Polyester synthases and polyester granule assembly

A thesis presented to Massey University in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Microbiology

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Dedicated to my parents

Meinen Eltern in Dankbarkeit gewidmet

The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" ("I found it!"), but rather, "hmm.... that's funny...."

Isaac Asimov (1920-1992), biochemist and science fiction author

Acknowledgements

Preface

This thesis is written according to the regulations of the latest version of the Handbook for Doctoral Study, published by the Doctoral Research Committee in April 2007 (GRS version 3). The thesis format complies with the format of a thesis based on publications, as described on page 61-62 under chapter "Submission of a thesis based on publications".

Below, all chapters which are published or submitted for publication are listed. These publications do not appear in chronological order.

Chapter I B:

Katrin Grage, Verena Peters, Rajasekaran Palanisamy and Bernd H. A. Rehm (2009). Polyhydroxyalkanoates: from bacterial storage compound via alternative plastic to bio-bead.

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Chapter II:

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Chapter IV:

Jane A. Brockelbank, Verena Peters and Bernd H. A. Rehm (2006). Recombinant *Escherichia coli* produces ZZ domain displaying biopolyester granules suitable for IgG purification.

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Chapter V:

Verena Peters and Bernd H. A. Rehm (2008). Protein engineering of streptavidin for *in vivo* assembly of streptavidin beads.

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Listing of research contributed to the publications/chapters by Verena Peters

Chapter I B: The book chapter was partly written by Verena Peters, with particular emphasis on structure, associated proteins, assembly process and biotechnological potential of PHA granules.

Chapter II: All experiment regarding the polar positional information of the PHA synthase were conducted by Verena Peters, including primer/vector design and cloning, mutagenesis of the PHA synthase, translational fusions to GFP and fluorescent microscopy imaging. The localisation experiments of the phasin protein were also performed by Verena Peters, including primer and vector design and cloning, expression experiments and fluorescent microscopy imaging. Dorit Becher did all in-frame linker/hemagglutinin insertion mutagenesis experiments including the mutant analyses. Verena Peters repeated the preparation of samples for GC/MS analyses of all linker PHA synthase insertion mutants and all hemagglutinin PHA synthase insertion mutants.

Chapter III: All experiments were performed by Verena Peters. Plasmid pBHR80AlacZ was constructed by Verena Peters during her diploma thesis. Jane Brockelbank is acknowledged for her SDS-PAGE analysis of whole cell extract of *Escherichia coli* expressing the fusion protein under lac promoter control. Isogenic marker-free Δ*phaC1-Z-ΔC2* deletion mutant of *P. aeruginosa* PAO1 was provided.

Chapter IV: Verena Peters designed all primers/vectors used in this study. The cloning strategy was developed by Verena Peters, and she also conducted the preparative SDS-PAGE analyses of fusion proteins for MALDI-TOF/MS analyses.

Chapter V: All experiments were done by Verena Peters. Natalie Parlene is acknowledged for her assistance in operating the FACS instrument.

A service provider was used for DNA Sequencing, TEM, MALDI-TOF/MS and GC/MS analyses.

This is to certify that the above mentioned research has been conducted by Verena Peters.

_____   _____
(Date, Signature)   (Date, Signature)
Prof. Bernd H. A. Rehm   Verena Peters

Abstract

PHAs are a class of biopolymers consisting of (R)-3-hydroxy-fatty acids and are produced by the majority of eubacteria and some archaeal bacteria as carbon storage material. In general, PHA is synthesised when a carbon source is available in excess while another essential nutrient is limited. The key enzyme of PHA biosynthesis, the PHA synthase, catalyses the polymerisation of the substrate (R)-3-hydroxyacyl-CoA to PHA accompanied by the release of coenzyme A. PHA is stored intracellularly as inclusions, the so-called PHA granules. When the external carbon source becomes exhausted, bacteria can metabolise these carbon inclusions by degradation of the polymer.

PHA granules are water-insoluble, spherical inclusions of approximately 50-500 nm in diameter which consist of a hydrophobic polyester core surrounded by a phospholipid layer with embedded and attached proteins. One could consider isolated PHA granules as bio-beads due to their structure and size. In this study we tested if the PHA synthase can be used as an anchor molecule in order to display proteins of interest at the PHA granule surface. Furthermore, these modified PHA granules were analysed for their potential applicability as bio-beads in biotechnological procedures. The concept of using the PHA synthase as granule-anchoring molecule for display of proteins of interest was established by the functional display of the β-galactosidase at PHA granules. This "proof of concept" was followed by the display of biotechnologically more interesting proteins. The IgG binding domain of protein A as well as streptavidin, which is known for its biotin binding ability, were fused to the PHA synthase, respectively, and therefore localised at the PHA granule surfaces during PHA granule assembly, resulting in functional bio-protein A -beads and bio-streptavidin-beads. Moreover, their applicability in biotechnological assays was demonstrated.

Recently, we fused the green fluorescent protein (GFP) to the PHA synthase and demonstrated that the PHA granule assembly does not start randomly distributed in the cytoplasm but occurred localised at or near the cell poles. To further investigate if the localisation of the PHA granule formation process is due to polar positional information inherent to the PHA synthase, different mutated versions of the PHA synthase of *Cupriavidus necator* were created and analysed for a potential alteration in localisation. Furthermore, the phasin protein PhaP1 of *C. necator* was fused to HcRed, a far-red fluorescent protein, and localisation studies were accomplished when the fusion protein was expressed under different conditions in *Escherichia coli*.

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Abbreviations

A	Alanine	Mw	Molecular weight
BChl	Bacteriochlorophyll	OD	Optical density
C	Cysteine	PAGE	Polyacrylamide gel
°C	Degrees Celsius		electrophoresis
CoA	Coenzyme A	PHA	Polyhydroxyalkanoate
D	Aspartic acid	PP	Polypropylene
GAP	Granule associated protein	RNA	Ribonucleic acid
GC	Gas chromatography	Rubisco	Ribulose-1,5-bisphosphate
GFP	Green fluorescent protein		carboxylase/oxygenase
Gm	Gentamycin	S	Serine
H	Histidine	SA	Streptavidin
DNA	Deoxyribonucleic acid	SDS	Sodium dodecyl sulphate
ELISA	Enzyme-linked	TEM	Transmission electron
	immunosorbent assay		microscopy
FACS	Fluorescence activated cell	WS/DGAT	Wax ester synthase /
	sorting		diacylglycerol
Fig.	Figure		acyltransferase
kDa	Kilo Dalton	(wt/vol) or	Weight per volume
LacZ	β-Galactosidase	(w/v)	
LB	Luria-Bertani	(vol/vol) or	Volume per volume
MALDI-TOF	Matrix-assisted laser	(v/v)	
	desorption/ionisation time-	YFP	Yellow fluorescent protein
	of-flight	SDS	Sodium dodecyl sulphate
MS	Mass spectrometry		

Chapter I A

Introduction

Prokaryotic inclusions

Prokaryotic inclusions

Prokaryotic inclusions can be found in a wide variety of eubacteria and archaeal bacteria. These inclusions differ in composition, size and shape as well as their physiological function. Most commonly, bacterial inclusions are located in the cytoplasm of the bacterial cell, but some inclusions have been found to reside between the cell wall and the cytoplasmic membrane. Several reviews have been dedicated to bacterial inclusions and attempts have been made to classify these inclusions. In the past, they have been divided into two groups based on the presence or absence of a surrounding membrane: cyanophycin granules, glycogen granules, polyphosphate granules and raphidosomes were grouped as nonmembrane-enclosed inclusions, and carboxysomes, polyhydroxyalkanoate granules, sulfur globules, triacylglycelrol and waxester granules as membrane-enclosed inclusions (Shively, 1974). More recently, an article published online in the encyclopaedia of life sciences divided prokaryotic inclusions with respect to their physiological role; as there exist inclusions as metabolic organelles (carboxysomes, chlorosomes), inclusions associated with cell mobility (gas vesicles, magnetosomes), inclusions as metabolic reserves (cyanophycin, glycogen, polyhydroxyalkanoate granules, polyphosphate, sulfur globules, triacylglycerol granules, waxester granules) and inclusions with unknown functions (rhapidosomes) (Shively *et al.*, 2006). This chapter shall introduce the diversity of bacterial inclusions and shall give a brief overview of their occurrence, emergence, biosynthesis, degradation, physiological role, and, where applicable, their biotechnological significance. Polyhydroxyalkanoate granules will only be briefly presented in this general chapter since the next chapter (chapter 1 B) aims to review in detail the current knowledge in the field of polyhydroxyalkanoates.

Chlorosomes

Chlorosomes are intracellular inclusions that occur in the cytoplasm of green sulfur bacteria and most members of the filamentous anoxygenic phototrophic bacteria. They act as specialised compartments that function as the main light harvesting systems and transfer absorbed light to photosynthetic reaction centres in the plasma membrane (Shively *et al.*, 2006). Chlorosomes possess a length of 50-200 nm and contain bacteriochlorophyll (BChl) *c*, *d* or *e*. The interior of the chlorosome is separated from the cytoplasm by a protein-containing unilayer membrane of approx. 3-4 nm thickness, which is mostly composed of the glycolipid monogalactosyl diglyceride; the fatty acid tails of the lipids are oriented towards the interior of the chlorosomes

(Staehelin *et al.*, 1980). The chlorosomes are connected to the cytoplasmic membrane by the baseplate, an array of pigmented proteins which is 5-6 nm thick (Staehelin *et al.*, 1980). The bacteriochlorophyll molecules inside the chlorosomes form large aggregates of rod-shaped structure known as rod elements. In addition to bacteriochlorophyll, considerable amounts of carotinoids and quinones are present in the chlorosomes.

In 2007, a hypothesis on chlorosome biogenesis in green photosynthetic bacteria was published (Fig. 1) (Hohmann-Marriott & Blankenship, 2007). They suggested that chlorosomes are derived from the cytoplasmic membrane and that no special machinery is required for chlorosome biogenesis. BChls, carotenoids and quinones accumulate between the two membrane leaflets of the cytoplasmic membrane (Fig. 1 A). Glycosyl diacylglycerols localise into the vicinity of the incorporated pigments because of their high affinity for them (Fig. 1 B). While the accumulation of BChls, carotenoids, quinines and glycosyl diacylglycerols continues, chlorosome associated proteins including the baseplate proteins localise to the nascent chlorosome (Fig. 1 C). With further accumulation of BChls, carotenoids, quinones, lipids and chlorosome proteins, the chlorosome grows out of the membrane to form a connected vesicle (Fig. 1 D). This chlorosome vesicle eventually becomes detached and forms a mature chlorosome, which comes in contact with the photosynthetic reaction centres (Fig. 1 E).

Carboxysomes

More than 40 years ago, carboxysomes were first discovered in cyanobacteria. Until now, they have been described to occur in all cyanobacteria as well as a limited number of other photosynthetic prokaryotes; they have also been detected in many chemolithoautotrophic bacteria (Shively *et al.*, 2006). Carboxysomes are polyhedral bodies of 100 nm or more in diameter that are filled with enzymes. The main enzymes in carboxysomes are the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and carbonic anhydrase. Hence, carboxysomes form subcellular microcompartments for highly efficient CO_2 fixation, where the carbonic anhydrase catalyses the generation of CO_2 which is subsequently used by Rubisco as substrate for the production of biomass (Badger & Price, 2003; Kaplan & Reinhold, 1999; Shively *et al.*, 1973). Carboxysomes are surrounded by an envelope of 3-4 nm thickness, formed by carboxysome associated proteins. The structure of carboxysomes and the genomic organization of their components have been studied in detail from two organisms: the cyanobacterium *Synechocystis* PCC6803 and the chemoautotrophic bacterium *Halothiobacillus neapolitanus*. Three genes coding for three homologous proteins have been identified in *H. neapolitanus* which were found to be the major components in the carboxysomal shell (English *et al.*, 1994).

BChls, carotenoids and quinones (green) accumulate between the bilayer. Glycosyl diacylglyceroles (red) localise into the vicinity due to their high affinity for the incorporated pigments.

Accumulation of BChls, carotenoids and quinones continues and chlorosome associated proteins (yellow) including the baseplate proteins (orange) become attached.

Further accumulation of BChls, carotenoids and quinones results in the formation of a connected vesicle.

The connected vesicle eventually becomes detached and forms a chlorosome which is in contact with the respective reaction center (blue for reaction center of filametous anoxygenic phototrophic bacteria and purple for reaction center of green sulfur bacteria).

Filamentous anoxygenic phototrophic bacteria

Green sulfur bacteria

Figure 1. Proposed model for the formation of chlorosomes (modified from Hohmann-Marriott & Blankenship, 2007).

Homologues of these carboxysomes shell proteins have also been found in *Synechocystis* PCC6803 and in other cyanobacteria that contain carboxysomes (Price *et al.*, 1993; Shively *et al.*, 1998). Crystal structures have been determined for four of the different homologous shell proteins, and they all show common features that has allowed a number of conclusions about the surrounding

layer of carboxysomes to be made (Kerfeld *et al.*, 2005; Tsai *et al.*, 2007). The major finding of all obtained structures was that the major shell proteins assemble to form hexameric building blocks. Three of the four analysed proteins showed an ability to further assemble and to form two-dimensional layers of protein molecules (Kerfeld *et al.*, 2005; Tsai *et al.*, 2007). These layers appear to represent the flat facets of the carboxysome shell, which is suggested to exhibit a roughly icosahedral shape (Schmid *et al.*, 2006). The fact that not all of the major shell proteins show the ability to assemble further into layers led to the hypothesis that different proteins fulfil different roles in the architecture of carboxysomes, e. g. proteins that cannot form an extended sheet may serve a role at the edges of the flat facets (Yeates *et al.*, 2007). The six subunits of each hexamer assemble closely together so that only a small pore of 4-7 Å remains down the central axis of symmetry. A current hypothesis is that substrates and products of the CO_2 fixation process are able to enter and leave the carboxysome by travelling through the pore. Furthermore, the pore is suggested to bear a positive electrostatic potential which could explain a preference for the passage of negatively charged molecules such as bicarbonate (Yeates *et al.*, 2007). It remains unknown how the flat self-assembled hexagonal layers that have been visualised so far are able to form into a closed shell. It is also not known how the shell is connected to its content and what molecular interactions are required for the enclosure of Rubisco molecules inside the protein shell. Interestingly, polyhedral inclusions have also been found in several heterotrophic enteric bacteria, e.g. in *Salmonella enterica*. These structures are smaller; appear to be less dense and commonly more irregularly shaped than carboxysomes of autotrophic bacteria. It is suggested that these polyhedral inclusions also form a microcompartment that provides catalytic advantage or protection for whatever enzyme is localised inside.

Gas vesicles

Gas vesicles are subcellular structures that contain a gas filled space. They occur commonly in prokaryotic microorganisms from aquatic habitats and their function is to confer buoyancy and to control cell depth. Gas vesicles have been described to occur mainly in phototrophic aquatic microorganisms, these include halophilic and methanogenic Archaea as well as different phyla of eubacteria (Shively *et al.*, 2006). Gas vesicles were first described in 1895 in cells of water-bloom forming cyanobacteria (Ahlborn, 1895) and further studies showed that these vesicles contain gas and provide buoyancy (Klebahn, 1922; Klebahn, 1925; Klebahn, 1929). Decades later, first electron microscopy images of cyanobacteria gave evidence for a layer of 1-2 nm in thickness that surrounds the gas vesicles, and that gas vesicles appeared to be made up of stacks of cylindrical vesicles measuring 75 nm in diameter and up to 1.0 µm in length (Bowen & Jensen, 1965). Gas

vesicles can vary widely in size, from 50 nm to $\geq$ 1 μm in length and from 30 nm to 250 nm in width. The surrounding layer is purely composed of proteins; and two proteins, GvpA and GvpC, have been identified as part of the gas vesicle surrounding layer. GvpA is the most abundant protein which forms striated structures on the surface of gas vesicles and serves as the major structural protein (Tandeau de Marsac *et al.*, 1985). GvpC is described as protein that binds to the outside of the gas vesicle shell and its main function is to strengthen the structure against collapse (Hayes *et al.*, 1988; Walsby & Hayes, 1989).

The formations of gas vesicles seems to be a complex process, e.g. in *Halobacterium* sp. NRC-1, 14 genes have been shown to be both necessary and sufficient for vesicle formation (Shukla & DasSarma, 2004). Functions of these gene products have been discussed, there are proteins speculated to play a role in the initiation of gas vesicle formation and proteins that may act as regulatory proteins. Further investigations will be necessary in order to elucidate the gas vesicle formation process.

Gas vesicles are mainly characterised by their great stability, rigidity, gas-permeability and absence of lipids; hence, genetic fusions to the outer, stabilising protein GvpC have been constructed for display of proteins/enzymes of interest at gas vesicle surfaces in order to use these micro-structures for biotechnological applications (Stuart *et al.*, 2001; Stuart *et al.*, 2004).

Magnetosomes

Magnetosomes are membrane-bound magnetic crystal inclusions that occur in magnetotactic bacteria and confer a magnetic dipole that enables the bacterial cell to use geomagnetic fields for sensing directions (a phenomenon described as magnetotaxis). Magnetotactic bacteria include a diverse group of motile microorganisms; they have been isolated from freshwater as well as marine environments and include bacteria belonging to the phylogenetic groups of α-Proteobacteria, δ-Proteobacteria, γ-Proteobacteria, and the Nitrospira (Delong *et al.*, 1993; Kawaguchi *et al.*, 1995; Simmons *et al.*, 2004; Spring *et al.*, 1993). To date no gram-positive or archaeal bacteria have been discovered to belong to the group of magnetotactic bacteria (Komeili, 2007). It is suggested that magnetotaxis together with chemotaxis help cells to locate and maintain an optimal position in vertical chemical concentration gradients in chemically layered environments by reducing a 3-dimensional search to a single dimension (Shively *et al.*, 2006). Magnetosomes consist of either iron oxide magnetite (Fe_3O_4) or iron sulfide greigite (Fe_3S_4) and are surrounded by a lipid bilayer membrane that contains magnetosome specific proteins. Typically arranged in chains along the cell's long axis, magnetosomes have a size of 20-120 nm and a variability of shapes from rounded to bullet shaped. These chains of

magnetosomes were found to be flanked by a network of cytoskeletal filaments, composed of MamK, a protein which is a homolog of the bacterial actin-like protein MreB (Komeili *et al.*, 2006).The magnetosome membrane is present before the magnetite is formed and is the site of its biomineralisation. Recently, electron cryotomographic imaging showed that all magnetosomes were invaginations of the cytoplasmic membrane regardless of their position in the chain (Komeili *et al.*, 2004; Komeili *et al.*, 2006). The majority of magnetosome specific proteins are encoded by genes which are localised within a distinct genomic region called the Magnetosome Island. These genes have been suggested to be involved in the formation of the magnetosome membrane, organization of the magnetosome chain and biomineralisation of magnetite (Ullrich *et al.*, 2005). A model for magnetosome formation has been suggested (Fig. 2) (Komeili, 2007): The first step is the synthesis of the magnetosome membrane and the targeting of magnetosome specific proteins to the membrane. The proteins responsible for the formation of this membrane have not yet been identified. It is hypothesised that the protein sorting to the magnetosome membrane may occur concurrently with the invagination of the cell membrane similar to eukaryotic membrane trafficking (Lee *et al.*, 2004). Protein sorting may also occur via "diffusion and capture" mechanisms after the magnetosome membrane is formed, as described for other bacterial systems (Rudner *et al.*, 2002). Once a magnetosome membrane invagination has been formed, the assembly into a chain has to occur. Proteins like MamK and MamJ have been shown to be part of the process (Komeili *et al.*, 2006; Scheffel *et al.*, 2006), but it is likely that more proteins are necessary for the chain arrangement. The final step of magnetosome formation is the biomineralisation of magnetite, which starts with the uptake of iron, followed by its oxidation to ferrihydrite in the periplasm and transport into the magnetosome and its final reduction to form magnetite. Because of their unique characteristics, size and shape, bacterial magnetosomes have been considered as magnetic nano-particles to be utilised in biotechnological applications. The surface of magnetosomes has been modified, chemically or with genetic approaches, and magnetosomes have been used successfully used for numerous procedures, such as the extraction of DNA and mRNA from biological samples (Yoza *et al.*, 2003a; Yoza *et al.*, 2003b), immobilisation of antibodies through a fusion of the antibody-binding ZZ domains of protein A to a magnetosome associated protein (Tanaka & Matsunaga, 2000), and fusion of reporter proteins like GFP and luciferase to magnetosome associated proteins (Lang *et al.*, 2007; Matsunaga *et al.*, 2000).
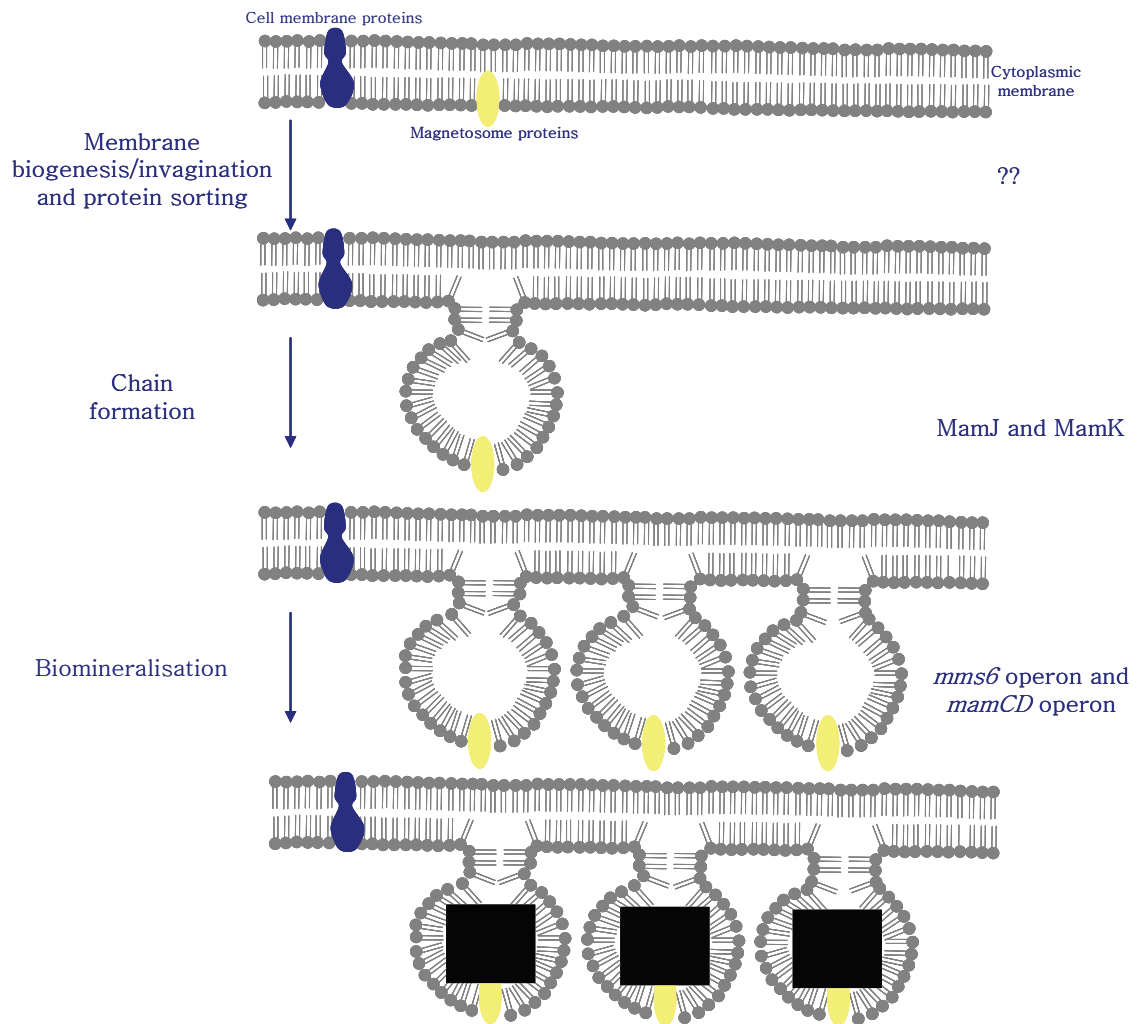
Figure 2. Model for magnetosome formation (modified from Komeili, 2007). Magnetosome formation occurs in 3 steps. The first step is the formation of a membrane invagination, and magnetsome proteins (yellow) are sorted away from cell membrane proteins (blue). The chain formation of magnetosomes follows which is achieved with the help of MamJ and MamK. The magnetosome formation is accomplished after the biomineralisation of magnetite crystals (black rectangles); genes of the *mms6* and *mamCD* operons are possibly involved in this last step.

Glycogen

Glycogen is a polysaccharide composed purely of glucose monomers, which are linked by α-1,4-glucosidic bonds and branched through α-1,6-glucosidic bonds. It is synthesised by a wide variety of microorganism, including bacteria, archaea and cyanobacteria (Chao & Bowen, 1971; Elbein & Mitchell, 1973; Konig *et al.*, 1982; Lang, 1968; Murray & Zinder, 1987; Orpin, 1973). Glycogen may be dispersed throughout the cytoplasm or, when a large amount of glycogen is accumulated, it may be deposited in form of granules in the cytoplasm of bacteria or in the photosynthetic thylakoid membranes in cyanobacteria. The size of these granules ranges from approx. 20-100 nm (Preiss, 2000). Glycogen is synthesised when excess carbon is available but another essential nutrient, e.g. nitrogen, is limiting (Elbein & Mitchell, 1973). Serving as carbon storage

material, glycogen can be catabolised when needed. Three enzymes are needed for bacterial glycogen synthesis (Ball & Morell, 2003): The ADP-glucose pyrophosphorylase catalyses the formation of ADP-glucose and utilises glucose-1-phosphate and ATP as substrate. Next, the activated glucose unit is transferred to the non-reducing end of a growing α-1,4-linked glucan mediated by glycogen synthase. The last step is performed by a branching enzyme which generates α-1,6-branches. For glycogen catabolism, the glycogen phosphorylase catalyses the phosphorolysis of the α-1,4-glucosidic bonds and a debranching enzyme mediates the hydrolysis of the α-1,6-glucosidic bonds. One important control point of glycogen biosynthesis is the allosteric regulation of the ADP-glucose pyrophosphorylase, which is activated by glycolytic intermediates and repressed by ADP and inorganic phosphate (Ballicora et al., 2003).

Wax ester bodies

Wax esters are esters of long-chain fatty alcohols with long chain fatty acids. They were first described to occur in gram-negative bacteria of the genus *Acinetobacter* (Gallagher, 1971), to date wax ester biosynthesis has been described to occur in other bacteria belonging to the phylogenetic group of γ-Proteobacteria, e.g. *Moraxella*, as well as to the phylogenetic group of Actinobacteria, e.g. *Mycobacterium* and *Corynebacterium* (Bacchin et al., 1974; Bryn et al., 1977; Wang et al., 1972).

The key enzyme of wax ester formation is the wax ester synthase/diacylglycerol acyltransferase (WS/DGAT), which catalyses the final step of wax ester formation as well as the final step of the biosynthesis of triacylglycerol (see below). This bifunctional enzyme was first discovered and characterised in *Acinetobacter calcoaceticus* ADP, a strain which accumulates both, wax ester and triacylglycerols. Experiments with mutants carrying a defective acyltransferase gene emphasized the key function of WS/DGAT since none or only weak accumulation of wax ester and triacylglycerol could be detected (Kalscheuer & Steinbüchel, 2003). During wax ester synthesis, the WS/DGAT uses the CoA thioester of an alkanoic acid as substrate for the esterification of a long chain fatty alcohol with the concomitant release of CoA (Wältermann et al., 2007). Wax esters are stored intracellularly as lipid bodies and serve as carbon storage material in bacteria. In *A. calcoaceticus*, generally only one or a few wax ester bodies occur in the cell. These wax ester bodies are often of spherical shape; although flat, disk-like or rectangular inclusions have also been observed in different *Acinetobacter* strains grown on different carbon sources (Ishige et al., 2002; Singer et al., 1985). Not only differences in shape, but also differences in the surrounding layer have been reported, ranging from no membrane structure at all, over a lipid monolayer membrane to a lipid bilayer membrane. Specific wax ester inclusion associated proteins as well as

proteins involved in mobilisation of wax esters have yet to be identified. In 2005, wax ester body formation as well as triacylglycerol body formation has been investigated, and a general model for prokaryotic lipid body formation has been proposed (Fig. 3) (Wältermann *et al.*, 2005). These studies were carried out in *A. calcoaceticus* and *Rhodococcus opacus*. The first step of lipid body formation is the attachment of WS/DGAT to the cytoplasmic membrane. The WS/DGAT starts the synthesis of the storage material, and small lipid droplets are formed which remain bound to the enzyme. The next step is the formation of a lipid pre-body, where several small lipid droplets become surrounded by a phospholipid monolayer membrane and are released into the cytoplasm. The formation of a lipid body is completed with the coalescence of the small lipid droplets inside the pre-body.

Triacylglycerol bodies

Triacylglycerol is a glyceride where the glycerol is esterified with three fatty acids. Triaacylglycerol inclusions were first identified, isolated and characterised in *Rhodococcus opacus* strain PD630 (Alvarez *et al.*, 1996), but have been described earlier as lipophilic inclusions in the genus *Mycobacterium* and later in *Streptomyces* as lipid inclusions with no further internal structure and a thin surrounding membrane (Brieger & Glauert, 1956; Burdon, 1946; Packter & Olukoshi, 1995). As mentioned above, the key enzyme of triacylglycerol biosynthesis is the bifunctional enzyme WS/DGAT which catalyses the final step of wax ester as well as triacylglycerol biosynthesis; in case of triacylglycerol synthesis the WS/DGAT uses an alkanoic acid-CoA thioester as substrate for the esterification of diacylglycerol (Wältermann *et al.*, 2007). Proteins specific for the triacylglycerol inclusion membrane and for mobilisation of triacylglycerol have yet to be identified. The above described model of lipid body formation is the proposed model for wax ester as well as triacylglycerol body formation (Fig. 3) (Wältermann *et al.*, 2005). A phasin protein, which is normally associated to polyhydroxyalkanoate granules, was shown to also bind to triacylglycerol bodies and could be used as anchor molecule to display proteins of interest at the surface of these lipid bodies (Hanisch *et al.*, 2006).

WS/DGAT (red) attaches to
the cytoplasmic membrane
and formation of small lipid
droplets (yellow) starts.

Cytoplasm

Small lipid droplets

WS/DGAT

These small lipid droplets
remain attached to
WS/DGATs and form an
oleogenous layer covered
with phospholipids.

Oleogenous layer

During lipid accumulation, the
lipid droplets leave the
WS/DGAT and conglomerate
to membrane-bound lipid
prebodies.

Lipid-prebody

After these lipid-prebodies
have reached a certain size,
they are released into the
cytoplasm. Inside the free
lipid-prebody, the lipid
droplets coalesce with each
other and a mature lipid-
body with a homogenous lipid
core is formed.

Released lipid-prebody

Mature lipid-prebody

Figure 3. Model for the formation of lipid bodies, such as wax ester and triacylglycerol bodies (modified from Wältermann & Steinbüchel, 2005).

Cyanophycin granules

Cyanophycin, or multi-L-arginyl-poly(L-aspartic acid), is a non-ribosomal polyamide which is composed of an aspartic acid backbone with arginine side groups. This polyamide is found as inclusions in all cyanobacterial groups; to date it has not been found in any other bacterial species

and therefore seems to occur exclusively in cyanobacteria (Oppermann-Sanio & Steinbüchel, 2002). Growth under stressful, non-optimal conditions such as starvation of sulfur, phosphorus and CO_2 results in an increase of cyanophycin production, suggesting that cyanophycin serves as nitrogen reserve material (Allen *et al.*, 1980). In heterocyst-forming cyanobacteria, the presence of cyanophycin granules in the nitrogen-fixing heterocysts has been linked with a possible function of cyanophycin as dynamic reservoir which buffers the heterocysts against environmental fluctuations (Li *et al.*, 2001; Picossi *et al.*, 2004; Stubbe *et al.*, 2005). Electron microscopy analyses of cyanophycin granules revealed an absence of a surrounding membrane, a non-uniform shape and a size ranging from 200-500 nm (Allen & Weathers, 1980; Lang, 1968). The synthesis of cyanophycin is catalysed by the cyanophycin synthetase. In *in vitro* experiments, cyanophycin is produced as soon as the synthetase is incubated with Mg^{2+}, ATP, aspartate, arginine and a primer like cyanophycin (Berg *et al.*, 2000). For utilisation, cyanophycin can be degraded by the cyanophycinase, which hydrolyses the polyamide to dipeptides of aspartate and arginine (Richter *et al.*, 1999).

Sulfur globules

Microorganisms capable of producing intracellular sulfur in form of sulfur globules include members of the family Chromatiaceae, the main family of purple sulfur bacteria, members of the gliding sulfur bacteria, e.g. *Beggiatoa* species, as well as other sulfur bacteria (Shively *et al.*, 2006). These sulfur inclusions are often formed as metabolic intermediates and vary in size from 0.1-1 µm in diameter (Shively *et al.*, 2006). One of the model organisms to study sulfur oxidizing bacteria is the anoxygenic phototrophic γ-proteobacterium *Allochromatium vinosum*, a purple sulfur bacterium belonging to the family of Chromatiaceae. The sulfur globules of *A. vinosum* are surrounded by a protein envelope, consisting of a 2-5 nm thick monolayer of three hydrophobic, structural proteins (Brune, 1995; Nicolson & Schmidt, 1971). Sulfur globules envelope forming proteins SgpA and SgpB are sequence homologues and can replace each other, while the absence of SgpC leads to the formation of smaller granules (Prange *et al.*, 2004). To specify the sulfur of different sulfur-accumulating bacteria, X-ray absorption near edge structure spectroscopy was applied and showed evidence for the occurrence of sulfur chains, for example present as polythionates in *Acidithiobacillus ferrooxidans* and as cyclooctasulfur in *Beggiatoa alba* and *Thiomargarita namibiensis* (Prange *et al.*, 2002).

