Novel polyhydroxyalkanoate beads for use as a vaccine against tuberculosis

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Abstract

Tuberculosis was in 1993 declared as a re-emerging disease by the World Health Organization. The only vaccine currently available, BCG, an attenuated strain of *Mycobacterium bovis*, does not protect adults against the pulmonary disease, which is the form of transmission. New vaccine candidates are being developed to provide protection against tuberculosis. Subunit vaccines offer a safer alternative than whole cell preparations and provide the possibility of utilizing only the components that mediate protective immune responses. This thesis describes the production of bacterially derived polyhydroxyalkanoate (PHA) beads for use as a delivery system for *Mycobacterium tuberculosis* reverse vaccinology antigens and immune modulators.

In the first study, the immunogenicity of beads derived from an endotoxin-free host, *Clear coli*, displaying *M. tuberculosis* antigens Rv1626, Rv2032 and Rv1789 was evaluated in mice. Beads displaying Rv1626 were selected for further studies based on the magnitude and specificity of the immune response elicited. In a final study, the immune modulators Cpe30, CS.$T3_{378\text{-}395}$ and Flagellin were co-displayed with Rv1626 antigen on beads and the immunogenicity of these functionalised beads evaluated in mice. Vaccinations with Rv1626 beads and the immune modulators Cpe30 and CS. $T3_{378\text{-}395}$ induced a Th1/Th17 skewed immune response. These beads were then assessed for their ability to protect mice against aerosol challenge with *Mycobacterium bovis*. Rv1626 beads reduced the bacterial loads in 0.48 $\log_{10}$ compared with the negative control group but the inclusion of immune modulators did not enhance the immunogenicity or protection induced by Rv1626 beads.

This study has demonstrated the potential of PHA beads delivering a single reverse vaccinology antigen for protection against tuberculosis infection in mice. While the co-display of immune modulators did not improve the protection induced by the antigen, further studies are needed to determine optimal doses for delivery of immune modulators to enhance protective immunity.

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Preface

This thesis is written according to the Graduate Research School regulations for PhD thesis by publications. The list below presents the publication status of each chapter.

Chapter 1A

Basic concepts in immunology, vaccines and tuberculosis.

This chapter was written by Patricia Rubio Reyes as an introductory section of this thesis and is not intended for publication

Chapter 1B

Self-Assembled Protein-Coated Polyhydroxyalkanoate Beads: Properties and Biomedical Applications. Natalie A. Parlane, Sandeep K. Gupta, Patricia Rubio Reyes, Shuxiong Chen, Majela Gonzalez-Miro, D. Neil Wedlock and Bernd H. A. Rehm. Published in ACS Biomaterials Science & Engineering. Special Issue: PHA Biomaterials (2016).

This review was written by all the authors. Patricia Rubio Reyes made a contribution on the section describing biomedical applications of polyhydroxyalkanoate beads.

Chapter 2

Immunogenicity of antigens from *Mycobacterium tuberculosis* self-assembled as particulate vaccines. Patricia Rubio Reyes, Natalie A. Parlane, D. Neil Wedlock and Bernd H.A. Rehm. Published in International Journal of Medical Microbiology, 306 (2016) 624–632.

All experiments were carried out by Patricia Rubio Reyes except mice vaccinations and processing of mice samples that were co-carried out with Natalie A. Parlane.

Chapter 3

Immunological properties and protective efficacy of a single particulate mycobacterial antigen displayed on polyhydroxybutyrate beads. Patricia Rubio Reyes, Natalie A. Parlane, Bryce M. Buddle, D. Neil Wedlock, Bernd H.A. Rehm. Published in Microbial Biotechnology (2017).

All experiments were carried out by Patricia Rubio Reyes. Natalie A. Parlane helped with mice vaccinations and processing of mice samples and Bryce Buddle assisted with challenge experiment and lungs histology.

Chapter 4

Conclusions

This chapter was written by Patricia Rubio Reyes as conclusions of this thesis and it is not intended for publication

Appendix 4

***In vivo* polyester immobilized sortase for tagless protein purification.** Iain D. Hay, Jinping Du, Patricia Rubio Reyes and Bernd H. A. Rehm. Published in Microbial Cell Factories (2015) 14:190.

Patricia Rubio Reyes made a contribution on the preparation of the plasmid pET14:PhaC-SrtA-Rv1626, purification of Rv1626 antigen and in the demonstration of the functionality of PhaC-SrtA-MBP beads and only those parts are submitted for examination. The entire publication is included for a better understanding of the methods used.

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Chapter 1: General introduction

Chapter 1A. Basic concepts in immunology, vaccines and tuberculosis.

Abbreviations:

APCs: antigen presenting cells, DCs: dendritic cells, NK: natural killer, PAMPs: pathogen associated molecular patterns, LPS: lipopolysaccharide, TLRs toll like receptors, IL: interleukins, TIR: Toll–IL-1-resistence, MYD88: myeloid differentiation primary-response protein 88, MAL: MYD88-adaptor-like protein, IRAKs: IL-1R-associated kinases, JNK: JUN N-terminal kinase, TRAFs: TNF receptor-associated factors, IFN: interferon, TNF: tumour necrosis factor, MHC: major histocompatibility complex, CD: cluster of differentiation, Th: helper, Tfh: follicular helper T cells, Abs: antibodies, Igs: immunoglobulins, LPS: lipopolysaccharide, GRAS: Generally Recognized As Safe, PLGA: Poly Lactic-co-Glycolic Acid, ISCOMs: Immunostimulating complexes, TB: Tuberculosis, HIV: human immunodeficiency virus, DC-SIGN:DC-specific intercellular adhesion molecule-3 grabbing nonintegrin, CFPs: Culture filtrate proteins, HspX: Heat shock protein X, ESAT6: Early secreted antigen, LAM: Lipoarabinomannan, HBHA: Heparin binding hemagglutinin, MmpL: mycobacterial membrane protein Large, ACAD: acyl coenzyme A dehydrogenase, OmpA, outer membrane protein A, Erp: Exported repeated protein, EspR: ESAT-6 secretion system secreted protein regulator, PPD: purified protein derivative, BCG: the Bacille Calmette-Guérin, IGRAs: IFN-γ release assays, ELISpot: enzyme-linked immunospot, BCG: Bacille Calmette-Guérin, RD: Region of Difference, Rv: Reverse vaccinology, Cpe: Clostridium perfringens enterotoxin, PHAs: Polyhydroxyalkanoates.

1.1 Host Immune Responses

The outcome of the constant exposure to inhaled or swallowed organisms and to symbionts depends on their virulence factors and on the host response mechanisms. Immunity is divided into two types characterized by the speed and specificity of the immune response [1]. Innate response elements recognize patterns that are shared by many microorganisms and toxins that are not present in the host and act rapidly after their encounter. Adaptive responses elements are specific for individual pathogens, toxins or allergens, take longer to develop and have the ability to manifest immune memory [2]. Appropriate interactions between both the innate and adaptive immune response are required to efficiently recognize and clear pathogens and inappropriate interactions can result in harmful immune responses like allergy and autoimmunity [3].

The innate immune system plays a crucial role in the early recognition and triggering of an inflammatory response to invading pathogens. It is composed primarily by phagocytic and antigen presenting cells (APCs) such as granulocytes, neutrophils, macrophages, dendritic cells (DCs) and cytotoxic natural killer (NK) cells [4]. Pathogen recognition receptors (PRRs) in these cells recognize pathogen associated molecular patterns (PAMPs). Examples of PAMPS include surface glycoproteins, DNA, RNA species, peptidoglycan, lipoproteins and lipopolysaccharide (LPS) that are invariant in classes of pathogens, are essential for their survival and can be distinguished from self-molecules [5].

The most studied PRRs are the family of toll like receptors (TLRs). To date, eleven members of the TLR family have been identified. The localization of TLRs in the cell is related to the PAMP they recognize [6]. TLR signalling is initiated by PAMP-induced dimerization of receptors followed by engagement of the Toll–IL-1-resistence (TIR) domain with domain-containing adaptor proteins (myeloid differentiation primary-response protein 88 (MYD88), MYD88-adaptor-like protein (MAL), or TIR domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM)). Adaptor molecules stimulate

downstream signalling pathways that involve interactions between IL-1R-associated kinases (IRAKs) and the TNF receptor-associated factors (TRAFs), that activate the JUN N-terminal kinase (JNK) and p38, and transcription factors. A major consequence of TLR signalling is the induction of pro-inflammatory cytokines, and in the case of endosomal TLRs, the induction of type I interferon (IFN) [7]. Cytokines, e.g. IFN, interleukins (IL), chemokines and tumour necrosis factor (TNF) family are pleiotropic proteins that mediate numerous immune effects [8]. On all cell surfaces and in the plasma, there is a system of proteins called complement, which recognize pathogen surfaces and lead to the generation of inflammatory mediators [9]. Cytokines and the complement system are functional bridges between innate and adaptive responses facilitating an integrated host defence to pathogens.

Inflammatory responses increase the flow of cells containing antigens; activated DC and other cells initiate the adaptive immune response by activating T lymphocytes [10]. T cell receptors recognize a complex consisting of an antigen derived peptide bound to major histocompatibility complex (MHC) molecules (produced in APCs) [11]. MHC binds peptides that have been synthesized within the cell (class I) or that have been ingested and processed (class II) [2].

T cells mature in the thymus, express T cell receptors and can be defined by the surface production of cluster of differentiation (CD) molecules, CD8 (cytotoxic) or CD4 (helper T cells) [12]. After encountering antigen, $CD4^+$ cells differentiate into different subsets: T helper (Th) 1, Th2, Th9, Th17, Th22, regulatory T cells (Tregs) and follicular helper T cells (Tfh) depending on the cytokines present at the activation site [13]. Once naive T cells are activated, these proliferate and differentiate into effector cells which migrate to the site of infection to eliminate the pathogen [14]. Each Th subset releases specific cytokines that can have either pro or anti-inflammatory, survival or protective functions. Th1 cells produce IFN-γ and TNF (pro-inflammatory cytokines); Th2 cells produce IL-4, IL-5 and IL-13; Th9, IL-9,

Tregs secrete IL-10 (immunosuppressive function) and Th17 cells produce IL-17 (important in host defence against bacteria, and fungi) [15].

Th2 cells stimulate B cells to produce antibodies (Abs). Abs belong to the superfamily of immunoglobulins (Igs) and are heterodimeric glycoproteins that can be divided functionally into variable domains (binds antigens) and constant domains (specify effector functions) and structurally into light and heavy chains [16]. The heavy-chain constant region (Fc region) defines the different isotypes of Abs, Fcγ-IgG, Fcα-IgA, Fcε-IgE, Fcμ-IgM and Fcδ-IgD, [17] and determines most of their functions. Abs play an important role in neutralization of pathogens, phagocytosis, cellular cytotoxicity and complement-mediated lysis of pathogens or pathogen-infected cells (humoral response) [18]. B cells also participate in T cell activation via antigen presentation and cytokine production [19].The immune system can generate persistent memory cells in response to pathogens, evoking an enhanced response if reinfection occurs, involving both, B and T lymphocytes [20, 21].

Effective host defence responses involve the interaction of innate and adaptive mechanisms to protect against infection and disease, but some pathogens are able to evade or alter host defences and establish infection. It is critical to understand the interactions between host and pathogens in order to develop preventative and therapeutic strategies [22].

1.2 Vaccines

A vaccine is an inactivated or attenuated pathogen or a component of the organism that when administered to the host, stimulates a protective immune response against the disease [23]. Most current vaccines act by inducing Abs that block infection.

Live attenuated vaccines are viable infectious organisms with a reduced virulence and inactivated vaccines are killed infectious organisms [24]. These types of vaccines have been effective against polio, yellow fever, measles, mumps, rubella, influenza, whooping cough, hepatitis A, and others, and their use led to the eradication of smallpox in the 1980s [25]. Despite their success, live attenuated vaccines have failed against hyper variable viruses

and more complex pathogens [26]. Identification of the components responsible for protective immunity allows simplification of vaccine formulations. Subunit vaccines offer an improved safety profile and the possibility to develop new vaccines against diseases where the classical approach to vaccinology has had limited success [27].

Subunit vaccines can consist of proteins, inactivated toxins (diphtheria and tetanus), carbohydrates (pneumococcus), or conjugates (meningococcus) [28] and are transported by different carriers or delivery systems like liposomes, proteoliposomes, virus-like particles, polymeric nanoparticles or inorganic nanoparticles (DNA vaccines) [29]. However, subunit vaccines are less immunogenic than whole cells preparations, needing adjuvants in their formulations to enhance the immune response to the co-administered antigens [30]. Adjuvants are compounds that enhance the immune response against an antigen [31]. Adjuvants are also used to reduce the dose of antigen or the number of vaccinations required to induce protective immunity. Based on this, any product or association of components that increases or modulates the immune response against an antigen can be considered an adjuvant. Some researchers refer to delivery systems as adjuvants [32] [33] while others consider immune modulators, e.g. molecules that activate specific immune cells as new adjuvants formulations [27, 34].

Adjuvants may be part of delivery systems or used to improve the efficacy of vaccines in specific groups within a population for example, neonates, elderly or immunocompromised individuals [35]. Until the 1990s, aluminium salts were the only adjuvant licenced for human vaccines but more recently a range of new adjuvants such as emulsions or combinations of these (AS03, AS04) are also being used [32].

Production of recombinant proteins for use as antigens or diagnostics

Heterologous systems are used for production of protein antigens, as it is often difficult or impossible to obtain high yields of the protein from natural sources [36]. *Escherichia coli,* a

Gram-negative bacterium, is one of the most widely used microorganisms for producing recombinant proteins as it is easy to genetically manipulate, can be cultured at a low cost, has rapid growth, and its genetic and metabolism have been well characterised [37]. Nevertheless, the processes of protein production and purification using this system are laborious and time consuming. Various methods have been developed to enrich proteins of interest from crude extracts. The most effective method is affinity purification by fusing the protein of interest to a partner that binds to an immobilized ligand [38]. Commonly used affinity tags are hexahistidine, which binds metal ions, glutathione S-transferase, which binds glutathione, streptavidin-binding peptide binding streptavidin and antibody systems. Many of these tags are polypeptides and may affect the structure and function of the target protein, often making tag removal necessary. Accordingly, a protease cleavage site is often engineered between the tag and the target protein, e.g. thrombin, enteropeptidase and tobacco etch virus protease [39] but residual amino acids may still impact the activity of the protein of interest.

The major component of the outer membrane of *E .coli* is lipopolysaccharide (LPS), also termed endotoxin. LPS is recognized by the immune system as an indicator of bacterial invasion and induces an inflammatory response that in extreme cases leads to endotoxic shock [40]. Due to this immune response to LPS, laboratories carry out different entotoxin-removal procedures such as ion-exchange chromatography, and use of affinity adsorbents, gel filtration chromatography, ultrafiltration and sucrose gradient centrifugation. However, the success of these techniques depends on the properties of the target protein [41]. An alternative to avoid the process of endotoxin removal is the use of Gram-positive microorganisms, like lactic acid bacteria, that are Generally Recognized As Safe (GRAS) organisms used for the industrial manufacture of milk products, vegetables, meat and wine [42]. However, *E. coli* is the first choice for the production of heterologous proteins, and there are numerous genetic engineering tools adapted to this microorganism. Recently, the company Lucigen (Lucigen Corporation, Middleton, WI, USA) developed an *E. coli BL21*

(DE3) based system, *Clear coli,* which allows for the production of proteins virtually free of endotoxins,. This strain incorporates genetic deletions that block the production of LPS. *Clear coli* cells grow at 50% of the rate of normal *BL21*, tend to aggregate because their envelope is hydrophobic, require vigorous shaking, and due to their outer membrane instability, are required to grow in media containing 1% NaCl [43].

Types of subunit vaccines

Particulate vaccines

Formulation of antigens in particles of the size range of pathogens can facilitate the uptake by APCs with co-delivery of the antigens and the adjuvant to the same APC [44]. In addition, antigens can be protected against degradation and particle-based vaccines can act as a depot for antigen release. A number of particulate antigen delivery systems are being developed.

Liposomes and liposome-based vaccines

Liposomes are spherical vesicles formed by phospholipid bilayers surrounding an aqueous compartment. Their lipid composition, charge, size, entrapment and location of antigens or adjuvants can be modified in order to elicit the desired immune response [45]. Water soluble compounds like proteins, nucleic acids or carbohydrates can be entrapped in the inner space while, lipophilic compounds like lipopeptides and lipophilic adjuvants can intercalate into the lipid bilayer. In addition, antigens can be adsorbed or chemically linked to the surface of liposomes [46].

Virus-like particles

Virus-like particles are multi-subunit self-assembled protein structures highly related to their corresponding native viruses that do not contain genetic material but are able to enter host cells [47]. They combine good safety profiles with strong immunogenicity, as it is possible to display heterologous antigens in high copy numbers on their surface [48].

Polymers

Poly Lactic-co-Glycolic Acid (PLGA) are a family of biodegradable and biocompatible polymers that have been studied as delivery systems for drugs, proteins, DNA, RNA and peptides. Their degradation can be employed for sustained vaccine delivery at desirable doses and it is possible to customize parameters such as polymer molecular weight, ratio of lactide to glycolide and vaccine concentration [49].

Immunostimulating complexes (ISCOMs)

ISCOMs are cage-like structures that are formed by the antigen, cholesterol, phospholipids and saponin. ISCOMs-based vaccines have been shown to promote antibody and cellular immune responses in experimental animal models [50].

Chitosan

Chitosan is a low cost, biodegradable and biocompatible mucoadhesive polymer derived from chitin. It is positively charged, allowing the opening of intercellular junctions which enables transport of drugs and vaccines, as it can encapsulate or adsorb antigens [51].

1.3 Tuberculosis (TB)

Tuberculosis (TB) is an infectious disease caused by organisms of the *Mycobacterium tuberculosis* complex [52]. In 2015 there were 10.4 million new cases of TB and 1.4 million deaths, with India, Indonesia, Nigeria, Pakistan and South Africa accounting for 60% of the new cases (Figure 1) [53]. In New Zealand in 2014, 302 cases of TB were notified [54]. Transmission occurs when a naïve individual inhales droplets of sputum from a patient with active TB [55]. Most of the infected individuals develop pulmonary TB, but up to 25% develop extrapulmonary disease, commonly involving lymph nodes, pleura and the osteoarticular system, though the disease can affect any organ [56]. It is estimated that nearly one-quarter of the world's population is latently infected with *M. tuberculosis* [57], although only 5-10% of people develop active disease in their life time, usually immunocompromised individuals, elderly and young children [58]. Co-infection with human

immunodeficiency virus (HIV) constitutes the main burden of infectious disease in developing countries, and in these individuals the risk of developing TB increases to 5-15% per year [59].

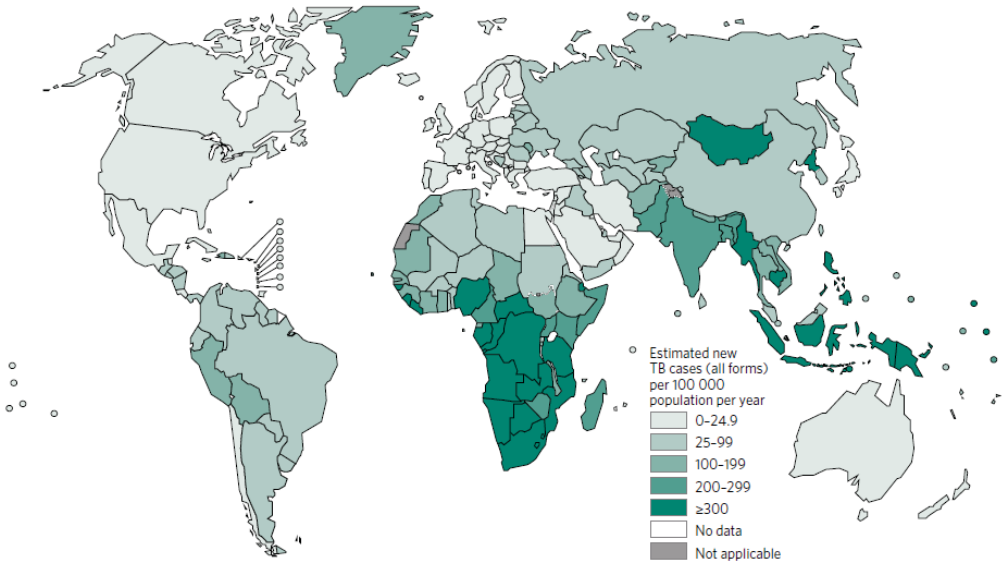

Figure 1.1. Estimated TB incidence rates in the world in 2015. New cases per 100,000 individuals are represented with a color code. Image taken from Global TB report 2016.

Pathogenesis

M. tuberculosis primarily interacts with alveolar macrophages, entering via complement, mannose receptors [60] and Fc receptors [61]. The pathogen can also infect DCs, entering via DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) [62]. Activated macrophages can transfer phagocytosed *M. tuberculosis* organisms to lysosomes but some mycobacterial lipids and proteins interfere with phagosome maturation [58] preventing phagosome-lysosome fusion, which enables intracellular survival of the mycobacteria [63]. The mode of entry to the macrophages can influence the outcome. When *M. tuberculosis* is phagocytosed via opsonizing antibodies (Abs), mycobacterial clearance is improved by enhancing phagosome maturation [64]. *M. tuberculosis* can survive within

phagosomes or escape into the cytosol, were mycobacterial antigens are presented to $CD8^+$ T cells [65].

TB-infected macrophages and DCs produce pro and anti-inflammatory cytokines and chemokines [66], including IFN-γ, TNF-α, IL-12 and IL-10, which act to recruit inflammatory and other cell types to the site of infection leading to the formation of granulomas [67]. Surrounding the core of infected macrophages there are foamy and epithelioid macrophages, monocytes and multinucleated giant cells that are also surrounded by fibroblasts, creating a defined structure [68]. Granulomas are the hallmark of the host-*M. tuberculosis* interaction [69], but the understanding of whether they are representative of protection or pathology is still incomplete [70] for while granulomas limit the growth of mycobacteria, they also provide a niche from where the organisms may disseminate [71]. *M. tuberculosis* can persist in granulomas for decades. Latent infection is a dynamic process of bacterial survival and immunologic control that is influenced by factors such as immunosuppression, malnutrition, tobacco smoke, air pollution, alcoholism, cancer, and other health conditions [72].

Virulence factors

Virulence factors are molecules that enable the bacteria to invade, replicate and disseminate within a host and evade its defence mechanisms [73]. *M. tuberculosis* virulence factors can be characterised based on their function, molecular features or cellular localization [74]. A number of virulence factors have been identified.

Culture filtrate proteins (CFPs)

CFPs are proteins present in the media used to culture *M. tuberculosis* [75]. A number of these proteins have been identified as virulence factors. Glutamine synthetase has a central role in nitrogen metabolism; it alters ammonia and pH levels in the phagosome and helps the mycobacteria to avoid phagosome-lysosome fusion [76]. Superoxide dismutase is a strong

superoxide radical scavenger and aids survival of *M. tuberculosis* in the phagocyte by counteracting reactive oxygen species [77]. Heat shock protein X (HspX) activates the transcription of genes in response to hypoxia and nitric oxide stress, where it can account for up to 25% of the proteins produced by the mycobacteria [78]. This protein is required for persistence of *M. tuberculosis* within the macrophage and plays an active role in the latency stage [79]. Early secreted antigen (ESAT6) and its chaperone, CFP10 prevent phagosome-lysosome fusion [80], ESAT6 inhibits T cell responses by affecting T cell receptor signalling pathways [81] and also causes Ca^{2+} influx followed by neutrophil necrosis [82]. CPF7 belongs to the ESAT6 family, which are highly homologous proteins, providing a mechanism for immune evasion through antigenic variation [83]. Another virulence factor found among CFPs is the 19 kDa lipoprotein, which binds mannose receptors promoting phagocytosis [84] and induces macrophage apoptosis through a mitochondrial mediated mechanism [85].

Cell surface constituents

The cell wall of *M. tuberculosis* is a complex structure composed of peptidoglycan, arabinogalactan and mycolic acids surrounded by a capsule of proteins and polysaccharides [86]. These components play an important role in pathogenesis, as they mediate signalling with the host and some are essential for cell viability [87] and proliferation.

Lipoarabinomannan (LAM) is one of the key virulence factors of *M. tuberculosis;* it contains short mannose caps that mediate binding to the mannose receptor on macrophages and can bind to TLRs [87] inducing proinflammatory responses [88]. Heparin binding hemagglutinin (HBHA) is a cell surface associated antigen [89] that acts as an adhesin molecule for nonphagocytic cells [90]. It is secreted in patients with latent TB and is required for extrapulmonary dissemination [91]. Mycobacteria have three mycolyltransferase enzymes [92] that play important roles in the biosynthesis of major components of the cell envelope [93]; Ag85 complex (i.e. Ag85A, Ag85B and Ag85C) are also predominantly secreted proteins that specifically interact with fibronectin contributing to adherence, invasion and dissemination in the host [94]. Proteins responsible for cell wall lipid transfer are important

for virulence. For example,mycobacterial membrane protein Large (MmpL) proteins mediate transfer of lipids across the membrane [95], account for *M. tuberculosis* impermeability to biocides and play a role in host-pathogen interaction [96]. Additional proteins associated with the cell wall that play a role in virulence include acyl coenzyme A dehydrogenase (ACAD), which regulates cholesterol catabolism modulating *M. tuberculosis* response to the host immune system [97], and hydroxymycolate synthase (MmaA4), which modulates IL-12 production though a modification to mycolic acid [98]. Exported repeated protein (Erp) is required for intracellular survival and cell wall integrity, [99] and outer membrane protein A (OmpA), a porin induced in acidic conditions, has a role in the response to low pH [100].

Other important virulence factors include enzymes involved in metabolism, which aid *M. tuberculosis* survival during starvation and stress conditions, and transcriptional regulators, discovered using direct mutations [75], such as the ESAT-6 secretion system secreted protein regulator (EspR) that negatively regulates the production of ESAT-6 to avoid the host immune response [101].

Role of the innate immunity in TB

The innate immune response against *M. tuberculosis* constitutes a critical part of the host defence, activating anti-microbial pathways that limit the development of the disease and are regulators of the adaptive immunity [102]. When *M. tuberculosis* organisms are inhaled they first encounter the respiratory mucosa lining the airways, which constitutes the first line of defence against the infection [103]. This interaction occurs primarily with the epithelium connective tissue, mucus, immune cells, IgA and innate immune factors, which form a barrier to prevent invasion, [104].

Macrophage receptors are not only involved in the phagocytosis of *M. tuberculosis*, but also in the activation of downstream pathways with anti-mycobacterial effect [102]. Engagement of TLR2 leads to inducible nitric oxide synthase gene transcription, and IFN-γ activated macrophages have augmented capacity for anti-mycobacterial functions [105], showing that

macrophages are capable of restricting mycobacterial growth upon appropriate activation signals from cells in the lung environment [102]. Neutrophils infected with *M. tuberculosis* secrete chemokines and pro-inflammatory cytokines that act to recruit other immune cells. Infected neutrophils can be phagocytised by infected macrophages, which leads to improved killing when the content of neutrophil granules fuses with phagosomes [104]. NK cells also play a role in the host immune response to *M. tuberculosis* infection. NK cells can lyse *M. tuberculosis* infected monocytes to a greater extent than uninfected ones, and link the innate and adaptive responses though enhancing $CD8^+$ T lymphocytes production of IFN-γ [106]. The role of DCs in the defence against *M. tuberculosis* infection is still unclear, as some studies show that DCs strengthen the cellular immune response while other studies that *M. tuberculosis* inhibits DC maturation and masks its presence impairing the ability of DC to stimulate antigen-specific T cells [107]. However, despite the contradictory experimental findings, DCs are crucial in the host response *to M. tuberculosis*, as it is only after they traffic to the mediastinal lymph node that antigen specific $CD4^+$ T cell expansion is detected [108]. Exposing the mycobacteria to a toxic environment containing reactive oxygen and nitrogen species and toxic metals like zinc is another mechanism that controls *M. tuberculosis* infection [104].

Role of the adaptive immunity in TB

Cell mediated immunity

$CD4^+$ T cells are activated with antigens presented via MHC II and released from mycobacteria within the phagosomes of the macrophages [109].Their main function is to evolve into Th1 effectors cells to produce IFN-γ, which is crucial to activate macrophages for controlling the infection or killing infected macrophages and to induce the synthesis of radical nitrogen intermediates to kill the bacteria [110]. IFN-γ produced primarily from $CD4^+$ T cells, rather than from other cells, is essential for controlling TB infection and is the major anti-*M.*

tuberculosis effector pathway [111]. The important role of $CD4^+$ T cells is evidenced by the increased susceptibility to develop TB in individuals that are HIV positive [112].

$CD8^+$ T cells are also required for defence against *M. tuberculosis* infection [65].$CD8^+$ T cells kill *M. tuberculosis* via a granule-mediated function or Fas ligand/receptor induced cell apoptosis. These cells are activated mostly upon presentation of protein antigens restricted by MHC I and CD1 restricted lipid antigen presentation [112].

Polyfunctional $CD4^+$ and $CD8^+$ T cells produce IFN-γ, TNF-α and IL-2, which can promote optimal protection as secretion of IFN-γ and TNF-α by the same cell mediates more efficient killing compared with either cytokine alone and the secretion of IL-2 promotes T cell expansion which can enhance $CD8^+$ T-cell memory function [113].

The role of Th1 cells in the control of *M. tuberculosis* proliferation is irrefutable; however, Th17 cells also have an important role in protection, mediated by the secretion of IL-17, which induces the recruiting of neutrophils to the infection site [114]. Understanding their participation is complex, as Th17 cells mediate both antibacterial and pro-inflammatory responses [115].

Role of Abs

Cell-mediated immunity has been classically described as the mechanism of defence against *M. tuberculosis*, as it is an intracellular pathogen. However, the organisms can be localized in the extracellular compartment at the beginning of the infection and after rupture of granulomas [116] and thus at stages were Abs have access. Abs contribute to the immunity against *M. tuberculosis* [117], as phagocytosis mediated by Abs promotes phagolysosomal fusion, and engagement of the Fcγ region triggers signals that increase the concentration of Ca^{2+} inside macrophages, intracellular killing and complement activation resulting in increased phagocytosis by macrophages [118].

Epidemiological and experimental evidence demonstrate a role for Abs in *M. tuberculosis* infection. Children with low serum IgG against mycobacterial antigens are predisposed to

dissemination of *M. tuberculosis* [119]. In a mouse model, administration of secretory IgA from human colostrum induces protection against tuberculosis [120]. Serum IgG against LAM induces bacteria internalization and the significant production of IFN-γ by CD4+ and CD8+ T cells [121]. When anti-HBHA Abs are used to coat mycobacteria before administration to mice, dissemination is inhibited [122], and Abs anti-HspX reduce bacterial loads [123].

Innate immunity, cell-mediated immunity and antibodies all contribute to the defence against *M. tuberculosis*, but host and pathogen genetics also play a role and can determine the outcome of the infection towards latency or active disease [124]. *M. tuberculosis* is a complex pathogen, and the immune response that this organism triggers is also complex. Thus while dividing the role of each immune component may be an efficient strategy for studying the disease, all aspects of immunity need to be taken into account in order to understand the complexity of the host-pathogen interaction that leads to different results upon infection.

Prevention and control of TB

TB diagnostics

While diagnostic tests for human TB are available, the inability of detecting new cases in a timely fashion is having a detrimental impact in controlling transmission of TB [125]. Diagnosis of tuberculosis in developing countries is particularly difficult due to the challenges for high cost, limited resources and the poor performance of diagnostic tests [126].

Diagnosis of pulmonary TB is a priority for the patient and for public health prevention. It can be done with a chest X-ray, which is not specific for TB and has to be confirmed with bacteriological culture that has a high sensitivity but requires from 2 to 6 weeks for interpretation. Sputum smear microscopy is fast, simple and inexpensive but has low sensitivity [127]. The tuberculin skin test was, until recently, the only method to diagnose latent infection. The most widely used skin test uses purified protein derivative (PPD) which

is derived from *M. tuberculosis* cultures and is injected intracutaneously. The diagnostic test is based on measuring the diameter of induration in the skin provoked by a delayed hypersensitivity reaction. The sensitivity and specificity of the test can be compromised by previous mycobacterial infections [126] and the use of the Bacille Calmette-Guérin (BCG) vaccine in children [128].

Recent advances in mycobacterial genomics and immunology have resulted in blood tests that detect *M. tuberculosis* infection by measuring IFN-γ release (IFN-γ release assays, IGRAs) in response to antigens only present in *M. tuberculosis* and absent in BCG and in most non-tuberculous bacteria [129]. The enzyme-linked immunospot (ELISpot), T.SPOT.TB uses ESAT6 and CFP10, and QuantiFERON-TB Gold In-Tube also includes the antigen TB7.7. T.SPOT.TB represents a significant improvement over the tuberculin skin test, as it is more specific and sensitive among immunosuppressed individuals [130]. QuantiFERON-TB Gold In-Tube is also more specific but cannot distinguish between latent and active TB and there is controversy on its use in immunocompromised patients and in monitoring treatment with anti-TB agents [131]. In the past 20 years there have been substantial improvements in the ability to detect *M. tuberculosis* regardless of the stage of the disease [132], however there is still the need for more sensitive, economic, easy and rapid point of care diagnostic methods, as the regions with the highest burden of TB usually lack the equipment, trained personnel, supplies and financial resources needed to effectuate the appropriate test [133].

Antibiotic treatment

Since the 1940s effective drugs have been developed to treat TB disease. The current treatment consists of a six month regimen of administering the antibiotics isoniazid, rifampicin, ethambutol and pyrazinamide, and has a success rate of at least 85% for new cases [53]. Nevertheless, drug intolerance and toxicity, particularly in co-infected HIV patients, can lead to interruption of treatment [134], poor patient management, and non-adherence to the regimen. This can lead to the emergence of drug resistant tuberculosis

[135]. Treatment of these drug resistant strains of *M. tuberculosis* is more complicated [136], can take up to 20 months and has reduced success rates [137]. New drugs are being developed in order to shorten the prolonged period needed for TB treatment and to treat drug resistant strains [138]. The possibility of combining immunotherapy with the common chemotherapy of TB is also been explored, considering that host specific therapies could provide new opportunities for managing latent and active disease [139].

Vaccination with BCG

Bacille Calmette-Guérin (BCG), a live attenuated strain of *M. bovis,* is the only vaccine available against TB. BCG was used for the first time in humans in 1921 and to the date, it is the most used vaccine worldwide [140]. During the early use of BCG, the vaccine reduced mortality by 90% [141]. It is clear that vaccination of neonates with BCG confers protection against severe forms of childhood disease but multiple studies have shown its efficacy at preventing adult pulmonary TB varies between 0 and 80% [142]. In addition to the variable efficacy in adults, use of BCG has safety issues when administered to immunocompromised individuals [143]. Understanding BCG mechanisms of protection and the reasons for its failure to protect against the main cause of new TB infections can give new insights to the strategies that can be developed to complement its induced immunity.

BCG vaccination stimulates IFN-γ production from $CD4^+$ T cells and activates cytotoxic T $CD8^+$ cells [144] but the precise mechanisms involved in protection conferred by BCG are incompletely understood [145].

