

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Towards a better understanding of the
polyhydroxyalkanoate synthase from *Ralstonia
eutropha*: Protein engineering and molecular
biomimetics**

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

Anika Carolin Jahns

2009

With love and gratitude to

Mum & Dad

And in memory of my grandmother

Acknowledgement

I would like to thank my supervisor Prof Bernd H. A. Rehm for excellent guidance and support in the last three years, and for giving me the opportunity to come to New Zealand and gain insights into an interesting topic with a bright future.

Many thanks also to my co-supervisor Dr. Jan Schmidt who did not get tired of listening to all – crazy – ideas.

Massey University and especially IMBS have been great hosts. A big thank you to everyone. In the beginning it was hard to find my way around but there was always someone willing to lend me a hand – thank you.

I am grateful for the financial support, granted by the New Zealand Foundation for Research Science and Technology. There were a lot of reports involved but I think we managed quite well.

Furthermore, I would like to thank all members of the Rehmlab. We had a lot of fun during the last three years and certainly did not talk only about work. A special thanks to the PHA group. I will miss our Wednesday cake rounds. Katrin and Jane kept me good company in the middle lab and did not flee even when I broke into song. Thank you.

Coming to a foreign country where I did not know anybody and where people spoke a strange language, certainly not English (at least that is what I thought initially) was a big step. I met a lot of very interesting people along the way, always friendly and helpful.

I would especially like to thank Sophia Macris and Murray Scott for their friendship and support, for making me laugh and enjoy life outside university.

I am indebted to Sophia for everything, but especially for proof-reading my thesis and still being my friend afterwards.

Without Murray, life would have been so boring. Thank you very much for countless hours at the movies and for sharing with me a variety of your great life stories.

Some old friends in Europe have been glued to the phone and email over the last three years. In particular, I would like to thank Janine for all the long-distance support and surprises that suddenly arrived at my doorstep.

These last three years have been an interesting experience not only for me but for my family as well. Without their constant support my New Zealand adventure would not have been possible. Vielen Dank für eure Liebe und Unterstützung, euer Vertrauen und Zuspruch. Unvergessen sind all die Pakete und Päckchen mit Leckereien aus der Heimat, auch wenn einige mit mehrmonatiger Verspätung eingetroffen sind. Ohne euch wäre dieses Abenteuer nicht möglich gewesen. Vielen Dank.

Preface

This thesis is written according to the regulations of the latest version of the Handbook for Doctoral Studies, version 5, published by the Doctoral Research Committee in January 2009. The format of this thesis complies with the format of a thesis based on publications as described in the chapter “Submission of a thesis based on publications” on page 64.

All chapters that are published or submitted for publication are listed below. These contributions do not appear in chronological order.

Chapter I B

Indira A. Rasiah, Natalie Parlane, Katrin Grage, Rajasekaran Palanisamy, Anika C. Jahns, Jane A. Atwood and Bernd H. A. Rehm (2009). Biopolyester particles: preparation and applications. **Encyclopedia of Industrial Biotechnology**: in press

Chapter I C

Katrin Grage, Anika C. Jahns, Natalie Parlane, Rajasekaran Palanisamy, Indira A. Rasiah, Jane A. Atwood and Bernd H. A. Rehm (2009). Bacterial polyhydroxyalkanoate granules: Biogenesis, structure and potential use as micro-/nano-beads in biotechnological and biomedical applications. **Biomacromolecules** **10(4)**: 660-669

Chapter II

Anika C. Jahns and Bernd H. A. Rehm (2009). The class I polyhydroxyalkanoate synthase from *Ralstonia eutropha* tolerates translational fusions to its C terminus: A new mode of functional display. **Applied and Environmental Microbiology**: in press

Chapter III

Anika C. Jahns, Richard G. Haverkamp and Bernd H. A. Rehm (2008). Multifunctional Inorganic-Binding Beads Self-Assembled Inside Engineered Bacteria. **Bioconjugate Chemistry** **19(10)**: 2072-2080

Chapter IV

Anika C. Jahns, Verena Peters and Bernd H. A. Rehm (2009). Engineering of bacterial polyester inclusions towards the display of ten lysine residues and potential applications. **Journal of Biomedicine and Biotechnology** – submitted for publication (2. Submission in revised form)

Listed below are all research contributions to the chapters/publications performed by Anika Jahns:

Chapter I B: The review was partly written by Anika Jahns, focussing on the background description and particle formation of polymalate and the applications of PHA particles.

