Appl Microbiol Biotechnol (2015) 99:8045–8053
DOI 10.1007/s00253-015-6719-6

Insights into the surface topology of polyhydroxyalkanoate synthase: self-assembly of functionalized inclusions

David O. Hooks[1] · **Bernd H. A. Rehm**[1,2,3]

Received: 3 March 2015 / Revised: 17 May 2015 / Accepted: 21 May 2015 / Published online: 7 June 2015

Abstract The polyhydroxyalkanoate (PHA) synthase catalyzes the synthesis of PHA and remains attached to the hydrophobic PHA inclusions it creates. Although this feature is actively exploited to generate functionalized biobeads via protein engineering, little is known about the structure of the PHA synthase. Here, the surface topology of *Ralstonia eutropha* PHA synthase was probed to inform rational protein engineering toward the production of functionalized PHA beads. Surface-exposed residues were detected by conjugating biotin to inclusion-bound PHA synthase and identifying the biotin-conjugated lysine and cysteine residues using peptide fingerprinting analysis. The identified sites (K77, K90, K139, C382, C459, and K518) were investigated as insertion sites for the generation of new protein fusions. Insertions of FLAG epitopes into exposed sites K77, K90, K139, and K518 were tolerated, retaining >65 % of in vivo activity. Sites K90, K139, and K518 were also tested by insertion of the immunoglobulin G (IgG)-binding domain (ZZ), successfully producing PHA inclusions able to bind human IgG in vitro. Although simultaneous insertions of the ZZ domain into two sites was permissive, insertion at all three lysine sites inactivated the synthase. The K90/K139 double ZZ insertion had the optimum IgG-binding capacity of 16 mg IgG/g wet PHA beads and could selectively purify the IgG fraction from human serum. Overall, this study identified surface-exposed flexible regions of the PHA synthase which either tolerate protein/peptide insertions or are critical for protein function. This further elucidates the structure and function of PHA synthase and provides new opportunities for generating functionalized PHA biobeads.

Keywords Polyhydroxyalkanoate · Immunoglobulin G · Biotinylation · Protein immobilization

Introduction

Polyhydroxylakanoates (PHAs) are a group of carbon storage polymers produced in some bacteria and archaea when subjected to nutrient-imbalanced conditions (Brandl et al. 1988; Hezayen et al. 2000). The final enzyme in the production pathway is PHA synthase (PhaC), which catalyzes the conversion of (*R*)-3-hydroxyacyl-CoA to the PHA polymer (Rehm and Steinbüchel 1999). PhaC remains covalently attached to the end of the nascent PHA chain, and these amphipathic PhaC-PHA chain molecules form spherical inclusions of approximately 50–500 nm diameter inside the cell (Grage et al. 2009). PhaC from *Ralstonia eutropha* (ATCC 17699) has been used as an anchor for fusion protein immobilization to the bead surface (Brockelbank et al. 2006). This process allows for the in vivo production of functional PHA biobeads which are extracted by cell lysis and centrifugation (Rehm 2003; Draper et al. 2013). Applications for these beads have included enzyme display, affinity purification, and medical uses such as vaccines and diagnostics. Until now, limited understanding of the structure of PhaC has confined

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6719-6) contains supplementary material, which is available to authorized users.

✉ Bernd H. A. Rehm
b.rehm@massey.ac.nz

[1] Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand

[2] MacDiarmid Institute for Advanced Materials and Nanotechnology, Wellington, New Zealand

[3] Polybatics Ltd, Palmerston North, New Zealand

protein engineering to fusions at the PhaC C-terminus and/or N-terminus (Brockelbank et al. 2006; Parlane et al. 2011; Blatchford et al. 2012; Hooks et al. 2013, 2014; Chen et al. 2014).

As the key enzyme in PHA biosynthesis, PhaC has been extensively studied, but no X-ray crystal structure has yet been obtained, perhaps in part due to its amphipathic nature. It is recognized that the protein has six conserved regions and the first 100 amino acids of the N-terminus are variable (Rehm 2003). Deletion of the first 78 amino acids of PhaC is permissive; however, deletions of the first 80 amino acids reduced in vitro activity by 50 % and deletion of the first 88 amino acids strongly impaired PHA accumulation in recombinant *R. eutropha* (Zheng et al. 2006; Ye et al. 2008). Despite the lack of topological information, the tolerance of PhaC to genetic manipulation through fusion to proteins of interest has previously been used in the design of functionalized PHA biobeads (Jahns and Rehm 2009).

The aim of this study was to provide insight into the topology of the inclusion-attached PHA synthase by specifically probing the surface of the synthase using conjugation of biotin labels. The structural flexibility of the identified exposed sites was further characterized by inserting functional peptides. This approach provides data in support of a topological model of the synthase and improves protein engineering capability for surface display of functional domains within PhaC itself.

Materials and methods

Reagents

Biotin conjugation chemicals sulfo-NHS-biotin, iodoacetyl-LC-biotin, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), and biotin-hydrazide were purchased from ProteoChem (Loves Park, IL). Streptavidin-HRP was purchased from Invitrogen (Carlsbad, CA), anti-PhaC antibodies were produced by Genscript Corporation (Picastaway, NJ), and Anti-FLAG-HRP conjugate was purchased from Abcam (Cambridge, MA). Other chemicals were procured from Sigma-Aldrich (St. Louis, MO).

Growth conditions

Propagation of plasmids was carried out in *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) cultivated in Luria-Bertani broth at 37 °C, 200 rpm. PHA production was performed with *E. coli* BL21(DE3) (Novagen, Madison, WI) containing the helper plasmid pMCS69 (Amara and Rehm 2003) in LB supplemented with 1 % glucose at 25 °C, 200 rpm. As required, ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL) were added to the media. Production of the fusion proteins was induced by the addition of IPTG to a final concentration of 1 mM. PHA beads were isolated from production cultures as described elsewhere (Hooks et al. 2013); briefly, cells were lysed, and the beads were recovered from the cell lysate by ultracentrifugation in a glycerol gradient.

