A study of recombinant *Plasmodium falciparum* PFC0760c

By

Jacqueline Ethel Viljoen

B.Sc. (Hons) (University of KwaZulu Natal)

Submitted in fulfilment of the

academic requirements for the degree of

Master of Science

in

Biochemistry,

School of Biochemistry, Genetics, Microbiology and Plant Pathology

University of KwaZulu-Natal

Pietermaritzburg

March 2011

FACULTY OF SCIENCE AND AGRICULTURE DECLARATION 1 - PLAGIARISM

I, Jacqueline Ethel Viljoen, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed……

PREFACE

The experimental work described in this thesis was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu- Natal, Pietermaritzburg, under the supervision of Professor J. P. Dean Goldring. The studies represent original work by the author and have not otherwise been submitted in any other form to another University. Where use has been made of the work of others it is duly acknowledged in the text.

_____

Jacqueline Ethel Viljoen

30 March, 2011

_____

Professor J. P. Dean Goldring

30 March, 2011

ABSTRACT

Malaria is a devastating disease caused by one of the world's most pathogenic parasites, *Plasmodium*. Five species of *Plasmodium* infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. *P. falciparum* is the most pathogenic and causes the greatest numbers of deaths. To date, no licensed vaccine against malaria is available, although there are numerous vaccine candidates in various stages of development.

Pca 96 is a 96 kDa *Plasmodium chabaudi adami* protein shown to have a protective property in mice challenged with *P. chabaudi adami*. Thus, a *P. falciparum* orthologue of *Pca* 96 may be useful in vaccine development. BLAST searches with the *Pca* 96 amino acid and nucleotide sequences revealed proteins with high sequence identity to *Pca* 96 including the hypothetical *P. falciparum* PFC0760c and *P. yoelii yoelii* PY05757 proteins. A peptide sequence FKLGSCYLYIINRNLKEI was found to be conserved in all homologues of *Pca* 96, including PFC0760c, PY05757 and in the sequences of proteins from 5 other *Plasmodium* species.

Bioinformatic approaches were explored to attempt to find a possible role of the protein and the possible importance of the conserved sequence. The conserved sequence was predicted to be an alpha helix and to contain possible HLA-DRB1*1101 and HLA-DRB1*0401(Dr4Dw4) T-cell epitopes (GSCYLYIINRNLKEI) in addition to a possible H2-Kd T-cell epitope (CYLYIINRNL). Protein-protein interaction predictions revealed that PFC0760c was likely to interact with proteins involved with nucleic acid binding. PFC0760c was predicted to have a domain found in proteins involved in the structural maintenance of chromosomes, which may suggest the protein is involved in chromatid cohesion during mitotic chromosome condensation. PFC0760c was also predicted to be located in the nucleus by the sub cellular prediction program, SubLoc.

Anti-peptide antibodies were raised against the conserved amino acid sequence and against a peptide specific for PY05757 (SDDDNRQIQDFE). Both antibodies detected native antigens with immunofluorescence microscopy. The fluorescent signal appeared throughout the parasite cytoplasm and as an intense signal in the parasite nucleus. These immunofluorescence data supports the predicted nuclear location of the protein.

A 822 bp portion of PFC0760c gene was expressed as a maltose-binding protein fusion protein (*Pf*33-MBP). *Pf*33-MBP was expressed and purified. Reducing SDS-PAGE and western blotting analysis revealed the fusion protein to be expressed at low levels as four bands (79, 60, 45 and 37 kDa). The purified fusion protein was cleaved with Factor Xa. MBP and *Pf*33 were of similar molecular mass after cleavage. To attempt to obtain better expression and purification, the 822 bp insert from pTS822 was sub-cloned into pGEX4T1. A glutathione-*S*-transferase (GST)-fusion protein (*Pf*33-GST) was expressed. The level of expression was poor and therefore not pursued.

To take the study further, potential proteins that interact with PFC0760c and *Pf*33 need to be identified. In addition, immunisation of mice with the protein and subsequent *Plasmodium* challenge needs to be performed to ascertain the protective potential of the protein.

ACKNOWLEDGEMENTS

I would like to express my eternal gratitude to the following people and organisations for their generous contribution to this study:

To Professor J. P. D. Goldring, for his outstanding supervision, continued encouragement, support, patience, understanding, humour and for all of the assistance with this thesis.

To Professor T. H. T. Coetzer, for her invaluable assistance, encouragement and support.

To Professor Alain Boulange, for his assistance with cloning and for making the laboratory an interesting place to work.

To Dr. Laura (Huson) Saunderson, for all of the assistance she gave me and especially for all the encouragement and cherished friendship.

To Dr. Bridgette Cumming, for her ongoing support, interest and treasured friendship.

To all of my laboratory colleagues and fellow post-graduate students throughout the years, especially Laura Saunderson, Bridgette Cumming, Dave Choveaux, Robert Krause, Davita Pillay, Richard Kangethe, Tammy Hiltunen, Ike Achilonu, Ramona Hurdayal, Hlumani Ndlovu, Lorelle Bizarré, Phillia Vukea, Parushka Maharaj, Laurelle Jackson, Celia Snyman, Sabelo Hadebe, Hermóges Muchache and Erastus Kakhunde, for your special friendships, valuable assistance, encouragement and for making a day in the lab all that much brighter! I wish you success in all of your future endeavors.

To my husband Russell Viljoen for making me laugh when all I wanted to do was cry, and for his continuous love, patience, encouragement and support.

To my furry boys Jack and Zarkie, for their love and for always making smile and cheering me up after a difficult day.

To my parents Amy and Peter Foster, for bringing me into this world, for all the opportunities they have given me in life, their unconditional love, ongoing interest, support and encouragement.

To my other parents Rose and Mike Viljoen, for their love, encouragement and interest.

To my family and friends, for their continued encouragement and interest.

To Charmaine Ahrens and Robyn Hillebrand: Thank you for your valuable assistance with all things administrative and for always having a smile on hand.

To Agnes Zondi, Goodman Zondi, and Richard Shabalala, who always had a cheerful smile for me and who kept the laboratories clean and well stocked with distilled water.

To Jessica Moodley, Yegan Pillay, Megan Brunkhorst, Denzil Lakay, and Kim Govender, for their valuable and cheerful assistance with equipment. Jess, thank you also for your friendship, encouragement and support.

To Mark Brown, for his assistance and advice with the mice and all the other rescued creatures I have brought to him.

To Ann, Francis, and the staff at Ukulinga, for their valuable assistance with the chickens. Baggy and Mitsy, thank you for always making a trip to the farm a happier experience.

To all the academic staff in the School of Biochemistry, Genetics and Microbiology for a good grounding in the field and for continued interest and support.

To the mice and chickens: Without you this study would have suffered greatly. Thank you.

To the National Research Foundation (Grant-Holders bursary) and the Medical research council for financial assistance.

To Professor Wim G.J. Hol (Howard Hughes Medical Institute and Department of Biological Structure, University of Washington, USA) for kindly providing the RIG plasmid and valuable information.