BCG is derived from *M. bovis*, which belongs to the *M. tuberculosis* complex, accounting for its protection against TB disease, but the close relationship between mycobacterial species has also negative aspects [146]. Pre-exposure to environmental mycobacteria can have a deleterious effect on the efficacy of BCG; it is possible that immunity to these mycobacteria already confers some level of protection against TB, to which BCG adds little or nothing [147], or that this pre-existing response to common mycobacterial antigens blocks BCG

replication and consequently vaccine intake [148]. It has been also hypothesized that variations in the efficacy of BCG are due to the variations between strains used for vaccination. Each BCG vaccine is prepared in a different way, and they differ in the proportion of viable cells per dose [149]. Attenuation of BCG has caused the loss of genes that are present in virulent strains of *M. bovis* and in *M. tuberculosis* but are not needed for the survival of the mycobacteria *in vitro* [150]. These genes are encoded in the Region of Difference (RD) 1 to RD16 [151] and their absence hampers the virulence of BCG as genes for ESAT6 and CFP10 [152] and other potential antigens are deleted.

Development of improved vaccines for TB

Tuberculosis is responsible for more human deaths than any other disease today, and BCG is clearly insufficient for its global control, therefore many research groups in the world are working on the development of new vaccines that either replace BCG (pre-exposure vaccines) or boost BCG's protective effects (post-exposure) [153]. The majority of the current vaccines in clinical trials (Table 1.1) induce Th1 cytokines like IFN-γ or TNF-α from $CD4^+$ or $CD8^+$ T cells [154].

Vaccine development is divided into two stages, pre-clinical and clinical development. In the pre-clinical stage the research uses animal models and in the clinical stage the vaccine is tested in humans. This second stage is divided into four phases. Phase I includes trials to assess the safety of the vaccine and the immune response it induces, phase II studies the safety of the vaccine and side effects in larger cohorts than in phase I, phase III evaluates the vaccine under natural disease conditions (endemic areas), and phase IV aims to detect rare adverse effects (long term efficacy) when the vaccine has been introduced into use [155].

Table 1.1 TB vaccine candidates in clinical stage in the global pipeline (modified from Global TB report 2016) [53]

Phase I	Phase II	Phase III
Ad5Ag85A	DAR-901	*M. vaccae* ™
TB/FLU-04L	RUTI	
MVA85A (Aerosol)	M72+AS01E	
MVA85A-IMX313	H1/H4/ H56:IC31	
ChAdOx1.85A/MVA85A	ID93+GLA-SE	
MTBVAC	VPM1002	

Vaccines in Phase I

Viral vectors

Ad5Ag85A is an adenovirus serotype 5 vector expressing Ag85A which shows improved protection over BCG in animal models when administered via the respiratory route and is safe in humans using the intramuscular route of immunization. It stimulates strong polyfunctional T cell responses in BCG vaccinated individuals [156]. TB/FLU-04 L is an influenza vector-based vaccine expressing Ag85A and ESAT-6 which shows increased antigen specific IFN-γ responses in animal models and is well tolerated when administered to humans as a nasal spray inducing IL-1β, TNF-α and IL-2 [157]. MVA85A (Aerosol) is a modified vaccinia virus Ankara expressing Ag85A [158] administered via the respiratory route to BCG vaccinated adults which produces Ag85A specific $CD4^+$ T cells [159]. MVA85A-IMX313 is a candidate using IMX313, a small protein domain that self-assembles into a nanoparticle with seven identical chains and was designed to improve cellular and mucosal immunity. The candidate is well tolerated and immunogenic [160]. ChAdOx1.85A/MVA85A is a chimpanzee adenovirus expressing Ag85A (ChAdOx1.85A)

followed by MVA85A administered either mucosally or systemically. It induces strong immune responses and is able to improve protective efficacy of BCG [161].

Mycobacterial whole cell

MTBVAC is a live attenuated vaccine based on a human isolate of *M. tuberculosis* developed as a BCG-replacement strategy in neonates. It is safe in newborn mice and shows better protection than BCG [162].

Vaccines in phase II

Recombinant proteins with adjuvant

H1:IC31, H4:IC31 and H56:IC31 are an Ag85B-ESAT6 fusion protein (H1) [163], an Ag85B-TB10.4 fusion protein (H4) [164] and an Ag85B-ESAT6-Rv2660c fusion protein (H56) [165] formulated with the adjuvant IC31. IC31 combines the immunostimulatory effect of an 11-mer antibacterial peptide and a synthetic oligonucleotide which is a TLR-9 agonist [166]. H1:IC31 is well tolerated and safe in HIV-infected adults and induces a specific durable Th1 response; H4:IC31 elicits persistent antigen-specific CD4+ T cells and triggers T cell expansion and production of IFN-γ and multifunctional Th1 cells; and H56:IC31 induces antigen-specific IgG responses, Th1 cytokine expressing CD4+ T cells and specific memory cells [163,164,165]. ID93+GLA-SE is a Rv2608-Rv3619-Rv3620-Rv1813 fusion protein (ID93) formulated in GLA-SE adjuvant, a TLR-4 agonist [167]. It is safe for use in BCG vaccinated adults and its safety and immunogenicity are being evaluated in HIV negative TB patients that have completed treatment for pulmonary disease [53]. M72+AS01E is a Mtb32A-Mtb39A fusion protein formulated in AS01, a liposome based adjuvant containing 3-*O*-desacyl-4'-monophosphoryl lipid A and the saponin QS-21 and promotes CD4+ T cells [168]. M72+AS01E mediates specific humoral and polyfunctional CD4+ T cells in adults treated for tuberculosis but high local reactogenicity has been observed in patients currently in treatment [169].

Mycobacterial whole cells or extracts

DAR-901 is a vaccine prepared based in SRL172 [170], a whole inactivated *Mycobacterium vaccae* vaccine, but it is manufactured using a new scalable method and will likely be used as a BCG booster as it induces protection in animals when compared with a BCG boost [171]. RUTI is a therapeutic vaccine generated from detoxified *M. tuberculosis* fragments which induce Th1 and Th2 responses and it is designed to shorten the treatment of latent TB infection [172]. VPM1002 is a recombinant BCG strain (rBCG Δ*ureC*::*hly*) which expresses the Hly protein from *Listeria monocytogenes* which acts to perforate the phagosomal membrane improving antigen presentation [173]. This vaccine also has the gene *ure*C deleted, which provides the optimum pH for Hly activity in the phagosome [174]. The vaccine is safe and triggers both B and T cell responses [175].

Vaccine in phase III

Mycobacterial whole cell

M. vaccae ™ is a heat inactivated non TB mycobacteria licensed in China to be used in conjunction with TB therapy. It is been assessed for prevention of TB disease in skin test positive individuals [176].

Animal models in TB vaccine research

It has not been possible to reproduce *in vitro* the induction of a protective or therapeutic immune response by a vaccine. The use of animal models is therefore unavoidable for the evaluation of vaccines efficacy [177]. Animal models allow analysing the mechanism, route and transmission of diseases, immune response to vaccination and infection, and characterizing vaccine induced protection [178].

In the evaluation of TB vaccines, three models are most commonly used in a sequential way: mice, followed by guinea pigs, and non-human primates as an optional model. The change

of model is based on the achievement of a better protection than the obtained with BCG or similar protection with improved safety [179].

Mice are the species of choice in biomedicine for immunological testing due to the similitude between the immune system of mice and humans [180]. In the case of TB vaccine research, mice offer the possibility of screening a high number of vaccine candidates at a low cost. Numerous reagents are available for testing immune responses in mice, and there are nude, combined immunodeficiency and gene knockout strains available. The disadvantage of the use of this model is that the protective mechanisms cannot be exactly reproduced in humans as mice are naturally resistant to the infection by *M. tuberculosis* and the composition and organization of granulomas is different to human ones [181]. Guinea pigs are susceptible to *M. tuberculosis* and the disease progress similarly to humans but this model has a high cost and the reagents to evaluate the immune responses in these species are limited [182]. The non-human primate model is advantageous because of the similarities with the evolution of TB in humans and the availability of reagents for immune evaluation. However, non-human primates have a high cost limiting the use of large number of animals, which interferes with statistical validations, restricting this model to the last part of pre-clinical studies [183].

Current knowledge in TB pathogenesis has been also derived from studies that involve animal challenge models with related mycobacteria like *M. bovis* [184]. *M. bovis* shows a high similarity at the genomic level with the other *M. tuberculosis* complex organisms [185] and shares 94 % of the transcriptome with *M. tuberculosis* during the exponential phase of growth [186]. The use of *M. bovis* is also justified as its manifestations in animal hosts mimic human TB [187]. In humans, *M. bovis* is also considered for studying vaccine efficacy; a BCG challenge model is under consideration for testing TB vaccines efficacy as ethical barriers impede challenging with *M. tuberculosis* [188].

Challenges in the development of new TB vaccines

An ideal new vaccine against TB needs to perform better than natural immunity in individuals resistant to the disease. These individuals are able to contain *M. tuberculosis* but are not able to eliminate the bacteria [189]. Blocking infection would be possible inducing the production of protective Abs. These Abs should recognize surface-associated molecules critical for *M. tuberculosis* virulence, should be present in the airways before infection and be of the correct isotype that triggers an appropriate immune protection. This would require a detailed knowledge about the molecular mechanisms of pathogenesis of *M. tuberculosis* and Abs to be long lived or be continuously secreted to the lungs [190]; making this approach unrealistic, considering the limited understanding about TB-host interaction and virulence factors.

It could be possible to achieve protection against *M. tuberculosis* infection with a vaccine that protects throughout induction of a cell mediated immunity considering that the protective efficacy of BCG against *M. tuberculosis* infection reaches a 20 % in children. But compared with the 70 % protection against TB disease [191], seems more feasible to accomplish the goal of the WHO of reducing by 2035 the incidence of TB by 90% compared with 2015 [137], with the development of a vaccine that protects against TB disease.

Post-exposure vaccines would prevent already infected individuals from developing active disease. Th1 cytokines play an important role in TB protection [192], especially IFN-γ, but the production of this cytokine is not sufficient for a protective response [193]. Th17 cytokines may be of high importance as well. In mice, the lack of Th17 memory cells results in loss of Th1 memory cells [194] showing that new vaccines should find a balance between Th1 and Th17 responses. There is also attention driven towards specific Tregs, which can limit the damage caused by the immune responses [190]. But even having some insight into possible protective responses, there are no true correlates of protection against TB and

there is the added challenge of not knowing how to identify the most promising vaccine candidates [195].

More challenging than developing a new TB vaccine with insufficient knowledge about BCG mechanisms of protection, TB pathogenesis and not having correlates of immune protection, is the impact of the disease worldwide. TB itself kills 5000 persons per day [189] and patients co-infected with HIV raise those statistics. The disease has different manifestations in different age groups, suggesting that different vaccines would be needed for each one. Also a vaccine is needed for preventing drug resistant strains, and for preventing the disease in immune-compromised individuals [195] evidencing that may be not one, but more vaccines are needed to prevent the spread of *M. tuberculosis*.

1.4 New approaches to vaccine development

Identification of vaccine antigen candidates by reverse vaccinology

Technology developments of the 21st century have enabled advances in the field of vaccinology. The possibility of using pathogen genome sequences, proteome analysis, structural biology and immuno-informatics tools have allowed the mapping of antigenic epitopes *in silico* to identify vaccine candidates [196]. Reverse vaccinology (Rv) was first defined by Rino Rappouli in 2000 and was described as the study of vaccine development *in silico* using genomic information, and with no need to cultivate the pathogen [197]. This approach led to the development of a vaccine against Meningococcus serogroup B (Bexero) [198] and after 17 years, has integrated DNA microarray technologies, comparative genomics, proteomics and experimental data to the original design [199]. This approach represents the possibility of analysing virtually all the protein antigens of pathogens regardless of their abundance or time of production during infection but has the disadvantage that non-protein antigens cannot be used, impeding the consideration of polysaccharides, lipopolysaccharides, glycolipids and other antigens.

Reverse vaccinology has been applied to tuberculosis vaccines research. Mining the whole genome of *M. tuberculosis* and screening for human MHC class I and II epitopes lead to 99.8% of the putative *M. tuberculosis* epitopes being tested *in vitro* [200]. Comparative genomics has permitted the characterization of cellular immune responses to RDs antigens considered for vaccine design, grouping them into two major categories; the antigens that induce secretion of IFN-γ and those that activate secretion of IL-10 [201]. Research groups are identifying the vaccine candidates after *in silico* selection and testing their protective efficacy *in vivo* [202-204]. *In silico* software recently used for epitopes prediction [205] are New Enhanced Reverse Vaccinology Environment (NERVE), which predicts vaccine candidates [206] and VaxiJen, which predicts protective antigens [207]. Reverse vaccinology represents a major advance in the strategies to identify vaccine candidates, but to date only 35% of the predicted candidates in *M. tuberculosis* have been tested for protection in animal models, and more studies are needed [208].

New adjuvants formulations

Most of the adjuvants already licenced, like alum or oil-in-water emulsions, were developed empirically, without considering the immune responses required for protection. Understanding the mechanisms that mediate their immunogenicity has given insights for the design of new adjuvants that act via stimulation of specific cell subsets or receptors [28].

Targeting DCs, as the most potent APCs, offers a means to achieve good vaccine efficacy. This can be achieved by incorporating PAMPs that target DCs expressed PRRs, using particulate delivery systems that mimic shape and size of pathogens or antibodies against DCs surface receptors [27, 209]. Upon stimulation, DCs undergo a process of maduration/migration that allows them to present the antigens and initiate the immune responses [210]. Activated DCs produce high levels of MHC molecules containing pathogen derived peptides, facilitating the engagement of T cell receptors and also produce co-stimulatory molecules which transmit signals important for T cell proliferation [211].

Adjuvants that target TLRs are bacterial lipoproteins (TLR2), lipopolysaccharides (TLR4), flagellin (TLR5) and nucleotide based (TLR3, 7, 8 and 9) [27]. Monophosphoryl lipid A is a component of the LPS derived from *Salmonella minnesota* that binds TLR4. It is substantially less toxic than LPS and has been incorporated into vaccines for hepatitis B and human papilloma virus [212].

Flagellin is the major structural protein of flagella of Gram-negative bacteria. It is the natural ligand of TLR-5, inducing DC maturation/activation [213]. Flagellin is an attractive candidate to be used in human vaccines as it is effective at low doses (1-10 μg) in non-human primates [214], does not produce allergic responses [215], prior immunity to Flagellin does not impair its adjuvant activity [216] and fusion partners can be inserted either at N or C-terminus without losing its signalling via TLR5 [217]. Flagellin has been used as an adjuvant for the development of vaccines for chicken, inducing mixed Th1 and Th2 responses [218] and as an adjuvant and carrier for cocaine vaccines stimulating anti-cocaine antibodies in mice [219]. In the context of TB vaccine research, Flagellin has been used as a carrier and adjuvant in combination with Rv2108 inducing the production of high levels of IFN-γ and cell proliferation [220]. Also, Flagellin has been used in conjunction with Ag85B, enhancing antigen-specific CD4+, CD8+ T cell and memory responses, correlating with protection against *M. tuberculosis* aerosol challenge [221].

A strategy to enhance immunogenicity of peptide vaccines is to use T cell epitopes. They represent an advantage over protein carriers as they are smaller and can be incorporated in specific sites of their protein partners [222]. A major limitation for using these epitopes comes from the polymorphism of the binding site of MHC molecules which implies that the epitopes presented vary between individuals. Promiscuous or universal epitopes have been identified that bind several MHC alleles in pathogens, allergens and tumour antigens [223]. Promiscuous T cell epitopes are capable of binding different classes of MHC II molecules and possess structural features that allow the peptide-MHC complex to be recognized by the

T cell receptor to produce cross reactive responses [224]. CS.T3 $_{378\text{-}395}$ is a peptide derived from the circumsporozoite protein of *Plasmodium falciparum.* This peptide is almost universally recognized by human and mouse T lymphocytes, produces immunological cross reactivity and potentiates immune responses [225], characteristics that makes it an ideal epitope to be used as an adjuvant for vaccine development.

Targeting immune cells present in specific sites is relevant for conferring immunity at the site of infection. Mucosal immune surveillance is largely achieved by specialized mucosal (M) cells. *Clostridium perfringens* enterotoxin (Cpe), binds to the protein Claudin 4 [226]. Claudin 4 is highly expressed in colon, the nasopharynx surface epithelia and M cells in Peyer's patch [227]. Cpe30, the C-terminal 30 amino acids of the Cpe, included in a chitosan vaccine against viral myocarditis enhanced mucosal IgA and T cell immune responses and protected mice against myocarditis better than the variant that did not contain Cpe30 [228].

New vaccine delivery systems

Polyhydroxyalkanoate bead-based vaccines

Polyhydroxyalkanoates (PHAs) are naturally occurring inclusions in many bacteria which serve as energy and carbon storage material. It is possible to produce protein vaccine candidates on the surface of PHAs in a one-step process. The most common PHA, Poly(3-hydroxybutyrate) (PHB), is a biocompatible polymer, evidenced by its lack of toxicity and compatibility in contact with tissue and blood [229]. PHB is also biodegradable *in vivo* by hydrolytic enzymes. These polymers are degraded by the action of non-specific lipases and esterases [230]. To date, PHB based vaccines have enabled delivery of antigens inducing immunity against tuberculosis [231, 232], hepatitis C [233] and pseudomonas [234]. Chapter 1B describes the formation of PHAs, their properties and applications.

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Chapter 1B: Self-Assembled Protein-Coated Polyhydroxyalkanoate Beads: Properties and Biomedical Applications

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Abstract

Polyhydroxyalkanoates (PHAs) are biological polyesters that can be naturally produced by a range of bacteria as water-insoluble inclusions composed of a PHA core coated with PHA synthase, structural, and regulatory proteins. These naturally self-assembling shell−core particles have been recently conceived as biomaterials that can be bioengineered as biologically active beads for medical applications. Protein engineering of PHA-associated proteins enabled the production of PHA-protein assemblies exhibiting biologically active protein-based functions relevant for applications as vaccines or diagnostics. Here we provide an overview of the recent advances in bioengineering of PHA particles toward the display of biomedically relevant protein functions such as selected disease-specific antigens as diagnostic tools or for the design of particulate subunit vaccines against infectious diseases such as tuberculosis, meningitis, pneumonia, and hepatitis C.

Abbreviations

PHA: polyhydroxyalkanoate, PHB: Polyhydrohybutyrate, EM: electron microscopy, AFM: Atomic force microscopy, TEM: transmission electron microscopy, PHASCL: short-chain-length monomers of PHA, PHAMCL: medium-chain-length PHA monomers, iPHA: intracellular PHA, eYFP: enhanced yellow fluorescent protein, GAPs: granule associated proteins, GRAS: Generally Regarded As Safe, DARPins: designed ankyrin repeat proteins, MOG: myelin oligodendrocyte glycoprotein, IL:interleukin, GFP: green fluorescent protein, TST: Tuberculin skin testing, MALDI TOF-MS: matrix-assisted laser desorption ionization–time-of-flight mass spectrometry ,HCV hepatitis C virus, PHBCo: PHB beads coated with the core protein (Co) from HCV.

1.6 Introduction

Polyhydroxyalkanoates (PHA) are naturally produced as intracellular polyester inclusions by many bacteria and archaea under conditions of unbalanced nutrient availability. PHAs are deposited in the cell cytoplasm as water-insoluble granules which have an amorphous biopolyester core and attached or embedded proteins at the granule surface [1] (Figure 1.2). PHAs natural function is to serve as a reserve polymer which can be used in times of carbon starvation using associated depolymerizing enzymes.

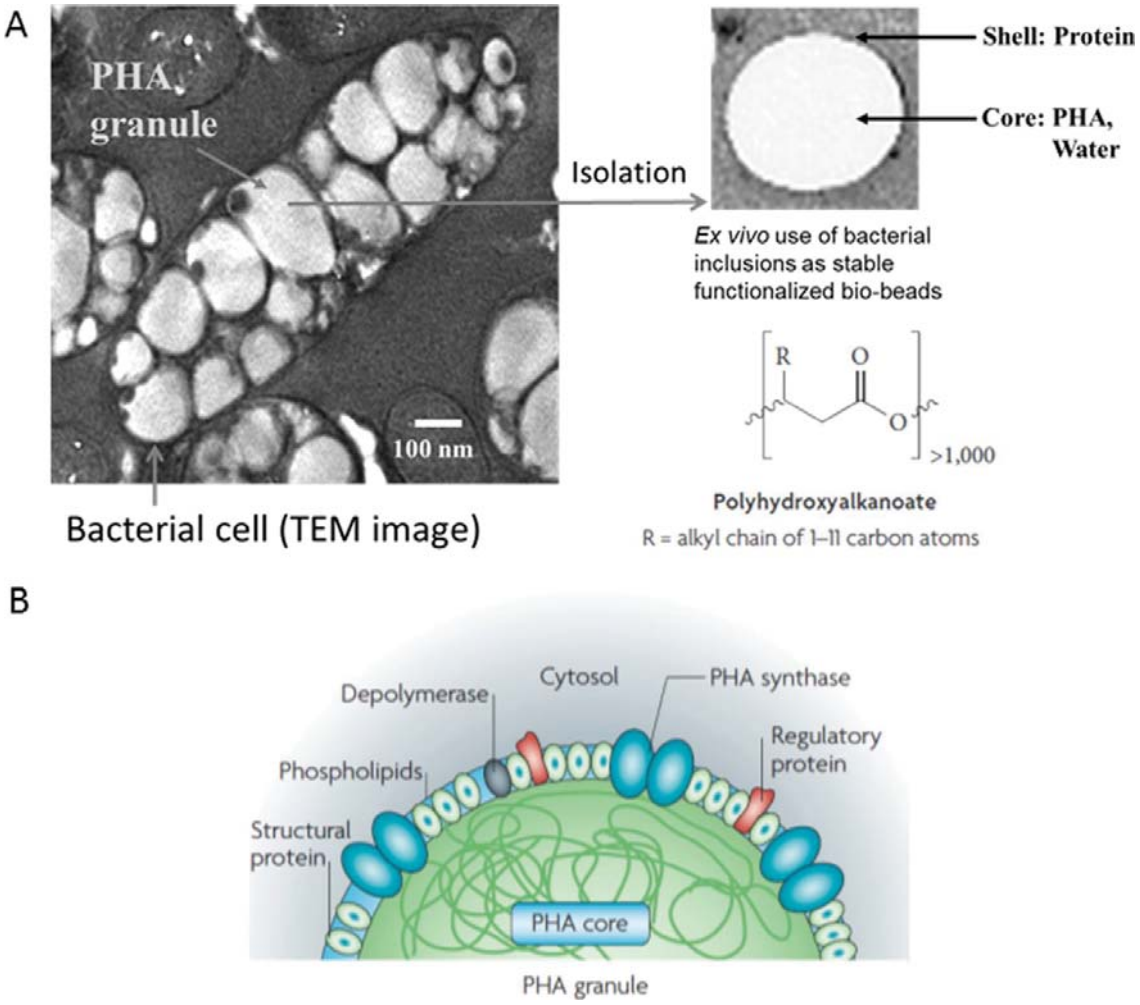

Figure 1.2. A, Polyhydroxyalkanoate (PHA=Biopolyester) inclusions; B, Schematic of PHA granule (Reprinted from [1])

Over 150 different PHAs have been described, which may occur as homopolymers or as co-polymers, but the most commonly produced polyester in bacteria is poly (3-hydroxybutyric acid) (PHB) which is synthesized from 3-hydroxybutyrate [2, 3] (Figure 1.3).

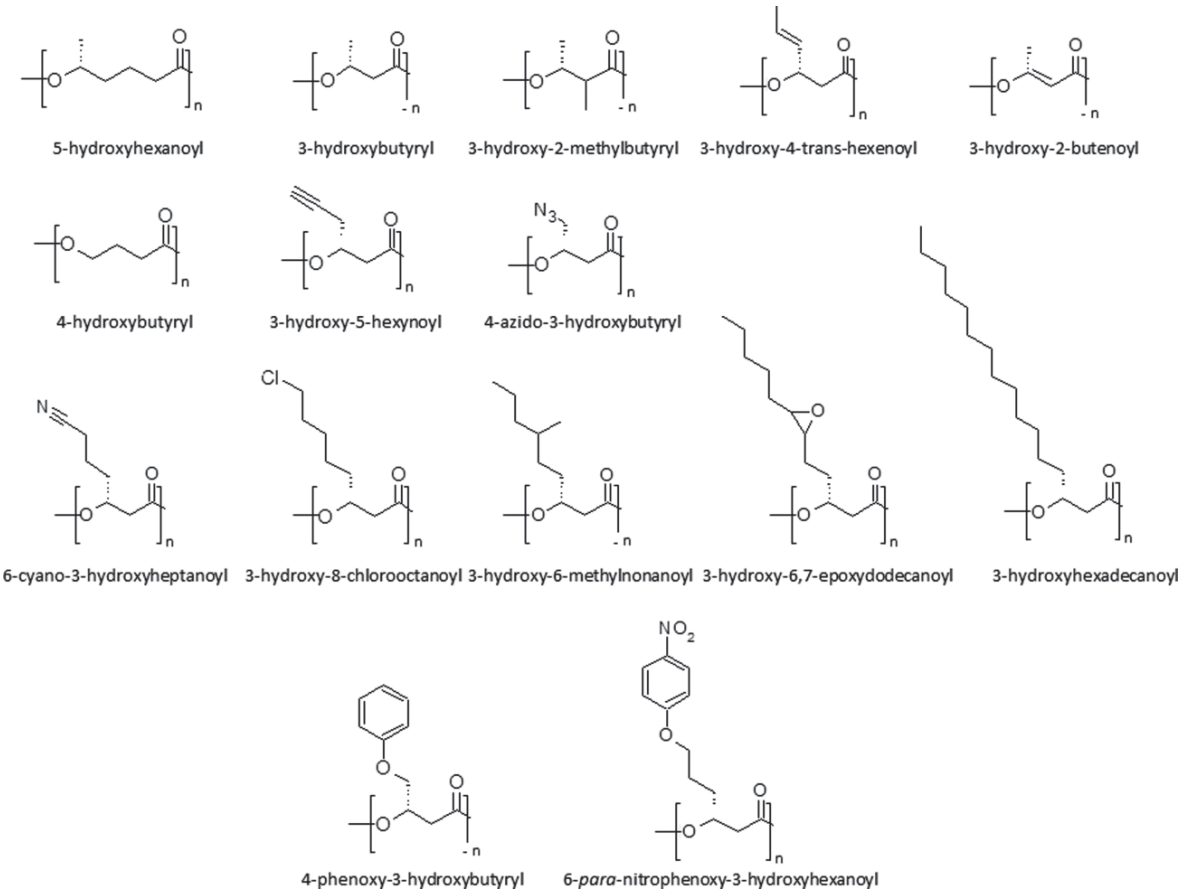

Figure 1.3. Representative constituents of PHAs. Numerous constituents of PHAs are known, suggesting a vast design space for the development of a range of PHAs exhibiting various material properties (adapted from reference 3). Only the R enantiomer is used in the biosynthesis of PHA.

Comonomers of PHAs include those with moderate side-chain modifications such as terminal unsaturated bonds and azide groups enabling click reactions toward chemical modifications [4, 5].

The diversity of side chains in the hydroxyacyl comonomers, their sequence and arrangement, the varying molecular weight of the PHA, and the possibility of chemical

modifications provide an enormous design space to synthesize a vast range of polyesters exhibiting numerous material properties. This opens up applications ranging from renewable replacements of oil based bulk-commodity plastics to biomaterials for tissue engineering.

The production and processing of bacteria producing PHAs will vary depending on the end use, but generally safe bacteria are cultured under appropriate conditions resulting in intracellular bead-like inclusions that can then be purified and used for many biotechnological and biomedical applications [6, 7]. PHAs can also be recombinantly produced by insertion of appropriate biosynthesis genes into alternative bacterial hosts or plants [8, 9]. PHAs can be used as alternatives to oil-based plastics [10, 11], but because of fermentation and purification costs, their principal niche use is in the biomedical field. The biocompatible and biodegradable properties of PHA [12−15] have enabled PHA-based materials to be used in sutures, repair patches, stents, bone scaffolds, and drug delivery systems [7, 16−19]. These biomaterials are based on extracted PHAs, isolation of which often requires enzymes, chemicals, and/or harsh solvents.

In contrast, production of functionalized PHA beads aims to retain the structural integrity of the PHA granule (Figure 1.2). This entails the incorporation of additional genes into the bacterial production host so that a polymer bead is produced that displays a surface-immobilized PHA-specific protein along with specific relevant functional proteins of choice [20].

Production of the protein-coated PHA assemblies occurs within the bacterial cell as a one-step process. Therefore, overall production costs are reduced because there is no need to first produce proteins and then chemically cross-link the proteins to carrier beads. These functionalized beads need a gentle isolation and purification process to maintain the bead shape and functionality.

These *in vivo*-assembled functionalized beads exhibit properties for a broad range of applications in agriculture, the biopharmaceutical and biotechnology industries, bioremediation, [21−24] and the field of biomedicine, which is the focus of this review.

This review summarizes the current knowledge of PHA biosynthesis, focusing on the molecular mechanisms of self-assembly of PHA inclusions and the recent bioengineering approaches to produce functionalized PHA beads applicable for biomedical applications such as diagnostics and vaccines.

1.7 Pha biosynthesis and self-assembly of PHA Granules

Many Gram-positive and Gram-negative bacteria are capable of forming PHA granules. The process of PHA biosynthesis is somewhat similar in all of these bacterial species. The three enzymes that play key roles in the formation of the most common PHA, PHB, are β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and polyester synthase (PhaC) (Figure 1.4).

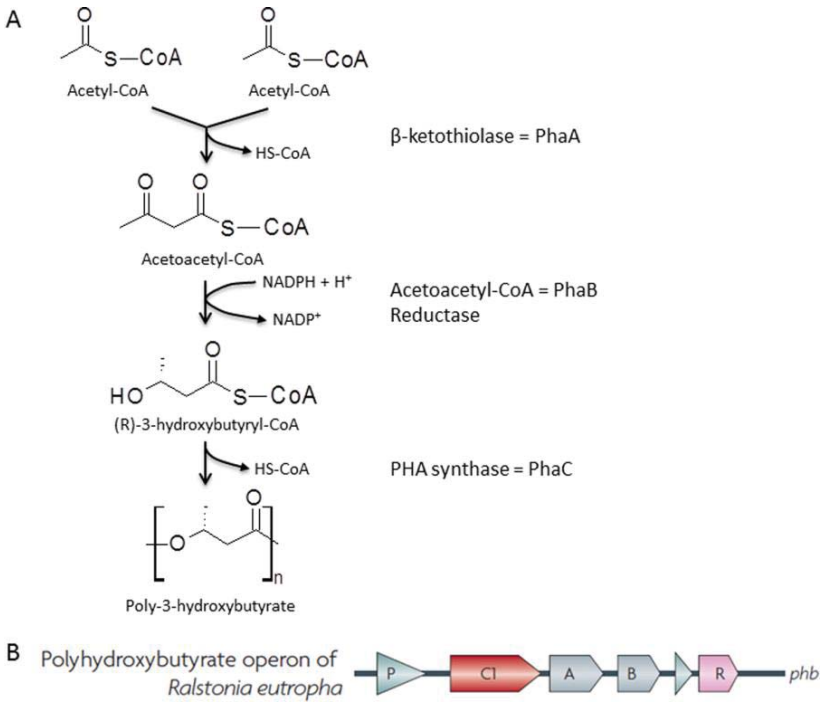

Figure 1.4. Biosynthesis and genetics of PHB production. (A) PHB biosynthesis pathway. (B) PHB biosynthesis operon (adapted from reference 1).

PhaA catalyses the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA, which is reduced by PhaB to form 3-hydroxyacyl-CoA. This is then polymerized by PhaC into

PHA with continuous release of CoA [2, 25−27]. During this process, PhaC remains covalently attached to the surface of the PHA granules [28]. Despite the considerable research on catalytic synthesis of PHA *in vitro*, the molecular events occurring during PHA granule assembly *in vivo* are not fully understood. Currently three models have been proposed for PHA granule assembly *in vivo*, namely, the micelle model, the membrane budding model, and the scaffolding model [6,29] (Figure 1.5).

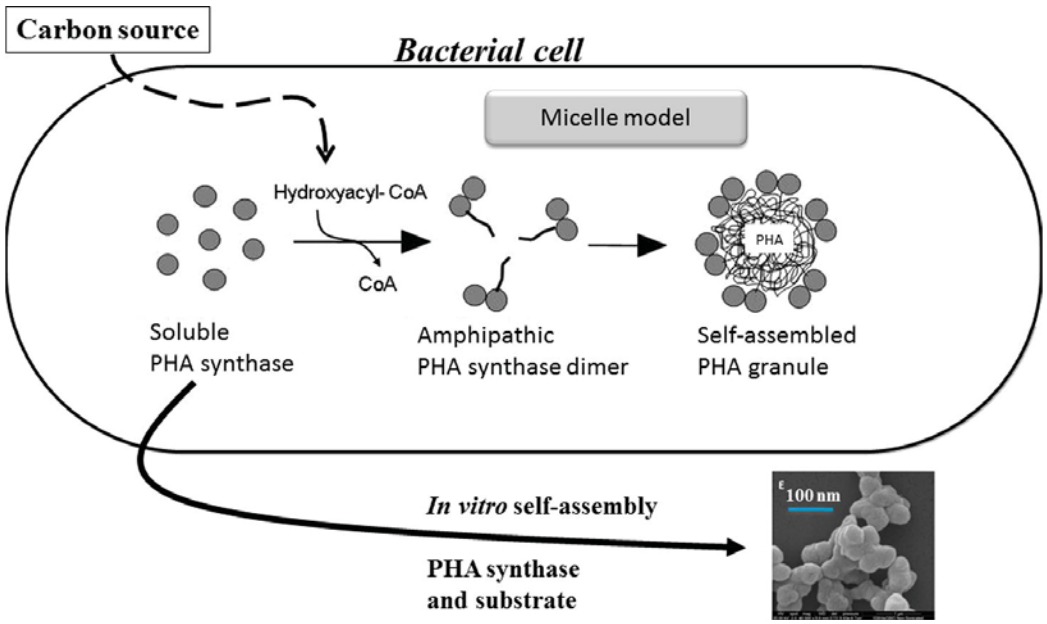

Figure 1.5. Schematic of self-assembly of PHA granules.

In the currently favoured micelle model, soluble PhaC interacts with the substrate in the cytoplasm to initiate the polymerization process, and several polymer molecules aggregate by hydrophobic interactions to form micelle-like structures [30−33] (Figure 1.5). Phasins and other granule-associated proteins (PhaZ and PhaR) bind to PHA granules, although it has been argued that the fusion of granules is prevented by proteins such as PhaP [34]. It has been proposed that this model requires a controlled elongation rate of PHA so that there is sufficient time for short PHA chains to form the micelle structure [35,36]. Recently, Cho *et al.* found that the elongation rate of PHA is higher than its initiation rate and observed high

levels of PhaP1 at an early stage of granule formation in *Ralstonia eutropha* (formerly *Cupriavidus necator*) [37]. In addition, the authors for the first time purified soluble PhaC in a complex with PhaP1. On the basis of these findings, they proposed a modified micelle model. In this model, the initial complex consists of a PhaC dimer covalently attached to one PHB chain with which four to nine PhaP1 proteins are associated in the early stages of granule formation. These complexes fuse together by hydrophobic interactions to form soluble precursor granules, which undergo a phase transition to form the mature granules.

