Recombinant *Escherichia coli* producing an immobilised functional protein at the surface of bio-polyester beads: A novel application for a bio-bead

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Abstract

Polyhydroxyalkanoates (PHAs) are polyesters, produced by many bacteria and some archaea. The most commonly characterised is polyhydroxybutyrate (PHB). Produced when nutrients are growth limiting and carbon available in excess, PHA serves as a carbon-energy storage material and forms generally spherical insoluble inclusions between 50-500nm in diameter in the cytoplasm. The key enzyme for PHA synthesis is the PHA synthase and this enzyme catalyses the polymerisation of (*R*)-3-hydroxy fatty acids into PHA. PHA synthase remains covalently attached to the growing polyester chain and is displayed on the surface of the PHA granule. Other proteins associated with PHA granules include depolymerases for mobilisation or degradation of granules, regulatory proteins and phasins, proteins that aid in PHA granule stability.

PHA bio-beads displaying an IgG binding protein were produced and used to purify IgG from serum demonstrating that the PHA synthase can be engineered to display functional synthase fusion proteins at the PHA granule surface. Correctly folded eukaryotic proteins were also produced and displayed at the PHA granule surface as phasin fusion proteins. Multiple-functionality was also achievable by co-expression of various hybrid genes suggesting that this biotechnological bead production strategy might represent a versatile platform technology.

The production of functional eukaryotic proteins at the PHA bead surface represents a novel *in vivo* matrix-assisted protein folding system. Protein engineering of PHA granule surface proteins provides a novel molecular tool for the display of antigens for FACS based analysis and offers promising possibilities in the development of future biotechnological production processes. Overall, the results obtained in this study strongly enhance the applied potential of these polyester beads in biotechnology and medicine.

Acknowledgements

Approval for the research has been obtained for this work (GMO 07/MU013).

I would like to thank my supervisors, Professor Bernd Rehm and Dr Zoe Jordens for the ideas, encouragement and for all of the support during my husband Paul's illness and after his death in November 2006. Thanks to the people at IMBS and to all in the Rehm workgroup at the time who also gave me their time, support, technical advice and expertise when needed. Thanks to Pat Munro for all the scientific discussion and networking during those trips between Levin and Science Tower C. Thanks to Chris Pennington for proofreading the final draft version and finding the word horseradish funny.

Thanks to my family, especially my parents Graeme and Daphne Brockelbank who did their best to start me off in the right direction, my daughter Caitlin and my husband Paul Atwood for his unending support, encouragement, and insistence that I complete this degree.

2006 was the year that I learned much about loss, about grief, about my own strengths and weaknesses and while I do not know how I managed, I do know that due to the support of my work colleagues, my friends and my family, I was able to continue. Finally, thanks to Rory who has been there for me this year.

Life is a precious gift and my advice to you is this:

MAKE EACH DAY COUNT

(And don't forget to take photos along the way).

"When operating earthmoving equipment…….get right back out of it"

(This work is dedicated to Paul Atwood 1957-2006)

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Abbreviations

AFM	Atomic force microscopy
BSA	Bovine serum albumin
CFA	Complete Freund's adjuvant
DMSO	Dimethyl sulfoxide
ELISA	Enzyme linked immuno-assay
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
GCMS	Gas chromatography mass spectrometry
GCPM	Gfp-PhaC and PhaP-MOG fusion protein construct
GPM	GFP-PhaP-MOG fusion protein construct
GFP	Green fluorescent protein
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
IL2	Interleukin 2
kDa	Kilo Daltons
MALDI-TOF	Matrix assisted laser desorption ionisation time-of-flight mass spectrometry
MCL	Medium chain length
MOG	Myelin oligodendrocyte glycoprotein
ORF	Open reading frame
PCR	Polymerase chain reaction
PHA	Polyhydroxyalkanoate
PhaC	PHA synthase
PhaE	Type III PHA synthase subunit
PhaP	Phasin
PhaR	Phasin regulatory protein
PhaY	Hydrolase (depolymerase)
PhaZ	PHA intracellular depolymerase
PHB	Polyhydroxybutyrate
scFv	Single chain variable fragment (antibody)
SCL	Short chain length
SDS-PAGE	Sodium dodecyl sulphate gel electrophoresis

CHAPTER 1: POLYHYDROXYALKANOATE

1.1 Bacterial polyester

Polyhydroxyalkanoic acids (PHAs) are polyesters. The first example of a bacterial polymer, poly(3-hydroxybutyric acid) (PHB) formed from (*R*)3-hydroxybutyryl-CoA was discovered in *Bacillus megaterium* in 1925 (Lütke-Eversloh *et al.* 2002; Zheng *et al.* 2006) and this is still the most commonly isolated PHA from bacteria (Campisano *et al.* 2008; Kikkawa *et al.* 2005; Taguchi and Doi 2004). Most genera of Bacteria and even members of the family Halobacteriaceae of the Archaea are known to synthesize PHA (Brandl *et al.* 1990; Hezayen, F.F. *et al.* 2000; Hezayen, F.F. *et al.* 2002a; Hezayen, F.F *et al.* 2002b) which is produced in conditions of nutrient limitation but where carbon is available in excess (Kim *et al.* 2004; Hoffman and Rehm 2005; Kuchta *et al.* 2007; Campisano *et al.* 2008). PHA serves as energy storage for bacteria and is deposited as insoluble spherical inclusions in the cytoplasm (Figure 1). Bacteria have been known to accumulate as much as 80% of their dry weight in PHA (Lee 1996; Madison and Huisman 1999) and in carbon starvation conditions the PHA polymerisation process can also be reversed due to intracellular depolymerases that remain attached to the PHA surface (Gao *et al.*

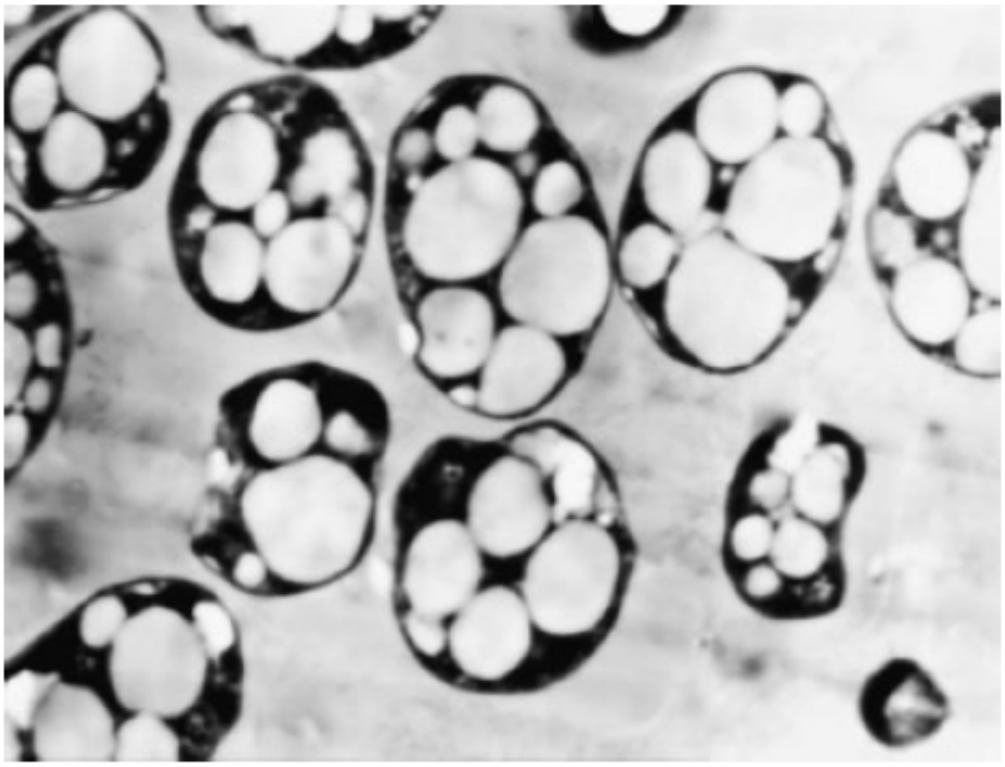
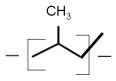
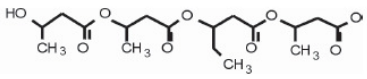
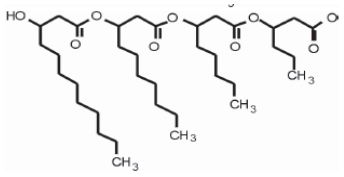


Figure 1. Cupriavidus necator accumulating PHA (Zou and Chen, 2007).

2001; Handrick *et al.* 2000). PHAs have some interesting properties including being biodegradable and thermoplastic, yet are similar in many characteristics to polypropylene (Table 1).

Table 1 Properties of PHA compared to polypropylene (modified from Rehm 2007).

	T_m(°C)	Crystallinity (%)	Elongation at break	Structure
Polypropylene (PP)	~170	60	400	C_3H_6 
PHA (SCL)	~175	70	5	
PHA (MCL)	61	30	300	

As Table 1 shows, the properties of the PHA polyhydroxybutyrate (PHB), varies depending on the length of the component hydroxyl fatty acids. Short chain length (SCL) 3-hydroxy fatty acids result in a polymer that is fairly hard or brittle and PHAs from medium chain length 3-hydroxy fatty acids have more elastomeric properties, less tensile strength and a lower melting temperature (Hazer and Steinbüchel 2007; Kaweda *et al.* 2003; Rehm 2003; Rehm 2007). To date, over one hundred and fifty monomers have been identified as precursors or constituents of PHAs and these include branched, unbranched, saturated, unsaturated and aromatic molecules (Kessler *et al.* 2001b; Steinbüchel and Valentin 1995). PHAs are completely biodegradable in aerobic or anaerobic conditions without special treatment (Tokiwa and Calabia 2004) and low specificity for substrate by the PHA synthase means many different variations of PHA can be produced by fermentation (Antonio *et al.* 1999).

PHAs have received a lot of attention in the past thirty years as an alternative to petroleum based plastics as they can be produced biologically and from renewable

energy sources (Anderson and Dawes 1990; Kikkawa *et al.* 2005; Manna and Paul 2000; Nomura and Taguchi 2006; Taguchi and Doi 2004; Zheng *et al.* 2006).

Due to the biodegradability, biocompatibility and elastomeric properties of PHAs, there is a lot of interest in the potential applications of biopolymers. For example, with some chemical modification, PHAs can be modified to be less brittle, more flexible yet retain tensile strength and have uses in industrial or medical applications and have been shown to be well tolerated by mammalian systems (Hazer and Steinbüchel 2007). In medical applications, PHAs could be used for implants like heart valves, stents and bone scaffolding (Furrer *et al.* 2007; Hazer and Steinbüchel 2007).

1.2 Isolating PHA from bacteria

Generally, PHAs for use as bioplastics are harvested from bacterial cultures by one of two methods. The first is by the addition of organic solvent to dried biomass usually with chlorination followed by methanol to precipitate the PHA from contaminating proteins and cell debris. The second employs the use of chemicals or enzymes to lyse cells and release the intracellular granules of PHA. Both may be used together to improve purity (Chen *et al.* 2001; Furrer *et al.* 2007; Kessler *et al.* 2001a). Purity may be critical for medical applications as the Federal Drug Administration (FDA) of the U.S. has stringent regulations applying to products synthesised from gram-negative bacteria because of the presence of lipopolysaccharides (LPS) which are pyrogenic. Endotoxin limits are therefore set low at 20 EU (endotoxin units) per medical device (Furrer *et al.* 2007).

PHAs are currently more expensive to produce than petroleum-based plastics, so to help decrease production costs, technology is being developed to enable higher PHA production and in a shorter time frame (Lee and Choi 2001; Zou and Chem 2007). Much of the research into PHA is based on its thermoplastic properties and many studies of the biogenesis of PHA have centred on enhancing PHA production (Lütke-Eversloh *et al.* 2002; Lütke-Eversloh and Steinbüchel 2003; Lütke-Eversloh and Steinbüchel 2004; Taguchi and Doi 2004; Tessmer *et al.* 2007; Tsuge *et al.* 2004; Zou and Chem 2007). This has included investigating and even engineering the substrate specificity of the key enzymes involved in PHA biosynthesis (Taguchi and Doi 2004; Normi *et al.* 2005a; Normi *et al.* 2005b; Park *et al.* 2005). Alteration to

saturated groups or functional side-chains also allows modification to the polyester properties such as melting point and crystallinity (Grage *et al.* 2009).

1.3 Classification of PHA synthases

The key enzyme for PHA synthesis is PHA synthase and this enzyme polymerises (*R*)3-hydroxyacyl CoA thioester monomers into polyester with the release of CoA (Figure 2).

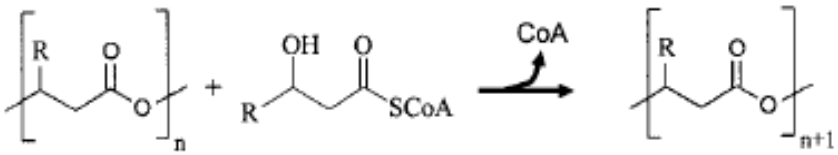


Figure 2. Reaction catalysed by the synthase PhaC (Rehm 2003).

Many different varieties of PHA are known to exist and as many as 88 different PHA synthases have been identified and cloned to date (Rehm 2007). The class of PHA synthase is currently classified based on the specificity of the synthase (Rehm 2003; Yuan *et al.* 2001) (Table 2).

Table 2. Polyester synthases are classified into four classes (Rehm, 2003).

Class	Subunits	Species	Substrate
I	PhaC ~60-73 kDa	*Cupriavidus necator*	$3HA_{SCL}$CoA (C3-C5)
II	PhaC ~60-65 kDa	*Pseudomonas aeruginosa*	$3HA_{MCL}$CoA (>C5)
III	PhaC PhaE ~40 kDa ~40 kDa	*Allochromatium vinosum*	$3HA_{SCL}$CoA(C3-C5) $3HA_{MCL}$CoA(C7-C8) 4HACoA 5HACoA
IV	PhaC PhaR ~40 kDa ~22 kDa	*Bacillus megaterium*	$3HA_{SCL}$CoA

As table 2 shows, class I and class II PHA synthases are comprised of a single subunit designated PhaC with a molecular mass between 61 and 73 kDa. Class I PHA synthases show substrate specificity preferentially using short chain length (SCL) CoA thioesters of (*R*)-3-hydroxy fatty acids (3-5 carbon atoms). Class II PHA synthases preferentially use medium chain length (MCL) (*R*)-3-hydroxy fatty acids (6-14 carbon atoms). Class III PHA synthases contain two subunits both of similar molecular mass (40 kDa), designated PhaC and PhaE and utilise both SCL and MCL (*R*)-3-hydroxy fatty acids. While class III synthase PhaC has some amino acid similarity to class I PhaC synthases (21-28%), PhaE does not. Class IV PHA synthases are similar to class III but with PhaR (molecular mass approximately 20 kDa) replacing PhaE and a substrate preference for MCL (*R*)-3-hydroxy fatty acids (Rehm 2003). There are exceptions to this classification but the overall finding has been that PHA synthases show broad substrate specificity (Amara and Rehm 2003; Antonio *et al.* 1999; Matsusaki *et al.* 2000; Rehm 2006; Steinbüchel and Hein 2001; Stubbe and Tian 2003; Yuan *et al.* 2001).

Closer examination of the 88 PHA synthases has highlighted eight conserved regions and an N terminal region that is highly variable (Figure 3). Mutational analysis has shown that the highly variable N terminal region containing up to the first 100 amino acids is dispensable (Rehm 2003). The α/β hydrolase fold region has been determined to be essential for enzyme activity (Pham *et al.* 2004) and the hydrophobic C terminus is highly conserved among all classes of synthase suggesting a role in attachment to the polyester core (Rehm 2003). As yet, the crystal structure of PHA synthase remains to be determined and models have been proposed based on homology to the *Burkholderia glumae* lipase (Rehm 2007; Rehm *et al.* 2002).

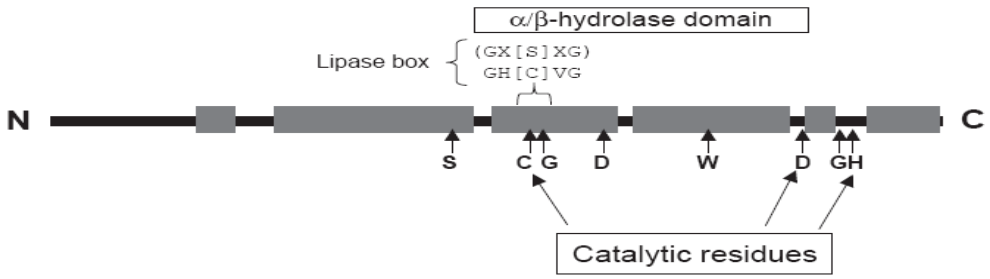


Figure 3. Conserved regions (grey rectangles) and variable N terminal region of PhaC from Cupriavidus necator with the eight conserved amino acid residues indicated by arrows (Rehm, 2007).

1.4 Biosynthesis and formation of PHA granules

PHA granule formation requires the activity of three essential enzymes designated PhaA (encoded by gene *phaA*), PhaB (encoded by gene *phaB*) and PhaC (usually encoded by gene *phaC* but can be *phaE* or *phaR* as Table 2 shows) (Qi 2001; Slater *et al.* 1998; Taguchi and Doi 2004). PhaA is a β-ketothiolase that condenses two available acetyl-CoA molecules to acetoacetyl-CoA that is in turn reduced by PhaB, an NADP-dependent acetoacetyl-CoA reductase, into (*R*)-3-hydroxybutyryl-CoA. This becomes polymerised in a reaction catalysed by PHA synthase which is comprised of a PhaC dimer forming the single subunit PhaC (or a PhaC and PhaE/PhaR for class III/IV synthase) into a growing linear chain at the same time releasing CoA.

In *Cupriavidus necator,* 3-hydroxyacyl CoA thioesters are generated from two acetyl-CoA molecules whereas for *Pseudomonas aeruginosa* that produce PHA_{MCL}, it is usually intermediates of fatty acid β-oxidation and/or fatty acid *de novo* synthesis that provides precursors that are converted into (*R*)-3-hydroxylacyl-CoA thioesters (Grage *et al.* 2009) (Figure 4).

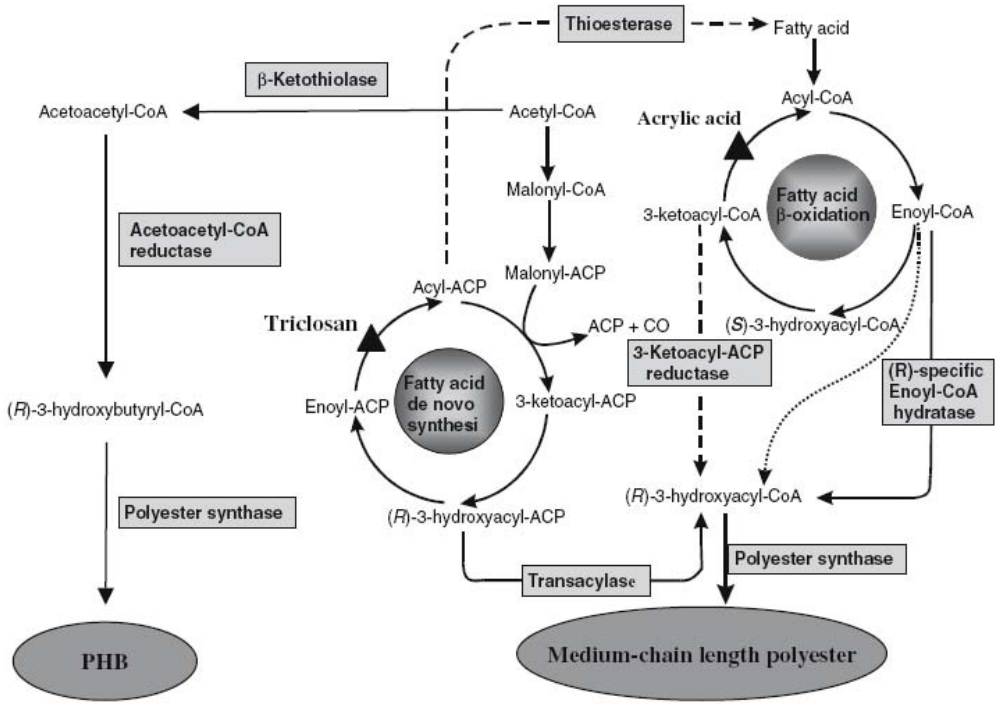


Figure 4. Metabolic routes toward PHA biosynthesis. The dashed arrows show steps only shown in recombinant systems (from Rehm, 2006).

In vivo, the biosynthesis of PHA granules begins as soon as the substrate (*R*)-3-hydroxyacyl-CoA thioesters become available intracellularly (Jurasek and Marchessault 2004; Peters and Rehm 2005; Rehm *et al.* 2002; Rehm *et al.* 1998). In *C. necator,* these genes are clustered together as part of the *phaCAB* operon (Rehm *et al.* 2002) and *phaC* is constitutively expressed so as soon as the conditions for PHA biosynthesis are permissive, PhaC dimerises and as active PHA synthase, begins to polymerise available substrate. Experimental data by Peters and Rehm (2005) has also indicated that emerging PHA granules actually locate to the cell poles and potential cell division sites rather than form randomly within the cytoplasm. The formed PHA granule core is amorphous with a layer surrounding it thought to be comprised of a phospholipid monolayer with attached or embedded proteins (Steinbüchel *et al.* 1995a). Water is thought to be found within the polyester core as well and act as a plasticiser (Anderson and Dawes 1990).

In vitro, a lag phase occurs and aggregates of PhaC catalyse the formation of the polyester chain. In contrast to *in vivo* granule formation where PHA synthase comprises two subunits of PhaC, studies using atomic force microscopy (AFM) found that, more than two PhaC molecules were required for *in vitro* polymerisation (Hiraishi *et al.* 2005) which could explain the lag phase seen as PhaC requires time to aggregate. Currently, the exact mechanism for granule assembly process is not fully understood but two models exist for granule assembly and they are the micelle model and the budding model (Figure 5).

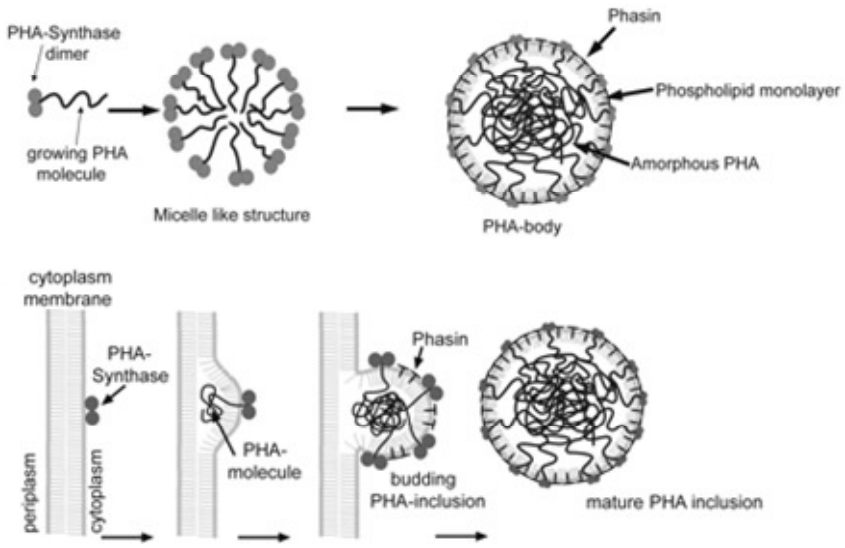


Figure 5. Budding and micelle model of PHA synthesis (Waltermann and Steinbüchel, 2005)**.**

In vitro granule assembly supports the micelle model where the growing polyester chain becomes increasingly hydrophobic and forms a granule with the PHA synthase covalently attached to the end continuously adding and polymerising the substrate (*R*)-3-hydroxybutyrate as it becomes available (Rehm *et al.* 2002). More synthase-polyester molecules aggregate together with the hydrophobic polyester forming the core of the inclusion body and the PHA synthase remaining attached on the surface exposed to the cytoplasm. In the budding model, it is thought that the synthase associates with the cytoplasmic membrane and eventually 'buds' off forming an inclusion body. The PHA inclusion is surrounded by a phospholipid monolayer in which proteins are attached or become embedded (Grage *et al.* 2009).

Whether formed *in vivo* or *in vitro*, PHA polyester chains aggregate to form a granule or an inclusion body, spherical in shape, of approximately 50 – 500 nm in diameter with the PHA polyester at the core and the PHA synthase remaining covalently attached and displayed at the surface. *In vivo,* the surface of the PHA granule is not completely covered by the polyester synthase and other proteins also associate with it (Figure 6) (Pötter and Steinbüchel 2005).

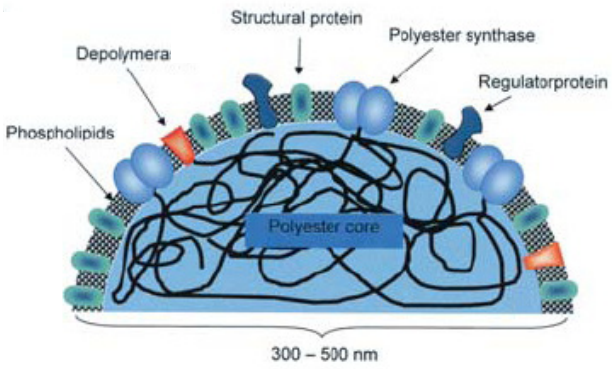


Figure 6. A schematic diagram showing a polyester granule (Rehm, 2003).

1.5 Granule associated proteins

In *C. necator*, PHA granules contain four designated classes of protein on the granule surface, namely; the polyester synthases (PHA synthase), depolymerase (PhaZ), regulatory proteins (PhaR) and phasin (PhaP).