Biotin conjugation

Conjugation of biotin to the PHA beads was performed using Sulfo-NHS-biotin, iodoacetyl-biotin, and biotin-hydrazide according to established protocols (Hermanson 2008). For labeling lysines, a 500-μL aliquot of 1.25 mg total protein/mL PhaC beads (as determined by the method of Bradford) was dispensed into a tube, and 30 μL of 10 mM sulfo-NHS-biotin was added. The labeling reaction proceeded for 2 h on a rotating mixer at 4 °C. Quenching was accomplished with Tris-HCl. Finally, the PHA beads were washed three times with phosphate buffer. For labeling cysteines, iodoacetyl-biotin was dissolved in dimethylformamide at a concentration of 2 mg/mL. PhaC beads at 4 mg total protein/mL were dispensed in a 200-μL aliquot and 45 μL of the iodoacetyl-biotin was added. The labeling reaction was incubated in the dark for 90 min on a rotating mixer at 25 °C. Finally, the PHA beads were washed three times with phosphate buffer. For labeling aspartic and glutamic acid, the PhaC beads were resuspended in 100 mM MES buffer and a 500-μL aliquot dispensed into a tube. Twenty-five microliters of biotin-hydrazide dissolved in DMSO at a concentration of 13 mg/mL was added to the labeling reaction, followed by addition of 12.5 μL of EDC dissolved in water at a concentration of 100 mg/mL. The labeling proceeded for 2 h on a rotating mixer. Finally, PHA beads were washed three times with 50 mM phosphate buffer to remove residual biotinylation compounds and resuspended in 50 mM phosphate buffer.

Biotinylated PHA samples (10 μL of 1 mg/mL wet weight) were electrophoresed through a 10 % polyacrylamide gel in MOPS buffer to separate the bead-associated proteins. PhaC was identified by Western blot using an anti-PhaC antibody and a horse radish peroxidase (HRP)-labeled secondary antibody. Biotinylation of amino acids was confirmed using streptavidin-HRP conjugate. Successfully biotinylated PhaC was excised from the gel and subjected to MALDI-TOF/TOF performed by the Centre for Protein Research (Otago, New Zealand). Biotinylation sites were identified by a mass shift corresponding to the molecular weight of the conjugated chemical.

Mutagenesis of *phaC* to create FLAG insertions

The exposed sites of PhaC, as identified by biotinylation, were subjected to mutagenesis to insert FLAG epitopes to confirm their surface exposure and assess their suitability for accepting

functional insertions. The FLAG epitopes were inserted into *phaC* carried on the plasmid pET14b:PhaC (Brockelbank et al. 2006) using site-directed ligation-independent mutagenesis (SLIM) as described elsewhere (Chiu et al. 2004, 2008). SLIM primers are specified in supplementary Table S1 in Online Resource 1. Primers and other synthesized DNA as required were purchased from Integrated DNA technologies (Coraville, IA), while *Taq* and Platinum *Pfx* were purchased from Invitrogen (Carlsbad, CA). Successful mutagenesis was confirmed by DNA sequencing performed by the Massey Genome Centre on a capillary AB13730 genetic analyzer (Palmerston North, New Zealand).

Microscopy

Production of PHA beads was observed by fluorescent microscopy after staining with the lipophilic dye Nile Red, as described previously (Peters et al. 2007).

Plasmid construction

The construction of plasmids containing *phaC* with inserted *zz* sequence (Brockelbank et al. 2006) was performed using methods described elsewhere (Sambrook et al. 1989). Primers were purchased from Integrated DNA Technologies (Coraville, IA) and polymerases from Invitrogen (Carlsbad, CA). Gene synthesis of the triple *zz*-inserted *phaC* was performed by Genscript Corporation (Picastaway, NJ), and inserted into pUC57 with *Nde*I and *Bam*HI restriction endonuclease sites for additional subcloning into a pET14b vector for protein expression (supplementary Table S1 in Online Resource 1). The synthesized triple *zz phaC* was generated such that the site K90 *zz* was flanked by *Spe*I restriction endonuclease sites, K139 by *Afl*II, and K518 by *Mfe*I. The double and single zz insertions were generated by subcloning restriction fragments of the pUC57 triple *zz* plasmid into pET14b:PhaC as outlined in supplementary Table S1 in Online Resource 1.

ELISA for detection of FLAG epitopes

The exposure and accessibility of the FLAG tags was assessed by enzyme-linked immunosorbent assay (ELISA). PHA Beads (10 mg/mL wet weight) were bound to microtiter plates by adding 100 μL of PHA bead suspension to each well and incubating overnight at 4 °C. After incubation, the wells were emptied and washed three times with PBST (PBS + 0.05 % Tween 20). Anti-FLAG-HRP antibodies were diluted 1:20,000 and 100 μL added to each well. The plate was then incubated in a dark humidified container for 30 min. After incubation, the wells were emptied and washed five times with PBST. Finally, 100 μL of *o*-phenylenediamine was added to each well, and after 15 min incubation in a dark humidified container, the color development was halted by addition of 100 μL H_2SO_4 (1 N). Using an ELX808 Ultra Microplate plate reader (Bio-Tek Instruments, Inc., Winooski, VT), substrate conversion was read at wavelength 490 nm. The extent of color development is based on the amount of anti-FLAG-HRP bound to the PHA beads and thus to the accessibility of the FLAG tag within the PhaC protein.

PhaC activity after insertion of FLAG epitopes and ZZ domains

The impact of FLAG tag and ZZ domain insertions into PhaC on overall PHA production was quantified by gas chromatography-mass spectroscopy (GCMS) after conversion into 3-hydroxy-methylesters by acid-catalyzed methanolysis as described elsewhere (Brandl et al. 1988). GCMS was performed by The New Zealand Institute for Plant and Food (Palmerston North, New Zealand).