The membrane budding model was proposed on the basis of its similarities with the synthesis of lipid bodies in eukaryotic systems [38, 39]. According to this model, PhaC and the phasin PhaP1 bind to the inner side of the cytoplasmic membrane along with PHB chains that are liberated into the bilayer of the membrane, forming a PHB granule at the cytoplasmic membrane. The granule subsequently buds off from the membrane surrounded by a phospholipid monolayer and PhaC and PhaP1 covering the surface of the granules. Several studies have shown convincing evidence in favour of the budding model. One study showed that PHB granules are predominantly located near the cell pole and cell wall using fluorescent techniques in three different PHA-granule-producing bacteria (*Rhodospirillum rubrum, R. eutropha*, and recombinant *Escherichia coli*) *in vivo* [40]. Another study using similar techniques demonstrated that nascent PHA granules localize to the cell poles in recombinant *Pseudomonas aeruginosa* and *E. coli* [20]. Using mukB mutants, these authors demonstrated that the localization requires proper nucleoid structure and segregation. In contrast to these observations, several studies have provided evidence indicating that the budding model is unlikely for granule formation. Studies demonstrated that various key genes associated with the biosynthesis of phospholipids such as phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin are downregulated in *R. eutropha* during PHB production [41, 42]. One would expect these genes to be upregulated if PHA granules need to be covered by a lipid monolayer as proposed in the budding model. A further study used electron cryotomography to generate high-resolution images of granule genesis in *R.*

eutropha in a near-native state [43]. These observations revealed a discontinuous surface layer and no lipid monolayer on the granule surface, which are inconsistent with the membrane budding model. Furthermore, observations by Cho *et al* [37] suggest that a PhaC-PhaP1-PHB complex represents soluble precursors for granule assembly, which is inconsistent with the budding model because lipid-coated PHB would not be soluble.

The scaffolding model was proposed on the basis of the localization of granules close to dark-stained features (so-called “mediation elements”) in the centre of the cells [44]. These mediation elements were proposed to serve as scaffolds by providing sites for PhaC to initiate granule formation. Studies using transmission electron microscopy (TEM) showed that early PHB granules are often located more or less in the middle of the cells near the mediation elements [44]. Although the nature of these structures remained obscure at that time, they are now believed to be bacterial nucleoid. Pfeiffer and Jendrosseck provided further evidence and showed that PhaM, which was copurified with PhaC of *R. eutropha*, specifically interacts with DNA and nucleoid both *in vitro* and *in vivo* [45]. These authors proposed that as a result of this interaction, PHB granules are attached to bacterial nucleoid. A further study by this group using TEM confirmed this interaction. They demonstrated that binding of PHB granules to the nucleoid is mediated by PhaM and that overexpression of this protein resulted in many small PHB granules attached to the nucleoid region in *R. eutropha* [46]. On the basis of these observations, it appears that these models of PHA synthesis may be species-specific or even that multiple models could be integrated at any given time point in PHA biogenesis.

1.8 Composition and structure of PHA Granules

Despite continuous research since their discovery almost a century ago, the precise composition and surface structure of PHA granules have yet to be fully elucidated. Early investigations showed that these PHA granules consist of polymer, proteins, and lipids. The chemical composition was first identified for the PHA granules of *Bacillus megaterium* [36].

The authors reported that the purified granules consisted of ~97.7% PHB, 1.87% protein, and trace amounts of lipids. The polyester core in the granules is largely amorphous [47, 48]. Several studies have shown that PHA granules are spherical and that their size varies among organisms. The diameters of these spherical subcellular organelles range from 200 to 500 nm, as suggested by TEM studies.

Early studies used electron microscopy (EM) to determine the structure of PHA granules and suggested that the polymer core of PHA granules is covered by a membrane with a thickness of 4 nm [49]. Another study confirmed these findings by determining the thickness of the PHB granule surface layer and that of the cytoplasmic membrane, which were found to be ~3.8 and 7.23 nm, respectively [50]. These findings suggested that PHA granules contain a phospholipid monolayer with embedded proteins. While this monolayer membrane structure of PHA granules is widely accepted, alternative membrane models have also been suggested. According to one model, the polyester core of PHA granules is surrounded by three layers in which a phospholipid layer is sandwiched between two separate protein layers [51]. In another model, Jendrossek *et al* used TEM and found the thickness of the surface layer of purified PHA granules from *Caryophanon lactum* to be 14 nm [52]. The authors also provided evidence that this layer consists of a paracrystalline-like layer of densely packed proteins with a diameter of ~8 nm and suggested that associated proteins cover most of the granule surface.

A number of other techniques have also been used to determine the structure of PHA granules. Russell *et al* used contrast-variation small-angle neutron scattering to study PHA granule organization, and their observations supported the protein and lipid layer model [53, 54]. Atomic force microscopy (AFM) has been used to investigate the PHA granule surface. This tool offers the ability to image at the nanoscale level with gentle sample preparation compared with EM. Using AFM, Dennis *et al* [55] demonstrated that the surface layer of PHA granules contains porin-like structures, and beneath these structures they observed parallel arrays consisting largely of phasin proteins. The authors suggested that the porin-like

structures are the gateway to the granule core and possibly the centres for synthesis and depolymerization. In a further study, these authors evaluated the role of phasin protein in the formation of a network layer on the surface of PHA granules. The network-like layer was absent on the surface of PHA granules produced in phaP-negative *R. eutropha,* confirming that PhaP protein is a part of the network layer [56]. While most of the studies to date have indicated the presence of a lipid layer in PHA granules, a recent study provided strong evidence for the absence of this lipid layer [43]. These authors produced high-resolution images of *R. eutropha* cells in a near native state using electron cryotomography and indicated that the surface of PHA granules is coated with proteins and not phospholipid. To confirm this hypothesis, very recently Bresan *et al* [57] expressed fusion proteins of DsRed2EC and other fluorescent proteins with the phospholipid-binding domain (LactC2) of lactadherin in three model PHA-accumulating organisms. Fluorescence microscopy revealed that the fusion proteins colocalized with the cytoplasmic membrane and did not colocalize with PHA granules. They concluded that PHB/ PHA granule surface layers in natural producers are free of phospholipids and consist of proteins only.

Collectively, these studies indicate that variations observed in the structural layer of PHA granules could be specimen preparation-induced artefacts. Often the preparation of PHA granules for analysis involves harsh treatments such as excessive sonication, freeze−thaw cycles, or exposure to solvents, detergents, or alkalis, which makes it hard to determine the exact structure and composition of PHA granules. To avoid this, a simpler and less abrasive technique such as mechanical cell lysis could be used to purify PHA granules without affecting their native structure. This technique should allow access to a more native structure of PHA granules.

1.9 Granule-associated proteins

Several proteins have been identified that are associated with the granule surface and play an important role in PHA granule formation and degradation. These known PHA-associated proteins are classified into four major classes as discussed below.

PHA Synthases

PHA synthase (PhaC) is the key enzyme in the biosynthesis of PHA granules that catalyzes the polymerization of hydroxyacyl-coenzyme A (CoA) to PHA. Advancements in genome sequencing technology have led to the identification of more than 90 PHA synthase genes in a number of different bacterial species. On the basis of primary structural and biochemical properties, PHA synthases have been classified into four major classes [2, 27, 58] that are distinguished mainly by subunit composition and sequence similarity.

PHA synthases of *R. eutropha* belong to class I, while PHA synthases of *P. aeruginosa* represent class II. Both class I and class II PHA synthases consist of only one type of subunit, with molecular weight ranging between 60 and 70 kDa [2, 59, 60]. Class III PHA synthases of *Allochromatium vinosum* consist of two 40 kDa heteromeric subunits [61]. These subunits are encoded by PhaC and PhaE with no homology to each other. Class IV PHA synthases are found in Bacillus species and also consist of two subunits, encoded by PhaC and PhaR, with masses of 40 and 20 kDa, respectively [62]. In terms of their substrate specificities, class I, III, and IV synthases catalyze the polymerization of shortchain-length monomers of PHA (PHASCL) [63], while class II PHA synthases utilize (*R*)-3-hydroxyacyl-CoAs containing 6-14 carbon atoms and produce medium-chain-length PHA monomers (PHAMCL) [64].

Comparison of amino acid sequences suggested that the PHA synthases share a conserved catalytic triad consisting of conserved cysteine, aspartic acid, and histidine residues (Figure 1.6). This is similar to hydrolases such as lipases, except that the serine in the catalytic site is replaced by cysteine in PHA synthase [65]. Bioinformatics analysis of PHA synthase

suggested that it contains an α/β hydrolase region, which was shown to be essential for its enzymatic activity [66, 67]. Studies have suggested that the growing PHA chain is covalently attached to the cysteine residue of the active site of PHA synthase, while aspartic acid residues play an important role in chain elongation during the process [28] (Figure 1.6).

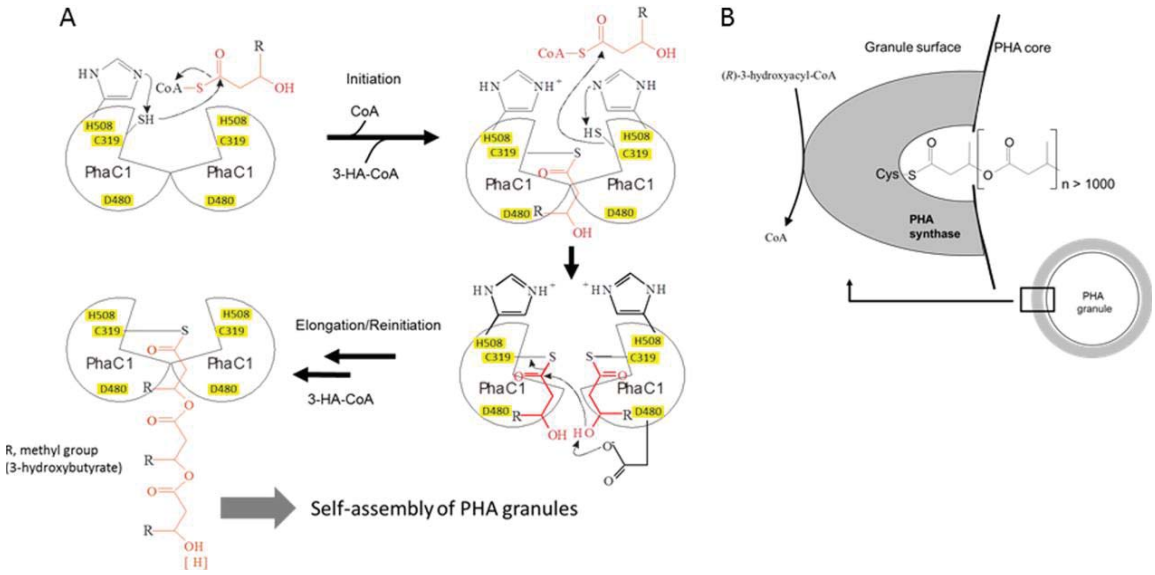

Figure 1.6. Proposed covalent catalysis mechanism for PHA synthesis. (A) Proposed reaction mechanism of PHA synthase (e.g., PhaC1 from *Ralstonia eutropha*) highlighting amino acid residues proposed to constitute the catalytic triade (adapted from reference 2). (B) Schematic depicting localization of PHA synthase at the PHA granule surface, attached via a covalent linkage to the PHA core.

This covalent linkage of the PHA synthase has been exploited to tightly coat PHA granules with functional proteins inserted into dispensable regions of the PHA synthases, as discussed below (Figure 1.7). No crystal structure of a PHA synthase is currently available. However, a recent study used chemical labelling of granule-bound PHA synthase to detect structurally more flexible surface-exposed regions that could be verified as sites permitting insertion of foreign protein functions [68] (Figure 1.7).

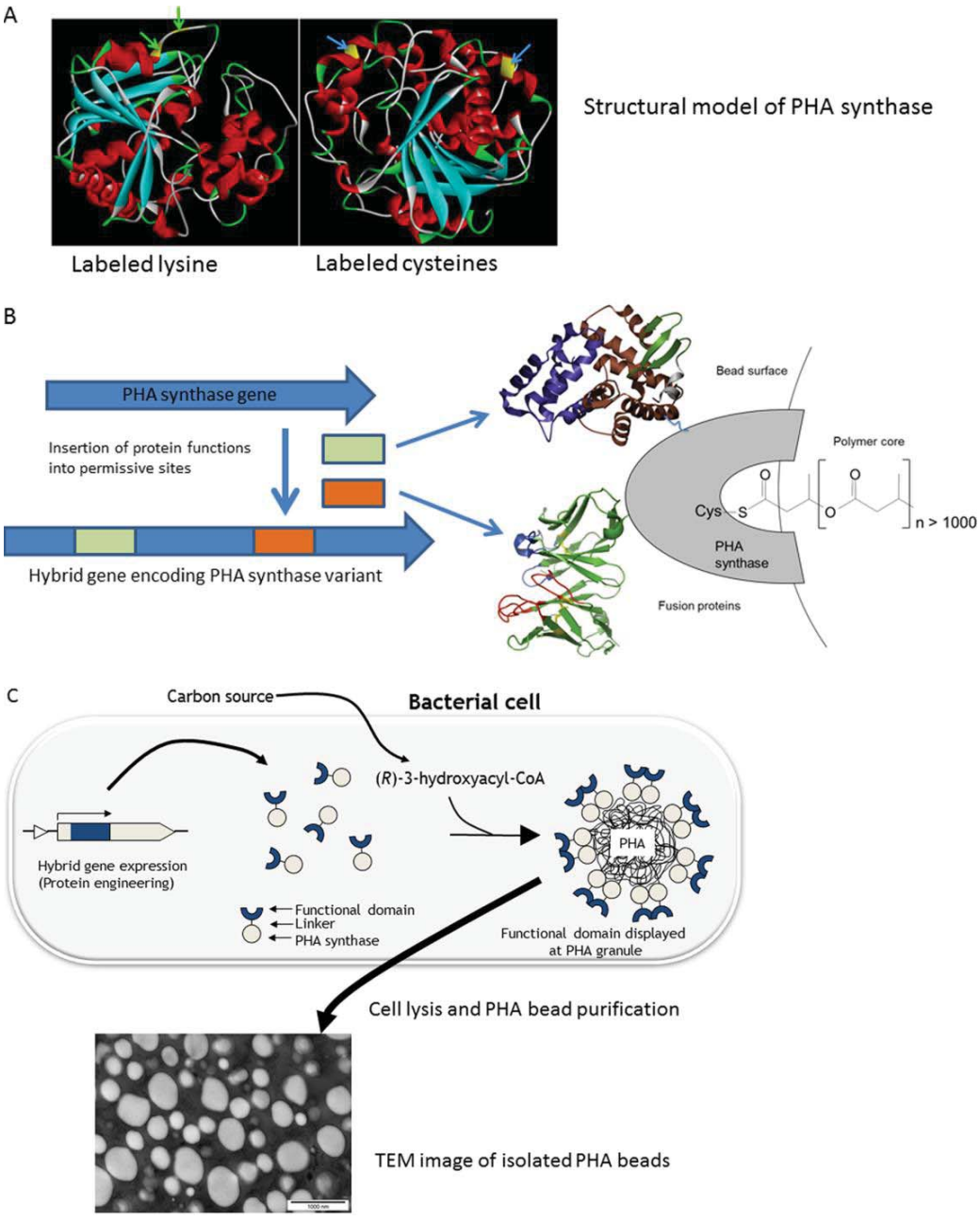

Figure 1.7. Topological model and engineering of PHA synthase (*Ralstonia eutropha*). (A) Structural models [68] showing surface-exposed amino acid residues as detected by chemical labelling (arrows indicate labelled sites). (B) Protein engineering of PHA synthase for display of various protein functions on the PHA bead surface. (C) Development of a production strain for production of functionalized PHA beads.

PHA Depolymerases (PhaZ)

PHA depolymerases are the main enzymes that degrade PHAs. The PHA depolymerases are classified into two groups, namely, intracellular (PhaZ) and extracellular depolymerases [69]. The intracellular PHA (iPHA) depolymerases are found on the surface of PHA granules and degrade the previously accumulated PHAs within the bacteria [67, 70]. The extracellular PHA polymerases are secreted extracellularly by a large number of bacteria to utilize extracellular (denatured) PHAs present in the environment [71, 72]. Analysis of the *R. eutropha* genome revealed the presence of seven iPHA depolymerases and two oligohydrolases [69, 73] On the basis of their amino acid sequence similarities, these enzymes were named PhaZa1−PhaZa5, PhaZd1, and PhaZd2 for iPHA depolymerases and PhaZb and PhaZc for oligohydrolases. Recently, two novel PHA synthase/depolymerase-like proteins with possible roles in PHB metabolism have been identified in PHB granules using comparative genome analysis of *R. eutropha* [74].

Phasins (PhaP)

Phasins are a group of proteins that are found on the surface of PHA granules [75−77]. The name “phasin” (PhaP) is based on the homology of these proteins with olesins of oil globules in some plant cells [78]. Phasins are the major proteins of the surface-associated proteins of PHA granules, constituting up to 5% (w/w) of the total cellular protein [76]. Several different phasins have been identified in PHA-synthesizing bacteria, such as PhaP1−PhaP7 from *R. eutropha* [76,79], PhaP from *B. megaterium* [80], GA14 from *Rhodococcus ruber* [75], PhaF and PhaI from *Pseudomonas putida* [81], and PhbP and FA8 from Azotobacter species [82,83]. PhaP1 of *R.eutropha* is the most studied member of this class of proteins.

Two main roles have been assigned for phasins; preventing the aggregation of PHA granules and inhibiting nonspecific attachment of other proteins to PHA granules [75,76,78]. In addition, these proteins have been proposed to participate in several other functions such as regulation of PHA synthesis and degradation, granule size, formation of networks on the PHA granule surface, and distribution of PHA granules during cell division [45,76,84−86]. A

number of studies have suggested that phasins form an amphiphilic layer on the surface of PHA granules to stabilize them and prevent merging of individual granules [76,77,87]. Studies have also suggested that phasins are not essential for PHA production, but experiments involving phasin mutants have indicated that these proteins affect the size and number of PHA granules. A study showed that mutation of the phaP1 gene in *R. eutropha* had no significant effect on the PHA granules [88]. Moreover, several studies have shown that phasins are produced only under PHA-accumulating conditions and that the synthesis and abundance of phasins are directly correlated to PHA levels in the cell [76,85,89]. These studies suggest that phasins influence PHA synthesis, possibly by preventing binding of other proteins to the PHA granule surface in a nonspecific manner.

Regulatory Proteins (PhaR, PhaF, PhaI).

There is evidence that phasin expression and PHA granule synthesis are tightly regulated by transcriptional repressor PhaR [89−92]. PhaR homologue genes are present in various PHASCL-producing bacteria, suggesting that PhaR play an important role in the regulation of PHA biosynthesis [77, 87, 93]. It has been shown that PhaR binds not only to PHA granules but also to DNA upstream of the phaP and phaR genes to modulate the regulation of PHA synthesis [89]. Those authors also demonstrated that PhaP is constitutively expressed in phaR-negative *R. eutropha* mutants, confirming that PhaR works as a repressor of PhaP. York *et al*. provided additional evidence and demonstrated that phaR-deletion mutants of *R. eutropha* accumulated PhaP levels higher than the wild-type strain, indicating that PhaR is a negative regulator of PhaP accumulation [92]. All of these findings led to the establishment of a widely accepted regulatory model for PhaP synthesis. PhaR binds to the phaP promoter region to inhibit transcription of the protein when conditions are not favorable for PHA biosynthesis. Once conditions are favorable for PHA synthesis, PhaR binds to the surface of the newly formed PHA granules, resulting in a low concentration of cytoplasmic PhaR to a point that is not sufficient to repress the transcription of phaP, which in turn leads to the synthesis of PhaP. During the PHA accumulation process, when PHA

granules have reached their maximum size and all of the binding sites for PhaP and PhaR have been occupied, excess soluble PhaR again binds to the DNA sequences upstream of phaP and phaR, resulting in the repression of PhaP and PhaR. This model suggests autoregulation of the phaR gene, allowing only the synthesis of a sufficient amount of PhaR that is required for adequate repression of phaP expression [94].

Other granule-associated proteins with phasin-like functions have been reported. The proteins PhaF and PhaI have been identified in *Pseudomonas oleovorans* with regulatory functions similar to those of the PhaP/PhaR system of *R. eutropha* [81]. Pfeiffer *et al.* identified the protein PhaM with function similar to that of PhaF in *R. eutropha* [45]. These authors observed that phaM-negative mutants of *R. eutropha* accumulate only one or two large PHB granules, indicating the role of this protein in determining the surface to volume ratio of PHB granules. These studies suggest that both PhaF and PhaM play an important role in the distribution of PHA granules in daughter cells and have DNA-binding ability, which appears to be very similar to PhaR.

Recently a structural model of PhaF from *P. putida* was developed and suggested that PhaF is an elongated protein containing a long amphipathic N-terminal helix mediating the hydrophobic interaction with PHA, while its superhelical C terminus was proposed to bind to chromosomal DNA [95].

Other Granule-Associated Proteins.

A recent study reported two entirely novel proteins in PHB granules of *R. eutropha* [74]. The first protein is a patatin-like phospholipase, an enzyme that has not been associated with PHB granules of any PHB-accumulating species to date. Another protein, named A2001, forms an operon with acetoacetyl-CoA reductase and PHB synthase. Using fusion proteins with enhanced yellow fluorescent protein (eYFP), these authors confirmed that these novel proteins localize on the PHB granule surface, proposing their possible role in PHB metabolism.

1.10 Biomedical applications of PHA-protein Assemblies

The increasing knowledge about the topology, structure, and biochemical properties of GAPs has opened up opportunities to rationally engineer these proteins by fusing or inserting various protein functions while retaining their ability to attach to PHA granules. This ultimately leads to PHA granules displaying desired protein functions, i.e., surface-functionalized PHA beads suitable for a variety of applications such as in biomedicine (Table 1.2). In this context, PHA synthase was found to be an advantageous target for protein engineering and PHA bead surface functionalization, as this enzyme remains covalently linked to the PHA core of the beads, i.e., tightly linking the function/activity to the PHA core, thereby avoiding leaching as was found for the other GAPs (Figure 1.7).

Table 1.2. Summary of recent developments in PHA-bead-based biomedical applications

Biomedical applications of PHA beads	Brief description of the process	Reference
protein production and purification	endotoxin removal, recombinant protein, immunoglobulins and antigens purification, therapeutic proteins	96, 6, 21,97-101,103,105-109,102-104
diagnostic tools	detection of specific antigens and molecular targets, flow cytometry, bioimaging, studies of protein-protein interactions	104,110-112
diagnostics for infectious diseases	tuberculosis-improved skin test reagent	114-115
Vaccines	tuberculosis, hepatitis	114,116-118, 98,119

Protein Production and Purification

Various proteins used for biomedical purposes such as therapeutic proteins, antigens, and antibodies often require purification from a complex mixture of proteins without

compromising the biological activity or changing the protein structure [120]. Such purification often requires multiple steps and can be complex and costly.

Proteins can be purified by exploiting affinity-based purification technologies, which utilize a specific interaction between the protein of interest and a solid support [121]. The interaction often requires fusion of the protein of interest to a purification tag, such as polyhistidine. However, a purification tag may lead to a change in the protein's intrinsic properties, such as solubility, net charge, and protein folding [121]. This issue may be solved by enzymatically removing the affinity tag from the protein of interest after purification [122]. However, the target protein may become insoluble after removal of the tag [123].

Accordingly, affinity-based protein purifications need to be optimized for each individual protein of interest. Moreover, enzymatic cleavage and complicated multiple separation steps make this technique expensive and time-consuming. Protein immobilization is often utilized to bind proteins of interest to a matrix. It has been shown that immobilization can enhance the stability and activity of the target proteins [124,125]. However, many traditional protein immobilization approaches need to use reagents to purify the proteins and their solid supports, and subsequently, the proteins are displayed on the support by nonspecific absorption or chemical cross-linking [126]. Hence, the multiple immobilization steps make this traditional immobilization technique expensive and time-consuming. Therefore, it would be desirable that a bacterial cell could produce both the target protein and the micro/nanoparticle (solid support) and that the protein could be directly displayed on the surface of the particle *in vivo*. The particles displaying the protein of interest could be purified readily by centrifugation after cell disruption [125]. Endotoxin removal is an important field for biomedicine, and it is noteworthy that PHA beads were produced from prior extracted PHA with the capability for removing endotoxins. This was achieved by adsorbing an engineered *R. eutropha* PhaP that was fused with human lipopolysaccharide binding protein onto PHA particles utilizing the natural hydrophobic interaction between PHB and PhaP [96].

In vivo production of PHA beads with engineered protein surfaces is proposed to be cost-effective and hence is suggested as a suitable platform for the purification and immobilization of target proteins [97, 98,120]. Target proteins can be purified and covalently immobilized on PHA bead surfaces by using a surface-associated protein, such as PhaC or PhaP, as an affinity tag to express and purify medically important recombinant proteins, including single-chain antibodies [21, 97, 99−101].

A further cross-linking step between the target protein and PHA bead is not required, as the protein is directly displayed on the surface of the PHA bead. The one-step production and simple extraction procedure for PHA beads displaying foreign proteins is suitable for large-scale industrial applications [120].

Therapeutic protein production has been established using *in vivo*-produced PHA beads utilizing the phasin as an affinity tag to produce recombinant human tissue plasminogen activator. The protein was released from the beads by treatment with thrombin [102].

Efficient purification of immunoglobulin G (IgG) from human serum has been demonstrated using PHA beads coated with an engineered PHA synthase which the N-terminus was fused to the ZZ domain of protein A from *Staphylococcus aureus* [107,108]. The binding capacity was similar to that of commercial protein A sepharose chromatography resins. A subsequent study produced the ZZ-domain-displaying beads in the Generally Regarded As Safe (GRAS) and endotoxin-free bacterium *Lactococcus lactis* in order to target biopharmaceutical-grade related purity criteria for antibody purification resins [109].

In another study, various protein-based binding domains such as camelid antibodies or designed ankyrin repeat proteins (DARPins) isolated from libraries by screening against a target compound were translationally fused with PHA synthase and functionally displayed on respective PHA beads [106]. The PHA beads showed performance as high-affinity purification resins and suggested the versatility of this approach to obtain custom made affinity purification resins.

Recently, PHA beads densely coated with a fusion protein composed of PHA synthase-sortase-target protein were produced as a new tool to produce pure tag-free recombinant protein [103]. After isolation of the PHA beads, the sortase was activated by addition of $CaCl_2$, which cleaved off the target protein. The pure target protein (e.g., the tuberculosis (TB) antigen Rv1626) was obtained as soluble protein in the supernatant while the remaining beads were separated by sedimentation.

Diagnostic Applications of Engineered PHA Beads.

The possibility of engineering labelled (e.g., fluorescent, gold) PHA beads with a surface specifically interacting with various molecular compounds has triggered various experimental approaches to implement PHA beads as diagnostic tools in various biomedical applications [104] (Figure 1.8).

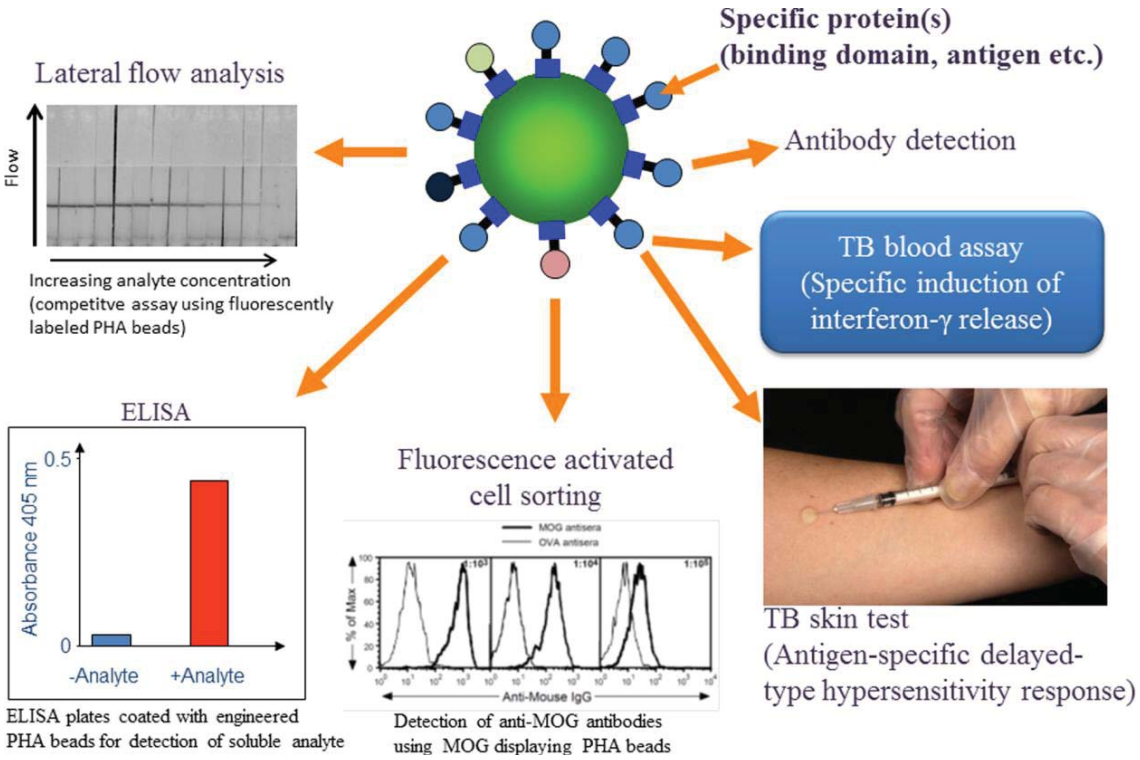

Figure 1.8. Diagnostic applications of engineered PHA beads. MOG denotes myelin oligodendrocyte glycoprotein. The image showing the fluorescence-activated cell sorting application of PHA beads was adapted from reference 104.

PHA beads have been engineered to display a range of proteins that specifically bind target molecules such as specific antibodies in sera or other proteins of interest. In addition, the display of specific antigens enables the production of PHA beads that are useful to stimulate T cell memory-mediated immune responses to diagnose infectious diseases such as TB, as discussed below. Overall this suggested the applicability of engineered PHA beads in various diagnostic applications, for which proof of concept was obtained (Figure 1.8). Display of single-chain antibodies on PHA beads has been demonstrated using the variable fragment (scFv) of an anti-β-galactosidase antibody and PhaC as an anchoring motif [110]. These beads provided a custom-made high-affinity purification matrix for the purification of β-galactosidase and could also be engineered as diagnostic beads to detect specific antigens.

PHA beads have been produced that displayed the cytokine interleukin-2 (IL-2) or the myelin oligodendrocyte glycoprotein (MOG) covalently bound to the PHA-granule-associated protein PhaP [104]. These beads were shown to bind specifically to their respective antibodies and could be used for flowcytometry-based diagnostics to determine antibody responses in vaccinated animals or humans.

Beads have been developed with two independent functionalities, namely, a fluorescently labelled protein and an antigen for antibody detection. These bifunctional PHA beads displayed green fluorescent protein (GFP) and MOG fused to either PhaC or the PhaP [111]. An application of these beads would be in diagnostics, where beads displaying both a fluorescent protein and a specific binding protein could be used to detect a specific molecular target. In addition to beads displaying GFP [101,105], HcRed fluorescent proteins have been developed [97], which could also be used for diagnostic imaging and assays.

Multifunctional PHA beads displaying an IgG binding domain and a special peptide sequence for binding inorganics (gold or silica) have been proposed as useful tools for bioimaging and diagnostics. The two functionalities reacted independently from each other,

and no inhibition or interference could be detected. These beads enabled antibody-based targeted delivery of a contrast agent to selected tissues [112].

The substrate-binding domain of PhaZ has been used to produce PHA microbeads that were used in immunoassays and could be used to study protein-protein interactions and develop assay systems [113].

There is a demand for more specific detection of human and bovine TB, which are caused by *Mycobacterium tuberculosis* and *Mycobacterium bovis*, respectively [127−129], infecting the lungs and other organs. The 2015 global TB report [130] estimated that 3.6 million cases of human TB were missed by health systems, and inaccurate diagnosis was considered to be one of the main factors causing this omission [128,130]. Therefore, a more effective and accurate diagnostic test is important for control of TB infection and transmission worldwide [114,115,131,132]. Tuberculin skin testing (TST) is a primary diagnostic tool for both human and bovine TB and is used in programs for eradication of the disease in cattle throughout the world. However, false positive results can be obtained if humans or animals are exposed to non-pathogenic environmental mycobacteria or vaccinated with BCG [133−135]. Therefore, a new, highly accurate diagnostic reagent containing specific TB antigens is required to overcome the low specificity of TST [135]. Recently, highly specific and sensitive TB skin test reagents based on the display of the specific TB diagnostic antigens CFP10, ESAT6, and Rv3615c from *M. tuberculosis* [136,138] on the surface of PHA beads have been developed [114,115]. Addition of a fourth TB antigen, Rv3020c, further increased the sensitivity for detection of cattle infected by *M. bovis*. A biotechnological production process has been developed for these beads as they offer a more specific and cost-effective diagnostic reagent compared with conventional tuberculin (Figure 1.9). These beads are currently undergoing field trials for use in diagnosis of bovine TB.

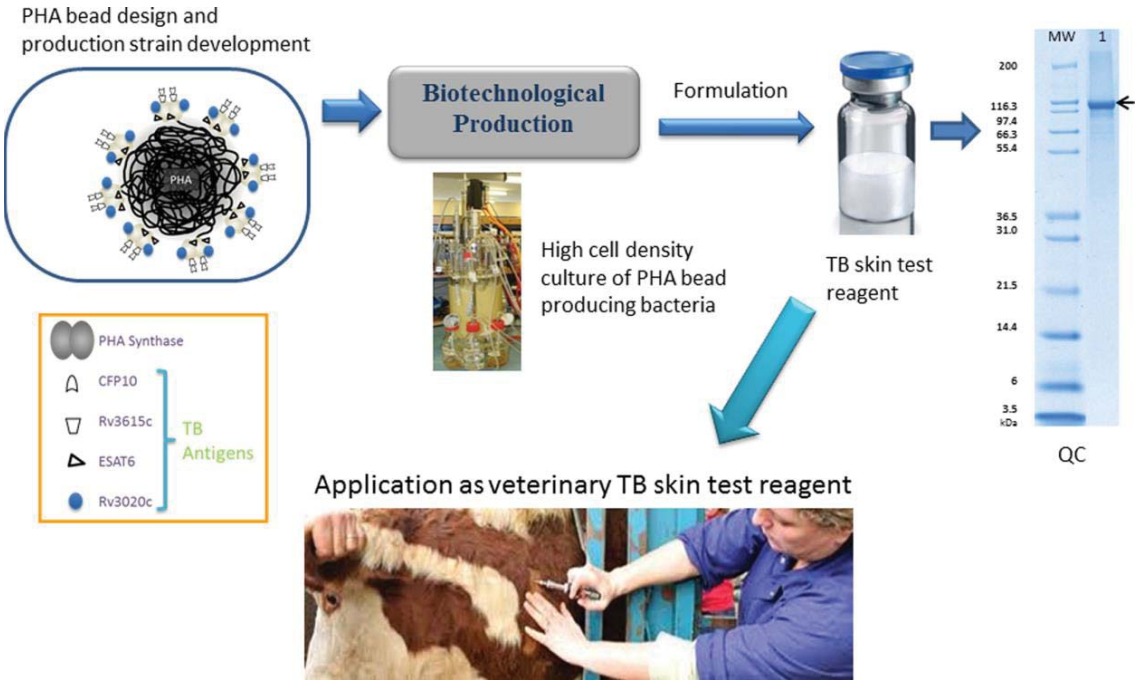

Figure 1.9. Development of a new veterinary TB skin test reagent based on TB-antigen-displaying PHA beads. QC denotes quality control, showing SDS-PAGE analysis of PHA bead associated proteins (lane 1). The arrow indicates full-length fusion protein confirmed by tryptic peptide fingerprinting using MALDI TOF-MS.