The type of PHA depolymerase found on the PHA granule surface differs from the extracellular PHA depolymerases made by many bacteria that are secreted to degrade PHA from the environment (Pötter and Steinbüchel 2005). Intracellular depolymerases like PhaZ found on the surface of PHA granules are important for the metabolism and mobilisation of PHA granules (Handrick *et al.* 2000). The nucleotide sequence of the gene encoding PhaZ1 from *C. necator* was first published in 2001 (Saegusa *et al.* 2001) and since then four more intracellular depolymerases of *C. necator* have been identified and designated PhaZ2-PhaZ5 (Schwartz *et al.* 2003; York *et al.* 2003). There exists experimental data indicating that *phaZ1* only appears to be expressed under nitrogen-starved, carbon-rich conditions (Pötter and Steinbüchel 2005). Another protein also known to be involved in depolymerisation is a 3-hydroxybutyrate-oligomer hydrolase, previously designated PhaZ2 but since renamed PhaY to avoid confusion with depolymerases already named PhaZ (Pötter and Steinbüchel 2005).

Phasins, the most abundant protein found at the PHA granule surface, are amphipathic with a hydrophobic domain that associates with the PHA granule and a hydrophilic domain that is exposed to the cytoplasm of the cell. Thought to stabilise PHA granules, phasins are also thought to influence the size and the number of PHA granules present (Kuchta *et al.* 2007; Pötter *et al.* 2002; Pötter *et al.* 2004; York *et al.* 2001a; York *et al.* 2001b). Four phasin gene homologs have so far been identified and confirmed to be intact in *C. necator* and have been designated *phaP1* to *phaP4* (Pötter and Steinbüchel 2005). Recent experimental evidence also suggests that PhaP1 is a planar triangular protein that occurs as a trimer (Neumann *et al.* 2008).

Experimental evidence shows that over-production of the PhaP1 protein, the main phasin of *C. necator,* results in large numbers of very small granules while mutant cells lacking PhaP1 accumulate only single larger granules in each cell (Kuchta *et al.* 2007). Phasins are abundant in granule-producing bacteria (Steinbüchel *et al.* 1995b) and are in turn regulated by an auto-regulated repressor PhaR which can bind to the promoter region of PhaP, the promoter region of PhaR or to granules (Pötter *et al.* 2002).

1.6 PHA granules as bio-nano beads

PHA beads isolated from recombinant bacterial cells could be adapted for use as beads for many different applications in science and biotechnology. As polymers of a size ranging from 50nm to 500nm they are versatile being fully biodegradable, non-toxic and tolerated by mammalian systems. Their size makes them small enough for fluorescent labelling, antigen display or drug delivery with an engineered immobilised protein displayed on the surface. By using bacteria to synthesise these bio-nano beads, PHA beads could potentially provide a novel one-step, self assembly and cost effective protein production system.

1.7 Protein production and immobilisation of functional proteins

Recombinant protein production is usually via 'microbial factories' where bacteria are genetically engineered to produce the protein of interest in excess Protein production usually requires purification steps and several protocols and systems exist for the purification of proteins that have an engineered binding domain of one protein referred to as a tag, fused to the protein of interest. There are many commercially available tags, the choice of which depends on the expression system used. Hexahistidine (His^{6x}) is commonly used whereby the DNA sequence of 18 bases coding for six histidine amino acids together is added to a gene of interest. The resulting protein can be purified based on the intrinsic charged properties of the hexahistidine or by binding to antibodies that recognise the His^{6x} structure. These affinity purification methods use peptides or antibodies chemically cross-linked (immobilised) to beads. The concept of immobilisation of a functional protein is not new and protein-coated beads are commercially available. There are many examples of methods available to bind and immobilise molecules on a matrix including biotinylation. Commercially available beads vary in size but are generally around 1-2.8 μM in diameter, two to twenty times larger than PHA granules. The cost of these commercial beads is high, around NZD500.00 for a 2 ml suspension containing biotinylated polymer beads at a concentration of 10^{10} beads per ml (Invitrogen 2007).

Engineering proteins with a tag for purification can be problematic. The addition of a tag may change the intrinsic properties of the protein of interest by affecting properties such as solubility, the net charge, or the ability to fold correctly. It is important that the fusion protein retains the activity of both constituent proteins and unfortunately, the addition of a tag to a protein may affect the activity of the protein of interest. This problem may be overcome by inclusion of a cleavable site to remove the tag after the purification or by selecting a tag that does not affect the activity of the protein. Misfolded protein produced may be refolded but this process is time consuming and tedious.

Another challenge to protein purification is that some purification methods using affinity and pH to elute the protein may in doing so permanently inactivate the protein or alter its native conformation.

When using bacteria as 'microbial factories' overproduction of the desired protein may be toxic to the production host. Over-production of a protein may also result in an increased tendency for the protein to misfold and form insoluble inclusions in the cytoplasm of the bacterial host. This may lead to the necessity for refolding as mentioned before or the misfolded protein may be toxic to the host.

The physical characteristics of PHA beads provide a possible platform for protein production within bacteria. PHA beads at around 50-500 nm are much smaller than commercial polymer beads so have relatively more surface area available, are biodegradable, tolerated by eukaryotic systems and contain a surface on which proteins can be immobilised by exploiting the PHA synthase PhaC or phasins such as PhaP1. *In vivo* immobilisation of PhaC and GFP fusions to PhaC has already been demonstrated (Peters and Rehm 2005) and PhaP1, the phasin protein abundant on the surface of PHA granules offers another possible protein for immobilisation of proteins (Banki *et al.* 2005; Barnard *et al.* 2005; Pötter and Steinbüchel 2005). As biological polyesters, they have elastomeric and plastic-like properties but are fully biodegradable. The synthase PhaC that remains covalently attached at the granule surface can tolerate N terminal fusions and could be engineered to display PhaC fused to another protein and immobilised at the granule surface. PhaP1, the surface-associated phasin protein is found in abundance and has already been shown to tolerate C terminal fusions (Barnard *et al.* 2005). Similarly, PhaP could also be engineered so that PhaP fusion proteins are immobilised at the granule surface. Both

systems offer advantages in protein production. There are possibly several benefits of using this method over conventional protein production and purification systems. Firstly, the protein production and immobilisation is part of a self-assembly process occurring within the bacterial host so there is no need to synthesise the protein then immobilise it on beads. Next, purification of the synthesised protein would be relatively simple, as simple as isolating the PHA beads. The desired protein could then be cut off by using a cleavable site (added during the design stages between the PhaC or PhaP and the protein of interest) recognised by an enterokinase.

Utilising the bio-beads for protein production, protein purification, or antigen display would be a novel use of PHA that could offer a much less expensive method than current commercially available systems.

1.8 The aim of this study

The purpose of this study is to generate bio-beads with functional immobilised surface proteins as a tool for protein purification in, for example, diagnostic tests (see appendix for a schematic).

We know that the protein from *Staphylococcus aureus* known as protein A binds to the Fc region of most classes of the immunoglobin G (IgG). A synthetic gene containing two copies of the region encoding the IgG binding domain of protein A was generated in 1987 (Lowenadler *et al.* 1987) and termed 'ZZ' and used as a tag to a protein of interest enabling affinity purification using commercially available IgG sepharose beads. The present study aims to fuse a 'ZZ' tag to the N-terminus of PhaC and produce PHA beads displaying the synthetic IgG binding domain to use as beads to purify IgG. Producing biopolymer beads inside bacterial cells in a one-step process offers a cost effective alternative to chemical cross-linking of beads.

This project also aims to produce PHA bio-beads displaying functional MOG or IL2. PHA synthase will be used for N terminal fusions with the IgG binding protein ZZ. Phasin protein will be used for C terminal fusions to MOG or IL2.

Confirmation of the fusion protein will be determined by matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF). Assessment of functionality of the proteins will be by the use of enzyme-linked immuno-assay

(ELISA) with conformational antibodies and fluorescent activated cell sorting (FACS) analysis. Assessment of PHA accumulation will be by gas chromatography mass spectrometry (G.C.M.S).

CHAPTER 2: MATERIALS AND METHODS

Unless otherwise indicated, reagents were purchased from Sigma, Ajax Finechem, or Merck.

2.1 Bacterial strains, culture conditions and chemicals

Table 3 contains the bacterial strains used and listed in Tables 5, 6 and 7 are the plasmids and oligonucleotides used in this thesis. All oligonucleotide primers used in this work were synthesised by Invitrogen.

Table 3. Bacterial strains used in this study

Strain	Relevant Characteristics	Reference
Escherichia coli:		
BL21 (DE3)	F $^-$;*omp*T *hsdS*$_B$(r_B^- m_B^-) *gal dcm*(DE3);pLysS(Cam^r)	Novagen/Merck
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)].*	Novagen
Cupriavidus necator		
H16	Wildtype (PHA+)	

2.1.1 Liquid media

Luria-Bertani (LB) media (Invitrogen) 20g in per litre of H_2O and the medium was autoclaved at 121°C for 20 minutes.

2.1.2 Solid media

10 g of Luria-Bertani (LB) media and 8 g of agar were dissolved in 500 ml H_2O. The medium was autoclaved at 121°C for 20 minutes.

2.2 Antibiotic stock solutions and final concentrations

The antibiotic solutions were prepared according to laboratory protocols (Sambrook *et al.* 1989), sterilised by filtration through a 0.22 µl filter and stored in aliquots at -20°C for further use. After autoclaving of the nutrient media and subsequent cooling to 50ºC, the antibiotics were added at the concentrations given below (Table 4).

Table 4. Antibiotic stock solutions and respective concentrations mg ml^{-1}.

Antibiotic	Concentration of stock solution (mg/ml) and diluent	Final concentration (µg/ml)
Ampicillin (sodium salt)	75 in H_2O	75
Chloramphenicol	50 in EtOH (95%)	50
Tetracycline	12.5 in EtOH (70%)	12.5

2.3 Preservation of strains

Strains were incubated overnight in LB liquid media at 37ºC on a Thermoline orbital shaker TLM510 (N.S.W, Australia) with the appropriate antibiotic and 1 ml of the resulting culture mixed with 60 µl sterile dimethylsulfoxide (DMSO) giving a final concentration of 7.5% (v/v) in 2 ml cryovial tubes and stored at -70°C for future use. For resuscitation of the strain 20 µl of this mixture was added to 20 ml of liquid LB media containing the appropriate antibiotics into a 150 ml conical flask and then incubated overnight at 37ºC on a Thermoline orbital shaker TLM510 (N.S.W, Australia).

2.4 Details of the cultivation conditions and plasmids

Cultivation in liquid cultures was performed in Erlenmeyer flasks and the ratio between the volumes of the flask to the volume of the liquid kept high (10:1). The cultivation was started with a 1% (v/v) inoculum from overnight-incubated cultures (tables 1 and 2). Cultivations were performed in liquid LB media containing the respective antibiotics and, when required, 1% glucose as the carbon source. Cultivations were at 37°C for *E.coli* XL1-Blue or 30°C for *E.coli* BL21(DE3) and

flasks were agitated on a Thermoline orbital shaker TLM510 (N.S.W, Australia) at approximately 200rpm.

Table 5. Plasmids and oligonucleotides used in the ZZ-PhaC study.

Plasmid name	Characteristics	Reference
pCWE	pBluescript SK(-) derivative containing the PHA synthase gene from *C. necator*	(Peters and Rehm 2005)
pCWE-ZZ(-)phaC	pCWE derivative containing the ZZ domain-encoding *Nde*I fragment lacking the signal sequence-encoding region	(Brockelbank et al. 2006)
pCWE-ZZ(+)phaC	pCWE derivative containing the ZZ domain-encoding *Nde*I fragment plus the signal sequence-encoding region	(Brockelbank et al. 2006)
pET14b	Ap^r; T7 promoter	Novagen
pET14b-ZZ (-)phaC	pET14b containing *Xba*I /*Bam*HI fragment comprising gene *ZZ-phaC* without the signal sequence-encoding region	(Brockelbank et al. 2006)
pET1b-ZZ(+)phaC	pET14b containing *Xba*I /*Bam*HI fragment comprising gene *ZZ-phaC*	(Brockelbank et al. 2006)
pBHR69-ZZ(-)phaC	pBHR69 derivative containing the hybrid PHA synthase gene from pCWE-ZZ(+)phaC upstream of genes *phaA* and *phaB* of *C. necator* co-linear to *lac* promoter	(Brockelbank et al. 2006)
pBHR69-ZZ(+)phaC	pBluescript derivative containing genes *phaA* and *phaB* of *C. necator* co-linear to *lac* promoter	(Brockelbank et al. 2006)
pEZZ18	Ap^r; Lac promoter, encoding the IgG binding domain ZZ from protein A	GE Healthcare
pBHR69	pBBR1MCS derivative containing genes *phaA* and *phaB* of *C. necator* co-linear to *lac* promoter	(Qi and Rehm 2001)
pMCS69	pBBR1MCS derivative containing genes *phaA* and *phaB* of *C. necator* co-linear to *lac* promoter	(Hoffman and Rehm 2005)

Primer name	Restriction site (underlined)	Sequence from 5' to 3'	
5'-ZZ(+)-NdeI	*Nde*I	GCGCG<u>CATATG</u>ACTTTACA AATACATACAGGGGGTATT AATTTG	(Brockelbank *et al.* 2006)
3'-ZZ-NdeI	*Nde*I	GTAAT<u>CATATG</u>GGGTACCG AGCTCGAATTCGCGTCTAC	

Table 6. PhaP-MOG/IL2 study.

Plasmid name	Characteristics	Reference
PCRII	Ap^r ColE1 origin	Invitrogen
PCR-phaP	pCRII containing phaP gene from *C. necator*	(Bäckström et al. 2007)
pHAS	pET-14b containing *Nde*I/*Bam*HI-inserted *phaCWe* gene from *C. necator*	(Yuan et al. 2001)
pHAS-phaP	pHAS containing the *phaP* gene inserted into *Xba*I /*Nde*I sites	(Bäckström et al. 2007)
pHAS-phaP-MOG	pHAS-PhaP containing the MOG encoding fragment inserted into *NdeI/BamH*I sites	(Bäckström et al. 2007)
pHAS-phaP-IL2	pHAS-PhaP containing the IL2 encoding fragment inserted into *NdeI/Bam*HI sites	(Bäckström et al. 2007)
pBHR68-phaP-IL2	DNA fragment encoding PhaP-IL2 fusion protein subcloned from pUC57-phaP-IL2 via *Xba*I and *Bam*HI into pBHR68	(Bäckström et al. 2007)
pBHR68-phaP-MOG	DNA fragment encoding PhaP-MOG fusion protein subcloned from pUC57-phaP-MOG via *Xba*I and *Bam*HI into pBHR68	(Bäckström et al. 2007)
pUC57IL2	DNA fragment encoding N terminal 60–169 amino acids of IL2 from mouse inserted in *Sma*I site of pUC57	(Bäckström et al. 2007)
pUC57MOG	DNA fragment encoding N terminal 1–173 amino acids of MOG from mouse inserted in *Sma*I site of pUC57	(Bäckström et al. 2007)
pUC57-phaP-IL2	DNA fragment encoding *phaP* inserted into *Xba*I and *Nde*I site of plasmid pUC57-IL2	(Bäckström et al. 2007)
pUC57-phaP-MOG	DNA fragment encoding *phaP* inserted into *Xba*I and *Nde*I site of plasmid pUC57-MOG	(Bäckström et al. 2007)

Primer name	Restriction site (underlined)	Sequence from 5’ to 3’	
5’ - phaP-*Xba*I	*Xba*I	AAAAA<u>TCTAGA</u>AAAAGG AGATATACGTATGATCC TCACCCCGGAACAAG	(Bäckström *et al.* 2007)
3’ - phaP-NdeI	*Nde*I	ACC<u>CATATG</u>GTGGTGAT GGTGATGCGAGC	

Table 7. Double functionality study.

Plasmid name	Characteristics	Reference
pBHR68 NS_EXTPhaP-MOG	Intermediate cloning vector containing the DNA fragment encoding EXTPhaP-MOG without a start codon (ATG)	(Atwood & Rehm, 2008)
pBHR68EXTPhaP-MOG	Intermediate cloning vector containing the DNA fragment encoding SpeI-EXTPhaP-MOG inserted at *Spe*I and *Bam*HI of plasmid pBHR68	(Atwood & Rehm, 2008)
pBHR68GPM	DNA fragment encoding GFP-EXTPhaP-MOG generated by inserting the GFP fragment subcloned from pBHR69gfpC *Spe*I site of pBHR68SpeEXTPhaP-MOG	(Atwood & Rehm, 2008)
pBHR68 SpeEXTPhaP-MOG	Intermediate cloning vector containing the DNA fragment encoding the fusion protein EXTPhaP-MOG	(Atwood & Rehm, 2008)
pBHR69-gfp-phaC	Intermediate cloning vector containing the DNA fragment encoding the fusion protein GFP-PhaC	(Atwood & Rehm, 2008)
pBHR69-gfp-phaC	Intermediate cloning vector containing the DNA fragment encoding the fusion protein GFP-PhaC from pCWEAgfp	(Atwood & Rehm, 2008)

Primer name	Restriction site (underlined)	Sequence from 5' to 3'	
5'- (-) ATG *Xba*-PhaP	*Xba*I	CGCTCTAGAAAAAGGAGATATACGTGCAATCCTCACC	
3'- Nde-PhaP	*Nde*I	ACCCATATGGTGGTGATGGTGATGCGAGC	(Atwood & Rehm, 2008)
5'-SpeEXTphaP-MOG	*Spe*I	CAAACTAGTCTCCTAAATAGCTATGACCATGATTACGCCAAGCGCGC	
3'-SpeEXTphaP-MOG	*Bam*HI	CGGGGGATCCTTAATCTTCAACTTTCAGTTCCATGGCGGC	(Atwood & Rehm, 2008)
5' - *Xba*-PhaP-MOG	*Xba*I	GGCCGCTCTAGAATAAAGGAGATATACGTATGATCCTCACCCCGG	
3' –*Xba*-PhaP-MOG	*Bam*HI	GATTCTAGATCTTCAACTTTCAGTTCCATGGCGGCTTCTTCCTGGTA	(Atwood & Rehm, 2008)

2.5 Oligonucleotide design and cloning strategy

The design of the oligonucleotides used as PCR primers, the cloning strategy, primer design and the construction of the initial expression vectors containing a ZZ fusion to PhaC was assisted by Verena Peters (Brockelbank *et al.* 2006), (Table 5) and all subsequent cloning steps performed by the author (see 2.5.1). Design of the oligonucleotides used for PCR primers, the cloning strategy and initial constructs used for the PhaP-MOG/PhaP-IL2 work was done in collaboration with Thomas Bäckström (Table 6) (Bäckström *et al.* 2007) and all subsequent cloning steps performed by the author (see 2.5.2). Design of the oligonucleotides used for PCR primers and the cloning strategy for further work on multifunctional PHA beads was done by the author (Table 7, section 2.5.3, Figure 9 and Figure 10).

2.5.1 ZZ-PhaC cloning method

The DNA fragment encoding the synthetic gene *ZZ* with or without the signal sequence was generated by PCR using primers 5'-ZZ(+)-NdeI and 3'-ZZ-NdeI and the commercially available vector pEZZ18 (Lowenadler *et al.* 1987) as template. The constructed plasmids were given their parent name and a (+) or (-) based on the presence or absence (+/-) of the signal sequence for ZZ. Vector pCWE was linearised at restriction site *Nde*I and the PCR products ligated at this *Nde*I restriction site generating plasmids pCWE-ZZ(+)phaC and pCWE-ZZ(-)phaC (Table 5, Figure 7).

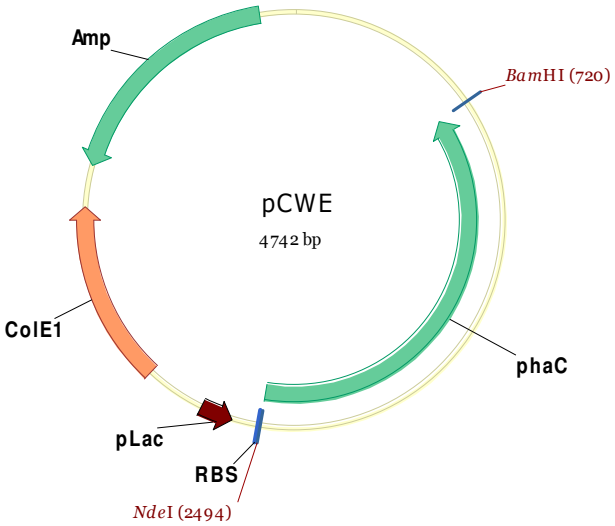


Figure 7. pCWE contains the gene *phaC* from *Cupriavidus necator* and an in-frame *Nde*I restriction site at the N-terminus of *phaC* to allow the addition of the ZZ fusion partner.

2.5.2 PhaP-IL2 Cloning Method

The region encoding the protein phaP was amplified by PCR from plasmid pCR-phaP using oligonucleotides 5' phaP-XbaI including an *E. coli* ribosomal binding site and 3' phaP-NdeI. The resulting PCR product was subcloned into the *Xba*I and *NdeI* sites of pHAS generating intermediate vector pHAS phaP. Either pUC57-MOG or pUC57-IL2 was hydrolysed with *Nde*I and *Bam*HI and the corresponding DNA fragments were subcloned into pHAS-phaP resulting in plasmids pHAS-phaP-MOG and pHAS-phaP-IL2, respectively. The respective fusion protein-encoding region was then subcloned into pBHR68 using *Xba*I and *Bam*HI sites downstream of the *lac* promoter and upstream of the PHB biosynthesis operon (Figure 8).

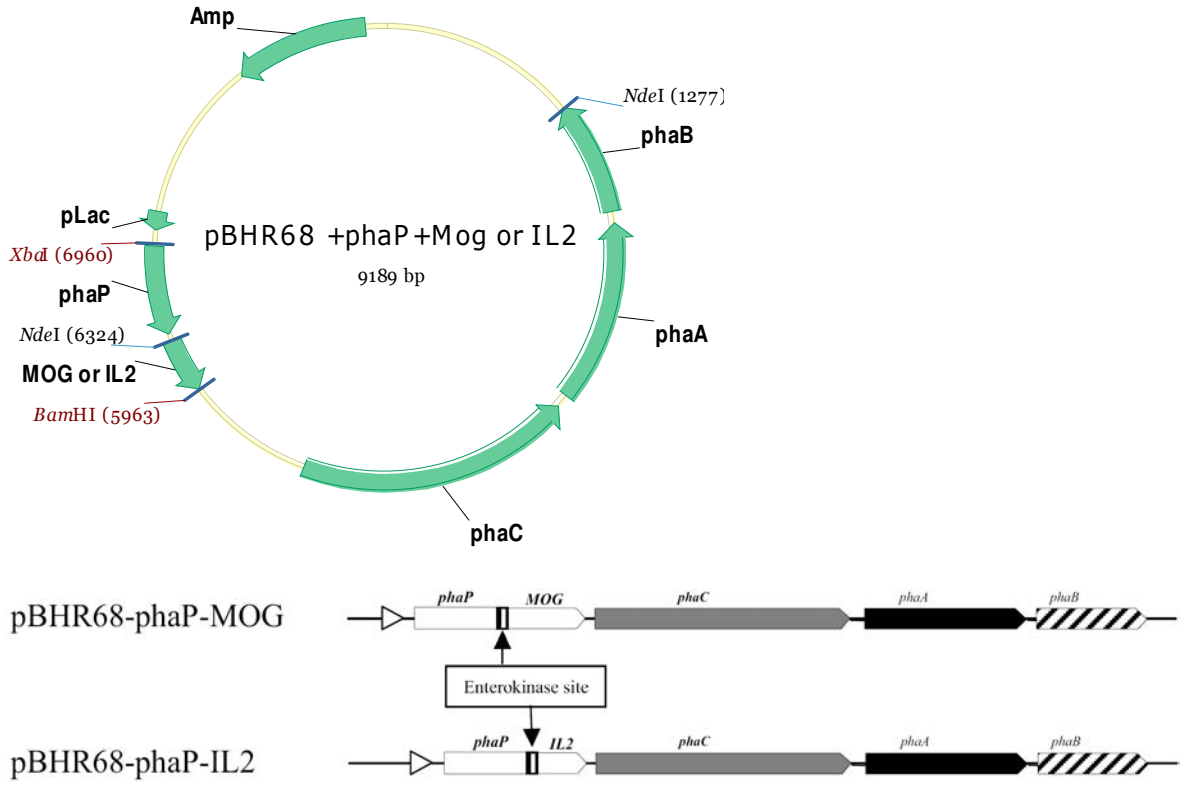


Figure 8. Vector map and schematic diagram of the fusion MOG and IL2 constructs (modified from Bäckström *et al.,* 2007).

2.5.3 Construction of PHA beads displaying two functional proteins

Two constructs were generated, one contained GFP fused N terminally and MOG fused C terminally to PhaP. The other construct contained the insertion of the DNA fragment encoding PhaP-MOG upstream of the DNA fragment encoding Gfp-PhaC in the vector pCWEA gfp (Figure 9 and Figure 10).