IgG binding assays for inserted ZZ domains

The ZZ antibody binding domain of protein A from *S. aureus* was inserted into the exposed sites successfully used to display a FLAG tag. The functionality of the inserted ZZ domain was quantified by an IgG binding assay as described previously (Brockelbank et al. 2006). Briefly, PHA beads were resuspended in 450 μL of PBS buffer at a concentration of 100 mg/mL (wet bead weight), and 500 μL of 10 mg/mL human immunoglobulin (IgG) was added. The IgG was allowed to bind to the PHA beads during 30 min incubation at 25 °C on a rotating mixer. After the incubation period, the beads were centrifuged at 6000×*g* for 4 min. The supernatant (unbound fraction) was removed, and the sediment was washed by resuspension in 1 mL of PBS. The washing steps were repeated three times in total to remove excess unbound IgG. The remaining beads were resuspended in 1 mL of 50 mM glycine (pH 2.7) and incubated at 25 °C for 5 min to elute bound IgG. The samples were pelleted by centrifugation at high speed (15,000×*g*, 4 min) and the supernatant transferred to a new tube. This elution fraction was then neutralized by the addition of 20 μL 1 M K_2HPO_4. The amount of IgG eluted was quantified by the method of Bradford using human IgG as a standard. The purification of IgG from human serum was conducted as outlined above, except the feed material consisted of 1 mL heat-treated human serum from Invitrogen (Carlsbad, CA)—enough to saturate the IgG binding capacity of the PHA bead.

Results

Chemical cross-linking of biotin to surface-accessible sites on PhaC

PHA beads were recombinantly produced in *E. coli* using the pET14b:PhaC and pMCS69 plasmids (described in supplementary Table S1 in Online Resource 1) to express the PhaA, PhaB, and PhaC enzymes from *R. eutropha*. The PHA beads were subjected to biotinylatin reactions to label surface-exposed amino acids. Three compounds with different amino acid specificities were used to probe a range of exposed amino acids. Sulfo-NHS-biotin reacts with primary amino groups found on lysine side chains and forms a stable amide bond. Iodoacetyl-biotin reacts with the reduced thiol group in cysteine residues to form an irreversible thioether bond. Finally, the cross-linker EDC is used to bond carboxyl groups to primary amines. Activation of carboxyl groups on glutamic and aspartic acid residues with EDC results in *o*-acylisourea intermediates allowing the amine-containing biotin-hydrazide to chemically bond via an amide linkage. PHA beads reacted with sulfo-NHS-biotin, iodoacetyl-LC-biotin, or biotin-hydrazide (after activation with EDC) showed successful biotinylation of bead-associated proteins (Fig. 1a). However, in the case of EDC and biotin-hydrazide, PhaC was lost from the beads during the reaction conditions and is absent on the Western blot using anti-PhaC antibodies (Fig. 1b); therefore, this method of biotinylation was not further explored.

Identification of labeled surface-accessible amino acids by MALDI-TOF mass spectrometry

Protein bands corresponding to successfully biotinylated PhaC were excised from the SDS-PAGE gel and subjected to MALDI-TOF mass spectrometry. Six amino acids along the length of PhaC were identified as having reacted with sulfo-NHS-biotin and iodoacetyl-LC-biotin: K77, K90, K139, K518/K520, C382, and C459. Some of these sites can be visualized on the Phyre-generated threading model (Fig. 2) (Kelley and Sternberg 2009), which maps amino acids 205–534 (55 %) of the PhaC C-terminal domain to the alpha/beta hydrolase domain from human epoxide hydrolase (d1zd3a2). As K518 and K520 are in close proximity, they were considered to be part of the same exposed region. K518 was chosen for further study as it was always detected as biotinylated when subjected to MALDI-TOF/MS, whereas K520 was not always detected as being biotinylated. This result showed K518 was more exposed and accessible to the biotinylation reagent than K520, and thus, K518 was selected for further investigation of this protein region.

Assessment of structural constraints of identified surface-exposed regions of bead-associated PhaC using FLAG epitope insertions

The *phaC* gene was mutagenized with site-directed ligation-independent mutagenesis (SLIM) to insert a FLAG epitope (DYKDDDDK) encoding sequence directly after each biotinylated amino acid in order to confirm surface exposure and to assess the structural flexibility of the region (Fig. 3). The effect of FLAG insertion on the ability of PhaC to produce polyhydroxybutyrate (PHB) was quantified by gas chromatography-mass spectroscopy (GCMS) and compared to wild-type PhaC (Fig. 4). There was no statistically significant difference between wild-type PhaC and PhaC with FLAG insertions at K139 or K518. However, insertion of FLAG at K77 or K139 did significantly reduce the amount of PHB to approximately 65 % of the wild-type amount ($p < 0.05$). Insertion of FLAG at sites C382 or C459 had a dramatic effect on PHB production, with neither variant able to mediate PHB production at levels significantly greater than zero when compared to wild-type production levels. Additionally, an

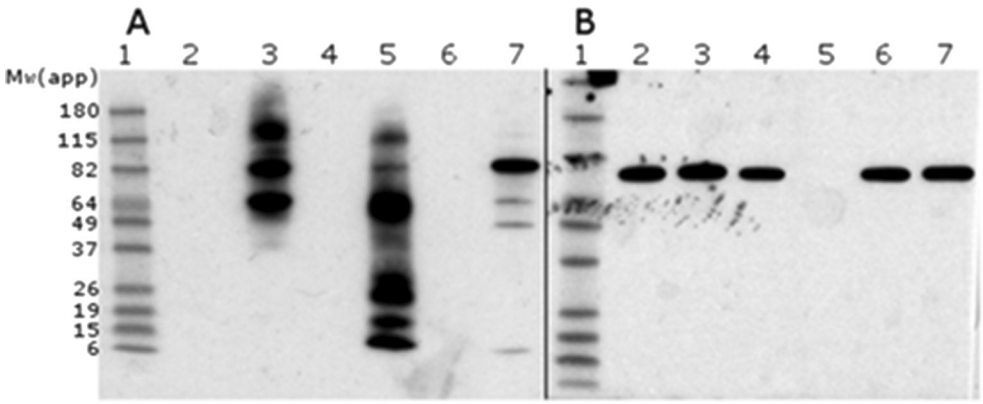

Fig. 1 Isolated PHA beads were reacted with biotin-containing reagents to label bead-surface proteins including PhaC. **a** Strepdavidin-HRP immunoblot to detect biotinylated bead-surface proteins. **b** Anti-PhaC immunoblot identifying the presence of PhaC after biotinylation treatment. *Lane 1*: BenchMark prestained molecular weight standard, *2*: unlabelled PhaC, *3*: iodoacetyl-biotin labeled, *4*: unlabelled PhaC, *5*: biotin-hydrazide labeled, *6*: unlabelled PhaC, *7*: sulfo-NHS-biotin labeled