A. vinosum uses only the polymeric fraction of elemental sulfur when grown on commercially available sulfur, which usually consists of polymeric sulfur and cyclo-octasulfur (Franz *et al.*, 2007). Furthermore, a direct cell-sulfur contact seemed to be necessary for the uptake; but how

the polymeric sulfur is converted into sulfur globules remains unknown (Franz *et al.*, 2007). For the oxidation of intracellular sulfur in *A. vinosum*, proteins encoded by genes of the *dsr* locus have been found to be required; however, a definite pathway for sulfur oxidation has not yet been formulated (Dahl *et al.*, 2005; Shively *et al.*, 2006).

Polyphosphate granules

Inorganic polyphosphate is a polymer which is composed of chains of many phosphate residues that are linked by high energy phosphoanhydrid bonds. Interestingly, polyphosphate is ubiquitous and found in every kingdom: bacteria, archaea, fungi, protozoa, plants and animals (Kornberg *et al.*, 1999). For decades polyphosphate has not only been dismissed as an unimportant, evolutionary remain, but it was also wrongly thought to be absent in some bacteria due to its regular absence during the exponential growth of bacterial cultures.

To date different roles have been proposed for polyphosphates in microorganisms (Kornberg *et al.*, 1999): these include a function as ATP substitute and energy source, as reservoir for inorganic phosphate, as chelator of metal ions, a function during cell capsule formation as well as regulatory functions (Rao & Kornberg, 1996; Rao & Kornberg, 1999; Skorko, 1989; Tinsley *et al.*, 1993; Voelz *et al.*, 1966).

For biosynthesis and degradation of polyphosphate, two enzymes are required: the polyphosphate kinase and the expolyphosphatase. In *E. coli*, genes encoding these enzymes are found in the same operon (Akiyama *et al.*, 1993). The polyphosphate kinase catalyses the conversion of ATP into polyphosphate by adding one phosphate residue for each ATP to the polymer. The exopolyphosphatase is required for mobilisation of polyphosphate and the reaction proceeds by removing orthophosphates from the polymer's end (Stubbe *et al.*, 2005). Polyphosphate granules are seen as highly refractive, crystalline spherical bodies of around 200 nm in diameter. They have been thought to lack a limiting membrane in bacteria, however, recently surrounding membranes have been detected in *Agrobacterium tumefaciens* and *Rhodospirillum rubrum* (Shively *et al.*, 2006). How these polyphosphate granules assemble and how they are rapidly mobilised remains to be elucidated.

Rhapidosomes

Rhapidosomes are inclusions which have been detected in a variety of bacteria and algae and which function has not yet been determined. They are of tubular shape and variable size. The diameter of the outer cylinder is approx. 25 nm, the central core shows a diameter of approx.

7.5 nm (Shively *et al.*, 2006). The composition of rhapidosomes from *Aquaspirillum itersonii* has been reported in 1993 and has been described as purely proteinaceous. Two polypeptides of 53 kDA and 29 kDa were detected when rhapidosomes were purified and analysed via SDS-PAGE (Pazirandeh & Campbell, 1993).

Polyhydroxyalkanoate granules

Polyhydroxyalkanoate (PHA) granules can be found in a wide variety of eubacteria as well as in some archaeal bacteria. PHA is a polymer which consists of (*R*)-3-hydroxy fatty acids and serves as carbon storage material. Polymerised by the PHA synthase, the polymers can be mobilised by an enzyme called PHA depolymerase. PHA granules show a diameter of around 50-500 nm and are surrounded by a phospholipid monolayer with PHA granule associated proteins (GAPs) embedded or attached. The next chapter presents a comprehensive review on the current knowledge of PHA research and covers occurrence, emergence, composition, genetics, proteomics as well as biotechnological significance of PHA and PHA granules.

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Chapter I B

Introduction

Polyhydroxyalkanoates: from bacterial storage compound via alternative plastic to bio-bead

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Polyhydroxyalkanoates: from bacterial storage compound via alternative plastic to bio-bead

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Abstract

Polyhydroxyalkanoates (PHAs) are organic polyesters composed of (*R*)-3-hydroxy fatty acids which are synthesised by most bacteria as a carbon and energy storage material in times of unbalanced nutrient availability. They are deposited intracellularly as insoluble spherical inclusions called PHA granules which consist of a polyester core surrounded by a phospholipid layer with attached proteins. One of these proteins is the PHA synthase, the key enzyme of PHA biosynthesis, which catalyses polyester formation from different (*R*)-3-hydroxyacyl-CoA precursors. The PHA synthase remains covalently attached to the polyester and thus to the PHA granule; other granule-associated proteins are involved in depolymerisation, regulation or structural stabilisation. This chapter provides a comprehensive overview of the current understanding of PHAs and PHA granules, including granule biogenesis and granule-associated proteins. In recent years, apart from investigating in particular the granule self-assembly process and the function of granule-associated proteins, a lot of research interest has been focused on the usability of this natural system. Accordingly, this review will also give an overview of metabolic engineering and large-scale production approaches, and discuss applications of PHAs as biocompatible and biodegradable plastics as well as the potential applicability of PHA granules as micro- / nano-beads.

Introduction

Most microorganisms live in environments where nutrients are available in an intermittent manner. Because of this "feast-or-famine" type of existence, many microorganisms possess systems that allow them to produce storage polymers as reserve materials. Excess nutrients available under favorable growth conditions are stored in these polymers in order to serve as a source of energy or building blocks in times of nutrient deprivation. The polymers are deposited as insoluble inclusions within the bacterial cell. One of the most common inclusions is poly(3-hydroxybutyric acid) (PHB), a carbon and energy storage compound synthesised from 3-hydroxybutyric acid (3HB) in situations when a carbon source is readily available but other nutrients (e.g. nitrogen or oxygen) are growth-limiting. Apart from PHB, hydroxy fatty acids of varying chain length are used by some microorganisms to generate a range of other

polyhydroxyalkanoates (PHAs). Other intracellular storage lipids found in bacteria are triacylglycerols (TAGs) and wax esters (for review see Wältermann & Steinbüchel, 2005). The polysaccharide glycogen is another example for a carbon and energy storage material deposited by prokaryotes (for review see Preiss & Romeo, 1989). An intracellular nitrogen reserve is provided by the polyamide cyanophycin (also referred to as cyanophycin granule polypeptide (CPG)) (for review see Oppermann-Sanio & Steinbüchel, 2002). In addition, polyphosphate and elemental sulfur are sometimes found.

The key enzyme of PHA biosynthesis is the PHA synthase (or polyester synthase). It catalyses the polymerisation of (R)-3-hydroxyacyl-CoA to the respective polyester, releasing coenzyme A during the reaction. Depending on the organism, one distinguishes between several classes of PHA synthases; and the (R)-3-hydroxyacyl-CoA precursors can be provided by different pathways. Nascent polyester chains assemble to form a spherical inclusion, termed PHA granule. The exact mechanism of the assembly process is still under debate; several models have been proposed which will be discussed below. Independent of the model for granule assembly, the PHA granule consists of a hydrophobic polyester core, surrounded by a phospholipid monolayer with proteins associated to its surface (Barnard & Sanders, 1989; Russell et al., 2007). As the PHA synthase remains covalently attached to the growing polyester chain, the initially soluble enzyme has to be converted into an amphipathic protein. Apart from the covalently bound synthase, proteins with regulatory functions (Prieto et al., 1999; Maehara et al., 2002; Pötter et al., 2002; York et al., 2002) or a structural role (phasin proteins) (Pieper-Fürst et al., 1995; York et al., 2001b; Jurasek & Marchessault, 2002) and proteins involved in PHA depolymerisation (Handrick et al., 2000; Gao et al., 2001; Saegusa et al., 2001; York et al., 2003) are attached to the granule surface. The PHA synthase as the key enzyme of PHA biosynthesis has been particularly well investigated, but a lot of research has also contributed to our understanding of the functions of the other granule-associated proteins.

Bacteria can accumulate up to 80% of their dry cell weight as PHA (Lee, 1996b; Madison & Huisman, 1999). Depending on the monomer composition, the properties of PHA polymers can range from thermoplastic to elastomeric. Since they are also biodegradable and biocompatible (Brandl et al., 1990; Brandl et al., 1995; Jendrossek et al., 1996) PHAs have been considered as a potential alternative to conventional synthetic / petrochemical-based plastics for several years, and considerable bioengineering effort has been invested into understanding and modifying the parameters that determine their physical and material properties, generating new homo- and copolymers as well as developing efficient bacterial production strains and large-scale fermentation methods (Anderson et al., 1990; Lee & Chang, 1995; Lee & Choi, 2001; Aldor & Keasling, 2003). Due to comparatively high production costs, examples of applications are so far

mainly found in the medical sector (Pouton & Akhtar, 1996; Williams *et al.*, 1999; Zinn *et al.*, 2001; Misra *et al.*, 2006). More recently, however, the polyester granules themselves have attracted increasing interest as functionalisable bionanoparticles with great potential for biotechnological and medical applications. PHA granules displaying various protein-based functions have been successfully generated by protein engineering and used for applications in e.g. protein purification, bio-separation and immunoassays (Barnard *et al.*, 2005; Brockelbank *et al.*, 2006; Peters & Rehm, 2006; Bäckström *et al.*, 2007; Grage & Rehm, 2008).

Occurrence and diversity of biopolyesters

PHAs, the most complex class of water-insoluble biopolyesters, are synthesised by a wide range of prokaryotic microorganisms. *Bacteria* from a variety of taxonomic and physiological groups have been found to accumulate PHAs as a storage polymer, with examples ranging from Gram-negative to Gram-positive, from aerobic to anaerobic and from heterotrophic to autotrophic. PHA accumulation has also been shown to occur in some members of the family *Halobacteriaceae*, which belong to the *Archaea* (Hezayen *et al.*, 2000; Hezayen *et al.*, 2002; Han *et al.*, 2007). As outlined above, PHAs are synthesised as carbon and energy storage compounds in situations of nutrient depletion when a carbon source is available in excess, and are deposited as insoluble cytoplasmic inclusions termed PHA granules. Under carbon starvation conditions granule-associated PHA depolymerising enzymes degrade the polymer. PHA degradation can delay the degradation of macromolecules and aid the survival of the organism. PHA can also be used as a carbon and energy source for sporulation in *Bacillus* and encystment in *Azotobacter* (Stevenson & Socolofsky, 1966; Thompson & Nakata, 1973), and in nitrogen-fixing prokaryotes it has been found to provide a substrate for oxidation in order to protect the enzyme nitrogenase in the absence of an external substrate (Senior & Dawes, 1971; Stam *et al.*, 1986).

PHB was the first PHA to be discovered in 1926 when intracellular accumulation of a polymer consisting of 3-hydroxybutyric acid was reported for *Bacillus megaterium* (Lemoigne, 1926), and it is also the most common PHA in nature. However, it is now well known that a variety of other substrates are also used by prokaryotes to synthesise a range of PHAs. Depending on the number of carbon atoms in the monomers, one distinguishes between short chain length PHAs (PHA$_{SCL}$, composed of (*R*)-3-hydroxy fatty acids with 3-5 carbon atoms), medium chain length PHAs (PHA$_{MCL}$, monomers with 6-14 carbon atoms) and sometimes long chain length PHAs (PHA$_{LCL}$, monomers with more than 14 carbon atoms). Most microorganisms synthesise either PHB or PHA$_{MCL}$. PHAs can occur as homopolymers consisting of just one type of hydroxyalkanoic acid or as copolymers of two or more types of monomers. Over 150 different

monomers have so far been discovered as constituents of PHAs, including straight and branched, saturated, unsaturated and aromatic molecules (Steinbüchel & Valentin, 1995; Kessler *et al.*, 2001; Kim & Lenz, 2001). Unsaturated groups and other functional groups in the side chains are of particular interest as they allow further chemical modification of the polyester. The monomeric composition of PHAs evidently depends on the carbon source available in the growth medium, but also on the producing microorganism. Thus the carbon source is a major factor impacting on molecular weight and material properties of the polyester (e.g. melting point and crystallinity). The exact mechanism that determines the molecular weight of a PHA is not yet completely understood, but apart from organism and carbon source cultivation conditions and isolation method also seem to play a role. The presence of PHA degrading enzymes and spatial/steric constraints within the cell could be factors limiting granule size. For PHB, molecular weights over 10^6 g/mol have been reported and average molecular weights range from 10^5 to 10^6. Molecular weights of other PHAs (PHA_{MCL}) are approximately one order of magnitude lower. PHA_{SCL} and PHA_{MCL} also differ in their thermal and physical properties. PHA_{SCL} (i.e. PHB) are typical thermoplastic polymers (relatively high T_m (melting temperature)) while PHA_{MCL} are in general elastomers (much lower T_m and T_g (glass transition temperature)) (Table 1). PHB can reach a much higher degree of crystallinity compared to PHA_{MCL} (for review on properties see van der Walle *et al.*, 2001). In addition, one also has to distinguish between PHAs or PHA granules in their native intracellular stage (mainly amorphous) and after extraction from the cell (higher degree of crystallinity). Considering this range of properties and modifiable parameters, the interest and amount of research that has been (and still is) contributed towards engineering bacteria to produce more and more new polyesters is not surprising.

Table 1. Material properties of two major classes of biopolyesters compared to polypropylene (PP)

Properties	PHA_{SCL}	PHA_{MCL}	PP
T_m (°C)	177	61	176
T_g (°C)	2	-36	-10
Crystallinity (%)	70	30	60
Elongation at break (%)	5	300	400

modified from Rehm, 2003

Apart from the PHAs produced by prokaryotic microorganisms, only plants synthesise other water-insoluble organic polyesters, cutin and suberin, which have a structural role and barrier function with regard to water, nutrients and pathogens (Kolattukudy, 2001; Heredia, 2003;

Franke & Schreiber, 2007). Some eukaryotic microorganisms have been shown to produce a water-soluble polyester, poly(malic acid). This polyester was first discovered in the slime mold *Physarum polycephalum*, where a regulatory function rather than a storage function has been proposed (Fischer *et al.*, 1989; Doerhoefer *et al.*, 2002). A very different form of PHA, low molecular weight PHB (also termed c-PHB as it occurs complexed to other macromolecules), has been found in both prokaryotic and eukaryotic cells (Reusch, 1989, 1992; Reusch *et al.*, 1992; Reusch, 1995). This c-PHB is present only in very low concentrations, and its physical and chemical properties are modified by complexation, such that forms of it occur both intracellularly and in membranes (Huang & Reusch, 1996). A complex of c-PHB and calcium polyphosphate detected in bacterial membranes could play a role in the formation of ion channels (Reusch, 2000). So far, no information is available about genes or enzymes involved in c-PHB biosynthesis, but they are expected to be different from those of the "normal" PHB biosynthesis pathway.

PHA biosynthesis and genes involved

As mentioned in the introduction, the key enzymes of PHA biosynthesis are the PHA synthases which are responsible for the actual polymerisation of (*R*)-3-hydroxyacyl-CoA thioester monomers to the respective polyester (Scheme 1). In the different groups of PHA-producing microorganisms, the PHA synthase is generally encoded by *phaC* or – if more than one type of subunit is involved – by *phaC* and either *phaE* or *phaR*. These different classes of synthases will be discussed in more detail below.
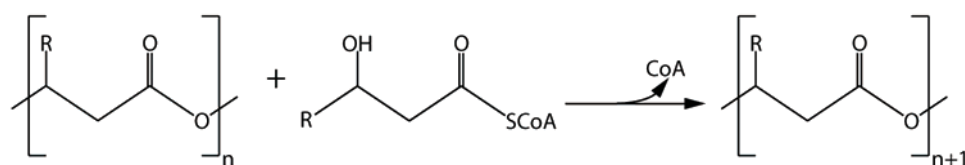
Scheme 1: Reaction catalysed by PHA synthase. R, alkyl chain.
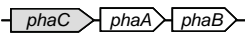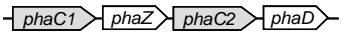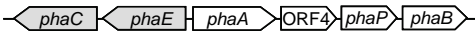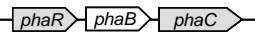
PHB biosynthesis in *Cupriavidus necator* (formerly *Ralstonia eutropha*) has been particularly well investigated. Here, 3-hydroxyacyl-CoA thioesters are generated from acetyl-CoA, involving the action of two other enzymes: β-ketothiolase (encoded by *phaA*) condenses two molecules of acetyl-CoA to acetoacetyl-CoA, and this is subsequently reduced to (*R*)-3-hydroxyacyl-CoA by the NADPH-dependent acetoacetyl-CoA reductase (encoded by *phaB*) (Figure 1).

Bacteria synthesising PHA$_{MCL}$ (pseudomonads like *Pseudomonas oleovorans, Pseudomonas fluorescens* and *Pseudomonas aeruginosa*) are utilising intermediates of fatty acid β-oxidation and/or

fatty acid *de novo* biosynthesis, depending on the carbon source (Figure 1). Related carbon sources (fatty acids, alkanes, etc.) enter the β-oxidation pathway, and certain pathway intermediates are then converted into (*R*)-3-hydroxyacyl-CoA thioesters (Lageveen *et al.*, 1988; Huisman *et al.*, 1989). Non-related carbon sources like gluconate, glucose or glycerol are metabolised to acetyl-CoA which enters the fatty acid *de novo* biosynthesis pathway (Haywood *et al.*, 1990; Timm & Steinbüchel, 1990). The intermediate (*R*)-3-hydroxyacyl-ACP (acyl carrier protein) is diverted to PHA biosynthesis and converted into (*R*)-3-hydroxyacyl-CoA by a transacylase (encoded by *phaG*) (Rehm *et al.*, 1998; Hoffmann *et al.*, 2000a, 2000b). Most pseudomonads are able to use both β-oxidation and fatty acid *de novo* biosynthesis for PHA production, but e.g. *P. oleovorans* uses only the β-oxidation pathway as its *phaG* gene seems to be silent (Hoffmann *et al.*, 2000a,).

It is quite common for the genes involved in PHA biosynthesis and other genes involved in PHA metabolism to occur in one or several clusters in the bacterial genome (Table 2) (for review see Rehm & Steinbüchel, 1999).

Table 2. Overview of the four classes of PHA synthases

Class	Representative organism(s)	Subunits	Substrate	Operon organization (in model organism)
I	*C. necator*	(PhaC) ~60-73 kDa	$3HA_{SCL}$-CoA ($\sim C_3$-C_5), $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA, $3MA_{SCL}$-CoA	*phaC* *phaA* *phaB*
II	*P. aeruginosa* *P. oleovorans*	(PhaC1) and (PhaC2) ~60-65 kDa	$3HA_{MCL}$-CoA ($\sim C_6$-C_{14})	*phaC1* *phaZ* *phaC2* *phaD*
III	*A. vinosum*	(PhaC) ~40 kDa (PhaE) ~40 kDa	$3HA_{SCL}$-CoA ($\sim C_3$-C_5), [$3HA_{MCL}$-CoA ($\sim C_6$-C_8), $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA]	*phaC* *phaE* *phaA* ORF4 *phaP* *phaB*
IV	*B. megaterium*	(PhaC) ~40 kDa (PhaR) ~22 kDa	$3HA_{SCL}$-CoA ($\sim C_3$-C_5)	*phaR* *phaB* *phaC*

modified from Rehm & Steinbüchel, 1999; Rehm, 2003

In *C. necator* and a large number of other PHB-producing organisms, the three PHB biosynthesis genes are organized in one operon, the *phaCAB* operon (Schubert *et al.*, 1988; Slater *et al.*, 1988; Peoples & Sinskey, 1989). There are also examples of bacteria where the genes are clustered but in a different order; whereas for example in PHB-accumulating α-proteobacteria such as *Caulobacter crescentus* or *Sinorhizobium meliloti* the *phaC* gene is situated in a different genome region of the genome than the other *pha* genes (Tombolini *et al.*, 1995; Qi & Rehm, 2001). PHA_{MCL}-accumulating pseudomonads have two *phaC* genes which occur in the order *phaC-phaZ-phaC*. In bacteria which possess a PHA synthase composed of two subunits, the genes for these are always localised together in the genome; either they are directly next to each other as in the case of *phaC* and *phaE* (Liebergesell *et al.*, 1992; Liebergesell & Steinbüchel, 1992), or they are separated only

by *phaB* as are *phaC* and *phaR* in the genus *Bacillus* (McCool & Cannon, 1999, 2001; Satoh *et al.*, 2002). Lastly, there are relatively few exceptions of organisms which exhibit no clustered gene organization at all.
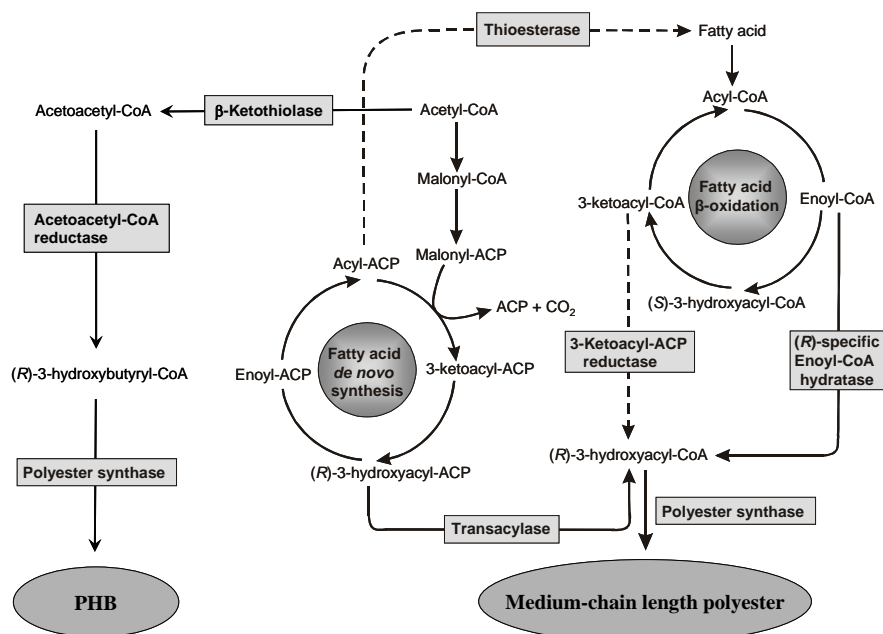
Figure 1. Metabolic routes towards PHA biosynthesis. Dashed arrows represent steps which have only been shown in recombinant systems. (modified from Rehm, 2006).

PHA granules

Structure/composition of PHA granules

PHA granules are water-insoluble spherical inclusions within the bacterial cell, the detailed composition of which has been under investigation for decades. Initial analyses of PHB granule composition revealed that they contain protein and lipid in addition to their major constituent PHB (Griebel *et al.*, 1968). In 1989, Barnard and Sanders published their high resolution ^{13}C NMR spectroscopy data of live cells, which showed that PHB is predominantly in a mobile state within the granule core, different from the solid (i.e. crystallised) state it is in after isolation (Barnard & Sanders, 1989). Furthermore, the data of Barnard and Sanders suggested that water may be an integral component of the PHB granule which acts as a plasticizer for the polymer (Barnard & Sanders, 1989). This amorphous PHA core is surrounded by a boundary layer, which has been suggested to be composed of a phospholipid-monolayer (Boatman, 1964; Preusting *et al.*, 1991) with embedded and attached proteins (Pieper & Steinbüchel, 1992; Steinbüchel *et al.*, 1995). At present, the embedded or attached proteins that are assigned to the granule-associated protein (GAP) group are the PHA synthase (Gerngross *et al.*, 1993; Liebergesell *et al.*, 1994; Valentin *et al.*, 1998), the intracellular PHA depolymerase (Handrick *et al.*, 2000; Gao *et al.*, 2001;

Saegusa *et al.*, 2001; Jendrossek & Handrick, 2002), phasin proteins, and regulator proteins (Prieto *et al.*, 1999; Maehara *et al.*, 2002; Pötter *et al.*, 2002; York *et al.*, 2002; Pötter *et al.*, 2005) (Figure 2).

Although alternative membrane models have been suggested (Stuart *et al.*, 1995; Stuart *et al.*, 1998), electron microscopy studies confirmed that the dimensions of the boundary layer are in agreement with a monolayer (Mayer & Hoppert, 1997). Recently, contrast-variation small-angle neutron scattering (SANS) was used to study PHA granule organization and the data obtained were consistent with and re-emphasising the phospholipid-monolayer model (Russell *et al.*, 2006; Russell *et al.*, 2007). Also recently, Dennis and coworkers studied PHA granules by atomic force microscopy (AFM) (Dennis *et al.*, 2003; Dennis *et al.*, 2007). Their observations supported the monolayer model and additionally enabled the discovery of complex structures at the granule surface. The authors suggested that the proteins covering the granule surface are organized in a cytoskeleton-like network (Dennis *et al.*, 2003) and showed that the phasin protein is involved in the formation of this scaffold (Dennis *et al.*, 2007). In addition, they reported the observation of porin-like structures at the granule surface which they suggested to be the sites of PHA metabolism.
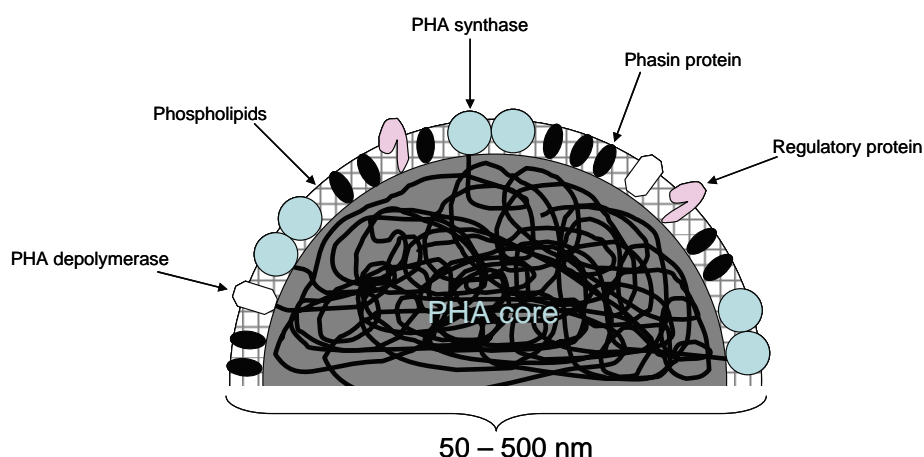
Figure 2. Schematic view of a PHA granule (modified from Rehm, 2003).

In vivo assembly of PHA granules

In vivo and under conditions permissive for PHA accumulation, the biosynthesis of PHA starts as soon as the substrate (*R*)-3-hydroxyacyl-CoA is available. Although the exact mechanism of PHA granule formation is still unknown and still under extensive investigation, there are currently two different models of PHA granule assembly discussed in the literature: one is called the "micelle" model (Figure 3A) and the other one is known as the "budding" model (Figure 3B).

The "micelle" model is based on the assumption that the PHA synthase is distributed randomly as a soluble enzyme in the cytoplasm. As soon as substrate is provided, the PHA synthase starts to synthesise polyester. Since the growing polyester chain stays covalently attached to the PHA synthase, it converts the initially soluble enzyme into an amphipathic molecule. Due

to hydrophobic interactions, the amphipathic molecules undergo a self-assembly process which is comparable to micelle formation. As the PHA inclusion increases in size, phospholipids as well as GAPs become incorporated into the PHA granule surface. The strongest support for the micelle model is provided by *in vitro* PHA granule formation (see below).

The "budding" model proposes that, as soon as substrate is available, the PHA synthase locates at the inner cytoplasmic membrane and starts synthesising PHA into the hydrophobic space between the phospholipid layers which causes a puffing of the plasma membrane. The puffing increases due to PHA synthase activity and results in budding of the PHA inclusions off the membrane. These PHA inclusions are surrounded by a phospholipid monolayer in which proteins become embedded or attached.
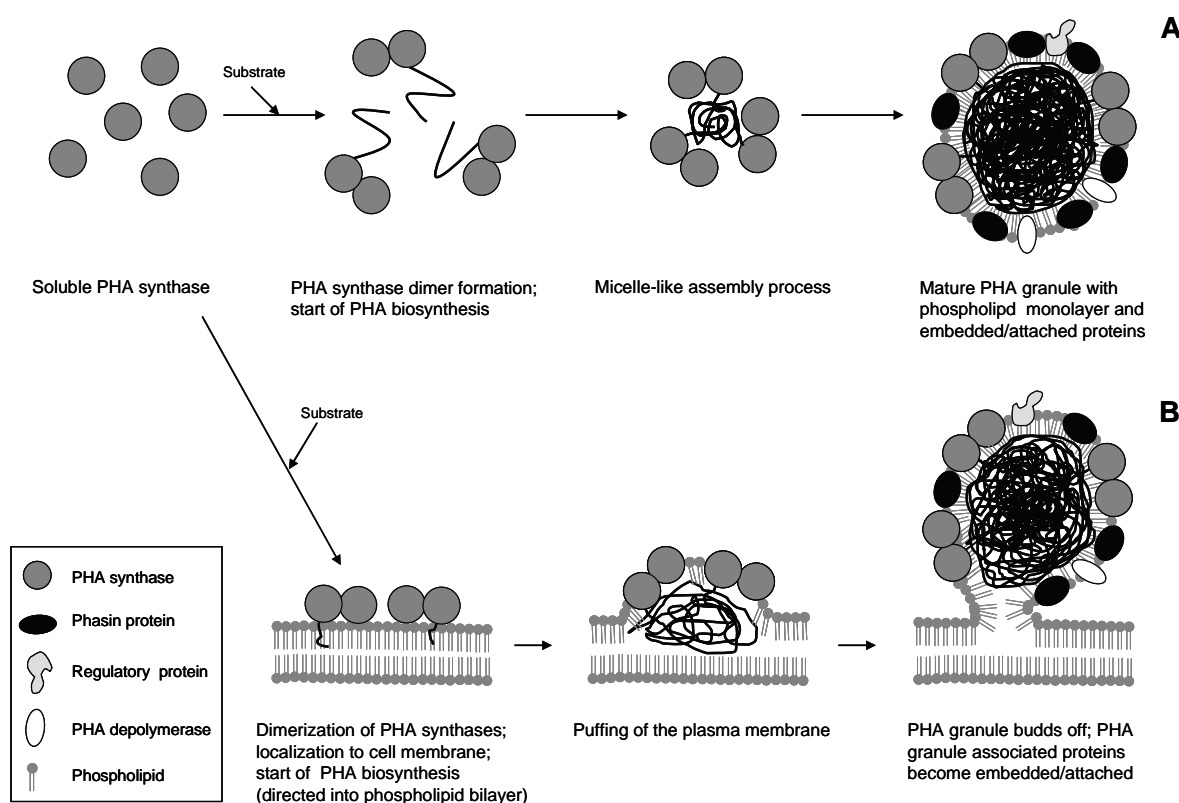
Figure 3. Current models for the PHA granule assembly process. A, "Micelle" model; B, "Budding" model. (modified from Rehm, 2006).

In 2005, Tian and co-workers published kinetic studies of PHB granule formation in *C. necator* using transmission electron microscopy (TEM) (Tian *et al.*, 2005b). They observed that PHA granule formation is neither randomly distributed nor located close to the cytoplasmic membrane. They found evidence that PHA granules emerge from mediation elements in the centre of the cell and proposed that these mediation elements serve as nucleation sites for PHA granule formation. In contrast, Jendrossek applied Nile Red as a hydrophobic fluorescent dye, which stains the PHA core, as well as YFP (yellow fluorescent protein)-PhaP fusion proteins to

observe PHA granule formation using fluorescence microscopy. This study showed that emerging PHA granules were frequently located close to the cell poles as well as to the cytoplasmic membrane (Jendrossek, 2005).

Fluorescence microscopy studies were also performed by Peters and Rehm using the GFP (green fluorescent protein) reporter protein fused to the N-terminus of the PHA synthase of *C. necator* and *P. aeruginosa* PAO1, respectively (Peters & Rehm, 2005). Since the synthase is the only essential protein required for PHA granule formation, a GFP-labelled synthase should enable observation of early stages of PHA granule formation and valuable insight into the assembly process. In this study it was observed that emerging PHA granules locate to cell poles and to mid-cell representing the future cell poles. Localisation of PHA synthases seemed to occur independently of Z-ring (septum) formation but was dependent on proper chromosome condensation. Further studies showed that even an inactive mutant of the *C. necator* PHA synthase localises to the cell poles, suggesting that the growing polyester chain does not impact on localisation (Peters *et al.*, 2007). In addition, the N-terminal as well as the C-terminal region of the PHA synthase could be ruled out as necessary for PHA synthase localisation. These findings led to the conclusion that the core region (amino acids 93-521) of the PHA synthase of *C. necator* is required for polar localisation. Meanwhile, more strains have been investigated with regard to the localisation of early-stage PHA accumulation, which suggested a common localisation either close to the cytoplasmic membrane, e.g. in *Caryophanum latum*, or close to the cytoplasmic membrane as well as at the cell poles, e.g. in *Beijerinckia indica* (Jendrossek *et al.*, 2007). These results provide further support for the budding model, backing the argument of a membrane-like layer surrounding the PHA granule.

The observation of Tian and co-workers, who suggested that early-stage PHA granules emerge from the centre of the cell, could not be confirmed by *in vivo* imaging using fluorescence microscopy, which suggested that PHA granule formation starts at the cell-poles and/or close to the membrane. However, both agree on a subcellular localisation of early-stage PHA granules rather than a random distribution of emerging PHA granules.