Vaccines for Infectious Diseases.

In vivo assembled PHA beads displaying disease-specific antigens obtained by engineering of PHA synthase were conceived as novel strategy for the design and production of particulate subunit vaccines. As these PHA beads are produced at a size of <1 µm while displaying antigens of viruses or pathogenic bacteria, they mimic the pathogen and hence might constitute safe and efficacious vaccines. As there is an unmet demand for vaccines against TB and hepatitis C, PHA bead design was initially aimed to develop vaccines against these two diseases (Figure 1.10).

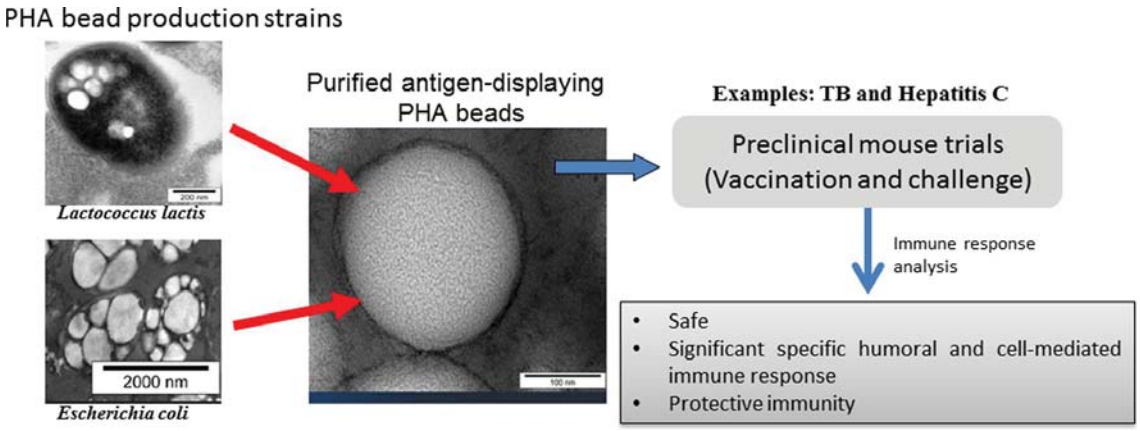

Figure 1.10. PHA beads displaying vaccine candidate antigens are immunogenic and show properties suitable for applications as particulate subunit vaccines. The TEM images of production strains are reprinted with permission from reference 117. Copyright 2012 American Society for Microbiology.

Considerable effort is being made to identify new and better vaccines for human TB, including subunit protein vaccines. The PHA-protein conjugates biocompatibility and biodegradability, a size range that facilitates uptake by antigen-presenting cells, and the simple and low-cost purification and one-step production of the antigen and carrier make them an advantageous system for vaccine delivery [116]. PHA-based subunit vaccines could be developed for both animals and humans, as they can be produced in naturally or engineered endotoxin-free hosts. TB vaccines based on the PHA platform have used PHA beads produced in *E. coli* [116] and in the GRAS organism *L. lactis* [117].

Hepatitis disease is commonly caused by infection with one or more hepatitis virus types (A, B, C, D, or E). Effective vaccines are available for the prevention of hepatitis A and B, and a vaccine against hepatitis E has been developed but is not available yet. However, no effective vaccine against the hepatitis C virus (HCV) has been licensed to date.

Recent HCV vaccine research has included a subunit vaccine approach involving recombinant viral envelope proteins E1 and E2 as antigens combined with MF59C.1 adjuvant (Novartis, phase I (NCT00500747)). PHA beads have been bioengineered for use as an HCV vaccine. *L. lactis* and *E. coli* were engineered to assemble PHB beads coated

with the core protein (Co) from HCV (PHBCo) [117]. Co is one of the most conserved molecules in the HCV virus, and results from other vaccine studies using this antigen had shown stimulation of a broadly protective immune response against the various HCV genotypes [138]. Results using the PHA vaccine antigen delivery systems in mice indicated successful induction of a specific antibody and Th1-type cell mediated immune responses associated with HCV clearance [139], but minimal antibody responses were shown in another study [98].

Recently, Martinez-Donato *et al.* investigated the immunological properties of lipopolysaccharide-free PHBCo by itself and in combination with E1 and E2 [118]. This study further confirmed that PHB beads displaying the Co antigen stimulate a specific and functional T cell immune response and provide control of viremia in mice.

Since induction of both cell immunity and neutralizing antibodies are important for immunity to hepatitis C, the ability of antigen-displaying PHB beads to induce both types of responses holds promise for the development of a PHA-bead based HCV vaccine.

1.11 Conclusions

Rapid progress in understanding the self-assembly and surface structure of PHA beads constituted of PHA-associated proteins has been made. The intrinsic properties of PHA granules are amenable to the development of a wide range of stable functionalized PHA beads for biomedical purposes, such as beads for facilitating production and purification of biologically active proteins. Recent developments in engineering of PHA beads indicate a growing application of specifically designed beads for use as new and improved diagnostics and efficacious vaccines for a wide range of infectious diseases.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): B.H.A.R. is the founding inventor, a shareholder, and Chief Science Officer of PolyBatics Ltd., which currently commercializes the PHA-bead-based TB diagnostic reagent.

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Thesis Scope

Problem statement and approach

TB has killed more people than any other infectious disease in the world. BCG, the only vaccine available against it, fails to prevent adult pulmonary TB, necessitating a new vaccine. The evaluation of vaccine antigens discovered by Reverse vaccinology offers the possibility to explore the protective capabilities of proteins that cannot be identified with traditional techniques. PHA beads used as the vaccine delivery system can enable co-delivery of antigen and immune modulators in the same particle.

Aims of the study

The aims were to 1. Evaluate the immunogenicity of PHA beads displaying Rv antigens and 2. Determine the ability of beads displaying selected Rv antigens and beads co-displaying Rv antigens and immune modulators to protect mice against experimental challenge with TB.

Specific objectives

1. Design and produce PHA beads displaying antigens discovered by Rv approaches.
   This objective involves cloning of selected *rv* genes upstream and downstream of *phaC* genes, production of PHA beads in *Clear coli* and characterization of the beads using SDS-PAGE, western blot, microscopy and size measurement techniques.
2. Evaluation of the immunogenicity of Rv beads in mice.
   This objective includes formulation of PHA vaccines, vaccination of mice and evaluation of their cellular and antibody-mediated immune responses by cytometry bead array (CBA) and Enzyme-Linked ImmunoSorbent Assay (ELISA).
3. Co-display immune modulators on the same bead with the most immunogenic antigen selected from the mouse immunology trial (conducted in objective 2).

This involves selection of immune modulators, cloning the genes of selected immune modulators, and producing and characterising beads displaying the immune modulators.

4. Evaluate immunogenicity induced by the immune modulators-Rv beads

   This objective includes a Rv beads dose response experiment and evaluation immune responses to Rv beads and immune modulators-Rv beads in mice using CBA and ELISA.

5. Evaluate protective immunity induced by the Rv beads, immune modulators-Rv beads, and soluble Rv antigen in a mouse model of TB infection.

   This involves formulation of the vaccines, vaccination of mice and challenge with *M. bovis*, evaluation of immune response using CBA and ELISA and evaluation of protection induced by the vaccines by bacteriological and histological analysis.

Chapter 2: Immunogenicity of antigens from *Mycobacterium tuberculosis* self-assembled as particulate vaccines

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Abstract

Traditional approaches to vaccine development have failed to identify better vaccines to replace or supplement BCG for the control of tuberculosis (TB). Subunit vaccines offer a safer and more reproducible alternative for the prevention of diseases. In this study, the immunogenicity of bacterially derived polyester beads displaying three different Rv antigens of *Mycobacterium tuberculosis* was evaluated. Polyester beads displaying the antigens Rv1626, Rv2032, Rv1789, respectively, were produced in an endotoxin-free *Escherichia coli* strain. Beads were formulated with the adjuvant DDA and subcutaneously administered to C57BL/6 mice. Cytokine responses were evaluated by CBA and antibody responses by ELISA. Specificity of the IgG response was assessed by immunoblotting cell lysates of the vaccine production strains using sera from the vaccinated mice. Mice vaccinated with beads displaying Rv1626 had significantly greater IgG1 responses compared to mice vaccinated with Rv1789 beads and greater IgG2 responses than the group vaccinated with Rv2032 beads ($p < 0.05$). Immunoblotting of anti-sera from these mice indicated the antibody responses were Rv1626 antigen-specific and there was no detectable immune response to the polyester component of the vaccine. Overall, this study suggested that selected TB antigens derived from reverse vaccinology approaches can be displayed on polyester beads to produce antigen-specific immune responses potentially relevant to the prevention of TB.

Abreviations

TB: tuberculosis, DDA: dimethyldioctadecylammonium bromide; CBA: cytometry bead array; BCG: Bacillus Calmette-Guerin; PHB: Poly(3-hydroxybutyrate); PhaA: β–ketothiolase, PhaB: acetoacetyl-CoA reductase; PhaC: PHB synthase; APCs: antigen presenting cells; OD: optical density; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PPDB: bovine purified protein derivative; LPS: lipopolysaccharide; SEM: scanning electron microscopy, HRP: horseradish peroxidase.

2.1 Introduction

It is estimated that in 2014, 9.6 million people developed TB and 1.5 million died from the disease, making it the leading cause of death from a single infectious disease worldwide [1]. BCG, the only vaccine available against TB, is insufficient for global control [2]. Subunit vaccines, administered with selected adjuvant and delivery systems designed to improve immune responses, represent a promising alternative to replace or supplement BCG.

Polyhydroxyalkanoates, a group of various biopolyesters comprised of natural 3-hydroxy fatty acids, are deposited as intracellular inclusions produced in bacteria as an energy and carbon storage material [3, 4]. PHB is the most extensive and well-characterized member of the polyhydroxyalkanoates. PHB can be produced heterologously by introducing the genes that encode for the enzymes PhaA, PhaB, and PhaC [5, 6] and then providing the appropriate conditions for bacterial cultivation [7]. Due to their properties of biodegradability, biocompatibility and ease of production from renewable resources, there is significant interest in applications of biopolyesters as functional biobeads [8]. On the bead surface it is possible to display the desired antigens, fused to PhaC, which is covalently anchored to the PHB core of the beads [9]. This approach has enabled over-production of desired proteins on the carrier bead, while the beads themselves have shown adjuvant properties such as enhancing the cell-mediated immune responses to the antigens [10]. This also allowed co-delivery of antigens and adjuvant as a single particle to APCs of the immune system. In addition, less antigen was required when compared to soluble antigens, which suggested this system promoted efficient antigen delivery to APCs.

Reverse vaccinology is an alternative to the traditional way of identifying vaccine candidates. The process uses the genomic sequence of the pathogen, allowing access (*in silico)* to all the proteins that the pathogen may express *in vivo.* Potential vaccine candidates are identified using bioinformatic tools including the identification of structural motifs and immunological epitopes [11]. In the case of *M. tuberculosis,* 271 antigens were

experimentally confirmed as being immunogenic, but only 35 % of these antigens have been tested in animal models so far [12]. Derrick *et al.* in 2013 tested sixteen of these antigens using challenge of mice with *M. tuberculosis*. Six of these antigens, Rv1626, Rv1735, Rv1789, Rv2032, Rv2220, and Rv3478 significantly reduced the colony forming units in the lungs of experimentally infected animals [13]. Previous studies showed that the display of various antigens on bacterial polyester beads enhanced their immunogenicity and stimulated a Th1 and Th2 immune response [9, 10]. Hence, in this study we investigated whether the display of these novel Rv antigens on polyester beads produced inside engineered *E. coli* will also be immunogenic.

This paper describes the design and construction of plasmids containing rv genes to engineer and produce antigen displaying beads in *Clear coli,* an endotoxin free strain of *E. coli*. Novel polyester beads displaying the antigens Rv1626, Rv2032 or Rv1789 were produced and their immunogenicity evaluated in C57BL/6 mice.

2.2 Materials and Methods

Generation of plasmids for production of beads displaying Rv antigens

Plasmids and oligonucleotides used in this study are described in Table 2.1. Genes encoding Rv antigens were synthesized by GenScript (USA). General molecular cloning procedures [14] were used to generate new plasmids and their sequences were confirmed by DNA sequencing.

Table 2.1 Strains, plasmids and oligonucleotides used

Strain, plasmid or oligonucleotide	Genotype or description	Source or reference
<u>*E. coli*</u> strains		
XL1-Blue	*endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)*	Stratagene
Clear coli BL21 (DE3)	F– *ompT hsdSB (rB- mB-) gal dcm lon* λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*]) *msbA148*	Lucigen

	Δ*gutQ*Δ*kdsD* Δ*lpxL* Δ*lpxM*Δ*pagP* Δ*lpxP* Δ*eptA*	
Plasmids		
pUC57-rv1626-rv3478-rv2220	Contains hybrid rv genes used for subcloning steps	GenScript
pUC57-rv1789-rv2032-rv1785	Contains hybrid rv genes used for subcloning steps	
pPOLYC-phaC	Amp^r. pET-14b derivative containing *phaC* from *Ralstonia eutropha* and *StuI, XhoI, XmaI* and *BamHI* sites downstream of *phaC*.	[15]
pMCS69	Cm^r. pBBR1MCS derivative containing genes *phaA* and *phaB* from *R. eutropha*	[16]
pPOLYC-phaC-rv1626	pPOLYC-phaC derivative containing *rv1626* inserted into *XhoI/BamHI* sites.	This study
pPOLYC-phaC-rv2032	pPOLYC-phaC derivative containing *rv2032* inserted into *XhoI/BamHI* sites.	This study
pPOLYC-phaC-rv1789	pPOLYC-phaC derivative containing *rv1789* inserted into *XhoI/BamHI* sites.	This study
pPOLYC-rv1626 - rv3478-phaC-rv2220	pPOLYC-phaC derivative containing *rv1626- rv3478* hybrid gene inserted into *XbaI/SpeI* sites and *-rv2220*	This study
pPOLYC-phaC-rv2220	inserted into *XhoI/BamHI* sites. pPOLYC-phaC derivative containing *rv2220* inserted into *XhoI/BamHI* sites.	This study
pPOLYC-phaC-rv1735	pPOLYC-phaC derivative containing *rv1735* inserted into *XhoI/BamHI* sites.	This study
pPOLYC-6His-rv1626	pPOLYC derivative containing *rv1626* and a 6xHis tag upstream.	This study
Oligonucleotides		
1626-XhoI	5'-GCTCTCGAGATGTACACAGGACCTACGACG-3'	This study
1626-BamHI	5'-CCGGATCCTGTATCTTTTGGTGTACCAAGCGTT-3'	This study
2220-XhoI	5'-GCTCTCGAGATGACGGAAAAAACGCCAGAT-3'	This study
2220-BamHI	5'-GCTGCTGGATCCCACATCATAATAAAGAGCAAATTC-3'	This study
3478-BamHI	5'-GCTGGATCCTCCAGCAGCAGGCGTTCTAGG -3'	This study
1789-XhoI	5'-GCTCTCGAGGATTTTGGTGCTCTTCCT -3'	This study
2032-BamHI	5'-GCTGGATCCTCCAGCAGCAGGCGTTCTAGG-3'	This study
1789-BamHI	5'-GCTGGATCCTCCAGCAAAAGGAGGTCGAGC-3'	This study
2032-XhoI	5'-GCTCTCGAGCCAGATACGATGGTG-3'	This study
2032-BamHI	5'-GCTGGATCCTCGATGATCTTTAGCTCTAAC-3'	This study
1735-XhoI	5'-GCTCTCGAGGGTGCTACGGCAATTACA-3'	This study
1735-BamHI	5'-GCTGGATCCCATTGGTTGGTTACGACGAAA-3'	This study
His1626-SpeI	5'-GCTACTAGTATGCATCATCACCATCACCACTACACAGGACCTACGACGGAT-3'	This study
1626-Xho stop	5'-GCTCTCGAGTTATGTATCTTTTGGTGTACCAAG-3'	This study

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 2.1. *E. coli* XL-1Blue was used for general cloning steps and was grown in Luria-Bertani (Acumedia, USA) broth, supplemented with ampicillin (100 µg/mL) at 37 °C. *Clear coli* was used for beads production and media were supplemented with *D*-glucose 0.2 % (w/v) (Merck, Germany) and NaCl (5 g/L) (Ajax Finechem, Australia). Cultures were grown in flasks in a shaking incubator at 37 °C at 200 rpm and the OD was monitored. Cultures were induced by addition of IPTG (1 mM) (Goldbio, USA) when an OD of 0.5 was reached and the incubation was continued at 25 °C and 200 rpm for 48 hours.

Bead isolation

Cells were harvested and resuspended in lysis buffer (50 mM Tris, pH 11, 10 mM EDTA, 0.08 % (w/v) SDS) [17] prior to disruption by using a microfluidizer (Microfluidics M-110P, USA). Beads were recovered after centrifugation of the whole cell lysate at 6000 x *g* during 30 minutes at 4 °C. Beads were washed twice with lysis buffer, treated with 70 % (v/v) ethanol to kill residual bacteria and stored in PBS buffer (10 mM Na_2HPO_4, 137 mM NaCl).

Bead characterization

Proteins attached to the beads were separated by using 8 % SDS-PAGE and stained with Coomassie Blue. The amount of fusion protein was calculated with Image Lab software version 3.0 using a standard curve of increasing BSA concentrations. Using BSA as the standard, the software estimates the amount of proteins run per lane on the gel and assigns a quantity to each protein band. This is used to determine the ratio: ng of fusion protein/ mg of beads. [15]. A Western Blot anti-PhaC protein was performed using a rabbit anti-PhaC antibody (Genscript, USA) and a goat anti-rabit IgG HRP conjugate (Abcam,UK). Beads were visualised by scanning electron microscopy (SEM).

Vaccine preparation and mice immunization

DDA was added to PBS at a concentration of 1.25 mg/ml and heated at 80 ^{o}C for 10 min with constant stirring until the formation of micelles. The emulsion was cooled to room temperature and added to the beads. Each vaccine dose contained 250 mg DDA. The particle size of the vaccine formulations was measured using a Mastersizer (Malvern Mastersizer 3000, UK). Wild-type (Wt) beads (5 µg of PhaC), Rv1626 beads (5 µg of Rv1626), Rv2032 beads (5 µg of Rv2032) or Rv1789 beads (1.5 µg Rv1789) were used for vaccination, respectively.

Female C57BL/6 mice were obtained from the animal breeding facility of AgResearch, Ruakura, Hamilton, New Zealand. Two hundred µL of the vaccine preparations were injected subcutaneously three times at 2 weeks intervals (6 mice per group). BCG was administered only once at a dose of 10^6 CFU. One group was administered DDA only as a negative control. Three mice per box (2 boxes per treatment) were allocated randomly in mouse boxes in a PC2 facility throughout the experiment and weighed before each vaccination and euthanasia. Animals were monitored daily and carefully observed after vaccinations. All animal experiments were approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand).

Preparation of mice samples and immunological assays

Three weeks after the last immunization, mice were sedated, bled, euthanized and a single cell suspension of splenocytes obtained as described by Parlane *et al.* in 2009. Mice splenocytes were stimulated with PPDB (5 µg/mL) (Thermofisher,USA) and cultured for 3 days in 10 % CO_2. Culture supernatant was removed and frozen at -20 ^{o}C until assayed. Cytokine release was measured using a BD CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, USA) adapted to Falcon V bottom polypropylene plates. Samples were acquired with a BD FACS Verse flow cytometer (BD Biosciences, USA) and data analysed with FCAP array software (BD Biosciences, USA).

IgG responses in sera were analysed by ELISA. Microlon high-binding plates (Greiner Bio-One, Germany) were incubated overnight at 4 °C with 5 µg of antigen (on beads) or 10 µg/mL of PPDB as a positive control in 0.05 M carbonate/bicarbonate buffer (pH 9.6). Plates were washed four times before blocking with 1% BSA in PBS (pH 7.4) for 1 hour at room temperature. After three washes, sera from individual mice diluted six times from 1000 to 32000-fold, were added and incubated for 1 hour. Four washes were conducted and goat anti-mouse IgG1 (diluted 15000-fold) or IgG2c (diluted 20000-fold). HRP-conjugated antibodies (ICLLab, USA) were used for detection of IgG1 and IgG2c antibodies, respectively. Six final washes were conducted and after addition of HRP enzyme substrate, the reactions were stopped with 0.5 M H_2SO_4. All washes were performed with PBS-Tween buffer (0.05% Tween 20) and carbonate/bicarbonate buffer was used as blank. In all steps, 50 µL of each solution per well was added, except in the blocking step, were 100 µL was used. Absorbance was measured at 450 nm in a VERSAmax tunable microplate reader (Molecular devices, USA). EC50 values were calculated adjusting the absorbance values to a four parameters logistic curve. EC50 represents the midpoint of the sigmoid (c coefficient of the curve).

To investigate the specificity of the IgG response induced by the vaccinations, pooled mice sera were diluted 1500-fold and used for immunoblotting against all bead proteins, total host cell proteins or purified soluble proteins. An anti-mouse IgG HRP-conjugate diluted 1000-fold in 1% BSA in PBS (pH 7.4) (Abcam, United Kingdom) was used for detection of bound IgG antibodies.

Statistical analysis

Dunn's test of multiple comparisons following a significant Kruskal-Wallis test was performed to analyze significant differences between the groups mean values from the CBA and ELISA assays using the program Minitab17. Statistical significance was considered at $p<0.05$.

2.3 Results

Construction of plasmids containing mycobacterial rv genes and their ability to mediate polyester bead production in *Clear coli.*

Different combinations of the rv genes fused to 5' and/or 3' ends of the *phaC* gene were generated and the resultant plasmids were transformed into the bead production strain *Clear coli*. Plasmids pPOLYC-rv1626-rv3478-phaC-rv2220, pPOLYC-phaC-rv2220, pPOLYC-phaC-rv1735, pPOLYC-phaC-rv1789, pPOLYC-phaC-rv1626 and pPOLYC-phaC-rv2032 each mediated PHA bead production. However, only plasmids pPOLYC-phaC-rv1626, pPOLYC-phaC-rv2032, pPOLYC-phaC-rv1789 and pPOLYC-phaC (Wt beads) mediated production of beads displaying the fusion protein as the dominant protein. The dominant bands observed corresponded to the expected molecular weights of the respective PhaC-fusion proteins. In addition, minor protein bands were observed and these were likely to be bacterial host cell proteins that co-purified with the beads and were not removed in the cleaning process (Figure 2.1A). An immunoblot using anti-PhaC antibodies (Figure 2.1B) further confirmed the stability and presence of the respective full-length fusion proteins. As Wt beads did not have antigen protein fused to PhaC, they were used as a positive control for the production of beads and as a negative control for immunizing mice.

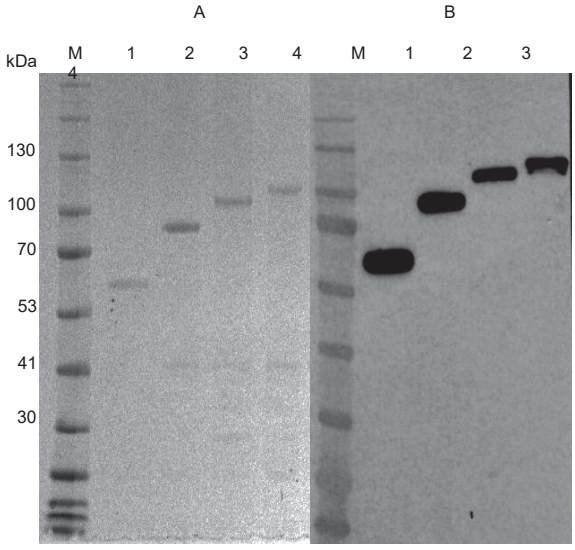

Figure 2.1. A, SDS-PAGE and B, Western Blot analysis of proteins attached to various polyester beads isolated from *Clear coli*. Lane 1, wild-type beads (PhaC: 64 kDa); lane 2, beads displaying Rv1626 (PhaC-Rv1626: 88.7 kDa); lane 3, beads displaying Rv2032 (PhaC-Rv2032: 102.4 kDa); lane 4, beads displaying Rv1789 (PhaC-Rv1789: 104.4 kDa).

Bead properties

The amount of fusion protein displayed on the various beads was calculated by densitometry using a BSA standard curve. This was used to deduce the amount of fusion protein and Rv antigen per bead mass for each type of bead (Table 2.2). SEM images (Figure 2.2) showed that, except for Rv2032 beads which appeared smaller than the other beads, bead size distribution (~ 500-750 nm) and surface morphology was similar for all other beads. Measurements of the particle size distribution of the formulations used for injection (i.e. after formulation with adjuvant) (Table 2.3) demonstrated that the beads aggregated in solution as the particle size increased after addition of adjuvant.

Table 2.2 Concentration of proteins in beads

Protein	Amount of PhaC-Rv antigen fusion /wet beads (ng protein/mg beads)	Amount of Rv antigen/ wet beads (ng protein/mg beads)	Molecular weight (kDa)
PhaC	695	-	64
PhaC-Rv1626	1210	393	88.7
PhaC-Rv2032	1183	409	102.4
PhaC-Rv1789	367	131	104.4

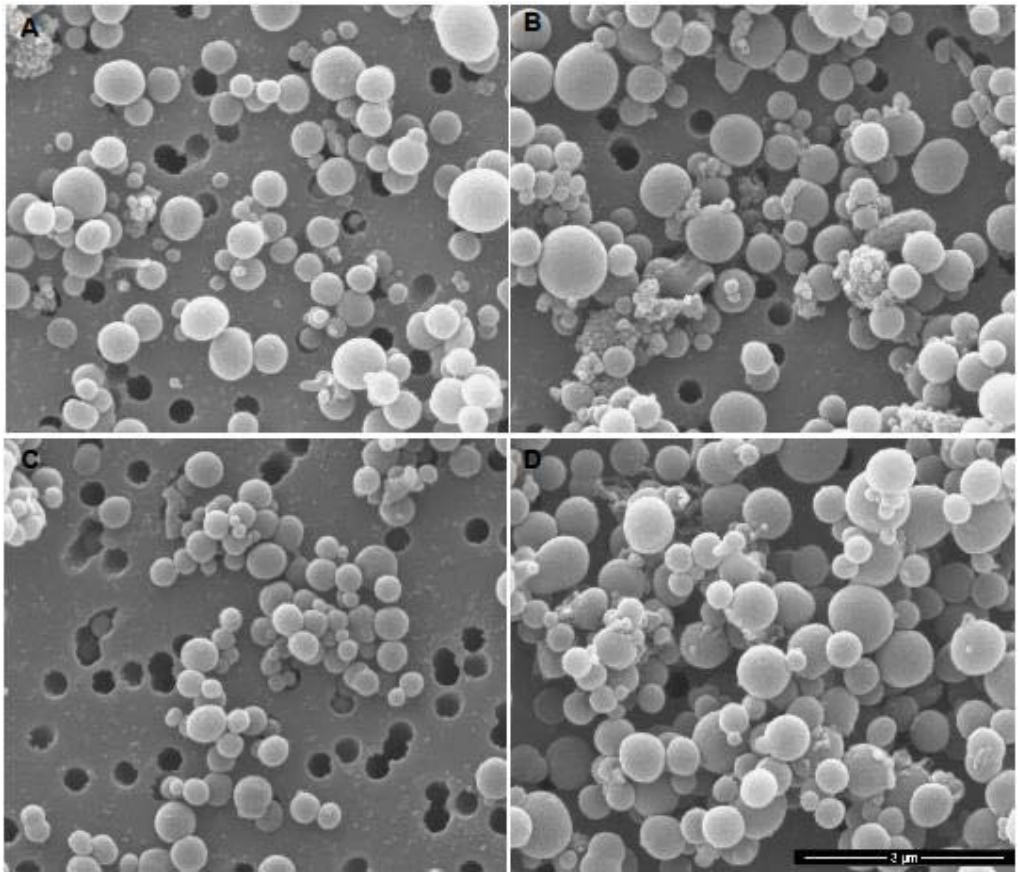

Figure 2.2. SEM of various PHA beads. A, Wt beads, B, Rv1626 beads, C, Rv2032 beads and D, Rv1789 beads.

Table 2.3 Size distribution of beads in vaccine formulations (µm) as measured by laser scattering. Dx represents the particle diameter corresponding to X% cumulative particle size distribution.

	D_{10}	D_{50}	D_{90}
Wt beads	3.42	8.74	65.9
Rv1626 beads	3.82	9.52	33.7
Rv2032 beads	4.18	13.7	89.0
Rv1789 beads	3.78	27.8	186.0

Vaccine formulation and mice immunization

Derrick *et al.* in 2013 used 5 µg of soluble recombinant protein for vaccination. The ratio of Rv antigen to bead was sufficiently high for mice to be immunized with a dose of beads containing 5 µg of Rv antigen in the case of beads displaying Rv1626 and Rv2032. However, due to the lower level of expression of Rv1789 antigen on beads, mice could only be immunized with 1.5 µg Rv1789. Control mice each received a dose of Wt beads containing 5 µg of PhaC.

No adverse effects due to immunisation were observed and animal weights gradually increased during the time course of the trial with no significant differences between the groups. Small lumps (~ 2 mm) were observed in mice vaccinated with beads but not with BCG or DDA alone, suggesting these lumps were due to an immune response to the beads.

Cytokine responses

All splenocytes were stimulated with PPDB and following incubation, IFN-γ, IL-10, IL-17A, Il-2, IL-4, IL-6 and TNF-α levels were measured in the culture supernantants (Figure 2.3, Appendix 1A). There was no detectable IL-4 released. TNF-α responses were significantly higher in all groups immunized with beads compared to the group given DDA alone. IFN-γ production was significantly higher for the BCG-vaccinated group than for groups immunized with Wt beads, Rv2032 beads and DDA.

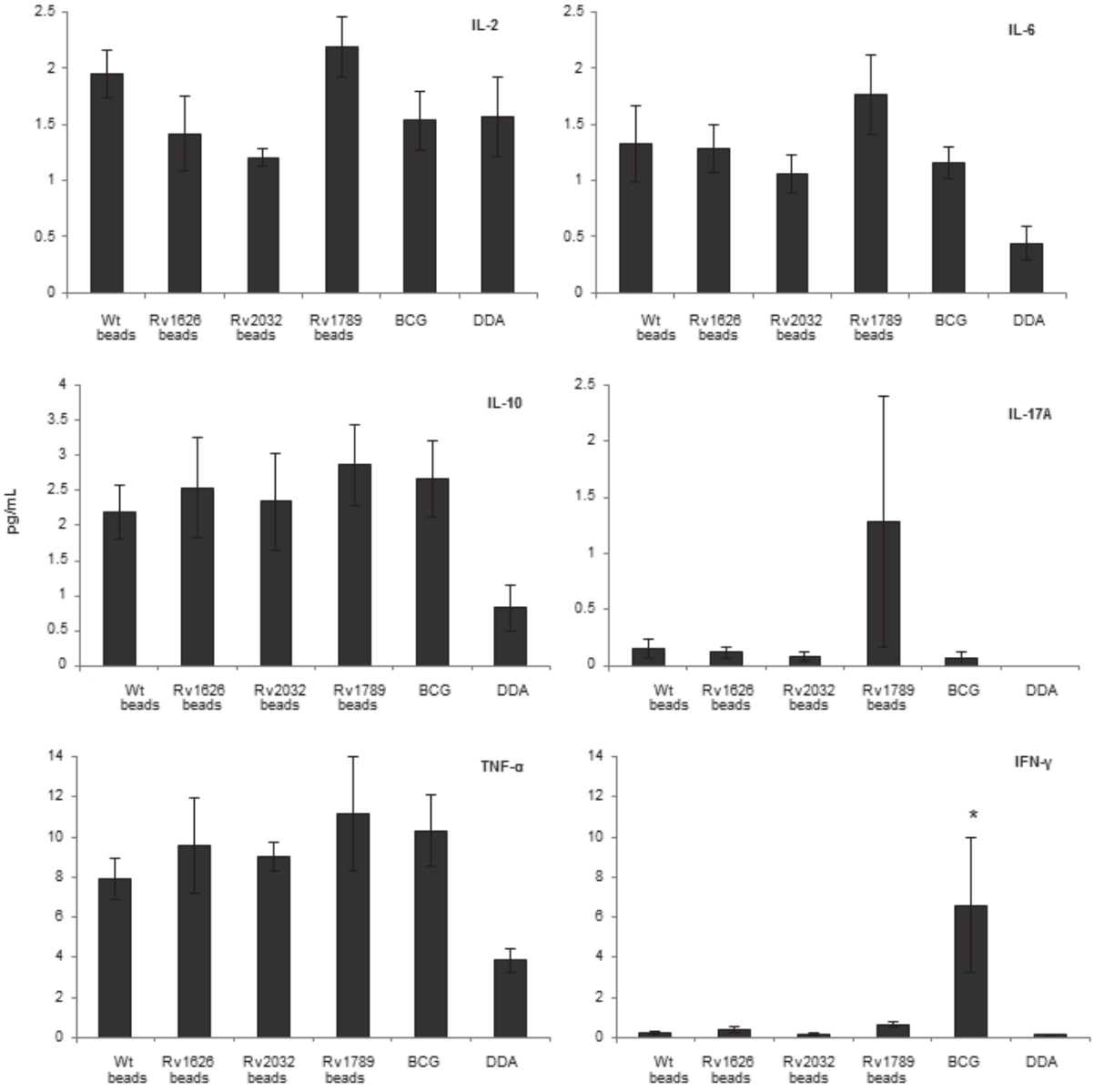

Figure 2.3. Cytokine responses of mice splenocytes upon stimulation with PPDB and analysed by cytometric bead array. Each bar represents the mean ± the standard error of the mean. Wt beads, beads negative control (beads without antigen); Rv number bead: beads displaying reverse vaccinology antigens; BCG (Bacillus Calmette–Guérin); DDA adjuvant used for vaccine formulation and as a negative control. *, significantly higher than wild type beads ($p < 0.05$).

Antibody responses

IgG1 and IgG2c responses were evaluated by ELISA (Figure 2.4, Appendix 1B) using microtiter plates coated either with beads or PPDB and using sera from immunized mice. PPDB contains a wide range of immunodominant mycobacterial proteins [18], and was used to determine antibody response in BCG-vaccinated mice as well as the mice vaccinated with beads. The results showed strong IgG1 and IgG2 responses to a Rv1626 bead preparation in mice vaccinated with the Rv1626 beads. The IgG1 responses were significantly higher ($p<0.05$) than IgG1 responses produced by mice vaccinated with Rv1789 beads and IgG2c responses were higher than those elicited by mice vaccinated with Rv2032 beads ($p<0.05$). The response to wild-type beads was similar in all groups and there were no differences in responses to the various antigen beads in this group. Minimal antibody responses to PPDB were measured in groups vaccinated with Wt, Rv1789 beads, BCG and DDA. Although not significantly different, the responses to PPDB by mice vaccinated with Rv1626 and Rv2032 were higher than responses induced by Wt beads.

