate should be lowered. The intracellular concentration of
oxaloacetate can be reduced by cutting off the supply of its direct precursor malate by blocking the glyoxylate pathway and/or by preventing processing of isocitrate by reducing isocitrate dehydrogenase activity. Therefore, inactivation of the glyoxylate pathway and downregulation of isocitrate dehydrogenase should result in an increased overall flux of acetyl-CoA into mcl PHA production.

In this paper we describe the mutant *P. putida* KT217 affected in the glyoxylate pathway and examine the potential of isocitrate lyase and the citric acid cycle in increasing mcl PHA levels in *P. putida*. 
Material and Methods

Bacterial strains, plasmids and growth conditions

_Pseudomonas putida_ KT2442 (3) is a strain, derived from _P. putida_ mt2, cured of the TOL plasmid, which is able to accumulate mcl PHA from various substrates. _P. putida_ KT217 is an aceA knock out mutant of _P. putida_ KT2442 generated in this study. _Escherichia coli_ strains JM109 (38), HB101 (25), S17-λpir (20) were used. Plasmids pGEC404 (15), pRK600 (7), pUC18 (38) and pUT mini-Tn5 tet (7), were utilized.

_Pseudomonas_ cells were grown in minimal medium 0.1 N E2 (17) at 30°C with addition of gluconate or a mixture of gluconate and heptanoate as indicated. _E. coli_ strains were grown at 37°C in complex Luria-Bertani (LB) medium (25). Cells were cultivated in Erlenmeyer flasks and incubated at 225 rpm. Antibiotics were added as needed: 12.5 μg/ml tetracycline, 30 μg/ml rifampicin. Media were solidified with 1.5% (w/v) agar for plate experiments. Cell dry weight (cdw) was determined gravimetrically (10) and spectrophotometrically at 450 nm (36). Cultures were harvested by centrifugation and washed with 10 mM MgSO₄. For determination of PHA the cell pellet was lyophilized.

DNA manipulation

Basic recombinant DNA techniques, such as preparation and purification of plasmid DNA, restriction endonuclease digestion, agarose gel electrophoresis and transformation of _E. coli_ were essentially carried out as described by Sambrook et al. (25).

Transposon mutagenesis was performed as described before (7). Southern blot analysis was performed by using a 2 kb _tet_ gene fragment, obtained by _SmaI_ digestion of pUT mini-Tn5 tet (7). The probe was labeled using the DIG labeling and detection kit (Boehringer Mannheim).
DNA sequencing and analysis of sequence data

DNA sequencing was performed with denatured double-stranded plasmid DNA using the Amersham thermo sequenase fluorescent labeled primer cycle sequencing kit. Standard primer (pUC18/19 -40 forward and -40 reverse primer) and primer binding to the mini-Tn5 derived tetracycline gene (primer A: 5' - GATGTTACCCGAGAGCTTACC-3' and primer B: 5' - TAAGCGTGCATAATAAGCCCTACA-3') were used. The homology search was done using the NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) programs.

PHA determination

Analysis of PHA accumulation and composition was performed as described before (16).

Enzyme assays

*Pseudomonas* cells were prepared as spheroplasts (37) and opened by two passages through a French pressure cell (20000 PSI). The isocitrate lyase was assayed by coupling the formation and subsequent reduction of glyoxylate to glycolate with the oxidation of NADH with lactate dehydrogenase (9). Each cuvette contained, in a final volume of 1 ml, 50 mM MOPS/NaOH, pH 7.3, 5 mM MgCl₂, 5 mM DL-isocitrate, 0.2 mM NADH, pig heart lactate dehydrogenase (0.1 mg/ml). The isocitrate lyase activity was corrected for non isocitrate lyase related NADH oxidation by subtracting the activity found in controls in which isocitrate was omitted. Isocitrate dehydrogenase was measured by monitoring its ability to reduce NADP. Each cuvette contained, in a final volume of 1 ml, 50 mM MOPS/NaOH, pH 7.3, 5 mM MgCl₂, 5 mM DL-isocitrate, 0.2 mM NADP. The isocitrate dehydrogenase activity was corrected for non isocitrate dehydrogenase related NADP reduction by subtracting the activity found in controls in which isocitrate was omitted. Protein concentrations were measured by using the Bradford assay (Bio-Rad Laboratories). All enzymes were assayed at 37° C and
specific activities are quoted per mg of crude supernatant protein. An enzyme unit was defined as the amount of protein necessary for the conversion of 1 μmol isocitrate per min.