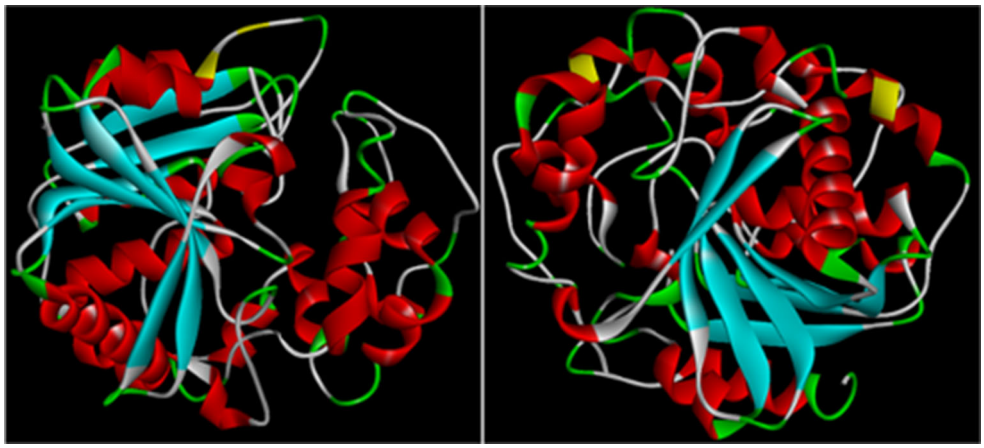

Fig. 2 Threading model of PhaC highlighting (*yellow*) the location of labeled residues. *Left*, lysines (K518, K520). *Right*, cysteines (C382, C459). Lysines in the N-terminal region of PhaC are not shown as they could not be mapped in the model (K77, K90, K139). This model was generated by Phyre based on the a/b-hydrolase fold of the C-terminal domain of human expoxide hydrolase (d1zd3a2)

immunoblot of respective cell lysates showed that while the other PhaC variants with FLAG insertion were produced at high levels, C382ΩFLAG appeared at lower levels and C459ΩFLAG was not detectable (Fig. 5). This suggests insertion of FLAG at C459 is more deleterious and the resultant PhaC is degraded within the cell, whereas the PhaC resulting from C382ΩFLAG insertion is produced within the cell but has low or absent activity. Overall, these results demonstrate the regions around K77, K90, K139, and K518 are more flexible, i.e., less structurally constrained and hence amenable to peptide insertion in order to add surface functionality.

FLAG insertions into accessible lysine sites are surface exposed in the bead-associated PHA synthase

PHA beads coated with synthase variants containing FLAG epitopes were subjected to enzyme-linked immunosorbent assay (ELISA) using anti-FLAG-HRP-conjugated antibodies (Fig. 6). PHA beads were normalized by weight and adsorbed overnight to the wells of 96-well plates. Excess beads were removed with repeated wash steps. Anti-FLAG-HRP antibodies were allowed to bind to the exposed FLAG epitopes and then reacted with *o*-phenylenediamine in a colorimetric assay. The negative control, PHA beads coated with wild-type PhaC, had no detectable absorbance at 490 nm whereas the FLAG-containing constructs had absorbance measured from 0.61 to 2.91. Of these, K90ΩFLAG, K139ΩFLAG, and K518ΩFLAG had approximately the same absorbance reading across the 6.25–50 μg bead mass range of 0.61–1.44. K77ΩFLAG had an approximately 70 % higher reading, from 1.39 to 2.91, at all bead mass levels compared to the other sites. These results demonstrated that the FLAG insertions at amino acids K90, K139, and K518 were all exposed to approximately the same level. Site K77 appeared to be more accessible to the anti-FLAG-HRP antibody, likely due to its location in the highly flexible N-terminal region of PhaC. All four of

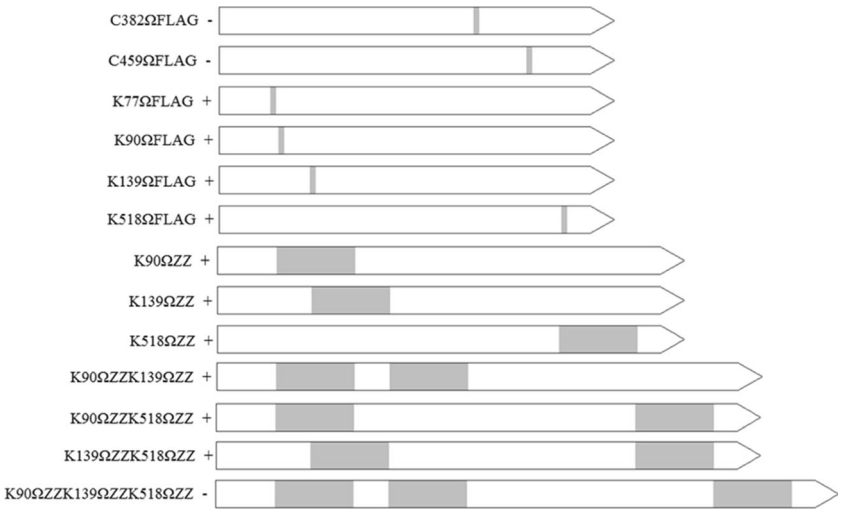

Fig. 3 Schematics of the PhaC insertions created in this study. *Shaded area* indicates the location and size of insertion. The *plus* or *minus sign* indicates whether the insertion was permissive for production of PHB. Of those that were permissive, PhaC insertion variants showed additional functionality by binding anti-FLAG-HRP antibodies (FLAG insertions) or binding and eluting human IgG (ZZ insertions)

the FLAG insertions showed both PhaC enzyme activity as well as functionalization of the PHA bead surface with a detectable epitope.