2.5.3.1 pBHR68GPM construction

The plasmid pBHR68GPM was constructed to enable production of polyester inclusions with an attached phasin protein showing an N and C terminal fusion. The N and C terminal fusion partners were the fluorescent protein GFP and the antigen murine myelin oligodendrocyte glycoprotein (MOG), respectively. The DNA fragment encoding the protein PhaP without a start codon (ATG) was generated by PCR amplification using oligonucleotides '5'(-)ATG_EXTPhaP containing the restriction site *Xba*I and with reverse primer '3' NdeI-PhaP'. This PCR fragment was ligated into intermediate vector pHAS PhaP-MOG at restriction sites *Xba*I and *Nde*I replacing the wildtype *phaP* gene. The *Xba*I-*Bam*HI fragment from the resulting plasmid pHAS EXTPhaP-MOG containing the hybrid gene encoding the fusion protein PhaP-MOG minus the start codon was subcloned into the *Xba*I-*Bam*HI restriction sites of pBHR68 resulting in plasmid pBHR68 (-)ATG PhaP-MOG. This vector was used as a template to make a PCR product that extended PhaP 34 amino acids and introduced a *Spe*I restriction site with primers 5'SpeEXTPhaP-MOG and 3'SpeEXTPhaP-MOG. The *Spe*I–*Bam*HI PCR fragment of SpeEXTPhaP-MOG was ligated back into pBHR68 generating plasmid pBHR68SpeEXTPhaP-MOG. Next, the enhanced GFP encoding *Spe*I DNA fragment (720bp) from plasmid pCWEAgfp was subcloned into the *Spe*I site of plasmid pBHR68SpeEXTPhaP-MOG resulting in pBHR68GPM.

2.5.3.2 pBHR69GCPM construction

The plasmid pBHR69GCPM was constructed to enable simultaneous production of the GFP-polyester synthase and phasin-MOG fusion protein attached to the polyester beads surface. The DNA fragment encoding the fusion protein GFP-PhaC was subcloned from pCWEAgfp into pBHR69 using restriction sites *Xba*I-*Bam*HI

generating plasmid pBHR69-gfp-phaC. *Xba*I restriction sites were added by PCR to generate a DNA fragment encoding PhaP-MOG while using pBHR68PhaP-MOG as template and primers 5' Xba-PhaP-MOG as well as 3' *Xba*-PhaP-MOG. The PCR product was ligated into the *Xba*I site of pBHR69-gfp-phaC resulting in plasmid pBHR69GCPM.

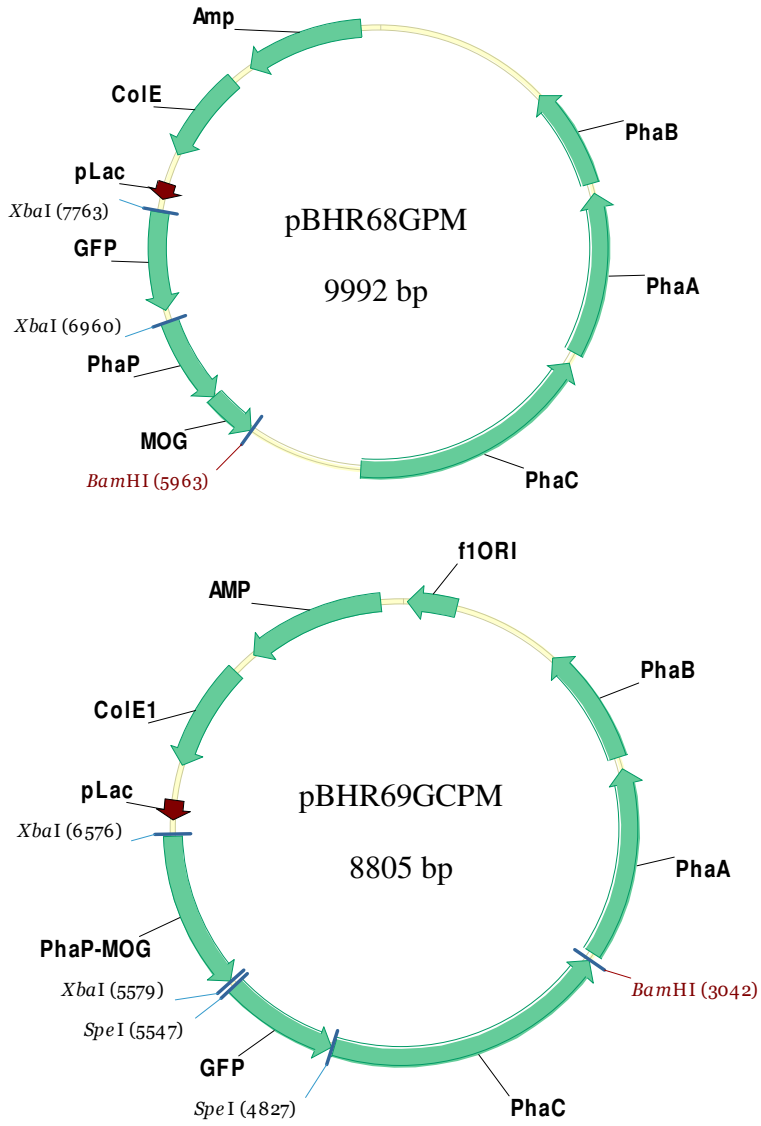


Figure 9. Vectors pBHR68GPM and pBHR69 GCPM.

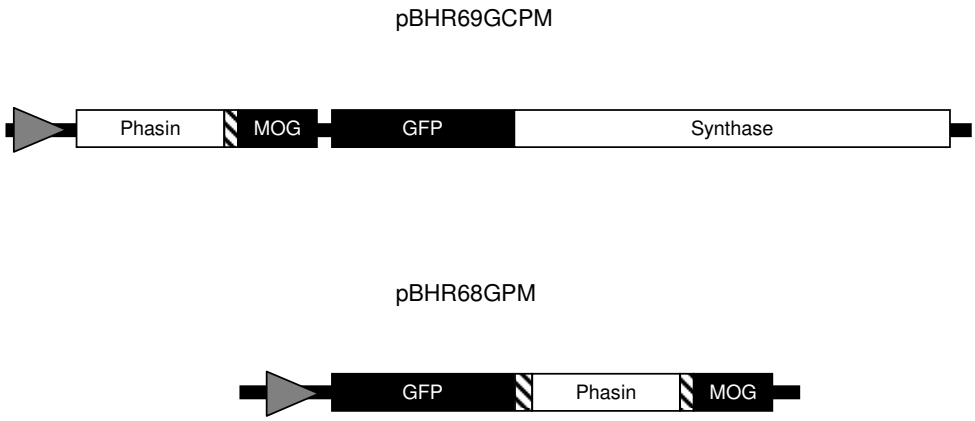


Figure 10. Schematic representation of hybrid genes for production of the respective fusion proteins. Triangle, *lac* promoter; diagonally striped rectangles, linker regions; MOG, murine myelin oligodendrocyte glycoprotein; GFP, green fluorescent protein; Synthase, polyester synthase (Atwood and Rehm, 2008).

2.6 Preparation of competent cells for long term storage

As previously described by Hanahan (Hanahan 1983; Hanahan 1985), the *E.coli* strain was cultivated in 50 ml LB liquid medium at 37°C until OD_{600} reached 0.3. After 10-15 minutes incubation on ice, the cells were harvested by centrifugation at 2250 x g in a Heraeus Multifuge 1 S-R, (Germany), resuspended in 18 ml RF1 solution and incubated on ice for a further 30 minutes. Cells were then again centrifuged for 15 minutes at 2250 x g (Heraeus Multifuge 1 S-R Germany) and resuspended in 4 ml of RF2 solution. The resulting competent cells were then aliquoted to 1.5 ml microfuge tubes (200 µl per tube) and stored at -70°C.

RF1 solution

100 mM	RbCl
50 mM	$MnCl_2$
30 mM	Potassium acetate
10 mM	$CaCl_2.6H_2O$

Adjust the pH to 5.8 with acetic acid.

RF2 solution

10 mM	RbCl
10 mM	MOPS
75 mM	$CaCl_2.6H_2O$
15% (v/v)	Glycerol

Adjust the pH to 5.8 with NaOH.

2.7 Transformation of *E.coli* cells

200 µl of competent cells were mixed thoroughly with 1-2.5 µl of plasmid DNA and incubated for 60 minutes on ice to allow the DNA to become adsorbed at the surface of the competent cells. For the uptake of the adsorbed DNA, the cells were heated at 42°C for 90 seconds and then placed back on ice for 5 minutes. For regeneration of the cells and for expression of the plasmid-encoded antibiotic resistance, 800 µl of LB was added and the cells incubated at 37°C for one hour. For isolation of the recombinant clones, the cells were pelleted by centrifugation for 2 minutes at 5000 x g (Heraeus Biofuge Pico, Germany) and resuspended in 350 µl fresh LB, 50-100 µl were spread onto solid media plates containing the appropriate antibiotic and the plates incubated overnight at 37°C.

2.8 Isolation of plasmid DNA

Strains containing the respective plasmid were incubated in 20ml LB medium with the appropriate antibiotics at 37°C overnight. 1 ml of culture in a microfuge tube was centrifuged at 10,000 x g for 3 minutes (Biofuge Pico, Heraeus, Germany), the supernatant was removed, 100 µl ice-cold GET solution was added to the pellet and the pellet resuspended. This was followed by the addition of 200 µl freshly prepared SDS-NaOH solution. After gently inverting the microfuge tube a few times, a clear solution was obtained indicating cell lysis was complete. The protein was precipitated by addition of 150 µl of HSS solution. After centrifugation at 10,000 x g for 30 minutes, the supernatant was transferred to a new microfuge tube and centrifuged at 10,000 x g for a further 15 minutes. The supernatant was transferred to

a new microfuge tube containing 350 µl ice-cold isopropanol and centrifuged at 10,000 x g for 30 minutes. The supernatant was poured off and 300 µl of 70% ice-cold ethanol added. The pellet was resuspended by vortexing and the tube centrifuged at 10,000 x g for a further 30 minutes. The supernatant was poured off, the tube allowed to dry at room temperature and the dried plasmid DNA dissolved in 30 µl of TER buffer and stored at -20°C. The recipes for the solutions used are given in Table 8.

Table 8. Reagents for DNA isolation.

GET solution:		
	25 mM	Tris/HCl, pH 8.0
	10 mM	EDTA
	50 mM	Glucose
SDS-NaOH solution:		
	200 mM	NaOH
	1% (v/v)	SDS
HSS solution		
	3M	Potassium acetate
	1.8M	Formic acid pH 4.8
RNase solution: to make TER buffer		
	150 mM	NaCl
	1% (v/v)	RNase A
T E Buffer: to make TER buffer		
	10 mM	Tris/HCl
	1 mM	EDTA, pH 8.0
TER Buffer	10 µl RNase solution: 990 µl TE buffer	

2.8.1 Determination of the size of DNA fragments

The size of the DNA fragments was estimated by comparison with fragments of known size on agarose gel electrophoresis. As a size standard (marker), phage lambda DNA previously digested with the restriction endonucleases *Pst*I to fragments with known size was used (Sambrook *et al.*, 1989).

2.8.2 Determination of the DNA concentration

The purity and concentration of the DNA solution was determined by at least one of two methods. The first was by agarose gel electrophoresis and/or measuring the absorption at 260 nm and 280 nm using the Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA).

2.8.3 Agarose gel electrophoresis (AGE)

The agarose gel electrophoresis was performed in horizontal slabs. For routine analysis of a DNA sample, 1 – 2 % agarose in TBE buffer was used. DNA samples containing the respective stop-mix (Table 9) was loaded into the wells in the gel. The gel electrophoresis conditions were 100-160 V for 30 – 60 minutes using a Biometra gel electrophoresis unit (Biometra, Germany). After electrophoresis, the gel was stained in ethidium bromide solution for approximately 15 minutes, rinsed briefly in distilled water and then detected by UV transilluminator at 254 nm. An image was generated using GELDOC software.

Table 9. TBE buffer and stop mix.

TBE Buffer	Stop-Mix (6 X)
50 mM Tris/HCl	4 M Urea
50 mM Boric Acid	50 mM EDTA
2.5 mM EDTA	50% (v/v) Sucrose
approx pH 8.5	0.1% Bromophenol Blue

2.8.4 DNA A-tailing procedure and ligation of fragments

To obtain A-tailed DNA from blunt-end PCR products a purified fragment generated by a proofreading polymerase (Pfx polymerase, Invitrogen) was used.

A typical reaction mixture contained the following:

1-6 µl PCR fragment

1 µl Taq polymerase(Invitrogen)

1 µl 10 x Taq reaction buffer without $MgCl_2$ (Invitrogen)

1 µl 50 mM $MgCl_2$ (Invitrogen)

1 µl dATP (0.2 mM) (Roche)

in a final reaction volume of 10 µl and incubated at 70°C for 30 minutes. 3.5 µl of this reaction was used in a ligation mixture containing the following:

3.5 µl A-tailed PCR product

1 µl pGEM-T easy ligase (Promega)

5 µl pGEM-T easy 2 x ligase buffer (Promega)

0.5 µl pGEM-T easy vector (Promega)

The final reaction volume was 10 µl. This was incubated at 4ºC by floating tubes in water (temperature approximately 10ºC) then transferring the container to a fridge and leaving overnight.

2.8.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed for the amplification of DNA fragments of template DNA which was flanked by specific oligonucleotide primers. The reaction was performed by repeated cycles of denaturation, annealing and extension or elongation in a Biometra T Personal Combi cycler (Germany). Platinum Pfx polymerase (Invitrogen) was used and the manufacturer's instructions followed accordingly. The reaction mixture is listed in Table 10.

Table 10. PCR reaction mixture.

In a 100 µl volume	Final Concentration
10 µl amplification buffer	1 x
5 µl 50 mM Magnesium Sulphate	2.5 mM
5 µl DMSO	5%
10 µl each of forward and reverse primers	0.1 µM of each
10 µl dNTPs of a mixture dATP, dTTP, dCTP and dGTP*	0.1 mM of each
1.5 µl Template DNA	up to 300ng
0.5 µl Pfx polymerase (1 unit)	0.4 - 1.0 µl = 1.0 - 2.5 units
48.5 µl Autoclaved distilled water	
* (Roche NZ Ltd)	

2.8.6 DNA sequencing

Sequencing of recombinant plasmids or amplified PCR products was performed by Allan Wilson Centre Genome Service using a capillary ABI3730 Genetic Analyzer, from Applied Biosystems Incorporated. Each sequencing reaction tube contained 300 ng of DNA and primers were added at a concentration of 3.2 pM in 15 µl. Results were provided in ABI format and analysed using software program Vector NTI version 9.

2.9 PHA extraction, preparation and analysis

2.9.1 Cell disruption and crude extract preparation

Cell harvesting from cultures was performed by centrifugation for 20 minutes at 4°C and 5000 x g in a Heraeus Multifuge 1 S-R (Germany). After the cells were harvested and washed with 50 mM potassium phosphate buffer (pH 7.5), they were centrifuged again for a further 20 minutes at 4°C and 5000 x g. Then the cell pellet was resuspended in 50 mM potassium phosphate buffer (recipe given below) (pH 7.5) and DNaseI (10 µg ml^{-1}) and lysozyme (300 µg ml^{-1}) was added and incubated

on ice for 30 minutes. This crude cell extract was then subjected to mechanical cell disruption (16 kPsi). A flow diagram sets out the method used for isolation of PHA from the resulting crude cell extract (Figure 11).

Potassium Phosphate buffer pH 7.5

50 mM K_2HPO_4 (base)

50 mM KH_2PO_4 (acid)

Mix the two above together adjusting the ratio as necessary to obtain a pH of 7.5.

2.9.2 Isolation of PHA from crude extract

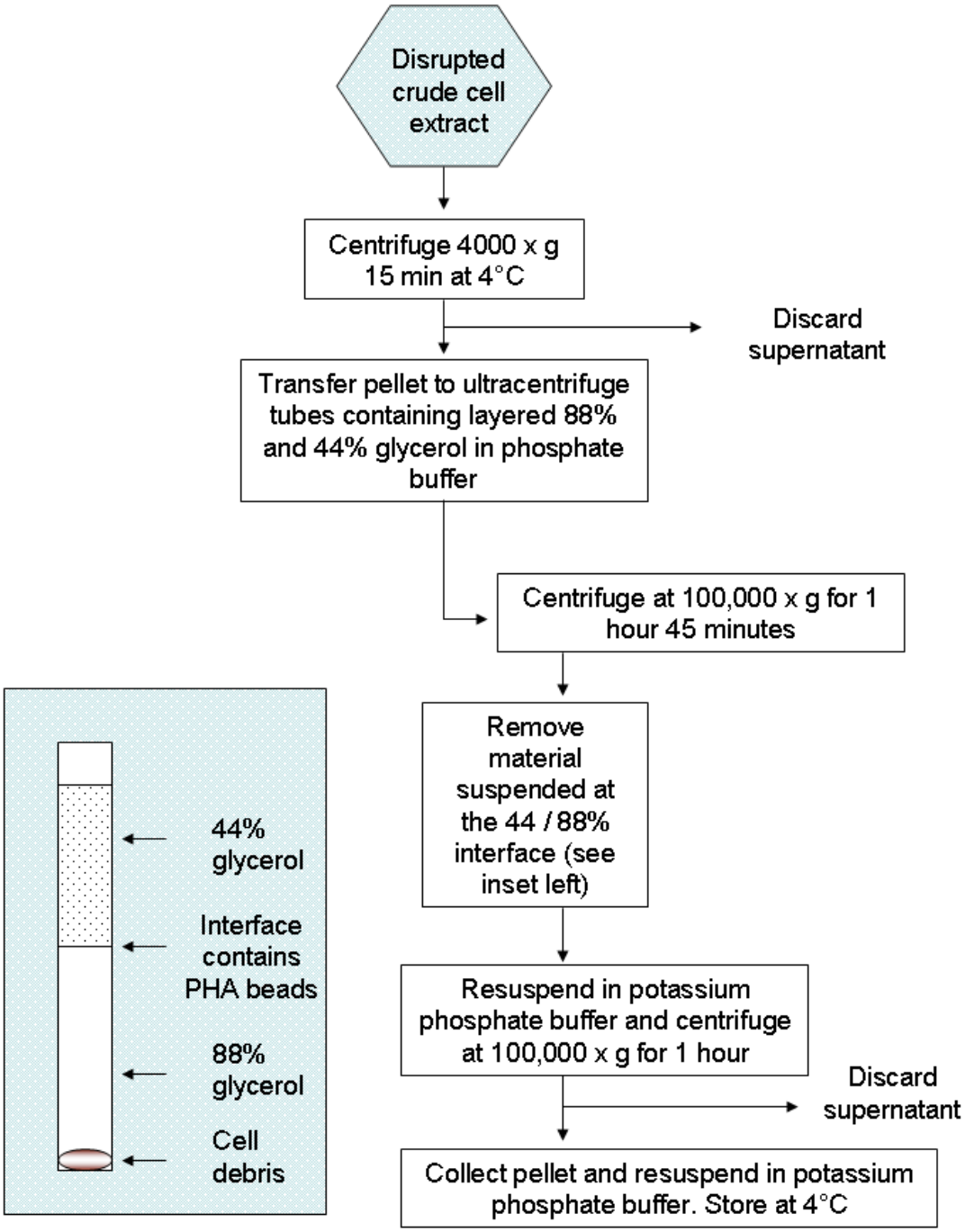


Figure 11. Diagram showing the process for isolation of PHA beads from crude cell extract. Inset (blue shaded box) is a schematic showing density separation of PHA beads after ultracentrifugation.

2.10 Detection of PHA accumulating colonies by staining with Nile Red

Screening for PHA accumulating colonies was performed according to Spiekermann *et al.,* 1999). For routine analysis, 0.002 vol. of a solution of 0.25 mg Nile Red (Sigma, St Louis, USA) per ml dimethylsulfoxide (DMSO) was added to the sterilised medium to give a final concentration of 0.5 µg dye per ml. These plates were then incubated with cultures of interest and incubated for 24-48 hours at 37ºC. The agar plates were then exposed to ultraviolet light (312nm) to detect accumulation of PHAs. Cultures in liquid media were prepared by centrifugation of a 1 ml aliquot in a microfuge tube. The cells were washed in potassium phosphate buffer (pH 7.5) and resuspended in 100 µl of potassium phosphate buffer (pH 7.5). 10 µl of the Nile Red stock solution (0.25 mg/ml DMSO) was added and the cells incubated at room temperature for 15 minutes. The cells were then washed in potassium phosphate buffer (pH 7.5) twice to remove unbound Nile Red and resuspended in 1 ml of potassium phosphate buffer. 2 µl was spotted on to a glass slide, a cover slip added and the slide was examined using fluorescent microscopy (Olympus) and Magnafire imaging software was used to capture images digitally.

2.11 Sample preparation for Gas Chromatography Mass Spectrometry analysis (GCMS)

10 mg lyophilised cells or PHA beads were suspended in 2 ml of chloroform and subjected to methanolysis in 2 ml methanol in the presence of 15% (v/v) sulphuric acid. The methanolysis was performed for 5 hours at 100°C in an oil bath, then the tubes were transferred to an ice bath. 2 ml water was added to the cooled mixture and mixed thoroughly by vortexing for 30 seconds. After phase separation, the resulting methyl esters of the corresponding fatty acid constituents were assayed by gas chromatography (GC) (Brandl *et al.* 1998; Timm and Steinbüchel 1992). The quantitative analysis of 3-hydroxyalkanoate methyl ester was performed by Hort Research (Palmerston North).

2.12 General methods for protein analysis

2.12.1 Protein concentration measurement (Bradford 1976)

200 µl of Bradford reagent was added to 10 µl of the sample to be measured (0.05-0.4 µg/ml) and the colour absorbance change measured at 595nm by an ultra microplate reader ELx808 IU (Biotek, Vermont USA). A standard curve was prepared with BSA (bovine serum albumin) in concentrations ranging from 0.05 µg – 0.4 µg ml $^{-1}$. Bradford reagent was purchased from Biorad Laboratories (California, USA) and was used according to the manufacturer's instructions.

2.12.2 Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

For characterisation and evaluation of proteins, SDS-PAGE was performed in an SDS-PAGE vertical slab gel electrophoresis apparatus as described by Laemmli *et al.* (1970) and Sambrook *et al*. (1989).

Each gel consisted of a stacking gel (4% polyacrylamide, pH 6.8) and a separating gel (8 – 12% polyacrylamide w/v, pH 8.9). The separating gel was prepared in a 0.8 – 1.5mm slab gel (ca. 20 x 20 cm) or vertical mini-gel (ca. 8.5cm x 7 cm).

The separating gel solution prior to polymerisation was degassed to remove gas and avoid formation of air bubbles by addition of approximately 0.01g of Na_2SO_3. Then 40 µl of 40% (w/v) ammonium persulfate and 20 µl of N, N, N', N'-tetramethylethyl-endiamin (TEMED) added to the solution. The solution was carefully poured between the glass plates and immediately overlaid with isopropanol. After 30 minutes for polymerisation, the isopropanol layer was poured off, the upper chamber washed with distilled water, and the stacking gel prepared.

Separating gel 8%

10.4 ml	Separating gel buffer
11.1 ml	30% Acrylamide
20.1 ml	Distilled water

41.6ml total volume

Stacking gel 4%

3.12 ml	Stacking gel buffer
1.88 ml	30% acrylamide
7.5 ml	Distilled water

12.5 ml total volume

Separating gel buffer

54.45 g	Tris/HCl
1.2 g	SDS
300 ml	Distilled water

Adjust pH to 8.9 with HCl.

Stacking gel buffer

18.14 g	Tris/HCl
1.2 g	SDS
300 ml	Distilled water

Adjust pH to 6.8 with HCl.

SDS denaturing buffer:

8.0 g	SDS
20 ml	b - mercaptoethanol
37.2 g	EDTA
40 ml	Glycerol
5 mg	Bromophenol blue

Make up to 100 ml with distilled water.

Electrode buffer:	Adjust with HCl pH to 8.5
28.8g	Glycine
6.0g	Tris
2g	SDS

Make up to 2 L with distilled water.

2.12.3 Preparation of protein samples for SDS-PAGE

2 volumes of the protein solution were mixed with 1 volume of the SDS denaturing buffer and incubated for 15 minutes at 95°C. The electrophoresis conditions were 15

mA in the stacking gel and 26 mA in the separating gel layer. Relative molecular weights of proteins were estimated by comparing the motility of the proteins with the motility of molecular weight standard proteins. The Coomassie blue prestained broad range protein marker (2-212 kDa) was used (New England Biolabs).

2.12.4 Protein staining with Coomassie Blue

(Weber and Osborn 1969). The SDS-PAGE gel was stripped from the gel plate, transferred to Coomassie blue staining solution, and stained on a slow shaker for 20-30 minutes. Then the gel was washed thoroughly with water and then destained until the background colour had gone.

Coomassie blue staining solution

4 g	Coomassie blue R 250
300 ml	Ethanol
100 ml	Acetic acid
600 ml	H_2O

Destaining solution

600 ml	Ethanol
200 ml	Acetic acid
1200 ml	H_2O

2.13 Determination of fusion protein activity

The bead suspensions were standardized and the activity of the fusion protein displayed on the surface determined by Enzyme Linked Immunosorbent Assay (ELISA) or Fluorescence Activated Cell Sorting (FACS) analysis as described at section 2.15 and section 2.16.

2.14 Standardisation of beads – quantification

The optical density OD_{600} was used to determine the number of beads per ml or the protein concentration of the beads was determined with Bradford's methods (see 2.12) to standardise samples. To calculate the number of PHA beads per ml, a suspension of beads with known optical density (OD_{600}) were stained with Nile Red (see 2.10), added to a cell haemocytometer and manually counted to obtain a standard curve.

2.15 Confirmation of functional protein at the surface of PHA beads : ELISA assay

Conformational antibodies were used in an ELISA assay to confirm that the fusion protein was active at the surface of the PHA beads.

Equal amounts of PHA bead protein (0.37 µg), corresponding to 2.6 µg polyhydroxybutyrate suspended in 100 µl PBS buffer pH 7.5 were added to each well of a high-binding capacity microtitre plate (catalogue # 6550610, Greiner bio-one) and incubated overnight at 4°C. The wells were then washed 3 times with PBS-tween buffer (blocked with the addition of 150 µl 3% BSA solution for 30 minutes.