To Leo Brady (Department of Biochemistry, University of Bristol, UK) for kindly providing the pKK223-3-*Pf*LDH and pKK223-3-*Pv*LDH plasmids.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

°C	degrees centigrade
~	approximately
2xYT	2 × yeast extract, tryptone
ABTS	2, 2-azino-di-[3-ethylbenzthiazoline sulfonate]
AMA-1	apical membrane antigen 1
ATP	adenosine triphosphate
BCCP	biotin carboxyl carrier protein
BLAST	Basic Local Alignment Search Tool
BLASTP	Basic Local Alignment Tool (protein to protein database)
BSA	bovine serum albumin
CDD	Conserved Domain Database
CD-Search	Conserved domain search
COGs	Clusters of Orthologous Groups
CSP	Circumsporozoite protein
C-terminal	carboxy terminal
DAPI	4', 6-diamide-2'-phenylindole dihydro chloride
DEAE	Diethylaminoethyl
dH_2O	distilled water
DMF	N, N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNTP	deoxynucleotide triphosphate
DTNB	5, 5'-Dithiobis (2-nitrobenzoic acid)
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
EBI	European Bioinformatics Institute
EBL	erythrocyte binding ligand
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay

EMP	erythrocyte membrane protein
EMP-1	Erythrocyte membrane protein 1
E-value	expectation value
EXP-1	exported protein 1
FITC	fluorescein isothiocyanate
g	gram
g	relative centrifugal force
GLURP	Glutamate-rich protein
GM-CSF	granulocyte macrophage colony stimulating factor
GPI	glycophosphatidylinositol
GST	glutathione *S*-transferase
h	hour
HBSS	Hanks balanced salt solution
HRPO	horseradish peroxidase
I. P.	intraperitoneal
IFN-γ	interferon gamma
IgG	immunoglobulin G
IgY	immunoglobulin Y
IL	interleukin
IPTG	isopropyl-beta-D-thiogalactopyranoside
KDa	kiloDalton
l	litre
LB	lysogeny broth
LMP	low melting point
LSA	liver stage antigen
LSP	long synthetic peptide
MBP	maltose binding protein
MBS	M-maleimidobenzoyl acid N-hydroxy succinimide ester
MEC	molecular exclusion chromatography
MHC	major histocompatibility complex
min	minute

ml	millilitre
M_r	molecular mass
MSP	merozoite surface protein
MW	molecular weight
NCBI	National Centre for Biotechnology
nm	nanometer
NMR	nuclear magnetic resonance
N-terminal	amino terminal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	Protein Data Bank
PEG	polyethylene glycol
PEXEL	*Plasmodium* export element
Pfam	Protein families
*Pf*EMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
*Pf*LDH	*Plasmodium falciparum* lactate dehydrogenase
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
*Pv*LDH	*Plasmodium vivax* lactate dehydrogenase
RBC	red blood cell
RESA	ring-stage infected-erythrocyte surface antigen
RIFIN	repetitive interspersed family
RNA	ribonucleic acid
RT	room temperature
s	second
SBP1	skeleton-binding protein 1
SBTI	soybean trypsin inhibitor
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERA	serine repeat antigen

SMART	Simple Modular Architecture Research Tool
SMC	structural maintenance of chromosomes
SOB	super optimal broth
SOC	super optimal catabolizer
SSP2	sporozoite surface protein 2
STE	sodium-tris-EDTA
STEVOR	subtelomeric variable open reading frame
TAE	tris-acetate-EDTA
TBH	Tris buffered Hanks balanced salt solution
TBLASTN	Basic Local Alignment Tool (protein to translated nucleic acid database)
TBS	tris buffered saline
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethyl ethylene diamine
T_m	melting temperature
TNF-α	tumour necrosis factor alpha
TPCK	N-tosyl-L-phenylalanyl chloromethylketone
TRAP	thrombospondin-related anonymous protein
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
UIS3	up-regulated in infectious sporozoites gene 3
UIS4	up-regulated in infectious sporozoites gene 4
µg	microgram
µl	microlitre
µm	micrometre

CHAPTER 1
Introduction

1.1 Overview of malaria

Malaria is a devastating disease caused by one of the world's most pathogenic parasites, *Plasmodium*. The *Plasmodium* parasite is transmitted by the bite of an infected female anophelene mosquito. There are four species of *Plasmodium* infecting humans: *P. falciparum*, *P. vivax, P. ovale* and *P. malariae*, with *P. falciparum* causing the greatest numbers of deaths (Girard *et al*., 2007). *P. falciparum* was estimated to have caused 451 (range 349-552) million infections worldwide in 2007 (Hay *et al*., 2010). In 2008 *Plasmodium* was estimated to cause 243 million cases of malaria, resulting in 863 thousand deaths (World Health Organisation, 2009). Approximately 85% (208 million) of the malaria cases were in Africa and resulted in the deaths of 767 thousand people, of which 88% were children under the age of 5 (World Health Organisation, 2009).

Malaria has in the past, been endemic all over the world. The use of insecticides saw its eradication from North America, Australia and Europe by 1950's. However, it is still prevalent in Africa, South East Asia and the Western Pacific region today (Girard *et al*., 2007). In South Africa, 10% of the population is at risk of contracting a malaria infection. Provinces in South Africa having the highest risks for malaria infection are Mpumalanga, Limpopo and Northern KwaZulu Natal. The statistics of malaria cases and related deaths provided by the National Department of Health show that Limpopo province experiences the greatest number of malaria infections and deaths, with 6 369 cases and 57 deaths recorded for 2006 (http://www.doh.gov.za/issues/malaria/statistics.html). The second and third most affected provinces are Mpumalanga and KwaZulu-Natal. In 2006, Mpumalanga experienced 4 558 malaria cases, of which 21 were fatal. KwaZulu Natal experienced 1 211 malaria cases and 11 malaria–related deaths in 2006.

1.2 Life cycle

The *Plasmodium* parasite is able to constantly change its gene expression (Bannister and Mitchell, 2003). This allows the parasite to exploit a number of different environments, such as the salivary glands, gut and vascular system of the mosquito, and the hepatocytes

(liver cells) and erythrocytes of humans (Figure 1.1). The life cycle essentially has two parts to it, the human stages and the mosquito stages. When a mosquito feeds on the blood of a human previously infected by a mosquito carrying *Plasmodium* parasites, it obtains parasites in the form of gametocytes. These gametocytes are fertilized to form a zygote, which divides and develops to produce large numbers of sporozoites (Figure 1.1). The sporozoites make their way into the salivary glands of the mosquito. When the mosquito feeds, it injects the sporozoites into the blood stream of a human. Sporozoites are transported in the blood stream to the liver, where they invade the liver cells (Figure 1.1). The sporozoites differentiate and replicate asexually as schizonts, producing tens of thousands of merozoites. The merozoites are released from the liver cells and enter the blood stream and infect erythrocytes. This stage of the life cycle is known as the asexual erythrocytic stage (Figure 1.1).

When a merozoite comes into contact with an erythrocyte, the parasite forms a close irreversible connection between itself and the erythrocyte and the parasite becomes completely enclosed in a membranous vacuole, known as the parasitophorous vacuole (Bannister and Mitchell, 2003).

Not all parasites within the infected erythrocytes enter into the cyclic asexual stage. Some differentiate into sexual forms known as gametocytes (Figure 1.1). When these gametocytes are taken up in the blood by a feeding mosquito, they transform themselves into gametes, and are eventually fertilized, leading to infection of the mosquito, and further transmission to human hosts.

Those parasites entering into the asexual erythrocytic stage spread into thin biconcave discs within the erythrocytes. These discs are thicker around their perimeter, due to the elongated nucleus, and thin in their centre, making them appear ring-shaped when stained with Giemsa stain (Bannister and Mitchell, 2003). The parasites feed and enlarge during this stage and synthesize specific proteins necessary for the duration of the asexual erythrocytic stage. The parasites export some of these proteins into the erythrocyte, which causes modification of the erythrocyte membrane, resulting in the erythrocytes adhering to the lining of blood vessels.