Targeting surface-exposed lysine sites toward functionalization of PHA beads

The IgG-binding domain ZZ based on protein A from *Staphylococcus aureus* was selected as a model for functionality, as its strong folding potential and hair-pin-like structure would potentially allow the internally modified PhaC to fold correctly (Nilsson et al. 1987; Löwenadler et al. 1987). Surface-exposed sites K90, K139, and K518 were targeted for insertion of the ZZ domain. K77 was not considered for further functionalization, as it is accepted that at least the first 78 amino acids of PhaC can be deleted without significant negative consequence on the ability of PhaC to produce PHB (Zheng et al. 2006; Ye et al. 2008). In addition to the single ZZ domain insertions at K90, K139, and K518, the three double and single triple ZZ insertion variants were also produced (Fig. 3). PHB production analysis by GCMS revealed that single insertions were tolerated well, with no discernible impact on PHB production; PHB content of cells ranged from 39 to 42 % PHB per milligram dry cell weight compared to 32 % PHB per milligram dry cell weight of the wild type (Fig. 7). In contrast, PhaC containing double ZZ domain insertions produced lower amounts of PHB, 3–22 % PHB per milligram dry cell weight, and the triple ZZ insertion strain produced no detectable PHB (Fig. 7). Immunoblot analysis of respective cell lysates revealed that the triple inserted PhaC protein was not produced in detectable amounts (Fig. 5). These results show that a single insertion of 116 amino acids at one of three identified surface-exposed lysine sites can be well tolerated by the PhaC enzyme, but further insertions of the same size at the other sites interferes with synthase activity. When three ZZ domains were inserted, the PhaC variant protein could not be detected and no PHB was produced, indicating a deleterious effect on protein production and/or stability within the cell.

Assessment of engineered PhaC surface functionality

The functionality of the ZZ inserted into the surface of attached PhaC was examined by incubating the respective ZZ-displaying beads with human immunoglobulin G (IgG) and measuring the amount of IgG which could be bound and subsequently eluted from the beads with a low pH treatment. All PHA beads coated by PhaC variants with ZZ domain insertions were able to bind and elute human IgG (Fig. 8), but the amount of IgG eluted varied depending on the number and positioning of the ZZ domains within the PhaC enzyme. Insertion of the ZZ domain at amino acid K90 resulted in the highest amount of eluted IgG, while insertion at amino acid K518 resulted in the lowest elution amount. Having a ZZ domain at site K518 appeared to have a negative impact on the quantity of IgG bound by the PHA beads possibly due to a less stable or lower amount of PhaC present on the bead surface. Single insertions of the ZZ domain bound between 9.1 and 13.6 μg IgG/mg PHA beads. Double insertions were not strictly additive, with K90ΩZZK139ΩZZ binding more at 16.0 μg IgG/mg bead while K90ΩZZK518ΩZZ and K139ΩZZK518ΩZZ bound less at 7.9 and 7.1 μg IgG/mg bead, respectively.

PhaC beads displaying immobilized ZZ domains fused directly to the N-terminus of PhaC have previously been shown to purify IgG from human serum to a similar quality as

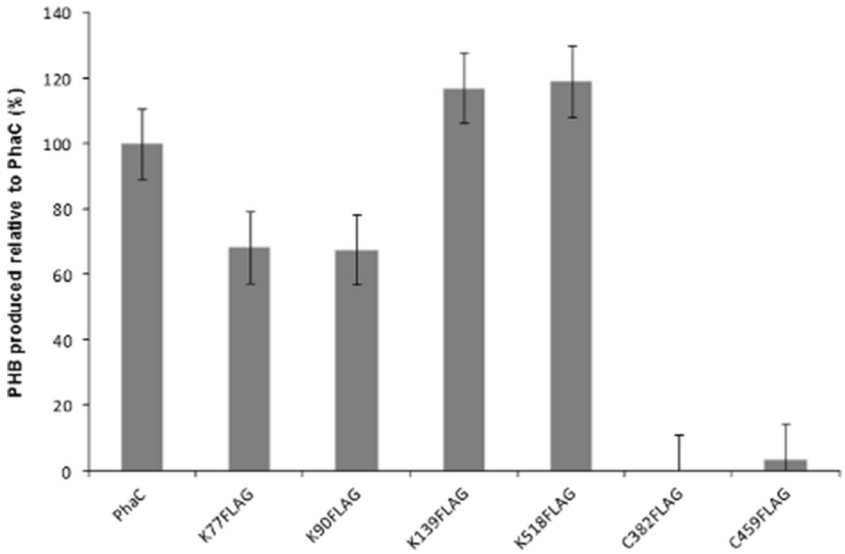

Fig. 4 PHB production by PhaC variants with FLAG insertions. FLAG insertion at amino acids K139 or K518 had no impact on PHB production, while insertion at amino acids K77 or K139 reduced the amount of PHB to ~65 % of the wild-type amount. Insertion of FLAG at sites C382 or C459 abolished PHB production. Tukey bars at experiment wide error rate of 0.05, $n = 3$