Chapter I C: The parts of the review article describing the phasins and the regulatory proteins were contributed by Anika Jahns.

Chapter II: All experiments were performed by Anika Jahns. Verena Peters is acknowledged for constructing the plasmid pCWE_{Spe}-Mpl-EC.

Chapter III: Except for AFM measurements, all experiments were performed by Anika Jahns. Richard G. Haverkamp obtained all AFM data.

Chapter IV: Verena Peters constructed the plasmids pHAS+phaPwt and pHAS+phaPpolylys. All further experiments regarding the granule isolation, characterization and identification of the respective proteins and the silica binding assays were performed by Anika Jahns.

DNA sequencing, MALDI-TOF/MS, GC/MS and TEM analyses were provided by external services.

This is to certify that the above mentioned research has been conducted by Anika Jahns.

(Date, Signature)

Prof. Bernd H. A. Rehm

(Date, Signature)

Anika Jahns

Abstract

Polyhydroxyalkanoates (PHAs) are polyesters composed of (*R*)-3-hydroxy-fatty acids. A variety of gram-positive as well as gram-negative bacteria and some archaea are able to produce these biopolymers as energy and carbon storage materials. In times of unbalanced growth, when carbon is available in excess but other nutrients are limited, PHA inclusions are formed. These granules are water-insoluble, stored intracellularly and can be maintained outside the cell as beads. The key enzyme for the formation of PHA inclusions is the PHA synthase PhaC, which catalyses the polymerization of (*R*)-3-hydroxyacyl-CoA to PHA with the concomitant release of CoA.

The PHA synthase from *Ralstonia eutropha* (currently *Cupriavidus necator*), which is covalently bound to the PHA granule surface, tolerates fusions to its N terminus without loss of activity. In this study it was investigated if it would also tolerate translational fusions to its C terminus. A specially designed linker was employed, aiming at maintaining the hydrophobic surroundings of the *R. eutropha* synthase C terminus to allow proper folding and activity. Two reporter proteins were tested as fusion partners, the maltose binding protein MalE and the green fluorescent protein GFP. As GFP is a hydrophobic protein itself, no additional linker between the PHA synthase and the reporter protein was necessary to produce PHA granules displaying the functional fusion protein on the surface. Principally, the PHA synthase PhaC tolerates translational fusions to its C terminus but the nature of the fusion partner influences the functionality.

Recently, PHA granules have often been acknowledged as bio-beads. A one-step production allows the formation of functionalised beads without the need for further cross-linking to impart desired surface properties. PHA beads displaying a gold- or silica-binding peptide at the N terminus of PhaC were constructed and tested for their applicability. Additionally, these beads were able to bind IgG due to the ZZ domain of the IgG binding protein A, which was employed as a linker sequence. These functionalised beads can be used as molecular tools in bioimaging and biomedicine, combining organic core with inorganic-binding shell structures.

In a different biomimetic approach, the display of ten lysine residues at the granule surface was achieved using the phasin protein PhaP as the anchoring matrix. Extensive work was performed in an attempt to also employ the synthase protein, but was unsuccessful. These positively charged bio-beads can be used for dispersion or cross-linking experiments as well as silica binding.

Table of Contents

Acknowledgement

Preface

Abstract	i
Table of Contents	iii
List of Figures	vi
List of Tables	vii
Abbreviations	viii

Chapter I

Chapter I A

Natural Polyesters	1
Natural Polyesters	2
Polymalic Acid.....	2
Cutin and Suberin	4
Bacterial Polyoxoesters.....	5
References.....	6

Chapter I B

Biopolyester particles: preparation and applications	13
Abstract	14
Introduction.....	14
Polyhydroxyalkanoates	16
Background	16
Preparation of PHA particles	17
Applications of PHA particles	20
Poly lactides	23
Background	23
PLA particles	24
Preparation of PLA particles.....	26
Applications of PLA particles.....	28
Poly(lactic-co-glycolic acid).....	30
Background	30
Preparation of PLGA nanoparticles	31
Application of PLGA in drug delivery	32
Polymalate.....	34
Background	34
Preparation of Polymalate particles	36
Applications of Polymalate particles	37
Conclusion	40
References.....	41

Chapter I C

Bacterial polyhydroxyalkanoate granules: Biogenesis, structure and potential use as micro-/nano-beads in biotechnological and biomedical applications	54
Abstract	55
Introduction	55
Structure of PHA Granules	57
PHA Granule Assembly	59
Granule-Associated Proteins	62
PHA synthase	62
PHA depolymerases	64
Phasins	65
Regulatory proteins	66
Applications of PHA Granules	67
Protein purification	68
Biological Nano- /Micro-beads	70
Targeted Drug Delivery	73
Outlook	74
References	74
Aims and Scope of the thesis	82