**Flux analysis I: Set up of metabolic network**

The biochemical reaction network for *P. putida* shown in Fig. 4 was constructed on the basis of established knowledge (29) and on genomic sequence analysis of the *P. aeruginosa* genome represented in the EMP database (http://wit.mcs.anl.gov/WIT2) (27, 28). Serial reaction steps were considered by lumped reactions, combining several reactions in one equation. The stoichiometric matrix was formulated accordingly. Based on thermodynamics and typical intracellular metabolite concentrations, the reactions catalyzed by the following enzymes were considered to be physiologically irreversible: pyruvate dehydrogenase, citrate synthase, 2-oxoglutarate synthase, malate dehydrogenase, malate synthase, the respiratory reaction as well as gluconokinase. Boundaries were imposed so that irreversible reactions had non-negative fluxes. The reaction from acetyl-CoA to mcl PHA was assumed to consist of the appropriate reactions of the fatty acid synthesis cycle and the polymerization reaction from 3-hydroxy acyl-CoA to the polymer. Conversion of NADPH to NADH via transhydrogenase reaction was assumed to be reversible (35). Precursor requirements for biomass formation were taken from Neidhardt and coworkers (22) considering the modifications proposed by Sauer et al. (26).

The principles of stoichiometric flux analysis have been extensively covered in the literature (33). Specific metabolite concentrations can be balanced according to the dynamic flux balance

$$\frac{dx}{dt} = S \cdot v - b \quad \text{(eq. 1)}$$

where \(x\) denotes the metabolite concentrations, \(S\) the stoichiometric matrix, \(v\) the intracellular reaction fluxes and \(b\) the withdrawal fluxes from the reaction system. Assuming balanced growth in the exponential growth phase of the batch cultures, the dynamic flux balance (eq. 1) reduces to the quasi steady state balance.
\[ S \ast v = b \]  \hspace{1cm} \text{(eq. 2)}

The dimension of the stoichiometric matrix was 22 x 22 with rank 20. The carbon dioxide as well as the oxygen balance was excluded. Flux through malic enzyme was assumed to be negligible (21) and the glyoxylate shunt was found to be inactive in the case of the mutant strain KT217, reducing the dimension of the stoichiometric matrix to 20 x 20 with rank 20, indicating a determined system. The linear equation system was solved using the linprog function from the MATLAB Optimization Toolbox (5). In case of the wild type KT2442 the system was underdetermined due to an active glyoxylate shunt, which did not allow to solve the system for an unambiguous solution.

**Flux analysis II: Solving the flux model**

In order to examine stoichiometric dependencies between fluxes of isocitrate dehydrogenase and specific mcl PHA production in wild type and mutant strains the complete system was considered without any constraints on the malic enzyme reaction and, in the case of the wild type strain, glyoxylate shunt activity. In this case the minimization of substrate conversion into PHA was used as objective function.
Results

Preparation of the transposon mutant *P. putida* KT217

Cells, mutagenised with the mini-Tn5 "tet" transposon, were screened on a 0.1 NE2 medium containing 15 mM octanoate, 30 µg/ml tetracycline and traces of LB. Colonies which grew poorly compared to wild type colonies, were plated on gluconate. Cells which showed slow growth on octanoate with LB traces, and normal growth on gluconate, similar to the wild type, were selected for further analysis. From 15,000 colonies 6 clones were selected and characterized in more detail. To determine whether the observed phenotype of the mutants was due to insertion of the mini-Tn5 "tet" into the bacterial chromosome, hybridization experiments were carried out. Chromosomal DNA of the 6 mutants was digested with *KpnI* and hybridized with the labeled fragment of the "tet" gene (data not shown). Since the mutants showed positive hybridization in Southern blot experiments, they were used for further characterization. One of the putative mutants, named KT217, was unable to grow on acetate and other Mcl fatty acids, such as hexanoic acid or decanoic acid as sole carbon source and was chosen for further analysis.