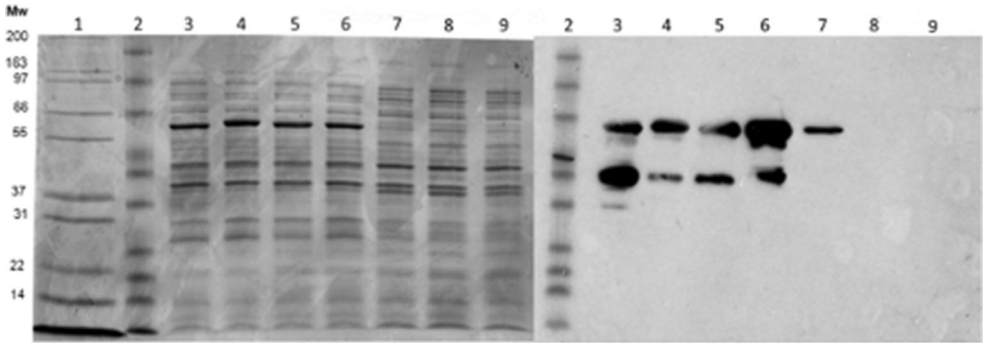

Fig. 5 Gel electrophoresis and immunoblot analysis of whole cell lysates from PhaC FLAG variants. *Left*, SDS-PAGE. *Right*, anti-PhaC immunoblot. *Lane 1*: mark 12 protein ladder, *2*: BenchMark prestained ladder, *3*: K77ΩFLAG, *4*: K90ΩFLAG, *5*: K139ΩFLAG, *6*: K518ΩFLAG, *7*: C382ΩFLAG, *8*: C459ΩFLAG, *9*: K90 Ω ZZK139ΩZZK518ΩZZ. On the SDS-PAGE gel, PhaC overproduction is visible (*arrows*) at ~64 kDa in *lanes 3–6* but not in *lanes 7–9*. For the anti-PhaC Western blot, PhaC is visible (*arrows*) in *lanes 3–7* indicating some amount of PhaC is produced in the C382ΩFLAG variant, even though it is not visible by SDS-PAGE

commercial protein A-sepharose beads (Brockelbank et al. 2006). In this study, these previously published ZZ-PhaC beads, used as a positive control, bound a similar amount of IgG to the lowest performing ZZ insertion beads of 6.6 μg IgG/mg bead (Fig. 8). Therefore, we assessed the ZZ insertion displaying beads with the highest binding capacity (K90ΩZZK139ΩZZ) for their ability to purify the IgG fraction from human serum (Fig. 9). SDS-PAGE analysis of eluted proteins revealed protein bands at ~50 and ~25 kDa, representing the heavy and light chains of human IgG, respectively. The protein elution profile of wild-type PHA beads suggests non-specific binding of IgG to the PhaC-only beads is not a significant issue. The proteins eluted at pH 2.7 from the K90ΩZZK139ΩZZ beads showed a high degree of purity demonstrating the inserted ZZ domains mediate IgG purification.

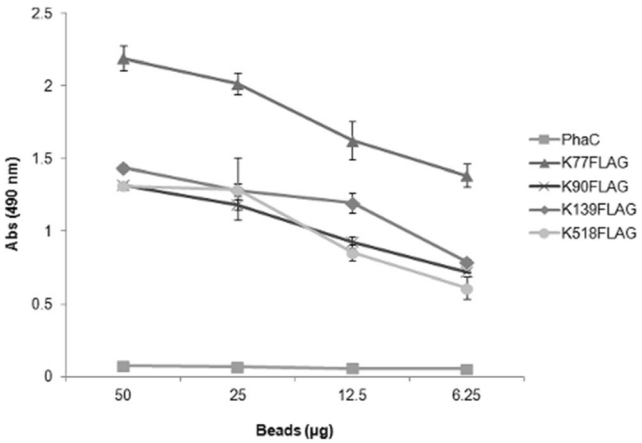

Fig. 6 ELISA using anti-FLAG-HRP antibodies to determine the exposure of the FLAG insertion for potential functional display. PHA beads containing FLAG tags at the indicated site were bound to microtiter plates and incubated with anti-FLAG-HRP antibodies. The HRP substrate *o*-phenylenediamine was added and color, allowed to develop for 15 min. Absorbance was read at 490 nm. A higher absorbance correlates with a higher level of and accessibility for the antibody. *Error bars* ±SEM, $n = 3$

Discussion

This study explored the surface topology of granule-associated PHA synthase by probing the folded protein with biotinylation reagents (Hermanson 2008) followed by identification of the biotinylated amino acid residues using MALDI-TOF/MS. Two different biotinylation reactions with sulfo-NHS-biotin and iodoacetyl-biotin resulted in identification of five lysine residues of interest, K77, K90, K139, K518, and K520, and two cysteine residues, C382 and C459. A Phyre model of PhaC based on a human epoxide hydrolase (d1zd3a2) covering 56 % of the PhaC sequence (Fig. 2) is

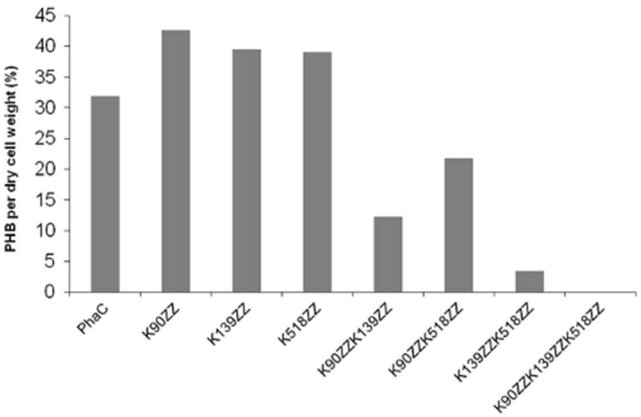

Fig. 7 PHB production mediated by PhaC variants with ZZ domain insertion. The 116 amino acid ZZ domain was inserted after the indicated amino acid and PHB production assessed. Single insertions of ZZ were well tolerated with no apparent impact on PHB production. PhaC containing double ZZ domain insertions decreased the amount of PHB produced, and the triple ZZ insertion strain produced no detectable PHB. $n = 1$