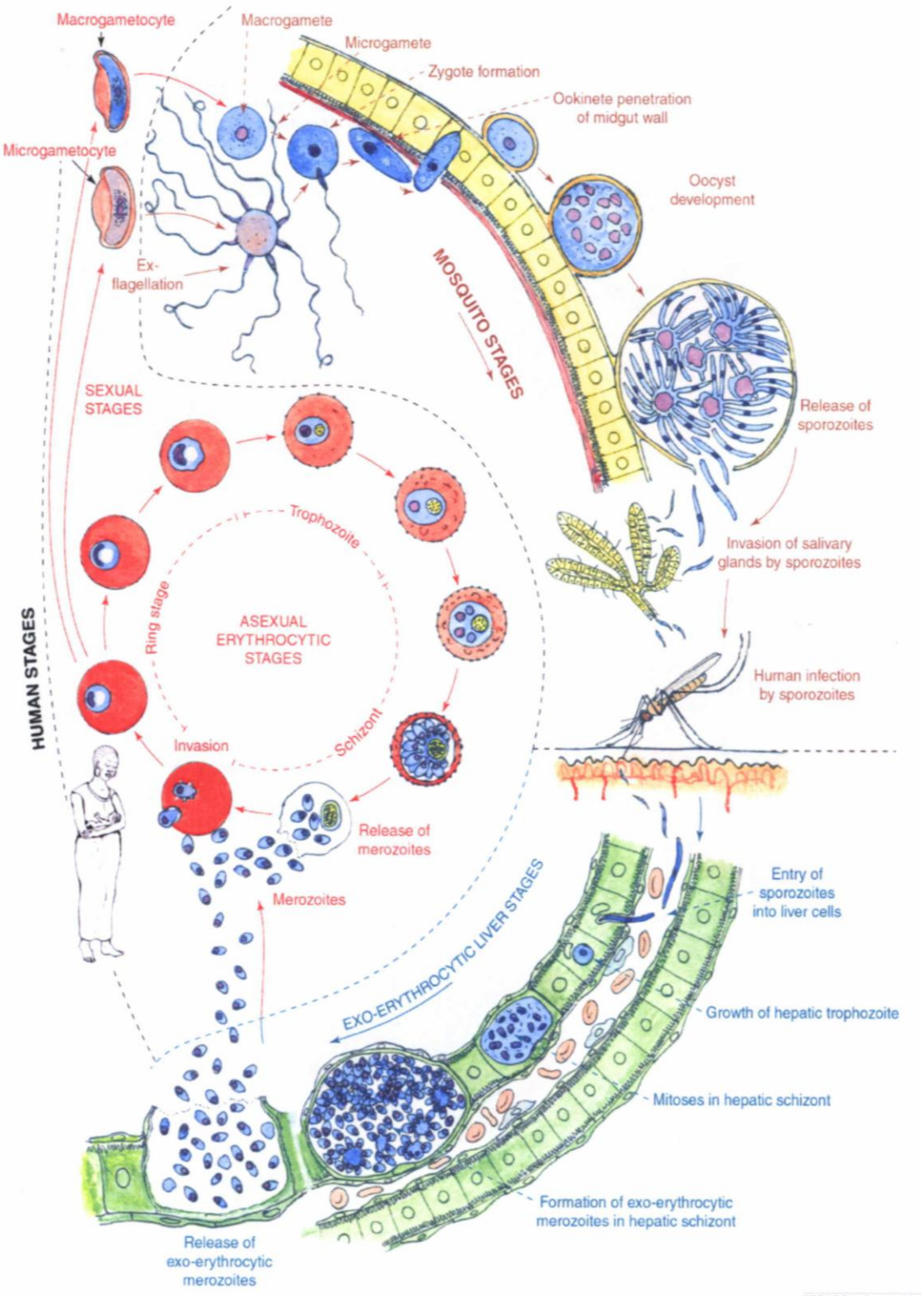


Figure 1.1. The life cycle of *Plasmodium falciparum.*
The liver stage, the erythrocytic sexual and asexual stages within humans, and the stages of parasite life within the salivary glands and gut of the mosquito are shown. (http://parasites.trends.com; Bannister and Mitchell, 2003)

The rings grow into rounded trophozoites, which undergo schizogony. Schizogony involves a number a nuclear divisions, generating approximately 16 nuclei from 1 schizont. These nuclei bud off from the central cytoplasmic body of the schizont. These are newly formed merozoites and they are released from the erythrocyte, to infect new erythrocytes, continuing the life cycle of the parasite.

Due to the parasites severe pathogenicity and ability to exploit these different environments, it is apparent that an effective method of protection from the disease is urgently needed.

1.3 Malaria control

Malaria is becoming increasingly problematic to control and combat. A number of strategies have been implemented to aid in combating malaria: anti-malarial drug treatments, long lasting insecticide-treated mosquito nets and residual indoor spraying with insecticides (World Health Organisation, 2008). However, surveys performed by the World Health Organisation, revealed that insecticide-treated mosquito nets and effective anti-malarial drugs were not reaching a sufficient portion of the malaria-affected population, especially in Africa. In addition, anti-malarial drugs are becoming ineffective due to *Plasmodium* developing resistance to the drugs (Baird, 2005; Mutabingwa, 2005), and the artemisinin-based combination therapies that are effective are very costly, especially for those who are economically disadvantaged (Mutabingwa, 2005). Furthermore, *Plasmodium*'s vector, the mosquito, is becoming increasingly resistant to insecticides (Bannister and Mitchell, 2003), thereby making insecticide spraying increasingly ineffective. Therefore it appears as though the most effective long-term solution would be to develop an effective, inexpensive vaccine against *Plasmodium*, which could be used in combination with mosquito nets and insecticides. However, developing a malaria vaccine is a complex undertaking. *Plasmodium* is a very complex parasite with great antigenic variation between species and strains and one has to identify suitable antigens from a genome of approximately 5300 genes (Gardner *et al*., 2002), of which less than half have been assigned a function (Tedder *et al*., 2010). Once suitable antigens have been identified, those antigens need to be recombinantly produced, which can be problematic due to a number of factors. Among the factors contributing to the difficulties of recombinantly

expressing the antigens are: the rich adenine and thymine content of the genome (Gardner *et al*., 2002); *Plasmodium* using a different set of codons to that of the expression hosts; the expressed antigens may not be correctly folded which raises problems for immunogenic conformational epitopes that may be on the native protein. The antigens chosen may also have a large degree of variability across species and strains, which may result in the immune response elicited by that antigen to be ineffective at protecting against a different strain or species. The adjuvant used with the antigen can also make large differences in whether the antigen elicits a strong protective response or not, as experienced with the RTS,S vaccine candidate (Stoute *et al*., 1997).

1.4 Immune response to malaria

Humans living in a malaria-endemic area are able to naturally acquire protective immunity to *Plasmodium*. However, obtaining this protective immunity is a slow process requiring repeated exposure to parasites and rarely results in sterile immunity, as low levels of parasites and rare occurrences of severe clinical disease are still observed (Perlmann and Troye-Blomberg, 2000; Hviid, 2005; Langhorne *et al*., 2008). Both B cells and T cells and their products are required for immunity against malaria. For the purpose of this study, the role of T cells in the immune response to malaria will be briefly discussed here.

Studies have been done with regards to the immune response to malaria in both B cell deficient and T cell deficient (athymic) mice (Weinbaum, *et al*., 1976; Grun and Weidanz, 1981; Grun *et al*., 1985; Cavacini *et al*., 1990; van der Heyde *et al*., 1994). Grun and Weidanz (1981) showed that in B cell deficient mice, *P. chabaudi adami* infection was resolved by a T cell dependent immune response, similar to that seen in immunologically intact mice. In contrast, athymic nude mice (mice without functioning T cells) were unable to resolve *P. chabaudi adami* infection (Grun *et al*., 1985; Brake *et al*., 1988; Cavacini *et al*., 1986; Cavacini *et al*., 1990). B cell deficient mice infected with *P. vinckei petteri* or *P. chabaudi chabaudi* resolved their infections in a manner similar to immunologically intact mice (Cavacini *et al*., 1990).