Characterisation of the genotype of KT217

Chromosomal DNA of mutant KT217 was digested with *KpnI* and ligated into *KpnI* digested pUC18 vector. Tetracycline resistant clones contained an insert of approximately 5.8 kb. The nucleotide sequence of the transposon flanking regions was determined by DNA sequencing. To determine the gene or chromosomal DNA stretch into which the mini-Tn5 "tet" transposon was integrated a homology search with the obtained nucleotide sequences was carried out. The two transposon flanking regions of KT217 showed highest homology to the aceA gene of *E. coli* (64% on amino acid sequence levels) indicating that we knocked out a gene encoding an isocitrate lyase. Moreover, analysis of the isocitrate lyase activity in crude extracts showed that *P. putida* KT217 contained only 1-5% of this enzyme
activity compared to *P. putida* KT2442, suggesting a block of the glyoxylate pathway (Table 4.1.). Determination of the *in vitro* activity of isocitrate dehydrogenase revealed a decrease in activity in the mutant KT217 by a factor of 2-5 dependent on the substrate compared with the wild type KT2442 (Table 4.1.). Apparently, inactivation of the aceA gene not only disrupted the glyoxylate pathway but also significantly reduced the isocitrate dehydrogenase activity.

**Accumulation of PHA in the aceA knock out mutant KT217 grown on gluconate**

To investigate whether the aceA mutation affects PHA biosynthesis, we determined the growth phase dependent PHA accumulation of *P. putida* KT217 and its parental strain *P. putida* KT2442. Table 4.2. shows that for growth on gluconate the cell dry weight (g l^1^) and polymer accumulation by KT217 were significantly higher at the end of the exponential phase and in the late stationary phase than for the wild type KT2442. The difference between the mean PHA accumulation in mutant and wild type strain was 52%, leading to an increase of mcl PHA accumulation at the end of the exponential phase in the mutant. In the late stationary phase the difference between the mean PHA accumulation in mutant and wild type strain was 8%. The PHA monomer composition was relatively constant during the observed period and showed 3-hydroxydecanoate as the predominant monomer in both strains (Table 4.2.).

**Accumulation of PHA in the aceA knock out mutant KT217 grown on mixtures of gluconate and fatty acids**

When we cultured cells on mixtures of gluconate and heptanoate, accumulation of PHA and the PHA monomer composition was dependent on substrate ratios. Fig. 4.1. A shows that incorporation of heptanoate derived monomers (C7) into PHA was low in mutant and wild type, when cells were cultured on low heptanoate concentrations (gluconate/ heptanoate ratio = 21) due to heptanoate substrate limitation. With increasing heptanoate concentrations (gluconate/ heptanoate ratio = 5 - 2), the C7 monomer incorporation into wild type PHA increased while the
Table 4.1.: Specific activities of isocitrate lyase and isocitrate dehydrogenase in crude extracts of *P. putida* KT2442 and *P. putida* KT217

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cultivation (hrs)</th>
<th>Specific activity <em>(nmol/min/mg)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Isocitrate lyase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KT2442</td>
</tr>
<tr>
<td>46 mM gluconate</td>
<td>24</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>35</td>
</tr>
<tr>
<td>23 mM gluconate, 10 mM heptanoate</td>
<td>24</td>
<td>321</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>147</td>
</tr>
</tbody>
</table>

1 Cells were cultivated in 0.1 N E2 minimal medium with gluconate or a mixture of gluconate and heptanoate at the concentrations indicated. The cells were harvested at the end of the exponential phase (24 hours) and at the late stationary phase (48 hours).