PBS-Tween buffer (10 x) pH 7.4	
H_2O	1000 ml
$N_a2HPO_4.12H_2O$	29 g
NaCl	80 g
KH_2PO_4	2 g
KCl	2 g
$Tween^{20}$	0.05%

After washing 3 times with PBS-tween buffer, 100 µl of a 1:1000 dilution of whole human serum was added and the plate incubated at room temperature in a humidified box for 60 minutes. The plate was then washed again 3 times with PBS-tween buffer and then a secondary antibody, goat polyclonal anti-human IgG–horseradish peroxidase

(HRP) conjugate (Sigma A0293), added at a dilution of 1:2000 for detection of bound human IgG. The plate was then incubated at room temperature in the dark for a further 30 minutes then washed with PBS-tween buffer 10 times. 100 µl of o-Phenylendiamin.2HCl (OPD) reagent (Abbot Laboratories) was added after making up according to the manufacturer's instructions and the plate incubated in the dark for 15 minutes. The reaction was stopped by the addition of 50 µl 0.75M H_2SO_4 and the plate was read at 490 nm. For binding-release experiments, 50 mM glycine pH 3.4 was used for elution of bound IgG.

2.16 FACS analysis

FACS analysis of PhaP-MOG and PhaP-IL2 beads was performed by Thomas Bäckström (Malaghan Institute for Cancer Research, Wellington) (Bäckström *et al.* 2007). Analysis of the ZZ-PhaC, GPM and GCPM beads was performed by Natalie Parlane (AgResearch, Hopkirk Centre, Palmerston North) using a FACScalibur after preparation by the author of samples as follows:

2.16.1 Materials, methods and reagents for FACS staining

FACS buffer - PBS + 1% FCS (filtered) + 0.01% sodium azide
Primary antibody
Primary antibody control/isotype control
Secondary antibody conjugate
FACS tubes

Beads were diluted to a suspension containing 1 mg/ml protein (2.12.1) then 25 µl was dispensed in to a 2 ml microfuge tube. FACS buffer was added to make a total volume of 2 ml then the tube was centrifuged for 5 minutes at 1000 x g at 4ºC. The supernatant was discarded and the primary antibodies diluted to the manufacturer's instructions (approximately 1:200) in cold FACS buffer. 50 µl of the diluted primary antibody was added to the relevant tubes, mixed by vortexing and incubated for 20-30 minutes on ice. The bead-antibody suspension was then washed twice with FACS buffer (2 ml volume). The secondary antibody was diluted in cold FACS buffer according to the

manufacturer's instructions then 50ul added and the beads incubated for 20-30 minutes on ice in the dark. The supernatant was discarded and the bead-antibody suspension washed two times with FACS buffer (2 ml volume). The supernatant was discarded and 100ul of FACS buffer added. Tubes were covered with foil to exclude light and stored at 4°C until analysis was performed with FACScalibur.

Controls were as follows: PHA beads prepared without addition of the primary antibody and another group without addition of the secondary labeled antibody. In addition, wildtype beads were used as a further control to exclude the possibility of unspecific antibody binding.

2.17 Production of PhaP fused to IL2 or MOG at the PHA bead surface

The two proteins MOG and IL2 derived from *Mus musculus* were fused to the C terminus of one copy of PhaP via construction of the respective hybrid gene (see method 2.5.2 and Figure 8). The DNA sequences encoding either MOG or IL2 were optimized with respect to the codon usage of *E. coli* and purchased as synthetic DNA fragment (Bäckström *et al.* 2007). An enterokinase recognition site plus six histidine residues was inserted as a linker region between PhaP and IL2 or MOG in order to facilitate independent folding of the fusion partner and to enable specific removal of the antigen from the fusion partner (see Figure 8). Plasmids pBHR68-phaP-MOG and pBHR68-phaP-IL2, which both comprise the entire PHB biosynthesis operon in addition to the respective hybrid gene encoding the PhaP fusion protein, were introduced into *E. coli* XL1 Blue. The respective recombinant strains were cultivated and PHA beads isolated as per method in 2.9. Proteins attached to these beads were separated by SDS-PAGE (see 2.12.2) and prominent proteins were subjected to peptide fingerprinting using MALDI-TOF/MS (see 2.18).

2.18 MALDI-TOF mass spectrometry

Mass spectrometric analyses of tryptic peptides relating to PhaP and IL2 fusions to MOG were undertaken by S. König (Integrated Functional Genomics, Interdisciplinary

Center for Clinical Research, University of Münster, Germany). Analyses were carried out on a MALDI VOYAGER DE-PRO time of flight mass spectrometer from PerSeptive BioSystems (Framingham, MA) utilizing 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 2000. 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% TFA.

For the fusion phasin protein GFP-PhaP-MOG, mass spectrometric analyses of tryptic peptides were performed as a service by Torsten Klefmann (Otago University). Excised protein spots/bands were subjected to in-gel digestion with trypsin essentially according to the method of Shevchenko et al. (Proc Natl Acad Sci U S A 93, 14440-14445, 1996). Eluted peptides were dried using a centrifugal concentrator. Peptides were re suspended in 30% [v/v] ACN (acetonitrile) and 0.1% [v/v] TFA (trifluoroacetic acid) in water. 1 µl of peptide solution was premixed with 2 µl of matrix (10 mg per ml alpha cyano-4-hydroxycinnamic acid (CHCA) dissolved in 65% [v/v] aqueous acetonitrile containing 0.1% [v/v] TFA and 10 mM ammonium dihydrogen phosphate). 0.8 µl of sample/matrix mixture were spotted onto a MALDI sample plate (Opti-TOF 384 well plate, Applied Biosystems, MA) and air dried.

For mass spectrometry, samples were analysed on a 4800 MALDI tandem Time-of-Flight Analyser (MALDI TOF/TOF, Applied Biosystems, MA). All MS spectra were acquired in positive-ion mode with 800-1000 laser pulses per sample spot. The 15 - 20 strongest precursor ions of each sample spot were used for MS/MS collision-induced dissociation (CID) analysis. CID spectra were acquired with 2000-4000 laser pulses per selected precursor using the 2 kV mode and air as the collision gas at a pressure of 2*E-7 torr.

2.19 N Terminal sequencing

N terminal sequencing was carried out by the Australian Proteome Analysis Facility (APAF). Proteins were passively eluted using gel elution buffer at 37ºC overnight then

transferred to PVDF and washed using a ProSorb cartridge (Applied Biosystems). The sample was subjected to 6 cycles of Edman N terminal sequencing and sequenced using the ‘pl PVDF 2’ protein method. Automated Edman degradation was carried out using an Applied Biosystems 494 Procise Protein Sequencing System.

CHAPTER 3: RESULTS

There are three parts to the results. The first involves the N terminal fusion of the PHA synthase PhaC to the IgG binding protein ZZ. The second involves two C terminal fusions of the phasin protein PhaP to the proteins MOG and IL2 respectively. The third part involves double fusions of the PHA synthase and phasin proteins.

3.1 Part I: ZZ-PhaC construct

The identity of the fusion protein ZZ-PhaC was confirmed by peptide fingerprinting using MALDI-TOF (see 2.18). ZZ(-)PhaC beads were made and isolated using the methods outlined at 2.9 and Figure 11. Electron microscopy and G.C.M.S as per method outlined in 2.11 confirmed there was PHA accumulation. An example of recombinant *E.coli* accumulating PHA is shown below in Figure 12.

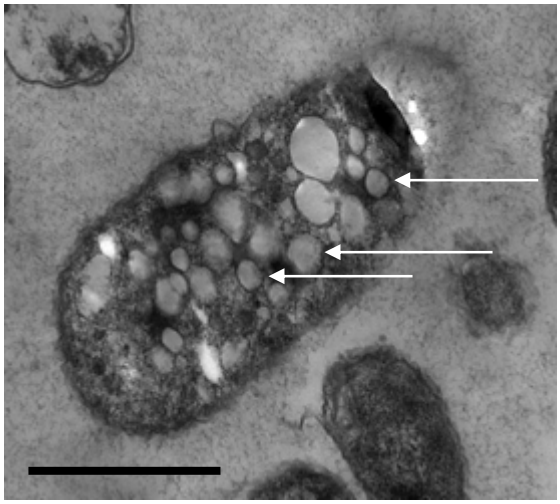


Figure 12. Electron microscopy of recombinant *Escherichia coli* with PHA accumulation appearing as spherical intracellular inclusions indicated by white arrows. The size bar represents 1000 nm

3.1.1 Display of the ZZ domain at the PHA bead surface and binding capacity of ZZ-PHAC beads

ELISA (see section 2.15) indicated that IgG bound specifically to ZZ-PhaC displaying PHA beads generated in both LacZ and the T7 expression systems. Beads displaying the fusion ZZ protein bound more IgG than wildtype beads with the highest binding (four-fold) seen with beads produced under control of the T7 promoter (Figure 13). Measurements were conducted in quadruplicate, and the mean value and the standard deviation are indicated.

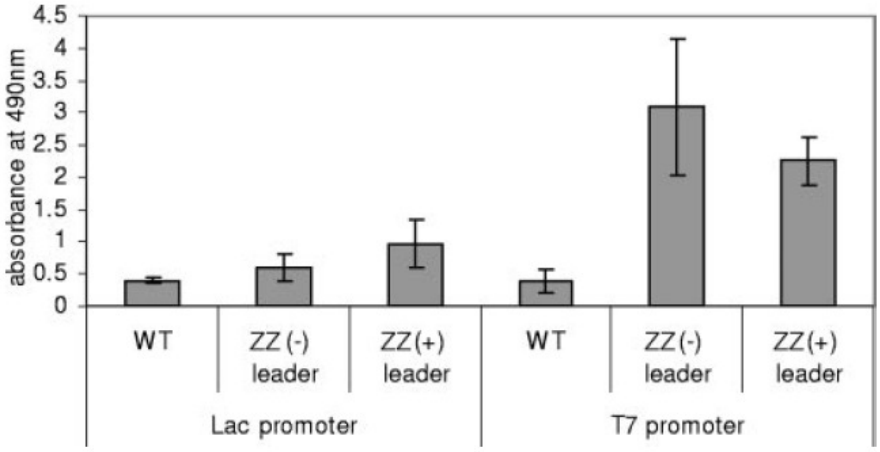


Figure 13. ELISA using various PHA beads and anti-IgG antibodies for the detection of IgG bound to PHA beads.

PHA beads generated from the LacZ and T7 promoter system were subjected to SDS-PAGE analysis as described in 2.12.2 confirming the presence of the fusion ZZ-PhaC protein. The construct pET14b(-)ZZ-PhaC had demonstrated IgG binding capability (Figure 13) and so strain BL21(DE3) (Table 3) harboring this plasmid was used for further work.

3.1.2 Purification of IgG from human serum with ZZ-PhaC beads

PHA beads displaying the IgG binding domain ZZ from protein A derived from pET14b(-)ZZ-PhaC (Table 5) were tested for their ability to bind and purify IgG from human serum by method 2.17. For comparison, protein A-Sepharose beads with

immobilised, recombinant protein A were also used to purify IgG from the same serum according to protein A-Sepharose 4B bead purification protocols (Sigma). SDS-PAGE analysis of proteins eluted from the beads showed that a protein with an apparent molecular mass of 50 kDa, representing the heavy chains of immunoglobulins, and a protein with an apparent molecular weight of 25 kDa representing the light chains were purified from human serum by the ZZ-PhaC beads (Figure 14). The immunoglobulins eluted from the beads at pH 2.7 (Figure 14, lane 2) and showed a high degree of purity, comparable to that of the commercially available protein A Sepharose beads (Figure 14, lane 4). In contrast, PHA beads formed by wild-type PHA synthase did not show elution of proteins (Figure 14, lane 3).

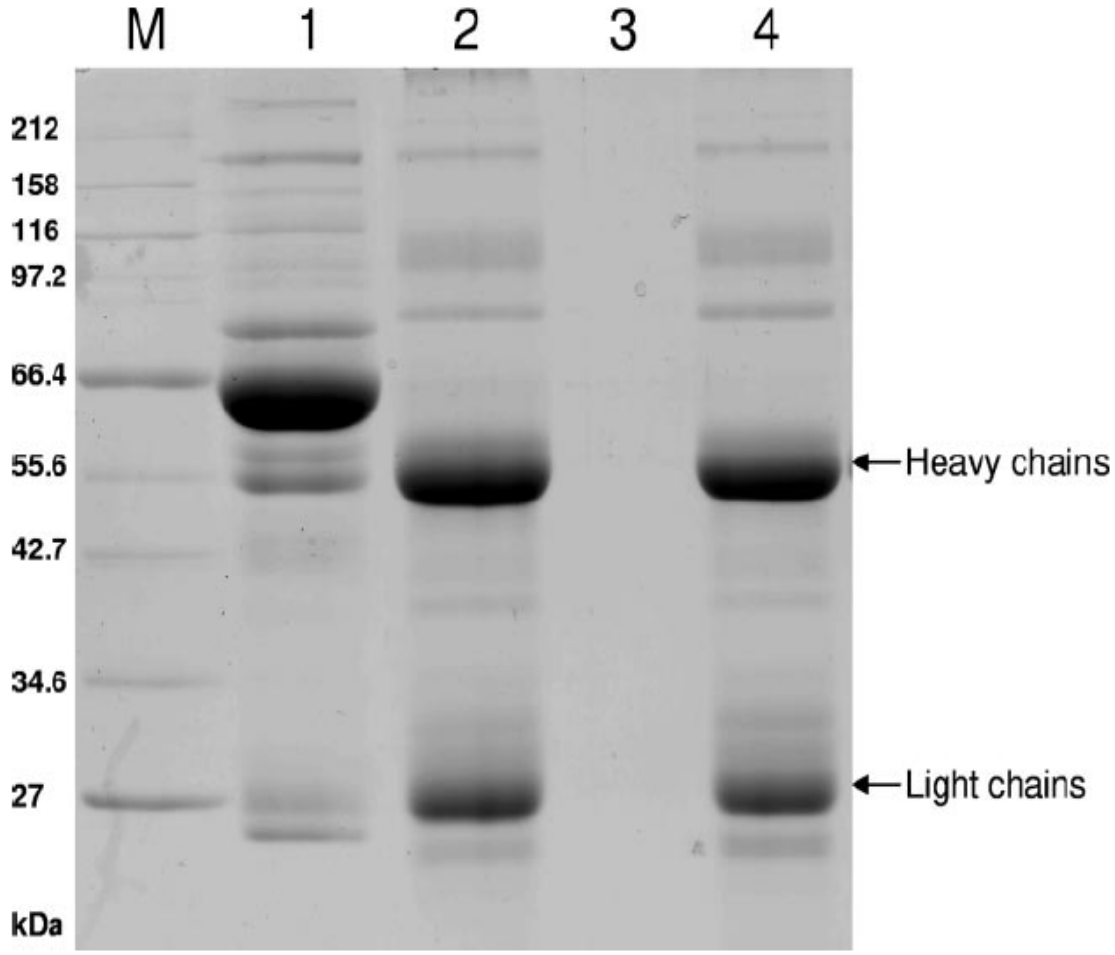


Figure 14. IgG purification from human serum. Lanes are marked as follows: 1: whole serum, 2: PHA beads ZZ(-)PhaC, 3: PHA beads WT, 4: Sepharose A beads (Sigma)

3.2 Part II: PhaP-MOG and PhaP-IL2

Genes encoding mouse interleukin 2 (IL2) and myelin oligodendrocyte glycoprotein (MOG) were each fused to the DNA regions encoding the C terminus of PhaP via an enterokinase site providing a linker region as described in the methods section 2.5.2 and shown in Figure 8. The hybrid genes expressed by PHA-accumulating recombinant *E. coli* produced proteins detected by SDS-PAGE as described in 2.12.2 and shown in Figure 15. To confirm the identity of MOG and IL2 fusion proteins attached to the surface of PHA beads MALDI-TOF/MS analysis (see section 2.18) and N terminal sequencing (see section 2.19) were undertaken as part of this study. Additionally FACS analysis was undertaken (Bäckström *et al.* 2007). PHA beads displaying either IL2 or MOG were subjected to FACS analysis using monoclonal anti-IL2 or anti-MOG antibodies conjugated to a fluorescent dye. All FACS analysis was performed by Thomas Bäckström (Malaghan Institute for Cancer Research, Wellington).

3.2.1 PhaP-MOG and PhaP-IL2 PHA beads

Plasmid pBHR68-phaP-MOG (Table 6) mediated the production of PHA beads showing four prominent proteins with apparent molecular weights of 36 kDa, 39 kDa, 41 kDa and 43 kDa (Figure 15). The 36 kDa and the 39 kDa protein were strongly overproduced and identified as PhaP-MOG fusion proteins using MALDI-TOF/MS analysis (section 2.18, Table 11). Both proteins were subjected to N terminal amino acid sequencing as described in method 2.19 and while the 36 kDa PhaP-MOG fusion showed the expected N terminal amino acid sequence MILTP, the more abundant 39 kDa PhaP-MOG showed an N terminus of MTMITP. The additional two more prominent proteins were subjected to MALDI-TOF/MS analysis and identified the 43-kDa protein as EF-Tu (accession no. AP004451), but no assignment for the 41 kDa could be obtained (Figure 15). Plasmid pBHR68-phaP-IL2 (Table 6) mediated the production of PHA beads showing two prominent and strongly overproduced proteins with apparent molecular weights of 39 kDa and 42 kDa (Figure 15). Both proteins were identified as PhaP-IL2 fusion proteins using MALDI-TOF/MS analysis (Table 11) and were subjected to N terminal sequencing as described in method 2.19. Similarly, to

PhaP-MOG, the 39 kDa PhaP-IL2 fusion showed the expected N terminus of MILTP, whereas the more abundant 42 kDa PhaP-IL2 showed an N terminus of MTMITP.

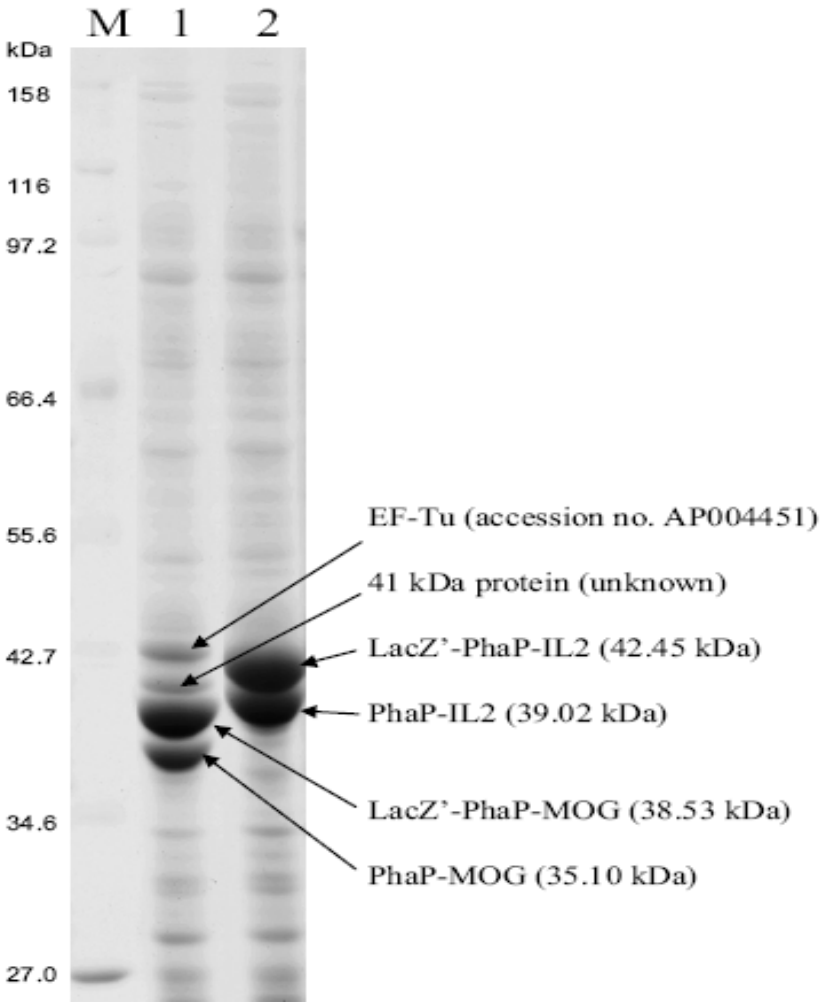


Figure 15. SDS-PAGE analysis of PHA bead-attached proteins

To test whether IL2 and MOG were expressed in native form on the surface of the respective PHA beads, antibodies that recognize correctly folded IL2 (PC61) or MOG (8-18C5) and FACS technology were used (Bäckström *et al.* 2007). The size of PHA beads produced in *E.coli* has been reported to be about 200-500 nm (Rehm 2003), and particles of this size were detected in the forward scatter (FSC) and 90° side scatter (SSC) detectors set to list mode at a 256-channel resolution (T.B, personal communication). PHA beads were incubated with unlabelled mouse anti-MOG antibodies, followed by APC-labeled anti-mouse IgG antibodies, or with PE-labeled anti-IL2 antibodies. The fluorescent intensity was then measured using FACS

technology (see method 2.16). FACS analysis showed significant and specific binding of respective antibodies (Figure 16).

Table 11. MALDI-TOF analysis of PhaP-MOG and PhaP-IL2

Protein	Peptide fragments
LacZ'-PhaP-IL2	A91-Y120, E96-A114, E121-E135, E190-T218
PhaP-IL2	A57-Y86, E62-A80, E87-E101, E156-T184, L300-G320
LacZ'-PhaP-MOG	N15-M34, W18-M34, L89-H118, A94-H118, L119-N155, L113-V133, A156-S169, T185-T216, A188-T216, T222-G232, G247-L255, T273-P283, V289-R308
PhaP-MOG	L55-H84, A60-H84, L85-N121, L79-V99, A122-S135, A154-T182, A173-T188, G247-L255, T273-P283, T275-V288, V289-R308

Results showed that the monoclonal antibodies specifically recognized PHA beads displaying the specific antigen, but not an un-related antigen. Both PC61 and 8-18C5 recognised only the respective native protein indicating that correctly folded IL-2 or MOG proteins were formed at the surface of the respective PHA beads. These PHA beads showed consistent performance in FACS analysis at least one year when stored at 4ºC (Bäckström *et al.* 2007).

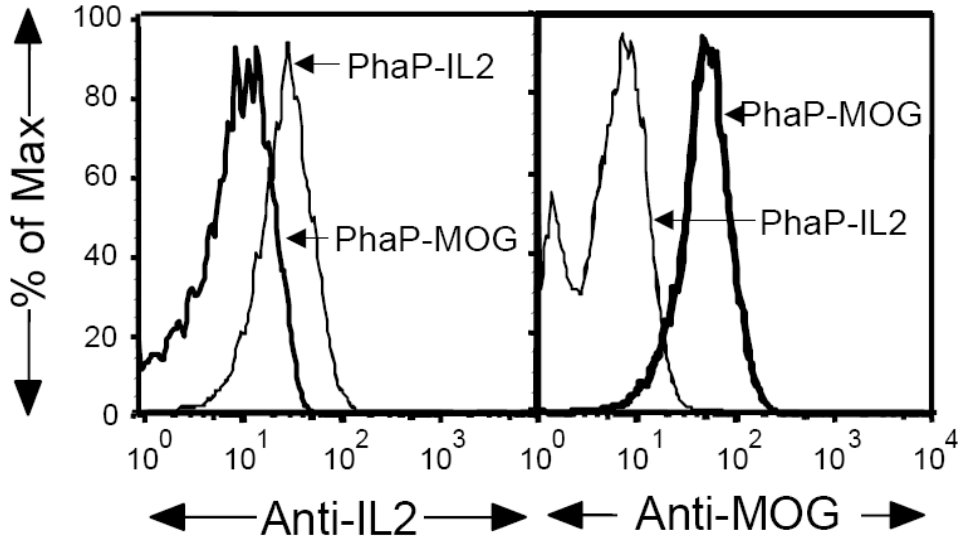


Figure 16. PHA beads display the native eukaryotic proteins (Bäckström et al., 2007)

PhaP-IL2 and PhaP-MOG fusion proteins containing the Asp-Asp-Asp-Asp-Lys recognition sequence are cleavable with enterokinase. To enable removal and/or purification of recombinant eukaryotic proteins, which form inactive inclusion bodies in *E. coli*, an enterokinase recognition sequence encoding for the Asp-Asp-Asp-Asp-Lys peptide had already been incorporated immediately downstream of PhaP (see section 2.52 and Figure 8). To determine if the recognition sequence was readily accessible, PhaP-IL2 and PhaP-MOG containing PHA beads were incubated for 16 hours with enterokinase. Samples of PhaP-IL2 and PhaP-MOG beads were removed before, after 1 hour and after 16 hours of incubation with enterokinase to determine the level of native protein remaining at the surface of the respective PHA bead. PhaP-IL2 and PhaP-MOG beads were incubated with PE-conjugated anti-IL2 and samples analysed by FACS. A similar pattern of decreased protein levels after incubation with enterokinase was found with PhaP-MOG beads incubated with anti-MOG monoclonal antibodies. The level of IL2 was reduced by ~80% after 1 hour incubation and after 16 hours incubation no significant levels of IL2 protein were detectable at the surface of the beads (Bäckström *et al.* 2007).

Since PHA beads can be detected using FACS technology, T. Bäckström (Malaghan Institute for Cancer Research, Wellington) tested the beads for their ability to detect and quantify the level of antigen-specific antibodies from immunised animals. For this purpose, mice were immunised with recombinant MOG or OVA protein in Complete Freund's adjuvant (CFA) to induce an antibody response to MOG and OVA, respectively. Twenty-eight days post immunisation, mice were tail-bled and immune sera collected. Sera from five different mice were pooled and tested for binding to PhaP-MOG beads. Results showed that MOG-immunised, but not OVA-immunised, anti-sera contained antigen-specific IgG antibodies that recognized the PhaP-MOG fusion protein. The sera could be diluted >1:100 fold and still showed a significant binding of IgG to the PhaP-MOG beads (Bäckström *et al.* 2007). Sera from OVA immunised mice did not bind significantly to PhaP-MOG beads. However, OVA immunised mice produced OVA-specific IgG, as microtitre wells coated with OVA, but not with MOG, showed bound IgG which was easily detectable using ELISA (Bäckström *et al.* 2007).