In order to identify the individual T cells, revised experiments were conducted using subsets of T cells. Adoptive transfer of spleen cells has also been investigated (Cavacini *et al*., 1986; Brake *et al*, 1988; Goldring *et al*., 1989; van der Heyde *et al*., 1994). In these

studies, non-immune and immune spleen cells or a cloned T cell line were adoptively transferred to athymic mice. Infections in mice adoptively transferred with immune spleen cells, resolved their infections earlier than mice adoptively transferred with normal spleen cells. In addition, athymic mice adoptively transferred with T cell enriched spleen cells were able to resolve their infections. In contrast, mice adoptively transferred with B cell enriched spleen cells succumbed to infection and died.

Severe combined immunodeficient (SCID) mice reconstituted either with immune spleen cells or immune T cells were able to suppress infection with *P. chabaudi adami* (van der Heyde *et al*., 1994). In contrast, unreconstituted SCID mice were unable to control their infections. Mice that received immune spleen cells had lower parasitemia than mice that received non-immune T cells (van der Heyde *et al*., 1994).

In addition, a T cell clone designated CTR2.1 was able to adoptively transfer protection against *P. chabaudi adami* infection, to athymic mice (Brake *et al*., 1988). Athymic mice adoptively transferred with the CTR2.1 T cell clone, produced antibodies against an array of *P. chabaudi adami* proteins (Goldring *et al*., 1989). Immmunologically intact mice produced a similar antibody response, whereas athymic mice not adoptively transferred did not. These antibodies were shown to not contribute towards protection in challenged mice. T cells exhibiting a Th1 phenotype have also been shown to provide protection against *P. yoelii* infection in adoptively transferred SCID mice (Makobongo *et al*., 2003).

1.5 Immune evasion by the parasite

The parasite is able to evade the immune system by a number of different means, including, among other strategies, antigenic variation, alternative red blood cell invasion pathways and the presence of cryptic epitopes.

1.5.1 Antigenic variation

The parasite uses antigenic variation to evade the host immune system. *P. falciparum* has large families of variant genes known as *var*, *rif*, *stevor* and *Pf*mc-2TCM. These genes are located at the sub-telomeres and respectively code for the erythrocyte membrane protein 1 (*Pf*EMP1), repetitive interspersed family (Rifin), subtelomeric variable

open reading frame (Stevor) and Mauer's cleft two transmembrane (*Pf*mc-2Tm) proteins (Casares and Richie, 2009). These proteins bind to host receptors, mediating rosetting and sequestration of the host erythrocytes, causing major mortality. Antibodies to these proteins form an important part of the host immune response to the parasite. As the parasite has many var, stevor, rif and *Pf*mc-2Tm genes, the parasite is able to evade the host immune system antibody response by simply expressing different variants of the proteins which will not be recognised by the antibodies (Casares and Richie, 2009).

1.5.2 Alternative erythrocyte invasion pathways

During the blood stage of the parasite life cycle, merozoites attach to erythrocytes randomly via merozoite surface protein-1 (MSP-1). Apical membrane antigen-1 (AMA-1) mediates the re-orientation of the merozoite in order for a tight junction to be formed between the parasite apical complex and the red blood cell, allowing the parasites hydrolytic enzymes to disrupt the red blood cell membrane. This tight junction is formed by erythrocyte binding ligands (EBLs) and reticulocyte binding protein homologues (RBLs) (Cortes, 2008). The EBL are encoded by the EBA175, EBA140/BAEBL and EBA181/JESEBL genes. The RBL genes express the *Pf*Rh1, *Pf*RH2a, *Pf*RH2b and *Pf*RH4 ligands. The parasite typically uses a sialic acid dependent invasion pathway, which uses the EBLs and *Pf*Rh1. However, antibodies against these proteins are able to prevent red blood cell invasion (Kinyanjui *et al*., 2004; Gao *et al*., 2008). The parasite is able to switch to using a sialic acid independent pathway, which utilise *Pf*Rh2a and *Pf*Rh4, thereby escaping the effects of the antibodies against the EBLs and *Pf*Rh1 (Stubbs, *et al*., 2005).

1.5.3 Cryptic epitopes

Immunogenic epitopes that are 'hidden' or 'invisible' to the host immune system are known as cryptic epitopes. Cryptic epitopes have been identified in *P. chabaudi adami* apical membrane protein 1 (AMA-1) (Amante *et al*., 1997), *P. falciparum* circumsporozoite protein (CSP) (Rathore *et al*., 2005). Cryptic epitopes that appear conserved across many species of *Plasmodium* have been identified in thrombospondin-related anonymous protein (TRAP) and CSP (Bharadwaj *et al*., 1998). These epitopes have been shown to induce invasion-blocking antibodies, although only when the epitopes are immunised in peptide

form, and not when the protein is in its native form or recombinantly produced, suggesting that these immunologically unresponsive epitopes are an evasion strategy for the parasite (Rathore *et al.*, 2005).

1.6 Vaccines

Malaria vaccine candidates typically target different stages of the parasite life cycle (Figure 1.2). There are numerous vaccine candidates in various stages of research, production and trials, against multiple *Plasmodium* targets. An overview of a few of the candidates will be discussed here.

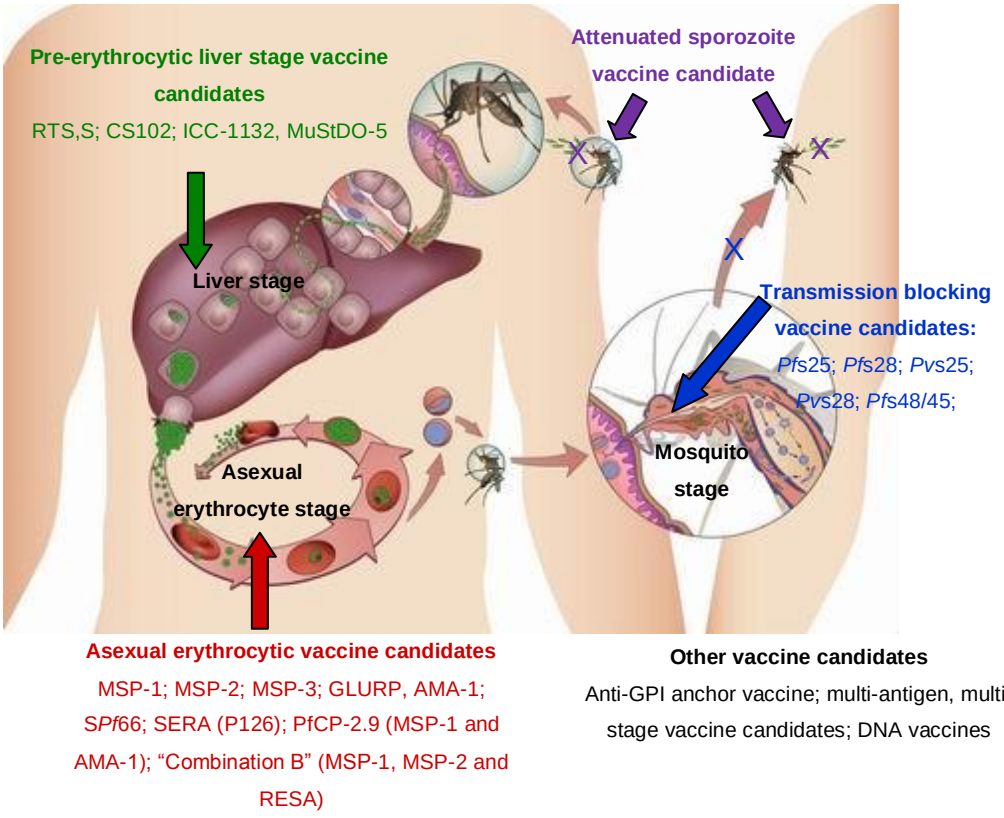


Figure 1.2. Malaria vaccine candidates and the stages of the parasite life cycle the vaccines target.
(Image adapted from http://sciencetrio.files.wordpress.com/2009/07/malaria-cycle1.jpg).