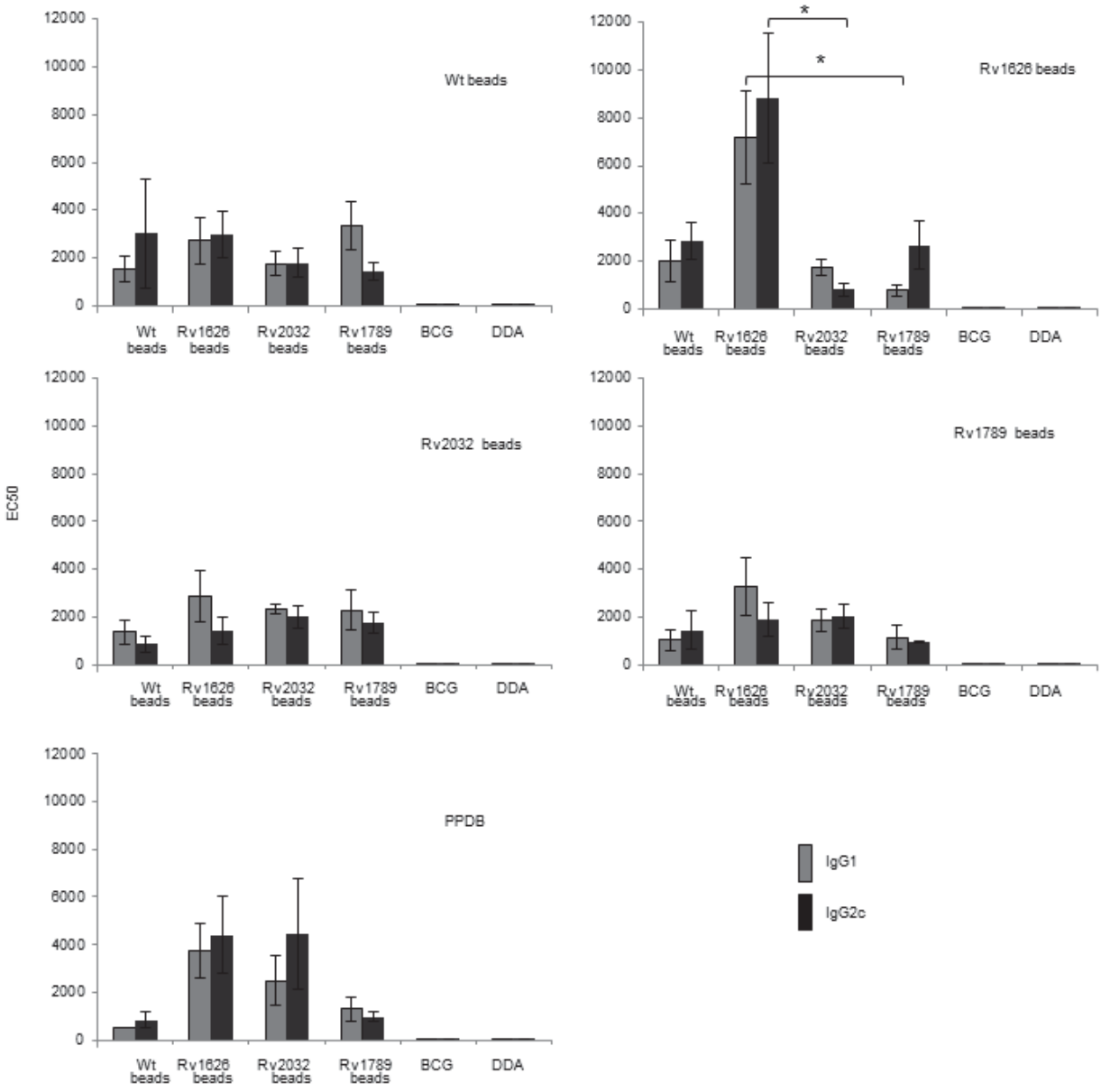

Figure 2.4. IgG1 and IgG2c titers expressed in EC50 values in response to various beads and PPDB analysed by ELISA for each immunized group. Each bar represents the mean ± the standard error of the mean. Wt beads, beads negative control (beads without antigen); Rv number bead: beads displaying reverse vaccinology antigens; BCG; DDA adjuvant used for vaccine formulation and as a negative control. *, significantly greater than other group vaccinated with beads ($p < 0.05$).

Specificity of IgG response

In order to confirm which proteins on the beads generated the immune response, an immunoblot was performed. Each of the beads used for immunising mice was tested for reactivity against sera produced from mice immunised with the various vaccines. No proteins were recognized by sera from mice immunised with wild-type beads, Rv2032 or Rv1789 beads. However, sera from mice vaccinated with Rv1626 beads specifically recognized a protein band corresponding to the expected molecular weight of the fusion protein PhaC-Rv1626 (Figure 2.5). Further immunoblot analysis with sera from mice immunized with Rv1626 beads showed a very specific IgG response as only Rv1626 was recognized in whole cell lysates of *Clear coli* bacteria producing Rv1626 beads and no antigen was detected in the cell lysate of *Clear coli* harbouring only the vector as the negative control (Figure 2.6). In these immunoblots, purified soluble Rv1626 served as a positive control.

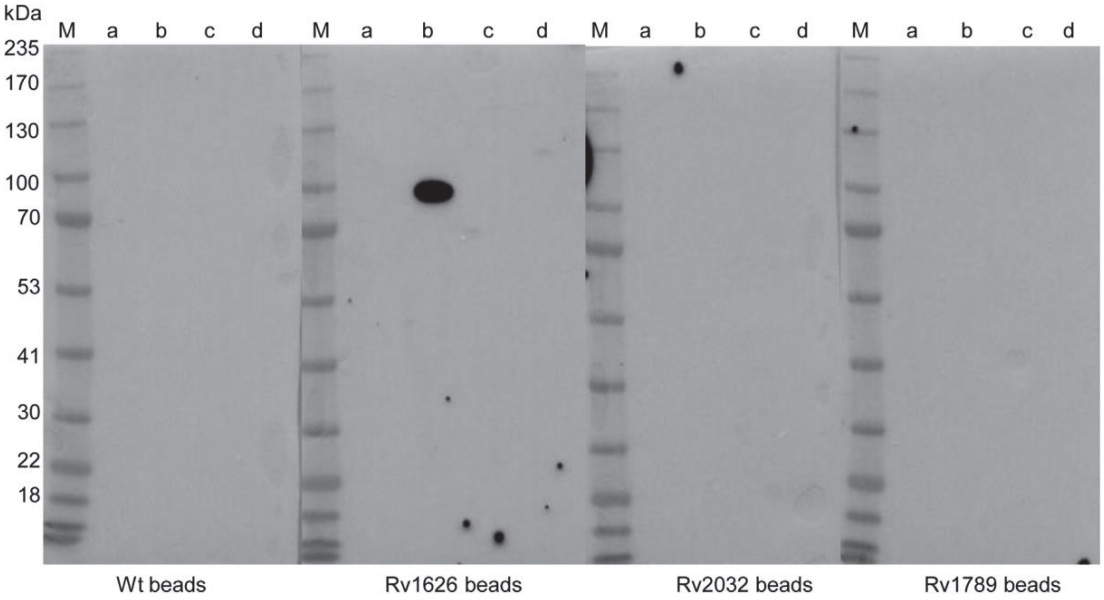

Figure 2.5. Immunoblot of beads associated proteins using pooled sera from mice immunized with the various beads. Beads used for immunization were indicated below the blot. Lanes a-d represent the protein profiles of the various beads as follows: Lane M, molecular weight standard, lane a, Wt beads (negative control without antigen); lane b, beads displaying Rv1626; lane c, beads displaying Rv2032 and lane d, beads displaying Rv1789.

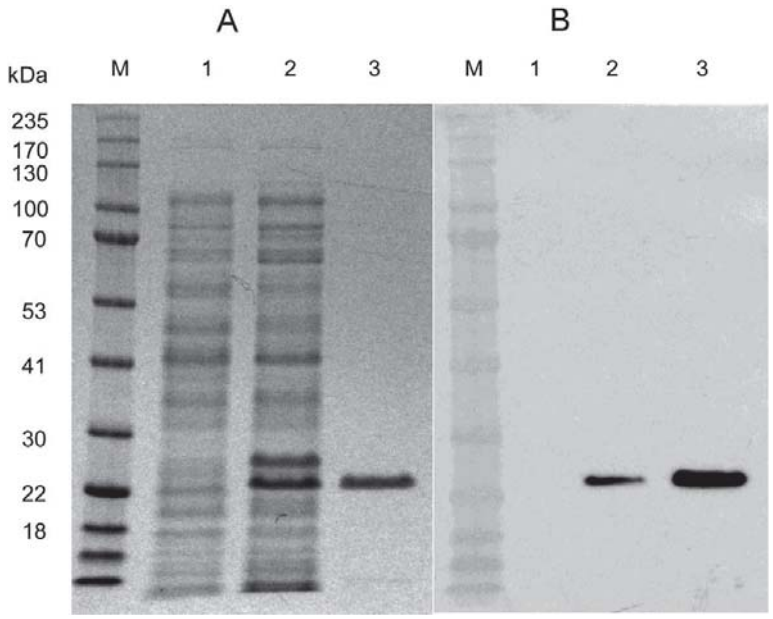

Figure 2.6. Specific recognition of the Rv1626 antigen by sera of mice immunized with beads displaying Rv1626. A, SDS-PAGE; B, immunoblot. Lane 1, negative control (lysate of *Clear coli* cells containing plasmid pPOLYC); lane 2, lysate of *Clear coli* cells producing recombinant Rv1626; Lane 3, purified recombinant Rv1626. Pooled sera from mice immunized with beads displaying Rv1626 were used for the immunoblot.

2.4 Discussion

Reverse vaccinology approaches which combine genomics/proteomics with vaccinology has enabled the successful development of a broadly protective protein-based vaccine against the serogroup B *Neisseria menigitidis* [19]. Potential vaccine candidates from *M. tuberculosis* have been identified by reverse vaccinology. When tested as soluble proteins, these antigens have been shown to stimulate protective immunity [13]. In the current study, beads were engineered to display three of these mycobacterial antigens, Rv1626, Rv1789 and Rv2032.

The various Rv antigens were translationally fused to different termini of PhaC in order to determine which fusion protein orientation was permissive i.e. produced a fusion protein which can be stably overproduced while mediating efficient bead assembly in *E. coli*. Although in some cases beads were produced, the fusion protein was not detectable by

SDS-PAGE analysis of the beads. Hence, a different fusion strategy was required to increase the level of antigen produced on the surface of the beads. For these preliminary investigations, it was determined that the most efficient design for each of the three Rv antigens was a C-terminal fusion with PhaC.

Another important consideration for bead vaccines is the amount of antigen protein that can be administered practically, due to the viscosity of bead suspensions. Hence, a high ratio of antigen-PhaC fusion to bead is preferred, as this provides the option of immunising animals with higher doses of antigen and lower bead content. In this study, it was possible to produce beads overproducing sufficient levels of Rv1626 and Rv2032 to administer the same dose (5 µg) as tested previously for the Rv1626 and Rv2032 proteins alone [13]. However, in the case of Rv1789, production of antigen fusion was only sufficient to allow administration of the equivalent of 1.5 µg of Rv1789. It is possible that if higher levels of antigen expression could be achieved for these beads, immunisation of mice with an increased antigen dose may have generated stronger immune responses.

Clear coli, is a novel strain derived from the commonly used BL21(DE3) strain. This host contains a genetically modified LPS which does not trigger an endotoxic response in human cells [20]. LPS is considered a major pathogenic element in Gram-negative septicaemia in humans [21] and it is usually removed from biotechnological preparations [22]. *Clear coli* is an expression system that allows production of virtually endotoxin-free recombinant proteins [20].

All the beads used for immunization showed particle sizes of <1 µm. However, after formulation, the various beads formed aggregates of different sizes, which might be due to the interaction of the different proteins on the surface of the beads with each other and with the adjuvant. The variation between beads in the extent of aggregation may account for the different immune responses they elicited. When comparing polystyrene beads conjugated with ovalbumin of 20, 40, 100, 500, 1000 and 2,000 nm, beads of 40 nm induced the

strongest IFN-γ and IgG responses [23]. However, when comparing poly-lactic acid particles of 600 to 26,000 nm, only the 4,000 nm particles were able to induce antibody production [24]. In a more recent study using the same particles, a particle size of 2,000-8,000 nm induced a greater IgG response than particles of <20,000, 10,000–70,000 and 50,000–150,000 nm [25]. Hence, it might be considered that Wt beads and Rv1626 beads exhibited a more suitable size range to induce stronger immune responses than the larger Rv2032 and Rv1789 beads. However, what happens after injection and how the morphology or size of the vaccines changes *in vivo* is unknown.

To assess the mode of immune response induced by the various polyester beads, splenocytes of vaccinate mice were stimulated with PPDB and their cytokine profiles were analysed. While BCG mediated a Th1-type immune response as indicated by IFN-γ and TNF-α production, both Wt and antigen displaying beads induced a strong TNF-α response but did not elicit induction of other cytokines. Hence, the cytokine profile analysis suggests the beads did not induce a strong Th1 and Th17-type immune response as was previously observed for beads displaying the TB antigens ESAT6 and Ag85A and beads displaying the Core antigen of HCV [9, 10, 26]. While tuberculin is known to contain Rv1626 [18], stronger cytokine responses may have been observed by using the specific antigens rather than PPDB.

The production of IFN-γ post-vaccination has been linked with the efficacy of the vaccine in humans [27]. Although IFN-γ, IL-2 and TNF-α produced by $CD4^+$ cells have been used to predict the capability of TB vaccines to induce protective immunity, recent vaccine candidates that generated considerable levels of multifunctional $CD4^+$ producing these cytokines were unsuccessful to confer enhanced protection [28].

The immunological properties of other *M. tuberculosis* Rv antigens such as e.g. Rv0577 [29], and Rv3425 [30] had been previously shown to induce a Th-1 like immune response. Rv0978c and Rv0754 induced maturation and activation of dendritic cells [31] and synthetic

peptides from Rv1733c improved the control of already-established infection after *M. tuberculosis* challenge [32].

In contrast to the minimal IFN-γ responses induced by the beads, immunisation of mice with the various beads, including the Wt beads induced antibody responses. The Rv1626 beads induced an antigen-specific IgG2c response significantly greater than responses in the groups vaccinated with Rv2032 beads, BCG or DDA alone and a greater IgG1 response than mice vaccinated with RV1789 beads, BCG or DDA alone. IgG levels were low for mice vaccinated with BCG, and were similar to levels produced in mice vaccinated with DDA adjuvant alone. The subclass of IgG induced after immunization is associated with the type of immune response. IgG1 production is induced by Th2-type cytokines and IgG2 by Th1-type cytokines [33]. The IgG response to Rv1626 beads was mixed, as indicated by induction of both Th1 and Th2 responses.

Vaccination with soluble Rv1626 and Rv2032 reduced the bacterial burden when compared with non vaccinated controls from 0.3–0.5 $\log_{10}$ in a challenge with *M. tuberculosis* and Rv1789 reduced it by more than 0.5 $\log_{10}$ [13]. Rv1789 is a member of the PPE protein family, which play an important role in *M. tuberculosis* virulence [34]. However, in this study, Rv1626 beads showed the strongest immunogenicity, which could infer that Rv1626 could be more protective antigen when this antigen is displayed on the surface of beads. It should be noted that the stronger immunogenicity could also be due to the more suitable size range of the respective Rv1626 beads for induction of an immune response as aforementioned.

The mice produced IgG responses to wild-type bead constituents and the sera from these showed a degree of antibody reactivity to the various antigen-displaying beads. This may be due to common impurities derived from the production host. These components present in beads may also have triggered the TNF-α responses observed in all the immunised groups of mice. Further assessment of the specificity of sera obtained from the immunised groups by immunoblotting against the various bead proteins clearly indicated that antibodies induced by the Rv1626 beads were specific for Rv1626. Since the other PhaC fusion

proteins were not detected, this could indicate that PhaC is not particularly immunogenic. Immunoblotting using sera against the Rv1626 beads against whole cell lysates of the production host, *Clear coli* in the absence or presence of soluble Rv1626 confirmed a specific response to Rv1626. Hence the bacterial host cell derived impurities did not induce measurable immune responses.

The Th1/Th2 paradigm assumes that *M. tuberculosis*, as an intracellular pathogen, induces cellular immune responses [35], and the classic vaccinology approach assumes that the natural immune response to infection is sufficient for protection against a future infection. Nevertheless, for all licensed vaccines with the exception of BCG and herpes zoster, antibodies mediate protection [36]. *M. tuberculosis* induces a humoral immune response to a wide variety of antigens despite being an intracellular pathogen [37] and numerous studies have shown that antibodies modify the course of experimental mycobacterial infection in mice to the benefit of the host [38]. In the context of TB infection, instead of mimicking natural immunity, vaccine induced protection might require “uncommon” or “unnatural” immunity [39]. Utilisation of the bead antigen display strategy could facilitate screening and identification of vaccine candidates and include components that trigger an immune response different to the response naturally induced by the pathogen. These alternative responses may be important for protective immunity.

In conclusion, the mycobacterial antigens, Rv1626, Rv1789 and Rv2032 identified previously from a reverse vaccinology approach were successfully displayed on polyester beads. Immunisation of mice with these beads confirmed Rv1626 as an immunogenic antigen mediating an antigen-specific antibody response. Polyester beads are efficient antigen delivery systems mediating specific immune responses to the delivered antigen. The potential of the Rv1626 antigen to induce protective immunity to tuberculosis will be investigated in future studies.

Author contributions

Performed the majority of the experiments: PRR, Designed the research study: PRR, NAP, DNW, BHAR. Contributed reagents/materials/analysis tools and assisted in post-mortemanalysis: NAP. Drafted the manuscript: PRR, BHAR. All authors read and approved the final manuscript.

Conflict of interest statement

BHAR is shareholder and CSO of PolyBatics Ltd that commercialises the polyester bead technology.

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Link to next Chapter

In chapter 2 it was demonstrated that Rv1626 antigen displayed on PHA beads mediated an antigen-specific antibody response. Chapter 3 describes the co-display of Rv1626 and various immune modulators on beads. Immune modulators-Rv1626 beads, Rv1626 beads and soluble Rv1626 antigen were used to vaccinate mice followed by challenge with *M. bovis*. For the study, soluble Rv1626 was obtained using a novel tagless protein purification technique (see Appendix 4 for description of the method). Immunogenicity and protection induced by vaccination with these beads was assessed.

Chapter 3: Immunological properties and protective efficacy of a single particulate mycobacterial antigen displayed on polyhydroxybutyrate beads

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Abstract

In 2015, there were an estimated 10.4 million new tuberculosis (TB) cases and 1.4 million deaths worldwide. Bacille Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, is the vaccine available against TB but it is insufficient for global TB control. This study evaluated the immunogenicity of the *Mycobacterium tuberculosis* antigen Rv1626 in mice while assessing the effect of co-delivering either Cpe30 (immunostimulatory peptide), $CS.T3_{378-395}$ (promiscuous T helper epitope) or Flagellin (TLR5 agonist) or a combination of all three immunostimulatory agents. Rv1626 and the respective immunostimulatory proteins/peptides were co-displayed on biopolyester beads assembled inside an engineered endotoxin-free mutant of *Escherichia coli*. Mice vaccinated with these beads produced immune responses biased towards Th1/Th17 type responses but inclusion of Cpe30, $CS.T3_{378-395}$ and Flagellin did not enhance immunogenicity of the Rv1626 protein. This was confirmed in a *M. bovis* challenge experiment in mice, where Rv1626 beads reduced bacterial cell counts in the lungs by 0.48 $\log_{10}$ compared with the adjuvant alone control group. However co-delivery of immunostimulatory peptides did not further enhance protective immunity.

Abbreviations:

PHAs: Polyhydroxyalkanoates, PHB: polyhydroxybutyrate, TB, tuberculosis, BCG: Bacille Calmette-Guérin, PAMPs: pathogen-associated molecular patterns, TLR: toll like receptor, ARP4: actin related protein 4, MALDI-TOF/MS: matrix-assisted laser desorption ionization-time-of-flight mass spectrometry, PC: physical containment, DDA: dimethyl dioctadecyl ammonium, ELISA: Enzyme-Linked ImmunoSorbent Assay, Wt: wild type, H&E: hematoxylin and eosin

3.1 Introduction

Polyhydroxyalkanoates (PHAs) are biopolyesters naturally produced as intracellular inclusions by many bacteria and archaea. The synthesis of the most common one, polyhydroxybutyrate (PHB), a short chain length PHA, involves three key enzymes, β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and polyester synthase (PhaC). The knowledge about topology, structure and properties of PhaC, which remains covalently linked to the PHB granules, has allowed translational fusion of foreign proteins to produce functionalized beads. These beads have been used for biomedical applications like protein production and purification as well as diagnostics and vaccines [1, 2]. Medical application were justified as beads are biocompatible and biodegradable, and their one step production of carrier and antigen is cost-effective [3].

An effective vaccine is needed for the global prevention of tuberculosis (TB). Such a vaccine needs to be affordable and accessible for developing countries, safe and able to elicit a longer lasting immunity than the current vaccine, Bacille Calmette-Guérin (BCG) [4]. PHB beads displaying Ag85A-ESAT-6 antigens from *Mycobacterium tuberculosis* induced stronger humoral and cellular immune responses than vaccination with soluble Ag85A-ESAT-6 [5] and resulted in protection against *Mycobacterium bovis* challenge [6].

New generation adjuvants are being developed for subunit vaccines e.g. molecular adjuvants, like promiscuous T helper epitopes that bind a number of HLA class molecules and allow entire populations to respond irrespective of MHC [7]. $CS.T3_{378-395}$, a peptide from the circumsporozoite protein of *Plasmodium falciparum* is a promiscuous T helper epitope almost universally recognized by human and mouse T lymphocytes, [8] producing immunological cross reactivity [9] and potentiating immune responses [10]. One of the strongest pathogen-associated molecular patterns (PAMPs) is flagellin, the monomeric subunit of the bacterial motility apparatus. Flagellin is the natural ligand of toll like receptor (TLR) 5 and the only protein TLR agonist identified to date [11]. Numerous studies have

described the adjuvant properties of flagellin in the context of a broad range of recombinant vaccines [12] inducing cellular responses, and humoral responses [13] even in a T cell restricted model [14]. Other peptides act as molecular adjuvants, like Cpe30 peptide which is derived from the C-terminus 30 amino acids of *Clostridium perfringens* enterotoxin. Cpe30 binds to the protein claudin-4, which is an endocytosis receptor on M cells [15]. It has been used in the context of mucosal vaccination along with other antigens, inducing strong IgG1, IgG2a and IFN-γ responses [16].

Previously, we demonstrated that beads displaying the Rv1626 antigen from *M. tuberculosis* were immunogenic, mediating an antigen-specific antibody response [17]. Rv1626 interacts with the actin related protein 4 (ARP4) of mammalian cells, the function of this interaction is not clear, but it is possible that Rv1626 modulates the host cell cytoskeleton enabling *M. tuberculosis* survival and protection [18]. There is strong production of Rv1626 in the early stages of *M. tuberculosis* growth [19] and it is constitutively produced and secreted [20].

In this study, the immunological properties of beads displaying Rv1626 and the molecular adjuvants, Cpe30, $CS.T3_{378-395}$ or Flagellin either alone or in combination was studied. The ability of these functionalised beads to protect mice against experimental challenge with *M. bovis* was determined.

3.2 Materials and Methods

Generation of vectors for beads production

Strains, vectors and oligonucleotides used in this study are described in Table 3.1. Genes encoding molecular adjuvants were synthesized by GenScript (USA) and contained in the plasmid pUC57. General cloning procedures [30] were used to generate plasmids and their sequences were confirmed by DNA sequencing.

Table 3.1. Strains, plasmids and oligonucleotides used

Strain, plasmid or oligonucleotide	Genotype or description	Source or reference
<u>*E. coli* strains</u>		
XL1-Blue	*endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)*	Stratagene
*Clear coli*BL21 (DE3)	F– *ompT hsdSB (rB- mB-) gal dcm lon* λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*]) *msbA148 ΔgutQΔkdsD ΔlpxL ΔlpxMΔpagP ΔlpxP ΔeptA*	Lucigen
<u>Vectors</u>		
pPOLYC-PhaC-Rv1626	pPOLYC-phaC derivative containing *rv1626* inserted into *XhoI/BamHI* sites	[19]
pPOLYC-Cpe30-Rv1626	pPOLYC-PhaC-Rv1626 derivative containing *cpe30* inserted upstream *rv1626* into *Xho*I/*BsrG*I sites	This study
pPOLYC-CS.T3-Rv1626	pPOLYC-PhaC-Rv1626 derivative containing two copies of *cS.T3*$_{378-395}$ inserted upstream *rv1626* into *Xho*I/*BsrG*I sites	This study
pPOLYC-Fla66-Rv1626	pPOLYC-PhaC-Rv1626 derivative containing *flagellin*$_{66-494}$ inserted upstream upstream *rv1626* into *Xho*I site	This study
pPOLYC-Cpe30-CS.T3-Fla66- Rv1626	pPOLYC-PhaC-Rv1626 derivative containing *cpe30,* two copies of *cS.T3*$_{378-395}$ and *flagellin*$_{66-494}$ inserted ustream *rv1626* into *Xho*I/*BsrG*I sites	This study
pMCS69	Cm^r . pBBR1MCS derivative containing genes *phaA* and *phaB* from *R. eutropha*	[33]
<u>Oligonucleotides</u>		
Fla66 XhoI fwd	5'-GCTCTCGAGATGAACGCGAACGACGGT-3'	This study
Fla66 XhoI rev	5'-GCTTGTACAAGGTGGTACGCAGCAGGCTCA-3'	This study

Production of PHA bead and soluble Rv1626

Strains used in this study are listed in Table 3.1. Cells were grown in Luria-Bertani (Acumedia, USA) broth, supplemented with ampicillin (100 µg/mL) and D-glucose 0.2 % (w/v) (Merck, Germany) and chloramphenicol (50 µg/mL). Beads production was induced with isopropyl-D-thiogalactopyranoside (IPTG) (1 mM) and cells cultured for 48 h at 25 °C. Cells were harvested and resuspended in lysis buffer [30] prior to mechanical disruption using a microfluidizer (Microfluidics M-110P, USA). Beads were isolated as previously described [19]. Briefly, beads were recovered after centrifugation of the whole cell lysate at 6000 x *g* for 30 minutes at 4°C, washed twice with lysis buffer, treated with 70 % (v/v) ethanol to kill residual bacteria and stored in PBS buffer (10mM Na_2HPO_4, 137 mM NaCl). Soluble Rv1626 was obtained using a sortase based tagless protein purification system as previously described [32].

Analysis of fusion proteins

Proteins attached to the beads were separated on an 8% SDS-PAGE gel and stained with Coomassie Blue. The amount of fusion protein was calculated using a standard curve with BSA. The identity of Cpe30-CS.T3-Fla66-Rv1626 was verified by subjecting the corresponding gel bands to tryptic peptide fingerprinting and using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF/MS).

Preparation of vaccine and vaccination of mice

Female C57BL/6 mice were obtained from the animal breeding facility of AgResearch, Hamilton, New Zealand. Throughout all vaccinations and the period prior euthanasia mice were allocated arbitrarily in mouse boxes (four mice per box, 2 boxes per treatment) in a PC2 facility during the course of the dose response experiment and the determination of the immunogenicity of Rv1626. For the challenge experiment the boxes were kept in biohazard animal cages designated for tuberculosis studies. Body weight was monitored before each vaccination and fortnightly after challenge. Animals were monitored daily and carefully

observed after vaccinations. All animal experiments were approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand).

Dose Response Experiment

To select a suitable dose of Rv1626 for testing the various molecular adjuvants in an immunology/*M. bovis* challenge trial, eight mice per group were vaccinated subcutaneously with 1.25, 2.5. 5 and 10 µg of Rv1626 displayed on beads and to Wt beads formulated with DDA. IgG1 and IgG2c responses were evaluated.

Determination of immunogenicity of Rv1626

The chosen dose of Rv1626 beads (2.5 µg of Rv1626) was combined with molecular adjuvants and formulated with DDA (1.25 mg/ml).These formulations were used to vaccinate eight mice per group subcutaneously. IgG1 and IgG2 and cytokines responses to vaccinations were evaluated.

Assessment of protective immunity induced by Rv1626 beads

Sixteen mice per group were vaccinated subcutaneously with BCG Pasteur 1173P2 (10^6 CFUs per mice), rec Rv1626, Rv1626 beads, Rv1626-Cpe30 beads, Rv1626-CS.T3 beads (adjusted to 2.5 µg of Rv1626) or DDA in a 200 µl dose. BCG was given only once and the rest of the vaccines were administered 3 times at 9 days intervals. Soluble Rv1626 was obtained using a sortase based tagless protein purification method [32].

Samples preparation and immunological assays

Three weeks after the last vaccination, mice were sedated, bled, euthanized and a single cell suspension of splenocytes obtained as described by Parlane *et al.* in 2012 [17]. Mouse spleen cells were stimulated with soluble Rv1626 or PPDB (5 µg/mL) and cultured for 72 hours in an atmosphere of 10 % CO_2 in air. Culture supernatants were removed and frozen at -20^{o}C until assayed. IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ release was measured

using a BD CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, USA) according to the manufacturer's instructions.

IgG responses in sera were analysed by ELISA. Microlon high-binding plates (Greiner Bio-One, Germany) were incubated overnight at 4 °C with 2.5 µg/mL of rec Rv1626. Sera from individual mice was added to the plates and goat anti-mouse IgG1 or IgG2c HRP-conjugate antibodies (ICLLab, USA) were used for detection of IgG1 and IgG2c antibodies, respectively as described previously [19]. Data were displayed as EC50.

M. bovis challenge trial

Three weeks after last vaccination half of the mice in each group were euthanized and samples processed for the immunology trial as described above. Six weeks later, remaining mice were challenged with *M. bovis* 83/6235 in a Madison chamber aerosol generation device calibrated to deliver 50 bacteria into the lungs. Five weeks after challenge, mice were euthanized and lungs and spleens removed. The right apical lung lobe was removed for histological processing. Sections were prepared and stained with H&E.

Culture of lungs and spleens

Remaining lungs and spleen samples were mechanically homogenized in 3 ml of PBS (pH 7.4) containing 0.5% Tween 80 using a Seward Stomacher 80 device (Seward, Norfolk, United Kingdom). Triplicate 10 µL of ten-fold dilutions ranging from non-diluted to 10^{-6} were plated on selective Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (Difco, BD, USA). Plates were sealed into plastic bags to prevent media dehydration and incubated at 37°C in humidified air. After three weeks incubation, 2-3 mm creamy, irregular, rough *M. bovis* colonies were observed. CFUs grown in non-confluent segments were counted. The number of colonies present in the highest dilution was multiplied by the dilution factor and the $\log_{10}$ of this value was reported.

Statistical analysis

Data from antibodies, cytokines responses and CFUs in lungs and spleens were analysed using a Dunn's test of multiple comparisons following a significant Kruskal-Wallis test. Significance level was considered at $p<0.01$.

3.3 Results and Discussion

Development of *Escherichia coli* strains assembling PHA beads displaying various antigen Rv1626 and immunomodulators

Genes *cpe30,* $cs.t3_{378\text{-}395,}$ $\text{flagellin}_{66\text{-}494}$ and $cpe30\text{-}cs.t3_{378\text{-}395}\text{-}flagellin_{66\text{-}494}$ were cloned separately upstream of *rv1626* into pPOLYC-Rv1626 plasmid. *Clear coli* was transformed with the various plasmids and used as the host for producing PHA beads for vaccination of mice. Beads were purified and bead protein profiles were analysed by SDS-PAGE (Figure 3.1). A prominent band corresponding to the expected molecular weight of the full-length fusion protein was observed in all cases and the identity of the band corresponding to PhaC-Cpe30-CS.T3-Fla66-Rv1626 was confirmed by MALDI-TOF/MS (Appendix 2). The amount of Rv1626 immobilized per mg of wet beads was 393 ng, 176.8 ng, 174 ng, 102 ng and 108 ng in Rv1626, Cpe30-Rv1626, CS.T3-Rv1626, Fla66-Rv1626 and PhaC-Cpe30-CS.T3-Fla66-Rv1626 beads respectively. There were 695 ng of PhaC in wild type beads. The various beads displaying the antigen and immunostimulatory molecules were used to study their immunogenicity in mice.

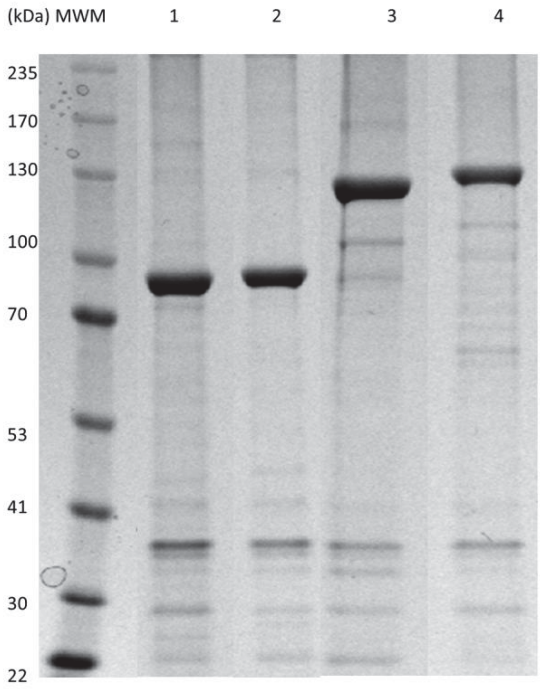

Fig.3.1. SDS-PAGE analysis of proteins attached to various polyester beads isolated from *Clear coli*. Lane 1, Cpe30-Rv1626 beads (92.5 kDa); lane 2, CS.T3-Rv1626 beads (93.1 kDa); lane 3, Fla66-Rv1626 beads (134 kDa); lane 4, Cpe30-CS.T3-Fla66-Rv1626beads (141 kDa). The linker VLAVAIDKRGGGGG (hydrophobic-charged amino acids) is included in this plasmid between PhaC and Rv1626 to facilitate display of the fusion partners. [25].

Dose response study

IgG1 and IgG2c responses in sera were analysed by ELISA (Figure 3.2, Appendix 1C). IgG1 responses to Rv1626 antigen were significantly higher in mice vaccinated with 2.5 µg and 5 µg of Rv1626 beads than responses in mice vaccinated with wild type (Wt) beads or given DDA alone. No significant differences were observed between mice vaccinated with the different doses of Rv1626. IgG2c responses of mice vaccinated with Rv1626 beads did not increase with the dose and responses were all significantly higher in mice vaccinated with 1.25 - 10 µg Rv1626 than mice vaccinated with DDA alone. These results differ from the ones obtained by Parlane *et al* in 2009, where higher doses significantly increased the immune response [5]. However, different antigens were used in these studies and it is possible that with the lower dose used of Rv1626 the antibodies responses were already

saturated. Different doses of Rv1626 on beads did not trigger significantly different IgG responses but IgG1 responses were higher in mice vaccinated with 2.5 µg and significantly different than the response of mice vaccinated with WT beads. Based on these results, a dose of 2.5 µg Rv1626 on beads was chosen for further immunological studies.