2 Crude extracts were prepared and assayed for isocitrate lyase and for isocitrate dehydrogenase activity.
Table 4.2: PHA content and monomer composition of *P. putida* KT2442 and *P. putida* KT217 cultivated on gluconate.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Time (hrs)</th>
<th>cdw (g)</th>
<th>PHA (g/l)</th>
<th>PHA (%)</th>
<th>C6</th>
<th>C8</th>
<th>C10</th>
<th>C12</th>
<th>C12:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT2442</td>
<td>24</td>
<td>0.12</td>
<td>1.7</td>
<td>17.2</td>
<td>4.3</td>
<td>3.2</td>
<td>0.9</td>
<td>15.3</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.87</td>
<td>0.7</td>
<td>13.9</td>
<td>3.9</td>
<td>7.5</td>
<td>1.4</td>
<td>14.8</td>
<td>0.18</td>
</tr>
<tr>
<td>KT217</td>
<td>24</td>
<td>0.12</td>
<td>1.7</td>
<td>17.2</td>
<td>4.3</td>
<td>3.2</td>
<td>0.9</td>
<td>15.3</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.87</td>
<td>0.7</td>
<td>13.9</td>
<td>3.9</td>
<td>7.5</td>
<td>1.4</td>
<td>14.8</td>
<td>0.18</td>
</tr>
</tbody>
</table>

1. cdw = cell dry weight
2. C6: 3-hydroxyhexanoate, C8: 3-hydroxyoctanoate, C10: 3-hydroxydecanoate, C12: 3-hydroxydodecanoate, C12:1: 3-hydroxy-5-cis-dodecenoate.

*Cellswere cultivated in 0.1 N E2 minimal medium with 1% gluconate. At the end of the exponential phase (24 hours) and at the late stationary phase (48 hours) cells were harvested.*
mutant incorporated less C7 into PHA than the wild type due to an unknown effect. At a gluconate/heptanoate ratio of 0.3 mutant cells incorporated much lower amounts of C7 monomers into PHA than the wild type (Fig. 4.1. A), indicating metabolic limitations because of limiting gluconate in combination with aceA gene inactivation.

Cells of KT217 grown on gluconate/heptanoate ratios between 2 - 21 produced higher amounts of PHA derived from gluconate than the wild type (Fig. 4.1. B), incorporated as monomers with even numbers of carbon atoms (3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate, 3-hydroxydodecenoate = Ceven). At low gluconate concentration (gluconate/heptanoate ratio = 0.3), both wild type and mutant strains incorporated low amounts of Ceven into PHA due to gluconate limitation (Fig. 4.1. B).

The results indicate that gluconate derived monomer incorporation into PHA was higher in the mutant KT217 than in the wild type, if gluconate was available in excess (Fig. 4.1. B). Increased incorporation of Ceven monomers into PHA might be due to the aceA knock out in KT217 and is in agreement with higher mcl PHA accumulation of KT217 compared to the wild type when grown on gluconate as sole carbon source. However, when one of the substrates gluconate or heptanoate was present in low concentrations, low amounts of the corresponding monomers (C7 for gluconate/heptanoate ratio = 21 (Fig. 4.1. A) and Ceven for gluconate/heptanoate ratio of 0.3 (Fig. 4.1. B) were incorporated into PHA in both mutant and wild type.
Fig. 4.1.: Incorporation of monomers into PHA by *P. putida* KT2442 (filled rectangles) and the aceA knock out mutant *P. putida* KT217 (open rectangles), grown on substrate mixtures of gluconate and heptanoate. Cells were cultivated on the following substrate mixtures in 0.1 N E2 minimal medium: 42 mM gluconate, 2 mM heptanoate (ratio gluc./hept. = 21); 32 mM gluconate, 6 mM heptanoate (ratio gluc./hept. = 5); 23 mM gluconate, 10 mM heptanoate (ratio gluc./hept. = 2); 5 mM gluconate, 18 mM heptanoate (ratio gluc./hept. = 0.3). Samples were harvested after 48 hours of cultivation, lyophilized and analyzed by GC.

A) Incorporation of C7 monomers (3-hydroxyheptanoate) into mcl PHA, relative to total cell dry weight.