1.6.1 Attenuated sporozoite vaccine

Attenuated sporozoites are able to infect the hosts liver, but maturation and differentiation into merozoites is hampered, resulting in the *Plasmodium* infection not developing into clinical malaria (Frevert and Nardin, 2005).

Sedegah *et al*. (2007) showed that protection could be achieved in mice against different *Plasmodium* strains. Mice immunised with radiation-attenuated *P. yoelii* sporozoites were 100% protected when challenged with *P. yoelii* sporozoites and 63% protected when challenged with *P. berghei* sporozoites. Mice immunised with *P. berghei* radiation-attenuated sporozoites were 100% protected when challenged with *P. berghei* sporozoites and 79% protected when challenged with *P. yoelii* sporozoites. Humans have also been shown to be protected with radiation-attenuated sporozoites: in total, 24 of 26 volunteers were protected against *P. falciparum* challenge (Hoffman *et al*., 2002). Those protected included volunteers challenged 23-42 weeks after their second immunisation and challenge with *P. falciparum*.

A concern with radiation attenuated sporozoites is that the radiation dose needs to be precise for the immunisation to work effectively and safely (VanBuskirk *et al*., 2009). Although there is concern about precise radiation dose for attenuated sporozoites, Chattopadhyay *et al*. (2009) have shown that increasing the minimal dose necessary to attenuate sporozoites for immunisation in mice can safely be doubled and still provide protection, thereby indicating that this may be possible for sporozoites for immunisation in humans which will ensure safety without reducing the efficacy.

Alternative methods for attenuating parasites exist. One way is to chemically attenuate them with the DNA binding drug, centanamycin, as Purcell *et al*. (2008) did. The immunised sporozoites provided sterile immunity and cross protection against heterologous challenge.

Another method of attenuation is genetic attenuation. The pre-erythrocytic stage of *P. berghei* has been genetically attenuated by deletion of genes called ‘up-regulated in infectious sporozoites gene 3’ (UIS3) (Mueller *et al*., 2005b) and UIS4 (Mueller *et al*., 2005a). Two other genes, P36 and P52, have been simultaneously deleted in *P. yoelii* sporozoites, resulting in the parasites not forming a parasitophorous vacuole within liver cells *in vitro* or *in vivo*. Genetically modified sporozites such as P36/52 deficient and UIS3 and UIS4 deficient sporozoites develop normally within the mosquito host and invade the liver, but cannot develop into liver stage parasites and therefore become arrested in the liver. The sporozoites do not develop into blood stage forms, irrespective of the number of sporozoites inoculated (Labaied *et al*., 2007). Mice immunised with UIS3 deficient

sporozoites or UIS4 deficient sporozoites, were completely protected when challenged with *P. berghei*, (Mueller *et al*., 2005b; Mueller *et al*., 2005a). Mice immunised with P36/P52 deficient sporozoites were also completely protected when challenged with infected sporozoites (Labaied *et al*., 2007). It is for this reason that these genes were deleted in *P. falciparum* sporozoites and used to inoculate a humanised mouse model (Van Buskirk *et al*., 2009). The parasites did not develop into blood stage forms.

Thus, an attenuated sporozoite vaccine appears to be the most promising vaccine for providing sterile immunity to malaria. However, there are concerns of attenuation efficacy as mentioned above. As the sporozoites are obtained from dissecting mosquito salivary glands a further concern is whether it will be possible to provide large quantities of sterile sporozoites that are not contaminated with mosquito debris (Luke and Hoffman, 2003; Fervert and Nardin, 2005). Additional concerns are the route of immunisation and cryo preservation of the sporozoites (Luke and Hoffman, 2003; Fervert and Nardin, 2005), especially considering that most of the people requiring the vaccine, live in climates with excessive heat and are unlikely to have the facilities to store the sporozoites.

1.6.2 Pre-erythrocytic vaccines

The aim of pre-erythrocytic or liver stage vaccines is to protect against malaria infection. Ideally these vaccines should prevent invading sporozoites from entering the liver, and alternatively, or additionally, inhibit sporozoite maturation. There are a number of pre-erythrocytic candidate vaccines in various stages of development. A few are listed in Table 1.1. The most advanced liver-stage candidate vaccine at the moment is the recombinant RTS,S vaccine, which consists of amino acids 207-395 of the C-terminus of *P. falciparum* circumsporozoite protein (CSP), fused to the hepatitis B surface antigen (Stoute *et al*., 1997). In initial phase I clinical trials, RTS,S was administered with AS02 adjuvant, which is a combination of an oil-in water emulsion, monophosphoryl lipid A and saponin isolated from the soapbark tree *Quillaja saponaria* (QS21). Six out of seven volunteers were protected against malaria challenge. At sixty days post-challenge, those protected individuals that were still malaria-free and asymptomatic remained so for more than three additional months (Stoute *et al*., 1997).

RTS,S has been tested with two different adjuvant systems:AS01 and AS02 (Bejon *et al*., 2008). AS02 consists of an oil-in-water emulsion, QS21 and monophosphoryl lipid A, and AS01 consists of a liposome-based related adjuvant containing monophosphoryl lipid A and QS21. The RTS,S vaccine given with the adjuvant AS01B was shown to have an efficacy of 50% in contrast to 32% for the RTS,S vaccine given with the adjuvant AS02A (Kester *et al*., 2009). Protected individuals were re-challenged 5 months after their initial challenge and 44% were protected. Protected individuals had higher CSP specific IgG titres and a higher number of CSP-specific, IFN-γ-producing, $CD4^+$ T cells. Sun *et al*. (2003) also observed the presence of CSP-specific $CD4^+$ and $CD8^+$ T-cells producing IFN-γ, during their studies of volunteers protected from malaria by RTS,S immunisation.

Efficacy of RTS,S/AS01 has been tested in young children of 5-17 months old. Of the 402 children who received RTS,S/AS01 vaccinations, 32 developed malaria, resulting in an adjusted vaccine efficacy of 56% (Bejon *et al*., 2008). In addition, Sacarlal *et al*. (2009) performed a Phase 2b trial in Mozambican children aged 1-4 years old. There was a notable decrease in malaria-related mortality in the group that were immunised with RTS,S/AS02 in comparison to the control group: over the 45 month surveillance period, 63 cases of severe malaria were experienced by volunteers who were immunised with RTS,S/AS02 (4.6%) in comparison to 83 cases in the control group (7%). Thus the RTS,S vaccine appears to be a promising vaccine candidate providing moderate protection.