Chapter II

The class I polyhydroxyalkanoate synthase from <i>Ralstonia eutropha</i> tolerates translational fusions to its C terminus: A new mode of functional display	84
Abstract	85
Introduction	85
Materials and Methods	86
Results	90
Discussion	93
Acknowledgement	95
References	95
Supplemental Material	100

Chapter III

Multifunctional Inorganic-Binding Beads Self-Assembled Inside Engineered Bacteria	102
Abstract	103
Introduction	103
Experimental Procedures	105
Results	111
Discussion	119
Acknowledgement	122
References	122

Chapter IV

Engineering of bacterial polyester inclusions towards the display of ten lysine residues and potential applications128
 Abstract129
 Introduction.....129
 Materials and Methods.....131
 Results.....134
 Discussion137
 Acknowledgement139
 References.....139

Chapter V

Conclusions.....144
 Conclusions.....145
 Outlook146

Chapter VI

Appendix.....149

List of Figures

Chapter I B

Figure 1: Overview of different biopolyester constituents	15
Figure 2: Schematic of biopolyester particle applications	40

Chapter I C

Figure 1: Different representations of PHA granules	58
Figure 2: Potential applications for PHA granules	71

Chapter II

Figure 1: Display of the maltose binding protein	91
Figure 2: IgG binding ability of the tripartite synthase fusion protein	93
Figure 3: Schematic overview of hybrid genes used in this study.....	94

Chapter III

Figure 1: Schematic overview of polymer bead assembly	105
Figure 2: Protein profile of polymer beads displaying gold binding peptides.....	112
Figure 3: Gold binding of various polymer beads	113
Figure 4: TEM images of polymer beads	114
Figure 5: Gold-binding to polymer beads.....	114
Figure 6: Scheme of the surface of a bifunctional polymer bead	115
Figure 7: Protein profile of polymer beads displaying silica binding peptides	116
Figure 8: Silica-binding to polymer beads.....	117
Figure 9: Protein profile of polymer beads displaying both binding activities.....	118
Figure 10: Incubation of different polymer beads with gold and silica.....	119
Figure 11: Schematic overview of hybrid genes used in this study.....	120

Chapter IV

Figure 1: Scheme of the polyester bead surface displaying polylysine residues.....	130
Figure 2: Protein profile of polyester beads displaying polylysines.....	134
Figure 3: Density dependent migration in a sucrose gradient.....	135
Figure 4: DNA-binding and elution from silica coated bio-beads.....	136

List of Tables**Chapter II**

Table 1: Bacterial strains, plasmids, and oligonucleotides	88
--	----

Chapter III

Table 1: Bacterial strains, plasmids, and oligonucleotides	107
--	-----

Table 2: Identified peptide fragments of proteins analyzed by MALDI-TOF/MS.....	111
---	-----

Chapter IV

Table 1: Bacterial strains, plasmids, and oligonucleotides	132
--	-----

Table 2: Identified peptide fragments of protein analyzed by MALDI-TOF/MS	134
---	-----

Abbreviations

AFM	Atomic Force Microscopy	LacZ	β -Galactosidase
Au	Gold (aurum)	LB	Luria-Bertani
BSA	Bovine serum albumin	M	Molar (mol/l)
$^{\circ}\text{C}$	Degrees Celcius	MALDI-TOF	Matrix-assisted laser desorption ionisation/time- of-flight
CoA	Coenzyme A	MalE	Maltose binding protein
<i>d</i>	Density	MS	Mass spectrometry
DEAE	Diethylaminoethyl cellulose	Mw	Molecular weight
DNA	Deoxyribonucleic acid	PAGE	Polyacrylamide gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay	PCR	Polymerase chain reaction
FACS	Fluorescence activated cell sorting	PHA	Polyhydroxyalkanoate
Fig.	Figure	PHB	Poly(3-hydroxybutyrate)
GAP	Granule associated protein	PLA	Polylactide
GC	Gas chromatography	PLGA	Poly(lactic-co-glycolic acid)
GFP	Green fluorescent protein	PMLA	Poly(β -L-malate)
GTP	Guanosine triphosphate	SDS	Sodium dodecyl sulphate
HPLC	High performance liquid chromatography	TEM	Transmission Electron Microscopy
IgG	Immunoglobulin G		
kDa	Kilo Dalton		