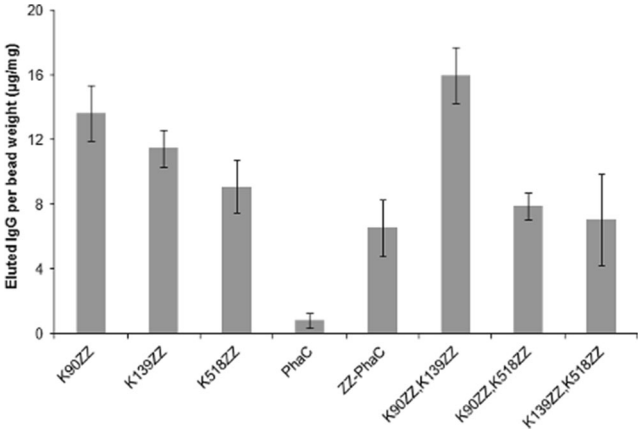

Fig. 8 Human IgG-binding capacity of PHA beads formed by PhaC with ZZ insertions. Beads (30 mg) containing the indicated ZZ domain insertions were incubated with 5 mg of IgG for 30 min at 25 °C. Bound IgG was eluted with glycine-HCl (pH 2.7) and measured by the method of Bradford. *Error bars* ±SEM, $n = 3$

consistent with surface exposure of the identified residues present in the model (K518, K520, C382, C459). Attempts at using ab initio structure prediction tools to visualize the remaining variable N-terminal structure were not convincing (data not shown).

Multiple alignments of PHA synthases have previously shown there are six conserved regions of the enzyme (Rehm and Steinbüchel 2001). Sites K77 and K90 are located before the first conserved region, K139 is located between conserved regions 1 and 2, C382 and C459 are located within the fourth conserved region, and K518 is located between conserved regions 5 and 6 (Rehm 2003). It is therefore reasonable that the non-conserved regions (K77, K90, K139, K518) could be modified with functional insertions without disrupting protein function, as opposed to the conserved regions (C382 and C459). In this work, only the lysine sites could be confirmed as surface-exposed and amenable to insertion by detectable FLAG sequences (Fig. 6). Previously, the tuberculosis-related antigens ESAT6, CFP10, and Rrv3615c were shown to be highly accessible to their respective antibodies in ELISA assays when directly fused to the N-terminus of PhaC (Chen et al. 2014). Additionally, the ZZ domain fused directly to the N-terminus of PhaC is accessible for antibody binding in ELISA assays (Brockelbank et al. 2006). Ultimately, three sites, K90, K139, and K518, were targeted by ZZ domain insertions which mediated binding of IgG (Nilsson et al. 1987; Löwenadler et al. 1987) by the respective isolated PHA beads (Fig. 8). The K90/K139 double ZZ insertion had the highest IgG-binding capacity and could selectively purify the IgG fraction from human serum (Fig. 9), indicating the suitability of these sites for functionalized insertions. Single insertions of either FLAG or ZZ had little impact on the ability of PhaC to synthesize PHA in vivo; however, multiple insertions did have a negative impact on PHA production (Figs. 5 and 8). Commercially available protein A resins include cross-linked agarose, porous glass, and other polymeric matrices ranging in size 50–300 μm in diameter. The dynamic binding capacity of these materials has been measured under operating conditions in a variety of column setups to be between 5 and 30 mg of IgG per mL of settled resin (Hahn et al. 2003). Previous ZZ-displaying PHA beads have been shown to bind up to 100 mg IgG per gram drained beads, and the use of internal ZZ display in conjunction with N- and C-terminal display could potentially increase overall IgG binding capacity.

Using these new surface-display sites will allow the immobilization of additional functionalities such as binding domains (e.g., biotin acceptor peptide) or multiple epitopes while leaving the more flexible N- and C-terminal binding sites free for larger fusion partners. This approach of using a single engineered PhaC as the sole protein anchor on PHA beads will maintain the simplicity of the PHA bead overexpression system and purification processes while allowing more complex applications, such as novel multivalent particulate vaccines, to be explored (Hooks et al. 2014).

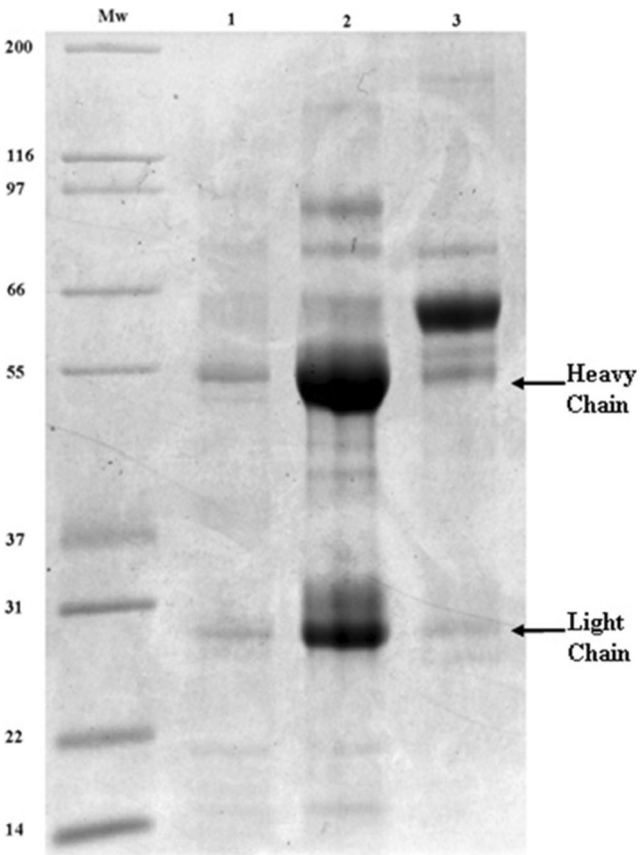

Fig. 9 SDS-PAGE of human serum proteins bound in vitro to wild-type or PhaCK90ΩZZK139ΩZZ beads and released after low pH elution, showing the purification ability of ZZ-inserted PHA beads. *Lane Mw*: mark 12 protein ladder; *1*, proteins eluted from wild-type PHA beads; *2*, proteins eluted from PhaCK90ΩZZK139ΩZZ beads; *3*, human serum (1:200). The heavy and light chains of human IgG are indicated

Acknowledgments This work was supported by the Institute of Fundamental Sciences and Massey University, Palmerston North, New Zealand. DOH received a PhD scholarship from Massey University.

Conflict of interest The authors declare that they have no competing interests.