1.6.3 Asexual erythrocytic stage vaccines

Asexual eryhtrocytic stage vaccines aim to protect against malaria by reducing the number of parasites and thereby reducing the number of deaths of infected people. Table 1.2 lists a number of the asexual erythrocytic stage candidate vaccines under investigation. Possibly the most advanced asexual erythrocytic stage vaccine candidates at the moment are those using the antigens glutamate-rich protein (GLURP), apical membrane antigen 1 (AMA-1) and the merozoite surface proteins 1 (MSP-1), 2 (MSP-2) and 3 (MSP-3).

Table 1.1. Pre-erythrocytic vaccine candidates

Vaccine candidate	Composition of vaccine	Protective efficacy
CS102	CS102 is a 102 amino acid peptide representing the C-terminus of CSP.	Immunisation of human volunteers with CS102 induced antibodies against the peptide and $CD8^+$ and $CD4^+$ T-cells (Prato *et al*., 2005). However the vaccine candidate has failed to protect against experimental malaria challenge in Phase I and IIa clinical trials (Girard *et al*., 2007).
ICC-1132/ Malariavax	A modified Hepatitis B virus core protein, expressing the central repeat regions of *P. falciparum* CSP. This region contains a B-cell epitope $(NANP)_3$ and a $CD4^+$T-cell epitope (NANPNVDPNANP), in addition to a universal T cell epitope (Gregson *et al*., 2008).	The ICC-1132 vaccine candidate is well tolerated (Oliveira *et al*., 2005; Walther *et al*., 2005; Gregson *et al*., 2008). T-cells secreting IFN-γ and IL-2, in addition to antibodies to CSP and Hepatitis B virus core protein, were detected (Nardin *et al*., 2004; Oliveira *et al*., 2005, Walther *et al*., 2005). However, no protection was observed in volunteers challenged with *P. falciparum* sporozoites (Walther *et al*., 2005). It was proposed that more potent adjuvants should be used to increase the immunogenicity of the vaccine candidate (Gregson *et al*., 2008).

Table 1.2. Erythrocytic stage vaccine candidates under development and their relative efficacies

Vaccine candidate	Composition of vaccine	Protective efficacy
SPf66	Synthetic peptide vaccine containing blood stage and sporozoite stage antigens, but is predominantly targeted against the blood stage of the parasite life cycle	Trials of SPf66 revealed the efficacy to be too low, especially in Africa. It was mildly protective in some trials in South America but was deemed to be too weak to warrant further testing (Acosta *et al*., 1999; Graves and Gelband 2006).
Glutamate-rich protein (GLURP)	A long synthetic peptide (AA 85-213 of GLURP)	A Phase I trial induced a cellular and humoral immune response with large quantities of antibodies, predominantly cryophilic IgG_1. Serum collected from volunteers thirty days after their last immunisation inhibited parasite growth in vitro in the presence of monocytes (Hermsen *et al*., 2007).
Merozoite surface protein (MSP)-1	The 42 kDa C-terminus of MSP-1(FMP1)	FMP1/AS02 was considered to not have sufficient potential as a monovalent vaccine candidate in a Phase IIb trial (Ogutu *et al*., 2009).
$MSP\text{-}1_{19}$	The 19 kDa C-terminus of MSP-1	Antibodies to $MSP\text{-}1_{19}$ were shown to play an important role in suppressing parasitemia in mice (de Koning-Ward *et al*., 2003) and in Ghanaian children (Dodoo *et al*., 2008)

Table 1.2. Erythrocytic stage vaccine candidates under development and their relative efficacies (continued)

Vaccine candidate	Composition of vaccine	Protective efficacy
MSP-2	Two long synthetic peptides (LSP) representing both allelic forms of MSP-2.	Peptide specific antibodies were associated with protection against malaria, and immunisation of mice with the peptides elicited a strong antibody response. The antibodies recognised native MSP-2 (Flueck *et al.*, 2009).
MSP-3	MSP-3 LSP	Phase Ib trial was found to be safe, well tolerated in Tanzanian children aged 12-24 months. The vaccine elicited cytophilic antibodies, thereby providing affirmation that the vaccine should be evaluated in a phase II trial to test MSP-3's ability of protecting against *P. falciparum* infection (Lusingu *et al*., 2009).
Apical membrane antigen (AMA)-1	Recombinant AMA-1	A Phase I/IIa efficacy trial revealed AMA-1 to be highly immunogenic, however, the immunogenicity did not translate into adequate protection against sporozoite challenge (Spring *et al*., 2009).

Table 1.2. Erythrocytic stage vaccine candidates under development and their relative efficacies (continued)

Vaccine candidate	Composition of vaccine	Protective efficacy
'Combination B'	The 'combination B' vaccine consists of MSP-1, MSP-2 and ring-stage infected-erythrocyte surface antigen (RESA).	A 62% reduction in parasites was observed in volunteers vaccinated with the 'combination B' vaccine candidate in a phase I/IIb trial (Genton *et al*., 2002). Only the one allelic form (3D7) of MSP-2 was used in the vaccine which resulted in breakthrough infections with parasites of the FC27 allele genotype, thereby indicating that both allelic forms of MSP-2 need to be included in the vaccine for it to be completely effective (Flück *et al*., 2004).

1.6.4 Transmission blocking vaccines

Transmission blocking vaccines aim to prevent the development of infectious sporozoites in the salivary glands of *Anopheles* mosquitoes, thereby preventing the infection of human hosts. These vaccines aim to achieve this by inducing the production of antibodies against sexual stage antigens (Lavazec and Bourgouin, 2008; Sutherland, 2009). Among the candidate transmission blocking vaccines in development at the moment are the *P. falciparum* ookinete surface antigens *Pf*s25 and *Pf*s28, the *P. vivax* ookinete surface antigens *Pv*s25 and *Pv*s28, *Pf*s230 and *Pf*s48/45.

Evaluation of *Pf*s48/45 immunity using membrane feeding assays in mice showed effective transmission blocking action (Outchkourov *et al.*, 2008; Chowdhury *et al.*, 2009). High antibody titres and transmission blocking activity of greater than 93% was experienced when *Pf*s48/45 was given to Olive baboons (Chowdhury *et al.*, 2009), indicating its potential as a promising transmission blocking vaccine candidate for humans.

Mice vaccinated with *Pv*s25 and *Pv*s28 produced antisera that recognised the antigens and completely blocked the transmission of parasites (Hisaeda *et al.*, 2000). In a Phase I human vaccine trial of *Pv*s25 adsorbed onto Alhydrogel®, transmission blocking immunity was observed although the level of transmission blocking was not sufficiently high for a feasible vaccine (Malkin *et al.*, 2005). It was thought that a more potent adjuvant would provide a more immunogenic result. Thus, *Pv*s25 and *Pf*s25 were formulated with Montanide ISA 51 and administered to humans during a Phase 1 trial. The trial had to be discontinued due to adverse systemic reactions experienced by the volunteers, possibly due to the combination of the antigen and the Montanide ISA 51 (Wu *et al.*, 2008). However, sufficient data was obtained from the individuals immunised with *Pf*s25 prior to the closure of the vaccine trial, indicating that *Pf*s25 was able to induce transmission blocking antibodies in humans. Thus suggesting that a *Pf*s25 vaccine would be feasible, provided that the systemic reactions could be minimised (Wu *et al.*, 2008).

1.6.5 Alternative vaccine approaches

1.6.5.1 Multi-stage, multi-antigen vaccines

Multi-stage, multi-antigen vaccine candidates are composed of a number of parasite antigens from many of the stages of the parasites life cycle. The benefits of such an approach

are the possibility of overcoming the problems associated with antigenic variation and the parasite evading the host immune system (Stanley, 1998; Girard *et al*., 2007). However, this approach may come with problems, including the possibility of the different components of the vaccine producing adverse reactions when combined, or interacting negatively with each other (Girard *et al*., 2007). Two of the multi-stage, multi-antigen vaccines that will be discussed briefly here, are the NYVAC-*Pf*7 (Tine *et al*., 1996) and FALVAC-1A vaccine candidates.