3.3 Part III: Multifunctional PHA beads

The additional fusion to the N terminus of PhaP generating a second larger fusion phasin protein was an unexpected result (see Figure 15). This indicated that not only did the phasin protein tolerate C terminal fusions but that it also remained functional when an alternative start codon led to an additional fusion of 33 amino acids to the N terminus (Bäckström *et al.* 2007). The plasmid pBHR68GPM which harbors all required polyester (polyhydroxybutyrate) biosynthesis genes was then constructed to mediate polyester inclusion formation while producing the GFP-phasin-MOG double fusion protein (see section 2.5.3.1, Figure 9 and Figure 10).

To test the possibility of exhibiting multiple fusion proteins on the surface of PHA beads in *E.coli,* PhaP-MOG and GFP-PhaC fusions were also combined in bead production (see section 2.5.3.2., Figure 9 and Figure 10). G.C.M.S confirmed the accumulation of PHA and the resulting beads from pBHR68GPM subjected to SDS-PAGE analysis with the fusion GFP-PhaP-MOG protein identified by MALDI-TOF analysis (see section 2.18 and Table 12).

Bead suspensions with a protein concentration of 1 mg per ml total protein as assessed by Bradford's method (see section 2.12.1) were subjected to ELISA (see section 2.15). The results indicated that antibodies to MOG (clone 8.18-C5 kindly provided by C. Bernard, Monash University, Melbourne) bound specifically to PHA beads displaying MOG as well as beads displaying both MOG and GFP together (Figure 17).

Wildtype PhaC, GFP-PhaC, PhaP-MOG, GCPM and GPM PHA beads were incubated with unlabelled mouse anti-MOG antibodies, followed by phycoerythrin (PE)-conjugated anti-mouse IgG antibodies, then with FITC-labeled GFP antibodies. The fluorescent intensity was then measured using FACS technology (see section 2.16). FACS analysis results confirmed that the monoclonal antibodies specifically recognised PHA beads displaying the specific antigen, but not an un-related antigen and that GCPM and GPM displayed both MOG and GFP at the surface (Figure 18).

Beads isolated and purified from recombinant *E. coli* either harboring pBHR69GCPM or pBHR68GPM were also assessed by fluorescence microscopy, which showed fluorescent labeling of beads suggesting display of GFP at the bead surface (Figure 19).

Table 12. MALDI-TOF analysis of pBHR68GPM

Protein/Protein sequence	**Peptide fragments assigned to the various protein regions**
GFP-phasin-MOG (MW: 65.3 kDa): MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGE GDATYGKLTLKFICTTGKLPVPWPTLVTTLTYGV QCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFK DDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDG NILGHKLEYNYNSHNVYIMADKQKNGIKVNFKI RHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHY LSTQSALSKDPNEKRDHMVLLEFVTAAGITHGM DELYKTSITPSAQLTLTKGNKSWSSTAVAAALEK GDIRAILTPEQVAAAQKANLETLFGLTTKAFEGV EKLVELNLQVVKTSFAEGVDNAKKALSAKDAQE LLAIQAAAVQPVAEKTLAYTRHLYEIASETQSEF TKVAEAQLAEGSKNVQALVENLAKNAPAGSEST VAIVKSAISAANNAYESVQKATKQAVEIAETNFQ AAATAATKAAQQASATARTATAKKTTAADDDD KRGSHHHHHHMGQFRVIGPGYPIRALVGDEAEL PCRISPGKNATGMEVGWYRSPFSRVVHLYRNGK DQDAEQAPEYRGRTELLKETISEGKVTLRIQNVR FSDEGGYTCFFRDHSYQEEAAMELKVED	**GFP**: F27-K41, S86-R96 **Phasin**: L304-K313, D331-K349, H356-K370, N382-K392, Q425-K443 **MOG**: M513-R519, N531-R544, F568-R579

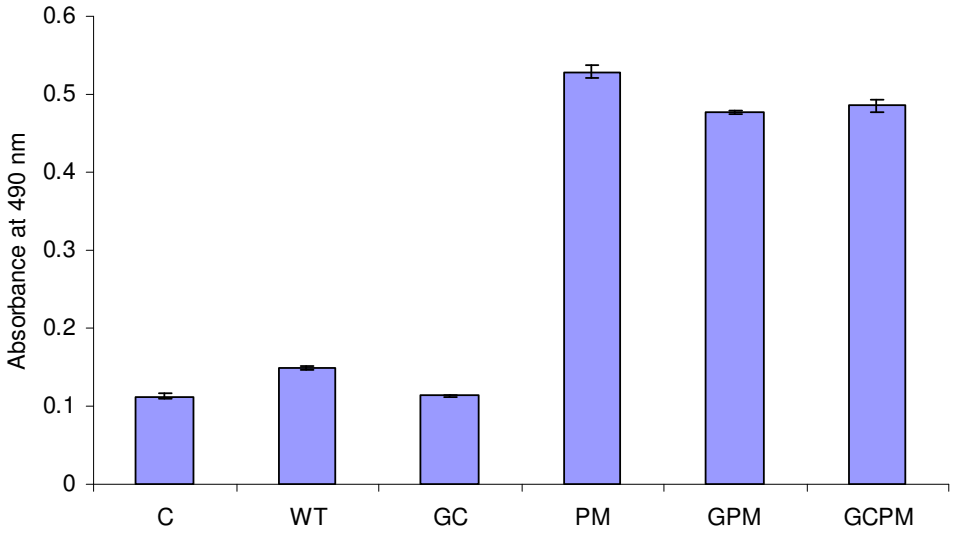


Figure 17. ELISA showing anti-MOG antibody binding to various beads. C, only secondary HRP-conjugated antibody; WT, wildtype beads (no fusion protein present); GC, beads with GFP-polyester synthase; PM, beads with phasin-MOG; GCPM, beads with GFP-polyester synthase and phasin-MOG; GPM, beads with GFP-phasin-MOG (Atwood and Rehm, 2008).

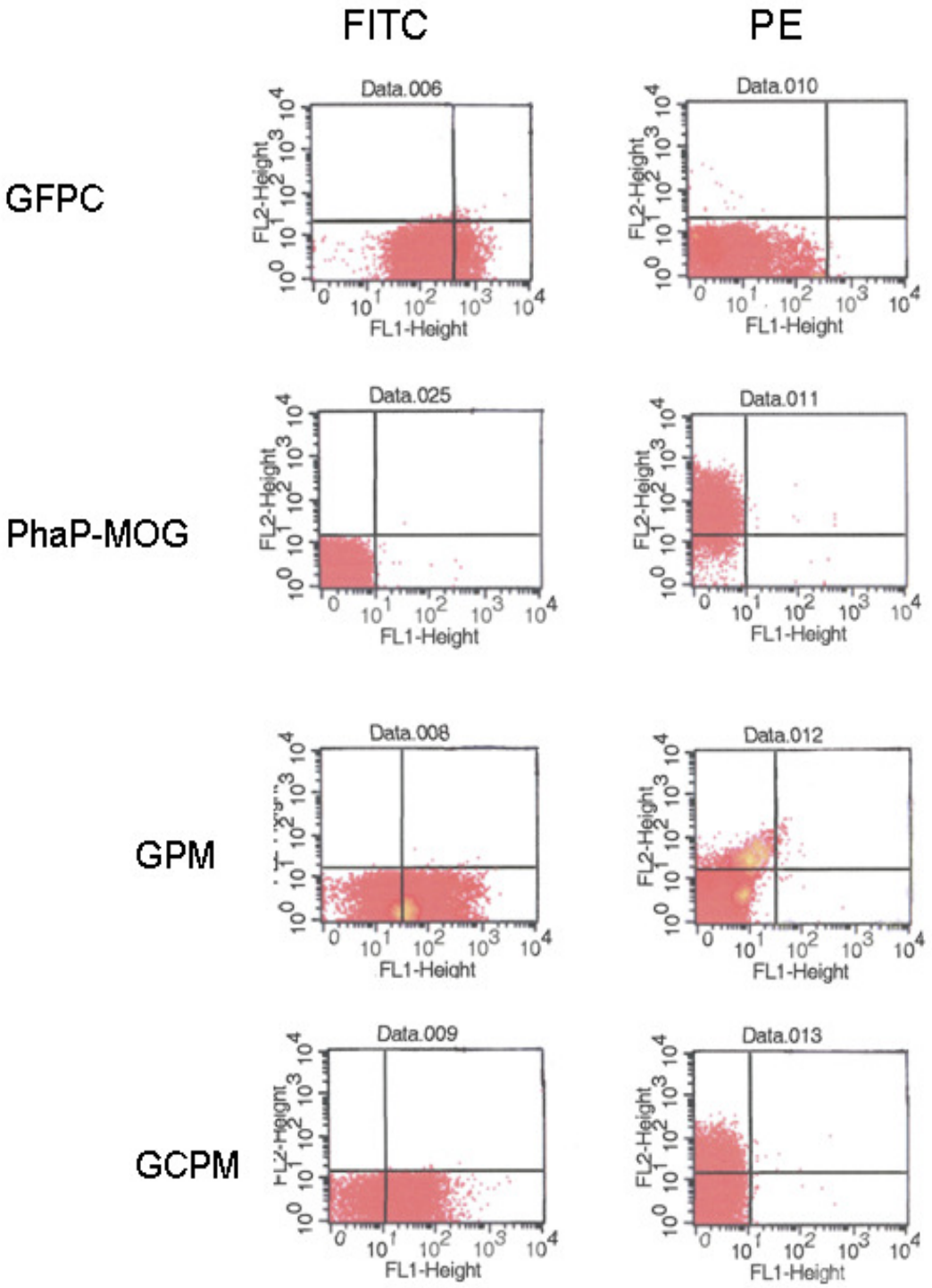


Figure 18. FACS results. Beads displaying GFP showed fluorescence when incubated with FITC conjugated anti-GFP antibodies whereas beads displaying MOG showed fluorescence when incubated with anti-mouse antibodies followed by PE conjugated anti-mouse antibodies followed by PE conjugated anti-mouse antibodies. Legend: FL1 = FITC, FL2 = Phycoerythrin (PE) (Atwood and Rehm, 2008).

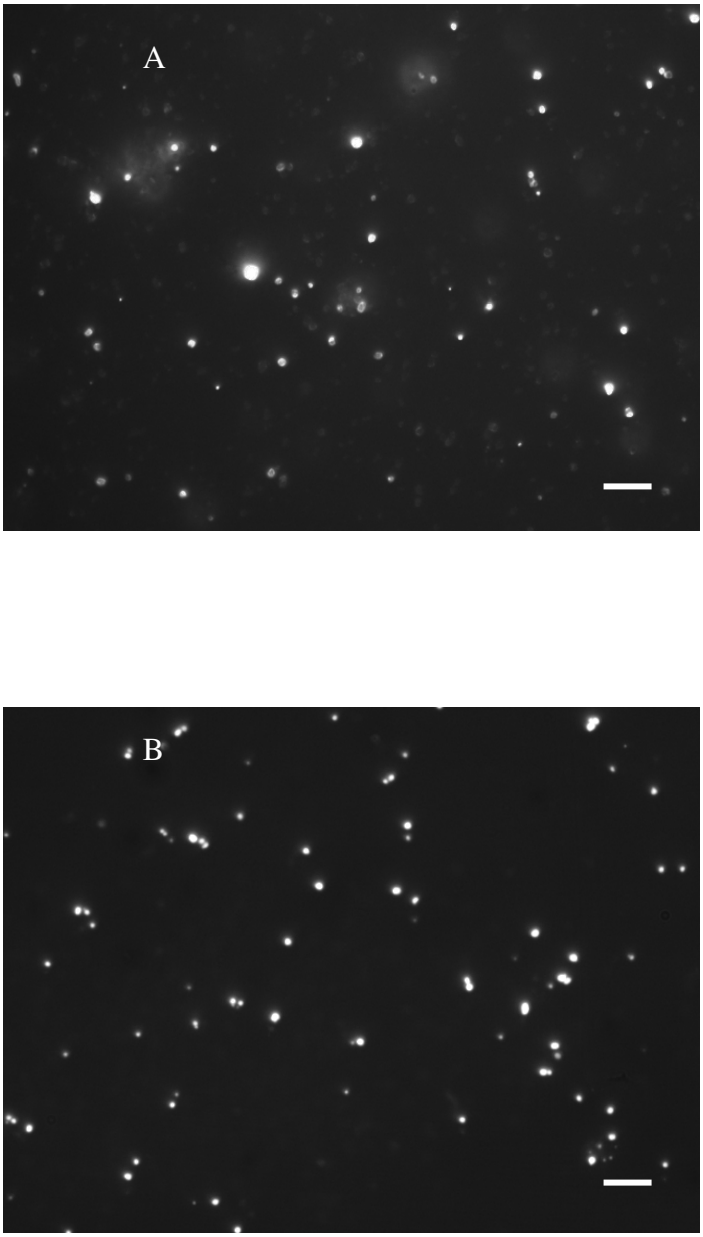


Figure 19. Fluorescence microscopy using a FITC filter demonstrated the presence of GFP on the surface of the beads. A. GCPM beads. B. GPM beads. Magnification, 1000x; Bar corresponds to 1 µm (Atwood and Rehm, 2008).

CHAPTER 4: DISCUSSION

4.1 Protein production and purification: A novel application for a bio-bead

Several protocols and systems are available commercially for the production of and purification of recombinant proteins. A novel application for bio-beads was envisaged where the PHA beads could be used to immobilise and display proteins or to capture, by affinity, another protein of interest much like commercially available beads, for example, Protein A Sepharose^{TM}, (Amersham, GE Lifesciences) and Dynabeads®, (Invitrogen). If successful, PHA beads offered several advantages over the commercial beads. PHA beads at 200-500nm in size are smaller than commercial sepharose beads and hence, the surface area is relatively larger. Chemically coupling the desired ligand to the bead is unnecessary because the ligand or analyte is produced attached to the polyester bead in a one-step process by the bacterial host. As beads for the purification of IgG, the ZZ-PhaC beads would be less expensive to produce than commercially available Protein A beads.

For protein purification, PHA beads offer the convenience of having the engineered protein of interest immobilised already on the bead surface and in the second part of this study, we focused on the novel use of engineered PHA beads for FACS-based diagnostics and protein production.

The third part of this study was to develop multi-functional PHA beads using a combination of the PhaC and PhaP fusion proteins. Combining PhaP-MOG (GCPM PHA beads) with GFP-PhaC was chosen because GFP fluorescence combined with an antigen on a bio-bead surface offered detection and antigen on the one bead, another novel use for PHA beads. The development of an N and C terminally fused PhaP (GPM PHA beads) was included in this study after the results from the PhaP fusion indicated that an N terminal fusion of 33 amino acids to PhaP did not interfere with the activity of PhaP.

4.2 PhaC fusion protein

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 bead surface. Based on the current model of PHA granule formation which suggests that the PHA synthase stays covalently attached to the emerging biopolyester bead (Peters and Rehm 2006; Rehm 2006a; Rehm 2006b), it was hypothesised that the ZZ domain would be exposed at the surface of the PHA bead when fused to PhaC. To investigate this hypothesis, PHA beads of *E. coli* harboring plasmid pCWE-ZZ(-)phaC, pCWE-ZZ(+)phaC, pET14b-ZZ(-)phaC, or pET14b-ZZ(+)phaC, as well as PHA beads produced by wild-type PHA synthase (pCWE or pHAS), were isolated and tested by enzyme-linked immunosorbent assay (ELISA).

The results of ELISA (see section 3.1.1) supported the current PHA granule formation model suggesting a functional display of the ZZ domain at the PHA bead surface. The presence or absence of the signal peptide did not affect IgG binding capacity. However, PHA beads whose formation was mediated by over-production of ZZ-PhaC due to expression under the T7 promoter system showed significantly increased binding capacity (Figure 13). Protein A Sepharose™ beads (LifeSciences) contain protein A immobilised by the CNBr method to Sepharose 4 Fast Flow and is used to isolate and purify classes, subclasses and fragments of immunoglobulins from biological fluids and from cell culture media (LifeSciences). PhaC-ZZ PHA beads used to purify IgG performed comparably to the commercially available Protein A Sepharose beads.

4.3 PhaP fusion protein

The second part of this study showed that by exploiting the granule-associated phasin protein PhaP, it was possible to display functional eukaryotic proteins MOG or IL2 on the surface of PHA beads for use in FACS-based diagnostics and for recombinant eukaryotic protein production.

The PhaP protein differs from PhaC in that it associates with the granule surface but is not covalently attached (Hanley 1999). The surface of PHA beads produced by recombinant *E. coli* were engineered to display natively folded eukaryotic proteins,

which could serve as antigens in diagnostic tests, or for protein systems. The eukaryotic proteins MOG and the cytokine IL-2 form inclusion bodies upon production in *E. coli* (Linnington *et al.,* 1984, Lowenthal *et al.,* 1985). A microbial protein production system avoiding the formation of inclusion bodies would have a huge advantage by circumventing the tedious process of solubilisation and refolding. In this second part of the study, the C terminal of protein PhaP was fused to either MOG or IL2 and a strong overproduction of the respective fusion proteins was observed under *lac* promoter control (Figure 15). Unexpectedly, the fusion proteins appeared in two versions with different apparent molecular weight. The smaller molecular weight version of the fusion protein showed the theoretical molecular weight of the respective fusion protein. The N terminal sequence (MTMITP) of the larger molecular weight version suggested that the N terminal 33 amino acid residues of LacZ (theoretical molecular weight of 3.4 kDa) were fused to the N terminus of the PhaP fusion protein, based on the start codon upstream of the start codon of the hybrid gene encoding the PhaP-MOG or PhaP-IL2. This was also in accordance with the apparent molecular weight difference of 3.5 kDa between the two versions. Interestingly, the LacZ'-PhaP-MOG/IL2 fusion proteins were more abundant than the respective single fusions (Figure 15). These data suggest that N terminal fusions to PhaP do not interfere with the binding to the PHA core of PHA beads and that the copy numbers could be increased. This was unexpected because the current phasin protein model (Hanley *et al.,* 1999) suggests the N terminus interacts directly with PHA beads so additional amino acids at the N terminus might be expected to interfere with this interaction. The presence of the other two *E. coli* proteins, the 41 kDa protein and EF-Tu, at the MOG displaying PHA bead surface might be due to a non-specific interaction with the PHA bead surface, as observed with the ZZ-PhaC beads and has been observed elsewhere (Peters and Rehm 2006). In contrast to a previous study where multiple copies of PhaP were required for binding of prokaryotic protein fusions (Banki *et al.* 2005), the present study showed that a single copy was sufficient using an enterokinase site linker and eukaryotic proteins.

FACS analysis using antibodies specifically recognizing the respective native protein folds (Figure 15) demonstrated that the natively folded eukaryotic proteins MOG and IL2 were at the PHA bead surface indicating that designer PHA beads could be used for FACS-based diagnostics (Bäckström *et al.* 2007). Although MOG and IL2 are

secreted proteins, the derived protein domains were properly folded attached to the PHA granule in the reducing cytosol of *E. coli*. Enterokinase treatment of MOG-displaying or IL2-displaying PHA beads and the resulting release of the antigen suggested that the respective antigen was exposed at the PHA bead surface (Bäckström *et al.* 2007).

To evaluate the suitability of the MOG and IL2 displaying PHA beads made in this study for qualitative and quantitative FACS-based antibody detection, mice were immunised with MOG or OVA (control) and induction of specific antibody production was assessed and confirmed by ELISA. This clearly indicated that anti-MOG antibodies can be specifically detected at least up to an antisera dilution of 1:100,000 (Bäckström *et al.* 2007).

These results demonstrated that correctly folded recombinant proteins can be produced as a fusion protein with PhaP and that the fusion partner can be completely removed by enterokinase treatment. This provides a platform for recombinant protein production whereby the protein of interest can be produced in excess as a fusion to the phasin protein and cleaved later at the enterokinase site.

4.4 Multiple functionality: display of MOG and GFP at the bead surface

The second fusion protein of either MOG or IL2 resulting from an N terminal fusion, which did not interfere with attachment to PHA was an unexpected result. As the PHA beads from constructs pBHR68 PhaP-MOG and pBHR68 PhaP-IL2 contained a fusion PhaP protein with 33 additional amino acids at the N terminus that did not affect function, the possibility of an N terminal and C terminal fusion to PhaP was explored further. GFP was fused to the N terminus of PhaP and MOG fused to the C terminus of PhaP via the construct named pBHR68GPM. Additionally, the gene encoding PhaP-MOG was inserted upstream of the gene for GFP-PhaC on the plasmid pBHR69+gfpC to engineer PHA beads displaying two separate functionalities. Fluorescent microscopy confirmed that the engineered PHA beads of pBHR69 GCPM and pBHR68GPM displayed active GFP protein (Figure 19) and ELISA confirmed that MOG was also present (Figure 17). In addition, FACS analysis of both types of engineered beads using antibodies specifically recognizing

the respective native protein folds, demonstrated the display of both natively folded proteins MOG and GFP (Figure 18).

Construction of beads displaying both GFP fused to the PHA synthase and a MOG fusion to phasin protein demonstrated that multiple-functionality of the PHA beads is possible (Atwood and Rehm, 2008). ELISA and FACS results demonstrated that the phasin protein PhaP N terminally fused to GFP and C terminally fused to MOG was associated with PHA beads and that both GCPM PHA beads and GPM beads displayed GFP and MOG. This opens up the possiblity of using engineered PHA beads for many applications. Biopolyester beads simultaneously displaying two functional proteins or antigens at the surface could be applied as a multi-valent vaccine or used in diagnostics where, for example, a fluorescent protein and a specific binding protein would enable the detection of a relevant analyte.

4.5 Conclusions

The engineered ZZ-PHAC beads performed equally well when compared with commercial protein A-Sepharose beads with respect to IgG purification. This opens up a new and interesting field of biotechnological applications for these biopolyester beads. This study demonstrated that genetic engineering of the PHA synthase provides a convenient and efficient network for covalent protein immobilisation. Commercial protein A beads require the *in vitro* production of polymer beads and subsequently the chemical cross-linking of purified protein A. In contrast, PHA granule-based beads with covalently attached functional proteins are produced in a one-step process by recombinant bacteria, suggesting a commercially viable biotechnological production process.

Correctly folded eukaryotic proteins can also be abundantly produced at the PHA bead surface as phasin fusion proteins. Isolated PHA beads displaying the respective eukaryotic proteins could be used as beads for specific and sensitive antibody detection using FACS technology. These native antigen-displaying PHA beads were manufactured by recombinant *E. coli* without the need for antigen purification and chemical cross-linking to independently produced beads. Often purification of these

proteins requires tedious refolding at low efficiency. The production of functional eukaryotic proteins at the PHA bead surface represents a novel *in vivo* matrix-assisted protein folding system, avoiding aggregation of protein folding intermediates. This study has opened up an alternative route for the production of protein-displaying beads harnessing nature's capacity to produce spherical polymer beads with surfaces that can be functionalised by engineering of specific bead-associating proteins.

Multiple-functionality was also achieved by co-expression of various hybrid genes suggesting that this biotechnological bead production strategy might represent a versatile platform technology. When compared to commercial beads, engineered PHA beads showed increased sensitivity with similar distribution of signal intensities in FACS.

Since this study, anti-β-galactosidase scFv-displaying polymer beads have been produced in engineered *E. coli* using the PHA synthase as a self-assembly-promoting fusion partner (Grage and Rehm 2008). *E. coli* have also been engineered to intracellularly manufacture PHA streptavidin beads (Peters and Rehm 2008) and even more recently, *E. coli* were engineered to manufacture multifunctional PHA beads for the simultaneous binding of inorganic substances and antibodies (Jahns *et al.,* 2008). These studies confirm that protein engineering of PHA bead surface proteins provides a novel molecular tool for the display of antigens for FACS-based analysis and offers promising possibilities for the development of future biotechnological production processes. Overall, the results obtained in this study strongly enhance the applied potential of these polyester beads in biotechnology and medicine.

Immunoglobulin G purification and antigen display using biopolyester beads

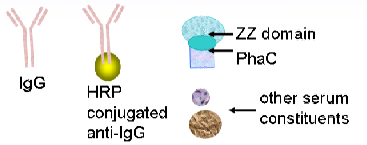
Jane A. Atwood, B. Thomas Bäckström, Verena Peters and Bernd H. A. Rehm

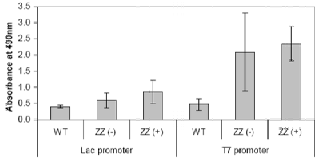
Polyhydroxyalkanoate is a biopolyester produced by many bacteria. The most common, PHB, forms insoluble inclusions in the cytoplasm. We produced recombinant PHA beads displaying functional proteins immobilised at the surface.