Regardless of the model for the initial stages of PHA granule formation, after assembly of the boundary layer-covered polyester granule and attachment of proteins, the PHA synthase continues to incorporate polymer and the PHA granule increases in size until the maximum size under the particular physiological conditions is reached (i.e. carbon source or space in cell depleted). In general, diameters of native granules range between 100 and 500 nm, and about 5-10 granules which can fill almost the entire cell are synthesised per cell under maximum PHA-accumulation conditions (Steinbüchel *et al.*, 1995). Marchessault and co-workers developed several computer programs of increasing complexity to simulate PHA granule formation,

including GAPs and certain parameters relating to regulation and degradation (Jurasek *et al.*, 2001; Jurasek & Marchessault, 2002, 2004).

In vitro PHA granule formation

In vitro PHA synthesis was first described by Gerngross and Martin in 1995 (Gerngross & Martin, 1995). They established the minimal requirements of PHB granule formation *in vitro* by exposing the purified class I PHA synthase of *C. necator* to (R)-3-hydroxybutyryl-CoA, which resulted in formation of PHA granules. The spherical granules obtained showed a significant increase in diameter of up to 3 μm when compared to *in vivo* synthesised PHA granules. Furthermore, the authors demonstrated the reverse proportionality between PHA synthase concentration and molecular mass of the polymer. A similar correlation was reported for the recombinant expression of the *C. necator phaCAB* operon in *Escherichia coli* (Sim *et al.*, 1997), but has so far not been observed in the native system. *In vitro* PHB synthesis was also obtained when recombinantly produced and purified class III PHA synthase of *Allochromatium vinosum* was provided with (R)-3-hydroxybutyryl-CoA (Jossek *et al.*, 1998). Different reaction mixtures were tested and an increased 3HB-CoA turnover was detected when salts, proteins (e.g. BSA) or detergents were added. Using (R)-3-hydroxydecanoate as precursor, *in vitro* PHA synthesis could also be shown for the class II PHA synthase of *P. aeruginosa*, but the *in vitro* activity of this PHA synthase was only about 37 mU and therewith about 3000-fold less when compared to class I and class III PHA synthase activities (Rehm *et al.*, 2001; Rehm, 2003). Since free CoA was found to inhibit PHA synthases, *in vitro* coupled-enzyme-assays were developed in which CoA was recycled (Jossek & Steinbüchel, 1998).

Based on the amount and molecular weight of PHA produced *in vitro*, it was calculated how many polyester chains were synthesised per molecule of PHA synthase. These calculations indicated that both class I synthase from *C. necator* and class II synthase from *P. aeruginosa* made on average less than one polyester chain per molecule of enzyme (Gerngross & Martin, 1995; Qi *et al.*, 2000), whereas class III synthase from *A. vinosum* synthesised multiple chains, i.e. chain transfer must have occurred (Jossek *et al.*, 1998). Other researchers have suggested chain transfer to also happen *in vivo* in *C. necator* (Kawaguchi & Doi, 1992; Tian *et al.*, 2005a) (see also below). If chain transfer and "recycling" of the PHA synthase represent the native situation, a lot of questions remain to be addressed, e.g. about the mechanism of chain termination and re-initiation. The existence of a chain transfer reagent has been proposed (Kawaguchi & Doi, 1992).

Immobilisation of an N-terminally decahistidine-tagged PHA synthase of C. *necator* onto different solid surfaces enabled surface-initiated polymerisation of (R)-3-hydroxybutyryl-CoA which resulted in formation of a polymer film with a uniform thickness. Atomic force microscopy (AFM) analysis showed evenly distributed granular structures over the entire surface.

The structures were uniform in shape and size and presumably derived from crystallization of polymer chains on the surface (Kim *et al.*, 2004). BSA was found to have a beneficial effect; apart from reducing non-specific adsorption of the synthase to the surface, it promoted growth of the PHB layer to a thickness of up to 1 μm. It was suggested that BSA stabilised the growing PHA structure by rendering it less hydrophobic (Niamsiri *et al.*, 2007).

The PHA synthase

Polyhydroxyalkanoate synthases are the key enzymes of PHA synthesis and catalyse the stereo-selective conversion of (*R*)-3-hydroxyacyl-CoA to polyoxoesters while releasing coenzyme A (Scheme 1) (for detailed review on the PHA synthase see Rehm, 2003).

Classification of PHA synthases

The growing number of sequenced genomes leads to an ongoing increase of the number of open reading frames potentially encoding PHA synthases. Meanwhile, at least 88 PHA synthase genes have been obtained and characterised, and the number is constantly rising (Rehm, 2007). In 2004, a potential PHA synthase gene candidate in the *Halobacteriaceae* was reported in the genome sequence of *Haloarcula marismortui* (Baliga *et al.*, 2004). Recently, this gene and a second gene under the same promoter were characterised as *phaE* and *phaC*, genes which encode two subunits of an active class III PHA synthase in *H. marismortui* (Han *et al.*, 2007). According to number of subunits and substrate specificity, PHA synthases have been divided into 4 major classes (Table 2):

PHA synthases belonging to class I exhibit one subunit (PhaC) of 60-73 kDa and synthesise PHA composed of short-chain-length monomers (PHA$_{SCl}$) (Schubert *et al.*, 1988; Slater *et al.*, 1988; Peoples & Sinskey, 1989; Qi & Rehm, 2001). Class II PHA synthases occur in bacteria of the genus *Pseudomonas* and are also composed of only one type of subunit (PhaC). In contrast to class I PHA synthases, they prefer (*R*)-3-hydroxyacyl-CoA composed of 6 to 14 carbon atoms as substrate in order to synthesise medium chain length PHA (PHA$_{MCl}$) (Langenbach *et al.*, 1997; Qi *et al.*, 1997). PHA synthases of class III possess two subunits: one PhaC subunit of about 40 kDa which exhibits strong similarity to class I and class II PHA synthases and one PhaE subunit (about 40 kDa) with no similarity to PHA synthases (Liebergesell *et al.*, 1992; Liebergesell & Steinbüchel, 1992). Class III and class IV PHA synthases synthesise PHA of short chain length (*R*)-3-hydroxyacyl-CoA. Class IV PHA synthases occur in bacteria of the genus *Bacillus* and are composed of a PhaC subunit (about 40 kDa) and a smaller PhaR subunit of about 20 kDa (McCool & Cannon, 2001). A few PHA synthases do not fit into the four-class model, e.g. PhaC1

and PhaC2 of *Pseudomonas sp.* 61-3, which catalyse the formation of a co-polymer consisting of (*R*)-3-hydroxybutyrate as well as MCL-(*R*)-3-hydroxy-fatty acids (Matsusaki *et al.*, 1998).

Investigations of the substrate specificity of PHA synthases have been mostly carried out *in vitro* or in recombinant *E. coli*. Interestingly, *in vitro* studies showed a very narrow substrate specificity (Haywood *et al.*, 1989a; Yuan *et al.*, 2001) whereas the substrate specificity in *E. coli* was rather broad (Langenbach *et al.*, 1997; Qi *et al.*, 1997; Antonio *et al.*, 2000).

Structure of PHA synthases

Comparison of PHA synthase primary structures led to the identification of six conserved blocks as well as eight identical amino acids (Rehm, 2003). The N-terminal regions of PHA synthases do not show any conserved sequences. Therefore, and since it was shown that the first 100 amino acids of the PHA synthase of *C. necator* were dispensable for a functional active enzyme (Rehm *et al.*, 2002), this region did not attract attention in the past. Recently, Normi and co-workers discovered that a single amino acid substitution at position four of the N-terminal region of PHA synthase of *C. necator* enhanced both the PHB yield as well as the level of PHA synthase (Normi *et al.*, 2005), suggesting a more important role of the N-terminus of PHA synthases as has been assumed until now. In contrast, the C-terminal region (about the last 40 amino acid residues) appears to be conserved in class I and class II PHA synthases and is composed of mainly hydrophobic amino acids. Deletion of five amino acid residues of the C-terminus of class I PHA synthase of *C. necator* resulted in abolished enzyme activity, indicating an important role of the hydrophobic C-terminus, probably by binding to the hydrophobic core of the polyester granule (Rehm *et al.*, 2002). In PHA synthases of class III and IV, it is the PhaE or PhaR subunit rather than the PhaC subunit that provides a very hydrophobic C-terminal region.

Multiple alignment analysis of the primary structure of PHA synthases showed the presence of a lipase box (GX[S/C]XG) in which the active site serine is replaced by a cysteine. Conserved-domain-homology searches were performed suggesting that PHA synthases - like lipases - belong to the family of α/β-hydrolase fold enzymes. Threading models of the class I PHA synthase from *C. necator* (Rehm *et al.*, 2002), of class II PHA synthase from *P. aeruginosa* (Amara & Rehm, 2003), and of class III PHA synthase from *A. vinosum* (Jia *et al.*, 2000) could be obtained. All these models show the α/β-hydrolase fold with its typical catalytic triad in the order nucleophile-acid-histidine. Another similarity to lipases is the increased activity of granule-bound PHA synthase compared to soluble enzyme (Gerngross & Martin, 1995). This resembles the interfacial activation of lipases at the lipid-water interface.

Catalytic mechanism

The conserved residues cysteine, aspartic acid and histidine (C149, D302 and H331 in *A. vinosum*; C319, D480 and H508 in *C. necator*) of the PHA synthase are believed to form a catalytic triad.

The current model proposes that two PHA-synthases form an active dimer (Figure 4). During initiation of PHA synthesis, the thiol groups of both subunits are activated by the conserved histidines, and perform a nucleophilic attack on the thioester bond of two (R)-3-hydroxyacyl-CoA substrates. At the end of this initiation step, both thiol groups of the active sites form a thioester bond to a 3-hydroxy-fatty acid. During elongation, the third conserved amino acid residue of the proposed catalytic triad plays an important role. The aspartic acid residue of one subunit activates the hydroxyl group of the bound 3-hydroxy-fatty acid, which then attacks the thioester bond between the cysteine and the hydroxyl-fatty acid of the second subunit. Therefore, the growing polyester chain is attached to one subunit of the active dimer, while the thiol group of the other subunit is free to bind a new 3-hydroxy-fatty acid substrate. It has been proposed that the growing polyester chain changes from one catalytic centre to the other with each additional monomer (mechanism similar to fatty acid biosynthesis/synthase (Witkowski *et al.*, 1997)). An alternative model (based on similarity to α/β-hydrolases (Schrag & Cygler, 1997)) suggests that only one active site cysteine might be required and that initially, before being incorporated into the polyester chain, hydroxyacyl-CoA monomers bind non-covalently elsewhere to the synthase.

Recent publications show evidence for this proposed catalytic mechanism as well as provide new insights into PHA homeostasis. Jia and co-workers used radioactively labelled, saturated trimers (sT-CoA) to analyse the first initiation step (Jia *et al.*, 2001). Incubation with class I and class III synthase resulted in one [3H]-label per synthase dimer, clearly supporting the conversion of the monomeric to the dimeric form as shown in the suggested model (Figure 4). Furthermore, a variant of the PHA synthase of *A. vinosum* was constructed in which the conserved aspartic acid residue was replaced by an alanine (Tian *et al.*, 2005d). Relative to the wild type synthase, the D302A synthase mutant showed a 1000-fold decreased PHB formation rate, indicating the important role of the conserved aspartic acid in chain elongation. Incubation of the D302A mutant with varying concentrations of substrate resulted in acylated intermediates of different length as shown by SDS-PAGE. Protease digestion of the (HB)$_n$-PHA synthase and analysis by HPLC resulted in a peptide containing the conserved cysteine and oligomers of 3-hydroxybutyrat with n=3-10 (Tian *et al.*, 2005d). These data provided evidence that the polyester chain stays covalently attached to the cysteine at the beginning of the elongation process. In addition, Tian and co-workers proposed an additional role of the PHA synthase of *A. vinosum* to be involved in chain termination and re-initiation (Tian *et al.*, 2005c). They found evidence to support a revised model of the synthase reaction mechanism, in which the PHA synthase after initiation and elongation can sense the length of the polymer chain. The authors suggested that the PHA synthase can catalyse the chain termination by transferring most of the polymer chain to a

second, surface exposed amino acid, which is causing hydrolysis of the polyester chain. A primed PHA synthase remains and starts a new cycle of PHA synthesis. (For further discussion of "chain transfer" versus "no chain transfer" see chapter on *in vitro* PHA granule formation.)
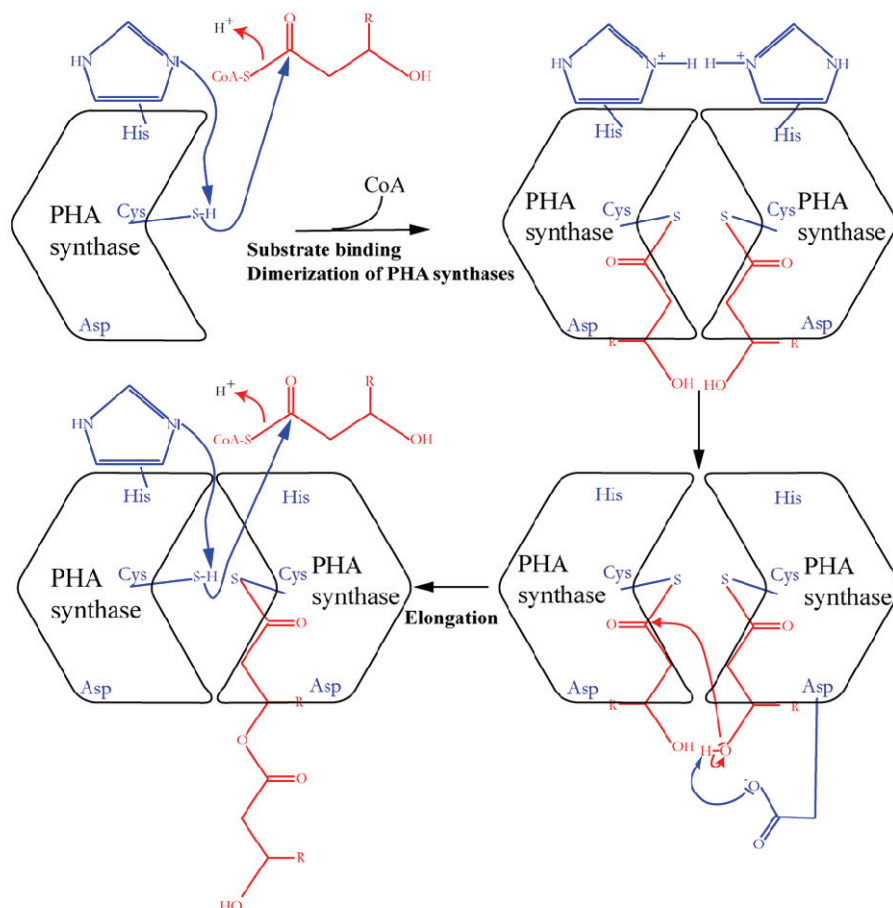
Figure 4. Proposed model of the catalytic mechanism of the PHA synthase. His, histidine; cys, cysteine; asp, aspartic acid; R, alkyl chain.

Other granule associated proteins

PHA depolymerases

Degradation of PHA can be achieved by either extracellular or intracellular PHA depolymerases (for rewiew see Jendrossek & Handrick, 2002).

The ability to use extracellular, partially crystalline PHA as carbon source is widely spread among bacteria and fungi and not limited to PHA-producing organisms. Extracellular PHA depolymerases are secreted into the environment and catalyse the degradation of PHA which becomes available e.g. from dead cells. These enzymes are generally composed of (i) a signal peptide necessary for secretion, (ii) a large catalytic domain at the N-terminal region, (iii) a linking domain between the catalytic N-terminal domain and the C-terminal domain, and (iv) a substrate binding domain in the C-terminal region (Jendrossek & Handrick, 2002). Most bacteria produce

extracellular PHA depolymerases that are specific for either PHA_{SCL} or PHA_{MCL}; however, there are exceptions (Schirmer *et al.*, 1995).

For mobilisation of PHA carbon storage material inside the bacterial cell, an intracellular PHA depolymerase is required (Handrick *et al.*, 2000). Intracellular PHA depolymerases are specific for the native, amorphous form of PHA and can be found at the surface of PHA granules. These enzymes have been less intensively studied than their extracellular counterparts, and much remains to be learned, e.g. about their regulation. In 1990, Doi and co-workers investigated the biosynthesis and degradation of PHA in *C. necator* and reported a cyclic nature of PHA metabolism (Doi *et al.*, 1990). The PHB cycle starts with the PHB biosynthesis via acetyl-CoA, acetoacetyl-CoA and 3-hydroxybutyryl (3HB)-CoA to PHB, catalysed by ketothiolase, acetoacetyl-CoA reductase and PHA synthase. The cycle closes with the PHB degradation from PHB via 3HB, acetoacetate, acetoacetyl-CoA to acetyl-CoA, catalysed by intracellular PHB depolymerase, 3HB dehydrogenase, acetoacetate:succinyl-CoA transferase, and ketothiolase (Oeding & Schlegel, 1973; Senior & Dawes, 1973). Other studies confirmed the simultaneous occurrence of PHB biosynthesis and degradation by measuring incorporation of ^{14}C-glucose into PHB (Taidi *et al.*, 1995) as well as the simultaneous presence of PhaC and PhaZ by western analysis (Tian *et al.*, 2005a).

The intracellular PHB depolymerase PhaZ of *C. necator* was cloned, sequenced and characterised by Saegusa and co-workers in 2001 (Saegusa *et al.*, 2001). Activity of PhaZ could be demonstrated by detection of degradation products when artificial amorphous PHB granules were used as substrate. PhaZ was expressed concomitantly with the synthesis of PHB and was localised at the surface of PHA granules (Saegusa *et al.*, 2001). In the meantime, several other (putative) PHA depolymerases have been identified in *C. necator*; the functions of most of them remain to be confirmed (Schwartz *et al.*, 2003; York *et al.*, 2003; Pötter *et al.*, 2004). To further complicate matters, an intracellular 3HB-oligomer hydrolase has also been found in *C. necator* and has also been designated PhaZ (Saegusa *et al.*, 2002; Kobayashi *et al.*, 2003).

Other intracellular PHA depolymerases that have been reported include PhaZ of *Paracoccus denitrificans* (Gao *et al.*, 2001) and the PHA depolymerase of *Rhizobium meliloti* (Charles *et al.*, 1997).

Phasin proteins

Phasin proteins are the most abundant proteins attached to PHA granules. They can contribute up to 5 % of the total cell protein (Wieczorek *et al.*, 1995; Hanley *et al.*, 1999). Phasins are non-catalytic and appear to serve a structural role constituting the boundary between the hydrophilic cytoplasm and the hydrophobic polyester core. The phasin protein coating of the PHA granule surface also prevents coalescence of free PHA granules (Jurasek & Marchessault, 2004).

While all phasin proteins seem to share the same function, they are not phylogenetically related as discussed by Hanley and co-workers (Hanley *et al.*, 1999); e.g. the phasin protein sequences of *C. necator* and *Rhodococcus ruber* share only 16.2 % identity. The phasin protein of *R. ruber* was shown to bind to PHA granules with the two hydrophobic domains at its C terminus (Pieper-Fürst *et al.*, 1995). In contrast, the phasin protein PhaP1 of *C. necator* does not bind to PHA granules with its C terminus as previously suggested, but presumably interacts via several hydrophobic regions including the C-terminal hydrophobic regions (Hanley *et al.*, 1999).

PhaP1 of *C. necator* is the most investigated phasin. Recent genome analysis of *C. necator* led to the identification of three PhaP homologues which await more detailed functional characterisation (Pötter *et al.*, 2004). Tian and co-workers correlated amounts of PhaP with cell and granule size and concluded that PhaP covers up to 30 % of the granule surface (PhaC and PhaZ each roughly 1 %) (Tian *et al.*, 2005a). Furthermore, it was shown that a disruption of the gene coding for the phasin protein results in the accumulation of one single large PHA granule (Wieczorek *et al.*, 1995). On the other hand, over-production of phasin protein resulted in several PHA granules which showed a smaller diameter when compared to wild type PHA granules (Pötter *et al.*, 2002). This effect of phasin protein on granule size has been demonstrated both *in vivo* and in *vitro* (Pieper-Fürst *et al.*, 1995; Jossek *et al.*, 1998). In addition, synthesis of PhaP1 in *C. necator* has been found to be closely correlated with and to promote PHB production (York *et al.*, 2001a, 2001b, 2002).

Hänisch and co-workers demonstrated that recombinantly produced *C. necator* phasin protein also bound to lipid (TAG) inclusions in *Rhodococcus opacus* and *Mycobacterium smegmatis* (Hänisch *et al.*, 2006). In addition, non-phasin proteins such as BSA have been repeatedly reported to have a phasin-like effect on *in vitro* PHA granule production (smaller granule size, enhanced PHA synthase activity) (Horowitz & Sanders, 1995; Jossek *et al.*, 1998; Qi *et al.*, 2000). More recently, the *E. coli* heat-shock protein HspA was shown to partially replace the phasin protein during PHB accumulation in recombinant *E. coli* by binding to the granule surface in large amounts and decreasing granule size (Tessmer *et al.*, 2007). In 2006, first crystals of the phasin protein PhaP of *Aeromonas hydrophila* were obtained (Zhao *et al.*, 2006).

PHA-specific regulatory proteins

PHA-specific regulator proteins (transcriptional regulators) have been reported to bind non-covalently to the surface of PHA granules. So far, a PhaR protein in *C. necator* and another PhaR protein in *Paracoccus denitrificans* have been shown to bind to the DNA upstream of the respective *phaP* and *phaR* genes (Maehara *et al.*, 2001; Maehara *et al.*, 2002; Pötter *et al.*, 2002). PhaR homologues are present in other organisms, e.g. *A. vinosum* and *S. meliloti* (Pötter & Steinbüchel, 2005). Another regulator, PhaF, has been found in pseudomonads and seems to be a negative

regulator of PhaC1 and PhaI (Prieto *et al.*, 1999; Hoffmann & Rehm, 2004). However, direct interaction with DNA has not been demonstrated so far. Regulation of PHA biosynthesis and degradation is complex and takes place on more than just the transcriptional level. It will be discussed in more detail below.

For several proteins involved in PHA-granule formation in pseudomonads, such as PhaD or PhaI, the exact function and whether they are granule-bound or not has not yet been fully clarified (Klinke *et al.*, 2000b). Besides the four major protein classes that can be found attached to or embedded in the PHA granule surface, Uchino and co-workers suggested that all enzymes required for PHA metabolism are located at native PHA granules (Uchino *et al.*, 2007). They reported that native, isolated PHB granules from *C. necator* are not only able to catalyse the formation of PHB from acetyl-CoA, but can also catalyse the degradation of PHB to acetyl-CoA.

Apart from the PHA-synthase, none of the other GAPs are necessarily required for PHA biosynthesis. As long as substrate provision is ensured, recombinant production of PHA e.g. in *E. coli* is thus feasible by expressing only the PHA synthase gene. Absence of phasin proteins and PHA depolymerising enzymes on the one hand, and the strength of induction of the PHA synthase on the other hand are factors influencing PHA granule size in this situation.

Regulation of PHA metabolism

Our understanding of the regulation of PHA metabolism is still limited, although (or maybe because) according to the information which is presently available, it is complex. PHA biosynthesis is regulated at the transcriptional and/or at the enzyme level; this can vary between organisms. In addition, regulation is connected to other metabolic processes in the cell.

Regulation at the enzyme level has - once again - been mostly studied in *C. necator*. In this organism, concentrations of both free coenzyme A and acetyl-CoA were found to have an impact on PHB biosynthesis. Free CoA was shown to inhibit β-ketothiolase (PhaA), the enzyme which catalyses the first step of PHB biosynthesis, the conversion of acetyl-CoA into acetoacetyl-CoA (Oeding & Schlegel, 1973). High levels of NAD(P)H or high NAD(P)H/NAD(P) ratios, on the other hand, have an enhancing effect on PHB accumulation (Lee *et al.*, 1995). This indicates a connection to the TCA cycle as citrate synthase is inhibited by NAD(P)H (Henderson & Jones, 1997). A *C. necator* mutant strain with decreased TCA cycle activity indeed exhibited an accelerated PHB biosynthesis rate (Park & Lee, 1996). Much less is known about regulation of the PHA synthase in organisms with a class II PHA synthase like pseudomonads which synthesise PHA precursors via the β-oxidation pathway or fatty acid *de novo* biosynthesis. Experimental evidence so far only indicates that the PHA synthase and enzymes of the fatty acid

β-oxidation pathway compete for substrate during growth on related carbon sources (e.g. fatty acids) (Langenbach *et al.*, 1997; Qi *et al.*, 1998). Competition with the TCA cycle has been suggested by another study in *P. putida* (Klinke *et al.*, 2000a).

The granule-associated protein PhaR has been suggested to be a transcriptional repressor of *phaP*. PhaP production appears to be tightly regulated, as there has never been any free (non-granule bound) phasin detected in the cell, indicating that only the amount of phasin that can bind to the granule surface is produced. As mentioned above, PhaR has been reported to interact with the promoter regions of *phaR* and *phaP* (= *phaP1*) in both *C. necator* and *P. denitrificans*. For *C. necator*, there is additional evidence from mutagenesis studies (York *et al.*, 2002): A *phaR* deletion strain produced more PhaP than the wild type which indicates that PhaR normally represses PhaP production. A *phaC* deletion strain did not synthesise PhaP, but in a *phaC/phaR* deletion strain PhaP was again detectable in large amounts, indicating that PhaP was no longer correlated with PHB production.

Based on the experimental evidence, the following regulatory model has been suggested for *C. necator* (Pötter *et al.*, 2002; York *et al.*, 2002): Under non-PHA-accumulating conditions, PhaR binds to the *phaP* promoter region and represses transcription. Under PHA-accumulating conditions, PHA granules are formed. Once they are large enough to provide binding surface for other proteins apart from PhaC, PhaR binds to the PHA granule surface, hence no longer repressing *phaP* transcription. The PhaP protein is produced and also binds to the growing PHA granule. No soluble PhaP is detectable in the cytoplasm. At later stages of PHA-accumulation when the PHA granules stop increasing in size, PhaP starts to displace PhaR from the granule surface. PhaR again binds to the DNA and represses the transcription of *phaP* and of its own gene. Additional experiments with *phaR* and *phaP/phaR* deletion mutants indicated that PhaR must have an additional function apart from regulating PhaP because it was shown to also impact on PHA biosynthesis in a PhaP-independent manner (York *et al.*, 2002).

Apart from the PHA-specific regulatory proteins, regulators of e.g. quorum sensing and nitrogen metabolism have been reported to also have an influence on PHA metabolism (Miyamoto *et al.*, 1998; Sun *et al.*, 2000; Hoffmann & Rehm, 2005; Campisano *et al.*, 2008). Regulation of PHA degradation has not been very well investigated. Synthesis of the PHA depolymerase was generally thought to be repressed as long as a soluble carbon source was available. However, reports of simultaneous PHA production and degradation (see above) have changed the picture. (For a more in-depth overview of the regulation of PHA metabolism see reviews by Kessler and Witholt (Kessler & Witholt, 2001) or Stubbe *et al.* (Stubbe *et al.*, 2005).

Metabolic engineering of PHA-producing organisms

PHAs comprise around 150 different monomer units of short and medium chain length hydroxylalkanoates including 3-hydroxyalkanoates of 3-12 carbon atoms with a large variety of R-pendant groups, 4-hydroyalkanoates of 4-8 carbon atoms, 5-hydroxypentanoate, 5-hydroxyhexanoate, and 6-hydroxydodecanoate (Steinbüchel, 2001; Steinbüchel & Lütke-Eversloh, 2003). The diversity of different monomers that can be incorporated into PHAs depends on the key enzymes produced by the host both for PHA biosynthesis itself and for the provision of precursors (Taguchi & Doi, 2004). Though the monomer supply pathways are crucial for the entire biosynthesis pathway, the monomer supply can be manipulated by using strategically planned fermentation systems (Madison & Huisman, 1999). The composition and physical properties of the product can be controlled by the use of carbon substrate mixtures under ideal growth conditions (Hartmann *et al.*, 2004, 2006; Zinn & Hany, 2005). Besides the monomeric composition, the average molecular weight and molecular weight distribution also depend on the producing bacteria (Taidi *et al.*, 1994; Quagliano *et al.*, 2001; Mothes *et al.*, 2007), metabolic pathways and on the available external carbon source (Mantzaris *et al.*, 2001; Aldor & Keasling, 2003).

Aims and advantages

Metabolic engineering is a relatively new area and has become a new approach for the more efficient production of desired bioproducts. It is an interdisciplinary science that has grown from numerous roots in molecular biology and biochemistry, genetics, chemical engineering, biotechnology, mathematical modelling, and systems analysis (Lee, 2006). Metabolic engineering is the directed improvement of product formation or cellular properties through the modification of specific metabolic pathways by using genetic engineering techniques and other molecular biological tools.

Metabolic engineering was frequently attempted to improve the efficiency of PHA production by changing the selectivity of a pathway to favour one product and/or eliminating undesirable metabolic routes to enrich the desired product (Aldor & Keasling, 2003). Having a single product also improves purification. At the same time, by adding new catabolic activities for degradation of toxic materials in the fermentation process, both the productivity (or production rate) and the yield can be greatly improved. Liu and Steinbüchel established an efficient non-natural pathway using recombinant *E. coli* carrying a plasmid containing the genes for a butyrate kinase (Buk) and a phosphotransbutyrylase (Ptb) from *Clostridium acetobutylicum* and a PHA synthase (PhaEC) from *Thiocapsa pfennigii* (BPEC: Buk, Ptb and PhaEC) to synthesise polyoxoesters (PHA) and polythioesters (PTE) (Liu & Steinbüchel, 2000). In addition, by using

this pathway with careful incorporation of precursor substrate at an appropriate growth phase, PHA and PTE were synthesised independently from the central metabolism of the host cell (Liu & Steinbüchel, 2000; Thakor *et al.*, 2005).

Metabolic engineering can also be used to replace a substrate if it is too expensive by generating a strain which grows on an inexpensive substrate (Tsuge, 2002). This has been achieved for the use of whey to produce PHA (Koller *et al.*, 2007). Large quantities of whey are produced as a by-product during the manufacture of cheese and casein, representing 80-90% of the volume of the processed milk, and this must be disposed of or processed in an environmentally acceptable way. Recombinant *E. coli* harbouring a plasmid containing the *C. necator* genes for PHA synthesis and the *E. coli ftsZ* gene was employed to produce PHB from whey. A PHB content as high as 80% of the cellular dry weight was obtained (Wong & Lee, 1998). These results suggest that cost-effective production of PHB is possible by fed-batch culture of recombinant *E. coli* using concentrated whey solution as a substrate. This is a prime example of a case study to change the economic outlook of the process of biomass conversion into PHA. Changing the substrate can also enable production of a desirable product with certain properties with regard to monomeric composition, chain length and co-polymer microstructure. Another novel application of metabolic engineering is a new metabolic strategy to generate PHA-hyper producer strains which deposit PHA extracellularly. Sabirova et al. reported that by inactivating a specific thioesterase that channels the hydroxyacyl-CoA intermediates towards PHA_{MCL} synthesis in *Alcanivorax borkumensis* (Sabirova *et al.*, 2006) they generated a strain which produced high amounts of extracellular PHA.

The most important aims of the metabolic engineering of PHAs include (i) engineering of novel pathways for PHB-polymers and copolymers with desirable properties such as monomeric composition, chain length and co-polymer microstructure; (ii) development of integrated systems for PHB copolymer production; (iii) re-engineering of the central metabolism to improve yield in low-cost, high productivity fermentations; (iv) extension of substrate range for growth and product formation; (v) addition of new catabolic activities for the degradation of toxic chemicals; (vi) selectively shutting down pathways that interfere with efficient PHA production and storage; (vii) engineering of production strains with high efficiency to produce high value co-products from mixed carbon sources which can can be induced to produce enzymes for cell lysis and ideally nuclease to reduce viscosity.

PHA production in recombinant *E. coli*

The great advances in biotechnology have allowed the use of very different natural or genetically modified organisms in the commercial production of PHAs (Lee, 1996b). In nature, PHAs are produced by many different bacteria (Lee, 1996b; Verlinden *et al.*, 2007), and only the (R)-isomers

serve as substrates for the PHA synthase. Although PHA synthases exhibit extraordinarily broad substrate ranges, obtaining PHAs with specific properties like polythioesters consisting of 3-mercaptopropionic acid or 3-mercaptobutyric acid (Lütke-Eversloh *et al.*, 2001) needed an efficient enzyme which could synthesise novel PHAs (Aldor & Keasling, 2003; Takase *et al.*, 2003).

Only a few organisms have been exhaustively characterised, and metabolic engineers rely heavily on two of these workhorses, *E. coli* and *Saccharomyces cerevisiae*. *E. coli* in particular is found to be an efficient bacterial strain to achieve both a high substrate conversion rate and close packing of PHAs granules in the host cell (Lee, 1997; Taguchi *et al.*, 2003; Sujatha & Shenbagarathai, 2006). Moreover, this organism is well studied and able to utilise a wide range of carbon sources, simple and cheap downstream processing techniques are available (Fidler & Dennis, 1992), and no degradation of PHB occurs once it has been synthesised (Lee, 1996c).

Wild type *E. coli* is unable to accumulate PHA as a storage compound; however, small amounts of low molecular weight PHB (c-PHB) are found in the plasma membrane (Huang & Reusch, 1996). Since the finding that recombinant *E. coli* harboring *C. necator* PHA biosynthesis genes could accumulate P(3HB) from glucose, *E. coli* has been metabolically engineered to produce various PHAs (Steinbüchel, 2001). Cultivated under specific conditions which allow reaching high cell densities, high content of intracellular polymer, and yields compatible with an industrial process, these bacterial strains can use different renewable raw materials. The production of PHA by recombinant *E. coli* can reach 80–90% of the dry cell weight (Lee *et al.*, 1994; Lee, 1996b).