NYVAC-*Pf*7 was constructed with an attenuated NYVAC vaccinia virus strain, expressing seven *P. falciparum* antigens. The antigens are from different stages of the parasite life cycle: CSP, sporozoite surface protein 2. (sporozoite stage); liver stage antigen 1 (liver stage); MSP-1, serine repeat antigen (SERA), AMA-1 (blood stage) and a 25 kDa sexual stage antigen (gametocyte stage) (Tine *et al*., 1996). NYVAC-*Pf*7 was shown to be safe in Rhesus monkeys and to produce antibodies against antigens in all stages of the parasite life cycle (Tine *et al*., 1996). Disappointing results were obtained in a phase I/IIa trial as NYVAC-*Pf*7 did not protect human volunteers, against *P. falciparum* infection (Ockenhouse *et al*., 1998), however the time from infection to appearance of parasites in the blood was significantly longer in volunteers immunised with NYVAC-*Pf*7, in comparison to volunteers receiving the control.

FALVAC-1A consists of 21 B-cell, cytotoxic T cell and helper T cell epitopes from *P. falciparum* proteins found in the sporozoite, liver, erythrocytic and sexual stages of the parasite life cycle, in addition to a tetanus toxoid helper epitope (Zhou *et al*., 2006). FALVAC-1A was shown to be highly immunogenic in rabbits and to induce in vitro sporozoite inhibiting antibodies (Zhou *et al*., 2006). In 2008, Ravi *et al*. showed that it was possible to produce industrial scale, clinical grade FALVAC-1A for clinical trials at a reasonable cost. Mice immunised with FALVAC-1A elicited antibodies capable of recognising *P. falciparum* sporozoites and blood stage parasites (Kaba *et al*., 2008). Further trials need to be completed to assess the efficacy and safety for human trials.

1.6.5.2 Glycophoshatidylinositol (GPI) anchor

The GPI anchor is involved in securing several *Plasmodium* antigens to the membrane and induces tumour necrosis factor (TNF)-α and interleukin (IL)-1 production, in addition to

hypoglycaemia. It is therefore postulated that GPI may have a role in the pathogenesis of malaria infections (Schofield *et al*., 1993) and therefore targeting the molecule may provide an anti-disease vaccine. Schofield *et al*. (1993) have shown that monoclonal antibodies to *Plasmodium* GPI were able to neutralise the toxicity of *Plasmodium* parasites. Mice immunised with a synthetic *P. falciparum* GPI glycan and subsequently challenged, were significantly protected (58.3-75% survival) in comparison to control mice (0-8.7% survival). This data suggests that an anti-GPI vaccine may be feasible (Schofield *et al*., 2002).

1.6.5.3 DNA vaccines

DNA-based vaccines allow expression of recombinant protein in mammalian cells. This is made possible by immunisation with a recombinant plasmid or viral vector encoding specific protective antigens (Ivory and Chadee 2004). The advantages of DNA-based vaccines are that they are considerably cheaper to produce, they do not require to be kept cold and they have a long shelf life. In addition, DNA-based vaccines are relatively easy to produce and induce strong T-cell responses, making them a promising avenue for producing a malaria vaccine (Le *et al*., 2000).

The majority of the malaria DNA candidate vaccines undergoing various stages of development involve vectors containing the gene expressing CSP. Sedegah *et al.* (1998) showed that BALB/c mice immunised with a plasmid expressing *P. yoelii* CSP were protected by the vaccines induction of $CD8^+$ T-cells and IFN-γ. Mice immunised with the plasmid DNA and boosted with recombinant vaccinia expressing the *P. yoelii* CSP were better protected (11/16 mice protected) than mice immunised with the plasmid DNA and boosted with the plasmid DNA (7/16 mice protected). Similarly, mice immunised with a multi-epitope *P. chabaudi adami* DNA vaccine were protected against *P.chabaudi adami* challenge via opsonising antibodies and IFN-γ production (Scorza *et al*., 2005). Intramuscular injection of *P. falciparum* CSP plasmid DNA in healthy adults was shown to be safe and well tolerated. No antigen-specific antibodies were detected in volunteers; however the vaccine did induce cytotoxic T-cell responses (Le *et al*., 2000).

Another DNA vaccine candidate in development is the multi-stage DNA vaccine operation (MuStDO) 5. MuStDO 5 is a multivalent plasmid DNA vaccine, composed of 5 plasmids encoding *P. falciparum* antigens and one plasmid encoding human granulocyte-

macrophage colony-stimulating factor (GM-CSF) (Parker *et al*., 2001). GM-CSF is included to enhance the immunogenicity of the vaccine. The other 5 plasmids encode CSP; sporozoite surface protein-2 (SSP2), also known as thrombospondin-related anonymous protein (TRAP); exported protein-1 (EXP-1); liver stage antigen-1 (LSA-1) and liver stage antigen-3 (LSA-3). The vaccine was shown to be safe in mice and rabbits as no severe pathology normally associated with GM-CSF was observed. The investigators believe that these results show the potential for healthy volunteers to be immunised with MuStDO 5 and subsequently challenged with *P. falciparum* in a phase I/II clinical trial (Parker *et al*., 2001).

1.7 Objective of the present study

The objective of the present study was to express and purify a 822 bp sequence of the *P. falciparum* gene PFC0760c. PFC0760c is of unknown function and has been shown to have high sequence similarity to a 96 kDa *P. chabaudi adami* protein (*Pca* 96) (Smallie, 2003). *Pca* 96 was shown to illicit a protective immune response in mice (Wanidworanun *et al*., 1987). Thus PFC0760c is of immunological interest. Additional objectives of the study were to identify the function of PFC0760c and to raise anti-peptide antibodies against the protein for use in characterisation of the protein.

CHAPTER 2

General materials and methods

General biochemical and molecular biology techniques used in the present study are detailed in this chapter. Experimental techniques more specific to particular chapters are described in their appropriate chapters.

2.1 Materials

The majority of the chemicals used in the present study were of analytical grade, purchased from Sigma (USA), Saarchem (South Africa), Roche Diagnostics (Germany), Merck (South Africa), Fluka Chemicals (Germany) and BDH (England). Distilled water (dH_2O) from the Milli-Q Plus ultra pure water system from Millipore (USA), was used throughout this study.

Coomassie brilliant blue R-250, Serva blue G, lauryl sulfate (sodium dodecyl sulfate), acrylamide, 4-chloro-1-naphthol, L-cysteine hydrochloride monohydrate, 3-hydroxy-4- [2-sulfo-4- (4-sulfo-phenylazo) phenylazo]-2,7-napthalenedisulfonic acid (Ponceau S), Yeast extract powder, Freund's complete and incomplete adjuvants, glutathione agarose, reduced glutathione, 3-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS), rabbit albumin, sodium borohydride ($NaBH_4$), sodium cyanoborohydride ($NaCNBH_3$), dithiothreitol (DTT), 5,5'-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent), modified Hanks balanced salt, RPMI-1640, chloramphenicol, pepstatin A, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), soy bean trypsin inhibitor (SBTI), sodium bicarbonate, agarose, peroxidase-conjugated avidin, peroxidase-conjugated rabbit anti-chicken IgG and Sephadex G10 were all purchased from Sigma (USA). Glutaraldehyde, isopropyl-β-D-thiogalacto-pyranoside (IPTG), ampicillin sodium salt, dimethylsulfoxide (DMSO), N, N-dimethylformamide (DMF) and phenol were from Fluka (Germany). Nunc-Immuno^{TM} 96-well plates were from Nunc Intermed (Denmark). Bovine serum albumin (BSA) fraction V, shrimp alkaline phosphatase, 2,2′-Azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulfonate (ABTS), Tris-base, DNTP mix, phenyl-methylsulfonylfluoride (PMSF) and 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) were purchased from Roche diagnostics (Germany). Peroxidase-conjugated rabbit anti-chicken IgY, peroxidase-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated donkey anti chicken IgY (IgG) were purchased from Jackson