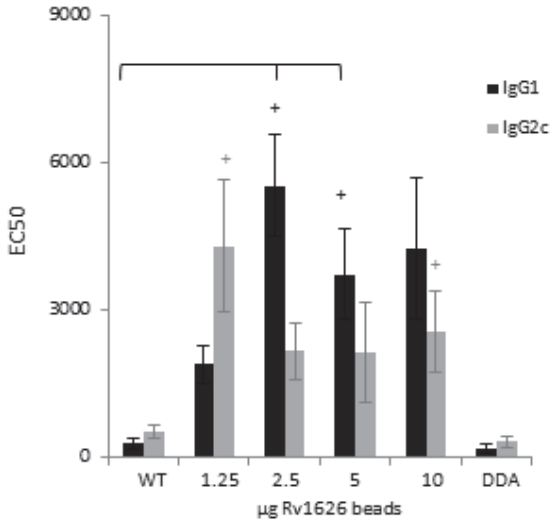

Figure 3.2. IgG1 and IgG2c titres expressed in EC50 values in response to Rv1626 of mice vaccinated subcutaneously with different doses of Rv1626 displayed on beads and Wt beads analysed by ELISA. Each bar represents the mean ± the standard error of the mean, +, significantly greater than DDA vaccinated group; groups between brackets, significantly different. ($p<0.01$).

Determination of immunogenicity of Rv1626 beads

Serum IgG1 and IgG2c responses to Rv1626 in mice vaccinated with Rv1626 beads with or without addition of immunomodulators were evaluated by ELISA (Figure 3.3, Appendix 1C). These responses were not significantly different between the groups but were increased compared to mice vaccinated with DDA alone. Mice splenocytes were stimulated with soluble Rv1626, and IL-2, IL-4, IL-6, IL-10, IL-17A, INF-γ and TNF-α levels were measured in culture supernatants. (Figure 3.4, Appendix 1D). Vaccination with the PHA beads triggered a Th1/Th17 skewed response but inclusion of the molecular adjuvants did not increase the immunogenicity of Rv1626 compared to Rv1626 beads alone.

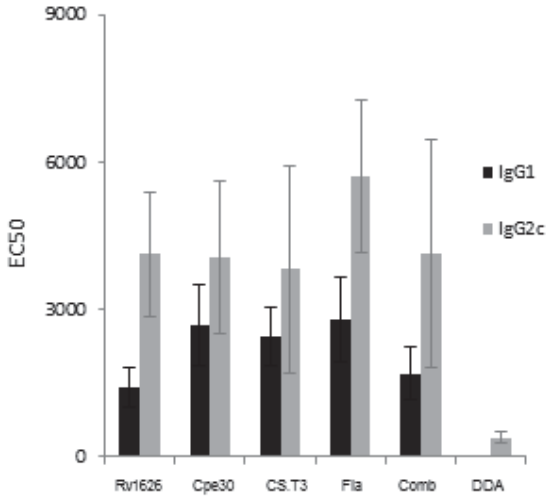

Figure 3.3. Serum IgG1 and IgG2c titres expressed in EC50 values in response to Rv1626 from mice vaccinated with different immune modulators. Each mouse received 2.5 μg of Rv1626 per vaccination. Antibody response was analysed by ELISA and each bar represents the mean of eight mice ± the standard error of the mean.

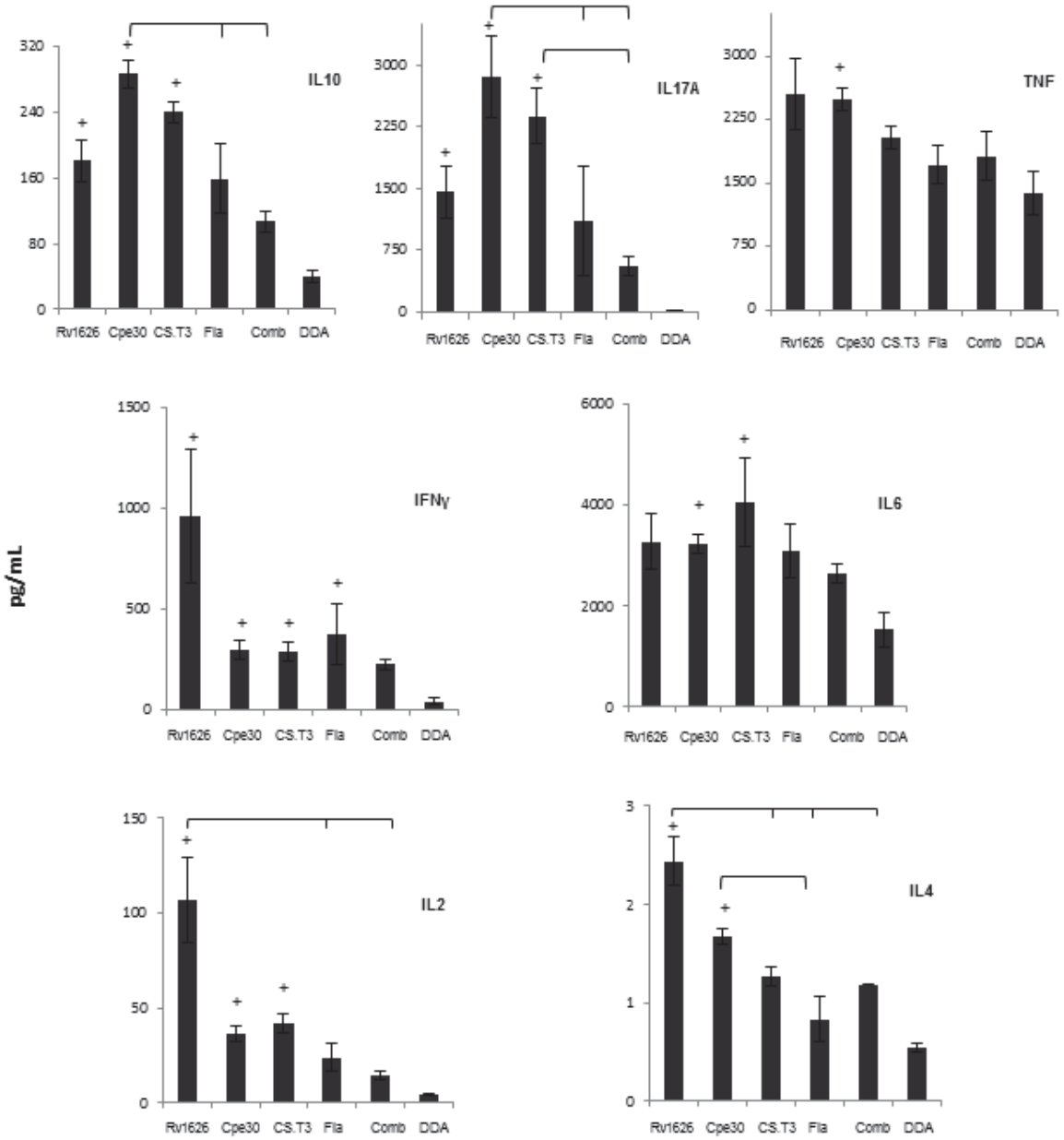

Figure 3.4. Cytokine responses of mice splenocytes upon stimulation with soluble Rv1626 (recRv1626) and analysed by cytometry bead array. Mice were vaccinated with Rv1626 beads or immune modulators-Rv1626 beads. Each bar represents the mean ± the standard error of the mean, +, significantly greater than DDA vaccinated group; groups between brackets, significantly different. ($p<0.01$).

Assessment of protective immunity induced by Rv1626 beads

As there are no confirmed correlates of protection for *M. tuberculosis* or *M. bovis* infection, the ability of Rv1626 displayed on beads and combined with immunomodulators Cpe30 or CS.T3 to protect against tuberculosis was evaluated in a *M. bovis* challenge experiment. These beads were selected from the immunogenicity experiment as they induced higher cytokines levels than the rest of the vaccines used.

Additional groups were given soluble recombinant Rv1626 or BCG while a control group received DDA. Antibody responses of mice vaccinated with the selected PHA bead based vaccines are shown in Figure 3.5 and Appendix 1C. Vaccination with soluble Rv1626 induced significantly higher levels of IgG1 than the negative control group while vaccination with Rv1626 beads higher levels of IgG2c than both the negative control group and the group vaccinated with soluble Rv1626.

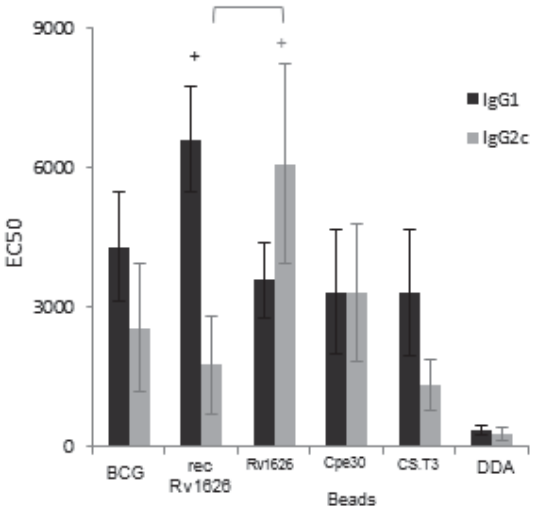

Figure 3.5. IgG1 and IgG2c titres expressed in EC50 values in response to Rv1626 of mice vaccinated subcutaneously with BCG, soluble Rv1626 (recRv1626), Rv1626 beads, Cpe30-Rv1626 beads, CS.T3-Rv1626 beads and DDA analysed by ELISA. Soluble Rv1626 was obtained using a sortase based tagless protein purification system as previously described [26]. Each bar represents the mean values of 8 mice ± the standard error of the mean, +, significantly greater than DDA vaccinated group; groups between brackets, significantly different ($p<0.01$).

The induced IgG subclasses provide an indication of the contribution of Th1 or Th2-type cytokines in the response, thus, release of IgG1 is related to induction by Th2-type cytokines, whereas production of IgG2c is related to induction by Th1-type cytokines.

Splenocytes from mice were stimulated with 5 μg/mL of soluble recombinant Rv1626 or PPDB (bovine protein purified derivative) and results are shown in Figure 3.6 and Appendix 1E. For splenocytes stimulated with soluble recombinant Rv1626, levels of IL-10, IL-17A, IL-2, IL-6 and IFN-γ were significantly higher in mice vaccinated with Rv1626, Cpe30-Rv1626 and CS.T3-Rv1626 beads than in mice vaccinated with DDA alone. Mice vaccinated with Cpe30-Rv1626 and CS.T3-Rv1626 beads produced significantly higher TNF-α levels than the negative control, and those vaccinated with Rv1626 and Rv1626-Cpe30 beads produced higher levels of IL-4. Differences were observed between mice vaccinated with beads and the soluble recombinant protein. The soluble Rv1626 induced significantly lower levels of TNF-α and IFN-γ than Cpe30-Rv1626 beads and lower IL-10 than CS.T3-Rv1626 beads. For splenocytes stimulated with PPDB, BCG vaccination induced significantly higher levels of IFN-γ than the negative control and higher IL-4 than all the other groups.

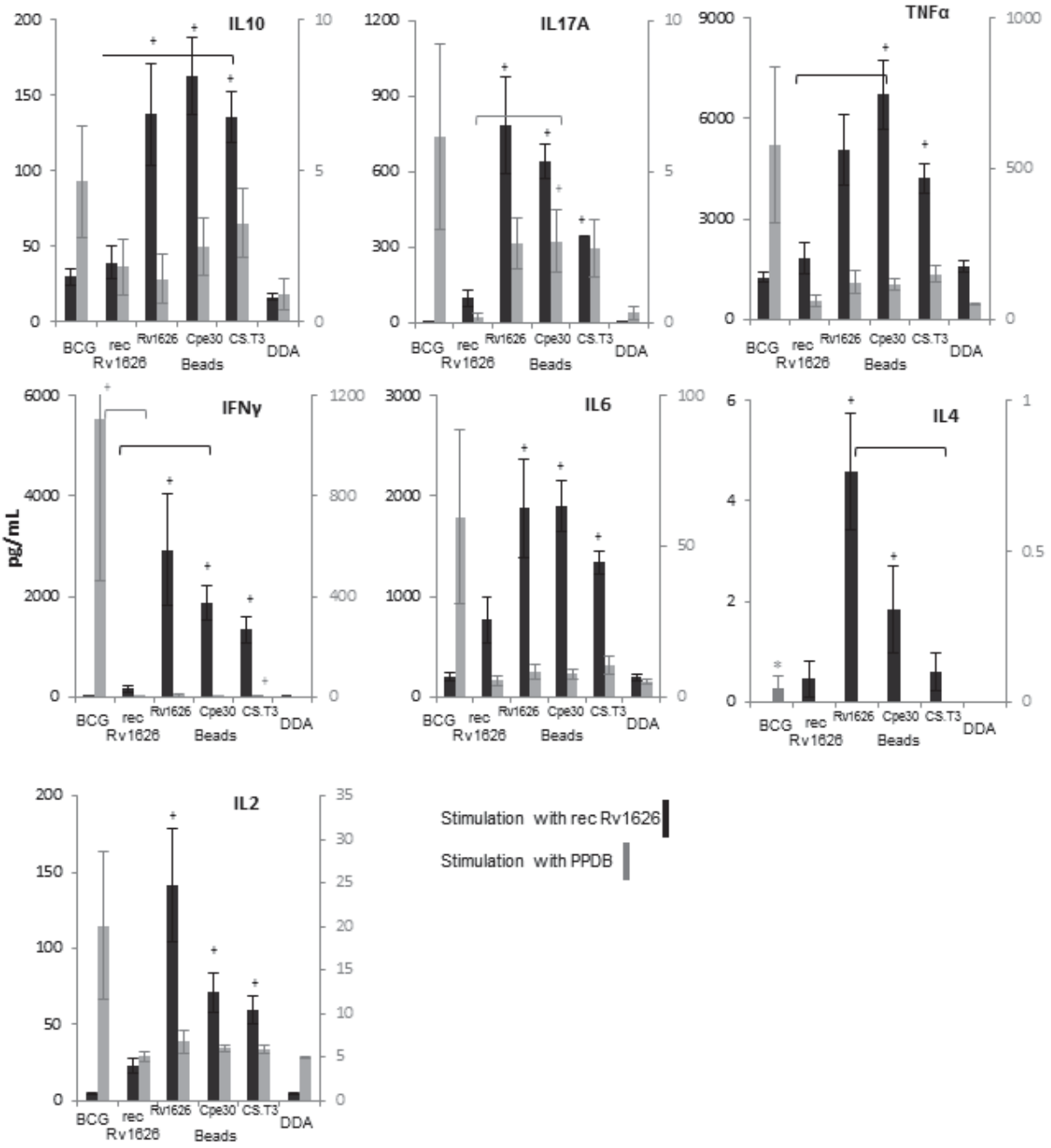

Figure 3.6 Cytokine responses of mice splenocytes upon stimulation with rec Rv1626 (left axis) or PPDB (right axis). Mice were vaccinated with BCG, rec Rv1626, Rv1626 beads, Cpe-Rv2626 beads, CS.T3-Rv1626 beads or DDA alone. Each bar represents the mean of eight mice ± the standard error of the mean, +, significantly greater than DDA vaccinated group; *, significantly different from all groups, groups between brackets, significantly different. ($p<0.01$).

BCG vaccination significantly reduced the bacterial burden in lungs compared with vaccination with soluble Rv1626, Cpe-Rv1626, CS.T3-Rv1626 beads or DDA and in spleen compared with all other groups (Table 3.2, Appendix 1F). Vaccination with BCG reduced bacterial cell counts in lungs and spleen by 1.35 $\log_{10}$ and 0.98 $\log_{10}$ respectively compared to the negative control (DDA). Vaccination with the Rv1626 beads reduced the lung bacterial counts by 0.48 $\log_{10}$, although this reduction was not significant compared to the negative control, in this group lung bacterial counts were not significantly different compared to that for the BCG-vaccinated group. Derrick et al, in 2013 reported a bacterial count reduction of 0.31 $\log_{10}$ after vaccination with soluble Rv1626 and the respective challenge. In the present study, soluble Rv1626 did not induce protection. Our data confirmed that the display of antigen on PHA beads increase induction of protective immunity compared to the soluble antigen as had been previously shown for other antigens [6, 21]. It may be possible to achieve greater protection with Rv1626 beads, by using the beads as a boost vaccine after primary vaccination with either BCG or ESAT6-Ag85B. Derrick *et al* achieved 1.12 $\log_{10}$ reduction after vaccinating mice with soluble Rv1626 following vaccination with ESAT6-Ag85B [22].

Table 3.2. Vaccine induced protection in lung or spleen after *M. bovis* aerosol infection

Vaccine	Lung (log_{10}) CFUs	Spleen (Log_{10}) CFUs
BCG	4.83 ± 0.09 (-1.35)[b]	3.86 ± 0.05 (-0.98)[b]
rec Rv1626	6.22 ± 0.24 (+0.04)[a]	4.90 ± 0.12 (+0.06)[a]
Rv1626 beads	5.70 ± 0.04 (-0.48)[ab]	4.73 ± 0.08 (-0.11)[a]
Cpe-Rv1626 beads	6.07 ± 0.09 (-0.12)[a]	4.72 ± 0.03 (-0.12)[a]
CS.T3-Rv1626 beads	6.46 ± 0.38 (+0.28)[a]	5.06 ± 0.23 (+0.22)[a]
DDA	6.18 ± 0.37[a]	4.84 ± 0.19[a]

Mean count of CFUs of 8 mice ± standard error (group mean-DDA group mean). Data was analysed using a Dunn's test of multiple comparisons following a significant Kruskal-Wallis test. Statistical significance is shown using a letter based system. Significantly different groups have different letters and groups with means not significantly different share the same letter ($p<0.01$).

Lung sections were stained with hematoxylin and eosin (H&E) (Figure 3.7). Lungs from the adjuvant control mice had multiple, coalescing granulomas composed predominantly of epithelioid macrophages and lymphocytes . Intracellular, acid-fast bacilli were observed in macrophages found in these lesions. The lungs of the BCG-vaccinated mice had less lesion involvement. The lesions were composed of loosely organised accumulations of inflammatory cells (arrows in Figure 3.7) within the perivascular areas, with a predominance of lymphocytes and fewer macrophages.

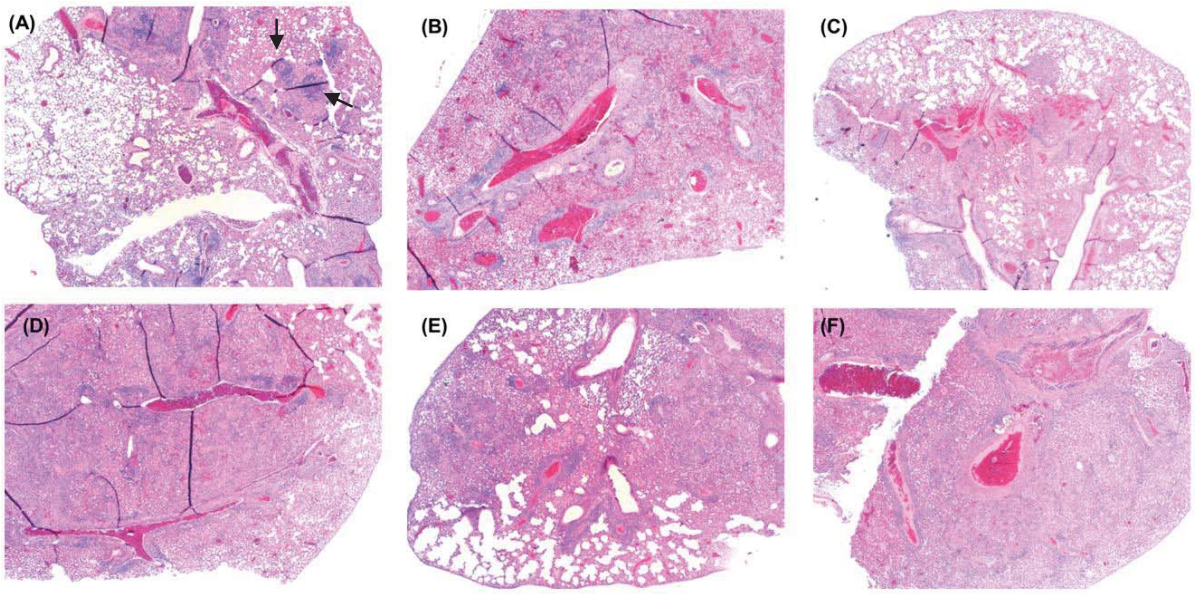

Figure 3.7. Histological appearance of lungs from mice that have the closest value to the CFUs mean after *M. bovis* challenge. Sections were stained with H&E. Groups: A, BCG; B, soluble Rv1626; C, Rv1626 beads; D, Cpe30-Rv1626 beads; E, CS.T3-Rv1626 beads; F, DDA alone. Appendix 3 contains the histological appeareance of all mice.

Assembling Cpe30 or CS.T3 with Rv1626 antigen on the same PHA bead did not significantly increase the immunogenicity of Rv1626, and may even have reduced protection. This could be due to a mis-folding of the fusion protein on the beads when the immune modulators were included in the same fusion protein, interfering with exposure of Rv1626 conformational epitopes to cell receptors [23]. The amount of immunomodulators on the various beads used in our study may not have been optimal as the primary concern was to have a similar dose of Rv1626 antigen in each type of vaccine. For example, too high levels of immunomodulator on the vaccine beads might have overstimulated cell receptors resulting in inflammation and tissue damage and possibly impaired protective immunity [24].

While further studies are needed to optimise the dose of immunomodulators on mycobacterial antigen displaying beads, this study demonstrated that a single *M. tuberculosis* antigen when displayed on PHA beads has the potential to protect against tuberculosis.

Acknowledgments

The authors would like to thank the Institute of Fundamental Sciences, Massey University and AgResearch for funding the research project.

Conflict of Interest

B.H.A. Rehm is founding inventor, Chief Technology Officer and shareholder of PolyBatics Ltd that commercialises the PHA bead technology.

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Chapter 4: General discussion

This thesis exploits PHAs as vaccine delivery systems against TB. PHAs are advantageous over other particulate vaccines systems like virus-like particles, liposomes and chitosan as antigens can be covalently fused; and as the particles are produced in bacteria this allows assembly of carrier and antigen in a one-step process, thus simplifying the production and purification phases [1].

Chapter 2 describes the attempts of producing beads co-displaying three different Rv antigens, and also single antigens when it proved difficult to produce the fusion protein. There are major advantages of using PHAs as the antigen delivery system but there are also disadvantages when using fusion proteins. Not all the fusions to PhaC permit the production of beads and when beads are produced, the ratio of antigen to beads has to be sufficiently high to allow vaccination with the desired dose of antigen as high viscosity of bead suspensions prevent its practical administration.

Prior to administration of products produced in *E. coli*, LPS needs to be eliminated as this molecule causes inflammation and septic shock in humans and experimental animals [2]. In this project, beads were produced in *Clear coli*. This strain allows the use of the well-known and easy to manipulate *E. coli* for production of human vaccines without the need to undergo LPS removal processes. Faster shaking, the use of baffled flasks and higher NaCl concentrations compared with those required for normal *E. coli* were required for cultivation. However, it is cost and time effective to use an endotoxin free strain instead of undergoing further steps to remove endotoxins from the preparations.

The results obtained in this thesis demonstrate the importance of challenge experiments in evaluating vaccine efficacy in animal models, especially for the case of TB vaccines development, were cytokines or Abs production cannot be correlated with a protective immune response. Rv1626 beads were chosen to evaluate their protective efficacy against

M. bovis challenge due mostly to the specificity of the antibodies responses they triggered. While Rv2032 and Rv1789 beads did induce IgG1 and IgG2c responses, those were not antigen-specific, as demonstrated in Figures 2.4 and 2.5. The specificity of mice IgG responses to Rv1626 was further confirmed (Figure 2.6) where this protein was selectively recognized in a cell lysate of the beads production host. Chapter 3 showed that Rv1626 beads induced partial protection against *M. bovis* challenge when compared with vaccination with BCG and the negative control (Table 3.2). This result shows the potential of Rv1626 beads as a vaccine candidate against TB as Rv1626 is a single mycobacterial antigen and BCG is a live vaccine and the protective response induced by them was not significantly different. Also emphasise the need of evaluating vaccine efficacy *in vivo*, as other vaccine candidates evaluated in this study induced the same cytokines profile that Rv1626 beads, however, only Rv1626 beads induced protection.

Splenocytes re-stimulated with soluble Rv1626 of mice vaccinated with Rv1626, Cpe30-Rv1626 and CS.T3-Rv1626 beads produced significantly higher levels of TNF-α and IFN-γ than mice vaccinated with DDA. CD4+ T cells expressing Th1-like cytokines define a response associated with control of *M. tuberculosis* infection [3]. IFN-γ is a principal mediator of macrophage activation and resistance to intracellular pathogens; mice unable to produce this cytokine are able to develop granulomas but do not produce reactive nitrogen intermediates and cannot restrict the growth of the bacilli [4]. TNF-α acts in synergy with IFN-γ stimulating production of reactive nitrogen intermediates, thus mediating macrophage function, stimulating migration of immune cells to the infection site and contributing to granuloma formation, ultimately controlling disease progression [5].

Also high levels of IL-6 and IL-17A were induced by these vaccinations (Figures 3.4 and 3.6). IL-6 participates together with TNF-α in the initiation of a pro-inflammatory response. IL-6 deficient mice are highly susceptible to *M. tuberculosis* infection and IL-6 is required for IFN-γ induced TB protection in mice [6]. IL-17 is capable of inducing chemokine expression

and recruitment of neutrophils to infected tissue. Protective response is altered by the absence of Th1 cells but not in the absence of Th17 cells. Nevertheless, memory responses in the lungs of vaccinated mice depend on IL-17 production, demonstrating the important role of this cytokine in the production of vaccine-induced memory cells [7].

High levels of antigen-specific IFN-γ, TNF-α, IL-6 and IL-17A were also obtained by Parlane *et al.* in 2012 after vaccinating with 30 μg of Ag85A-ESAT6 on beads formulated in the adjuvant emulsigen. This vaccination also induced protection against *M. bovis* infection in mice [8]. Ag85A-ESAT6 and Rv1626 when displayed on beads induced the same cytokines profile, however, performed differently inducing protection against *M. bovis* challenge. This difference may be due to that different antigens and adjuvants were used. Nevertheless, these results suggest that *E. coli* derived polyester beads displaying mycobacterial antigens induce a Th1/Th17 skewed immune response.

When soluble Rv1626 was used to boost a vaccination with ESAT6-Ag85B in mice, protection was equivalent to BCG-induced and greater than the protection induced by ESAT6-Ag85B alone [9]. This study used DDA adjuvant in combination with trehalose 6,6-dibehenate (TDB). TBD is a glycolipid immunomudulator which is a synthetic variant of the cord factor located in the mycobacterial cell wall [10]. The two-component liposomal adjuvant system (DDA-TBD) exhibits more stability and enhances the immune response induced by DDA [11]. Considering the results obtained by Parlane et al. [8] and Derrick et al. [9], the future work using Rv1626 beads could consider them to be used as a boost vaccination, immunizing first with Ag85A-ESAT6, but beads should be formulated in DDA-TBD.

It is not possible to predict the importance of these responses in the protection against human TB. Multifunctional CD4+ T cells, producing usually TNF-α, IFN-γ, IL-17A, and IL-2, have been correlated with immune protection [12]. However, the contribution of these cells to mycobacterial immunity may not be as relevant as it was thought. MVA85A vaccine,

developed as an intranasal booster to BCG increases multifunctional T cells compared to BCG alone vaccinated individuals. However, this vaccine does not enhance BCG protection. [13].

The failure of MVA85A vaccine to enhance protection induced by BCG constituted a step back for the TB vaccines pipeline, perhaps more focus should be put into combining the already known protective vaccine candidates with suitable adjuvants and into exploring different routes of immunization.

In regard to this approach, this thesis explores the inclusion of immune modulators on the beads. However, including immune modulators did not increase the partial protection induced by Rv1626. A possible explanation could be that the immune modulators caused over-activation of the immune system. Hyperactivation of immune cells induces a pro-inflammatory environment that causes the release of lipid factors and reactive oxygen species that amplify the inflammatory responses. Malfunction of regulatory mechanisms can result in tissue damage and uncontrolled inflammation [14]. Also it is possible that the display of immune modulators on the beads surface was not optimum; recently, Noble *et al* demonstrated that the density of immune modulator on the particle surface dictates signalling of co-stimulatory molecules needed to drive APC maturation and promote adaptive responses, rather than the total particle number or adjuvant mass delivered [15].

Due to the size of CS.T3 peptide, two copies where included in CS.T3-Rv1626 beads to facilitate cloning procedures. It is possible that using only one copy of this immunomodulator reduces the negative effect caused (Chapter 3). One copy of Cpe30 and Flagellin per Rv1626 was included, meaning that it is not possible to reduce the dose of immunomodulator in those beads. Future experiments could evaluate the immunogenicity of beads co-displaying immune modulators and antigens without the addition of another adjuvant. Also it is possible to evaluate N-terminus fusions to PhaC, to determine how antigen folding is affected by the inclusion of the immune modulators.

The nasal route is the primary route of *M. tuberculosis* infection. Delivering a vaccine directly by aerosol to the respiratory mucosa might offer an immunological advantage over the subcutaneous route [16]. Mucosal innate and adaptive immune systems at the site of pathogen entry may be required for protection against tuberculosis and may be generated by mucosal vaccination. Optimum mucosal vaccination leading to compartmentalized mucosal immune responses might ensure that the appropriate cells are ready to respond immediately to infection and to confer protection [17].

BCG vaccination via the intratracheal route or the intranasal route compared to vaccination via the subcutaneous route can confer superior protection against tuberculosis infection in mice [18] [19]. Aerosol vaccination with attenuated *M. tuberculosis* induces central memory responses and protects against tuberculosis [20]. However antigens administered at mucosal surfaces are less immunogenic and tend to induce tolerance, the efforts to develop effective mucosal vaccines have focused on developing the appropriate adjuvants or immune modulators that provide protective immunity against mucosal pathogens, with the advantage that provide secretory antibody-mediated protection [21]. Cpe30 immunomodulator could be considered for mucosal vaccination as it targets mucosal cells and various routes, intratracheal, intranasal and aerosol can be explored.

In conclusion, the findings in this thesis support the use of PHA beads as a vaccine delivery system for antigens to induce protective immunity against tuberculosis in a mice model. Further studies are needed to effectively incorporate immunomodutalors into this system.