Large-scale production of PHAs

Polyhydroxyalkanoates (PHAs) represent an environmentally effective alternative to synthetic thermoplastics for use in medicine, drug-delivery, agriculture and horticulture, the fiber industry, and consumer products. In particular PHA_{MCL} have attracted considerable attention due to their potential applicability in medicine and industry and as sources of chiral monomers (Witholt & Kessler, 1999; Ueda & Tabata, 2003); however, current production practices are not sustainable. There are two routes to biotechnological production of PHA, i.e. microbial fermentation and plant-based production systems, each with its own merits and demerits. Currently, commercial efforts to produce PHA employ microbial fermentation processes rather than plant-based production systems which are still in the research and development stage (Poirier, 1999; Scheller & Conrad, 2005).

Scaling-up

The overriding factor that propels biotechnology is profit. Without profit, there would be no remuneration for research and development, and consequently no new products. The large-scale cultivation of cells is central to the production of a large proportion of commercially important biological products. Not surprisingly, the maximization of profits is closely linked to optimizing product formation by cellular catalysts, i.e. producing the maximum amount of product in the shortest time at the lowest cost. Since the PHAs are accumulated as intracellular inclusion bodies in the native system as well as in recombinant *E. coli* the productivity is directly proportional to the cell density in the fermentation process.

High-cell-density culture techniques for culturing *E. coli* have been developed to improve productivity (Wang & Lee, 1998), and also to provide advantages such as reduced culture volume, enhanced downstream processing, reduced wastewater, lower production costs and reduced investment in equipment. However, high-cell-density techniques have several drawbacks, including substrate inhibition, the high oxygen demand during fermentation due to the high cell concentration obtained, and formation of growth-inhibiting by-products like acetate (Lee, 1996a). A well defined medium that contains a non-inhibitive concentration of nutrients allows growth of *E. coli* to a cell density of about 15 g/L dry cell weight (DCW) (Lee, 1996a). The oxygen transfer rate in a fermentor varies with design, size, and the supply of oxygen. Bauer and Shiloach indicated that during growth of *E. coli* if glucose is used as the substrate, the yield based on oxygen is 1.06 g cell/g O_2 (Bauer & Shiloach, 1974). In an ideal fermentation system at $25^{\circ}C$ oxygen supply can be 1.5 $mol^{-L \cdot h}$ when aerated with pure oxygen. An *E. coli* culture can theoretically reach a density of 350g/L DCW at a growth rate of 0.2 g/h (Shiloach & Fass, 2005). Earlier research indicated that acetate excretion in *E. coli* under aerobic conditions resulted from an excess of carbon source, especially glucose (Lee, 1996a; Eiteman & Altman, 2006). Acetate is undesirable because it retards growth even at concentrations as low as 0.5 g/L (Nakano *et al.*, 1997); and it inhibits protein production as acetate production costs the carbon source that might otherwise have been utilised for generating biomass or protein production (March *et al.*, 2002). According to Eiteman and Altman, acetate formation could be controlled in high density culture of *E coli* either by process modification or by genetic modification (Eiteman & Altman, 2006). In order to lower acetate formation by process modification, one has to reduce the growth rate by supplying the glucose slowly by using either a fed batch or chemostat culture system. Another option is to adapt recombinant strategies to decrease acetate biosynthesis, increase acetate utilisation and divert glucose from glycolysis to other pathways (Shiloach & Fass, 2005).

Scaling up from a lab-scale experiment to an industrial scale is a difficult task for many reasons. Cell culture systems are extremely complex and there are many inputs and many outputs.

Unlike most chemical systems, the catalysts themselves are self propagating. In PHA production, to assist in both understanding and quantifying cell culture systems mathematical models have often been used. Based on the mathematical model the processing system has to be checked at lab scale. Maintaining sterility and homogeneity is essential, which pose difficulties for industrial scale operations. Sterilisation time has to be adjusted based on the volume, and due to length of sterilisation changes in medium composition could be expected which has to be monitored with care. During large-scale cultivation, microorganisms experience gradients in temperature, dissolved O_2 concentration, pH and substrate concentration and thus experience an increasing pressure to mutate. For this reason, the number of generations for recombinant organisms must be tested experimentally. Especially when industrial-grade nutrients are used to reduce costs, the commercial substrates from different sources have to be checked on a small scale, and it is important to do optimization experiments under conditions similar to the large-scale system. The selection of design of the bioreactor should be based on commercial scale economics. Industrial operations are carried out in >1000 litre capacity fermentation systems; in such cases oxygen demand for cell growth must be met.

Up to now, PHA production by wild type and recombinant strains is usually performed in two-stage fed-batch fermentative processes, which consist of (i) a cell-growth phase in a nutritionally enriched medium to yield sufficient biomass during early cultivation and (ii) a PHA production phase. Based on the culture conditions required for PHA synthesis, bacteria can be divided into two groups. In the first group of bacteria, growth is limited by depletion of some nutrients such as nitrogen, phosphorus, oxygen, or magnesium (Haywood *et al.*, 1989b), and once exponential growth stops this acts as a trigger for the metabolic shift to cease replication and start the sequential PHA production phase. In the second group of bacteria such as *Alcaligenes latus*, *Azotobacter vinelandii* and recombinant *E. coli* nutrient limitation is not necessary for the synthesis of PHA, i.e. they can accumulate PHA during growth. These growth-associated properties of the second group of PHA producing bacteria enabled the development of industrial PHA production (Choi *et al.*, 1998; Haage *et al.*, 2001).

Bioreactors

Bioreactors are commonly monitored with traditional pH, temperature, and dissolved oxygen sensors. Whether the biopolymer mass turns out water-repellent, brittle, elastic or sticky depends on the type of microorganism and the growth conditions in the bioreactor. For example recombinant *E. coli* harboring *C. necator* PHA biosynthesis genes (*phaCAB*) can produce an ultra-high-molecular weight PHB when the culture pH is maintained in the range of 6.0-6.5 (Kusaka *et al.*, 1997). Special growth conditions of microorganisms allow the metabolically engineered

biosynthesis of novel PHAs consisting of chiral monomers with functional groups (Ren *et al.*, 2005).

A reactor is called continuous or chemostat (chemically constant) when the feed and product streams are continuously being fed and withdrawn from the system. In principle, a reactor can have a continuous recirculating flow by which the substrate concentration can be maintained at a desired level. Zinn and Hany used chemostat cultivation to maintain the enriched medium concentration till they attained the desired cell concentration (Zinn & Hany, 2005). An initial growth phase in nutritionally enriched medium yielded sufficient biomass, followed by a product formation phase in carbon and nitrogen-depleted medium. By limiting the C and N substrates in the continuous culture they were able to demonstrate that it was possible to produce poly (3-hydroxybutyrate-co-3-hydroxyvalerate (PHB/HV)). The content of 3-hydroxyvalerate in PHB/HV could be controlled reproducibly (Zinn & Hany, 2005).

Fed-batch cultures have many advantages such as high cell densities, and they are widely used in the bio-industry (Lee, 1996a). In fed-batch cultures, a fresh nutrient (the feeding-up intensity is commonly connected with the growth or biosynthesis rate) is supplied in the bioreactor continuously or in portions. Cell mass and productivity are maximized by controlling culture conditions such as temperature and pH, the composition of the feed media, and the substrate feed rate. Cells are allowed to grow exponentially without supplying any extra nutritional supplements until nutrients are somewhat depleted and cells are approaching the stationary growth phase. At this point, specific substrates are added to manipulate the properties of the polymer produced. In some cases, while cells are still growing exponentially but nutrients are becoming depleted, carbon substrate precursors are added to supply specific carbon sources, allowing for a further increase in cell concentration, the length of the production phase as well as the desired products.

For the production of poly(3MP) Thakor and co-workers used a metabolically engineered *E. coli* harbouring plasmid pBPP1 containing *buk*, *ptb* and *phaEC*, and the precursor substrate was added at the end of the exponential phase (Thakor *et al.*, 2005). The metabolic trigger to produce the biopolymer occurred when the carbon source in the medium was depleted by the end of the exponential phase, and the addition of the precursor 3-mercaptopropionic acid (3MP) was timed accordingly to manipulate the cells to take up the substrate and produce poly(3MP).

In the batch process, the bioreactor is supplied with fresh nutrient medium, and thereby the inoculum is also added. At the end on the fermentation process, the content is passed on to the separation stage, the reactor is cleaned and sterilised to be ready for the next process. Batch-culture bioreactors are mainly used in lab-scale experiments. Although this is the second most commonly used type of bioreactor, it has got advantages and disadvantages of its own. In fed-

batch and chemostat reactors cultures are maintained longer, and formation of secondary products (i.e. PHA) is repressed by growth of the cells; whereas in batch culture the whole fermentation process is completed within a short time period. In addition, chemostat cultivation will place strong selection pressure on the most rapidly growing cells, thus creating genetic instability. However for an industrial set-up batch-culture bioreactors are not feasible because of the increased non-productive time due to sterilising, filling and cooling. Besides, the high frequency of sterilisation causes greater stress on instruments as well as additional expense of preparing and maintaining stock cultures.

For PHA production to be economically competitive, the polymer must be produced on a large scale and from inexpensive raw materials. Several factors including the production organism, substrate, yield, the complexity of the technology and product isolation influence the production costs, and each must be considered in order to minimize the costs, particularly when the fermentation process is to be scaled up to an industrial level (Byrom, 1987). Substrate cost is one of the most important economic factors (Tsuge, 2002), which means that identifying the cheapest carbon sources is of upmost importance if large-scale production is to take place. The use of alternative, renewable substrate feedstock such as soy molasses (Full *et al.*, 2006), whey (Wong & Lee, 1998; Koller *et al.*, 2005a; Koller *et al.*, 2007), sugar cane molasses (Dias *et al.*, 2006), green grass juice and silage juice (Koller *et al.*, 2005b), palm oil (Tan *et al.*, 1997), palm oil mill effluent (Redzwan *et al.*, 1997), atmospheric CO_2 (Ishizaki *et al.*, 2001) and activated sludge is being investigated for its potential to lower the substrate costs associated with the use of relatively expensive carbon sources including glucose, sucrose, and starch.

Isolation of PHAs

Success in producing PHAs on a commercial scale depends not only on high levels of production of well-processed end products suitable for specific applications but also on the development of efficient extraction and purification strategies that keep costs, especially energy costs, low (Lee, 1996b; van Wegen *et al.*, 1998). There are many different methods in practice to achieve the digestion of aqueous cell suspensions containing PHA. In lab-scale experiments PHA isolation is being carried out by mechanical cell disruption to disintegrate the physical cell wall barrier to release the PHA. Industrially, there are two major extraction methods which are being used (Furrer *et al.*, 2007; Suriyamongkol *et al.*, 2007): (i) lyophilisation (freeze-drying) of the cell biomass followed by extraction with solvents such as chloroform and methylene chloride; (ii) treating the wet cell biomass with cell wall dissolving enzymes and removing the cell wall debris. What is left is a plastic-like polymer mass that possesses highly specific properties and is ready for use in a wide variety of industrial and medical applications.

In most cases it is essential to attain sufficiently high purity of an end product with desired properties. It was determined that if dry *P. putida* biomass containing PHA_{MCL} was washed in 20 volumes of methanol for 5 min followed by Soxhlet extraction in 10 volumes of acetone for 5 h, almost all of the PHA could be recovered with no detectable loss of molecular weight (Jiang *et al.*, 2006). The solvent has to be heated above 70°C to solubilise the biopolymer, so the longer the polymer remains exposed to this temperature during the processing, the more it will degrade, which can irremediably impair its thermoplastic properties. The lesser alteration the PHA suffers during the extraction process, the wider will be the range of its possible commercial applications. Commercial processing using solvents requires large quantities of solvents which means high production costs. Since some of the halogenated solvents are highly aggressive to the environment and to human health the use of those solvents should be avoided. Apart from the solvent problem, it is desirable to have an adequate process which does not degrade the product thermally. However, the solvent step is essential if a product of high purity is desired, especially when the end product is aimed at medical applications.

Alternatively, cells are treated with a detergent, Triton x-100, at a high pH, thus dissolving the cell wall components, to then expose the PHA to bleach very briefly for purification. This significantly degrades the cells, releasing the PHA and destroying or denaturing most other cell components. To purify the PHA alkaline solutions or oxidising agents such as sodium hydroxide, sodium hypochlorite and hydrogen peroxide are applied (Yu & Chen, 2006). Although these oxidising agents are essential for pyrogen removal, they are very aggressive to the environment and cause polymer degradation (Taniguchi *et al.*, 2003). In addition, they require additional expensive processing steps like ultracentrifugation and microfiltration (Marchessault *et al.*, 1995; de Koning & Witholt, 1997). The processing method has to be chosen carefully to ensure that the disintegrated cell debris is not of similar size than the end product if the end product is to be isolated either by filtration or centrifugation.

Another preferred way of isolating PHA is flocculating the cells by either acidifying with sulphuric or phosphoric acid or by adding an alkalizing agent such as calcium hydroxide. Then, after removing the fermentation medium, the cell flocculants are subjected to solvent extraction or enzyme based extraction. The flocculation operation comprises a step of coagulating the cellular biomass either by acidification of the diluted cellular biomass to obtain a pH of about 2-3 or by addition of the alkalizing agent to adjust the pH of the diluted cellular biomass suspension to between pH 7 and 12. During the flocculation process the PHA containing cells form bridges with the cell walls by exerting a positive charge, and they aggregate, leading to the formation of a stable flake with a density higher than that of the surrounding liquid. Thus, the process allows the partial removal of the extracellular impurities dissolved in the fermented culture medium. To

reduce processing costs Thakor and co-workers used a novel *in situ* isolation procedure (Thakor *et al.*, 2005). In this study they used an SDS solution at an optimum concentration ratio of SDS to DCW to lyse the cells without prior cell harvesting while they were still in the bioreactor and obtained almost 100% poly(3MP). The biopolymers are heat sensitive, and they tend to degrade irreversibly and lose their molecular weight when submitted to temperatures above a certain limit, which can definitely affect the properties that characterise them as thermoplastics. However, Yu and co-workers recently reported a study in which the dry cell mass was heated to and maintained at 80-125°C in an acidic solution for up to 14 hours, and the molecular weight of the PHA could be maintained (Yu & Chen, 2006).

Furthermore, although only a small amount of enzyme is required for enzymatic cell disruption, these enzymes are expensive and they cannot be recycled as in the case of using solvents. In addition, the generation of large amounts of effluent is unavoidable during the process, which creates additional costs for treating the effluents before introducing them into the environment. PHA granules could also be separated from cells without using any solvent or enzyme if recombinant *E. coli* was engineered to lyse in response to a temperature signal, releasing the PHA inclusions into the culture medium from which they could be precipitated using ice-cold methanol or ethanol.

Potential applications of PHA

PHA as alternative plastic ("bulk" applications)

The interest to establish PHA as an alternative plastic to conventional petrochemical-based plastics was first sparked because it can be produced from renewable carbon sources. Both researchers and industry also increasingly recognized the material's potential based on its biodegradability and biocompatibility. Initial efforts to commercialise PHA as a bioplastic for i.e. packaging materials (one of the pioneers was ICI, a UK-based company who in 1982 introduced Biopol, a PHB-PHV copolymer) were not successful because production and isolation costs were too high, and the range of material properties that could be obtained was not ideal. However, the interest remained, and – with the growing understanding of the genetics behind PHA metabolism - huge progress has been made with regard to metabolic engineering of PHAs and PHA production. A range of new polymers, copolymers and blends of PHA with other materials have been obtained (examples below). Considerable research effort has also been invested into developing more economical large-scale production techniques (see above), but production costs are still higher than for conventional plastics. Today, PHAs are therefore mainly considered for specialist, i.e. (bio)medical, applications such as drug delivery systems (Koosha *et al.*, 1989;

Pouton & Akhtar, 1996) and tissue engineering (wound management, vascular system devices, orthopedics, etc.) (Hazari *et al.*, 1999a, 1999b; Williams *et al.*, 1999; Sodian *et al.*, 2000). Depending on the type of application, different surface properties, stability/flexibility and durability/degradation rate are desired. New compositions and blends of PHA have been created and tested, including blends of PHA with other polymers (e.g. PHB with poly(caprolactone) (Kumagai & Doi, 1992) or poly(lactic acid) (Koyama & Doi, 1995, 1997)), and composite materials, i.e. blends of PHAs and other materials like bioactive glasses/ceramics (Misra *et al.*, 2006), hydroxyapatite (Doyle *et al.*, 1991; Wang *et al.*, 2005) or silicates (Maiti *et al.*, 2007). Composites of polymers are a general concept in tissue engineering because they represent a tool to (i) reinforce polymers (e.g. for bone grafts) or (ii) create porous scaffolds to allow tissue ingrowth (Bonfield *et al.*, 1981; Williams *et al.*, 1999; Sodian *et al.*, 2000). Investigations into cytotoxicity, pyrogenicity and pyrogen (endotoxin) removal have been carried out with some promising results (Saad *et al.*, 1999; Williams *et al.*, 1999). The interest in this field has been growing extremely fast, as has the number of publications. For further information, please refer to more specialised reviews and references therein (e.g. Pouton & Akhtar, 1996; Williams *et al.*, 1999; Zinn *et al.*, 2001; Chen & Wu, 2005; Misra *et al.*, 2006).

Companies involved in developing PHA-derived products are e.g. Metabolix (present owner of Biopol patents), Tepha (Metabolix sister company and specialised in medical applications), Procter & Gamble and Meredian (producer of other biopolymers, new to PHA market).

An entirely different application of PHA was recognized by several research groups who used PHA metabolism for the preparation of enantiomerically pure *R*-(-)-hydroxycarboxylic acids. These serve as chiral building blocks for the synthesis of fine chemicals, e.g. antibiotics or vitamins. De Roo and co-workers isolated PHA via solvent recovery, hydrolyzed it by acid methanolysis and obtained the *R*-(-)-hydroxyalkanoic acids by saponification of the methyl esters (de Roo *et al.*, 2002). Similar approaches were already previously investigated by Seebach and co-workers (Seebach & Zuger, 1982). Lee and co-workers utilised the natural depolymerisation capacity of PHA accumulating organisms and established a method for the *in vivo* depolymerisation of PHB at a low pH (where depolymerase activity was maximal and obtained *R*-(-)-hydroxybutyric acid was not further degraded) (Lee *et al.*, 1999). Gao and co-workers reported the synthesis of D-(-)-hydroxybutyric acid from glucose in *E. coli* by recombinant expression of the genes for β–ketothiolase, acetoacetyl-CoA reductase, phosphotransbutyrylase and butyrate kinase (Gao *et al.*, 2002).

PHA granules as beads

The potential use of PHA granules in their native form as bio-nano/micro-beads has been neglected for many years because it was assumed that these inclusions would crystallise outside the cell and would thus be unstable. However, recent findings related to PHA granule stability after isolation from the cell have encouraged research activities in this particular field (Rehm, 2007). The list of diagnostic and therapeutic applications of nano-particles in biology and medicine reads long, ranging from fluorescent biological labelling to drug and gene delivery and to detection of pathogens and proteins (for review see Salata, 2004). Nano-particles are usually composed of an inorganic or polymeric core, often surrounded by a membrane or a protective layer, which can be coated with bio-functional components. If e.g. proteins are immobilised on these beads, covalent interaction and binding in a directed (i.e. non-random) orientation are desirable to ensure a stable and sensitive performance.

PHA granules with their polymeric core, the surrounding phospholipid monolayer and their spherical shape showing a diameter of about 100-500 nm possess properties suitable for applications as functionalised nano-particles (Figure 5). An N-terminal fusion of GFP to the class I PHA synthase of *C. necator* and to the class II PHA synthase of *P. aeruginosa* resulted in the formation of PHA granules showing GFP fluorescence and demonstrated that protein engineering of the PHA synthase can be used for functionalisation of the PHA granule surface (Peters & Rehm, 2005). Other examples of proteins successfully fused to the PHA synthase by constructing and expressing the respective hybrid gene served as additional proof of concept. A β-galactosidase (Peters & Rehm, 2006), the IgG-binding domain of protein A (Brockelbank *et al.*, 2006) and a single-chain antibody (Grage & Rehm, 2008) were displayed at the surface of PHA granules, with both the PHA synthase and the respective fusion partner retaining their functionality in all cases. These bio-beads could be stably maintained outside the cell, and they can be produced either *in vivo* or *in vitro*. An established system for *in vivo* production of functionalised PHA granules is a recombinant *E. coli* strain which expresses the genes (e.g. *phaA* and *phaB*) to provide substrate for the PHA synthase as well as the gene encoding the PHA synthase fusion protein. Since the PHA synthase remains covalently attached to the growing polyester chain and thus to the granule, it is the preferred partner for stable surface modification (see above). Nevertheless, there are also examples for the use of other GAPs, such as the use of the PhaP phasin protein from *C. necator* as an immobilisation tags for the functional display of eukaryotic proteins on beads as a diagnostic tool for fluorescence activated cell sorting (FACS) (Bäckström *et al.*, 2007). For *in vitro* production of surface-modified PHA granules, the PHA synthase fusion protein would have to be purified and then exposed to its substrate. The resulting PHA granules would be of high purity, displaying only the protein of interest. Other proteins

could be added for additional modification of the granule surface, such as phasin proteins or "phasin-replacements" like BSA. However, if large-scale bio-bead production is of interest, *in vivo* synthesis will certainly be the method of choice.

A different approach to functionalise the PHA granule surface has also been described. Proteins of interest were fused to the substrate-binding domain (SBD) of the PHA depolymerase (of *A. latus*), the fusion proteins purified from recombinant *E. coli* and subsequently bound to previously isolated PHA granules via the SBD (Lee *et al.*, 2005). Apart from their potential as bio-nano-particles, the application of PHA granules for protein production and purification has also been investigated. Moldes and co-workers used the N-terminal domain of the PhaF phasin protein from *Pseudomonas putida* as a polypeptide tag to anchor fusion proteins to PHA granules (Moldes *et al.*, 2004). The fusion proteins could be released from purified granules by simple detergent treatment, suggesting that this could be an alternative method for large-scale, low-cost production of fusion proteins. The groups around Wood and Gerngross developed a similar, yet slightly more sophisticated method for protein purification from *C. necator* or *E. coli*. A protein of interest was tagged for immobilisation at the PHA granule surface by creating a fusion to PhaP phasin protein from *C. necator*. Granule production and purification were followed by intein-mediated self-splicing to release the target protein (Banki *et al.*, 2005; Barnard *et al.*, 2005).
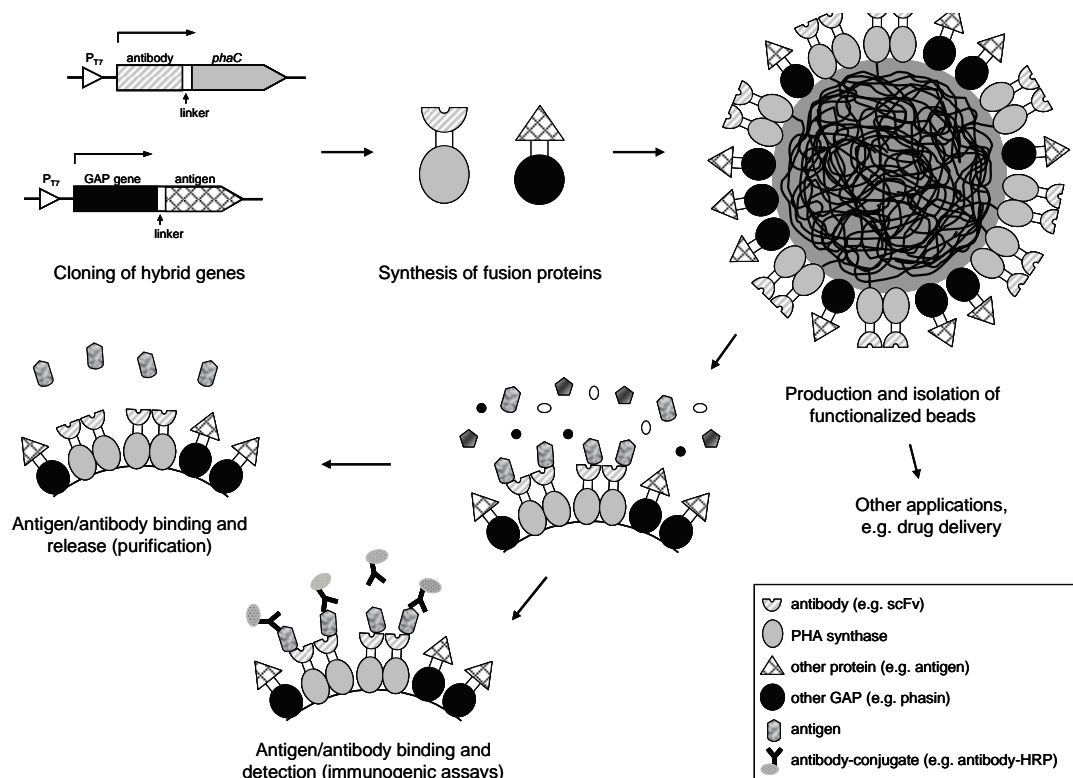
Figure 5:   Surface modification of PHA granules using GAPs as anchors / immobilisation tags and possible applications of these functionalised beads. GAP, granule associated protein.

Future directions

Recently, different workgroups dedicated their research to study the self-assembly process of PHA granules. In times of fast-developing microscopy and imaging technologies, hopes are rising for an elucidation of this process in the near future.

The recent findings that the PHA granule assembly starts at localised areas in the cell have given rise to even more questions. Is there a bacterial mechanism to localise the PHA granule assembly? Is the bacterial cytoskeleton involved in localisation? Does the PHA synthase interact specifically with other bacterial proteins to be localised? Or is the localisation of emerging PHA granules a passive process which is caused by space limitations in the bacterial cell, such as nucleoid occlusion? The study of the localisation of early-stage PHA granules offers a spectrum of new opportunities to apply the newest molecular-biological tools: from chip technology to see if the expression of any genes of interest are up/down-regulated to protein-protein interaction assays to test for specific interactions with the PHA synthase. Application of these techniques might provide further experimental evidence to either support the "budding-membrane" model or "micelle formation" model or might even lead to a completely different model which could explain PHA granule formation.

Decades of study have been dedicated to the composition and material properties of PHA in its crystallised form. A new perspective for industrial applications of PHA could be the use of native, isolated PHA granules as nano/micro-beads in biotechnology and medicine (Figure 5). As discussed above, the PHA granules exhibit all important features of shell-core nano-particles; their surface was demonstrated to be easily modified and the activity of surface exposed proteins of interest could be shown. The advantages of PHA granules as beads are obvious: (i) the PHA polyester can be generally considered as safe, non-toxic and biodegradable; (ii) the surface modification can be achieved in one step with bead production, avoiding the need for a two-step surface modification via tagging and coating or chemical cross-linking; (iii) stable beads with a high binding capacity can be obtained because the surface modification (e.g. protein immobilisation) occurs via covalent binding and in a directed orientation. Since a certain and uniform size of these bio-nano/micro-beads may be desirable, strain/synthesis development to control PHA granule size *in vivo* and *in vitro* might be necessary. In addition, methods for pyrogen removal have to be adapted. The potential use of PHA granules as beads provides a new perspective on applied PHA research, but it will mainly depend on the interest of the biotechnological industry whether these beads will replace existing standard beads in established processes.

Future research implementing the molecular mechanism of PHA granule assembly and biochemical analysis of involved proteins will provide further insight on the potential applicability of PHA granules as beads.

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Chapter I

Introduction

Aims and scope of the thesis

Aims and scope of the thesis

In 2005, we published fluorescence microscopy studies in which the green fluorescent protein (GFP) was fused to the PHA synthase of *Cupriavidus necator* and of *Pseudomonas aeruginosa*, respectively, in order to investigate the PHA granule assembly (Peters & Rehm, 2005). We observed that the PHA synthase and therefore emerging PHA granules locate to cell poles and to mid-cell representing future cell poles. In the same year, Jendrossek published his fluorescence microscopy data of early-stage PHA granules formation using a fluorescent dye as well as a fusion of the yellow fluorescent protein (YFP) to a phasin protein; he also detected localisation of emerging PHA granules to the cell poles and close to the cytoplasmic membrane (Jendrossek, 2005).

My thesis aims to provide further knowledge about the localisation of the PHA synthase and consequently the localisation of early stage PHA granules. The question we seek to answer is if a specific part of the PHA synthase possesses the polar positional information, for instance through interaction with another protein that directs the PHA synthase to the cell poles. In order to do so, we aim to mutate the PHA synthase of *C. necator* and study the localisation of these mutated proteins when fused to GFP and expressed in *Escherichia coli*. We aim to generate an inactive PHA synthase, where the active site histidine is substituted by an alanine to see whether the covalently attached, growing PHA chain is required for localisation. Additionally, we would like to delete the N-terminal part of the PHA synthase, which has been shown to be unnecessary for PHA synthase activity, in order to see if the N-terminus is involved in the localisation process. The C-terminal part of the PHA synthase, which is necessary for PHA synthase activity and thought to act as an anchor, will also be deleted to determine whether the C-terminal part plays a role in localisation of the PHA synthase.

Furthermore, the most abundant granule associated protein, the phasin protein of C. necator shall be fused to a reporter protein, expressed in *E. coli* and localisation of this protein shall be investigated in order to ascertain if other granule associated proteins are also directed to the cell poles.

Due to the granule's shape, size and material properties, one could consider isolated PHA granules as bio-nano/micro-beads with interesting characteristics suitable for applications in biotechnology. Synthetic bio-beads usually possess a core-shell structure where the surface becomes modified in a secondary procedure. PHA granules could be secondarily modified, for instance through cross-linking. However, it would be even more desirable if PHA granules could be stably modified in one step by expressing a translational fusion of a granule associated protein with the protein of interest.

My thesis aims to provide knowledge whether the PHA synthase can be successfully used as an anchor molecule in order to display proteins of interest at the PHA granule surface. The PHA synthase protein has been chosen because it stays covalently attached to the PHA surface and provides a stable attachment site. Furthermore, we would like to demonstrate the applicability of PHA granules as bio-beads by employing these functionalised bio-beads in different biotechnological assays.

The first protein that we aim to fuse to the PHA synthase is the β-galactosidase, a commonly used reporter enzyme, followed by analyses for PHA granule formation and β-galactosidase activity. If successful, other biotechnologically more interesting proteins will be fused to the PHA synthase in order to produce bio-beads for potential application in biotechnological procedures/assays, such as streptavidin, known for its high-affinity binding of biotin, and the IgG-binding domain of protein A. The applicability of these bio-beads shall be demonstrated.

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Chapter II

The inherent property of polyhydroxyalkanoate synthase to form spherical PHA granules at the cell poles: The core region is required for polar localisation

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The inherent property of polyhydroxyalkanoate synthase to form spherical PHA granules at the cell poles: The core region is required for polar localisation

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Abstract

Only the PHA synthase is required for formation of spherical intracellular PHA granules emerging at cell poles. This study aims to assign the polar targeting signal in the PHA synthase and to provide insight into molecular mechanisms of granule formation. Random in-frame insertion mutagenesis indicated dispensable and essential regions suggesting that only the N terminus (<100 aa) is dispensable and forms a random coil structure. The inactive PHA synthase (C319A) is still localised to cell poles, indicating that the nascent PHA chain does not serve as an anchor or signal for subcellular localisation and granule formation. Deletion of the N terminus did neither affect subcellular localisation nor PHA granule formation. The deletion of the hydrophobic C terminus (68 aa) did not impact on subcellular localisation of the PHA synthase, but abolished PHA synthase activity. The structural protein PhaP1 was found to be not required for subcellular localisation and initiation of granule formation. PhaP1 only localises to the cell poles, when PHA granules are formed. These data suggested that the PHA synthase itself localises to the cell poles via its core region (93–521 aa), which is structurally constraint and comprises the polar positional information for self-assembly of PHA granules at the cell poles.

Keywords: *Cupriavidus necator*; PHA synthase; PHA; Polyhydroxyalkanoate; Polyhydroxybutyrate; PHB; Mutagenesis; Polar localisation

Introduction

Polyhydroxyalkanoates (PHAs) are common carbon energy storage materials produced by numerous bacterial species (Madison and Huisman, 1999; Rehm, 2003). PHAs are now being under intensive investigation because of their inherent property as biodegradable thermoplastics. PHA granules are formed inside bacterial cells due to the activity of PHA synthases, which are considered as key enzymes of PHA biosynthesis (Rehm, 2006a). These enzymes catalyse the stereo-selective polymerisation of (R)-3-hydroxyacyl-CoA to PHA while concomitantly releasing CoA (Madison and Huisman, 1999; Rehm, 2003). As soon as the substrate, (R)-3-hydroxyacyl-

CoA thioester, is intracellularly provided, the PHA synthase starts to catalyse the formation of a high molecular weight PHA molecule ($n > 1000$). The growing PHA chain remains attached to the enzyme and converts the soluble enzyme into an amphipathic molecule ultimately resulting in the formation of the PHA granule with the PHA synthase presumably covalently attached to the surface (Rehm, 2003).

The PHA synthase from *Cupriavidus necator* has been extensively investigated by site-specific mutagenesis and site-directed evolution, which led to the identification of amino acid residues impacting on enzyme activity as well as substrate specificity (Hoppensack *et al.*, 1999; Normi *et al.*, 2005; Rehm *et al.*, 2002; Rehm, 2003; Taguchi *et al.*, 2002; Tsuge *et al.*, 2004). Fusion protein analysis indicated that fusion points located at the N terminus of the conserved α/β-hydrolase fold region, and at a variable surface-exposed loop based on the current protein model, are tolerated by PHA synthases (Rehm *et al.*, 2001, 2002). The N terminus, i.e. the first about 100 amino acid residues relative to the *C. necator* PHA synthase, represents the most variable region of the protein and has been found to be dispensable (Rehm, 2003; Schubert *et al.*, 1991).