Bacterial cell

Purification of IgG from serum

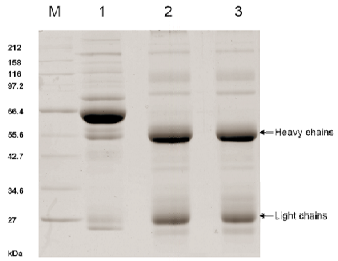
Isolated IgG binding domain (ZZPhaC) displaying PHA beads





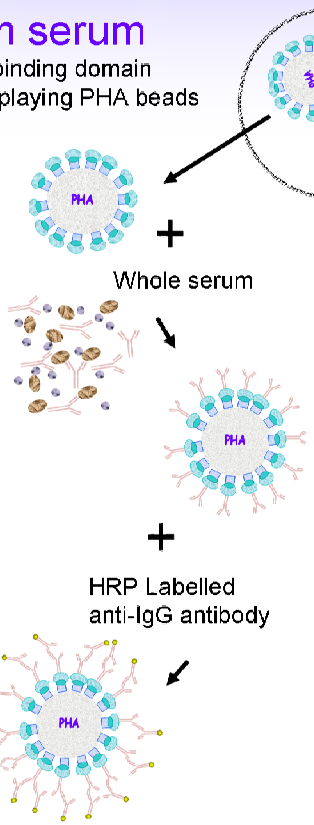
IgG binding ELISA

Beads displaying ZZ + or – signal sequence bind IgG.



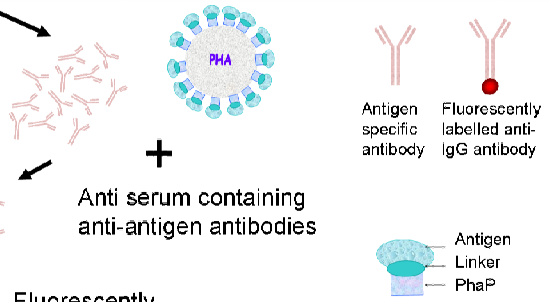
IgG purification

1. Whole serum 2. Eluted from commercial sepharose beads 3. PHA WT beads 4.Eluted from PHA beads displaying IgG binding domain (ZZ)

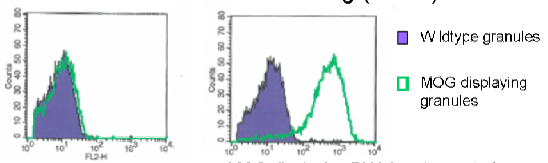


Functional display of antigen

Isolated antigen (PhaP-MOG) displaying PHA beads



Fluorescence-activated cell sorting (FACS)



PHA beads displaying MOG do not cross react with antibodies to interleukin-2 (IL2)

MOG displaying PHA beads reacted to conformational antibodies to myelin oligodendrocyte glycoprotein (MOG)

IMBS Massey University

APPENDIX

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Publications

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Recombinant *Escherichia coli* Strain Produces a ZZ Domain Displaying Biopolyester Granules Suitable for Immunoglobulin G Purification▽

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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.

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 immobilized 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).

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 NdeI sites at each end of the PCR product (Table 1). Each PCR product was then inserted into the NdeI site of plasmid pCWE, resulting in plasmids pCWE-ZZ(+)phaC and pCWE-ZZ(−)phaC, respectively (Table 1). Each hybrid gene was subcloned into XbaI/BamHI 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)/pLysS harboring pMCS69 (*phbA phbB*). The PHA synthase function of the fusion proteins was assessed by analyzing 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

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

Strain, plasmid, or oligonucleotide	Description[a]	Source or reference
E. coli strains		
BL21(DE3)/pLysS	F^-; *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm*(DE3); pLysS (Cam^r)	Invitrogen
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 NdeI/BamHI-inserted *phaC*$_{We}$ gene from *C. necator*	11
pET14b	Ap^r; T7 promoter	Novagen
pET14b-ZZ(−)phaC	pET14b containing XbaI/BamHI fragment comprising gene *ZZ-phaC* without the signal sequence-encoding region	This study
pET14b-ZZ(+)phaC	pET14b containing XbaI/BamHI fragment comprising gene *ZZ-phaC*	This study
pCWE	pBluescript SK(−) derivative containing the PHA synthase gene from *C. necator*	6
pCWE-ZZ(−)phaC	pCWE derivative containing the ZZ domain-encoding NdeI fragment lacking the signal sequence-encoding region	This study
pCWE-ZZ(+)phaC	pCWE derivative containing the ZZ domain-encoding NdeI fragment	This study
pBHR69-ZZ(−)phaC	pBHR69 derivative containing the 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 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
Oligonucleotides		
5′-ZZ (+)-NdeI	5′-GCGCGCATATGACTTTACAAATACATACAGGGGGTATTAATTTG-3′	This study
3′-ZZ-NdeI	5′-GTAATCATATGGGGTACCGAGCTCGAATTCGCGTCTAC-3′	This study

[a] Restriction enzyme recognition sites are underlined.

and pET14b-ZZ(−)phaC, encoding ZZ-PhaC with and without the signal peptide, respectively, mediated overproduction of ZZ-PhaC at the PHA granule 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 immobilized 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 localize 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).

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

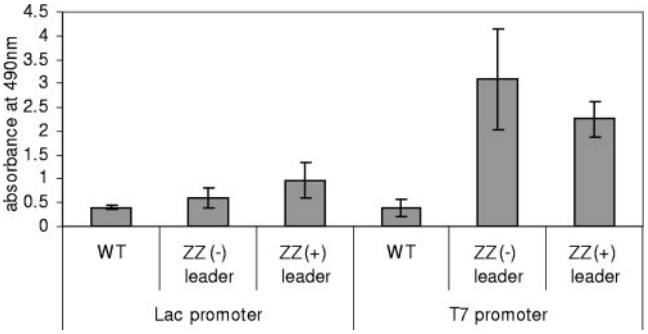

FIG. 1. ELISA using various PHA granules and anti-IgG antibodies for the detection of IgG bound to PHA granules. PHA granules were isolated from recombinant *E. coli* harboring various plasmids. Plasmids contained either the *lac* promoter or the T7 promoter for gene expression. The following versions 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.

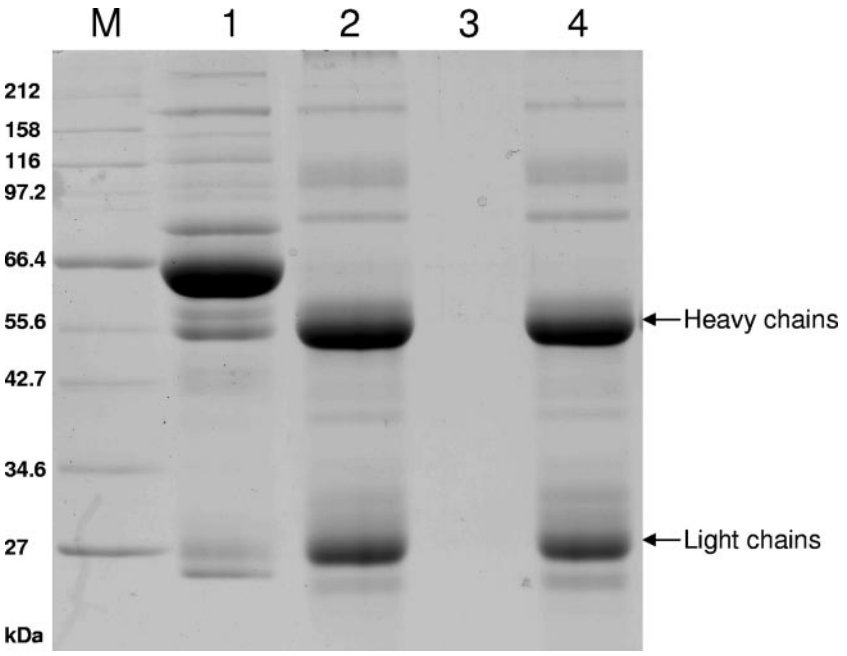

FIG. 2. SDS-PAGE analysis of proteins bound in vitro to either ZZ-PHA granules or protein A-Sepharose and released after elution. Lanes: M, molecular weight standard; 1, human serum; 2, proteins eluted from protein A-Sepharose beads; 3, proteins eluted from wild-type PHA granules; 4, proteins eluted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

human serum. For comparative analysis, protein A-Sepharose beads with immobilized, 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.

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 immobilization (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 functionalization of PHA granules (10).

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BMC Biotechnology

Research article

Recombinant *Escherichia coli* produces tailor-made biopolyester granules for applications in fluorescence activated cell sorting: functional display of the mouse interleukin-2 and myelin oligodendrocyte glycoprotein

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Abstract

Background: Fluorescence activated cell sorting (FACS) is a powerful technique for the qualitative and quantitative detection of biomolecules used widely in both basic research and clinical diagnostic applications. Beads displaying a specific antigen are used to bind antibodies which are then fluorescently labelled using secondary antibodies. As the individual suspension bead passes through the sensing region of the FACS machine, fluorescent signals are acquired and analysed. Currently, antigens are tediously purified and chemically cross-linked to preformed beads. Purification and coupling of proteins often renders them inactive and they will not be displayed in its native configuration. As an alternative, we genetically engineered *Escherichia coli* to produce biopolyester (polyhdroxyalkanoate=PHA) granules displaying diagnostically relevant antigens in their native conformation and suitable for FACS analysis.

Results: Hybrid genes were constructed, which encode either the mouse interleukin-2 (IL2) or the myelin oligodendrocyte glycoprotein (MOG) fused via an enterokinase site providing linker region to the C terminus of the PHA granule associated protein PhaP, respectively. The hybrid genes were expressed in PHA-accumulating recombinant *E. coli*. MOG and IL2 fusion proteins were abundantly attached to PHA granules and were identified by MALDI-TOF/MS analysis and N terminal sequencing. A more abundant second fusion protein of either MOG or IL2 resulted from an additional N terminal fusion, which did surprisingly not interfere with attachment to PHA granule. PHA granules displaying either IL2 or MOG were used for FACS using monoclonal anti-IL2 or anti-MOG antibodies conjugated to a fluorescent dye. FACS analysis showed significant and specific binding of respective antibodies. Enterokinase treatment of IL2 displaying PHA granules enabled removal of IL2 as monitored by FACS analysis. Mice were immunized with either MOG or OVA (ovalbumin) and the respective sera were analysed using MOG-displaying PHA granules and FACS analysis showing a specific and sensitive detection of antigen-specific antibodies within a wide dynamic range.

Conclusion: *E. coli* can be genetically engineered to produce PHA granules displaying correctly folded eukaryotic proteins and which can be applied as beads in FACS based diagnostics. Since PHA granule formation and protein attachment occurs in one step already inside the bacterial cell, microbial production could be a cheap and efficient alternative to commercial beads.

Background

Polyhydroxyalkanoate (PHA) granules (biopolyester particles) are formed inside bacterial cells based on the activity and biochemical properties of the PHA synthases and specific biosynthesis enzymes which are involved in PHA precursor supply [1,2]. Biologically, PHA serves as a reserve material. The PHA granule core is composed of PHA and the surface of a phospholipid membrane with embedded or attached proteins. Amphipathic phasin proteins are one group of proteins specifically and hydrophobically interacting with the PHA core (for review see [1,2]). The functional role of the phasins impacting on PHA granule structure has been studied in detail [3-5].

Phasins and their fusion proteins have been increasingly considered for protein production at the PHA granule surface [2,6-8]. Recently, PHA synthase engineering enabled production of the beta-galactosidase and GFP fusion proteins, respectively, at the PHA granule surface [9,10]. The GFP-PHA synthase fusion even enabled monitoring of *in vivo* PHA granule formation indicating that PHA granule formation starts at the cell poles. Only recently, PHA granules have been considered as spherical biopolyester particles which can be stably maintained outside the bacterial cell exerting a size range from about 100 nm to several μm [2,8].

Here we co-expressed the PHA biosynthesis operon from *Cupriavidus necator* with a hybrid gene encoding a phasin fusion protein in *Escherichia coli* in order to mediate the formation of PHA granules efficiently displaying the respective fusion partner. In this study, eukaryotic antigen displaying PHA granules were designed and their application performance with respect to diagnostic applications using fluorescence activated cell sorting (FACS) was evaluated. The displayed myelin oligodendrocyte glycoprotein (MOG) was used as an example depicting diagnostic analysis of the autoimmune disease multiple sclerosis (MS). It was for the first time observed that surface-engineered and antigen displaying PHA granules can be efficiently used for FACS based diagnostics. Thus designed PHA granules, which combine cheap one step production with facilitated folding of proteins, might in future replace commercial beads.

Results

Production of PhaP fused to IL2 or MOG at the PHA granule surface

The two immunologically and medically relevant proteins MOG and IL2 derived from *Mus musculus* were each produced as PhaP fusion protein via construction of the respective hybrid gene. The DNA sequences encoding either MOG or IL2 were optimized with respect to the codon usage of *E. coli* and purchased as synthetic DNA fragment [see Additional file 1]. An enterokinase recognition site plus six histidine residues were inserted as linker region between PhaP and IL2 or MOG in order to facilitate independent folding of the fusion partner and to enable specific removal of the antigen from the fusion partner. Plasmids pBHR68-phaP-MOG and pBHR68-phaP-IL2, which both comprise the entire PHB biosynthesis operon in addition to the respective hybrid gene encoding the PhaP fusion protein, were introduced into *E. coli* XL1 Blue (Fig. 1, Table 1). The respective recombinant strains were cultivated and PHA granules isolated. Proteins attached to these granules were separated by SDS-PAGE and prominent proteins were subjected to peptide fingerprinting using MALDI-TOF/MS. Plasmid pBHR68-phaP-MOG mediated the production of PHA granules showing four prominent proteins with apparent molecular weights of 36 kDa, 39 kDa, 41 kDa and 43 kDa (Fig. 2). The 36 kDa and the 39 kDa protein were strongly overproduced and identified as PhaP-MOG fusion proteins using MALDI-TOF/MS analysis (Fig. 2, Table 2). Both proteins were subjected to N terminal amino acid sequencing and the 36 kDa PhaP-MOG fusion showed the expected N terminal amino acid sequence MILTP, whereas the more abundant 39 kDa PhaP-MOG showed an N terminus of MTMITP. The additional two more prominent proteins were subjected to MALDI-TOF/MS analysis and identified the 43 kDa protein as EF-Tu (accession no. AP004451), but no assignment of the 41 kDa could be obtained (data not shown). Plasmid pBHR68-phaP-IL2 mediated the production of PHA granules showing two prominent and strongly overproduced proteins with apparent molecular weights of 39 kDa and 42 kDa. Both proteins were identified as PhaP-IL2 fusion proteins using MALDI-TOF/MS analysis (Table 2) and were subjected to N terminal sequencing and the 39 kDa PhaP-MOG fusion showed the expected N terminus of MILTP, whereas the more abundant 42 kDa PhaP-MOG showed an N terminus of MTMITP.

Batch cultivations led to the production of about 1 × 1011 PHA granules/L cultivation broth corresponding to about 3.0 g protein displayed at the PHA granules surface.

Native IL2 and MOG proteins were displayed on PHA granules

To test whether IL2 and MOG were expressed in native form on the surface of the respective PHA granules, antibodies that recognize correctly folded IL2 (PC61) or MOG (8-18C5) and FACS technology were used [11,12]. The size of PHA granules produced in *E. coli* has been reported to be about 100–500 nm [1], and were detected in the forward scatter (FSC) and 90° side scatter (SSC) detectors set to list mode at a 256-channel resolution (data not shown). PHA granules were incubated with unlabelled mouse anti-MOG antibodies, followed by APC-labelled anti-mouse IgG antibodies, or with PE-labelled anti-IL2

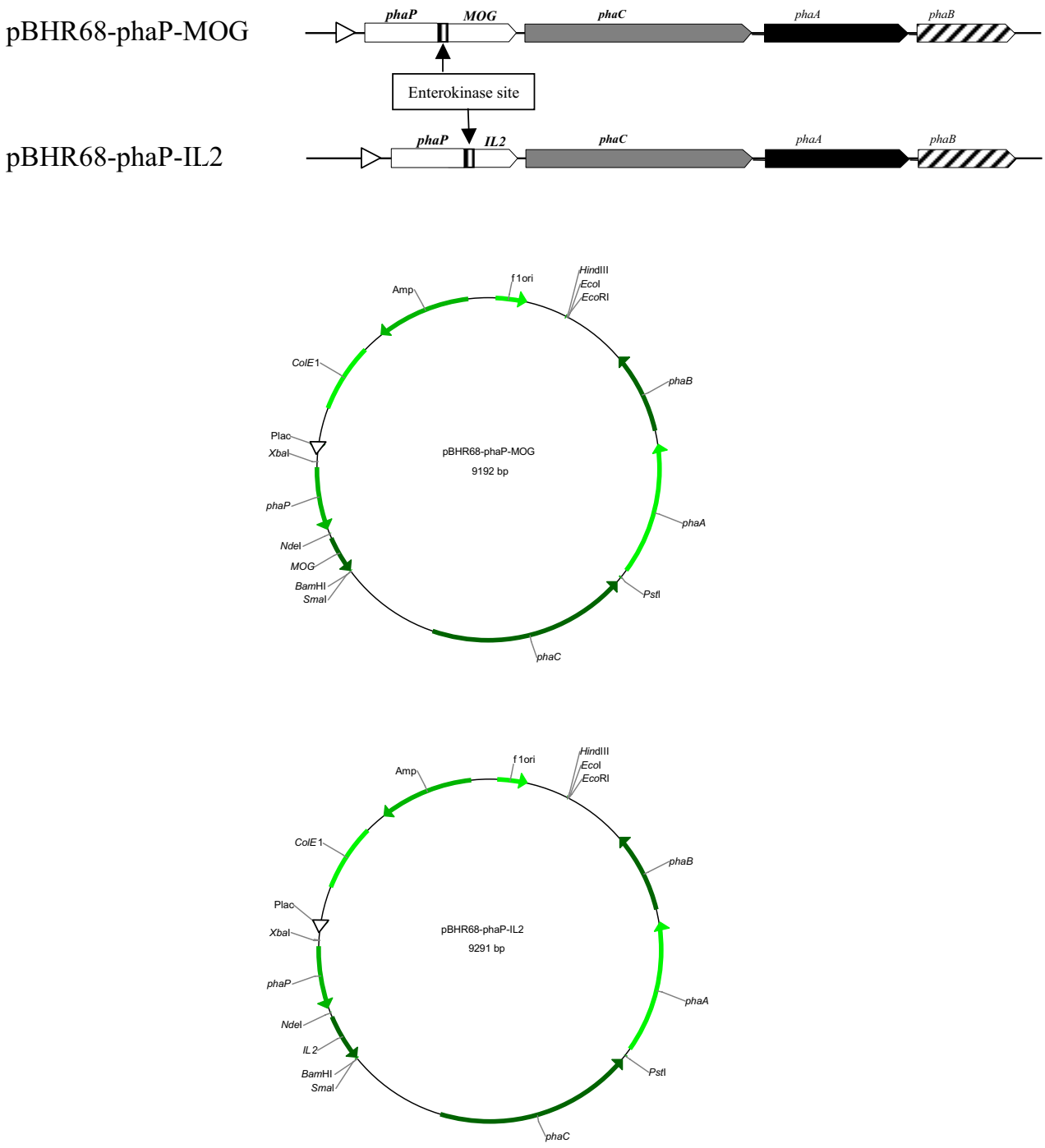

Figure 1
Schematic view of plasmid constructs mediating production of antigen displaying PHA granules in *E. coli*. Triangle represents the *lac* promoter. *phaP*, phasin gene; IL2, interleukin 2 gene; MOG, myelin oligodendrocyte glycoprotein encoding gene; *phaC*, PHA synthase encoding gene; *phaA*, gene encoding β-kethothiolase; *phaB*, gene encoding acetoacetyl-CoA reductase.

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

Strains	Genotype	Source or reference
Escherichia coli	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_k^-, m_k^+),	[16]
XL1-Blue	*supE44*, *relA1*, -, *lac* [F', *proAB*, *lacI*^{q}, *lacZ*Δ*M15*, Tn*10*(Tc^r)]	
Cupriavidus necator H16	Wildtype (PHA$^+$)	DSMZ 428
Plasmids		
pCRII	Amp, ColE1 origin	Initrogen
pBHR68	PHB biosynthesis operon from *C. necator* in pBluescriptSK$^-$	[17]
pHAS	pET-14b containing *Nde*I/*Bam*HI inserted *phaC* gene from *C. necator*	[18]
pUC57-MOG	DNA fragment encoding N terminal 1–173 amino acids of MOG from mouse inserted in *Sma*I site of pUC57	This study
pUC57-IL2	DNA fragment encoding N terminal 60–169 amino acids of IL2 from mouse inserted in *Sma*I site of pUC57	This study
pCR-phaP	pCRII containing *phaP* gene from *C. necator*	This study
pHAS-phaP	pHAS containing the *phaP* gene inserted into *Xba*I/*Nde*I sites	This study
pHAS-phaP-MOG	pHAS-phaP containing the MOG encoding DNA fragment inserted into *Nde*I/*Bam*HI sites	This study
pHAS-phaP-MOG	pHAS-phaP containing the IL2 encoding DNA fragment inserted into *Nde*I/*Bam*HI sites	This study
pUC57-phaP-MOG	DNA fragment encoding PhaP inserted into *Xba*I and *Nde*I site of plasmid pUC57-MOG	This study
pUC57-phaP-IL2	DNA fragment encoding PhaP inserted into *Xba*I and *Nde*I site of plasmid pUC57-IL2	This study
pBHR68-phaP-MOG	DNA fragment encoding PhaP-MOG fusion protein subcloned from pUC57-phaP-MOG via *Xba*I and *Bam*HI into pBHR68	This study
pBHR68-phaP-IL2	DNA fragment encoding PhaP-IL2 fusion protein subcloned from pUC57-phaP-IL2 via *Xba*I and *Bam*HI into pBHR68	This study
Oligonucleotides		
phaP-*Xba*I-*Nde*I	5'-aaaaatctagaaaaaggagatatacatatgatcctc-accccggaacaag-3'	This study
phaP-*Xba*I	5'-aaaaatctagaaaaaggagatatacgtatgatcctc-accccggaacaag-3'	This study
phaP-*Nde*I	5'-aaaaacatatggtggtgatggtgatgcgagccgcgt-ttatcatcatcatcggcagccgtcgtcttc-3'	This study

antibodies. The fluorescent intensity was then measured using FACS technology. Results show that the monoclonal antibodies specifically recognized PHA granules displaying the specific antigen, but not an un-related antigen (Fig. 3). Both PC61 and 8-18C5 recognize only the respective native protein indicating that correctly folded IL-2 or MOG proteins were formed at the surface of the respective PHA granules. These PHA granules showed consistent performance in FACS analysis at least one year when stored at 4°C.

PhaP-IL2 and PhaP-MOG fusion proteins containing the Asp-Asp-Asp-Asp-Lys recognition sequence are cleavable with enterokinase

To enable removal and/or purification of recombinant eukaryotic proteins, which form inactive inclusion bodies in *E. coli*, an enterokinase recognition sequence encoding for the Asp-Asp-Asp-Asp-Lys peptide was incorporated immediately downstream of PhaP. To determine if the recognition sequence was readily accessible, PhaP-IL2 and PhaP-MOG containing PHA granules were incubated for 16 h with enterokinase. Samples of PhaP-IL2 and PhaP-MOG granules were removed before, after 1 h and after 16 h of incubation with enterokinase to determine the level of native protein remaining at the surface of the respective PHA granule. PhaP-IL2 and PhaP-MOG granules were incubated with PE-conjugated anti-IL2 and samples analysed by FACS (Fig. 4A). A similar pattern of decreased protein levels after incubation with enterokinase was found with PhaP-MOG granules incubated with anti-MOG monoclonal antibodies (data not shown). The level of IL2 was reduced by ~80% after 1 h incubation and after 16 h incubation no significant levels of IL2 protein were detectable at the surface of the granules (Figure 4B). Overall, results shown in Figures 3 and 4 demonstrate that correctly folded recombinant proteins can be expressed as a fusion protein together with PhaP and that the fusion partner can be completely removed by enterokinase treatment.