4.3 References

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Appendix 1:

Raw Data used to elaborate charts and tables

1A. Cytokine responses of mice splenocytes upon stimulation with PPDB and analysed by cytometry bead array

Vaccine	IFN	IL-10	IL-17A	IL-2	IL-4	IL-6	TNF
BCG	2.54	4.28	0	2.67	0	0.99	9.57
BCG	0.22	2.94	0	1.4	0	1.51	9.77
BCG	0.53	2.74	0	0.84	0	1.28	10.29
BCG	13.15	3.7	0	1.87	0	1.17	13.49
BCG	22.25	2.36	0	1.62	0	1.51	16.46
BCG	0.86	0	0.41	0.8	0	0.49	2.31
Rv1626 b	0.36	0.79	0.13	2.09	0	1.3	7.64
Rv1626 b	0.53	5.06	0.39	2.13	0	1.79	15.57
Rv1626 b	0.44	3.51	0.06	1.51	0	1.43	18.59
Rv1626 b	1.08	3.7	0.14	2.06	0.02	1.82	8.95
Rv1626 b	0	2.17	0	0.72	0	1.04	4.75
Rv1626 b	0	0	0	0	0	0.33	2.07
Rv1789 b	0.31	4.67	0	2.2	0	2	17.35
Rv1789 b	1.32	3.7	7.4	3.36	0	1.64	20.4
Rv1789 b	0.62	2.74	0.32	2.11	0	1.49	16.02
Rv1789 b	0.31	3.32	0.01	1.16	0	1.23	4.84
Rv1789 b	0.6	2.74	0	2.41	0	3.52	6.16
Rv1789 b	0.75	0	0	1.9	0	0.74	2.07
Rv2032 b	0.25	2.36	0	1.72	0	1.04	4.29
Rv2032 b	0.12	1	0	0.62	0	0.57	5.69
Rv2032 b	0	0.79	0	0.84	0	0.4	5.21
Rv2032 b	0.08	0.79	0	2.06	0	0.6	3.32
Rv2032 b	0.34	0	0	3.18	0	0	2.72
Rv2032 b	0	0	0	0.99	0	0.04	1.75
Wt b	0.22	1.78	0	2.27	0	0.63	5.31
Wt b	0.34	2.55	0.58	1.9	0	1.17	11.14
Wt b	0.71	3.32	0.13	2.71	0.02	3.06	11.56
Wt b	0	1.78	0	1.4	0	0.66	6.65
Wt b	0	3.13	0.17	1.16	0	1.56	7.44
Wt b	0.08	0.58	0.05	2.27	0	0.88	5.4
DDA	0.25	2.36	0	1.72	0	1.04	4.29
DDA	0.12	1	0	0.62	0	0.57	5.69
DDA	0	0.79	0	0.84	0	0.4	5.21
DDA	0.08	0.79	0	2.06	0	0.6	3.32
DDA	0.34	0	0	3.18	0	0	2.72
DDA	0	0	0	0.99	0	0.04	1.75

1B. IgG1 and IgG2c titers expressed in EC50 values in response to various beads and PPDB analysed by ELISA

	IgG1 response to:					IgG2c response to:				
Vaccine	**Wt b**	**Rv1626 b**	**Rv2032 b**	**Rv1789 b**	**PPDB**	**Wt b**	**Rv1626 b**	**Rv2032 b**	**Rv1789 b**	**PPDB**
BCG	1150	500	500	500	500	500	500	500	500	286
BCG	2760	500	500	1070	500	500	500	500	500	500
BCG	500	500	500	500	500	500	3950	500	500	500
BCG	500	4450	1750	500	500	500	3240	500	500	500
BCG	461	484	1130	177	500	500	2980	2650	793	500
BCG	3880	5480	3920	3440	500	15600	5950	600	5950	2840
Rv1626 b	500	8230	6950	500	500	3930	13100	500	500	2980
Rv1626 b	500	5700	500	4070	2470	4500	18600	500	50	5950
Rv1626 b	500	16600	500	500	1010	500	500	500	500	4480
Rv1626 b	3400	500	3160	500	8230	6950	14000	500	5010	12000
Rv1626 b	5950	5950	500	7310	6600	1520	4030	2530	2860	500
Rv1626 b	5460	6170	5590	6830	3770	500	2670	4090	2540	500
Rv1789 b	1940	2750	2050	3920	6630	500	500	500	500	3010
Rv1789 b	3200	508	2590	1360	1260	4690	922	2660	2150	16500
Rv1789 b	500	2150	2200	2400	500	2390	390	3010	3630	500
Rv1789 b	3680	753	2120	2470	5470	816	396	3520	3380	500
Rv1789 b	780	2130	1760	500	500	1840	500	1760	2080	5740
Rv1789 b	500	2260	3260	500	500	500	2150	600	500	500
Rv2032 b	3160	2000	2590	500	3920	2470	500	2390	1020	1060
Rv2032 b	3860	785	5870	500	500	927	1990	1580	500	500

Rv2032 b	500	500	207	500	500	500	7930	500	500	500
Rv2032 b	500	500	500	4010	500	1570	949	500	500	500
Rv2032 b	4340	500	500	934	1880	2790	2380	2110	1460	1670
Rv2032 b	7820	406	4140	500	500	500	2180	3590	1690	1640
Wt b	50	50	50	50	50	50	50	50	50	50
Wt b	50	50	50	50	50	50	50	50	50	50
Wt b	50	50	50	50	50	50	50	50	50	50
Wt b	50	50	50	50	50	50	50	50	50	50
Wt b	50	50	50	50	50	50	50	50	50	50
Wt b	50	50	50	50	50	50	50	50	50	50
DDA	50	50	50	50	50	50	50	50	50	50
DDA	50	50	50	50	50	50	50	50	50	50
DDA	50	50	50	50	50	50	50	50	50	50
DDA	50	50	50	50	50	50	50	50	50	50
DDA	50	50	50	50	50	50	50	50	50	50
DDA	50	50	50	50	50	50	50	50	50	50

1C. IgG1 and IgG2c titres expressed in EC50 values in response to different vaccinations

Experiment →	Dose Response		Challenge			Immunogenicity		
Vaccine	**IgG1**	**IgG2c**	**Vaccine**	**IgG1**	**IgG2**	**Vaccine**	**IgG1**	**IgG2c**
Wt b	0	105	**BCG**	9820	1080	**Rv1626 b**	2480	8800
Wt b	0	357	**BCG**	3240	7500	**Rv1626 b**	741	0
Wt b	100	783	**BCG**	4000	0	**Rv1626 b**	2330	974
Wt b	556	117	**BCG**	8880	10700	**Rv1626 b**	53.3	8480
Wt b	948	888	**BCG**	4400	120	**Rv1626 b**	3250	2200
Wt b	106	242	**BCG**	4000	0	**Rv1626 b**	599	2770
Wt b	531	844	**BCG**	0	1030	**Rv1626 b**	1740	1180
Wt b	0	925	**BCG**	0	0	**Rv1626 b**	0	8600
1.25 µg	697	1050	**rec Rv1626**	7530	991	**Cpe30 b**	0	0
1.25 µg	913	8260	**rec Rv1626**	8190	0	**Cpe30 b**	4080	0
1.25 µg	4250	9360	**rec Rv1626**	9520	0	**Cpe30 b**	4280	4090
1.25 µg	1150	585	**rec Rv1626**	7350	0	**Cpe30 b**	6210	9170
1.25 µg	2120	1900	**rec Rv1626**	9420	4000	**Cpe30 b**	521	9990
1.25 µg	1410	513	**rec Rv1626**	0	0	**Cpe30 b**	361	9270
1.25 µg	2940	3280	**rec Rv1626**	8130	0	**Cpe30 b**	3360	0
1.25 µg	1680	9570	**rec Rv1626**	2810	9000	**Cpe30 b**	3360	0
2.5 µg	8980	2360	**Rv1626 b**	5260	19500	**CS.T3 b**	2540	0
2.5 µg	4850	411	**Rv1626 b**	7690	1740	**CS.T3 b**	3190	8140
2.5 µg	8610	0	**Rv1626 b**	2750	10500	**CS.T3 b**	1700	0
2.5 µg	784	1480	**Rv1626 b**	0	1150	**CS.T3 b**	4240	0
2.5 µg	4690	5180	**Rv1626 b**	3800	3150	**CS.T3 b**	0	17900
2.5 µg	2730	2200	**Rv1626 b**	905	8900	**CS.T3 b**	0	0
2.5 µg	9580	1730	**Rv1626 b**	4960	1290	**CS.T3 b**	4640	0

2.5 µg	4140	4010	**Rv1626 b**	3250	2480	**CS.T3 b**	2880	4520
5 µg	4990	4000	**Cpe30 b**	1170	0	**Fla b**	1480	9120
5 µg	8920	553	**Cpe30 b**	4000	7290	**Fla b**	3450	9290
5 µg	4120	0	**Cpe30 b**	4000	10500	**Fla b**	8750	0
5 µg	0	8950	**Cpe30 b**	0	8030	**Fla b**	1340	0
5 µg	1970	2230	**Cpe30 b**	5640	0	**Fla b**	2760	8880
5 µg	4730	0	**Cpe30 b**	0	0	**Fla b**	1200	9190
5 µg	1160	0	**Cpe30 b**	0	707	**Fla b**	446	0
5 µg	3960	1520	**Cpe30 b**	11800	0	**Fla b**	2850	9320
10 µg	4120	7230	**CS.T3 b**	0	0	**Comb b**	0	1580
10 µg	7290	441	**CS.T3 b**	734	273	**Comb b**	2680	20800
10 µg	4560	3690	**CS.T3 b**	8540	0	**Comb b**	1590	97.9
10 µg	12700	4890	**CS.T3 b**	0	4430	**Comb b**	480	4070
10 µg	0	2290	**CS.T3 b**	0	3220	**Comb b**	4650	0
10 µg	4960	664	**CS.T3 b**	7800	959	**Comb b**	0	1120
10 µg	373	1100	**CS.T3 b**	8240	0	**Comb b**	1380	5530
10 µg	0	169	**CS.T3 b**	1160	1730	**Comb b**	0	0
DDA	0	417	**DDA**	856	0	**DDA**	0	115
DDA	107	919	**DDA**	315	958	**DDA**	0	0
DDA	0	884	**DDA**	642	143	**DDA**	0	648
DDA	804	117	**DDA**	0	207	**DDA**	0	102
DDA	425	0	**DDA**	105	0	**DDA**	0	332
DDA	0	0	**DDA**	0	862	**DDA**	0	984
DDA	102	0	**DDA**	366	0	**DDA**	0	103
DDA	110	248	**DDA**	426	0	**DDA**		864

1D. Cytokine responses of mice splenocytes upon stimulation with rec Rv1626 and analysed by cytometry bead array

Vaccine	IL10	IL17	TNF-α	IFN-γ	IL6	IL4	IL2
Rv1626 b	177.37	1,561.48	1,856.37	444.73	2,932.88	3.21	105.02
Rv1626 b	222.11	2,981.42	2,913.17	486	3,517.53	1.86	61.58
Rv1626 b	65.18	452.77	1,267.24	436.89	1,516.15	1.71	66.59
Rv1626 b	85	1,199.69	1,309.24	223.72	1,563.13	1.75	75.88
Rv1626 b	170.79	236.48	1,723.55	120.53	1,809.91	2.11	51.41
Rv1626 b	174.9	2,589.92	3,235.48	1,175.03	4,040.02	2.37	113.34
Rv1626 b	263.05	1,273.81	3,192.24	3,034.65	4,518.71	3.66	121.25
Rv1626 b	288.22	1,327.03	4,962.72	1,771.80	6,353.07	2.86	261.34
Cpe30 b	258.03	1,320.33	2,939.34	184.07	2,884.93	2.06	48.55
Cpe30 b	347.1	4,005.68	2,389.92	466.87	3,717.96	1.61	26.09
Cpe30 b	219.61	2,240.66	2,597.12	154.68	3,006.56	1.63	48.16
Cpe30 b	341.21	3,614.35	2,058.27	178.63	3,462.23	1.48	22.7
Cpe30 b	221.28	2,085.22	2,194.17	166.48	2,498.46	1.33	18.69
Cpe30 b	313.43	2,927.29	2,206.98	310.84	3,295.37	1.97	45.03
Cpe30 b	297.46	5,573.73	3,202.12	543.96	4,243.80	1.76	35.94
Cpe30 b	289.9	1,048.87	2,251.93	376.38	2,737.88	1.52	49.26
CS.T3 b	302.5	2,089.99	1,420.91	286	2,612.26	1.11	40.87
CS.T3 b	270.59	4,363.92	1,810.81	234.07	3,620.62	1.71	40.42
CS.T3 b	204.65	2,300.05	2,098.60	273.05	3,774.54	0.86	24.6
CS.T3 b	212.96	2,733.29	1,873.01	393.7	10,241.25	1.46	36.76
CS.T3 b	219.61	2,034.24	1,966.21	102.7	2,648.70	1.03	30.16
CS.T3 b	280.66	1,453.71	2,446.29	534.98	4,379.65	1.24	35.58
CS.T3 b	190.57	1,307.69	1,913.60	115.35	1,580.88	1.2	55.81
CS.T3 b	236.28	2,687.30	2,722.36	379.49	3,484.29	1.56	72.73

Fla b	189.74	430.92	2,341.35	469.41	3,539.91	0.78	14.25
Fla b	75.01	75.18	1,316.00	96.04	3,027.18	0.14	7.77
Fla b	130.89	472.86	897.47	65.78	1,726.80	0.1	9.85
Fla b	83.45	226.05	1,399.76	49.54	2,407.04	0.61	14.66
Fla b	160.13	1,574.35	2,383.07	436.2	2,934.31	1.67	27.97
Fla b	63.68	383.93	914.68	45.54	1,231.49	1.03	22.25
Fla b	460.81	5,532.50	2,677.65	1,391.45	6,575.39	1.99	77.14
Fla b	110.09	121.94	1,827.06	447.63	3,368.73	0.36	19.47
Comb b	81.91	394.77	1,251.05	160.79	2,228.03	0.55	6.86
Comb b	171.61	673.98	3,482.50	287.74	3,516.89	1.54	12.14
Comb b	100.61	675.32	1,668.05	323.76	2,895.48	1.41	10.71
Comb b	119.65	96.19	1,638.98	330.86	3,084.71	1.03	13.73
Comb b	166.69	246.32	1,111.86	306.87	2,360.37	1.33	13.16
Comb b	169.97	822.73	1,390.14	140.65	1,948.16	1.28	24.88
Comb b	105.34	1,042.04	2,169.95	353.46	2,531.06	1.18	22.91
Comb b	42.48	1.85	2,455.25	37.41	1,892.70	0.59	5.26
DDA	54.05	6.35	2,266.50	110.05	2,343.58	0.7	5.67
DDA	77.3	16.15	1,628.46	52.53	2,519.88	0.7	5.78
DDA	54.05	19.55	1,864.52	129.44	3,135.22	0.61	4.84
DDA	18.68	2.53	560.3	3.18	577.94	0.38	5.73
DDA	24.57	1.09	870.13	1.12	475.33	0.39	3.55
DDA	24.57	2.83	606.08	4.01	707.89	0.49	4.05
DDA	17.39	1.12	729.36	1.42	631.65	0.49	3.7

1E. Cytokine responses of mice splenocytes upon stimulation with rec Rv1626 or PPDB.

	IL10		IL17A		TNF-α		IFN-γ		IL6		IL4		IL2	
Vaccine	**rec**	**PPDB**	**rec**	**PPDB**	**rec**	**PPDB**	**rec**	**PPDB**	**rec**	**PPDB**	**rec**	**PPDB**	**rec**	**PPDB**
BCG	43.41	14.24	0.9	17.38	1,796.06	1,665.62	14.78	3,622.25	407.02	173.07	0	0.35	6.72	46.78
rec Rv1626	55.23	2.26	277.76	0	1,853.00	43.44	77.9	0	638.56	4.75	2.64	0	34.4	5.28
Rv1626 b	198.73	5.67	1,156.69	2.15	6,731.18	122.31	6,896.30	1.48	2,361.21	5.53	6.52	0	253.64	6.89
Cpe30 b	205.11	7.14	502.13	1.87	9,869.31	217.24	2,800.60	1.38	2,220.08	12.24	2.71	0	34.05	6.03
CS.T3 b	181.83	7.86	141.8	0.69	3,480.85	138.77	760.43	1.48	962.28	7.86	0	0	27.62	6.56
DDA	14.95	4.1	0	0	1,054.01	43.44	0.32	0	94.03	4.07	0	0	5.47	4.86
BCG	59.57	0	0.21	0	2,216.50	0	2.59	500	354.29	0	0	0	6.03	0
rec Rv1626	74.75	5.67	105.75	0.42	2,413.60	93.84	156.01	0	1,128.05	10.21	0.96	0	26.04	5.85
Rv1626 b	177.64	0	958.92	5.79	6,141.39	118.4	1,488.91	0.96	1,667.59	8.75	9.57	0	111.07	7.52
Cpe30 b	310.75	4.1	844.58	2.29	10,942.85	128.73	3,519.89	1.07	2,979.28	14.52	3.14	0	58.54	7.21
CS.T3 b	162.03	8.57	89.7	0.69	4,612.32	299.63	783.66	2.59	1,363.29	14.18	0	0	22.13	7.05
DDA	28.26	2.26	2.08	1.65	2,050.07	59.28	8.47	0	318.61	9.09	0	0	7.37	4.86
BCG	37.7	4.9	0.69	3.15	1,373.99	598.78	1.28	228.56	106.05	54.53	0	0	5.47	20.72
rec Rv1626	91.47	6.41	191.22	0.99	3,538.01	171.81	699.64	1.38	1,972.37	15.67	2.12	0	53.36	8.97

Rv1626 b	192.37	4.9	1,341.29	6.71	8,115.26	364.14	8,808.00	49.2	3,795.91	21.87	4.44	0	73.48	16.19
Cpe30 b	176.6	0.65	740.55	10.08	7,856.69	110.27	2,749.03	0	2,725.15	7.75	7.73	0	125.47	6.72
CS.T3 b	183.94	2.26	827.21	3.02	3,823.07	92.8	2,004.84	2.35	1,623.95	28.32	2.32	0	85.95	5.07
DDA	24.46	0.65	0.8	0	2,312.77	59.94	2.43	0	245.9	9.31	0	0	5.67	5.47
BCG	27.5	8.57	1.49	24.16	1,188.52	1,926.32	5.29	4,880.07	311.33	218.4	0	0.87	5.28	71.63
rec Rv1626	38.51	0	105.81	0	4,070.75	109.92	243.99	0	1,420.49	5.64	0.07	0	24.57	4.86
Rv1626 b	316.29	0.65	1,677.33	3.47	9,731.90	193.88	4,334.58	0	4,214.40	15.67	9.02	0	352.64	6.03
Cpe30 b	201.91	5.67	850.03	2.76	9,216.97	176.94	1,835.27	0.32	2,452.14	10.21	0.46	0	26.91	6.03
CS.T3 b	198.73	4.1	716.45	8.98	7,145.52	227.31	2,994.14	3.89	1,939.23	17.18	2.45	0	96.62	8.12
DDA	9.99	0	0.99	0.9	1,302.64	52.1	3.67	0	155.83	5.31	0	0	5.47	5.07
BCG	9.28	0	0	0	1,025.44	68.25	1.84	12.41	76.55	4.3	0	0	4.64	6.03
rec Rv1626	10.7	0	13.7	0	673.95	18.38	5.22	0	191.31	1.51	0	0	7.21	4.64
Rv1626 b	57.82	0	313.24	0	3,988.59	39.04	798.47	0	1,179.32	2.43	3.02	0	97.58	4.18
Cpe30 b	122.49	0	513.32	0.69	4,834.56	54.7	1,309.30	0	1,161.03	3.5	0.57	0	70.15	3.65
CS.T3 b	84.9	3.25	242.35	0	3,121.31	83.15	1,138.28	0	1,024.67	4.75	0	0	64.23	5.85
DDA	9.99	0	1.65	0	911.14	26.82	2.1	0	120.51	3.27	0	0	5.28	5.47
BCG	14.24	0	0	0	927.15	0	0	0	91.86	0	0	0	3.03	0

rec Rv1626	0	0	0	0	12.5	21.24	0	0	1.06	1.51	0	0	3.65	3.65
Rv1626 b	81.18	0	549.38	1.26	3,050.58	25.64	680.14	0	831.52	4.52	2.89	0	143.64	4.18
Cpe30 b	89.58	2.26	607.6	2.08	3,358.07	113.8	809.41	0	1,128.17	6.09	0.07	0	84.87	6.21
CS.T3 b	96.21	0	274.13	3.22	4,485.42	172.54	1,094.29	1.18	1,334.68	5.2	0	0	43.98	6.56
DDA	14.95	0	0	0	1,197.43	46.3	1.18	0	130.88	2.79	0	0	4.18	4.64
BCG	28.26	9.28	3.98	4.67	760.98	187.24	0.54	52.76	138.44	16.48	0	0	4.86	9.51
rec Rv1626	21.48	0	49.03	0	1,234.75	22.11	56.03	0	375.14	1.65	0	0	20.9	4.42
Rv1626 b	0	0	0	0	0	80.42	0	0	0	5.98	0	0	0	3.92
Cpe30 b	120.5	0	793.65	1.79	3,479.53	90.72	1,218.06	0.32	1,312.57	4.52	0	0	126.63	7.05
CS.T3 b	83.03	0	178.24	1.49	3,527.45	84.53	940.94	0	989.68	2.91	0	0	85.22	4.18
DDA	11.4	0	0.9	0	1,906.30	57.31	0	0	185.32	3.96	0	0	4.18	4.18
BCG	13.53	0	0	0	821.38	171.45	0	36.47	123.07	11.45	0	0	3.92	6.39
rec Rv1626	17.82	0	36.16	0	1,013.78	25.93	22.33	0	404.9	2.91	0	0	14.54	3.03
Rv1626 b	72.94	0	306.21	1.72	2,887.61	33.77	450.18	0	968.19	2.55	1.3	0	98.98	5.28
Cpe30 b	73.84	0	268.31	0	4,119.97	38.1	745.18	0	1,264.12	1.92	0	0	43.36	5.67
CS.T3 b	88.64	0	289.32	1.57	3,493.59	101.15	1,004.07	1.38	1,486.46	4.19	0	0	52.24	3.92
DDA	12.82	0	1.08	0	1,909.22	58.3	10.04	0	319.36	1.92	0	0	3.36	5.28

1F. Vaccine induced protection (log $_{10}$ CFUs) in lung or spleen after *M. bovis* aerosol infection

Vaccine	lungs	spleens
BCG	5.477121	4.021189
BCG	4.732394	4.093422
BCG	4.69897	3.732394
BCG	4.799341	3.819544
BCG	4.838849	3.897627
BCG	4.612784	3.826075
BCG	4.681241	3.623249
BCG	4.832509	3.845098
rec Rv1626	5.763428	4.812913
rec Rv1626	5.579784	4.653213
rec Rv1626	6.826075	4.826075
rec Rv1626	6	4.886491
rec Rv1626	5.724276	4.944483
rec Rv1626	7.544068	5.748188
rec Rv1626	6.748188	4.819544
rec Rv1626	5.579784	4.531479
Rv1626 b	5.740363	4.681241
Rv1626 b	5.477121	4.255273
Rv1626 b	5.70757	4.732394
Rv1626 b	5.69897	4.763428
Rv1626 b	5.763428	4.716003
Rv1626 b	5.740363	5.146128
Rv1626 b	5.892095	4.78533
Rv1626 b	5.612784	4.770852
Cpe30 b	5.740363	4.70757
Cpe30 b	6.50515	4.770852
Cpe30 b	6.113943	4.653213
Cpe30 b	6.278754	4.740363
Cpe30 b	5.826075	4.869232
Cpe30 b	6.033424	4.662758
Cpe30 b	6.176091	4.623249
Cpe30 b	5.851258	4.740363
CS.T3 b	6.322219	4.857332
CS.T3 b	5.681241	4.672098
CS.T3 b	5.869232	4.892095
CS.T3 b	5.662758	4.477121
CS.T3 b	6.20412	4.80618

CS.T3 b	5.690196	4.60206
CS.T3 b	7.30103	5.60206
CS.T3 b	8.954243	6.556303
DDA	6.462398	4.880814
DDA	5.792392	5.113943
DDA	5.690196	4.255273
DDA	5.623249	4.623249
DDA	5.792392	6.079181
DDA	5.462398	4.633468
DDA	5.732394	4.623249
DDA	8.90309	4.50515

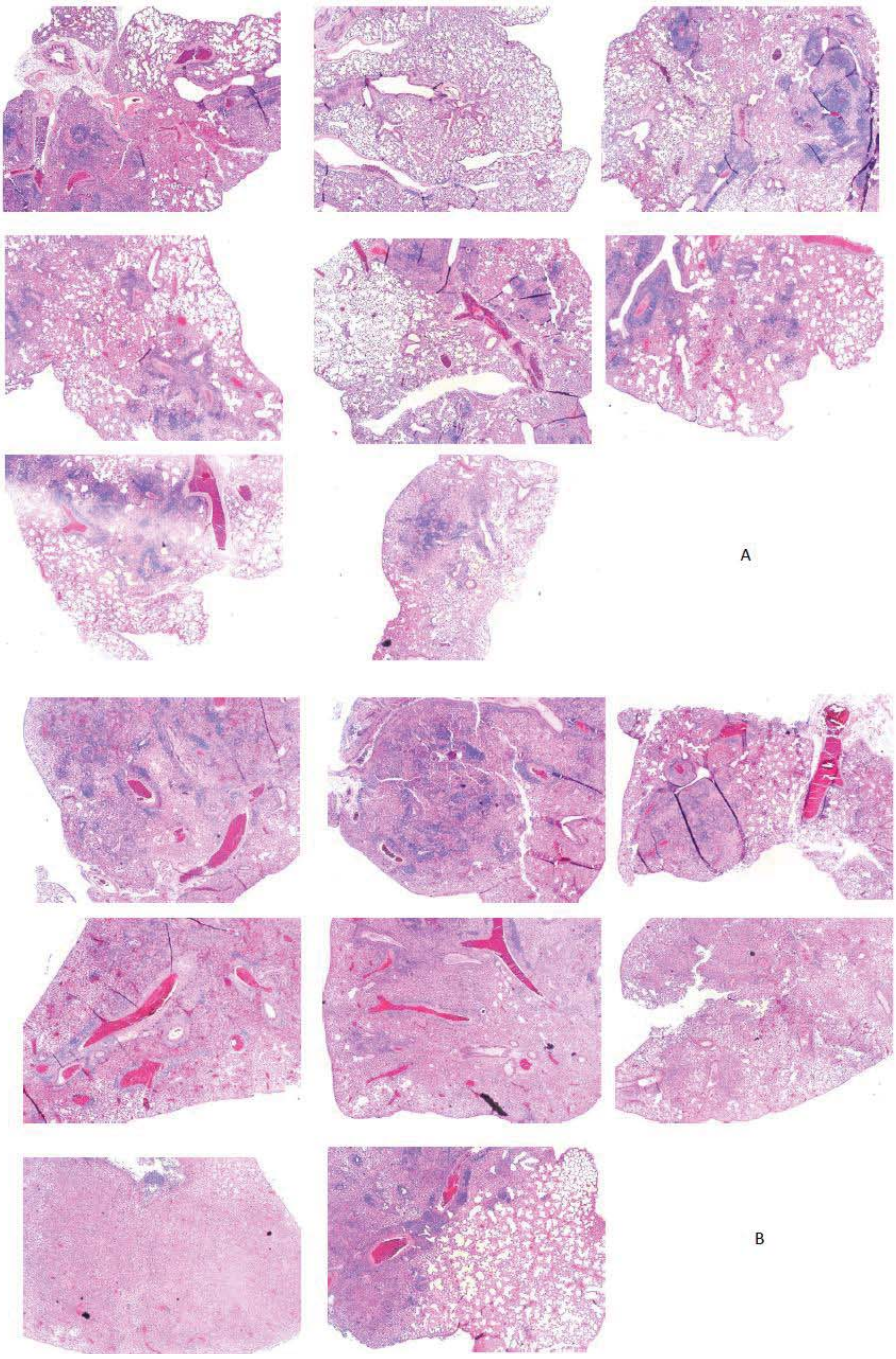
Appendix 2:

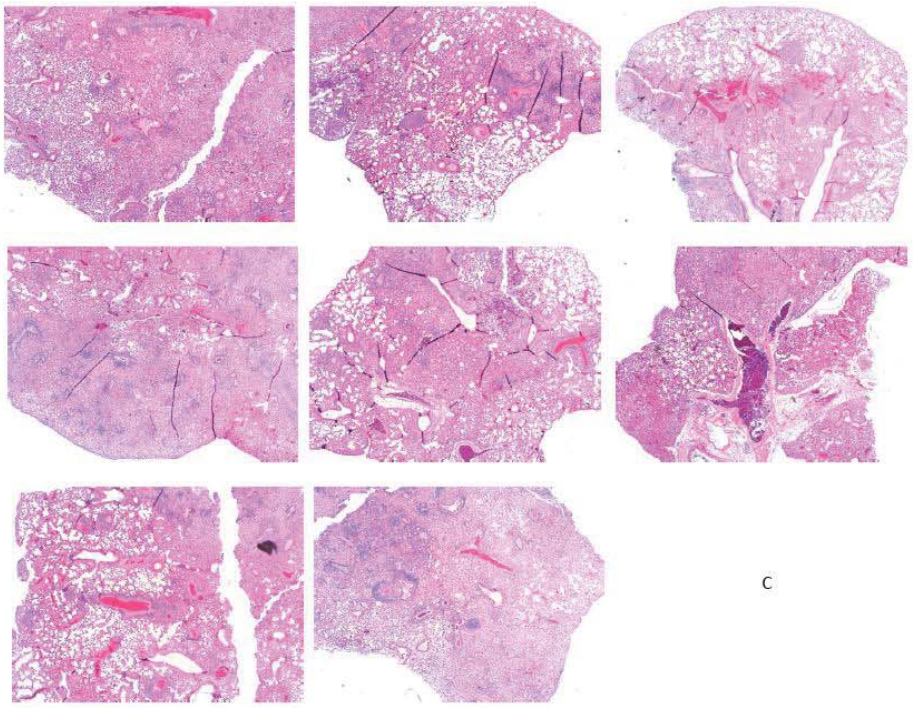
Amino acid sequence of protein PhaC-Cpe30-CS.T3-Fla66-Rv1626. In bold, peptides identified by MALDI-TOF/MS. Protein sequence coverage: 70 %.

1 MATGKGAAAS TQEGKSQPFK **VTPGPFDPAT WLEWSRQWQG TEGNGHAAAS**
51 **GIPGLDALAG VKIAPAQLGD IQQRYMKDFS ALWQAMAEGK AEATGPLHDR**
101 **RFAGDAWRTN LPYRFAAAFY LLNARALTEL ADAVEADAKT RQR**IR**FAISQ**
151 **WVDAMSPANF LATNPEAQRL LIESGGESLR AGVRNMMEDL TRGKISQTDE**
201 **SAFEVGRNVA VTEGAVVFEN EYFQLLQYKP LTDKVHARPL LMVPPCINKY**
251 **YILDLQPESS LVRHVVEQGH TVFLVSWRNP DASMAGSTWD DYIEHAAIR**A
301 IEVARDISGQ DKINVLGFCV GGTIVSTALA VLAARGEHPA ASVTLLTTLL
351 DFADTGILDV FVDEGHVQLR **EATLGGGAGA PCALLR**GLEL ANTFSFLRPN
401 DLVWNYVVDN YLKGNTPVPF DLLFWNGDAT NLPGPWYCWY LR**HTYLQNEL**
451 **KVPGKLTVCG VPVDLASIDV PTYIYGSRED HIVPWTAAYA STALLANKLR**
501 **FVLGASGHIA GVINPPAKNK** RSHWTNDALP ESPQQWLAGA IEHHGSWWPD
551 WTAWLAGQAG AK**RAAPANYG NARYRAIEPA PGRYVKAKAV LAVAIDKRGG**
601 **GGGLEMSLDA GQYVLVMKAN SSYSGNYPYS ILFQKFDIEK K**IAKMEK**ASS**
651 **VFNVDIEKKI AK**MEKASSVF NVNANDGISI AQTTEGALNE INNNLQR**VRE**
701 **LAVQSANSTN SQSDLDSIQA EITQRLNEID RVSGQTQFNG VK**VLAQDNTL
751 TIQVGANDGE TIDIDLK**QIN SQTLGLDTLN VQQKYKVSDT AATVTGYADT**
801 **TIALDNSTFK ASATGLGGTD QKIDGDLKFD DTTGKYYAKV TVTGGTGKDG**
851 **YYEVSVDKTN GEVTLAGGAT SPLTGGLPAT ATEDVKNVQV ANADLTEAKA**
901 **ALTAAGVTGT ASVVKMSYTD NNGKTIDGGL AVKVGDDYYS ATQNKDGSIS**
951 **INTTK**YTADD GTSK**TALNKL GGADGKTEVV SIGGK**TYAAS K**AEGHNFKAQ**
1001 **PDLAEAAATT TENPLQKIDA ALAQVDTLRS DLGAVQNRFN SAITNLGNTV**
1051 **NNLTSARSRI EDSDYATEVS NMSR**AQILQQ AGTSVLAQAN QVPQNVLSLL
1101 R**TTLYTGPTT DADAAVPRRV LIAEDEALIR MDLAEMLR**EE GYEIVGEAGD
1151 GQEAVELAEL HKPDLVIMDV KMPR**RDGIDA ASEIASKRIA PIVVLTAFSQ**
1201 **RDLVER**ARDA GAMAYLVKPF SISDLIPAIE LAVSR**FREIT ALEGEVATLS**
1251 **ER**LETRKLVE R**AKGLLQTKH GMTEPDAFKW IQR**AAMDRRT TMK**RVAEVVL**
1301 **ETLGTPKDTG S**

Appendix 3:

Histological appearance of lungs from mice vaccinated with A, BCG; B, rec Rv1626; C, Rv1626 beads; D, Cpe30-Rv1626 beads; E, CS.T3-Rv1626 beads; F, DDA alone. Lung sections were stained with H&E.





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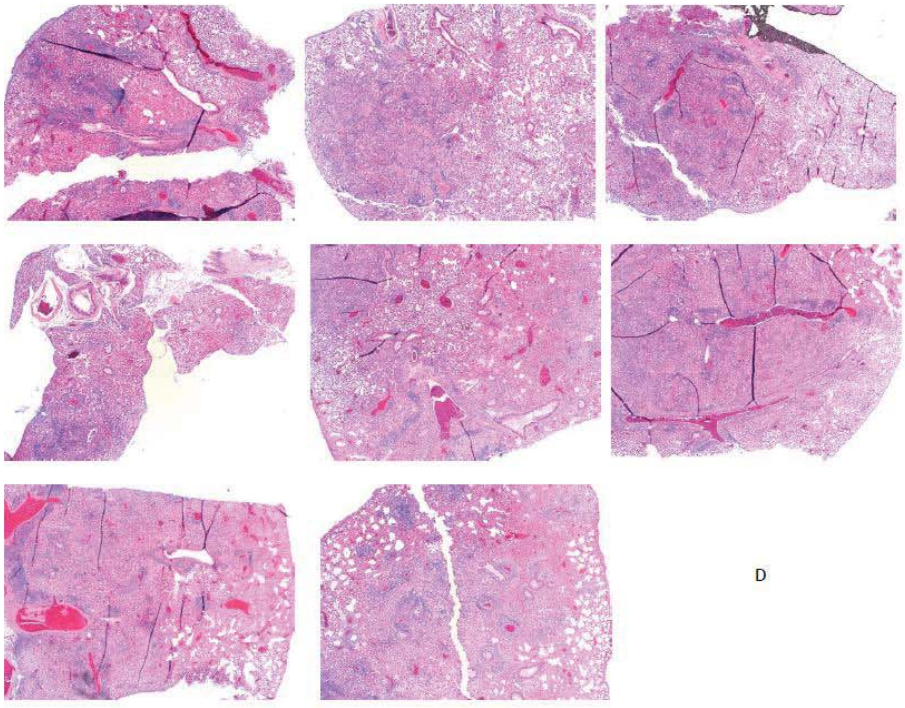

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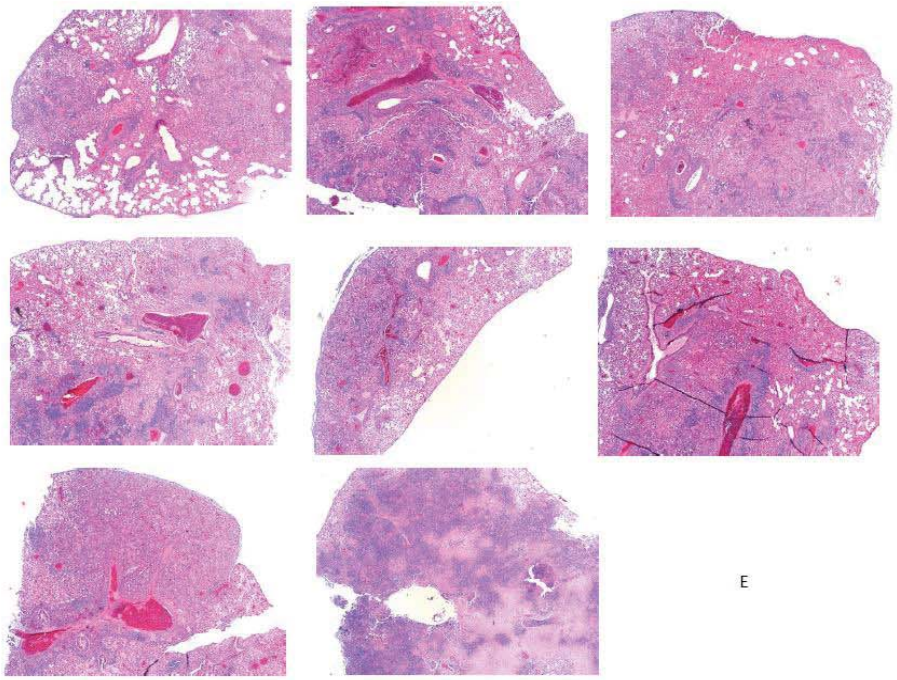

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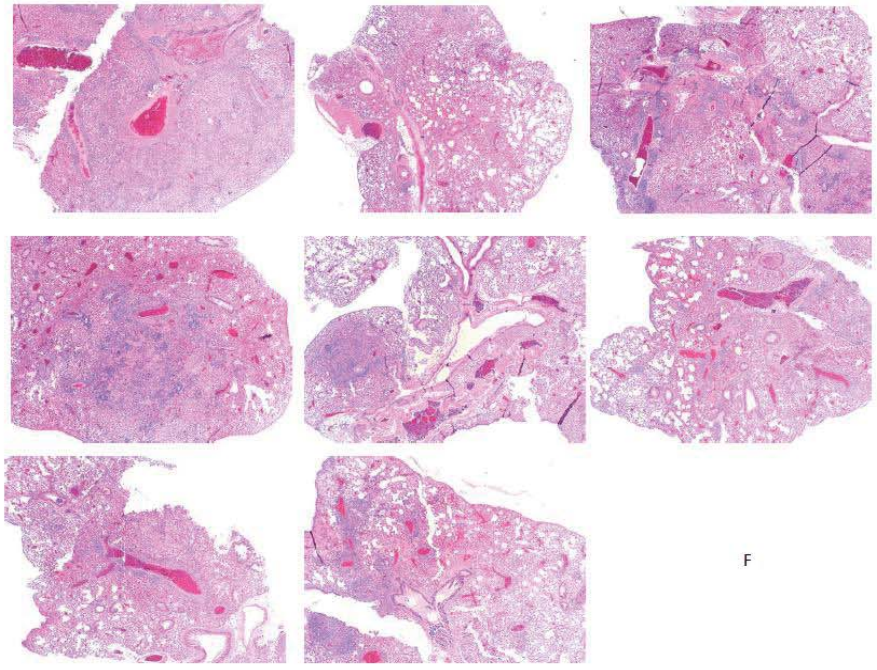

F

Appendix 4 :

In vivo polyester immobilized sortase for tagless protein purification

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Abstract

Background: Laboratory scale recombinant protein production and purification techniques are often complicated, involving multiple chromatography steps and specialized equipment and reagents. Here it was demonstrated that recombinant proteins can be expressed as covalently immobilized to the surface of polyester (polyhydroxyalkanoate,PHA) beads in vivo in *Escherichia coli* by genetically fusing them to a polyester synthase gene (*phaC*). The insertion of a self-cleaving module, a modified sortase A (SrtA) from *Staphylococcus aureus* and its five amino acid recognition sequence between the synthase and the target protein led to a simple protein production and purification method.