Recently, Peters and Rehm (2005) fused GFP to the N terminus of PHA synthases and the fusion protein still led to formation of PHA granules with GFP-labeled surface. Other examples of fusion proteins functionally displayed at the PHA granule surface comprise the *Escherichia coli* β-galactosidase, the ZZ domain of protein A from *Staphylococcus aureus* and various eukaryotic proteins (Baeckstroem *et al.*, 2007; Brockelbank *et al.*, 2006; Peters and Rehm, 2006). Emerging PHA granules and the PHA synthase were recently found to be localised to the cell poles using fluorescent microscopical analysis (Jendrossek, 2005; Peters and Rehm, 2005).

In this study, we used for the first time a random in-frame insertion approach to identify essential and dispensable less structurally constraint regions in the PHA synthase, which might contribute to polar localisation of the PHA synthase and thus PHA granule formation. Moreover, truncated PHA synthase was N-terminally fused to GFP and subcellular localisation as well as PHA synthase function was analysed. To investigate whether a PHA polymer chain is required as potential anchor mediating polar localisation, an inactive PHA synthase mutant was fused to GFP. The role of PhaP1 in polar localisation was investigated using the PhaP1-HcRed fusion and fluorescent microscopical analysis.

Materials and methods

Oligonucleotides and *Pfx* DNA polymerase were from Invitrogen (Carlsbad, CA); restriction enzymes and ligases were from New England Biolabs (Beverly, MA); and chemical reagents were from Sigma (St. Louis, MO).

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown in LB medium. All *E. coli* strains were maintained at 37 °C. When required, antibiotics were used at the following concentrations: ampicillin, 75 µg/ml; chloramphenicol, 20 µg/ml; kanamycin, 50 µg/ml; tetracycline, 12.5 µg/ml.

Induction of gene expression was achieved by addition of IPTG to the culture medium to 1 mM, when gene was under control of *lac* promoter. In case of the arabinose promoter, gene expression was initiated by adding up to 0.5 % (w/v) of arabinose.

In order to obtain PHA-accumulating growth conditions, 1 % (w/v) glucose or 1 % (v/v) glycerol was added to cultures expressing the genes *phaA* and *phaB* encoded by plasmid pMCS69 in addition to *phaC* (Table 1).

Isolation, analysis and manipulation of DNA

Insertions and insertion sites were confirmed by DNA sequencing according to the chain termination method using the model ABI310 automatic sequencer. All other genetic techniques were performed as described elsewhere (Sambrook *et al.*, 1989). Plasmids used in this study are listed in Table 1.

Construction of expression vectors

The gene encoding PhaP1 was amplified without a stop codon from genomic DNA of *C. necator* using primers 5'phaP1-NcoI and 3'phaP1-SacI and cloned into plasmid pBAD/HisB resulting in plasmid pBAD-phaP1. The gene *hcred* was amplified from plasmid pHcRed1 using primers 5'hcred-SacI and 3'hcred-PvuII generating a PCR-product missing the start codon, which could then be used for translational fusion to *phaP1* in plasmid pBADphaP1, thereby generating plasmid pBAD-PhaP-HcRed.

Plasmid pJO5-CAB was constructed by subcloning an *Xba*I/*Hin*dIII fragment of plasmid pBHR68 encoding *phaCAB* of *C. necator* into plasmid pBBR1JO-5. Plasmid pMCS2-phaC was generated by subcloning the gene *phaC* of plasmid pCWE into pBBR1MCS-2 using restriction sites *Xba*I and *Pvu*I. Plasmids pJO5-CAB and pMCS2-phaC were compatible with plasmid pBAD-PhaP1-HcRed1 and could be used for localisation studies.

Plasmid pCWE was converted to plasmid pCWE$_{Spe}$ by exchanging the *Xba*I/*Nco*I fragment of plasmid pCWE with a newly amplified *Xba*I/*Nco*I fragment which provided a *Spe*I restriction site for translational fusions and lacked a start codon.

For deletion of the N terminus of PhaC, the vector pCWE was hydrolyzed with endonucleases *Xba*I and *Age*I. The *gfp* gene was amplified from plasmid pPROBE-NT with primers 5'gfp-XbaI and 3'gfp-AgeI and cloned upstream of the truncated *phaC*, resulting in plasmid pCWEgfpΔ1–93aa encoding GFP fused to the N terminus of PhaCΔ1–93aa.

To construct pCWEgfpΔ521–589aa, an amplicon of *phaC* was created which lacks the DNA region encoding the last 68 amino acids of the PHA synthase by providing an earlier translational stop. After amplification using primers 5'phaC-AatII and 3'phaC-BamHIΔ68aa, the amplicon and plasmidpCWE$_{Spe}$GFP were hydrolyzed with the respective endonucleases and ligated, which resulted in plasmid pCWEgfpΔ521–589aa.

Plasmid pSKgfpadd521–589aa was constructed by amplifying the *gfp* gene from pCWE$_{Spe}$GFP with primers 5'gfp-XbaI and 3'gfp-BamHI and by cloning the resulting PCR product into vector pBluescriptSK⁻. A fragment encoding the C-terminal 68 amino acids of PhaC could be amplified using 5'phaCBamHI_521aa_plus and 3'phaC-HindIII as primers, and was inserted downstream of the *gfp* gene to generate a translational fusion.

To generate plasmid pSKgfp-phaC_C319A, a PCR product was obtained using primer 5'phaC-Nde_wt and the mutated primer 3'phaCC319A. This PCR product was subcloned into pHAS. The *Nde*I/*Bam*HI fragment encoding PhaC_C319A was then inserted into *Nde*I/*Bam*HI sites of pCWE and *gfp* was inserted upstream of *phaC* with the aid of an adaptor fragment as described before (Peters and Rehm, 2005).

Site-specific mutagenesis was applied to remove the single *Not*I restriction site in the coding sequence of *phaC*. The nucleotide substitution was performed by overlapping PCR as described elsewhere (Rehm and Hancock, 1996; Hoffmann *et al.*, 2002) and by using primers 5'phaC-Nde_wt, 5'phaCMut, 3'PhaCMut and 3'PhaC-BamHI_wt. The resulting amplicon was subcloned into pHAS and the *Xba*I/*Bam*HI fragment encoding PhaCΔNotI fused N-terminally to a 6×His-tag was then inserted into pUC19, which resulted in plasmid pCΔNotI.

Fluorescence microscopy

Bacterial cells were resuspended in 50 mM potassium phosphate buffer and mounted onto an agarose pad (1 % (w/v)) on a glass slide. Slides were examined using a fluorescence microscope (Olympus, type BX51) with an Olympus U-RFL-T burner. Images were taken with an Optronics (Goleta, C, USA) digital camera connected to a standard PC.

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study.

Strains	Genotype	Source or reference
E. coli XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsd*R17 (r_k^-, m_k^+), *supE44*, *relA1*, -, *lac* [F`, *proAB*, *lacI^q*, *lacZ*ΔM15, Tn*10*(Tc^r)]	(Bullock *et al.*, 1987)
E. coli Top10	F^-, *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lacZ*ΔM15, Δ*lacX74*, *recA1*, *ara*Δ139 Δ(*ara-leu*)7697, *galU*, *galK*, *rpsL* (Str^R), *endA1*, *nupG*	Invitrogen

Plasmids	Characteristics	Source of reference
pBAD/HisB	Amp^R, arabinose-inducible promoter	Invitrogen
pBBR1JO-5	pBBR1MCS-5 with MCS from pBluescript SK^-	(Peters & Rehm, 2005)
pBBRMCS-2	Broad host range vectors	(Kovach *et al.*, 1994)
pBBRMCS-5	Broad host range vectors	(Kovach *et al.*, 1994)
pBHR68	pSK^- containing PHB synthesis operon of *C. necator*	(Spiekermann *et al.*, 1999)
pCWE	pBluesrcriptSK^- containing *Nde*I/*Bam*HI-fragment comprising *phaC* from pHAS	(Peters & Rehm, 2005)
pCWEAgfp	pCWEA containing *Spe*I-inserted *gfp* gene derived from pPROBE-NT by PCR	
pHAS	pET-14b containing *Nde*I/*Bam*HI inserted *phaC* gene from *C. necator*	(Yuan *et al.*, 2001)
pHcRed1	Amp^R, containing *hcred* gene	BD Biosciences Clontech
pMCS69	pBBR1MCS derivative containing genes *phaA* and *phaB* from *C. necator*	(Amara & Rehm, 2003)
pPROBE-NT	pBBR1MCS-5 containing promoter probe cassette plus *gfp* reporter gene	
pUC19	Amp^R, lac promoter	Fermentas
pBAD-phaP1	pBAD/HisB containing gene *phaP*1	This study
pBAD-PhaP-HcRed	pBAD/HisB containing gene coding for PhaP1 fused N-terminally to HcRed	This study
pCWEgfpΔ1-93aa	pSK^- containing gene coding for GFP fused to an N-terminal deletion mutant of PhaC	This study
pCWEgfpΔ521-589aa	pSK^- containing gene coding for GFP fused to an C-terminal deletion mutant of PhaC	This study

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study (continued).

Plasmids	Characteristics	Source of reference
pSKgfpadd521-589aa	pSK containing gene coding for GFP fused to amino acids 521-589 of PhaC	This study
pSKgfp-phaC_C319A	pSK⁻ containing gene coding for PhaC_C319A fused to GFP	This study
pCWE_{Spe}	vector for 5'-end fusions to PhaC using the provided *Spe*I site	This study
pCWE_{Spe}GFP	pCWE_{Spe} containing *Spe*I-inserted *gfp* gene derived from pPROBE-NT by PCR	This study
pCΔNotI	pUC19 containg gene coding for PhaC with deleted *Not*I restriction site and N-terminal 6xHis	This study
pJO5-CAB	pBBR1JO-5 containing *Xba*I/*Hind*III fragment from pBHR68	This study
pMCS2-phaC	pBBRMCS-2 derivative with *Xba*I/*Pvu*I inserted fragment from pCWE	This study

Oligonucleotides	Sequence in 5'-3' direction	Source of reference
5'hcred-SacI	ggc<u>atgagc</u>tcgtgagcggcctgctgaaggagagtatg	This study
3'hcred-PvuII	att<u>cagctg</u>tcagttggccttctcgggcaggtcgctgtac	This study
5'phaP1-NcoI	ggaac<u>ccatgg</u>aataaggagattctagaatgatcctcacccc cggaacaagttgcagcagcgc	This study
3'phaP1-SacI	aaatt<u>gagctc</u>ggcagccgtcgtcttctttgccgtggcc	This study
5'gfp-XbaI	gcgcg<u>tctaga</u>aataaggagatatatgaatgagtaaaggag aagaacttttcactggagttg	This study
5'gfp-XbaI	ccgc<u>tctaga</u>atataggagat<u>actagt</u>atgagtaaaggaga agaactttc	This study
3'gfp-AgeI	ggact<u>accggt</u>ttttgtatagttcatccatgccatgtgtaatc	This study
3'gfp-BamHI	ctagt<u>ggatcc</u>tttgtatagttcatccatgccatgtg	This study
5'phaC-AatII	catcctc<u>gacgtc</u>tttgtcgacgagggccatg	This study
5'phaC-BamHI_521aa_plus	tcgaa<u>ggatcc</u>agccactggactaacgatgcgctgc	This study
5'phaC-Nde_wt	aatat<u>catatg</u>gcgaccggcaaaggcgcggcagc	This study
5'phaCMut	ctgaaggcaacggccacgcggcggcgtc	This study
5'phaC-Xba-Spe	aattg<u>tctaga</u>atataggagat<u>actagt</u>gcgaccggcaaag gcgcggcagcttccacgcag	This study
3'phaC-BamHI_wt	gagtag<u>ggatcc</u>tcatgccttggc	This study
3'phaC-HindIII	ctagc<u>aagctt</u>tcatgccttggctttgacgtatcg	This study
3'phaCMut	gacgccgccgcgtggccgttgccttcag	This study
3'phaCC319A	cagcgcggtc<u>gagac</u>aatggtgccgcccacggcgaagcc gagcacgttgatcttgtc	This study
3'phaC-BamHIΔ68aa	ctagc<u>ggatcc</u>tcagcgcttgttcttggccggcgggttg	This study
3-phaC-NcoI	ctcgg<u>ccatgg</u>cctgccacagcgctgagaag	This study

Random insertion mutagenesis

The EZ::TN™ In-Frame Linker Insertion Kit (Epicentre, Wisconsin, USA) has been used for random insertion mutagenesis of plasmid pCΔNotI containing the PHA synthase gene lacking the *Not*I site. The *in vitro* transposon insertion reaction was conducted according to the manufacturer's protocol (Epicentre, Wisconsin, USA) and using pCΔNotI. To select for insertions in the PHA synthase gene, mutagenized plasmids were transformed into *E. coli* harboring plasmid pMCS69, which mediates provision of (*R*)-3-hydroxybutyryl-CoA, and screened for PHA accumulation on Nile Red medium as previously described (Amara *et al.*, 2002). Mutants carrying an insertion in the ampicillin resistance gene were eliminated by adding ampicillin to the screening medium.

Non-fluorescent colonies representing PHA synthase gene insertion mutants, which harbor the transposon including the 1.2 kb kanamycine resistance gene cassette leading to inactivation of the PHA synthase, were selected. The respective plasmids were isolated and after removal of the resistance cassette by *Not*I hydrolysis, plasmids were ligated leaving a 57 bp in-frame insertion comprising a single *Not*I site. Mutants were again screened on Nile Red medium and 15 fluorescent permissive and 15 non-fluorescent non-permissive insertion mutants were isolated. Insertion sites were determined by DNA sequencing. According to the three reading frames, three different oligonucleotide pairs were designed encoding the hemagglutinin epitope YPYDVPDYA and containing *Not*I ends. After dimerisation, the oligonucleotide pairs were inserted into the *Not*I site of the permissive mutants. All resulting modified PHA synthases were analysed with respect to *in vivo* activity using GC–MS and *in vivo* PHA granule formation using Nile Red-mediated fluorescence microscopy analysis.

In vivo PHA synthase function

In vivo PHA synthase activity was obtained by analysing PHA content of the respective bacterial cells. The amount of accumulated PHA corresponds to the relative *in vivo* PHA synthase activity. The PHA contents were qualitatively and quantitatively determined by gas chromatography/mass spectrometry (GC/MS) after conversion of the PHA into 3-hydroxymethylester by acid-catalysed methanolysis (Brandl *et al.*, 1988).

PHA granule isolation

Cells were cultivated under PHA-accumulating conditions and harvested by centrifugation. Cells were resuspended in 50mM potassium phosphate buffer, pH 7.5 and subjected to cell lysis using a French press. The whole cell lysate was subjected to centrifugation at 4,000×*g* for 20 min to sediment PHA granules. To enhance purity, PHA granules were again resuspended in potassium phosphate buffer and loaded on top of a linear glycerol gradient. This gradient was obtained from a discontinuous gradient prepared from 5 ml of 88 % and 6 ml of 44 % glycerol in

potassium phosphate buffer (pH 7.5). After centrifugation (2.5 h, 100,000×*g*, 10 °C), a granule layer was obtained at the boundary between 88 % and 44 % glycerol. The granules were isolated from this region and were washed with 50 mM potassium phosphate buffer (pH 7.5) by centrifugation (1 h, 100,000×*g*, 10 °C).

ELISA

Isolated PHA granules, which formation was mediated by hemagglutinin epitope (YPYDVPDYA) insertion mutants of the PHA synthase, were subjected to ELISA using antihemagglutinin antibodies. ELISA was conducted as previously described (Brockelbank *et al.*, 2006).

Results

Random in-frame linker insertion mutagenesis

In order to screen for transposon insertions located in the coding region of the PHA synthase gene in pCΔNotI, the mutagenized plasmids were transformed into *E. coli* harbouring plasmid pMCS69. Plasmid pMCS69 contains the genes *phaA* and *phaB*, encoding the β-ketothiolase and the (*R*)-specific acetoacetyl-CoA reductase, respectively, downstream to the *lac* promoter (Amara and Rehm, 2003). These two enzymes catalyse diversion of acetyl-CoA to (*R*)-3-hydroxybutyryl-CoA, which is substrate for the PHA synthase and in the presence of a functional PHA synthase PHA is accumulated. Transformands harboring plasmid pMCS69 and mutagenized pCΔNotI were screened on selective solid medium containing the fluorescent dye Nile Red, which enables discrimination between PHA accumulating and non-PHA-accumulating cells (Spiekermann *et al.*, 1999). Accordingly, an insertion of the entire 1.2 kb transposon into the coding region of the PHA synthase gene will result in an inactivation of this gene. Thus, mutagenized pCΔNotI not mediating PHA accumulation was isolated from single colonies, the kanamycin resistance cassette was removed by *Not*I hydrolysis and after religation transformed into *E. coli* (pMCS69). Transformands were screened on selective solid medium containing Nile Red. More than 100 mutagenized plasmids were isolated and initially analysed by PCR to obtain mutants with different insertion site, respectively. From these mutants 15 permissive and 15 non-permissive mutants were isolated based on fluorescent staining of colonies on Nile Red medium.

Analysis of PHA synthase linker insertion mutants

All 30 linker insertion mutants were cultivated in LB medium containing 1 % (w/v) glucose for 48 h at 37 °C. The PHA content of the respective recombinant cells harboring modified PHA synthase was determined by GC/MS analysis. The PHA content represented the functionality of the respective modified PHA synthase, when compared to the wildtype enzyme. All 30 mutants

were subjected to DNA sequencing to exactly locate the insertion site. Four of the 15 presumably permissive mutants, which were isolated based on Nile Red-mediated colony fluorescence, did not accumulate PHA. The remaining 11 permissive mutants, which mediated PHA accumulation, showed a transposon insertion at amino acid position <100, whereas 19 non-permissive mutants showed insertion at amino acid position >100, except for the insertion after lysine 77 (Fig. 1). Electron microscopy analysis of cells harboring modified PHA synthases (linker insertion) mediating PHA accumulation did not indicate obvious differences in shape and size of PHA granules, when compared with the wildtype (data not shown). Moreover, *in vivo* formation of emerging PHA granules was assessed after cultivation for 3 h and fluorescence microscopy using Nile Red staining. All permissive insertion mutants mediated PHA granule formation at the cell poles (data not shown).
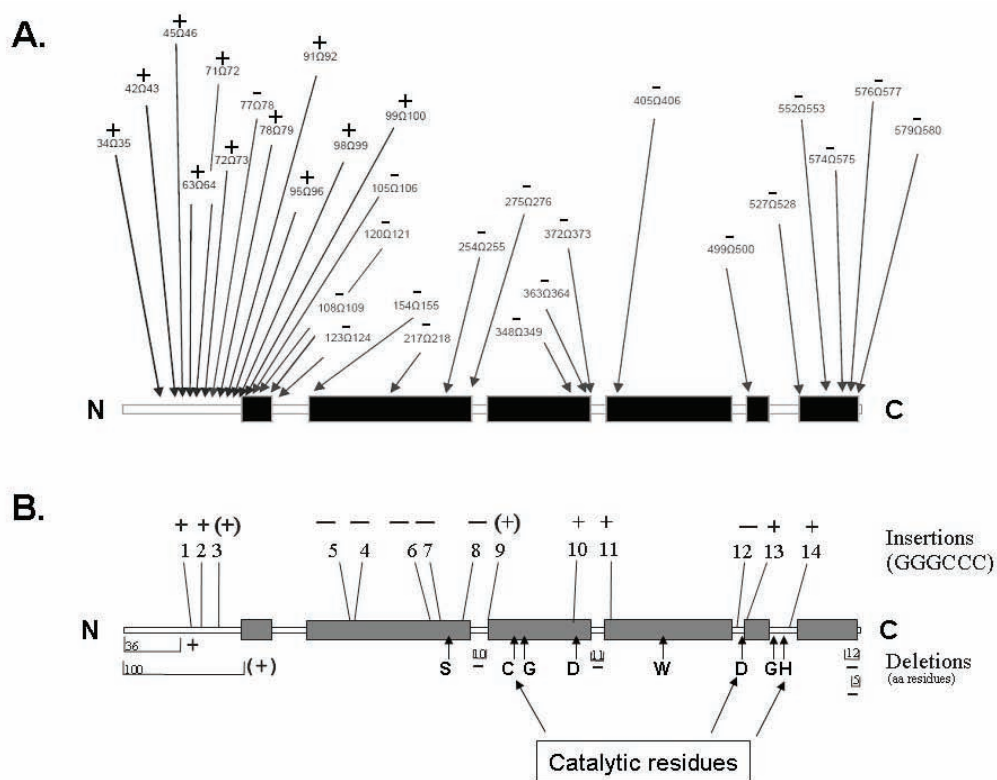

Figure 1. Permissive and non-permissive linker insertion sites in the PHA synthase of *Cupriavidus necator* obtained by random linker insertion mutagenesis. A, Insertion sites are indicated by arrows and number indicated the amino acid position. B, Localisation of mutation sites obtained in independent studies (modified according to (Kalousek, 1992; Rehm *et al.*, 2002; Rehm, 2006a; Rehm, 2006b)). Rectangular boxes indicate conserved regions. The eight conserved amino acid residues by single letter amino acid code. +, permissive mutation (active PHA synthase); (+), PHA synthase activity is strongly impaired; (-), non-permissive mutation (inactive PHA synthase).

Analysis of hemagglutinin PHA synthase insertion mutants

To further investigate whether the insertion site is variable and structurally flexible the hemagglutinin epitope encoding DNA fragment was inserted into the insertion sites (*Not*I) of the permissive mutants, respectively. Moreover, immunological analysis using monoclonal anti-hemagglutinin antibodies should enable detection of PHA granule surface-exposed domains of the PHA synthase. Immunological analysis using anti-hemagglutinin antibodies did not indicate a granule surface exposure of the epitopes residing in the N-terminal protein region (data not shown). Electron microscopy analysis of cells harboring modified PHA synthases (hemagglutinin insertion) mediating PHA accumulation did not indicate significant differences in shape and size of PHA granules, when compared with the wildtype.

The polar positional information is provided by the PHA synthase

Since emerging PHA granules were found to localise to the cell poles, it was assumed that the PHA synthase might mediate this polar localisation (Jendrossek, 2005; Peters and Rehm, 2005). An inactive site-specific mutant C319A of the GFP-PHA synthase fusion was generated, in order to exclude that the nascent PHA chain, which remains attached to the PHA synthase, is required for polar localisation. This mutation has been previously shown to inactivate the enzyme (Gerngross et al., 1994). The modified GFP-PHA synthase (C319A) did not mediate PHA accumulation, but was localised to the cell poles (Figs. 2 and 5). This is first evidence that only the PHA synthase and not emerging PHA granules localise to the cell poles. To investigate whether the N (1–93 aa) or the hydrophobic C terminus (521–589aa) comprises the polar positional information, the respective deletion mutants were generated, but modified PHA synthases still localised to the cell poles (Figs. 3–5). Only the C-terminal 68 amino acid residues did not mediate polar localisation (Figs. 4B and 5). The C-terminal deletion as well as only the C terminus did not mediate PHA accumulation, whereas as expected the N-terminal deletion mutant still showed PHA synthase function (Fig. 5).
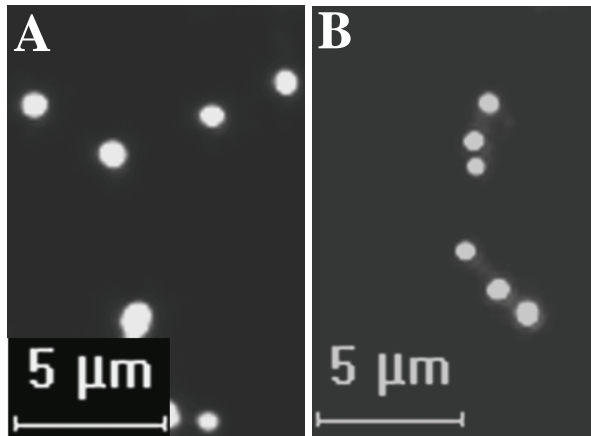
Figure 2. Polar localisation of the wildtype PHA synthase and the inactive mutant C319A. Fluorescence microscopy images of *E. coli* XL1-Blue cells expressing either (A) wild-type GFP-PHA synthase or (B) inactive variant GFP-PhaC_C313A.
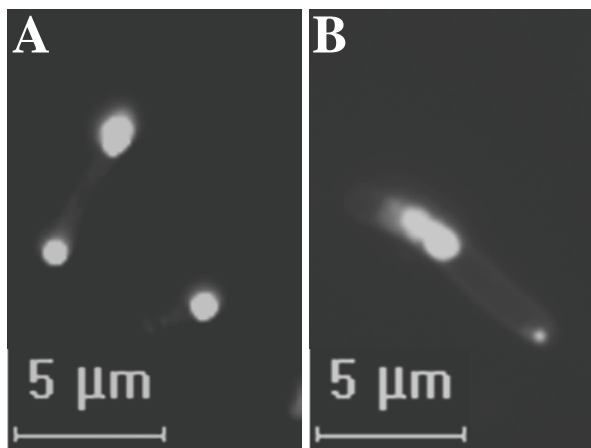

Figure 3. Fluorescence microscopy images of *E. coli* XL1-Blue cells expressing GFP-PhaCΔ1-93aa under (A) non-PHA accumulating conditions or (B) PHA accumulating conditions.
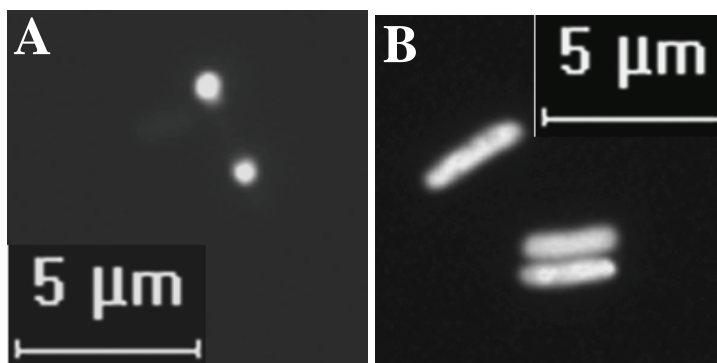

Figure 4. Localisation of (A) GFP-PhaCΔ521-589aa to the cell poles and (B) lack of polar localisation indicated by random distribution of fluorescence in cells producing GFP fused to amino acids 521-589 of the PHA synthase.
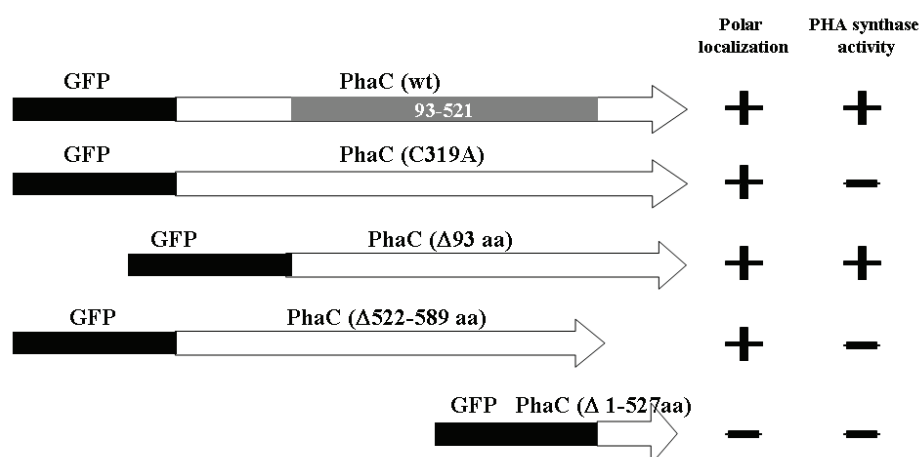
Figure 5. Correlation between polar localisation and *in vivo* PHA synthase activity of wildtype and modified PHA synthases. Grey box indicates the region comprising the polar positional information.

Does PhaP1 contribute to polar localisation?

In order to investigate whether phasins are localised to the cell poles in the absence of PHA granules, PhaP1 was fused to the N terminus of HcRed and produced in *E. coli* lacking PHA granule formation. Fluorescence microscopy analysis showed fluorescence of the entire cell and no polar localisation (Fig. 6A). Co-production of the PHA synthase in the absence of PHA precursor supplying enzymes (PhaA, PhaB), i.e. in the absence of PHA granules, showed no polar localisation of PhaP1-HcRed (Fig. 6B). However, PhaP1-HcRed showed polar localisation upon the formation of PHA granules (Fig. 6C).
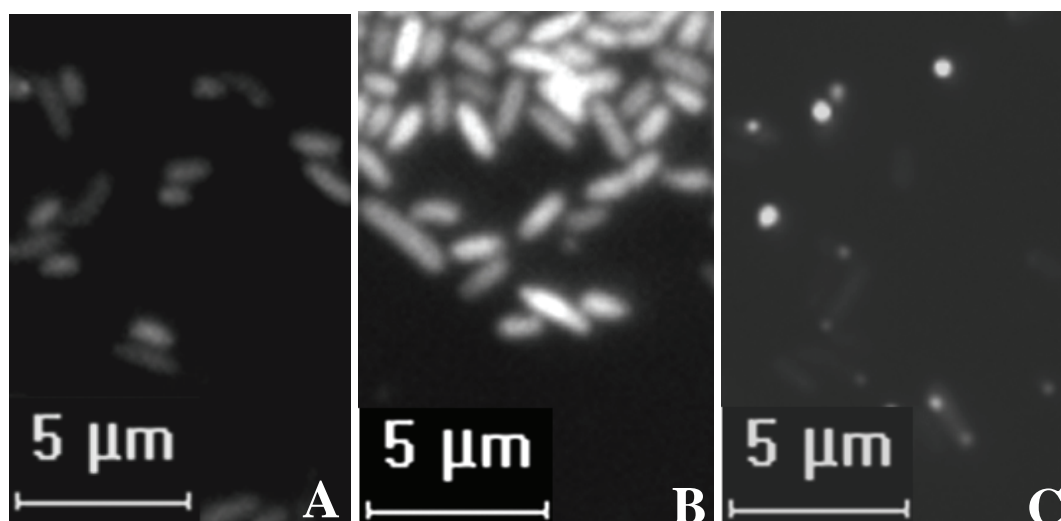

Figure 6: Fluorescence microscopy images of *E. coli* Top10 cells expressing PhaP1-HcRed under various conditions (A-C). (A) Cells expressing only PhaP-HcRed. (B) Cells expressing PhaP-HcRed as well as the PHA synthase under non-PHA accumulating conditions and (C) cells expressing PhaP-HcRed under PHA accumulating conditions.

Discussion

Previous studies indicated that the PHA synthase and emerging PHA granules are localised to the cell poles (Jendrossek, 2005; Peters and Rehm, 2005). This was observed in bacteria naturally producing PHA granules as well as in recombinant *E. coli* producing PHA granules. In contrast, a transmission electron microscopy study indicated that PHA granules are formed at central mediation elements (Tian *et al.*, 2005). The composition of these mediation elements remains to be elucidated.

In this study, the polar localisation of emerging PHA granules at the cell poles was confirmed and investigated in recombinant *E. coli* as model system, presumably reflecting conditions present in natural PHA granule producers (Peters and Rehm, 2005).

The PHA synthase gene from *C. necator* was subjected to transposon-mediated random linker insertion mutagenesis to identify structurally flexible regions and to identify regions which might contribute to polar localisation. GC/MS analysis showed that 11 of the 15 isolated presumably permissive mutants mediated PHA accumulation, suggesting that Nile Red staining might lead to false positive results due to unspecific staining. All permissive insertion sites were located in the N-terminal region (<100 aa) which was consistent with previous studies, indicating that the N terminus is highly variable and dispensable (Rehm *et al.*, 2002; Schubert *et al.*, 1991). Since 28 amino acids comprising insertions were tolerated in the N terminus, this protein region seems to be unstructured and highly flexible (Fig. 1). The non-permissive insertion sites did mostly reside in the amino acid region between 100 and 589 (Fig. 1). However, Kalousek *et al.* (1992) analysed the PHA synthase by insertion of two amino acid residues, which were also found to be tolerated in the core region of the protein (Fig. 1). Unfortunately, the exact insertion positions were not determined (Kalousek *et al.*, 1992). Interestingly, the 19 amino acid residues comprising insertion after amino acid 77 resulted in a loss of PHA synthase function (Fig. 1). Since insertions directly surrounding this nonpermissive insertion are permissive it is hard to explain how inactivation occurred. However, it might be conceivable that this particular insertion interacts with the remainder of the enzyme in such a mode which results in a loss of enzyme activity. It has been recently shown that mutations in the N terminus can even enhance PHA synthase activity (Normi *et al.*, 2005; Zheng *et al.*, 2006). Overall, this random insertion mutagenesis approach suggests that the PHA synthase is structurally constraint, i.e. does not tolerate 19 amino acids comprising insertions, except for the variable N terminus presumably forming a random coil structure. Since the hemagglutinin epitope inserted into the N terminus could not be detected by ELISA, the N-terminal region might not be extensively exposed at the PHA granule surface.

However, fusions of functional proteins to the N terminus of the PHA synthase enabled functional display of the respective protein (Rehm, 2007).

In order to understand how PHA granule formation is localised to the cell poles, we analysed mutants of the PHA synthase N-terminally fused to GFP by fluorescence microscopy. A site specific mutant, in which the catalytic C319 was replaced by A to generate an inactive enzyme as has been demonstrated elsewhere (Gerngross *et al.*, 1994), was used to exclude that PHA chains or small PHA granule formation causes polar localisation (Fig. 2). Moreover, it has been assumed that PHA granules emerge from the inner leaflet of the cytoplasmic membrane according to a budding model (Dunlop and Robards, 1973; Jensen and Sicko, 1971; Lundgren *et al.*, 1964; Mayer *et al.*, 1996). The nascent PHA chain extruding the PHA synthase could serve as a membrane anchor initiating granule formation according to the budding model (Rehm, 2006a). The catalytically inactive mutant of the PHA synthase clearly showed polar localisation strongly suggesting that PHA synthesis is not required for polar localisation and that the PHA synthase possesses the polar positional information for PHA granule formation to begin at the cell poles. To our knowledge no conserved motif or polar targeting signal has been identified among proteins which are localised to the cell poles.