B) Total mass of Ceven monomers (3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate and 3-hydroxydodecanoate, 3-hydroxydodecenoate), incorporated into PHA, relative to total cell dry weight.
**Discussion**

In this study we obtained a transposon mutant of *P. putida* which showed increased levels of mcl PHA. Analysis of the mutant revealed that by knock out of the *aceA* gene the glyoxylate pathway was inhibited and the activity of isocitrate dehydrogenase was reduced, resulting in increased carbon flux towards mcl PHA. We hypothesize that the increased mcl PHA accumulation in the mutant KT217 is caused by the following mechanism: Knock out of the *aceA* gene in KT217 results in the glyoxylate pathway being disrupted. As a result of transcriptional stop elements, flanking the transposon inserted in the *aceA* gene, transcription of the *aceK* gene encoding isocitrate dehydrogenase kinase/phosphatase would be prevented. As it was shown before (18), inactivation of the *aceK* gene can lead to reduced activity of isocitrate dehydrogenase. Due to this polar effect of the *aceA* gene knock out the isocitrate dehydrogenase activity in KT217 is reduced corresponding to a reduction in carbon flux through the protein (34). Consequently, flux of acetyl-CoA into the citric acid cycle will be diminished and flux into the fatty acid synthesis pathway will be increased leading to increased amounts of mcl PHA in KT217.

Surprisingly, the reduction of the isocitrate dehydrogenase activity did not lead to a reduced growth of the mutant. As a possible explanation for this result, we tested the hypothesis that the mutant excreted less secondary metabolites than the wild type (e.g. acetate). Since we could not detect significant amounts of metabolites in the culture broth supernatant neither in the mutant nor in the wild type (data not shown), this hypothesis was found not to be valid. However, we concluded that the flux of carbon through the citrate cycle in the wild type was not a limiting factor for the growth rate. Presumably, other metabolites important for growth-relevant precursor production (e.g. acetyl-CoA), which might be increased by the *aceA* mutation, led to the fast growth in the mutant.

Stoichiometric flux analysis of *P. putida* KT217 cultivated in batch culture on gluconate was carried out (Fig. 4.2.). The model predicted increased flux into PHA.
Fig. 4.2.: Flux distribution of P. putida KT217 during exponential growth on gluconate. Fluxes (numbers in boxes) are given in percent of the specific gluconate uptake rate. Malic enzyme is considered to use NAD as cofactor. Gluconeogenetic reactions and the non-oxidative branch of the pentose phosphate cycle are not shown. Abbreviations: EDP = Entner Doudoroff pathway, EMP = glycolysis, FS = fatty acid biosynthesis, GC = glyoxylate cycle, GNG = gluconeogenesis, PPC = pentose phosphate cycle, TCC = citric acid cycle. A = phosphoenolpyruvate carboxylase, B = malic enzyme, C = pyruvate dehydrogenase, D = malate synthase, E = isocitrate lyase, F = isocitrate dehydrogenase, G = 2-oxoglutarate synthase. T3P = triose-3-phosphate, PEP = phosphoenolpyruvate, PYR = pyruvate, ACoA, acetyl-CoA. OAA = oxaloacetate, GLY = glyoxylate, ICI = isocitrate, SUC = succinate, MAL = malate, * FADH2 is treated as NADH.
if flux through isocitrate dehydrogenase is reduced in combination with zero flux through the glyoxylate shunt. Since in vivo flux data were not experimentally available, we used in vitro enzyme assays. This is a reasonable approach because isocitrate dehydrogenase is one of the rate-controlling enzymes in the citric acid cycle (34), so that we expected a positive correlation between activity and flux through the enzyme. Indeed, we determined the activity of isocitrate dehydrogenase to be reduced by a factor of 2-5 in the mutant (Table 4.1.). Thus, stoichiometric flux analysis corroborated our hypothesis of enhanced carbon flow into mcl PHA in the mutant KT217.

At high ratios of gluconate/ heptanoate mixtures the mutant accumulated higher amounts of gluconate derived PHA than the wild type (Fig. 4.1. B). In agreement with our pathway hypothesis, the data on mixed substrates indicate that incorporation of gluconate derived monomers into PHA was increased in the mutant KT217 compared to the wild type, if the carbon source gluconate was available in excess.