PHA granules displaying PhaP-MOG fusion proteins were used in FACS-based assays to detect antigen-specific serum antibodies

Since PHA granules can be detected using FACS technology, we tested whether PhaP-MOG granules could be used to detect and quantify the level of antigen-specific antibodies from immunized animals. For this purpose, mice were immunized with recombinant MOG or OVA protein in CFA to induce an antibody response to MOG and OVA, respectively. Twenty-eight days post immunization, mice were tail-bled and immune sera collected. Sera from five different mice were pooled and tested for binding to PhaP-MOG granules. Results in Figure 5A showed that MOG-immunized, but not OVA-immunized, anti-sera contained antigen-specific IgG antibodies that recognized the PhaP-MOG fusion protein. The sera could be diluted >1:10^5 fold and still showed a significant binding of IgG to the PhaP-MOG granules (Figure 5B). Sera from OVA-immunized mice did not bind significantly to PhaP-MOG granules. However, OVA immunized mice produced OVA-specific IgG, as micro-titre wells coated with OVA, but not with MOG, showed bound IgG which was easily detectable using ELISA (Figure 5C). These results illustrate that recombinant eukaryotic proteins displayed with

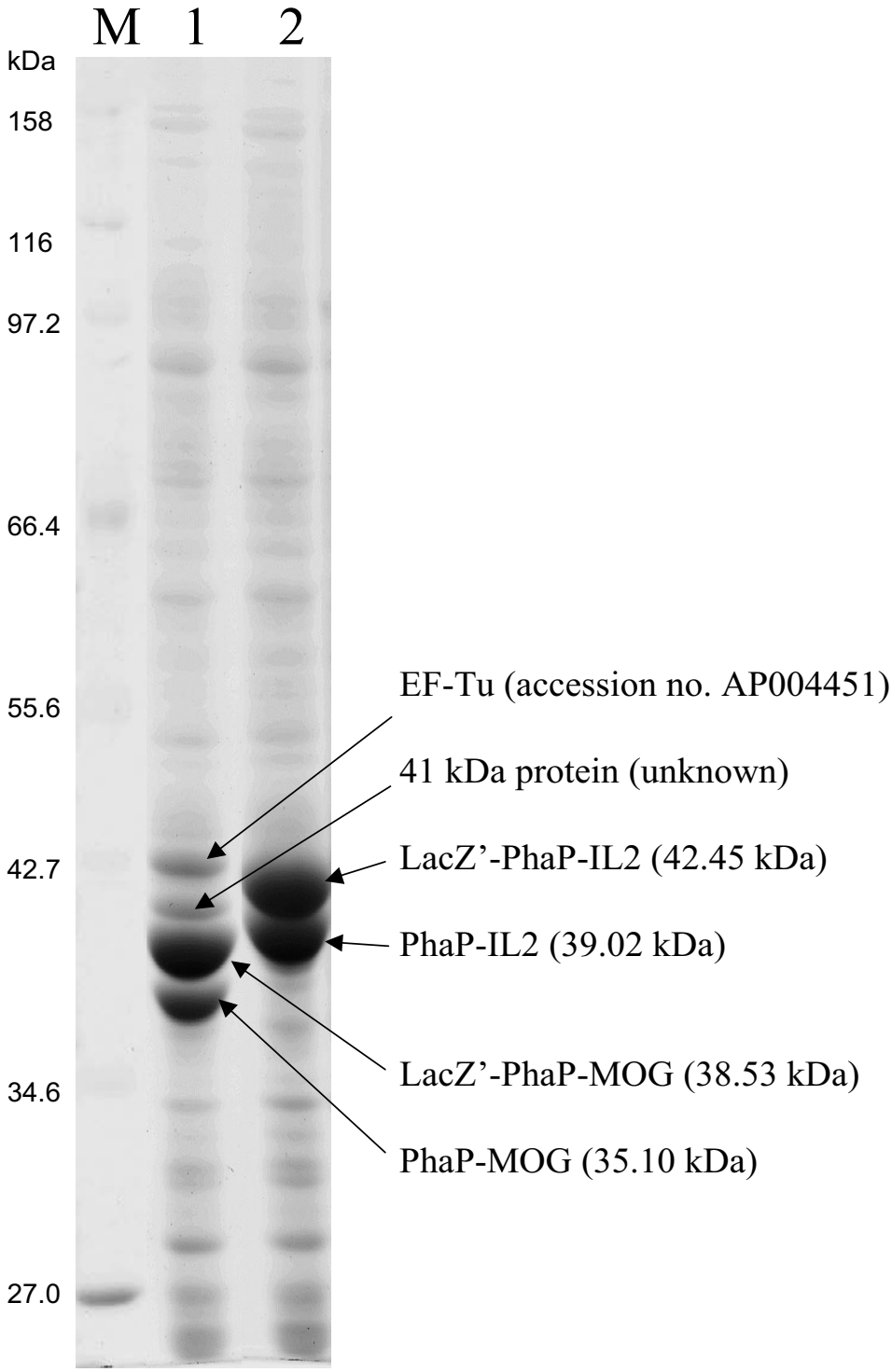

Figure 2
SDS-PAGE analysis of PHA granule attached proteins. Proteins were identified by peptide fingerprinting using MALDI-TOF/MS and N terminal amino acid sequencing. LacZ' represents the N terminal 33 amino acid residues of LacZ. M, molecular weight standard; 1, PHA granules isolated from *E. coli* harbouring plasmid pBHR68-phaP-MOG; 2, PHA granules isolated from *E. coli* harbouring plasmid pBHR68-phaP-IL2.

Table 2: Identified peptide fragments of proteins analyzed by MALDI-TOF/MS

protein	peptide fragments
LacZ'-PhaP-IL2	A91-Y120, E96-A114, E121-E135, E190-T218,
PhaP-IL2	A57-Y86, E62-A80, E87-E101, E156-T184, L300-G320
LacZ'-PhaP-MOG	N15-M34, W18-M34, L89-H118, A94-H118, L119-N155, L113-V133, A156-S169, T185-T216, A188-T216, T222-G232, G247-L255, T273-P283, V289-R308
PhaP-MOG	L55-H84, A60-H84, L85-N121, L79-V99, A122-S135, A154-T182, A173-T188, G247-L255, T273-P283, T275-V288, V289-R308.

PhaP as a fusion protein can be applied using FACS technology to detect and quantify the level of antigen-specific antibodies from vaccinated animals.

Discussion

In this study, the surface of PHA granules produced by recombinant *E. coli* was subjected to protein engineering to display natively folded eukaryotic proteins, which serve as antigens in diagnostics (Fig. 6). Both chosen eukaryotic proteins MOG and the cytokine IL-2 form inclusion bodies upon production in *E. coli* [13,14]. A microbial protein production system avoiding the formation of inclusion bodies would have a huge advantage by circumventing the tedious process of solubilization and refolding. The PHA granule attached structural protein PhaP was N terminally fused to either MOG or IL2 (Fig. 1). A strong overproduction under *lac* promoter control of the respective fusion proteins could be observed (Fig. 2). The fusion proteins could be identified by peptide fingerprinting using MALDI-TOF/MS analysis and by N terminal amino acid sequencing. Interestingly, the fusion proteins appeared in two versions with different apparent molecular weight. The smaller molecular weight version of the fusion protein showed the theoretical molecular weight of the respective fusion protein. The N terminal sequence (MTMITP) of the larger molecular weight version suggested that the N terminal 33 amino acid residues (theoretical molecular weight of 3.4 kDa) of LacZ were fused to the N terminus of PhaP fusion protein based on start codon upstream of the start codon of the hybrid gene encoding the PhaP-MOG or PhaP-IL2. This was also in accordance with the apparent molecular weight difference of 3.5 kDa between the two versions. Interestingly, the LacZ'-PhaP-MOG/IL2 fusion proteins were more abundant than the respective single fusions (Fig. 2). These data suggest that N terminal fusions to PhaP do not interfere with the binding to the PHA core of PHA granules and that the copy numbers can be even enhanced. This is unexpected considering the current phasin protein model [5]. The presence of the two *E. coli* proteins 41 kDa and EF-Tu at the MOG displaying PHA granule surface might be due to an unspecific interaction with PHA granule surface, which has been already observed elsewhere [10]. In contrast to a previous study, where multiple copies of PhaP were required for binding of prokaryotic protein fusions, a single copy was sufficient using an enterokinase site linker and eukaryotic proteins [6].

Engineered PHA granules were subjected to FACS analysis using antibodies specifically recognizing the respective native protein folds, which showed the display of the natively folded eukaryotic proteins MOG and IL2 at the PHA granule surface as well as the applicability of designed PHA granules for FACS-based diagnostics (Fig. 3). Although MOG and IL2 are secreted proteins, the derived protein domains were properly folded attached to the PHA granule in the reducing cytosol of *E. coli* while avoiding the formation of protein inclusion bodies. Although *E. coli* strains (e.g. Origami) are available, which provide an oxidative cytosol, protein overproduction often leads to inclusion body formation. Enterokinase treatment of MOG or IL2 displaying PHA granules and the complete release of the antigen suggested that the respective antigen was exposed at the PHA granule surface (Fig. 4). This surface exposure enables efficient antigen specific antibody binding. To evaluate the suitability of MOG or IL2 displaying PHA granules for qualitative and quantitative FACS-based antibody detection, mice were immunized with MOG or OVA (control) and induction of specific antibody production was assessed and confirmed by ELISA (Fig. 5). These MOG or OVA antisera were analysed using the MOG displaying PHA granules and FACS technology, which clearly indicated that anti-MOG antibodies can be specifically detected at least up to an antisera dilution of 1:100,000 (Fig. 5). In this study, it was demonstrated that eukaryotic proteins can be functionally displayed at the PHA granule surface using protein engineering of PhaP. Evidence was provided that the fusion proteins (antigens) were exposed at the PHA granule and thus PHA granules could be used to capture antibodies. This feature in combination with the particle properties of the PHA granules led to an outstanding performance in FACS based diagnostics, particularly considering the signal to noise ratio and dynamic range of antibody detec-

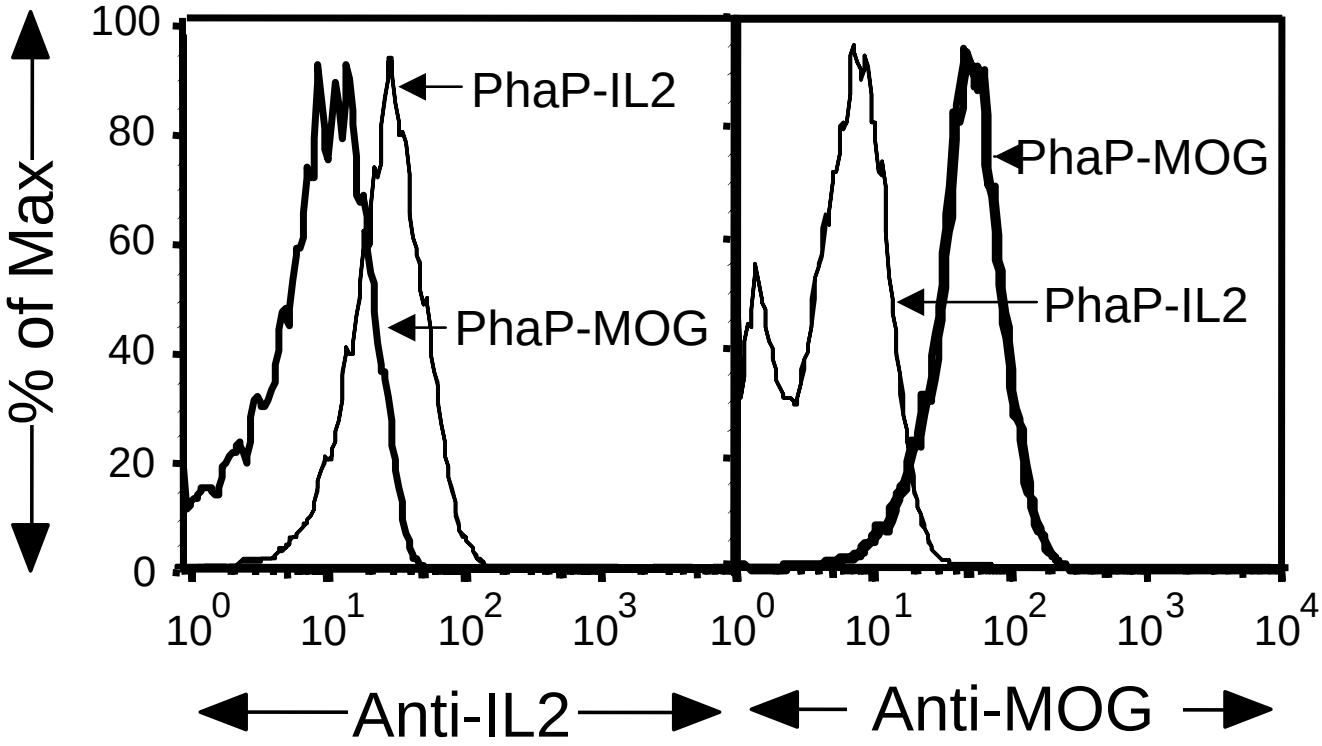

Figure 3

PHA granules display the native eukaryotic proteins. PhaP-IL2 and PhaP-MOG granules were produced as described in Materials and Methods. Approximately 5 × 10^8 PhaP-IL2 or PhaP-MOG granules were incubated with PC61 (anti-IL2) or 8-18C5 (anti-MOG) antibodies. Samples were collected using a FACsCalibur and then analyzed on FlowJo software. The data were depicted in a "normalized" fashion (% of Maximum). The % of Max is the number of cells in each bin divided by the number of cells in the bin that contains the largest number of cells. One representative experiment of at least three independent experiments, which showed no significant differences, is shown.

tion. It was recently shown that ZZ domain displaying PHA granules, which were produced via protein engineering of the PHA synthase, were suitable for IgG purification [15]. When compared to commercial beads, engineered PHA granules showed increased sensitivity with similar distribution of signal intensities in FACS. Overall, protein engineering of PHA granule surface proteins provides a novel molecular tool for the display of antigens for FACS-based diagnostics.

Conclusion

In this study, it was demonstrated that correctly folded eukaryotic proteins can be abundantly produced at the PHA granule surface as phasin fusion proteins. Isolated PHA granules displaying the respective eukaryotic proteins could be used as beads for specific and sensitive antibody detection using FACS technology. These native antigen displaying PHA granules were manufactured by recombinant *E. coli* without the need of antigen purification and chemical cross-linking to independently produced beads. Often purification of these proteins requires tedious refolding at low efficiency. The production of functional eukaryotic proteins at the PHA granule surface represents a novel *in vivo* matrix-assisted protein folding system avoiding aggregation of protein folding intermediates. Moreover, PHA granules could be stored for at least one year at 4°C without loss of performance in FACS supporting their potential use in diagnostic applications.

This work opens up an alternative route for the production of protein displaying beads harnessing nature's capacity to produce spherical polymer beads which surface can be functionalized by engineering of specifically bead associating proteins (Fig. 6). Multiple functionality might be easy achievable by co-expression of various hybrid genes suggesting that this biotechnological bead production strategy might represent a versatile platform technology.

Methods

Bacterial strains and growth conditions

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *Cupriavidus necator* and *E. coli* XL1 Blue were grown at 37°C. When required, ampi-

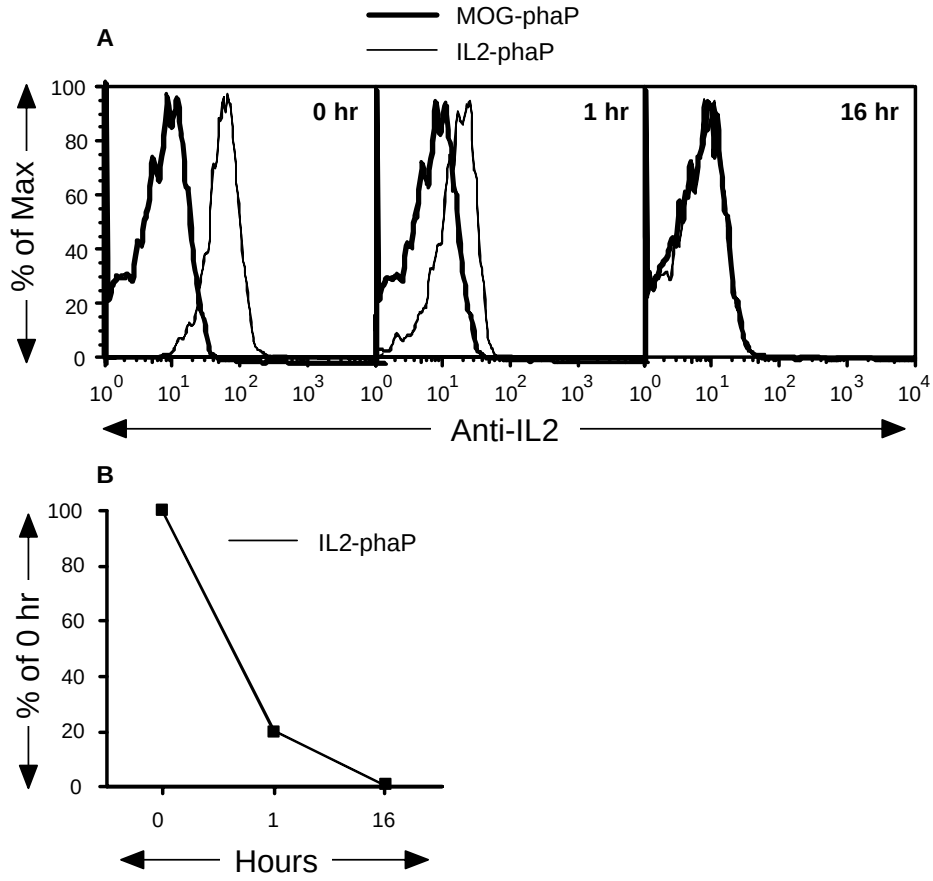

Figure 4

Enterokinase treatment of antigen displaying PHA granules and FACS analysis. PhaP-IL2 and PhaP-MOG granules were incubated with enterokinase for the indicated period of time, washed and then tested for the amount of native proteins remaining at the surface of the granules. In (A), the anti-IL2 PC61 and anti-MOG 8-18C5 monoclonal antibodies were used for this purpose as described for Figure 1. In (B), the percentage of IL2 protein detected after 1 and 16 hours incubation with enterokinase is shown. The data was depicted in a "normalized" fashion (% of Max). One representative experiment of at least three performed experiments, which showed no significant differences, is depicted. After overnight incubation no detectable staining using fluorescent anti-IL2 antibodies could be observed.

cillin 75 μg/ml was added. All chemicals were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Isolation, analysis and manipulation of DNA

General cloning procedures and isolation of genomic DNA were performed as described elsewhere [19]. DNA primers, deoxynucleoside triphosphate, *Taq* and Platinum *Pfx* polymerases were purchased from Invitrogen™ (CA, USA). DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using the model ABI310 automatic sequencer. Plasmids used in this study are listed in Table 1.

Construction of plasmids mediating production of either IL2 or MOG displaying PHA granules

The DNA fragments encoding either the full length mature IL2 protein (amino acids 60–169, accession no. AAN38301) or the extracellular part of the MOG protein (amino acids 1–117, accession no. Q61885) from mouse were synthesized by GenScript Corp. (USA). The codon usage was optimized for expression in *E. coli* [see Additional file 1]. Each DNA fragment contained an *Nde*I site at the 5' end and a *Bam*HI site at the 3' end. These DNA fragment were inserted into the *Sma*I site of pUC57 directly after synthesis by GenScript Corp. (USA) resulting in plasmids pUC57-MOG or pUC57-IL2, respectively. The

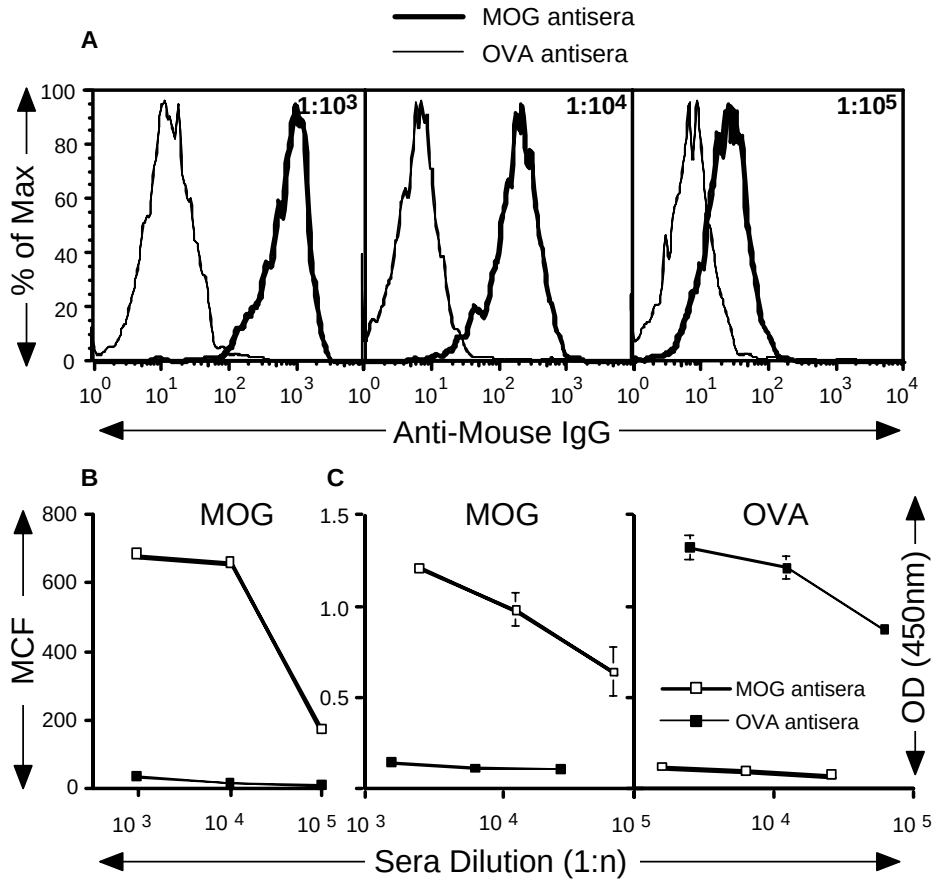

Figure 5

FACS-based detection of antigen-specific antibodies using antigen displaying PHA granules. In (A), MOG-phaP granules were incubated with serial dilutions of pooled antisera from five MOG or OVA immunized mice. Granules were extensively washed and then incubated with biotinylated anti-mouse IgG, followed by PE-conjugated streptavidin. The data was depicted in a "normalized" fashion (% of Max). One representative experiment, of at least two experiments performed, is depicted. In (B), the mean channel fluorescent for each dilution of antisera from MOG or OVA immunized mice binding to MOG-phaP was measured and depicted. In (C), ELISA was performed on anti-sera from MOG- and OVA-immunized mice. Serial dilutions of the anti-sera were added to MOG- or OVA-coated wells and incubated for 30 minutes. Biotinylated anti-mouse IgG was then added to the washed wells, followed by HRP-conjugated streptavidin and TMB substrate. The optical density was read at 450 nm and the results from one representative experiment, of at least two experiments performed, are shown. In (C), data are displayed as mean ± SEM from triplicate samples.

coding region of *phaP1* gene was amplified by PCR from chromosomal DNA of *C. necator* using oligonucleotides phaP-XbaI-NdeI and phaP-NdeI listed in Table 1 and introducing an *Xba*I site at the 5' end and an *Nde*I site including an enterokinase site at the 3' end. The PCR product was subcloned into TA cloning plasmid pCRII. The *phaP* coding region was again amplified from plasmid pCR-phaP using oligonucleotides phaP-XbaI including an *E. coli* ribosomal binding site and phaP-NdeI. The resulting PCR product was subcloned into the *Xba*I and *Nde*I sites of pHAS.

Either pUC57-MOG or pUC57-IL2 was hydrolyzed with *Nde*I and *Bam*HI and the corresponding DNA fragments were subcloned into pHAS-phaP resulting in plasmids pHAS-phaP-MOG and pHAS-phaP-IL2, respectively. The respective fusion protein encoding region was then subcloned into pBHR68 using *Xba*I and *Bam*HI sites down-

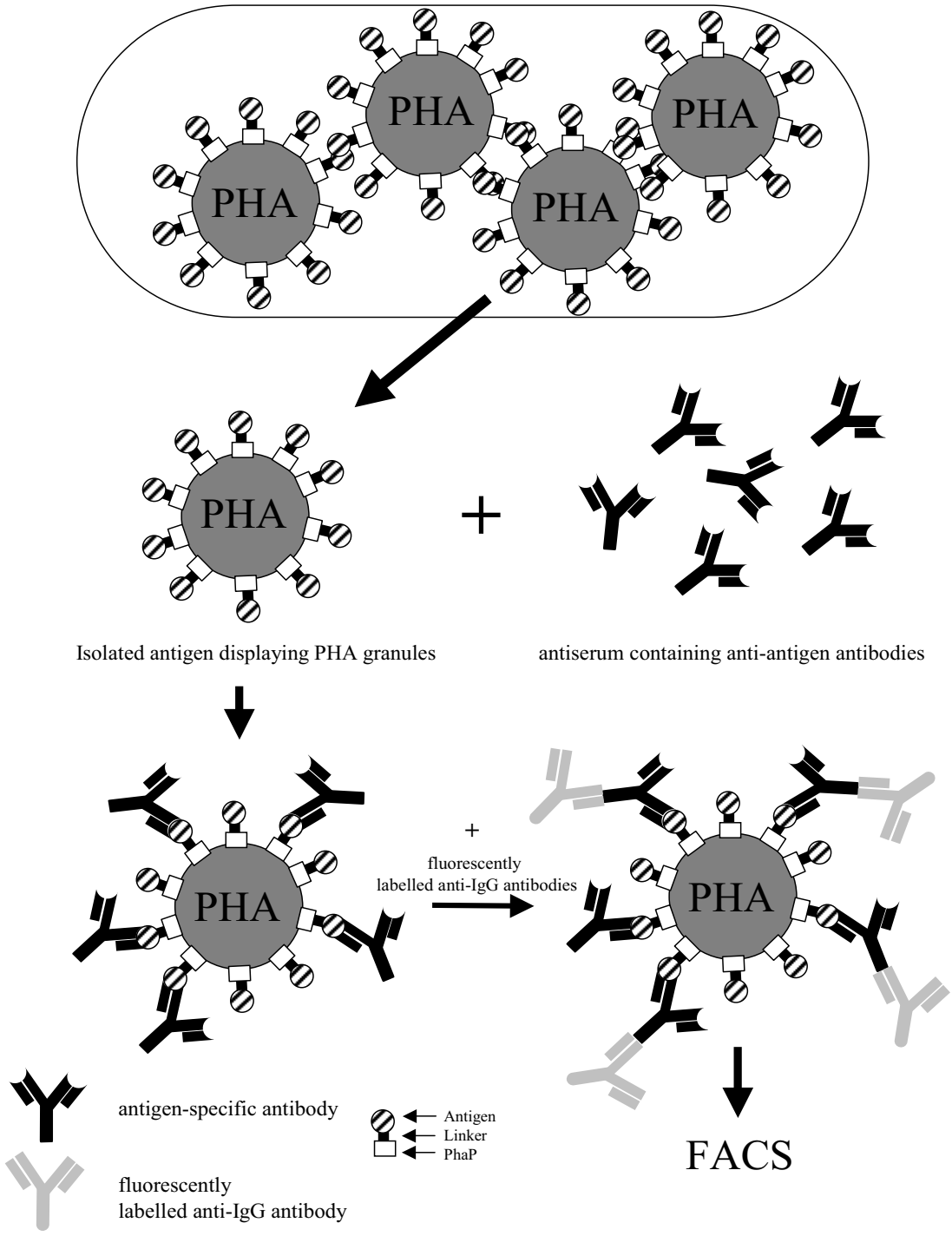

Figure 6
Schematic view of the microbial production of antigen displaying PHA granules and their use in FACS analysis

stream of the *lac* promoter and upstream of the PHB biosynthesis operon (Fig. 1).