To identify the protein region involved in polar targeting of the PHA synthase various deletion mutants were generated and fused to the C terminus of GFP. Deletion of the dispensable N terminus did neither impair polar localisation nor PHA synthesis (Fig. 3). Removal of the hydrophobic C terminus (68 amino acid residues), which was proposed to hydrophobically attach the PHA synthase to the PHA core or to anchor the PHA synthase to the cytoplasmic membrane, abolished PHA synthase activity but did not impact on polar targeting (Rehm *et al.*, 2002; Rehm, 2003) (Fig. 4). As a control, the anchor region only did not mediate polar targeting (Fig. 4).

Thus, the polar targeting resides presumably in the protein region between 100 and 521 amino acid residues, which comprises the highly conserved α/β-hydrolase domain (Jia *et al.*, 2000; Rehm, 2003) (Fig. 5). This suggests that the core structure of the PHA synthase contains polar targeting information.

The question how the structural phasin proteins contribute to early granule formation was investigated producing the PhaP1-HcRed fusions in the absence of PHA synthase and the presence of PHA synthase, respectively. PhaP1-HcRed did only show polar localisation in the presence of PHA synthase, when PHA granules could be formed (Fig. 6). This data suggested that the phasins do not interact with the polarly localised PHA synthase unless granules are formed, i.e. PHA is available for attachment.

Acknowledgements

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Chapter III

In vivo enzyme immobilisation by use of engineered polyhydroxyalkanoate synthase

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In vivo enzyme immobilisation by use of engineered polyhydroxyalkanoate synthase

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Abstract

This study demonstrated that engineered polyhydroxyalkanoate (PHA) synthases can be employed as molecular tools to covalently immobilise enzymes at the PHA granule surface. The β-galactosidase was fused to the N terminus of the class II PHA synthase from *Pseudomonas aeruginosa*. The open reading frame was confirmed to encode the complete fusion protein by T7 promoter-dependent overexpression. Restoration of PHA biosynthesis in the PHA-negative mutant of *P. aeruginosa* PAO1 showed a PHA synthase function of the fusion protein. PHA granules were isolated and showed β-galactosidase activity. PHA granule attached proteins were analysed and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization–time of flight mass spectrometry. Surprisingly, the β-galactosidase–PHA synthase fusion protein was detectable at a high copy number at the PHA granule, compared with PHA synthase alone, which was barely detectable at PHA granules. Localisation of the β-galactosidase at the PHA granule surface was confirmed by enzyme-linked immunosorbent assay using anti-β-galactosidase antibodies. Treatment of these β-galactosidase–PHA granules with urea suggested a covalent binding of the β-galactosidase–PHA synthase to the PHA granule. The immobilised β-galactosidase was enzymologically characterised, suggesting a Michaelis-Menten reaction kinetics. A K_m of 630 μM and a V_{max} of 17.6 nmol/min for orthonitrophenyl-β-*D*-galactopyranoside as a substrate was obtained. The immobilised β-galactosidase was stable for at least several months under various storage conditions. This study demonstrated that protein engineering of PHA synthase enables the manufacture of PHA granules with covalently attached enzymes, suggesting an application in recycling of biocatalysts, such as in fine-chemical production.

Introduction

Polyhydroxyalkanoate (PHA) granules (biopolyester particles) are formed inside bacterial cells based on the activities and biochemical properties of PHA synthases. PHA synthases are the key enzymes of PHA biosynthesis and PHA granule formation. These enzymes catalyse the stereoselective polymerisation of (R)-3-hydroxyacyl coenzyme A to PHA while concomitantly releasing coenzyme A-SH (17). Biologically, PHA serves as a carbon and energy reserve polymer.

The core of the PHA granules is composed of high-molecular weight PHA, for which more than 150 constituents have been identified. PHA granules are surrounded by a phospholipid membrane with embedded or attached proteins consisting of PHA synthase, intracellular PHA depolymerase, amphiphilic phasin proteins, PHA-specific regulator proteins, and additional proteins with yet-unknown functions (for a review, see references 17 and 19). Among these proteins, only the PHA synthase is required for PHA granule formation and only the PHA synthase was suggested to be covalently attached to the PHA granule core (5). Phasin proteins have recently been subjected to protein engineering in order to enable purification of proteins fused to these proteins, which hydrophobically attach to the preformed PHA granules (3, 4). A previous study showed that the fusion of green fluorescent protein (GFP) to the N terminus of the PHA synthase did not interfere with PHA granule formation (14). In this study, we targeted the PHA synthase with respect to immobilisation of an enzyme at the PHA granule surface. Only the PHA synthase is supposed to mediate covalent attachment to the PHA granule surface and hence would enable the design of a robust particle-based recycling system for biocatalysts (20, 21).

Material and Methods

Bacterial strains and growth conditions

The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. *Escherichia coli* BL21 (DE3) strains were grown in LB medium at 30 °C. All other *E. coli* strains were grown at 37 °C. When required, antibiotics were used at the following concentrations: ampicillin, 75 μg/ml; gentamicin (Gm), 10 μg/ml; and chloramphenicol, 50 μg/ml. *Pseudomonas aeruginosa* PAO1 strains were grown in mineral salt medium (MSM) (23) at 37 °C and, if required, antibiotics were added to appropriate concentrations. The antibiotic concentrations used for *P. aeruginosa* strains were as follows: gentamicin, 150 μg/ml; carbenicillin, 300 μg/ml. To achieve PHA granule formation in *P. aeruginosa* strains, nitrogen-dependent regulation of substrate provision was used (8). Cells were grown in MSM with sodium gluconate as a carbon source under nitrogen limitation with 0.05 % (wt/vol) NH_4Cl. All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo.).

Isolation, analysis, and manipulation of DNA

General cloning procedures were performed as described previously (22). DNA primers, deoxynucleoside triphosphate, and *Taq* and Platinum *Pfx* polymerases were purchased from Invitrogen (CA). The DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using the model ABI310 automatic

sequencer. The plasmids used in this study are listed in Table 1. The plasmids used to produce a LacZ-PhaC1 (β-galactosidase–PHA synthase) fusion were constructed as follows. A *Spe*I site-containing adaptor, encoding the linker region, was generated by hybridisation of the oligonucleotides adaptor and adaptor reverse (Table 1). The adaptor was inserted into the *Nde*I site of pBHR80. The *Spe*I site was used to insert the *lacZ* gene in frame with the respective PHA synthase gene. The *lacZ* gene coding region was amplified by PCR from genomic DNA of *E coli* S17-1 using the oligonucleotides 5'-lacZ-SpeI and 3'-lacZ-SpeI, which provided *Spe*I sites. To investigate LacZ-PHA synthase in the natural host, a broad-host-range construct was generated by subcloning the *Xba*I/*Bam*HI DNA fragment from pBHR80AlacZ into the respective sites of pBBR1JO-5 (14), resulting in plasmid pBBR1JO5-lacZphaC1. To achieve overexpression of LacZ-PhaC1 and PhaC1, the *Xba*I/*Bam*HI DNA fragments from pBHR80 and pBHR80AlacZ were subcloned into the respective sites of pET14b and transformed into strain BL21(DE3)/pLysS.

Overexpression of *phaC1* and *lacZ-phaC1*

Cells of *E. coli* BL21(DE3)/pLysS were transformed with plasmids pET14b-phaC1 and pET14b-lacZphaC1. The transformants were grown at 30°C to an optical density at 600 nm (OD_{600}) of 0.6 and then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After growth for an additional 5 h, the cells were harvested by centrifugation and stored at -80°C.

Excision of the Gm cassette

P. aeruginosa Δ*phaC1-Z-C2* contained a Gm cassette inserted into the PHA biosynthesis gene cluster. The Gm cassete was removed to avoid polar effects on other genes involved in PHA granule formation. *E. coli* S17-1 was used to transfer the Flp recombinase-encoding vector pFLP2 (7) into *P. aeruginosa* Δ*phaC1-Z-C2* strains, and after 24 h of cultivation on MSM containing 5 % (wt/vol) sucrose, gentamicin- and carbenicillin-sensitive cells were analysed by PCR for loss of the Gm resistance cassette.

Complementation of an isogenic-marker-free *P. aeruginosa* Δ*phaC1-Z-C2* mutant

For complementation of the PHA-negative mutant, plasmid pBBR1JO5-lacZphaC1 was transferred into *P. aeruginosa* Δ*phaC1-Z-C2*, and transconjugants were selected on MSM containing 150 µg/ml gentamicin (6). The cells were then grown under PHA-accumulating conditions, and the PHA content was determined by gas chromatography/mass spectrometry (GC/MS) analysis.

Determination of PHA synthase functionality

The functionality of the PHA synthase was investigated by analysing the PHA contents of the respective bacterial cells. The amount of accumulated PHA corresponds to the functionality of

the PHA synthase. The PHA contents were qualitatively and quantitatively determined by GC/MS after conversion of the PHA into 3-hydroxymethylester by acid-catalysed methanolysis.

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strains, plasmids or oligonucleotides	Description	Source or reference
Strains		
P. aeruginosa		
PAO1	Prototrophic wild-type strain;	(9)
Δ*phaC1-Z-C2*	*Pseudomonas aeruginosa* PAO1 Δ*phaC1-phaZ-phaC2*::GM; Gm-cassette excised; PHA-negative	(15)
E. coli		
BL21 (DE3) pLysS	F⁻; *ompT*; hsdS$_B$(r$_B^-$ m$_B^-$); *gal*; *dcm* (DE3); pLysS (Camʳ)	Invitrogen™ CA, USA
S17-1	*thi*-1; *proA*, *hsd*R*17* (r$_k^-$, m$_k^+$), *recA1*, *tra*-gene of plasmid RP4 integrated in chromosome	(24)
XL1-Blue	*E. coli* cloning strain	Stratagene
Plasmids		
pBBR1JO-5	Gmʳ; Broad-host-range vector; P$_{(lac)}$	(14)
pBHR80	*Nde*I-*Bam*HI fragment comprising gene *phaC1* (with translationally fused 6xHis-tag)	(16)
pET14b	Apʳ; T7-promotor	Novagen, WI, USA
pBBR1JO5- lacZphaC1	*Xba*I-*Bam*HI fragment comprising gene *lacZ-phaC1* inserted into vector pBBR1JO-5	This study
pBHR80A	pBHR80 containing *Spe*I adaptor in *Nde*I site	This study
pBHR80AlacZ	pBHR80A containing *Spe*I-inserted *lacZ* gene derived from genomic DNA of *E. coli* S17-1 by PCR	This study
pET14b-lacZphaC1	pET14b containing *Xba*I-*Bam*HI fragment comprising gene *lacZ-phaC1*	This study
pET14b-phaC1	pET14b containing *Xba*I-*Bam*HI fragment comprising gene *lacZ-phaC1*	This study
Oligonucleotides[a]		
5'-lacZ-*Spe*I	5'-GG<u>ACTAGT</u>ATGACCATGATTACGGATTCACTGG-3'	This study
3'-lacZ-*Spe*I	5'-CA<u>ACTAGT</u>TTTTTTGACACCAGACCAACTGGTAATTG-3'	This study
adaptor	5'-P-<u>TATGG</u>CTCTGC<u>ACTAGT</u>CACTGC-<u>CA</u>-3'	This study
adaptor reverse	5'-P-<u>TATGG</u>CAGTG<u>ACTAGT</u>GCAGAG-<u>CA</u>-3'	This study

ᵃ, Restriction enzyme recognition sites were underlined.

Isolation of PHA granules

Cells were harvested by centrifugation for 15 min at 5,000 x g and 4 °C. The sediment was washed and suspended in 3 volumes of 50 mM phosphate buffer (pH 7.5). The cells were passed through a French press three times at 8,000 lb/in^2, and 0.8 ml of the cell lysate was loaded onto a glycerol gradient (88 % and 44 % [vol/vol] glycerol in phosphate buffer). After ultracentrifugation for 2 h at 100,000 x g and 4 °C, granules could be isolated from a white layer above the 88 % glycerol layer. The PHA granules were washed with 10 volumes phosphate buffer (50 mM; pH 7.5) and centrifuged at 100,000 x g for 30 min at 4 °C. The sediment containing the PHA granules was suspended in phosphate buffer and stored at 4 °C.

β-Galactosidase activity assays

β-Galactosidase enzymatic assays were performed as described elsewhere (12). β-Galactosidase activity is given in Miller units (MU) (12) by using the OD_{600} of a PHA granule suspension instead of calculating the cell density. To measure the activity of β-galactosidase at isolated PHA granules, the PHA granule suspension was diluted to an OD_{600} between 0.3 and 0.4. The results are given as average values of at least three independent experiments.

Kinetics of β-galactosidase–PHA synthase fusion protein.

A PHA granule suspension with an overall protein concentration of 3.7 µg/ml was used to analyse the enzyme kinetics of LacZ-PhaC1. One unit of β-galactosidase activity corresponds to the conversion of 1 µmol orthonitrophenyl-β-D-galactopyranoside (ONPG) to orthonitrophenol per min at 20 °C.

ELISA

For enzyme-linked immunosorbent assay (ELISA), the wells of microtiter plates were coated with 200 µl of a PHA granule suspension and incubated overnight at 4 °C. After being blocked with 3 % (wt/vol) bovine serum albumin for 1 h, each well was incubated with polyclonal anti-β-galactosidase antibody conjugated to horseradish peroxidase (Abcam Inc., MA). After each step, the wells were washed several times with phosphate-buffered saline. As a substrate, 200 µl of an *o*-phenylenediamine solution (Abbott Diagnostics, IL) was added to each well, and after 30 min, the reaction was stopped by adding 0.5 volume of 3 N H_2SO_4. The amount of substrate conversion was measured at a wavelength of 405 nm using a microtiter plate reader.

SDS-PAGE

Protein samples were routinely analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described elsewhere (11). The gels were stained with Coomassie brilliant blue G250. Protein bands of interest were cut off the gel and analysed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS).

MALDI-TOF mass spectrometry

Mass spectrometric analyses of tryptic peptides were carried out on a MALDI VOYAGER DE-PRO time of flight mass spectrometer from PerSeptive BioSystems (Framingham, MA) utilising a nitrogen laser emitting at 337 nm and an accelerating voltage of 25 kV. Measurements were performed in the delayed-extraction mode using a low mass gate of 2,000. The mass spectrometer was used in the positive ion detection and linear mode. Samples of the digestion mixture were placed directly on a 100-position sample plate and allowed to air dry after the addition of an equal volume of saturated solution of 3,5-dimethoxy-4-hydroxycinnaminic acid (sinapinic acid) in 50 % acetonitrile and 0.3 % trifluoroacetic acid.

Results

Construction of plasmids mediating production of functional LacZ-PhaC1

The *lacZ* gene from *E. coli* S17-1 was inserted in frame upstream of the *phaC1* gene from *P. aeruginosa* PAO1 under *lac* promoter control, resulting in plasmid pBHR80AlacZ (see Materials and Methods) (Fig. 1). This in-frame gene fusion should enable production of LacZ fused to the N terminus of the PHA synthase separated by a linker plus six histidine residues, which should facilitate independent and functional folding of the two protein domains. The hybrid gene was subcloned into overexpression vector pET14b downstream of the strong T7 promoter in order to investigate the expression of the hybrid gene encoding the fusion protein. The hybrid gene was also subcloned into the broad-host-range vector pBBR1JO-5 in order to investigate functional production of the fusion protein, i.e., enzymatic activity of LacZ, as well as the PHA synthase function (Table 1).

Overexpression of *phaC1* and *lacZ-phaC1*

To investigate whether the entire open reading frame encoding the fusion protein could be efficiently expressed, the plasmids pET14b-phaC1 and pET14b-lacZphaC1 were transformed into *E. coli* BL21(DE3) pLysS. Proteins of whole-cell extracts were analysed by SDS-PAGE (Fig. 2). PhaC1 plus the N terminal six histidine residues has a predicted molecular mass of 63.3 kDa and appeared to be the predominant protein (Fig. 2). The identity of this protein was confirmed by peptide fingerprinting using MALDI-TOF/MS (Table 2). In crude extracts of cells harboring pET14b-lacZphaC1, an additional major protein with an apparent molecular mass of 180 kDa could be detected. The predicted molecular mass of LacZ-PhaC1 is 180.9 kDa. This protein was subjected to MALDI-TOF/MS analysis, which confirmed that it represented the fusion protein LacZ-PhaC1 (Table 2). Thus, both open reading frames could be efficiently and completely

expressed in *E. coli*. LacZ-PhaC1 could also be detected in whole-cell lysates of *E. coli* XL1-Blue harboring plasmid pBHR80ALacZ when produced under *lac* promoter control (Fig. 2).
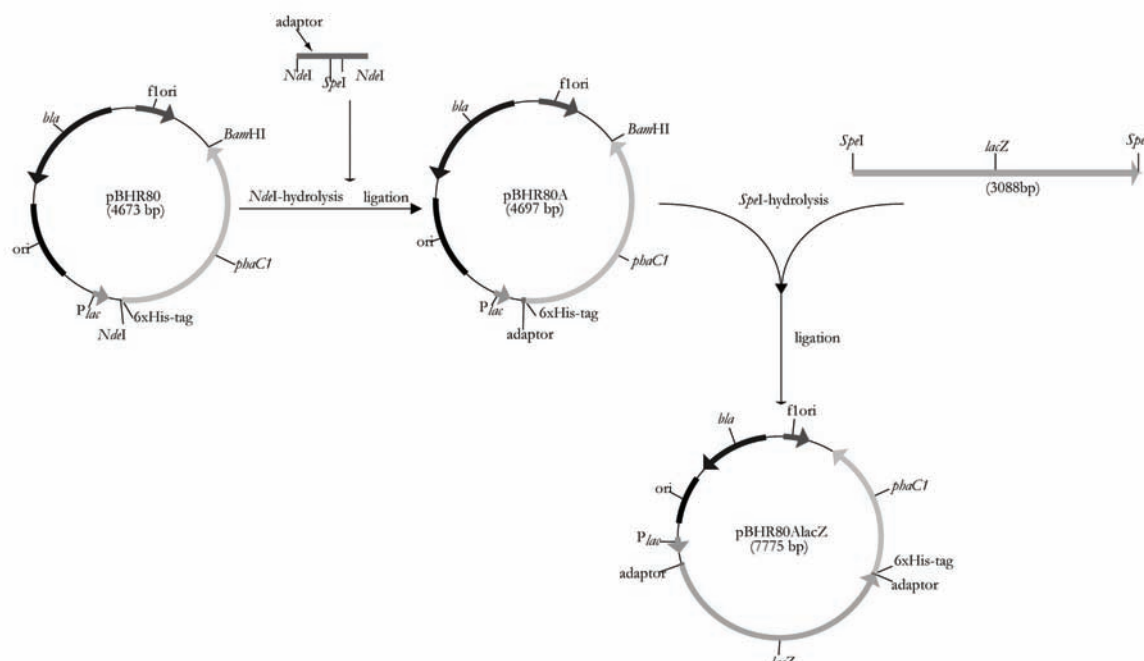

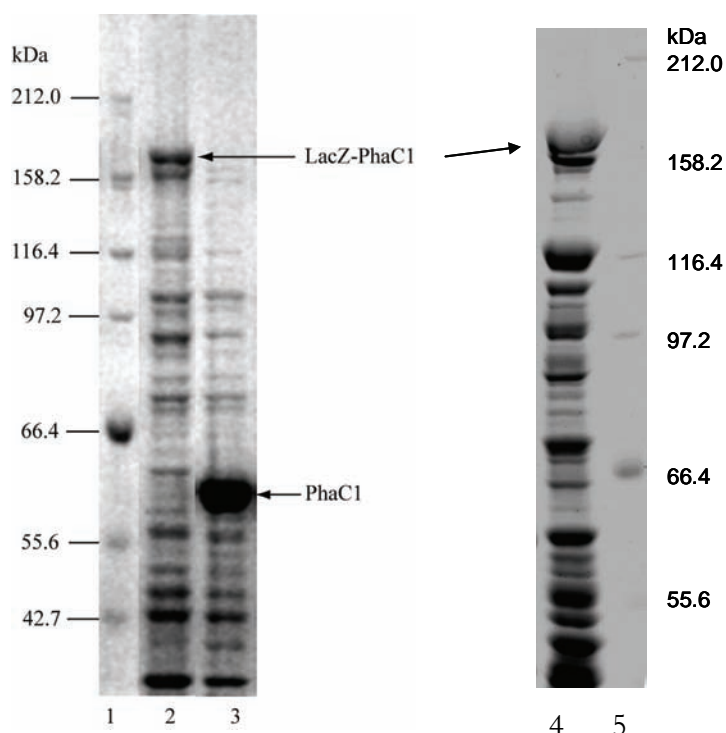**Figure 1.** Construction of plasmid pBHR80AlacZ.

Figure 2. SDS-PAGE analysis of overproduced PhaC1 and LacZ-PhaC1 in *E. coli* BL21(DE3). Lane 1, molecular mass standard (New England Biolabs, United Kingdom); lane 2, whole-cell lysate of *E. coli* BL21(DE3) harboring pET14b-LacZ-PhaC1; lane 3, whole-cell lysate of *E. coli* BL21(DE3) harboring pET14b-phaC1; lane 4, whole-cell lysate of *E. coli* XL1-Blue harboring pBHR80ALacZ; lane 5, molecular mass standard (New England Biolabs, United Kingdom). The arrows indicate proteins confirmed by peptide mass fingerprinting using MALDI-TOF/MS (Table 2).

Table 2. Identified peptide fragments of proteins analysed by MALDI-TOF/MS

protein	peptide fragments
LacZ-PhaC1	L33-R43, L173-R184, V262-R288, L299-R316, I343-R358, H628-R636, L637-R651, Q684-R705, V706-R727, Q781-R792, T854-R863, V906-R915, W924-R944, Q1052-R1067, L1444-K1457, S1458-K1477
LacZ	M1-R20, D21-R32, L173-R184, V262-R288, I343-R358, D453-R479, T618-R636, L637-R651, Q684-R705, Q781-R792, E949-R967
PhaC1	Q18-K35, N68-R84, N192-R203, F234-R247, S424-K443, T550-R565

Complementation of the isogenic-marker-free *ΔphaC1-Z-C2* deletion mutant of *P. aeruginosa* PAO1

To investigate whether the PHA synthase PhaC1 from *P. aeruginosa* PAO1 N-terminally fused to the C terminus of LacZ retains its enzymatic activity, as well as its capability to form PHA granules, the broad-host-range plasmid pBBR1JO5-LacZ-PhaC1 carrying the gene coding for LacZ-PhaC1 was transferred into PHA-negative *P. aeruginosa* Δ*phaC1-Z-C2*. The functionality of the PHA synthase was determined by analysing the PHA content of dried cells using GC/MS, which indicated a PHA content of 70.5 % compared with the wild type. The PHA composition showed an increased molar fraction of 3-hydroxydecanoic acid amounting to an additional 15.5 mol% (data not shown).

Localisation and display of LacZ at the PHA granule surface

Since the current model of PHA granule formation suggests that the PHA synthase stays covalently attached to the emerging polyester granule (6, 17), LacZ is presumably exposed at the surface of the PHA granule. To localise LacZ at the PHA granule surface, PHA granules of *P. aeruginosa* Δ*phaC1-Z-C2* harboring plasmid pBBR1JO5-lacZphaC1 and PHA granules produced by the wild-type *P. aeruginosa* PAO1 were isolated and used for ELISA. Specific binding of anti-LacZ antibodies to PHA granules isolated from *P. aeruginosa* Δ*phaC1-Z-C2* harboring pBBR1JO5-lacZphaC1 was suggested by a twofold increase in absorption at a wavelength of 405 nm compared to the wild-type PHA granules. PHA granules formed via LacZ-PhaC1 and PhaC1 mediated absorbances of 0.21 ± 0.015 and 0.1 ± 0.008, respectively.

β-Galactosidase activity assays. The LacZ activity was analysed in order to determine whether LacZ remains active when fused with its C terminus to the N terminus of the PHA synthase and immobilised at the PHA granule surface. LacZ activity could be detected and showed an average of 68,000 MU.

Determination of kinetic parameters of β-galactosidase immobilised at the PHA granule surface

In order to determine enzyme kinetics, the β-galactosidase activities of isolated PHA granules were monitored for 10 min (data not shown). The ONPG concentrations ranged from 50 μM to 500 μM. The correlation of reaction velocity with the substrate concentration could be fitted to Michaelis-Menten kinetics with the aid of nonlinear regression analysis (Sigma Plot enzyme kinetics; systat software, Inc.). A K_m of 630 μM and a V_{max} of 17.6 nmol/min could be derived.

Mode of protein-PHA granule interaction.

A typical protein profile of PHA granules isolated from the wild-type *P. aeruginosa* PAO1 is shown in Fig. 3. In contrast, the protein profile of PHA granules produced by *P. aeruginosa* Δ*phaC1-Z-C2* harboring pBBR1JO5-lacZphaC1 shows an additional major protein with an apparent molecular mass of 180 kDa. MALDI-TOF/MS analysis of derived peptides of this 180 kDa protein enabled the identification of LacZ-PhaC1 (Table 2). Interestingly, an additional protein occurred at 116 kDa, which was identified as LacZ by MALDI-TOF/MS analysis (Table 2). The nonfused PhaC1 could be detected as a minor protein, which was confirmed by MALDI-TOF/MS analysis (Fig. 3). Consistent with previous studies, the PHA synthase attached to PHA granules from wild-type *P. aeruginosa* PAO1 is only present at low copy numbers and could not be detected as a distinct protein band in SDS-PAGE analysis (10) (Fig. 3). Thorough washing with phosphate buffer containing 0.1% to 1% SDS (wt/vol) did not alter the amount of LacZ attached to PHA granules (data not shown). Harsh conditions (8 M urea, 10 mM dithiothreitol [DTT]) were applied to remove all non-covalently attached proteins. Figure 4 shows an SDS-PAGE analysis of PHA granules produced by *P. aeruginosa* Δ*phaC1-Z-C2* harboring pBBR1JO5-lacZphaC1, which were subjected to solubilisation using 8 M urea and 10 mM DTT. LacZ, as well as other non-covalently attached proteins, was completely removed from the PHA granules, while about 50% of LacZ-PhaC1 was still attached to the PHA granules.

Enzyme stability

The stability of LacZ immobilised at PHA granules under various storage conditions was investigated. LacZ activity was monitored over a period of 12 weeks. The PHA granule suspension was stored at 4 °C either with added protease inhibitor cocktail (Roche Diagnostics, IN) or with protease inhibitor cocktail plus 20 % (vol/vol) glycerol. The addition of 20 % (vol/vol) glycerol resulted in the best storage stability, with 91 % of the initial activity after 4 weeks. No addition of 20 % (vol/vol) glycerol resulted after 4 weeks in a reduction of LacZ activity to 84% of the initially determined activity (Table 3). Interestingly, after 12 weeks, 87 % of the initial activity was still detectable in the PHA granule suspension containing glycerol, while

the LacZ activity of the PHA granule suspension without glycerol already showed a reduced activity of 75 % of the initial LacZ activity (Table 3).
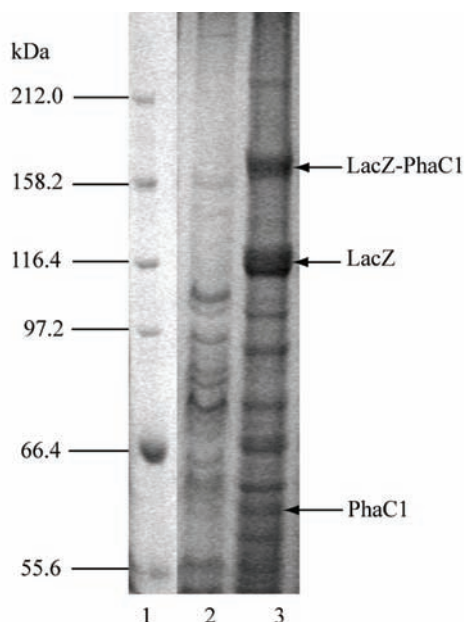

Figure 3. SDS-PAGE analysis of PHA granules. Lane 1, molecular mass standard (New England Biolabs, United Kingdom); lane 2, PHA granules from wild-type *P. aeruginosa* PAO1; lane 3, PHA granules from *P. aeruginosa* Δ*phaC1-Z-C2*(pBBR1JO5-lacZphaC1). The arrows indicate proteins confirmed by peptide mass fingerprinting using MALDI-TOF MS (Table 2).
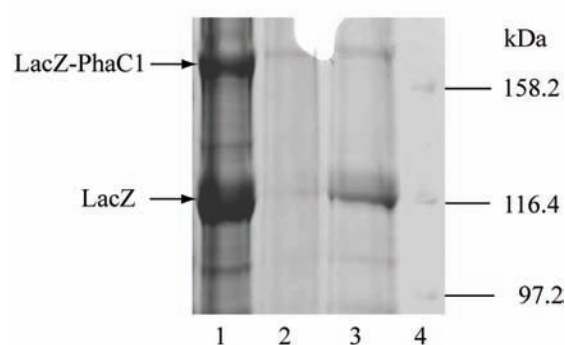

Figure 4. SDS-PAGE analysis of PHA granules before and after treatment with 8 M urea. Lane 1, PHA granules from *P. aeruginosa* Δ*phaC1-Z-C2*(pBBR1JO5-lacZphaC1) before treatment with 8 M urea; lane 2, PHA granules (insoluble fraction) after treatment with urea; lane 3, proteins (soluble fraction) released from PHA granules after treatment with 8 M urea; lane 4, molecular mass standard (New England Biolabs, United Kingdom). The arrows indicate proteins confirmed by peptide mass fingerprinting using MALDI-TOF/MS (Table 2).

Table 3. Determination of enzyme stability of β-galactosidase at PHA granule surface

Storage time (weeks)	β-galactosidase activity (protease inhibitor)[a] [MU]	β-galactosidase activity (protease inhibitor/20% (v/v) glycerol)[a] [MU]
0	77400	77400
3	65000	68900
4.5	60900	70300
12	58194	67287

[a], Protease inhibitor and 20% (v/v) glycerol were added to the PHA granules suspension at time point 0. PHA granules were stored at 4°C. β-galactosidase activity was measured on isolated PHA granules.

Discussion

This study elaborated the potential use of PHA synthase to mediate stable immobilisation of an enzyme at the PHA granule surface via protein engineering. Only the PHA synthase, which can be functionally produced in a wide range of organisms, is required for the formation of PHA granules. Thus, the PHA synthase might serve as a valuable molecular tool to covalently attach enzymes and/or other protein functions to PHA granules (20). Previously, it was demonstrated that GFP could be fused to the N termini of PHA synthases, enabling the production of GFP-labeled PHA granules (14).

LacZ was chosen in this study as an example of an enzyme to be immobilised. The LacZ was fused via a linker region to the N terminus of the PHA synthase PhaC1 from *P. aeruginosa* PAO1, which has been studied in detail (1). Production of the complete fusion protein was confirmed using T7 promoter-based overproduction in *E. coli* and the proper fusion protein, LacZ-PhaC1, was detected by the apparent molecular mass of 180 kDa and MALDI-TOF/MS analysis of the respective tryptic peptides (Fig. 1 and Table 2). The lower expression level of the fusion protein versus PhaC1 could be due to the threefold-larger molecular mass of the fusion protein and/or reduced stability of the fusion protein in the absence of PHA granule formation.

LacZ-PhaC1, produced from plasmid pBBR1JO5-lacZphaC1 under *lac* promoter control, restored PHA biosynthesis in the PHA-negative *P. aeruginosa* Δ*phaC1-Z-C2* (15). GC/MS analysis indicated that the PHA content was reduced by only about 30 % compared with the wild-type PHA synthase, indicating a PHA synthase function of the fusion protein.

PHA granules, whose formation was mediated by LacZ-PhaC1 in the PHA-negative *P. aeruginosa* Δ*phaC1-Z-C2*, were isolated, and LacZ could be localised at the PHA granule surface by ELISA using anti-LacZ antibodies. These PHA granules showed LacZ activity, suggesting that LacZ had been functionally attached to the PHA granule surface. The enzyme kinetics of immobilised LacZ was analysed by obtaining the reaction velocities at various substrate (ONPG) concentrations, which indicated a Michaelis-Menten reaction kinetics. The K_m of 630 µM

suggested a high binding affinity, compared with LacZ covalently attached to gold-coated devices (2).

The protein profile of PHA granules mediated by LacZ-PhaC1 showed, in comparison with wild-type PHA granules, three additional prominent proteins, which could be identified as LacZ-PhaC1, LacZ, and PhaC1. Interestingly, the copy numbers of LacZ-PhaC1 and PhaC1 were unusually high, particularly considering that PhaC1 is not detectable by SDS-PAGE analysis of wild-type PHA granule proteins (Fig. 3). Normi *et al.* (13) described a slight increase in PHA synthase production after introducing a G4D N-terminal mutation in the *Cupriavidus necator* PHA synthase, which suggested a role of the N terminus in controlling the copy number of the PHA synthase. This study showed that the N-terminal fusion might have stabilised the protein or that the insertion of the *lacZ* gene at the 5' end of the *phaC1* gene might have caused stabilisation of the respective mRNA (25). The additional occurrence of LacZ and PhaC1 at the PHA granule surface suggested that the fusion protein might be susceptible to proteolytic digestion in *P. aeruginosa* (Fig. 3), while the fusion protein is stable in *E. coli* (Fig. 2). The large amount of LacZ attached to the PHA granules could be due to a stable formation of a functional LacZ heterotetramer with one subunit contributed by LacZ-PhaC1 and three subunits represented by LacZ. This ratio has been suggested by SDS-PAGE analysis (Fig. 3).