In summary, this study demonstrates that modifying carbon flux can help in increasing accumulation of bacterial products and therefore can be a powerful tool in biotechnological strain improvement. However, since it is known that cellular systems often do only show no or minor phenotype changes upon mutations of a single gene unless a certain set of other genes is simultaneously altered (4), combination of the aceA knock out with other mutations might affect mcl PHA production even more seriously. Therefore, in our future work we will look more closely at the generation of strains with multiple mutations to further increase the mcl PHA production in Pseudomonas.

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CHAPTER 5

General Conclusions
Why generation of mcl PHA producing plants?

The concern for environmental issues, which has developed during the past 30 years in western societies, has led to a growing demand for sustainable processes and products. As part of this trend the potential of biopolymers has begun to be explored, since compared to conventional thermoplastics these polymers have the advantage of being biodegradable and of being produced from renewable resources. PHAs, as one of the most interesting groups of biodegradable polymers, share these advantages but have not yet succeed as commercial products, mainly because of their high price. The price for PHB produced by a fermentation process has decreased to 15 US$/kg compared to 1 US$/kg or less for standard polymers like polyethylene or polyvinylchloride (5). Thus, instead of producing mcl PHAs by fermentation, an EU research project with the goal of generating crop plants capable of producing mcl PHA was launched. An interdisciplinary group was built to tackle this task. As biotechnologists/microbiologists our part of the project included the development of recombinant PHA producing microorganisms and the generation of knowledge how PHA accumulation can be increased.

Feasibility of the generation of high-level mcl PHA producing plants

Mcl PHA producing plants of commercial interest have to accumulate between 15-20% (w/w) of the polyester (1). Although Poirier et al. showed that production of polyester including mcl PHA is basically feasible in plants (2-4), their results also pointed to the hurdles in this project. One of the challenges to production of PHAs in plants is to metabolically engineer target plants so as to generate the appropriate intermediates for mcl PHA production. These intermediates must originate from common carbon metabolite pools found in the plant, and thus require the design of novel pathways that take into consideration a number of factors. These include the pool of carbon available during different plant developmental stages, the specific plant tissue targeted for production (such as seed and leaf), and the targeted subcellular location for biosynthesis.
Chapter 5 111 General Conclusions

Keeping in mind the complexity of the task, it is not surprising that in the last three years of the EU research project, only the preliminary work (e.g. generation and transfer of constructs, testing for mcl PHA polymerase expression in transgenic plants etc.) has been done. A time horizon between 5-10 years till the first mcl PHA producing plant of commercial interest is generated might be realistic. This time will also be dependent on engagement of industry in this project. Since Monsanto closed down its mcl PHA transgenic plant research project and its PHA Biopol plant in 1998, the overall speed of transgenic plant development might decrease.

Applicability of Results for mcl PHA transgenic plant generation

One possibility to modify the plant metabolism for the production of PHA precursors could include the approach presented in Chapter 2. In that case mcl PHA synthase and the thioesterase must be targeted to the peroxisome, the cellular compartment where fatty acid synthesis and β-oxidation are mainly located in plants. That mcl PHA production in this organelle is feasible was shown recently (2).

Once transgenic plants that produce 5-10% mcl PHA are obtained, addition of genes which further increase the mcl PHA yield would be useful. Such genes could include the phaD and phaF gene, since it was shown in Chapter 3 that lack of expression of these genes leads to a significant decrease in mcl PHA accumulation. Modification of carbon flux through the citrate cycle in order to increase the precursor flux towards mcl PHA production might also be transferable to transgenic plants (Chapter 4).

Summarizing the results obtained thus far, they have the potential to be applied in generation of mcl PHA producing plants. A combination of 2 or 3 approaches might even multiply the positive effects on polyester production in plants.

Outlook

There is a long road to go, since the interest in sustainable products is only at its dawn. However, with decreasing oil reserves, increasing natural resource prices, and reduced space for landfill, the development of sustainable and also cheap material
like mcl PHA will dominate academic and industrial laboratories and sooner or later they will appear as biopolymers on the marketplace.
References


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