References

Amara AA, Rehm BHA (2003) Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochem J 374:413–421. doi:10.1042/BJ20030431

Blatchford PA, Scott C, French N, Rehm BHA (2012) Immobilization of organophosphohydrolase OpdA from *Agrobacterium radiobacter* by overproduction at the surface of polyester inclusions inside engineered *Escherichia coli*. Biotechnol Bioeng 109:1101–1108. doi:10.1002/bit.24402

Brandl H, Gross RA, Lenz RW, Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly(beta-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl Environ Microbiol 54:1977–1982

Brockelbank JA, Peters V, Rehm BHA (2006) Recombinant *Escherichia coli* strain produces a ZZ domain displaying biopolyester granules suitable for immunoglobulin G purification. Appl Environ Microbiol 72:7394–7397. doi:10.1128/AEM.01014-06

Chen S, Parlane NA, Lee J, Wedlock DN, Buddle BM, Rehm BHA (2014) New skin test for detection of bovine tuberculosis on the basis of antigen-displaying polyester inclusions produced by recombinant *Escherichia coli*. Appl Environ Microbiol 80:2526–2535. doi:10.1128/AEM.04168-13

Chiu J, March PE, Lee R, Tillett D (2004) Site-directed, ligase-independent mutagenesis (SLIM): a single-tube methodology approaching 100 % efficiency in 4 h. Nucleic Acids Res 32:e174. doi:10.1093/nar/gnh172

Chiu J, Tillett D, Dawes IW, March PE (2008) Site-directed, ligase-independent mutagenesis (SLIM) for highly efficient mutagenesis of plasmids greater than 8 kb. J Microbiol Methods 73:195–198. doi:10.1016/j.mimet.2008.02.013

Draper J, Du J, Hooks DO, Lee JW, Parlane NA, BHA R (2013) Polyhyroxyalkanoate inclusions: polymer synthesis, self-assembly, and display technology. In: BHA R (ed) Bionanotechnol. Biol. Self-assembly Its Appl. Caister Academic Press, Norfolk, pp. 1–36

Grage K, Jahns AC, Parlane N, Palanisamy R, Rasiah IA, Atwood JA, Rehm BHA (2009) Bacterial polyhydroxyalkanoate granules: biogenesis, structure, and potential use as nano-/micro-beads in biotechnological and biomedical applications. Biomacromolecules 10:660–669. doi:10.1021/bm801394s

Hahn R, Schlegel R, Jungbauer A (2003) Comparison of protein A affinity sorbents. J Chromatogr B Anal Technol Biomed Life Sci 790:35–51. doi:10.1016/S1570-0232(03)00092-8

Hermanson GT (2008) Bioconjugate techniques. Bioconjug Tech 506–545. doi:10.1016/B978-0-12-370501-3.00011-4

Hezayen FF, Rehm BHA, Eberhardt R, Steinbüchel A (2000) Polymer production by two newly isolated extremely halophilic archaea: application of a novel corrosion-resistant bioreactor. Appl Microbiol Biotechnol 54:319–325

Hooks DO, Blatchford PA, Rehm BHA (2013) Bioengineering of bacterial polymer inclusions catalyzing the synthesis of N-acetylneuraminic acid. Appl Environ Microbiol 79:3116–3121. doi:10.1128/AEM.03947-12

Hooks DO, Venning-Slater M, Du J, Rehm BHA (2014) Polyhydroyxalkanoate synthase fusions as a strategy for oriented enzyme immobilisation. Molecules 19:8629–8643. doi:10.3390/molecules19068629

Jahns AC, Rehm BHA (2009) Tolerance of the *Ralstonia eutropha* class I polyhydroxyalkanoate synthase for translational fusions to its C terminus reveals a new mode of functional display. Appl Environ Microbiol 75:5461–5466. doi:10.1128/AEM.01072-09

Kelley LA, Sternberg MJE (2009) Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4:363–371. doi:10.1038/nprot.2009.2

Löwenadler B, Jansson B, Paleus S, Houngren E, Nilsson B, Moks T, Palm G, Josephson S, Philipson L, Uhlén M (1987) A gene fusion system for generating antibodies against short peptides. Gene 58:87–97. doi:10.1016/0378-1119(87)90032-1

Nilsson B, Moks T, Jansson B, Abrahmsén L, Elmblad A, Holmgren E, Henrichson C, Jones TA, Uhlén M (1987) A synthetic IgG-binding domain based on staphylococcal protein A. Protein Eng Des Sel 1:107–113. doi:10.1093/protein/1.2.107

Parlane NA, Grage K, Lee JW, Buddle BM, Denis M, Rehm BHA (2011) Production of a particulate hepatitis C vaccine candidate by an engineered *Lactococcus lactis* strain. Appl Environ Microbiol 77:8516–8522. doi:10.1128/AEM.06420-11

Peters V, Becher D, Rehm BHA (2007) The inherent property of polyhydroxyalkanoate synthase to form spherical PHA granules at the cell poles: the core region is required for polar localization. J Biotechnol 132:238–245. doi:10.1016/j.jbiotec.2007.03.001

Rehm BHA (2003) Polyester synthases: natural catalysts for plastics. Biochem J 376:15–33. doi:10.1042/BJ20031254

Rehm BHA, Steinbüchel A (1999) Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. Int J Biol Macromol 25:3–19

Rehm BHA, Steinbüchel A (2001) Biopolymers. Wiley-VCH, Heidelberg

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, NY

Ye Z, Song G, Chen G, Chen J (2008) Location of functional region at N-terminus of polyhydroxyalkanoate (PHA) synthase by N-terminal mutation and its effects on PHA synthesis. Biochem Eng J 41:67–73. doi:10.1016/j.bej.2008.03.006

Zheng Z, Li M, Xue X-J, Tian H-L, Li Z, Chen G-Q (2006) Mutation on N-terminus of polyhydroxybutyrate synthase of *Ralstonia eutropha* enhanced PHB accumulation. Appl Microbiol Biotechnol 72:896–905. doi:10.1007/s00253-006-0371-0