To investigate how LacZ interacts with LacZ-PhaC1 at the PHA granule surface, the PHA granules were subjected to solubilisation with 8 M urea in the presence of 10 mM DTT. The resulting solubilised proteins and PHA granules were analysed by SDS-PAGE (Fig. 4), which indicated an almost complete removal of LacZ, while about 50 % of LacZ-PhaC1 remained attached to the PHA granules. Thus, LacZ might interact with LacZ-PhaC1 at the PHA granule surface via protein-protein interaction. These data also suggest that one subunit of LacZ-PhaC1, which presumably forms a homodimer (18, 26), remains covalently attached, whereas the other subunit is not covalently attached and can be removed. PHA granules harboring LacZ-PhaC1 were stored for a few months in the presence of protease inhibitor plus or minus 20 % (vol/vol) glycerol to investigate the stability of the immobilised LacZ. LacZ activity only dropped to 87 % of the initial activity in the presence of glycerol, which suggested that LacZ immobilised at the PHA granule surface remains stable for at least a few months. The purified native LacZ has been described by various commercial producers as stable at 4 °C for up to 6 months (http://www.worthington-biochem.com/BG/default.html; http://www.merckbiosciences.com/docs/PDS/345788-000.pdf).

This study demonstrated that protein engineering of PHA synthase provides a platform technology for efficient covalent enzyme/protein immobilisation. The PHA synthase contains all the inherent properties required for PHA synthesis, as well as PHA granule formation, and can

be produced in a variety of organisms (17). These unique properties and covalent binding to the PHA granule make these enzymes ideal tools for functionalisation of PHA granules (20) (Fig. 5).
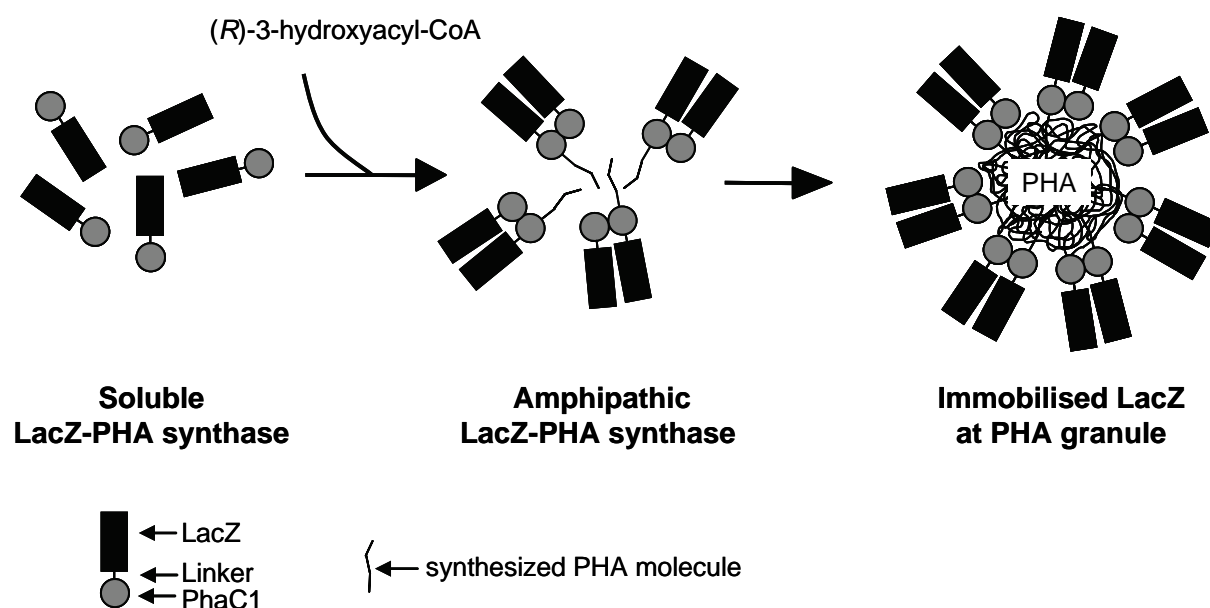

Figure 5. Model of *in vivo* enzyme immobilisation using engineered PHA synthase. CoA, coenzyme A.

Acknowledgements

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Chapter IV

Recombinant *Escherichia coli* strain produces a ZZ domain displaying biopolyester granules suitable for immunoglobulin G purification

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Recombinant *Escherichia coli* strain produces a ZZ domain displaying biopolyester granules suitable for immunoglobulin G purification

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Abstract

The immunoglobulin G (IgG) binding ZZ domain of protein A from *Staphylococcus aureus* was fused to the N terminus of the polyhydroxyalkanoate (PHA) synthase from *Cupriavidus necator*. The fusion protein was confirmed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry and mediated formation of ZZ domain-displaying PHA granules in recombinant *Escherichia coli*. The IgG binding capacity of isolated granules was assessed using enzyme-linked immunosorbent assay and could be enhanced by the overproduction of the ZZ-PHA synthase. ZZ-PHA granules enabled efficient purification of IgG from human serum.

Introduction

The polyhydroxyalkanoate (PHA) synthase is the key enzyme of PHA biosynthesis and PHA granule formation. PHA granules (biopolyester particles) are formed inside bacterial cells, based on the activity and biochemical properties of the PHA synthases (8, 9). The PHA granule core is composed of high-molecular-weight PHA, which is biodegradable and biocompatible. The surface of the PHA granule is composed of a phospholipid membrane with embedded or attached proteins. Evidence was provided that the PHA synthase is covalently attached to the PHA granule core (5). Recently, the enzyme β-galactosidase and green fluorescent protein (GFP) were immobilised to the PHA granules by use of PHA synthase engineering (5, 6). Phasin proteins have been also subjected to protein engineering in order to enable purification of proteins fused to these proteins, which hydrophobically attach to the preformed PHA granules (1, 2). In this study, we targeted the PHA synthase with respect to display of the immunoglobulin G (IgG) binding domain ZZ of protein A at the PHA granule surface. Only the PHA synthase provides covalent attachment to the PHA granule surface and hence provides a robust particle-based purification system (8, 10).

Results and Methods

Construction of plasmids mediating ZZ-PHA granule production in *Escherichia coli*.
The plasmid pCWE, encoding the PHA synthase from *Cupriavidus necator*, and plasmid pEZZ18

(GE Healthcare) (providing the ZZ domain- and signal peptide-encoding sequences; GenBank accession no. M74186) were used to generate plasmids encoding the respective PHA synthase fusion proteins (Table 1) (4). The DNA regions encoding the ZZ domain with or without the signal peptide were amplified from vector pEZZ18 by using oligonucleotides introducing *Nde*I sites at each end of the PCR product (Table 1). Each PCR product was then inserted into the *Nde*I site of plasmid pCWE, resulting in plasmids pCWE-ZZ(+)phaC and pCWE-ZZ(-)phaC, respectively (Table 1). Each hybrid gene was subcloned into *Xba*I/*Bam*HI sites of plasmid pBHR69 upstream of the genes *phbA* and *phbB*, which mediate provision of the activated precursor for polyhydroxybutyrate synthesis (3). This resulted in plasmids pBHR69-ZZ(+)phaC and pBHR69-ZZ(-)phaC (Table 1). To investigate whether the entire open reading frame encoding the respective fusion protein could be overproduced at the PHA granule surface, the respective hybrid genes were also subcloned into overexpression vector pET14b downstream of the strong T7 promoter (Table 1). The resulting plasmids pET14b-ZZ(+)phaC and pET14b-ZZ(-)phaC, encoding ZZ-PhaC with or without the signal peptide, respectively, were transformed into *E. coli* BL21(DE3) harboring pMCS69 (*phbA, phbB*). The PHA synthase function of the fusion proteins was assessed by analysing PHA accumulation of respective cells by gas chromatography-mass spectrometry analysis as previously described (6). No major differences in PHA accumulation could be detected compared to cells harboring pCWE or pHAS and pMCS69 as a control (data not shown). These data suggested that the ZZ-PHA synthase fusion protein mediates PHA biosynthesis and PHA granule formation. The presence or absence of the signal peptide did not affect PHA synthase function.

Production of the ZZ-PhaC fusion proteins.

The ZZ domain of protein A was chosen in this study as an example of a binding domain to be covalently attached to the PHA granule surface. PHA granules, whose formation was mediated by the respective fusion proteins, were isolated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis as previously described (5). ZZ-PhaC plus the N-terminal signal peptide has a theoretical molecular weight of 83,981 and a protein with an apparent molecular mass of 84 kDa could be detected as the predominant protein (data not shown). Without the signal peptide the fusion protein has a theoretical molecular weight of 79,338, and a protein with an apparent molecular mass of 80 kDa appeared as the predominant protein (data not shown). The identities of these proteins were confirmed by peptide fingerprinting using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Thus, both open reading frames could be efficiently and completely expressed in *E. coli*. The plasmids pET14b-ZZ(+)phaC and pET14b-ZZ(-)phaC, encoding ZZ-PhaC with and without the signal peptide, respectively, mediated overproduction of ZZ-PhaC at the PHA granule

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strains, plasmids or oligonucleotides	Description	Source or reference
Strains		
E. coli		
BL21 (DE3)	F⁻; *ompT*; hsdS$_B$(r$_B^-$ m$_B^-$); *gal*; *dcm* (DE3)	Invitrogen™ CA, USA
XL1-Blue	*E. coli* cloning strain	Stratagene
Plasmids		
pEZZ18	Ap^r; Lac promoter, encoding the IgG binding domain ZZ from protein A	GE Healthcare
pHAS	pET-14b containing *Nde*I/*Bam*HI inserted *phaC$_{We}$* gene from *C. necator*	(11)
pET14b	Ap^r; T7-promotor	Novagen, WI, USA
pET14b-ZZ(-)phaC	pET14b containing *Xba*I-*Bam*HI fragment comprising gene *ZZ-phaC* without the signal sequence encoding region	This study
pET14b-ZZ(+)phaC	pET14b containing *Xba*I-*Bam*HI fragment comprising gene *ZZ-phaC*	This study
pCWE	pBluesrcriptSK⁻ derivative containing the PHA synthase gene from *C. necator*	(6)
pCWE-ZZ(-)phaC	pCWE derivative containing the ZZ domain encoding *Nde*I fragment lacking the signal sequence encoding region	This study
pCWE-ZZ(+)phaC	pCWE derivative containing the ZZ domain encoding *Nde*I fragment	This study
pBHR69-ZZ(-)phaC	pBHR69 derivative containing the hybrid gene from hybrid PHA synthase gene from pCWE-ZZ(-)phaC upstream of genes *phaA* and *phaB* of *C. necator* colinear to *lac*-promoter	This study
pBHR69-ZZ(+)phaC	pBHR69 derivative containing the hybrid gene from hybrid PHA synthase gene from pCWE-ZZ(-)phaC upstream of genes *phaA* and *phaB* of *C. necator* colinear to *lac*-promoter	This study
pBHR69	pBluescript derivative containing genes *phaA* and *phaB* of *C. necator* colinear to *lac*-promoter	(7)
pMCS69	pBBR1MCS derivative containing genes *phaA* and *phaB* of *C. necator* colinear to *lac*-promoter	(3)
5'-ZZ (+)-*Nde*I	5'-GCGCG<u>CATATG</u>ACTTTACAAATA CATACAGGGGGTATTAATTTG -3'	This study
3'-ZZ-*Nde*I	5'-GTAAT<u>CATATG</u>GGGGTACCGAGC TCGAATTCGCGTCTAC-3'	This study

^{a}, Restriction enzyme recognition sites were underlined.

surface. Overall, these findings were consistent with previous studies, which demonstrated that GFP and LacZ could be fused to the N terminus of PHA synthases, enabling production of GFP-labeled PHA granules as well as PHA granules with immobilised LacZ (5, 6).

Display of the ZZ domain at the PHA granule surface and binding capacity of ZZ-PHA granules. Since the current model of PHA granule formation suggests that the PHA synthase stays covalently attached to the emerging biopolyester granule (5, 8–10), the ZZ domain is presumably exposed at the surface of the PHA granule. To localise the ZZ domain at the PHA granule surface, PHA granules of *E. coli* harboring plasmid pCWE-ZZ(+)phaC, pCWE-ZZ(-)phaC, pET14b-ZZ(+)phaC, or pET14b-ZZ(-)phaC, as well as PHA granules produced by wild-type PHA synthase (pCWE or pHAS), were isolated and used for enzyme-linked immunosorbent assay (ELISA) as previously described (5). Specific binding of IgG to PHA granules isolated from *E. coli* harboring any plasmid encoding a ZZ-PHA synthase fusion protein was suggested by at least a twofold increase in absorption at a wavelength of 490 nm compared to the wild-type PHA granules (Fig. 1). These data suggested a functional display of the ZZ domain at the PHA granule surface. The presence or absence of the signal peptide did not affect IgG binding capacity. However, PHA granules whose formation was mediated by overproduction of ZZ-PhaC showed significantly increased binding capacity (Fig. 1).
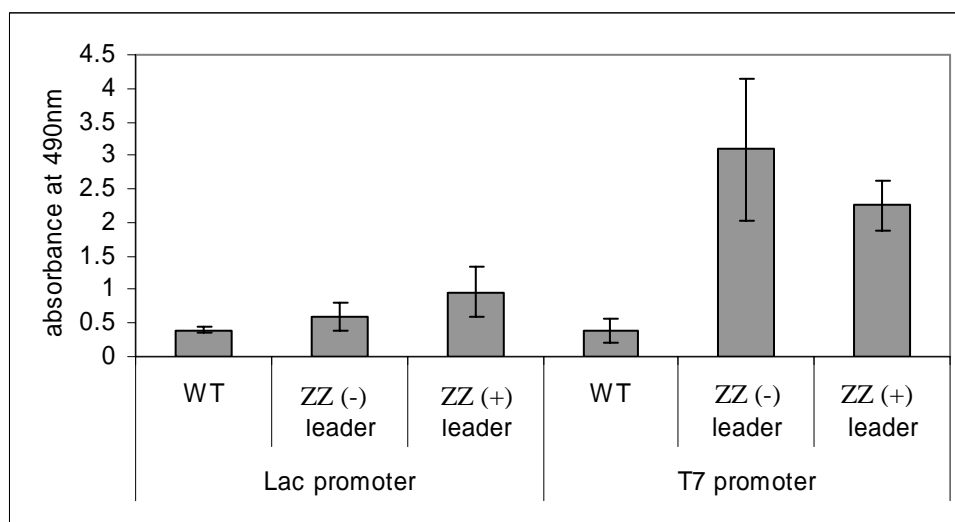

Figure 1. ELISA using various PHA granules and anti-IgG antibodies for the detection of IgG bound to PHA granules. PHA granules were isolated from recombinant *E. coli* harboring various plasmids. Plasmids contained either the *lac* promoter or the T7 promoter for gene expression. The following versions of the PHA synthase mediated production of PHA granules: WT, wild-type PHA synthase; ZZ(-), ZZ-PHA synthase without signal peptide; ZZ(+), ZZ-PHA synthase plus signal peptide. Goat polyclonal anti-human IgG–horseradish peroxidase conjugates were used for detection of bound human IgG. Equal amounts of PHA granule protein (0.37 µg), corresponding to 2.6 µg polyhydroxybutyrate, were added to each well. Measurements were conducted in quadruplicate, and the mean value and the standard deviation are indicated.

Purification of IgG from human serum by using ZZ-PHA granules and stability of ZZ-PHA granules.

PHA granules displaying the IgG binding domain ZZ from protein A derived from pET14b-ZZ(-)phaC were used for IgG purification from human serum. For comparative analysis, protein A-Sepharose beads with immobilised, recombinant protein A were also used to purify IgG. IgG purification was conducted according to protein A-Sepharose 4B bead purification protocols (Sigma). SDS-PAGE analysis of eluted proteins showed that the immunoglobulins (a protein representing the heavy chains, with an apparent molecular mass of 50 kDa, and a protein representing the light chains, with an apparent molecular weight of 25 kDa) were purified from human serum by using the ZZ-PHA granules displaying the ZZ domain as part of the PHA synthase on the surfaces of the granules. The immunoglobulins eluted from PHA granules at pH 2.7 and showed a high degree of purity comparable to that of the commercially available protein A sepharose beads (Fig. 2). PHA granules formed by wild-type PHA synthase did not show elution of proteins, suggesting that unspecific binding of serum proteins does not interfere with IgG purification and that the ZZ domain mediates IgG purification (Fig. 2). ZZ-PHA granules were subjected to repeated purification cycles, demonstrating consistent purification performance and strong stability (data not shown). Temperature stability was tested by subjecting ZZ-PHA granules to increasing temperatures and then assessing the IgG binding capacity by ELISA. At 60 °C, the binding capacity was dropping to 60 %, suggesting that the ZZ domain was partially unfolding (data not shown). Control PHA granules containing only wild-type PHA synthase showed only low levels of unspecific binding which were temperature independent.
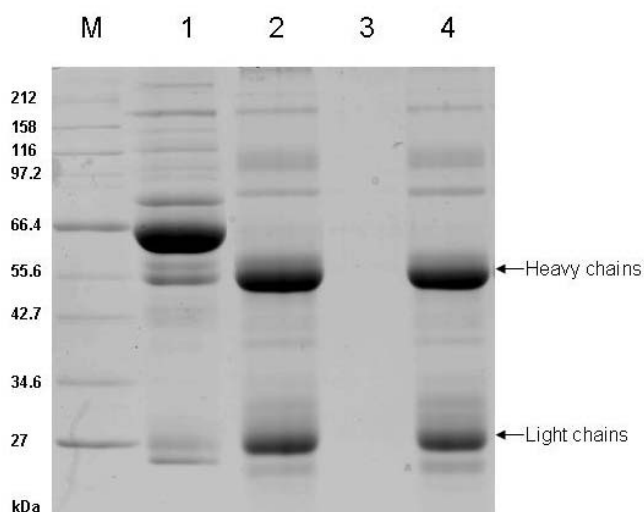

Figure 2. SDS-PAGE analysis of proteins bound *in vitro* to either ZZ-PHA granules or protein A-sepharose and released after elution. Lanes: M, molecular weight standard; 1, human serum; 2, proteins eluted from protein A-Sepharose beads; 3, proteins eluted from wild-type PHA granules; 4, proteins eluted from ZZ-PHA granules displaying the ZZ domain without signal sequence. The heavy and light chains of IgG are indicated.

Discussion

To our surprise, the engineered ZZ-PHA granules performed equally to commercial protein A-sepharose beads with respect to IgG purification (Fig. 2). This result in combination with the strong stability of the ZZ-PHA granules outside the bacterial cell opens up a new and interesting field of biotechnological applications for these biopolyester particles.

This study demonstrated that protein engineering of the PHA synthase provides a platform technology for efficient covalent enzyme/protein immobilisation (5). Commercial protein A beads require the *in vitro* production of polymer beads and subsequently the chemical cross-linking of purified protein A. PHA granule-based beads with covalently attached protein function are produced in one step by recombinant bacteria, suggesting a commercially viable biotechnological production process (5). The PHA synthase contains all the inherent properties required for PHA synthesis as well as PHA granule formation and can be produced in a variety of organisms (9). These unique properties and covalent binding to the PHA granule make these enzymes an ideal tool for functionalisation of PHA granules (10).

Acknowledgement

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Protein engineering of streptavidin for *in vivo* assembly of streptavidin beads

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Protein engineering of streptavidin for *in vivo* assembly of streptavidin beads

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Abstract

Escherichia coli was engineered to intracellularly manufacture streptavidin beads. Variants of streptavidin (monomeric, core and mature full length streptavidin) were C-terminally fused to PhaC, the polyester granule forming enzyme of *Cupriavidus necator*. All streptavidin fusion proteins mediated formation of the respective granules in *E. coli* and were overproduced at the granule surface. The monomeric streptavidin showed biotin binding (0.7 ng biotin/μg bead protein) only when fused as single-chain dimer. Core streptavidin and the corresponding single-chain dimer mediated a biotin binding of about 3.9 and 1.5 ng biotin/μg bead protein, respectively. However, biotin binding of about 61 ng biotin/μg bead protein with an equilibrium dissociation constant (K_D) of about 4×10^{-8} M was obtained when mature full length streptavidin was used. Beads displaying mature full length streptavidin were characterised in detail using ELISA, competitive ELISA and FACS. Immobilisation of biotinylated enzymes or antibodies to the beads as well as the purification of biotinylated DNA was used to demonstrate the applicability of these novel streptavidin beads. This study proposes a novel method for the cheap and efficient one-step production of versatile streptavidin beads by using engineered *Escherichia coli* as cell factory.

Keywords: full length streptavidin; core streptavidin; biotin; streptavidin beads; polymer inclusions

Introduction

Streptavidin is a tretrameric protein which binds the small molecule biotin with a remarkably high affinity ($K_D \sim 10^{-15}$M) representing one of nature's strongest non-covalent bindings, which makes it an interesting tool for diverse biotechnological applications where tight binding of biotinylated molecules is required (Hunt, 2005; Scholle *et al.*, 2004). Since biotinylation of biological molecules is easily achievable, the streptavidin-biotin system has been widely established as a tool for bio-molecule labelling, purification and immobilisation. Of particular interest are streptavidin displaying beads which provide an enhanced surface for the interaction with bio-molecules required in a large number of applications. Streptavidin beads are well established in diagnostic laboratories and are often used displaying immobilised biotinylated antibodies to detect specific

antigens. Currently, the production of streptavidin beads requires the production and purification of streptavidin. Then polymeric beads have to be generated to which the purified streptavidin is chemically cross-linked.

Recently, it was demonstrated that various proteins can be overproduced at the surface of intracellular polymer inclusions by C-terminally fusing the protein of interest to the polyester inclusions forming enzyme PhaC (Brockelbank *et al.*, 2006; Peters & Rehm, 2006). PhaC is the key enzyme in reserve polymer biosynthesis and catalyses the polymerisation of (R)-3-hydroxyacyl-CoA to the respective polyester. The nascent polyester remains attached to PhaC and the soluble enzyme converts into an insoluble molecule resulting in the assembly of polymer granules with PhaC covalently attached to the surface (Rehm, 2003; Rehm, 2006). These polyester granules possess a diameter of 50 –500 nm and have been recently considered as bio-(nano)beads (Rehm, 2007). Protein engineering suggested that proteins fused to or inserted into the N terminus of PhaC do not interfere with polymer inclusion formation and the fusion partner is displayed at the polymer inclusion surface (Peters & Rehm, 2005; Peters *et al.*, 2007). These inclusions have been isolated and successfully maintained as beads outside the cell while displaying the respective protein function at high density (Brockelbank *et al.*, 2006; Peters & Rehm, 2006). The overproduction of functional proteins as fusion partner of PhaC at the surface of these polymer inclusion has been considered as an *in vivo* matrix assisted protein folding system in which folding intermediates are spatially separated avoiding formation of inactive protein aggregates (Baeckstroem *et al.*, 2007).

In this study we propose not only the successful, recombinant intracellular overproduction of engineered streptavidins, but also the simultaneous immobilisation of streptavidin to polymer inclusions, resulting in "bio-streptavidin-beads". Different variants of engineered streptavidin, monomeric streptavidin (Wu & Wong, 2005; Wu & Wong, 2006), a minimized core streptavidin (Pahler *et al.*, 1987; Sano *et al.*, 1995) and mature, full length streptavidin (Sorensen *et al.*, 2003) were each C-terminally tagged with PhaC. This enabled the intracellular production of streptavidin displaying beads in one step. Isolated beads were stable and applicable for ELISA, DNA purification, enzyme immobilisation and flow cytometry.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *E. coli* BL21 [DE3] strains were grown in LB medium at 25°C, supplemented with 1 % glucose (w/v). Antibiotics were used at the following concentrations: ampicillin, 75 µg/ml and 30 µg/ml

chloramphenicol. Cells were grown for 4 h at 25 °C before polymer bead formation was induced by adding 1 mM IPTG. Growth was continued for 44 h.

In vivo polymer synthesis activity of various streptavidin fusion proteins

In vivo polymer synthesis activity was obtained by analysing the polyhydroxyalkanoate content of the respective bacterial cells. The amount of accumulated polymer corresponds to the relative *in vivo* synthesis activity. The polymer contents were qualitatively and quantitatively determined by gas chromatography/mass spectrometry (GC/MS) (Brandl *et al.*, 1988).

Isolation of polymer beads

Beads were isolated after mechanical cell disruption using a glycerol gradient ultracentrifugation step as previously described (Peters & Rehm, 2006).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted as previously described (Peters & Rehm, 2006). Blocking was achieved by incubation with 3 % (w/v) BSA. HRP-biotin (Zymed, San Francisco, CA, USA) or biotinylated antibody were used to study biotin binding of SA-beads. Bound biotinylated antibody was detected using a secondary HRP-labeled antibody. Bound HRP-biotin or secondary HRP-labeled antibody were quantified using O-phenylenediamine solution (OPD; Abbott Diagnostics, IL, USA) as HRP substrate according to the manufacturer's protocol. A competitive ELISA with increasing biotin concentrations at constant HRP-biotin concentration as well as a standard curve with purified streptavidin (Zymed, San Francisco, CA,USA) were used to calculate the amount of biotin bound to the respective streptavidin displaying beads. All antibodies were purchased from Abcam (Cambridge, MA, USA).

Protein analysis

Protein samples were routinely analysed by SDS-PAGE as described elsewhere (Laemmli, 1970). Protein bands of interest were cut off the gel and were identified by tryptic peptide fingerprinting using matrix assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF/MS).

Isolation, analysis and manipulation of DNA

General cloning procedures were performed as described (Sambrook *et al.*, 1989). DNA sequences of new plasmid constructs were confirmed by DNA sequencing. Plasmids used and constructed in this study are listed in Table 1.

DNA purification with SA-PhaC beads

A PCR with biotinylated primer 5'-phaCBiotin and primer 3'-phaC-NcoI resulted in a 5'-biotinylated PCR-product of 229 bp length using vector pCWE$_{Spe}$ as template (Table 1). One ml of the bead suspension (OD$_{600}$=7.5, PBS) was centrifuged and the sediment was suspended in PBS containing 3 % BSA (w/v) and incubated for 3 h at room temperature. Beads were

sedimented and resuspended in 900 µl PBS containing the biotinylated PCR-product and 1.5 % BSA (w/v) and mixed for 15 sec. Beads were washed in TBS (50mM Tris-HCl, 150mM NaCl, pH 7.4) then twice in 50mM Tris-HCl, 500mM NaCl, pH 7.4 and twice in TBS. Beads were suspended in 800 µl TBS and 160 µl of a 40 mg/ml (w/v) Protease K stock solution (TBS, 0.1 % (w/v) SDS) were added and incubated at 60 °C for 20 min. Three quarter volumes of isopropanol were added to either the whole bead suspension or to only the supernatant after sedimentation of beads. After 30 min of centrifugation, the pellet was washed in 70 % (v/v) ethanol, dried and suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Half a volume of protein denaturing buffer was added and samples were heated for 15 min at 95 °C, and then subjected to agarose gel-electrophoresis.

Fluorescence activated cell sorting (FACS)

To qualitatively and quantitatively assess the biotin binding of the beads, beads were subjected to FACS analysis as previously described (Baeckstroem *et al.*, 2007). At least 100,000 events were collected and analysed.

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study.

Strains, plasmids or oligonucleotides	Description	Source or reference
Strains		
E. coli BL21 (DE3)	F⁻; *ompT*; hsdS$_B$(r$_B^-$ m$_B^-$); *gal*; *dcm* (DE3)	Invitrogen™ CA, USA
Plasmids		
pHAS	plasmid encoding 6xHis-PhaC	(Yuan *et al.*, 2001)
pET14b	Ap^r; T7-promotor	Novagen, WI, USA
pETC	Plasmid encoding PhaCwt under T7 promoter control	This study
pET14b-SAphaC	plasmid coding for SA-PhaC fusion under T7 promoter control	This study
pET14b-cSAphaC	plasmid coding for cSA-PhaC fusion under control of T7 promoter	This study
pET14b-dcSAphaC	plasmid coding for dcSA-PhaC fusion under control of T7 promoter	This study
pET14b-mSAphaC	plasmid coding for mSA-PhaC fusion under control of T7 promoter	This study
pET14b-dmSA-phaCC	plasmid coding for dmSA-PhaC fusion under control of T7 promoter	This study
pCWE$_{Spe}$	vector for 5'-end fusions to PhaC using the provided *Spe*I site	(Peters *et al.*, 2007)
pCWE$_{Spe}$SA	plasmid coding for SA-PhaC fusion under lac promoter control	This study
pET-15b-I-SA	Plasmid encoding streptavidin fused to solubility factor	(Sorensen *et al.*, 2003)
Oligonucleotides		
5'-Strep-SpeI	5'-GCACTAGTATGACCACGGTCTCGATT-3'	This study
3'-Strep-SpeI	5'-TAACTAGTCTGCTGAACGGCGTC-3'	This study
5'-corestrep-SpeI	5'-TGCAAACTAGTATGGCCGGCATCACCGGCACCTGGTACAAC-3'	This study
3'-corestrep-SpeI	5'-GGTCAACTAGTCGGCTTCACCTTGGTGAAGGT-3'	This study
5'-phaCBiotin	5'-GGAAGGCAAGTCCCAACCATTCAAG-3'	This study
3'-phaC NcoI	5'-CTCGGCCATGGCCTGCCACAGCGCTGAGAAG-3'	This study

<div align="center">

Results
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Construction of plasmids for production of streptavidin fusion proteins

For the construction of plasmid pCWE$_{Spe}$SA which encodes the mature full length wildtype streptavidin fused to the polymer inclusion forming protein PhaC of *C. necator*, the gene

streptavidin was amplified from plasmid pET-15b-I-SA using 5'-strep-SpeI and 3'-strep-SpeI as primers (Table 1) and sub-cloned into the *Spe*I site of vector pCWE$_{Spe}$ (Peters *et al.*, 2007). For overexpression of the hybrid gene encoding SA-PhaC under control of the strong T7 promoter, the SA-PhaC gene was sub-cloned from pCWE$_{Spe}$SA into pET14-b using *Xba*I/*Bam*HI sites resulting in plasmid pET14b-SAphaC (Table 2). To display core streptavidin at the surface of the polymer inclusions, the DNA fragment encoding core streptavidin (Sano *et al.*, 1995) was amplified from pET14b-SAphaC and cloned into the *Spe*I site of vector pET14b-SAphaC. Clones were screened for single and double insertion of the DNA fragment encoding core streptavidin which resulted in plasmids pET14b-cSAphaC and pET14b-dcSAphaC (Table 1 and 2). The same cloning strategy was used to generate plasmids pET14b-mSAphaC and pET14b-dmSAphaC which resulted in an N-terminal fusion of monomeric streptavidin and a single-chain dimer of monomeric streptavidin to PhaC. The gene encoding monomeric streptavidin was synthesised by GeneScript Corp. (Piscataway, USA). This gene encodes mature full length streptavidin containing 4 amino acid substitutions (T76R, V125R, V55T, L109T) that had resulted in a soluble, monomeric form of streptavidin as described elsewhere (Wu & Wong, 2005) (Table 1 and 2). Furthermore plasmid pETC was constructed which encodes only wild-type PhaC of *C. necator* under control of the T7 promoter. All constructs were confirmed by DNA sequencing.

In vivo polymer synthase activity of various streptavidin fusion proteins

In order to investigate whether the different fusion proteins mediate the formation of intracellular polymer inclusions, each of the above described plasmids was transformed into *E. coli* BL21 [DE3] harboring plasmid pMCS69 (Amara & Rehm, 2003). Plasmid pMCS69 encodes for a beta-ketothiolase (PhaA) and an acetoacetyl-CoA reductase (PhaB), proteins that catalyse the provision of the precursor *R*-3-hydroxybutyryl-CoA for polymer synthesis. GC/MS analyses showed polymer production mediated by all fusion proteins (Table 2). Electron microscopy confirmed the presence of spherical inclusions inside bacterial cells (data not shown).

Protein analyses of proteins attached to polymer beads

Cells of *E. coli* BL21 [DE3] (pMCS69; pHAS (control)) and *E. coli* BL21 [DE3] (pMCS69; pET14b-SAphaC) were cultivated under polymer inclusion forming conditions and respective beads were isolated and subjected to SDS-PAGE analysis. In the protein profile of beads formed by SA-PhaC, the fusion of full length streptavidin to the polymer forming enzyme, an additional protein with an apparent molecular weight of 82 kDa appeared when compared to the protein pattern of the control beads. This molecular weight corresponded to the predicted molecular weight of the fusion protein (Figure 1 A). The identity of this additional protein with the SA-PhaC fusion protein was confirmed by MALDI-TOF/MS analysis (Table 2).

Table 2. Characteristics of the various streptavidins fused to PhaC.

Name of fusion protein	Predicted molecular weight in kDa	Confirmed by MALDI-TOF/MS	Bead forming/ polymerisation activity[a]	Biotin binding activity[b]	Hybrid genes encoding fusion proteins
SA-PhaC	82	+	+	+	2286 bp — full-length streptavidin (amino acids +11-159), phaC
mSA-PhaC	81	+	+	-	2253 bp — monomeric streptavidin (amino acids 1-159), phaC
dmSA-PhaC	98	+	+	+	2739 bp — 2x monomeric streptavidin, phaC
cSA-PhaC	77	+	+	+	2139 bp — Core streptavidin (amino acids 15-135), phaC
dcSA-PhaC	90	+	+	+	2511 bp — 2x Core streptavidin, phaC

[a], Bead forming/polymerisation activity was obtained by detection of accumulated polyester using GC/MS analysis; [b], The biotin binding activity was obtained using ELISA.