Results: The generation of hybrid genes encoding tripartite PhaC-SrtA-Target fusion proteins, enabled immobilization of proteins of interest to the surface of PHA beads in vivo. After simple cell lysis and isolation of the PHA beads, the target proteins could be selectively and efficiently released form the beads by activating the sortase with $CaCl_2$ and triglycine. Up to 6 mg/l of soluble proteins at a purity of ~98 % could be isolated in one step with no optimization. This process was used to produce and isolate three proteins: Green fluorescent protein, maltose binding protein and the *Mycobacterium tuberculosis* vaccine candidate Rv1626.

Conclusions: We have developed a new technique for easy production and purification of recombinant proteins. This technique is capable of producing and purifying high yields of proteins suitable for research application in less than 2 days. No costly or specialized protein chromatography equipment, resins, reagents or expertise are required.

Keywords: Protein purification, Polyhydroxyalkanoate, Sortase, Self-cleavage

Abbreviations

PHA: polyhydroxyalkanoate; GFP: green fluorescent protein; MBP: maltose binding protein; IPTG: isopropyl β-d-1-thiogalactopyranoside; DABCYL: 4-((4 (dimethylamino) phenyl) azo)

benzoic acid; EDANS: 5-((2-Aminoethyl) amino)naphthalene-1 sulfonic acid; EGTA: ethylene glycol tetraacetic acid; FRET: Förster resonance energy transfer.

Introduction

The expression and purification of a target protein of interest is a common undertaking in many research laboratories. This task is often complicated by the multiple chromatography steps required to obtain a product of acceptable purity. Furthermore, most lab-scale purification techniques require an affinity tag such as His, Strep, or GST tag to be engineered into the protein [1]. These tags require specific and often costly chromatography resins to isolate the target protein. Where tags may affect the structure, function or immunogenicity of the target protein (e.g. protein crystallography or antigens for antibody generation), they may need to be removed after purification by engineering a site-specific protease recognition site between the tag and the target protein. To remove the tag the purified protein is treated with a site-specific protease such as TEV protease; the released tag and added protease must then be removed from the sample by further chromatography steps [2]. This introduces additional steps to the workflow, decreasing the yield, and increases the risk of protein loss/degradation. Furthermore, the cleavage can often result in a "scar" (additional residual amino acids) which may negatively impact the target protein.

An alternative approach is to utilize a self-cleaving affinity tag. This approach utilizes a hybrid module composed of an affinity tag and an auto-processing protease or a modified intein. The target protein is genetically fused to this module and bound to the affinity resin, the auto-cleaving reaction can be activated by the addition of a cofactor (e.g. metal ions) or a shift in temperature or thiol for intein auto processing. The target protein can be eluted from the resin while the self-cleaving affinity tag remains bound [3]. An advantage of this technique is that many of these auto-processing domains result in no or minimal scars on the target protein after cleavage.

A modified form of the well-characterized cell surface sortase transpeptidase A (SrtA) from *Staphylococcus aureus* [4] represents one of such auto processing modules. In Gram

positive bacteria sortase proteins are responsible for linking specific secreted proteins to the cell wall peptidoglycan. SrtA does this by recognising a five amino acid “sorting signal” in the target protein, cleaving this signal and linking the C-terminus of the protein to pentaglycine in the cell wall. A soluble form of SrtA has previously been modified to self-cleave in the presence of Ca^{2+} by including the sorting signal on its C-terminus. By fusing a His tag to the N-terminus and a protein of interest immediately after the sorting signal a simple purification technique was developed [5, 6]. Cleavage occurs between T and the G of the sorting signal leaving a single additional G on the N-terminus of the C terminally fused target protein.

Here we utilized the ability to covalently immobilize proteins to the surface of bacterially produced polyester beads in vivo. This approach exploits the ability to genetically fuse proteins or protein domains to the PHA synthase from *Ralstonia eutropha* (PhaC). When the PHA synthase is supplied with *R*-(3)-hydroxybutyryl-CoA by the PhaA and PhaB enzymes it produces an insoluble polyester in the form of polyhydroxyalkanoate inside the cell. The PHA synthase remains covalently attached to the nascent PHA chain, which self-assembles into beads in the bacterial cytosol with a diameter of 100–500 nm. When the synthase is overexpressed in *Escherichia coli* it densely coats the surface of these beads [7, 8]. This technique has been employed to display various proteins and domains on the surface of PHA beads [9–14]. By combining this technique with the sortase based auto-cleavage process described above (replacing the His tag with the PHA synthase) we remove the requirement to use costly affinity resins, as the fusion protein is covalently attached to PHA beads that can be easily isolated from the cell (Figure 1).

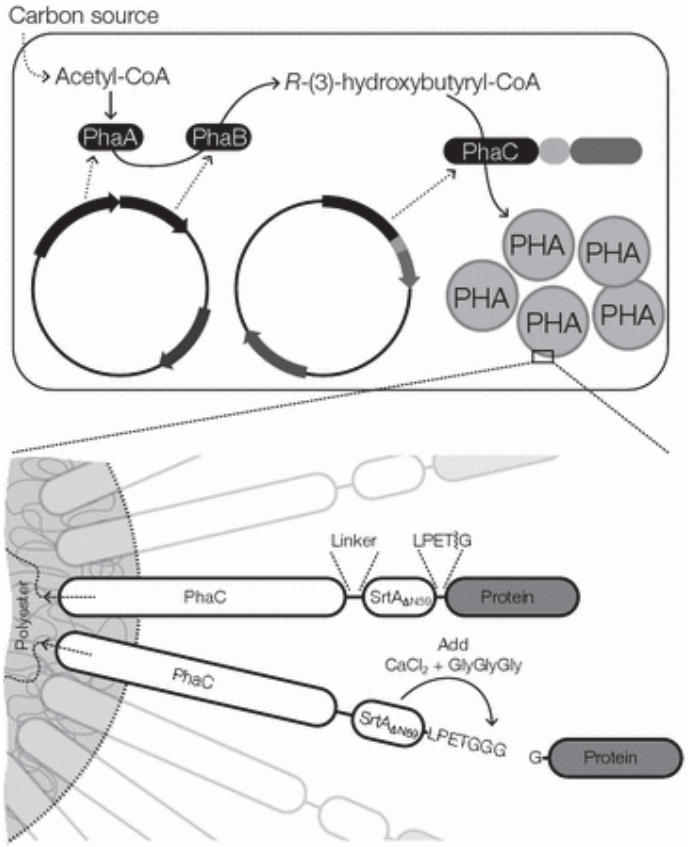

Figure 1 Schematic representation of the PHA immobilized sortase protein production and purification method described in this study

We demonstrated the practicality of this technique by recombinantly expressing and purifying three proteins at laboratory scale. This method can produce high yields of proteins without the need to modify the protein with cumbersome affinity tags. The proteins were purified to a level adequate for most common research laboratory needs without the need for specialised protein purification equipment or expertise.

Materials and Methods

Bacterial strains and growth conditions

All *E. coli* strains and plasmids used in this study are listed in Table 1. Strains were grown in Terrific Broth (12 g/l tryptone, 24 g/l yeast extract, 4 ml/glycerol, 17 mM KH_2PO_4, 72 mM

K_2HPO_4) at 37 °C. Where required, antibiotics were used at the following concentrations: ampicillin, 75 µg/ml; chloramphenicol, 34 µg/ml. For PHA bead and protein production, *E. coli* strains were first transformed with the PhaA and PhaB encoding plasmid pMCS69 [28], and subsequently with the fusion protein expressing plasmid. Cultures were grown in TB with 37 °C to an optical density at 600 nm (OD_{600}) of 0.4–0.5, induced with 1 mM IPTG (isopropyl- β-d-thiogalactopyranoside), and allowed to grow for approximately 16 h.

Table 1. List of all *E. coli* strains and plasmids used in this study.

Strain/Plasmid	Description	Reference
E. coli BL21 DE3	F− ompT hsdSB (rB− mB−) gal dcm (DE3)	Novagen
Clear coli	ΔgutQ ΔkdsD ΔlpxL ΔlpxM ΔpagP ΔlpxP ΔeptA. msbA148. Modified LPS with no endotoxic response	Lucigen
pMCS69	pBBR1MCS with phaA and phaB	28
pETC	pET14b containing wildtype phaC	14
pET14:PhaC-linker-MalE	pET14b encoding a PhaClinker-MalE fusion protein	12
pET14:PhaC-SrtA	pET14b encoding a PhaClinker-SrtAΔN59 fusion protein	This study
pET14:PhaC-SrtA-GFP	pET14b encoding a PhaClinker-SrtAΔN59-LPETG-GFP fusion protein	This study
pET14:PhaC-SrtA-MBP	pET14b encoding a PhaClinker-SrtAΔN59-LPETG-MBP fusion protein	This study
pET14:PhaC-SrtA-Rv1626	pET14b encoding a PhaClinker-SrtAΔN59-LPETGRV1626 fusion protein	This study

Plasmid construction

The PhaC-SrtA expressing plasmid (pET14:PhaC-SrtA) was constructed as follows: The srtA gene minus the N-terminal 59 amino acid membrane anchor region flanked by XhoI and

*Bam*HI sites was synthesized by Genscript, the product was cleaved with *Xho*I and *Bam*HI and ligated into the corresponding sites on the plasmid pET14b:phaC-linker-MalE [12].

To make the tripartite fusion protein expressing plasmids (pET14: PhaC-SrtA-GFP, pET14: PhaC-SrtAMBP, pET14: PhaC-SrtA-RV1626) the srtAΔN59 region was amplified from pET14: PhaC-SrtA with the primers SrtAN59_F and SrtAΔTAA_LPETG_R—this product does not have a stop codon and has LPETG coding region added on the C-terminus (with the TG encoded by an *Age*I site). This was ligated into pGEMteasy according to manufactures instructions. The GFP, MBP, and Rv1626 coding regions were synthesized by Genscript or amplified from existing sources with the start codon replaced with an *Age*I site and a *Bam*HI site after the stop codon. The target genes were ligated into the *Age*I and *Bam*HI sites of the pGEMteasy-SrtA plasmid described above. The srtA-target genes were cut from the pGEMteasy backbone with *Xho*I and *Bam*HI and ligated into the corresponding sites on the plasmid pET14b: phaC-linker-MlaE.

PHA bead isolation

Cells were harvested by centrifugation at 6000 g and washed once in TBS (50 mM Tris–Cl, pH 7.8, 150 mM NaCl) with 10 mM EGTA. Cell pellets were suspended in 1/3 culture volume TBS, 10 mM EGTA, 50 µg/ml lysozyme, 10 µg/ml DNase, 1X Bugbuster (Novagen) and incubated for 15 min at room temperature after which they were sonicated (20 on 20 s off) for a total of 1 min sonication. After lysis the insoluble PHA material was collected by centrifugation at 6000*g* and resuspended in fresh TBS, 10 mM EGTA, 0.05 % Tween20 with brief sonication. This washing step was repeated twice. Finally the beads were resuspended to 20 % slurry in TBS, 10 mM EGTA, 0.05 % Tween20.

Activation of sortase beads and isolation of target protein

To activate the sortase beads the slurry was centrifuged at 6000*g* and the pellet resuspended to a 20 % slurry in TBS, 0.05 % Tween20, 5 mM $CaCl_2$, 10 mM Gly-Gly-Gly. The beads were incubated on a rotary mixer at 37 °C for 1–24 h. To isolate the released

soluble target protein the mixture was centrifuged at 13,000 *g* for 10 min and the supernatant analyzed by SDS-PAGE.

Sortase A assay

To assess the function of the PhaC-SrtA beads a synthetic DABCYL-LPETG-EDANS substrate (Anaspecwas used). The peptide was dissolved in DMSO and added at a final concentration of 5 μM to a 5 % slurry of beads in TBS with 5 mM $CaCl_2$. Fluorescence was monitored over time using a FLUOstar Omega (BMG labtech) microplate reader with the sample shaking between readings.

Results and discussions

Sortase transpeptidase from *S. aureus* can be functionally immobilized on PHA beads

To first assess whether the sortase A transpeptidase for *S. aureus* (SrtA) could be functionally immobilized on the surface of PHA beads in vivo we made genetic fusions of the soluble (non-membrane anchored) form of SrtA (SrtAΔN59) [15] to the C-terminus of PhaC from *Ralstonia eutropha*. A “linker” (VLAVIDKRGGGGG) is included between the two proteins to allow them to fold and function independently [12]. When this hybrid gene was introduced into *E. coli* strains harboring the plasmid pBBRMCS69 (containing *phaA* and *phaB* to provide the *R*-(3)-hydroxybutyryl-CoA substrate for PhaC PHA synthase) it mediated production of similar yields of both PHA and fusion protein when compared to the unmodified PhaC gene (data not shown). To assess the peptidase activity of the immobilized SrtA we employed a synthetic fluorescently self-quenched peptide FRET substrate, composed of the fluorophore (EDANS) and a quencher (DABCYL) separated by the 5 amino acid *S. aureus* sorting signal (LPETG). If the sorting signal is cleaved then the fluorophore is separated from the quencher and its fluorescence can be detected. The substrate was added to a suspension of the PHA beads and incubated. No significant activity could be detected from

the PhaC beads, whereas significant activity could be detected from the PhaC-SrtA beads (Figure 2). The activity was dependent on the presence of $CaCl_2$ and could be removed by denaturing the protein on the beads at 95 °C for 15 min before conducting the assay, indicating that this activity is the result of the immobilized sortase.

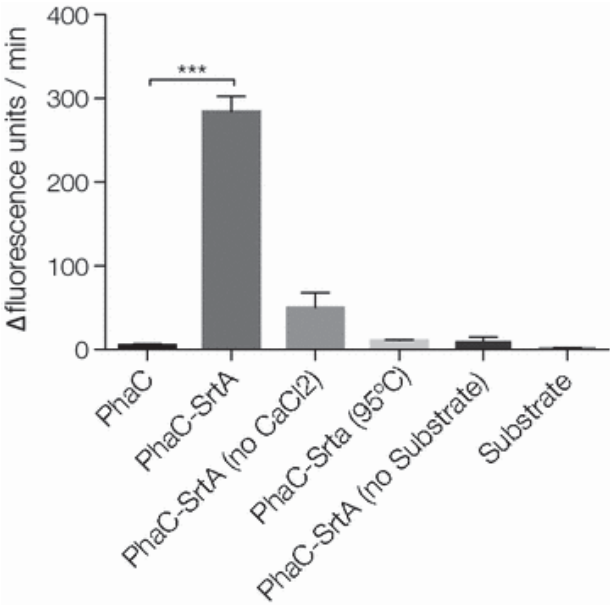

Figure 2 Activity of the PhaC-SrtA PHA beads measured via cleavage of the synthetic sortase FRET substrate. Fluorescent units are arbitrary. ***p = 0.0006

Proof of concept-Tripartite PhaC‑SrtA−Target fusions can be used for recombinant protein production and purification

To utilize the immobilized sortase beads for recombinant protein production and purification we generated two tripartite fusion protein encoding hybrid genes to prove the concept of this recombinant protein purification technique. The fusion proteins were designed to have SrtA fused to the C terminus of PhaC as above. A LPETG sorting signal was included on the C terminus of SrtA, the target protein would then be fused immediately after the sorting signal.

Green fluorescent protein (GFP) and maltose binding protein (MBP) were used as target proteins for proof of concept experiments probing the utility PhaC-SrtA fusions attached to

PHA beads for the production and purification of recombinant proteins. Tripartite PhaCSrtA-GFP or MBP translational fusions were generated. In order to minimize the scar on the target protein an *Age*I restriction site was used to ligate the target gene to SrtA (an in-frame *Age*I-ACCGGT site encodes the amino acids TG in the LPETG sorting signal). The sortase function of the fusion protein cleaves between the T and the G of the sorting signal and thus the target protein will be released with a single additional G on its N-terminus.

Although *E. coli* has low cytosolic levels of free Ca^{2+} (generally reported to be maintained in the range of 100– 300 nM [16, 17]), we modified the previously reported growth and PHA isolation process [18] to minimize the risk of premature autoprocessing of the fusion protein during the growth phase: Cells were grown in Terrific broth at 37 °C for 16 h instead of in LB with glucose at 25 °C for 48 h as previously reported; EGTA was added to the lysis and wash buffers to chelate any extracellular Ca^{2+} which may otherwise activate the sortase; The cells were lysed with commercial blend of detergents (Bug- Buster®, Novagen), and sonication. The reported glycerol gradient ultracentrifugation methods was replaced by washing in Tween 20 with sonication steps. This resulted in faster lysis and isolation of fusion protein displaying PHA beads and removed the need for specialized equipment (ultracentrifuge).

Both the PhaC-SrtA-GFP and the PhaC-SrtA-MBP fusion mediated cell densities and PHA yields similar to that mediated by unmodified PhaC (Table 1). PHA beads could be isolated from both strains producing the respective fusion protein and a dominant protein corresponding to the PhaC-StrA-Target could be detected attached to the beads by SDS-PAGE (PST band Figure 3). Little premature cleavage (PS band PhaC-SrtA) could be detected with GFP as target protein (Figure 3a) but moderate levels could be detected with MBP target protein (Figure 3b). Sortase A requires Ca^{2+} for hydrolysis activity and an oligo-glycine nucleophile for optimal kinetics [19]. Thus the sortase could be activated by washing the EGTA off the beads and by adding $CaCl_2$ and triglycine. When $CaCl_2$ and triglycine are not added to the buffer no released target protein (GFP or MBP) could be detected and the

PhaC-SrtA-Target fusion protein stays intact and attached to beads after 24 h of incubation. Whereas when $CaCl_2$ and triglycine were included in the buffer >60 % of the available target protein was released within 1 h (Figure 3). An increase in target product and a shift of the PhaC-SrtA-Target (PST) band to the PhaCSrtA (PS) band could be observed over 6 h. In both cases prolonged incubation for 24 h resulted in higher yields of the target protein (T) and almost complete conversion of PhaC-SrtA-Target into PhaC-SrtA plus the soluble target protein (Figure 3). In both cases little to no contaminating proteins were observed in the supernatants on SDS-PAGE (Figure 3). Identities of the target protein bands were confirmed by MALDI-TOF/MS analysis of tryptic peptides. Densitometry indicates the target proteins were present in the supernatant in >98 % purity in both cases.

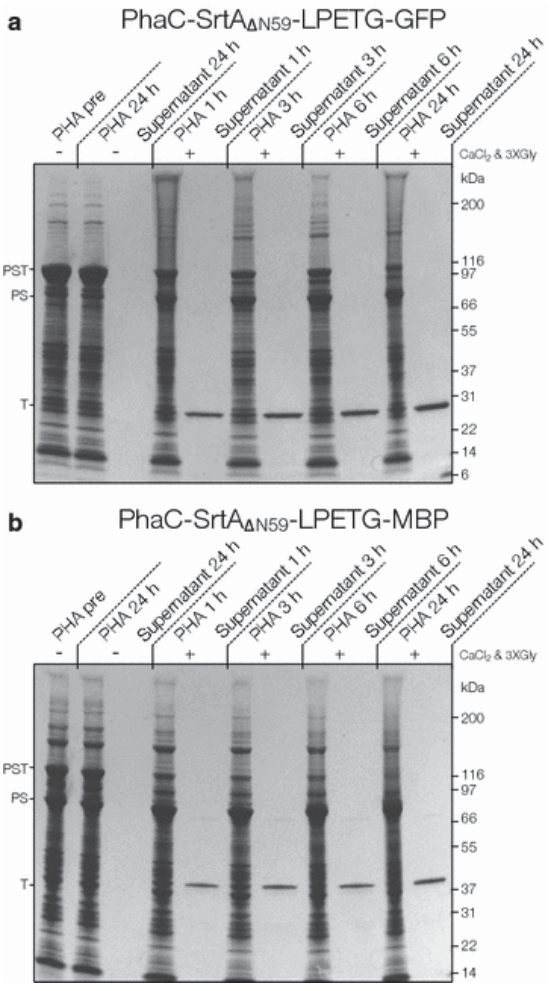

Figure 3 Purification of GFP (A) and MBP (B), from the PhaC-SrtA-Target beads. SDS-PAGE of the PHA bead and soluble fraction before and after activation with $CaCl_2$ and triglycine at different time points. *PST* PhaC-SrtA-Target band (pre cleavage); *PS* PhaC-SrtA band (post cleavage); *T* target protein.

For the GFP fusion protein 2.84 mg of target GFP could be released from 1 g of wet beads, this corresponds to 106.0 nmoles of GFP per gram of wet beads. The MBP fusion protein beads released 2.23 mg (55.3 nmoles) of MBP per 1 g of wet beads. In lab scale shake flasks this corresponds to 6.08 mg (226 nmoles) and 5.00 mg (123.85 nmoles) of purified target protein (GFP and MBP respectively) produced per liter without optimization (Table 2). Fluorescence from the GFP on the un-activated beads and in the purified supernatant could be detected, and all the MBP from the supernatant could be bound to an amylose column (Figure 4) suggesting that these proteins were produced in a functional form. This method produces high yields of soluble proteins without the need to modify the protein with affinity tags that can negatively affect the structure or function of the target protein. Target proteins are produced with a single G scar on the N-terminus; G's properties (uncharged and the smallest possible amino acid) should make it a relatively innocuous addition to most proteins. The proteins are purified to a level adequate for most common research laboratory needs and can be produced and isolated without the need for specialised or costly protein purification/chromatography equipment, reagents, resins or expertise. All equipment and reagents required are commonly found in most life science laboratories. For downstream use, proteins can be easily rebuffered/dialysed to remove the $CaCl_2$ and triglycine. The production and isolation of a soluble highly purified target protein can be completed in less than 2 days with this method.

Table 2. Yield and binding capacity of the proof of concept PhaC-SrtA-Target PHA beads

Construct	mg target protein eluted/ g wet bead	MW of target (g/mole)	nmoles target/ g wet bead	g Wet beads/ L	mg target protein/L	nmoles protein/L	g dry cells/L	% PHA by GCMS
PhaC-Srt-LPETG-GFP	2.84	26,82	106.03	2.14	6.08	226.9	3.87	27.50
PhaC-Srt-LPETG-MBP	2.23	40,25	55.29	2.24	5.00	123.85	3.74	20.66
PhaC	0.00	–	–	2.68	–	–	3.48	30.72

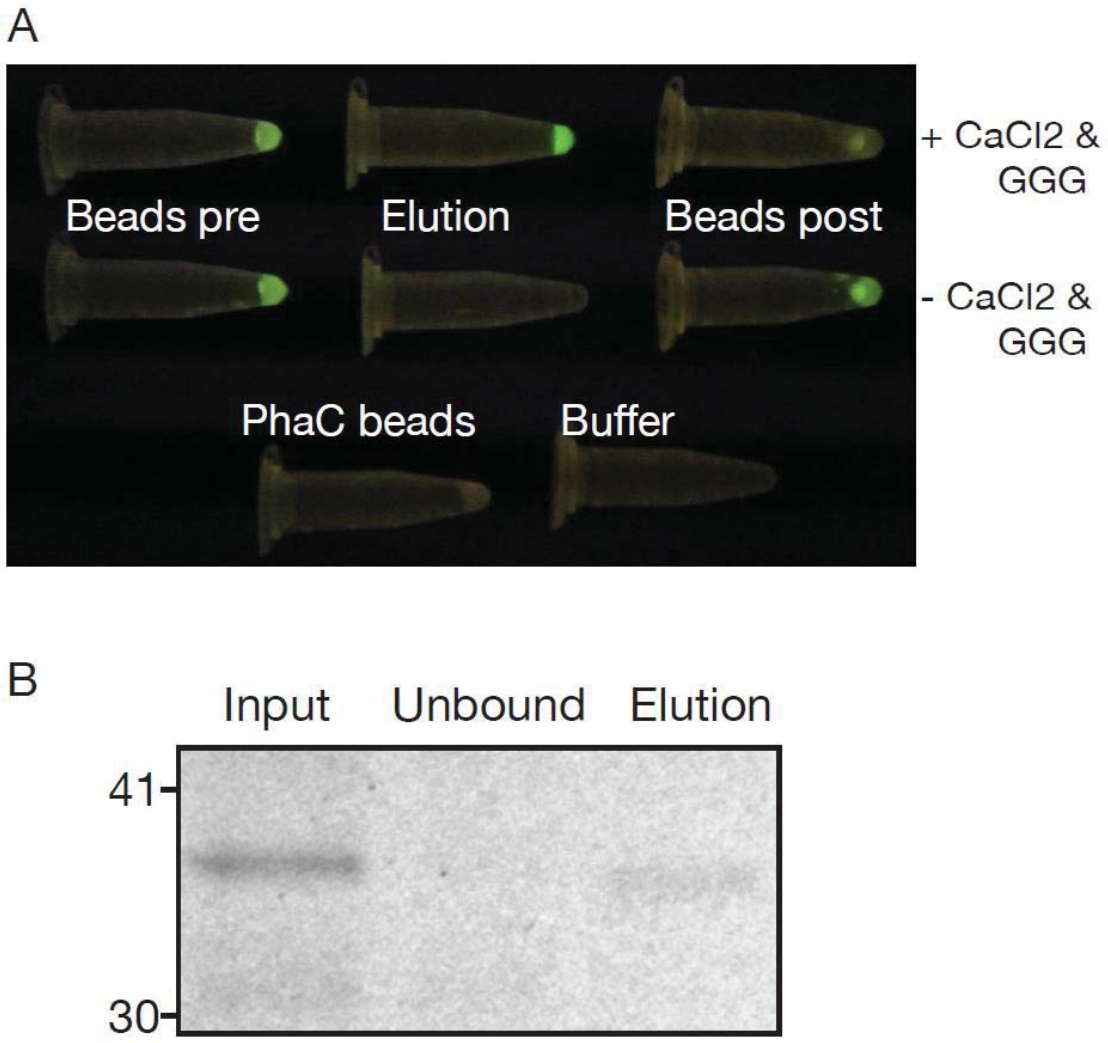

Figure 4. (A) GFP fluorescence can be detected on the PhaC-SrtA-GFP beads and retains its fluorescence once cleaved from the beads. Beads or purified proteins were placed on a UV transilluminator and imaged. (B) MBP produced and purified with the PhaC-SrtA-MBP beads is functional. The purified supernatant was applied to an amylose resin and eluted with maltose indicating the maltose binding function of the protein was retained.

Grage *et al*. [20] used PhaC engineering for target protein purification by translationally fusing the target protein to the N-terminus of PhaC including an enterokinase cleavage site as linker. Enterokinase cleavage of respective beads resulted in release of the pure soluble target protein. However, the costly use of the enterokinase and the low cleavage efficiency

of about 20 % made this approach less attractive. In another approach two PHA surface proteins: the PhaF phasin and the regulatory protein PhaR were used in combination with intein mediated self-cleavage module for protein release [21– 23]. However, these methods had two main drawbacks: the binding between the phasin/PhaR and the PHA beads are less stable non-covalent interactions, and the lack to tightly control the intein mediated cleavage in vivo and once the beads are isolated. Phasins bind to the hydrophobic surface of the PHA and other hydrophobic plastics and the binding is dependent on the salt concentration of the buffer, typically when eluting proteins from a resin one uses a relatively high salt concentration to avoid other proteins non-specifically binding to the resin. Yet under high salt concentrations the phasin can dissociate from the PHA beads and result in contamination of the target protein. Under low salt concentrations the phasin is tightly bound to the PHA beads but non target proteins also loosely bind to the PHA beads and can be present in the elution supernatant [21]. In the present system the PhaC PHA synthase is used as a scaffold, PhaC remains covalently attached to the PHA via a thioester linkage as it is synthesized [7], thus the fusion protein remains attached to the beads regardless of salt concentrations. Intein mediated cleavage reactions typically involve activation by a reducing agent such as DTT (which can disrupt native disulphide bonds in the target protein) or relatively small pH changes (lowering the pH below 7.0), thus the fusion protein cannot be in environments with a pH below approximately 7. The pH of the *E. coli* cytosol is reported to be in the range of 7.2–7.8 and can be influenced during growth by the extracellular environment particularly high levels of acetate [24]. As media acidification and acetate production are common by-product of lab scale bacterial fermentation [25] the premature in vivo and post lysis activation of inteins is a common problem [3], indeed significant premature cleavage was present in all three examples of the phasin/phaR-intein method [21–23]. By using the tighter control of the SrtA auto-processing module instead of inteins for the cleavage reaction the premature cleavage and target loss can be minimized.

An additional benefit comes from the physical immobilization of the proteins during growth. It is widely accepted that the immobilization of proteins can have a beneficial effect on the stability and solubility of proteins, thus the immobilisation of recombinant fusion proteins on the surface of PHA beads in vivo may aid in the functional folding of difficult proteins (i.e., those prone to inclusion body formation). Furthermore, the N-terminal SrtAc tag has been found to enhance the solubility and stability of its fusion partner [5].

Extending the technique to produce and purify real world targets

To further assess the broad applicability of this method to produce and purify a range of different proteins we targeted the antigen Rv1626 which is an emerging *Mycobacterium tuberculosis* vaccine candidate [26, 27]. The PhaC-SrtA-Rv1626 fusion-protein expressing plasmid was generated as above. The protein was produced and isolated as above with minor modifications. The Rv1626 containing fusion protein was produced in *Clear coli* BL21(DE3) *E. coli* cells (has proprietary mutations in LPS genes which eliminates the immunogenic endotoxin) to minimize the endotoxin in the final antigen product. PHA beads could be isolated from the respective recombinant bacteria and the PhaC-SrtA-Target fusion protein could be detected as the dominant protein on the beads (Figure 5). Levels of premature cleavage (PhaC-Srt) were similar to that of the MBP displaying PHA beads. Activation of the beads with $CaCl_2$ and triglycine resulted in 0.407 mg (17.95 nmoles) of Rv1626 protein per gram of wet PHA beads after 24 h or 2.04 mg (89.99 nmoles) Rv1626 per L culture.

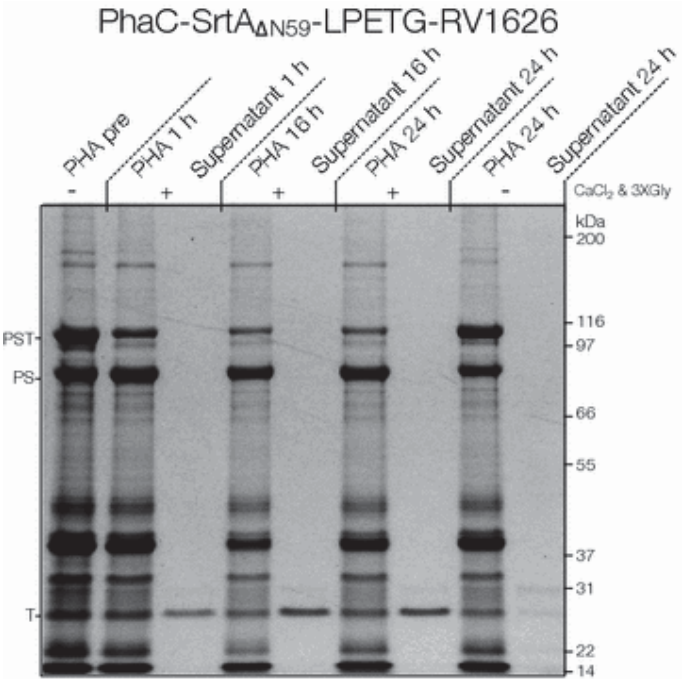

Figure 5. Purification of Rv1626 from the PhaC-SrtA-Target beads. SDS-PAGE of the PHA bead and soluble fraction before and after activation with $CaCl_2$ and triglycine at different time points. *PST* PhaC-SrtA-Target band (pre cleavage); *PS* PhaC-SrtA band (post cleavage); *T* target protein

Conclusions

Here it was demonstrated that the ability to functionally co-immobilize Sortase A from *S. aureus* together with a protein of interest on the surface of PHA beads in vivo i.e., production and purification of the protein of interest. Sortase A from *S. aureus* could be functionally immobilized in vivo to the surface of PHA beads. These beads could be used to cleave the LPETG peptide. This activity was used to design a system for recombinant protein production and their tag free purification. The recombinant protein of interest is genetically fused to the PhaC-SrtA fusion protein via a sortase recognition site. The protein is produced as immobilized on the surface of PHA beads in vivo. The respective beads can be easily isolated and the protein released from the beads with the addition of $CaCl_2$ and triglycine. As the PHA production itself has been established as commercially scalable process it provides the foundation for scalable and industrial PHA bead-based protein production [8]. After

protein release PHA could be recovered from beads as added value. The plasmids required for this technique are generated by a simple restriction enzyme cloning step. This technique requires no costly protein purification or chromatography equipment or expertise and no additional resin or expensive/uncommon chemicals are required. The soluble target proteins can be functionally isolated, without an extensive tag, at high concentration at a purity of up to 98 % in a single step. This is adequate for many common life science research applications including protein production for functional analysis, protein crystallography, or antigen generation for antibody generation.

Authors' contributions

IDH and BHAR conceived the study and designed the experiments. IDH, JD and PRR conducted the experiments. IDH and BHAR wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

BHAR is shareholder and CSO of PolyBatics Ltd that is commercialising the PHA bead technology.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Patricia Rubio Reyes

Name/Title of Principal Supervisor: Prof. Bernd Rehm

Name of Published Research Output and full reference:
Self-Assembled Protein-Coated Polyhydroxyalkanoate Beads: Properties and Biomedical Applications

In which Chapter is the Published Work: Chapter 1B

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 15 %
  and / or
- Describe the contribution that the candidate has made to the Published Work:

Candidate's Signature — 12/04/2017 Date

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Name of Candidate: Patricia Rubio Reyes

Name/Title of Principal Supervisor: Prof. Bernd Rehm

Name of Published Research Output and full reference:
Immunogenicity of antigens from Mycobacterium tuberculosis self-assembled as particulate vaccines

In which Chapter is the Published Work: Chapter 2

Please indicate either:

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Name of Candidate: Patricia Rubio Reyes

Name/Title of Principal Supervisor: Prof. Bernd Rehm

Name of Published Research Output and full reference:
Immune modulators did not enhance protective immunity against tuberculosis induced by a single particulate mycobacterial antigen

In which Chapter is the Published Work: Chapter 3

Please indicate either:

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  and / or
- Describe the contribution that the candidate has made to the Published Work:

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Name of Candidate: Patricia Rubio Reyes

Name/Title of Principal Supervisor: Prof. Bernd Rehm

Name of Published Research Output and full reference:
In vivo polyester immovilized sortase for tagless protein purification

In which Chapter is the Published Work: Appendix

Please indicate either:

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