Production of phasin fusion proteins at the PHA granule surface

Cells of *E. coli* XL1 Blue were transformed with plasmids pBHR68-PhaP-IL2 and pBHR68-PhaP-MOG, respectively. Transformants were grown at 37°C and induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After growth for 48 h, cells were harvested by centrifugation and subjected to PHA granule isolation.

Isolation of PHA granules

Cells were harvested by centrifugation for 15 min at 5,000 × g and 4°C. The sediment was washed and suspended in 3 volumes of 50 mM phosphate buffer (pH7.5). Cells were passed through French Press four times at 8000 psi. The cell lysate (0.75 ml) was loaded onto a glycerol gradient (88 % and 44 % (v/v) glycerol in phosphate buffer). After ultracentrifugation for 2.5 h at 100,000 × g and 10°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, pH7.5) and centrifuged at 100,000 × g for 30 min at 4°C. The sediment containing the PHA granules was suspended in phosphate buffer and stored at 4°C.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)

Protein samples were routinely analyzed by SDS-PAGE as described elsewhere [20]. The gels were stained with Coomassie brilliant blue G250. Protein bands of interest were cut off the gel and analyzed 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) utilizing 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 2000. 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% TFA.

Mice

C57BL/6 mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained by the Biomedical Research Unit, Malaghan Institute of Medical Research, Wellington, New Zealand. Experimental protocols were approved by the Victoria University of Wellington Animal Ethics Committees and performed according to the guidelines of their guidelines. In all experiments, sex and age matched mice were used between 8–12 weeks of age.

Mouse serum IgG responses

C57BL/6 mice were immunized with 150 μg recombinant mouse MOG protein consisting of amino acid 1–117 (MOG_{1-117}) of the matured protein [21] or 150 μg ovalbumin protein (OVA, Sigma-Aldrich) emulsified in Complete Freund's adjuvant (CFA), containing 500 μg *Mycobacterium tuberculosis* (both DIFCO Laboratories). The emulsion (100 μl) was injected subcutaneously over the flanks. Blood was collected from tail bleeds 28 days post-immunization, centrifuged at 13,000 g for 1 min, and then the top layer of serum was removed and stored at -20°C. Serum was tested for antigen-specific total IgG, using the PhaP-MOG granules and flow cytometry or ELISA (see below).

Cleavage of IL2 from IL2 displaying PHA granules

The enterokinase recognition sequence Asp-Asp-Asp-Asp-Lys was introduced between PhaP and IL-2 to enable cleavage of the IL-2 protein from the granules. Twenty-five μg of bovine enterokinase (Sigma-Aldrich, E-5144) in 10 mM Tris/HCl, 10 mM $CaCl_2$ (pH8) were incubated with 2 × 10^{10} PhaP-IL2 or PhaP-MOG granules at 37°C, and at indicated time points samples were removed, washed with PBS, and stored at 4°C until use. The relative amount of native IL2 and MOG at the surface of the granules was determined using flow cytometry.

Flow cytometry

PhaP-MOG or PhaP-IL2 granules (~5 × 10^8/well) were added to 96-well round-bottom plates (Becton, Dickinson and Company) and washed twice with FACS-buffer (PBS, 1% Foetal calf serum, 0.1% sodium azide, 5 mM EDTA, pH8). IL2-phaP or MOG-phaP granules were then incubated with phycoerythrin conjugated anti-IL2 monoclonal antibody (clone PC61, PharMingen) or a mouse anti-MOG monoclonal antibody (clone 8.18-C5, kindly provided by C. Bernard). Following 15 minutes incubation at ambient temperature, granules were washed twice in fluorescence activated cell sorting (FACS) buffer. Allophycocyanin-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) polyclonal antibodies were added to the MOG displaying PHA granules and incubated for 15 minutes, and then washed twice. At least 10,000 events for each sample were collected and analysed, using a BD FACsCAlibur and FlowJo analysis software (Tree Star, San Carlos, CA, USA). For detection of MOG-specific antibody responses, sera from MOG or

OVA immunized mice were diluted in FACS-buffer and then incubated for 30 minutes at ambient temperature with MOG-phaP granules. The granules were then washed and incubated with biotinylated anti-mouse total IgG, followed by PE-conjugated streptavidin. At least 10,000 events for each sample were collected and analysed on a BD FACsCAlibur and FlowJo software (Tree Star).

ELISA

Round-bottom 96-well plates (Becton, Dickinson and Company) were coated overnight at 4°C (50 μl/well) with 3 μg/ml of recombinant mouse MOG_{1-117}or OVA protein (Sigma-Aldrich) in PBS. Supernatant was discarded and wells blocked by adding 1% BSA in PBS (100 μl/well) for 1 h at ambient temperature. Plates were then wash 3 times with 10 mM Tris/HCl, pH 7.5, 0.05% Tween 20 (ELISA buffer). Diluted sera (50 μl/well) in 0.1% BSA/PBS from MOG_{1-117} or OVA immunized mice were added and after 2 h, plates were washed 3 times with ELISA buffer. Biotin-conjugated anti-mouse IgG antibodies (Southern Biotechnology, Ltd) diluted 1:4,000 in 0.1% BSA/PBS were added for 1 h, and wells then wash 3 times with ELISA buffer. Amdex Streptavidin-HRP (Amersham Biosciences) diluted 1:3,000 in 0.1% BSA/PBS, was added to each well (50 μl/well) and incubated for 30 minutes. Plates were then wash 3 times and 3,3',5,5'-tetramethylbenzidine (TMB) added for 5–30 minutes. Colour development was stopped using 2 M H_2SO_4 and the ELISA plates were then read at 450 nm on a Benchmark microplate reader (Bio-Rad Laboratories Inc. Hercules, CA, USA).

Authors' contributions

TB designed and conducted all immunological experiments including FACS analysis and identification of the antigens to be used as examples. TB wrote a part of the manuscript. JB carried out all cloning, PHA granule production/isolation and SDS-PAGE analysis. BR conceived the experimental strategy for the production of the engineered PHA granules and their analysis as well as wrote the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

DNA sequence of synthetic DNA fragments encoding either MOG or IL2 with optimized codon usage for expression in E. coli.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1472-6750-7-3-S1.doc]

Acknowledgements

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ORIGINAL RESEARCH PAPER

Protein engineering towards biotechnological production of bifunctional polyester beads

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Abstract Microbial polyester inclusions have previously been demonstrated to be applicable as versatile beads outside the bacterial cell. Engineering of proteins selectively binding to the polyester inclusions was conceived to produce polyester beads simultaneously displaying two protein-based functions suitable for applications in, for example, fluorescence activated cell sorting (FACS). The polyester synthase and the phasin protein were fused to the green fluorescent protein (GFP) and the murine myelin oligodendrocyte glycoprotein (MOG), respectively, or GFP and MOG were fused to the *N*- and *C*-terminus, respectively, of only the phasin. In both cases, fusion proteins were found to be attached to isolated polyester inclusions while displaying both functionalities per bead. Functionalities at the bead surface were assessed by ELISA, FACS and fluorescence microscopy. The respective double fusion protein was identified by peptide fingerprinting using MALDI-TOF/MS.

Keywords Bio-beads · Biopolyester · Nanoparticle · Polyhydroxyalkanoate

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Introduction

Polyesters composed of (*R*)-3-hydroxy fatty acids are accumulated by various bacteria in response to nutrient starvation but carbon source excess (Madison and Huisman 1999; Rehm and Steinbüchel 1999). These polyesters serve as reserve polymers and are deposited as spherical cytosolic inclusions with the core composed of the hydrophobic polyester and surface composed of specific proteins such as e.g., the polyester synthase and phasins (Rehm 2006, 2007). The polyester synthase catalyses the synthesis of the polyester and is the essential enzyme for polyester inclusion formation (Rehm 2003). The polyester synthase remains covalently attached to the synthesized polyester at the surface of the inclusion while the structural phasins attach hydrophobically to the polyester core during polyester inclusion formation (Hanley et al. 1999; Hezayen et al. 2002; Peters and Rehm 2006; Peters et al. 2007; Tian et al. 2005). Both proteins have been subjected to extensive protein engineering enabling display and production of functional proteins at the polyester inclusion surface and the respective engineered polyester beads were found to be applicable in diagnostics, affinity chromatography, protein production and enzyme immobilization (Barnard et al. 2005; Brockelbank et al. 2006; Grage and Rehm 2008; Peters and Rehm 2005, 2006, 2008). However, none of these previous investigations attempted to produce beads with two independent functionalities, such as e.g., a labeling

fluorescent protein and an antigen for antibody detection, attached to the same bead. In this study two approaches were evaluated with respect to the display of two different functionalities at the polyester bead surface: (1) Simultaneous production of two different fusion proteins and (2) production of one double fusion protein.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli XL1-blue was grown in LB medium (Sigma) containing 75 μg ampicillin/ml and 12.5 μg tetracycline/ml to an OD_{600} of 0.3 then induced with the addition of 1 mM IPTG. The strains were then cultivated at 25°C with shaking for 72 h.

Isolation, analysis and manipulation of DNA

General cloning procedures were performed as described elsewhere (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.

The plasmid, pBHR68GPM, was constructed to enable production of polyester inclusions with an attached phasin protein showing an *N*- and *C*-terminal fusion. The *N*- and *C*-terminal fusion partners were the green fluorescent protein (GFP) and the antigen murine myelin oligodendrocyte glycoprotein (MOG), respectively. The DNA fragment encoding the protein PhaP without a start codon (ATG) was generated by PCR amplification using oligonucleotides '5′(-)ATG_EXTPhaP containing the restriction site *Xba*I and with reverse primer '3′ NdeI-PhaP'. This PCR fragment was ligated into intermediate vector pHAS PhaP-MOG at restriction sites *Xba*I and *Nde*I replacing the wildtype *phaP* gene. The *Xba*I-*Bam*HI fragment from the resulting plasmid pHAS EXTPhaP-MOG containing the hybrid gene encoding the fusion protein PhaP-MOG minus the start codon was subcloned into the respective sites of pBHR68 resulting in plasmid pBHR68(-)ATG PhaP-MOG. This vector was used as a template to make a PCR product that extended PhaP 34 amino acids and introduced a *Spe*I restriction site with primers 5′SpeEXTPhaP-MOG and 3′SpeEXTPhaP-MOG. The *Spe*I–*Bam*HI PCR fragment of SpeEXTPhaP-MOG was ligated back into pBHR68 generating plasmid pBHR68SpeEXTPhaP-MOG. Next the enhanced GFP encoding *Spe*I DNA fragment (720 bp) from plasmid pCWEAgfp was subcloned into the *Spe*I site of plasmid pBHR68SpeEXTPhaP-MOG resulting in pBHR68GPM.

The plasmid pBHR69GCPM was constructed to enable simultaneous production of the GFP-polyester synthase and phasin-MOG fusion protein attached to the polyester beads surface. The DNA fragment encoding the fusion protein GFP-PhaC was subcloned from pCWEAgfp into pBHR69 using restriction sites *Xba*I–*Bam*HI generating plasmid pBHR69-gfp-phaC. *Xba*I restriction sites were added by PCR to generate a DNA fragment encoding PhaP-MOG while using pBHR68PhaP-MOG as template and primers 5′ Xba-PhaP-MOG as well as 3′ Xba-PhaP-MOG. The PCR product was ligated into the *Xba*I site of pBHR69-gfp-phaC resulting in plasmid pBHR69GCPM.

Polyester formation and polyester bead isolation

The formation of the polyester, polyhydroxyalkanoate, by recombinant *E. coli* cells was qualitatively and quantitatively determined by GC/MS as previously described (Brandl et al. 1988).

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

Enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted as previously described (Peters and Rehm 2008). Blocking was achieved by incubation with 3% (w/v) BSA. The MOG protein was detected using monoclonal mouse anti-MOG antibodies clone 8.18-C5 (Linnington et al. 1984). Bound anti-MOG antibody was detected using a secondary HRP-labeled antibody (Abcam, Cambridge, MA, USA). Bound HRP-labeled antibody was quantified using *o*-phenylenediamine solution (OPD; Abbott Diagnostics, IL, USA) as HRP substrate according to the manufacture's protocol. Samples were in quadruplicate and the plate suspension was diluted 3-fold for reading at 490 nm in a Biotek ELX808 microplate reader (Biostrategy, Auckland NZ).

Table 1 Plasmids and oligonucleotides used in this study

Plasmids		
pBHR68	Polyester biosynthesis operon from *C. necator* in pBluescriptSK-	(Spiekermann et al. 1999)
pBHR68-phaP-MOG	DNA fragment encoding phasin-MOG fusion protein subcloned from pUC57-phaP-MOG via *Xba*I and *Bam*HI sites into pBHR68	(Bäckström et al. 2007)
pHAS-PhaP-MOG	pHAS-PhaP containing the MOG encoding fragment inserted into *NdeI/BamH*I sites	(Bäckström et al. 2007)
pBHR69	pBBR1MCS derivative containing genes *phaA* and *phaB* of *C. necator* colinear to *lac* promoter	(Qi and Rehm 2001)
pCWEAgfp	pBluescriptSK-containing the polyester synthase gene and a *Spe*I-inserted *gfp* gene derived from pPROBE-NT by PCR	(Peters and Rehm 2005)
pBHR69-gfp-phaC	Intermediate cloning vector containing the DNA fragment encoding the fusion protein GFP-PhaC from pCWEAgfp	This study
pBHR68 NS_EXTPhaP-MOG	DNA fragment encoding EXTPhaP-MOG without start codon ATG	This study
pBHR68EXTPhaP-MOG	DNA fragment encoding EXTPhaP-MOG inserted into *Xba*I and *Bam*HI site of plasmid pBHR68	This study
pBHR68GPM	DNA fragment encoding GFP-EXTPhaP-MOG generated by inserting the GFP fragment subcloned from pCWEAgfp *SpeI* site of pBHR68SpeEXTPhaP-MOG	This study
pBHR68SpeEXTPhaP-MOG	Intermediate cloning vector generated by inserting the fusion protein(-)ATG PhaP-MOG at SpeI-BamHI sites of pBHR68	This study
pBHR69GCPM	DNA fragment encoding GFP-PhaC and PhaP-MOG generated by inserting PhaP-MOG into the *Xba*I site of pBHR69-gfp-phaC	This study

Oligonucleotides (Gene/plasmid)	Restriction site	Sequence from 5′ to 3′	
5′-(-)ATG_EXTPhaP	*Xba*I	CGCTCTAGAAAAAGGAGATATACGTGAATCCTCACC	This study
3′-Nde-PhaP (*phaP*/pBHR68GPM)	*Nde*I	ACCCATATGGTGGTGATGGTGATGCG AGC	This study
5′-SpeEXTPhaP-MOG	*Spe*I	CAAACTAGTCTCCTAAATAGCTATG ACCATGATTACGCCAAGCGCGC	This study
3′-SpeEXTPhaP-MOG (*phaP-MOG*/pBHR68GPM)	*Bam*HI	CGGGGGATCCTTAATCTTCAACTTTC AGTTCCATGGCGGC	This study
5′-Xba-PhaP-MOG	*Xba*I	GGCCGCTCTAGAATAAAGGAGATATACGTATGATCCTCACCCCGG	This study
3′-Xba-PhaP-MOG (*phaP-MOG*/pBHR69GCPM)	*Xba*I	GATTCTAGATCTTCAACTTTCAGTTCCATGGCGGCTTCTTCCTGGTA	This study

Protein analysis

Protein samples were routinely analyzed by SDS-PAGE (see 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). Excised protein bands were subjected to in-gel digestion with trypsin. Peptides were spotted onto a MALDI sample plate (Opti-TOF 384 well plate, Applied Biosystems, MA). Samples were analysed on a 4800 MALDI tandem Time-of-Flight Analyzer (MALDI TOF/TOF, Applied Biosystems, MA). The 15–20 strongest precursor ions of each sample were used for MS/MS collision-induced dissociation (CID) analysis. CID spectra were acquired with 2000–4000 laser pulses per selected precursor using the 2 kV mode and air as the collision gas at a pressure of $2 \times E^{-7}$ torr.

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 (Bäckström et al. 2007). At least 100,000 events were collected and analysed.

Results and discussion

Plasmid construction and mediation of polyester inclusion formation

The two plasmids, pBHR69GCPM and pBHR68 GPM, were constructed in order for each to mediate in recombinant *E. coli* formation of polyester inclusions displaying the GFP protein as well as the antigen MOG. The one-step production of already fluorescently labeled polyester beads which display an antigen for antibody capture would be applicable in FACS-based diagnostics. These beads would not require staining with fluorescent dyes for detection of the entire bead population. Thus the GFP labeled beads displaying the antigen should enable FACS-based diagnostics based on detection of the bead fraction which bound antibody (analyte) and was labeled by a specific secondary phycoerythrin (PE)-conjugated antibody as compared to the total bead population. To achieve the display of two functionalities on the same polyester bead the two strategies employing either two independent fusion proteins or fusing different protein functions to the *N*- and *C*-terminus, respectively, of the same proteins were conceived and evaluated.

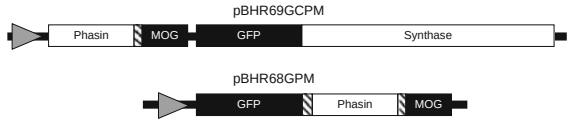

Fig. 1 Schematic representation of hybrid genes relevant for production of the respective fusion proteins. Both plasmids, pBHR69GCPM and pBHR68GPM, also contain the *Cupriavidus necator* gene *phaA* and *phaB* required for polyester precursor synthesis. Triangle, lac promoter; diagonally striped rectangles, linker regions; MOG, murine myelin oligodendrocyte glycoprotein; GFP, green fluorescent protein; Synthase, polyester synthase

Plasmid pBHR69GCPM (Fig. 1) which harbors all required polyester (polyhydroxybutyrate) biosynthesis genes was constructed to mediate polyester inclusion formation while simultaneously producing the GFP-polyester synthase and the phasin-MOG fusion protein. Both proteins, the polyester synthase and the phasins, are known to constitute the surface of polyester inclusions and the respective fusions have been shown to not interfere with protein functionality (Bäckström et al. 2007; Peters and Rehm 2005).

Phasin not only tolerates *C*-terminal fusions but also remains functional when an alternative start codon led to an additional fusion of 33 amino acids to the *N*-terminus (Bäckström et al. 2007). Thus plasmid pBHR68GPM (Fig. 1), which harbors all required polyester (polyhydroxybutyrate) biosynthesis genes, was constructed to mediate polyester inclusion formation while producing the GFP-phasin-MOG double fusion protein.

Both plasmids mediated polyester (polyhydroxybutyrate) accumulation amounting to about 40% (w/w) of cellular dry weight as determined by GC/MS analysis. Polyester inclusion formation was also confirmed by TEM analysis which was performed as previously described (Grage and Rehm 2008).

Polyester bead analysis and performance in FACS-based diagnostics

Beads isolated and purified from recombinant *E. coli* either harboring pBHR69GCPM or pBHR68GPM

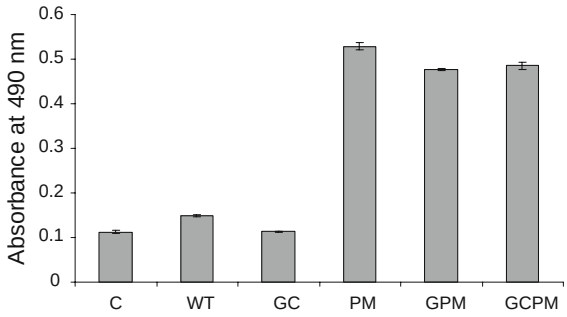

Fig. 2 ELISA showing anti-MOG antibody (clone 8.18-C5) binding to various beads. Bound antibody was detected using a HRP-conjugated secondary antibody. C, only secondary HRP-conjugated antibody; WT, wildtype beads (no fusion protein present); GC, beads with GFP-polyester synthase; PM, beads with phasin-MOG; GC-PM, beads with GFP-polyester synthase and phasin-MOG; GPM, beads with GFP-phasin-MOG

were assessed by fluorescence microscopy, which showed fluorescent labeling of beads suggesting display of GFP at the bead surface (see Supplementary Fig. 1). The same beads were subjected to ELISA using the monoclonal mouse anti-MOG antibody (clone 8-18C5) (Fig. 2). The ELISA data strongly suggested that the respective beads display the correctly folded MOG antigen. Proteins attached to the beads were analysed by SDS-PAGE analysis demonstrating the presence of the respective fusion proteins (data not shown). The hitherto not demonstrated double fusion protein GFP-phasin-MOG was shown as prominent protein which was identified by tryptic peptide fingerprinting using MALDI-TOF/MS (Table 2).

Beads displaying GFP and MOG were subjected to FACS analysis (Fig. 3). Beads either only displaying GFP or only MOG were used as control beads. Polyester inclusions were incubated either with unlabeled mouse anti-MOG antibodies, followed by PE-labeled anti-mouse IgG antibodies or with rabbit monoclonal anti-GFP antibodies (Abcam, Sapphire Biosciences, Australia), followed by goat FITC conjugated anti-rabbit polyclonal antibodies (Jackson Immunoresearch, Abacus ALS, New Zealand). The fluorescent intensity was then measured using FACS technology. Different filters were used to assess the two fluorescence signals emitted by the same bead population. Results show that the monoclonal anti-MOG and anti-GFP antibodies specifically recognized beads displaying the specific antigen, respectively, but not an un-related antigen (Fig. 3). Monoclonal antibody 8-18C5 recognizes only the respective native protein indicating that correctly folded MOG proteins were formed at the surface of the respective bead. The FACS analysis clearly demonstrated that both protein fusion approaches led to beads which were fluorescently labeled by GFP and that the same beads displayed an antigen suitable to detect an antigen-specific antibody. This study provides proof of concept regarding the microbial one-step production of polyester beads simultaneously displaying two

Table 2 Identified peptide fragments of GFP-phasin-MOG analyzed by MALDI-TOF/MS

Protein/Protein sequence	Peptide fragments assigned to the various protein regions
GFP-phasin-MOG (MW: 65.3 kDa): MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATY GKLTLKFICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHM KRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEG DTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMAD KQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVL LPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHG MDELYKTSITPSAQLTLTKGNKSWSSTAVAAALEKGDIR AILTPEQVAAAQKANLETLFGLTTKAFEGVEKLVELNLQ VVKTSFAEGVDNAKKALSAKDAQELLAIQAAAVQPVAE KTLAYTRHLYEIASETQSEFTKVAEAQLAEGSKNVQALV ENLAKNAPAGSESTVAIVKSAISAANNAYESVQKATKQA VEIAETNFQAAATAATKAAQQASATARTATAKKTTAAD DDDKRGSHHHHHHMGQFRVIGPGYPIRALVGDEAELPC RISPGKNATGMEVGWYRSPFSRVVHLYRNGKDQDAEQA PEYRGRTELLKETISEGKVTLRIQNVRFSDEGGYTCFFRD HSYQEEAAMELKVED	GFP: F27-K41, S86-R96 Phasin: L304-K313, D331-K349, H356-K370, N382-K392, Q425-K443 MOG: M513-R519, N531-R544, F568-R579

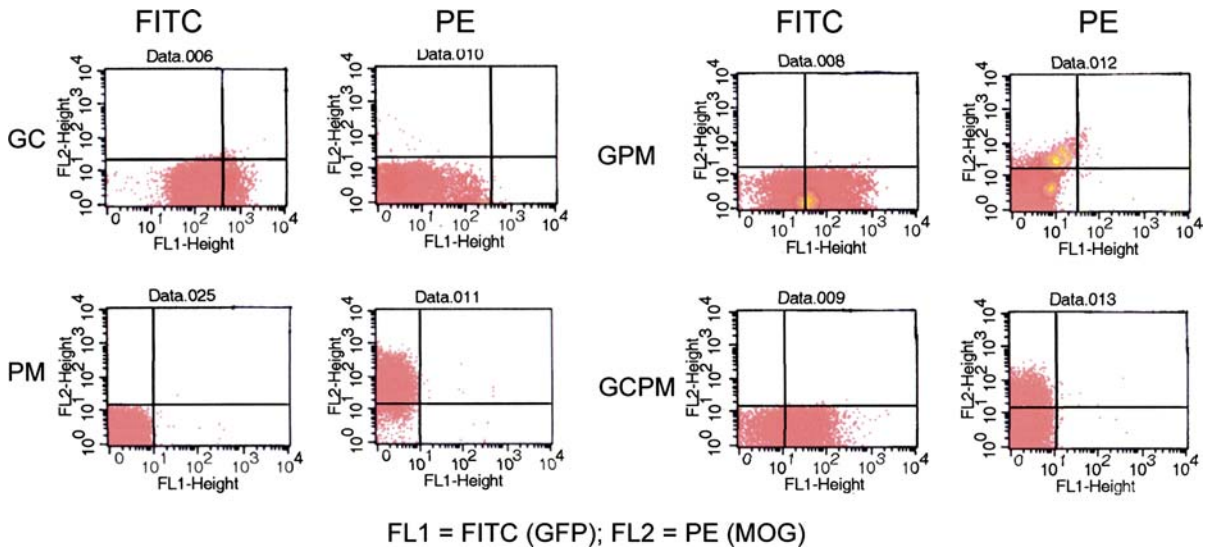

Fig. 3 FACS analysis of various polyester beads using either a FITC filter or a PE filter. GC, beads with GFP-polyester synthase; PM, beads with phasin-MOG; GCPM, beads with GFP-polyester synthase and phasin-MOG; GPM, beads with GFP-phasin-MOG

protein based functionalities. Biopolyester beads simultaneously displaying two functional proteins or antigens at the surface could be applied as multivalent vaccine or used in diagnostics where for example a fluorescent protein and a specific binding protein enables detection of a relevant analyte. Overall, the results obtained in this study strongly enhance the applied potential of these polyester beads in biotechnology and medicine.

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