Beads formed by cSA-PhaC, where core streptavidin is fused to PhaC and dcSA-PhaC, a single-chain dimer of core streptavidin fused to PhaC, showed additional protein bands, matching the predicted molecular weights of the respective fusion proteins of about 77 kDa and 90 kDa when compared to the protein profile of the control beads (Figure 1 B). The identity of both additional proteins with the cSA-PhaC and dcSA-PhaC fusion protein, respectively, was confirmed by MALDI-TOF/MS analysis of tryptic peptide fragment (Table 2). Beads formed by mSA-PhaC, the fusion protein of monomeric streptavidin and PhaC, showed the respective protein band at ~81 kDa as demonstrated by SDS-PAGE analysis (Figure 1 C) and identity could be confirmed by MALDI-TOF/MS analysis. Although expressed under T7 promotor control, only a small amount of mSA-PhaC could be detected. An additional protein band could be found in the protein pattern of these beads at ~ 36 kDa (Figure 1 C) as well as in the respective whole cell extracts. This protein represented a part of the fusion protein, probably due to some proteolytic degradation. Beads formed by PhaC fused C-terminally to the single-chain dimer of monomeric streptavidin, dmSA-PhaC, showed an additional protein band at ~ 90 kDA, and the identity of that additional protein with dmSA-PhaC was confirmed by MALDI-TOF/MS analysis.
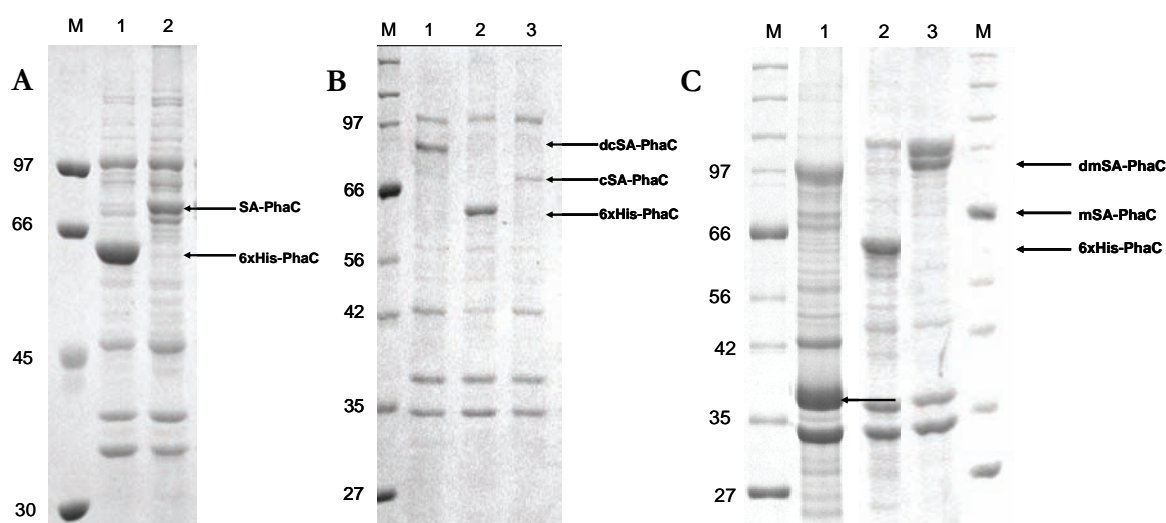

Figure 1. Protein profiles of engineered beads displaying various streptavidin variants fused to PhaC. Proteins were analysed by SDS-PAGE. M, molecular weight standard. A: Proteins of control beads (lane 1) and SA-PhaC beads (lane 2). B: Protein pattern of beads produced by dcSA-PhaC (lane 1), cSA-PhaC (lane 3) and control beads (lane 2). C: Protein pattern of beads produced by mSA-PhaC (lane 1), dmSA-PhaC (lane 3) and control beads (lane 2). Arrow indicates a proteolytic degradation product of mSA-PhaC. All fusion proteins indicated by an arrow were confirmed by MALDI-TOF/MS.

Binding of biotinylated horse radish peroxidase (HRP-biotin)

HRP-biotin was used to detect the biotin binding ability of the beads displaying streptavidin.

Beads either produced by PhaC or a hexahistidine tagged version of PhaC served as negative controls. A total of 100 ng of HRP-biotin was applied to each well of ELISA plates which were coated with beads and processed as described in Material and Methods. Beads were adjusted based on their total amount of protein as determined by Bradford analysis. Measurements were done over a wide range of bead protein concentrations, and the highest absorption in the linear range was used to deduce the biotin binding capacity. As shown in Figure 2a, the HRP-biotin binding ability of SA-PhaC beads could be demonstrated by a 41-fold increase of absorption at 490 nm when compared with the control beads and when 312.5 ng of bead protein was applied to each well. These data correspond to a biotin binding capacity of 61 ng biotin/µg bead protein.

The biotin binding ability of beads displaying monomeric or the single-chain dimer of monomeric streptavidin was also analysed by ELISA using HRP-biotin. Protein amounts ranging from 403 ng to 1.6 µg were applied. Activity could not be detected at any protein concentration with beads displaying the mSA-PhaC. However beads displaying the dmSA-PhaC showed 2.1-fold biotin binding capacity when compared to the control beads and when 1.6 µg bead protein was applied (Figure 2b). These data correspond to a biotin binding capacity of 0.7 ng biotin/µg bead protein.

ELISA using HRP-biotin binding was also applied to beads displaying cSA-PhaC or dcSA-PhaC fusions. At protein amounts of 5.3 µg per well, a 6.1-times increase in biotin binding for dcSA-PhaC and an 11.2-times increase in biotin binding for cSA-PhaC could be detected when compared to the control beads (Figure 2c). These data corresponded to a biotin binding capacity of 3.9 ng biotin/µg bead protein for the cSA-PhaC beads and 1.5 ng biotin/µg bead protein for the dcSA-PhaC beads.

Since the highest biotin-binding ability could be detected for SA-PhaC beads, further characterisation and application performance testing was done with these streptavidin beads.
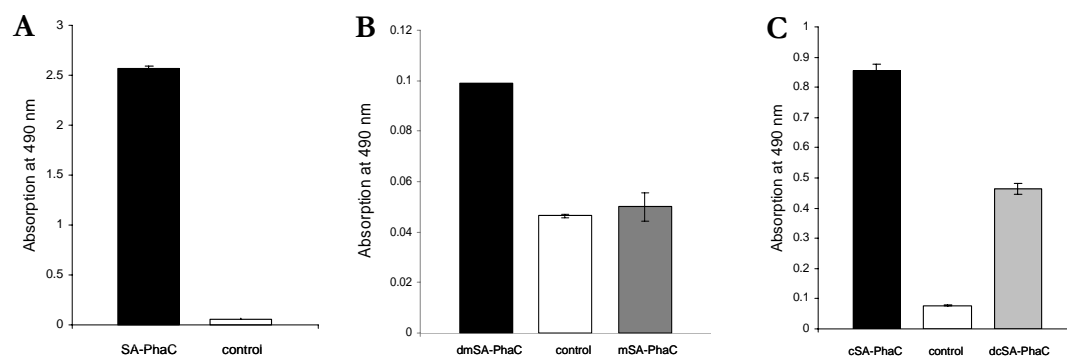

Figure 2. HRP-biotin binding to beads displaying various streptavidin variants fused to PhaC. ELISA was used to quantify binding of biotin to the various beads. As control beads served beads produced by either the wildtype PhaC or by hexahistidine tagged PhaC. A, SA-PhaC beads (312.5 ng protein/well); B, mSA-/dmSA-PhaC beads (1.6 µg protein/well); C, cSA-/dcSA-PhaC bead (5.3 µg protein/well).

Biotin binding characteristics

The apparent equilibrium dissociation constant (K_D) of SA-PhaC displayed on the beads was determined by ELISA using varying concentrations of HRP-biotin (0.3125 ng up to 625 ng per well) incubated for 1 h to reach equilibrium followed by a washing step and incubation with the HRP substrate OPD. ELISA plate wells were coated with 200 ng bead protein, respectively. The mean absorption at 490 nm was measured and these data were fitted to a monovalent binding model to determine the apparent equilibrium dissociation constant (K_D). An absorption maximum was measured for amounts $\geq$ 125 ng HRP-biotin (Figure 3), which is approximately the maximum amount of HRP-biotin which can be bound to 200 ng bead proteins. The half-maximum binding capacity corresponded to an absorption of about 0.8 (Figure 3), which was obtained when about 10 ng of HRP-biotin were used.
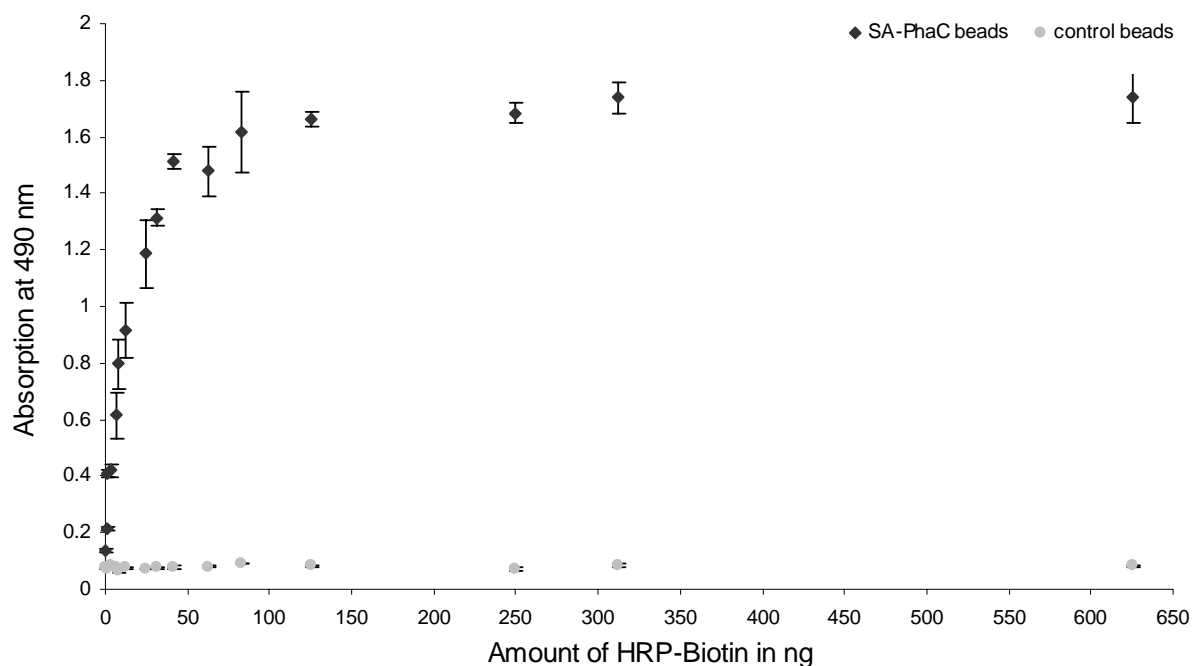

Figure 3. Determination of the equilibrium dissociation constant. ELISA plates were coated with SA-PhaC beads and control beads using 200 ng bead protein/well. Different amounts of HRP-biotin were applied to each well. An absorption maximum could be observed when beads were incubated with $\geq$ 125 ng HRP-biotin.

To determine the amount of biotin that is needed to block all free biotin binding sites provided by SA-PhaC beads as well as to determine the amount of biotin equalling 125 ng HRP-biotin, a biotin competition assay was performed. 200 ng of SA-PhaC bead protein was used in each ELISA plate well. The biotinylated HRP was used constantly at 125 ng per well in the presence of varying concentrations of D-biotin, ranging from 500 μM to 0.2 μM. As a positive control, the biotinylated HRP was applied in the absence of free biotin where the maximum HRP-biotin binding was measured (Figure 4). To saturate half of the free biotin binding sites in

the competitive ELISA, D-biotin had to be added at a concentration of about 0.5 µM. Thus 0.5 µM is corresponding to 125 ng HRP-biotin. This conversion was used to deduce from Figure 3 the apparent equilibrium dissociation constant (K_D) of 4×10^{-8} M.
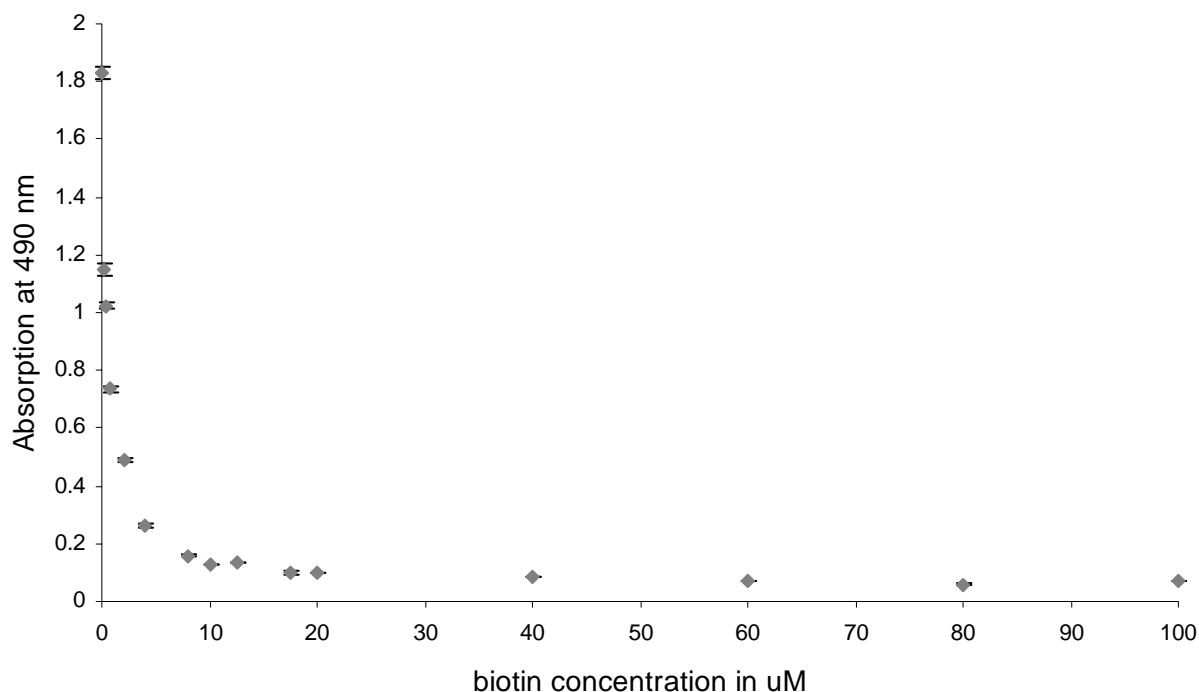

Figure 4. Biotin competition assay. ELISA plates were coated with 200 ng of bead protein. HRP-biotin (125 ng protein/well) was used in the presence of increasing concentrations of D-biotin.

Application performance of the streptavidin beads

Immobilisation of antibodies. In contrast to IgG-binding ZZ domain-displaying beads (Brockelbank *et al.*, 2006), the streptavidin beads enable a much stronger binding of biotinylated antibodies as well as of any other biotinylated molecule, which provides an enormous flexibility of the biotin-streptavidin system. To demonstrate the possibility of immobilisation of antibodies to the streptavidin beads, the binding of a biotinylated antibody was assessed. ELISAs using a biotinylated goat polyclonal to rabbit IgG H&L antibody and a secondary HRP conjugated rabbit polyclonal to goat IgG H&L antibody were performed with bead suspensions adjusted according to their total protein concentration. Biotinylated antibody immobilisation could be demonstrated by a strong increase of absorption at 490 nm. The maximum antibody binding was observed at 312.5 ng bead protein per well which indicated an about 30-fold increase when compared to the control beads (Figure 5).
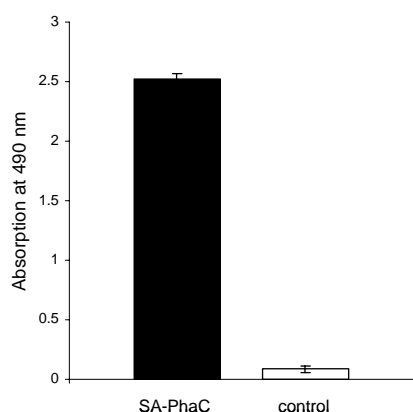
Figure 5. Binding of biotinylated antibodies to SA-PhaC beads. A biotinylated goat polyclonal to rabbit IgG H&L antibody was used to bind to the beads. Bound antibody was detected and quantified using a HRP-labelled rabbit polyclonal to goat IgG H&L antibody in ELISA.

DNA binding. To demonstrate that these recombinantly produced streptavidin beads can be applied for various laboratory methods requiring biotin binding properties, the possibility of binding biotinylated DNA to the beads was investigated. For this purpose, a small biotinylated DNA-fragment of 229 bp was incubated with control beads and SA-PhaC beads, respectively, which were adjusted according to their optical density at 600 nm. After several washing steps, immobilisation of biotinylated DNA to the streptavidin beads was analysed using agarose-gel electrophoresis (Figure 6), which indicated that the biotinylated PCR-product could be successfully immobilised to SA-PhaC beads. (Figure 6).
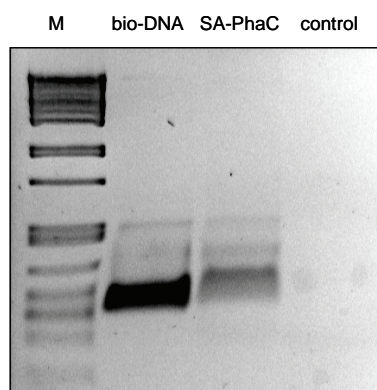

Figure 6. Immobilisation of biotinylated DNA. Beads were incubated with a biotinylated DNA fragment and subjected to agarose gel electrophoresis. Bio-DNA, biotinylated DNA fragment (229 bp); SA-PhaC, SA-PhaC beads incubated with biotinylated DNA; control, control beads incubated with biotinylated DNA; M, molecular weight standard (*Pst*I digested lambda-DNA).

FACS performance. To further demonstrate applicability of the beads, particularly harnessing the bead nature, the beads were subjected to fluorescence activated cell sorting (FACS). For this purpose, the biotinylated goat polyclonal to rabbit IgG H&L antibody and a secondary FITC conjugated rabbit polyclonal to goat IgG H&L were incubated with beads. A strong increase of fluorescence intensity when using SA-PhaC beads could be detected (Figure

7B) when compared to the fluorescence intensity of control beads (Figure 7A). From 100 % of all counted events, only 14 % were located in the marker region (M1) when control beads were used, which increased up to 78 % in the marker region when SA-PhaC beads were used. Further controls included incubation of SA-PhaC beads with only the biotinylated antibody, which led to 0.04 % of events located in the marker region, as well as incubation with only the FITC-labelled antibody, which showed 11 % of all events in the marker region (data not shown).
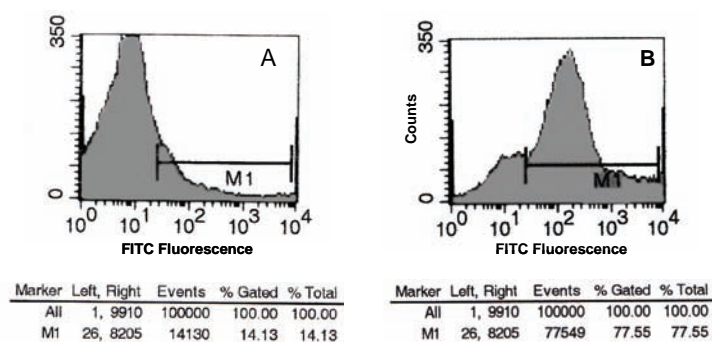

Marker	Left, Right	Events	% Gated	% Total
All	1, 9910	100000	100.00	100.00
M1	26, 8205	14130	14.13	14.13

Marker	Left, Right	Events	% Gated	% Total
All	1, 9910	100000	100.00	100.00
M1	26, 8205	77549	77.55	77.55

Figure 7. The use of SA-PhaC beads in fluorescence activated cell sorting. Beads were incubated with a biotinylated goat polyclonal to rabbit IgG H&L antibody and a FITC-labeled rabbit polyclonal to goat IgG H&L antibody and fluorescence was monitored. Counts of fluorescent event were plotted as a function of fluorescence intensity. Results are summarized in a table below the graph. A: control beads; B: SA-PhaC beads.

Discussion

Currently, the production of streptavidin beads requires the production and purification of streptavidin which is then chemically cross-linked to prepared beads. In this study we propose a novel strategy for the one step production of streptavidin beads by using engineered *E. coli* expressing a streptavidin fusion protein which mediates formation of polyester inclusions displaying SA. The various SA fusion proteins were successfully overproduced at the polymer inclusion surface (Figure 1). Proteins other than PhaC found attached to the control beads had been previously identified as *E. coli* proteins which bind unspecifically to the bead surface. The engineered monomeric streptavidin (Wu & Wong, 2005) was considered as the ideal streptavidin variant for the *in vivo* production of streptavidin beads because it does not require the formation of a homotetrameric molecule to constitute biotin binding sites. Although the monomeric streptavidin had been successfully overproduced in the cytoplasm of *E. coli* (Wu & Wong, 2006), the complete fusion protein mSA-PhaC was only produced at low levels at the bead surface (Figure 1). The additional presence of a truncated version of this fusion protein suggested proteolytic digestions, which might be the reason for the loss of biotin binding activity. To further investigate the lack of biotin binding activity, a single-chain dimer of the monomeric streptavidin was fused to PhaC (Table 2). This single-chain dimer of monomeric streptavidin

fused to PhaC mediated the formation of beads showing weak biotin binding (Figures 1 and 2). Since no truncated version of this fusion protein could be detected, it appeared to be more stable with respect to proteolytic digestions.

Streptavidin is produced by *Streptomyces avidinii* as a polypeptide of 159 residues, which is rapidly cleaved by proteases to yield several truncated versions of the protein (Sano *et al.*, 1995). The most stable and well studied form of the protein contains residues 13–139 and is called core streptavidin which shows enhanced solubility and little tendency to aggregate (Pahler *et al.*, 1987; Sano *et al.*, 1995). In this study, a core streptavidin and a single-chain dimer of the core streptavidin, respectively, were fused to PhaC. Both fusion proteins mediated formation of polymer beads displaying biotin binding sites (Figure 1 and 2). The core streptavidin displaying beads showed an about 5.6 times increased binding capacity, when compared to the single-chain dimer of the engineered monomeric streptavidin.

The mature full length streptavidin is characterised by a poor solubility and strong tendency to form higher-order aggregates (Argarana *et al.*, 1986; Bayer *et al.*, 1986; Bayer *et al.*, 1989). Consistently, mature full length streptavidin forms inactive inclusion bodies when produced in *E. coli* (Humbert *et al.*, 2005). Several reports suggested that streptavidins truncated at the C terminus display enhanced binding affinity for large biotinylated macromolecules (Bayer *et al.*, 1989; Sano *et al.*, 1995). In a recent study the structure of a full length streptavidin has been determined, which showed that the 20 residue extension (residues 139 to 159) at the C terminus forms a well-ordered polypeptide loop on the surface of the tetramer (Le Trong *et al.*, 2006). The residues 150–153 of this extension were bound to the ligand-binding site, possibly competing with exogenous ligands. This observation provided insight into the molecular mechanisms related to the reduced binding affinity of biotinylated macromolecules to mature full length streptavidin. In this study, the C terminus of mature full length streptavidin was fused to the N terminus of PhaC, which serves upon polymer synthesis as an immobilisation tag. Thus the C terminus of the full length streptavidin would not be available to interfere with binding of biotin or biotinylated macromolecules (Figures 2-5). This immobilisation of the C terminus and *in vivo* binding to polymer inclusion (Figure 1) resulted in assembly of beads with the highest biotin binding capacity of about 61 ng biotin per μg of bead protein (Figures 2 and 3). Further analysis of these streptavidin beads revealed an equilibrium dissociation constant K_D of about 4×10^{-8} M for biotin (Figure 4 and 5), showing a similar binding affinity as was found for the engineered monomeric streptavidins (Wu & Wong, 2005). The applicability of these streptavidin beads for laboratory uses was further evaluated by demonstrating binding of a biotinylated antibody using ELISA (Figure 5), immobilising of biotinylated DNA using agarose gel-electrophoretic analysis (Figure 6) as well as using these beads for FACS analysis (Figure 7).

In this study it was demonstrated that engineered streptavidin fusion proteins can be produced enabling the intracellular production of streptavidin displaying polymer beads in one step (Figure 8). Isolated engineered polymer beads were stable and could be successfully applied in ELISA, DNA purification, enzyme immobilisation and flow cytometry.
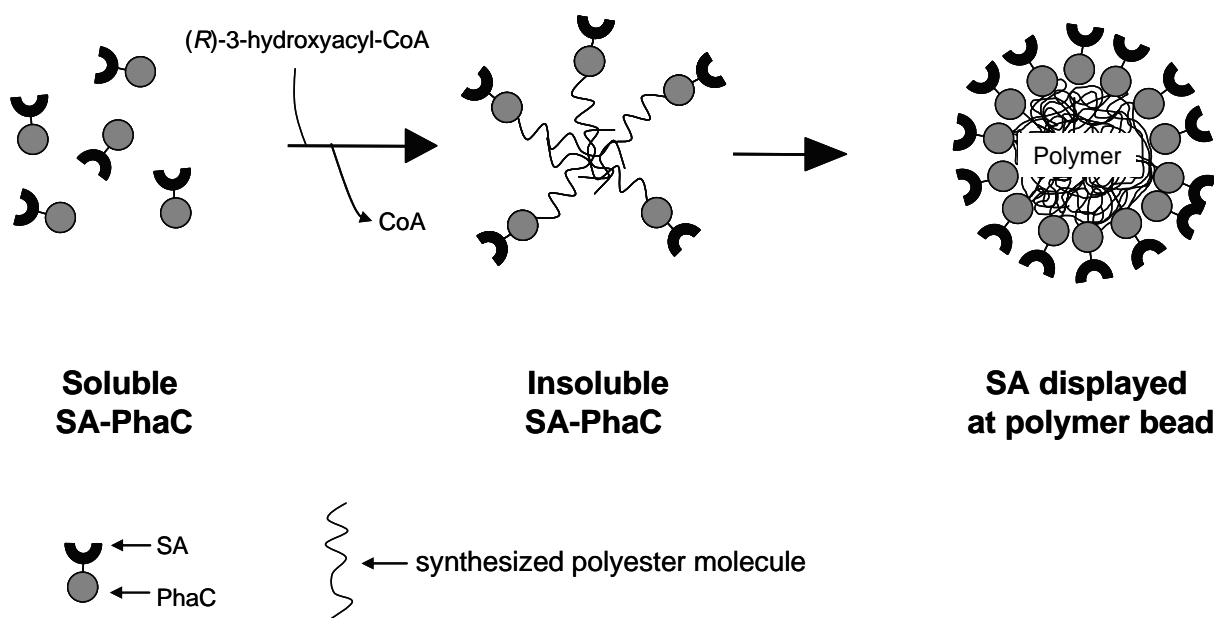

Figure 8. Schematic view of the *in vivo* streptavidin beads assembly. SA, streptavidin, PhaC, polyester inclusion forming enzyme.

Acknowledgements

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Chapter VI

Conclusions

Localisation of the PHA synthase

My thesis aimed to provide further knowledge about the localisation of the PHA synthase and consequently the localisation of early stage PHA granules. This was achieved by mutating the PHA synthase of *C. necator* and studying the localisation behaviour of these mutated versions when fused to GFP. Our results as stated and discussed in chapter II allowed us to draw the following conclusions:

Firstly, our results suggested that a growing polyester chain, which stays covalently attached to the PHA synthase during PHA synthesis, is not required for the localisation of the PHA synthase to the cell poles. This conclusion could be drawn due to our finding that the inactive PHA synthase PhaC_C319A was still located to the cell poles. Furthermore, this finding indicates that the localisation of the PHA synthase is an active process, probably driven by an interaction with one or several other proteins, and not only a passive phenomenon which is due to PHA biosynthesis and a limited amount of space. Secondly, we concluded that the N-terminal 93 amino acids are not involved in the localisation process of the PHA synthase, because PhaCΔ1-93aa, an N-terminal deletion mutant, could still be detected localised at the cell poles. Thirdly, the result that GFP-PhaCΔ521-598aa, a C-terminal deletion mutant of the PHA synthase, did not show any alteration in the localisation behaviour of the synthase, led us to reason that the C-terminal region is also not necessary for a successful localisation. In summary, these localisation studies of different mutated versions of the PHA synthase indicated that only the core polyester synthase (amino acids 93-521) is required for polar positional information.

Another aim of my studies was to ascertain if other granule associated proteins would also be directed to the cell poles. Our fluorescence microscopy studies of PhaP-HcRed, a fusion of HcRed to a phasin protein, did not reveal a localisation of the phasin protein to the cell poles. Hence, we concluded that granule associated proteins are not usually located to the cell poles and that the PHA synthase as key enzyme of PHA biosyntheses may present a particular exception.

Modification of the PHA granule surface using the PHA synthase as anchor

Moreover, my thesis aimed to clarify whether PHA granule surfaces can be successfully modified by using the PHA synthase as an anchor molecule to attach proteins of interest to the granule surface. The applicability of PHA granules as bio-beads should be evaluated by employing these functionalised bio-beads in different biotechnological assays. Our results as stated and discussed in chapter III, chapter IV, and chapter V gave rise to the following conclusions:

We deduced from the successful display of a functional β-galactosidase at the PHA granule surface that the idea to functionalise PHA granules by utilising the PHA synthase as anchor molecule is feasible. Additionally, the storage of these modified PHA granules for several months without a strong decrease of β-galactosidase activity led us to conclude that PHA granules are promising candidates for a potential use as bio-beads. The applicability of PHA granules as bio-beads displaying streptavidin and the IgG binding domain of protein A, respectively, was demonstrated in antibody/enzyme immobilisation, DNA immobilisation/purification and for fluorescence activated cell sorting (FACS). Recapitulated, we drew the conclusion that PHA granule surface can be successfully modified by the utilisation of the PHA synthase as an anchor molecule. In addition, we concluded that these functionalised bio-beads show strong ability to be applied in biotechnological procedures and assays.

Outlook

Localisation of the PHA synthase

The PHA synthase localisation studies with various mutated version of the synthase led to the conclusion that the core region of the PHA synthase (amino acids 93-521) is required for positional information. Since this region exhibits the α/β-hydrolase fold as well as it shows six conserved blocks and eight identical amino acids as analysed by multiple alignments of PHA synthases, deletions in this region may result in total loss of the protein structure and any altered localisation would not enable us to draw any further conclusions.

There are several other experimental techniques that should be applied in future and which could result in the elucidation of a potential "core PHA synthase" interacting protein. On the one hand the PHA synthase could be immobilised at a column, followed by incubation of the column with whole cell lysate. With removal of the PHA synthase from the column, a potential PHA synthase interacting protein would be co-eluted and could be analysed by SDS-PAGE and by MALDI-TOF/MS. On the other hand, PHA synthase interacting proteins could be identified by expression of the PHA synthase followed by a cross-linking treatment. Interacting proteins would be cross-linked and co-purified. Specific interaction of potential candidates with the PHA synthase could be shown with the help of yeast-two-hybrid experiments. Furthermore, DNA chip technology could be applied in order to detect any differences in gene expression in strains expressing the PHA synthase gene. An up/down-regulation of a specific gene that can be confirmed by relative RNA quantification may suggest an interaction of the gene product with the PHA synthase.

Not only these experiments, but also the likely substantial progress of microscopy imaging devices in the near future may help elucidate the localisation of the PHA synthase as well as the assembly process of PHA granule.

Modification of the PHA granule surface using the PHA synthase as anchor

As concluded above, the studies presented in my thesis showed that PHA granules can be easily modified by anchoring proteins of interest to the surface via the PHA synthase. Furthermore, it could be successfully demonstrated that these surface-modified PHA granules exhibit applicability as bio-beads. Moreover, studies displaying other proteins of interest, e.g. an antibody, as well as studies using different anchor-proteins/domains, e.g. a phasin protein, reinforce the potential use of PHA granules as bio-beads. The advantages of bio-beads are obvious; to begin with the polyester core can be generally considered as safe, non-toxic and biodegradable. In addition, the surface modification is achieved in one step simultaneously with the bead production, thus avoiding the necessity of a second-step modification such as chemical cross-linking. In my opinion, the concept of PHA granules as bio-beads has been proven sufficiently, and the future of bio-beads depends on the interest of the biotechnological industry. Whether these beads will replace existing standard beads in established processes will depend on funding made available for research that focuses on decreasing potential production costs, scaling-up, optimising of downstream processing and marketing. The immediate research projects that should follow and are currently being investigated to some extent by myself and/or other workgroup members are as follows: First of all, an efficient bio-bead production strain will be generated, which possesses the required genes for proteins involved in bio-bead production integrated in the genome. Secondly, we seek to exploit the number of different proteins of interest that can be displayed simultaneously at the PHA granule surface. Different fusions of distinguishable proteins of interest to various granule associated proteins will be created and expressed simultaneously during PHA biosynthesis, followed by detection of the different displayed proteins. It may also be necessary for some applications to produce bio-beads of a certain size. The possibility to influence the size of bio-beads should be explored by using different strains which are grown under different culturing conditions, followed by transmission electron microscopy analyses and measurement of bio-beads. During my doctoral thesis, I have already accomplished several studies about the size of bio-beads in different strains grown under various conditions. These studies are currently being completed and may be made public at a later stage depending on confidentiality agreements. Last but not least, the modification of bio-beads by generating translational fusions to granule associated proteins should be further explored and more proteins of interest should be tested of functional display, for instance a fusion of an active receptor would present a technique of directing bio-beads to a target site.

As for every potential new product, there are several possible drawbacks which need to be addressed. Modified bio-beads produced in *E. coli* not only show the expected protein of interest, but also exhibit several *E. coli* proteins that attach to the bio-bead surface in an unspecific manner. In case it is aimed to apply bio-beads in human medicine, these proteins hinder an accurate measurement and therefore dosage of the protein of interest. Besides, bio-beads produced in *E. coli* will have to be tested for their endotoxin level in order to use them safely for medical purposes. Moreover, it has to be considered that fluctuations in the amount of protein per bio-bead may occur, either due to variation of the protein expression level during bio-bead production or due to removal of the protein of interest during mechanical cell disruption. The potential loss of protein of interest through mechanical disruption has to be addressed especially in cases where granule associated protein are used which show only moderate of weak binding to the PHA granule surface.