Immunochemicals (USA). Triton X-100, NNN'N'-tetramethylethlenediamine (TEMED), chloroform and NN'-methylenebis-acrylamide were purchased from BDH (England). Poly-Prep® chromatography columns, SDS-PAGE molecular weight standards (low range) and Precision Plus molecular weight markers were from Bio-Rad (USA). Whatman No. 1 filter paper was from Whatman International Ltd (UK). Leupeptin was purchased from Cambridge Research Chemicals (England). The plasmid pGEX4T1 was from GE Healthcare (USA). SnakeSkin® pleated dialysis tubing and AminoLink® and SulfoLink® resins were from Pierce Chemical Company (USA). The DNA MassRuler™ marker was from Fermentas (Lithuania). Heparin coated Vacutainer™ tubes were from BD Vacutainer Systems (UK). Sephadex® G25 fine was purchased from Pharmacia Biotech (Sweden). Tween 20, polyethylene glycol (PEG) 6000, PEG 20 000, maltose, propan-2-ol, glycerol and D (+) glucose monohydrate were purchased from Saarchem (South Africa). Hybond-C extra nitrocellulose was purchased from Amersham Life Sciences (UK). FIREPol® DNA polymerase I, 10X PCR buffer (Mg^{2+} and detergent free) and $MgCl_2$ were purchased from Solis Biodyne (Estonia). Glycine, 2-mercaptoethanol, methanol (99.8%), acetic acid (98%), tryptone powder (pancreatic digest of casein) and bacteriological agar were purchased from Merck (South Africa). Low melting point (LMP) agarose, ethidium bromide solution, 1 kb DNA step ladder, Wizard® PCR Preps DNA Purification System, *Eco*R1, Wizard® DNA Clean-Up System, and the Wizard® *Plus* SV Minipreps DNA Purification System were purchased from Promega Corporation (USA). DNAse-free RNAse A was purchased from Boehringer Mannheim (Germany). Amylose resin, rabbit anti-MBP antiserum, pMAL-c2X, *Sac*I, *Bam*HI, *Acc*I and Factor Xa were purchased from New England Biolabs (USA). Biotinylated mouse monoclonal anti-GST Tag IgG was from AnaSpec Incorporated (USA). Terumo® U-100 insulin 1cc 27 Gauge x ½" syringes were from Terumo medical corporation (USA). BALB/c mice were from the National Health Laboratory Service Animal Unit (South Africa). Syringes (2 ml) were from Promex (South Africa) and hypodermic 20G x 1½" needles were from Tae-Chang Industrial Company (Korea). Glass slides were purchased from Separations (South Africa) and Ziploc® bags were from S. C. Johnson & Son (Pty) Ltd. (South Africa). The peptides FKLGSCYLYIINRNLKEI and CFKLGSCYLYIINRNLKEI were synthesized by GenScript Corporation (USA) and the peptide SDDDNRQIQDFEC by Auspep (Australia). DNA primers were synthesized by Integrated DNA Technologies Inc. (USA). Slides (8 mm, 8-well) were purchased from MP

Biomedicals (USA). Elite fat-free powdered milk was from Clover (South Africa). Professor Wim G.J. Hol (Howard Hughes Medical Institute and Department of Biological Structure, University of Washington, USA) kindly provided the RIG plasmid. The plasmids pKK223-3-*Pf*LDH (expressing *Plasmodium falciparum* lactate dehydrogenase) and pKK223-3-*Pv*LDH (expressing *Plasmodium vivax* lactate dehydrogenase) were kindly supplied by Leo Brady (Department of Biochemistry, University of Bristol, UK).

Images of western blots, SDS-PAGE gels and agarose gels were captured using the VersaDoc™ imaging system and Quantity One software from Bio-Rad (USA). The absorbance of ELISA plates were read with a Versamax tunable microplate reader using SOFTmax®PRO software from Molecular Devices (USA). Polymerase chain reaction (PCR) was performed using the GeneAMp® PCR system 2700 or 9700 from Applied Biosystems (USA). Sonication of bacterial cells was performed with the VirSonic 60 Virtis from The Virtis Company (USA). Absorbance readings were taken using the Ultrospec 2100 pro UV/visible spectrophotometer from Amersham Biosciences (UK) and SUPRASIL® quartz cuvettes from Hellma® (Germany). Centrifugation was performed with the following centrifuges: Biofuge *pico* microcentrifuge from Heraeus Sepatech (Germany), Sigma 3K20 from Sigma Laboratory centrifuges (USA), the J2-21M and J2-21 from Beckman (USA), and the Avanti J-26 XPI from Beckman Coulter (USA). SDS-PAGE gels were run using the Mini-Protean® II equipment and western blotting was performed using the Mini Trans-Blot® cell, both from Bio-Rad (USA). Agarose gels were run using the GES and mini-GES electrophoresis equipment from Wealtec Corporation (USA). Incubation of western blots with antibodies and silverstaining was performed on The Belly Dancer (Stovall Life Science Inc., USA),

Ethical approval for the animal procedures performed in the present study was obtained from the University of KwaZulu-Natal animal ethics committee (025/08/animal).

2.2 Bradford protein assay

The Bradford method (Bradford, 1976) of protein concentration determination was used routinely to determine the amount of protein present in samples. The assay involves the dye reagent binding predominantly to arginine residues of proteins (Compton and Jones, 1985). The Bradford assay is rapid, highly sensitive and produces reproducible results. Read and

Northcote (1981) modified the assay, resulting in increased sensitivity and less variation in response to different proteins. The dye can be dissolved in a solution of ethanol: phosphoric acid (1:2) which is stable for 1-2 weeks kept in the cold, or 2% (v/v) perchloric acid which is the preferable method due to the dye being indefinitely stable (Scopes, 1994).

2.2.1. Reagents

Bradford dye reagent. Coomassie brilliant blue G-250 (0.6 g) was dissolved in 2% (v/v) perchloric acid (1 l) by stirring for approximately 1 h. The dye was filtered through Whatman no. 1 paper and stored in an amber bottle at room temperature. If precipitate was observed, the dye was filtered and the standard curve was recalibrated.

2% (v/v) perchloric acid. Perchloric acid (27.8 ml) was made up to 1 l with dH_2O.

Ovalbumin stock solution (1 mg/ml). Ovalbumin (0.001 g) was dissolved in 1 ml dH_2O.

2.2.2 Procedure

Ovalbumin standards (0-100 µg) were used to construct a standard curve (Figure 2.1). Quintuplicate samples of 0-100 µl of the 1 mg/ml ovalbumin solution were made up to 100 µl with dH_2O. Bradford dye reagent (900 µl) was added to each of the ovalbumin samples (100 µl) and vortexed. The samples were left for 5 minutes before reading the absorbance at 595 nm. Protein samples of unknown concentration were made up to 100 µl with dH_2O and mixed with Bradford dye reagent (900 µl) as explained for the standard ovalbumin samples. The standard curve (Figure 2.1) was then used to determine the protein concentration, from the absorbance readings obtained at 595 nm.

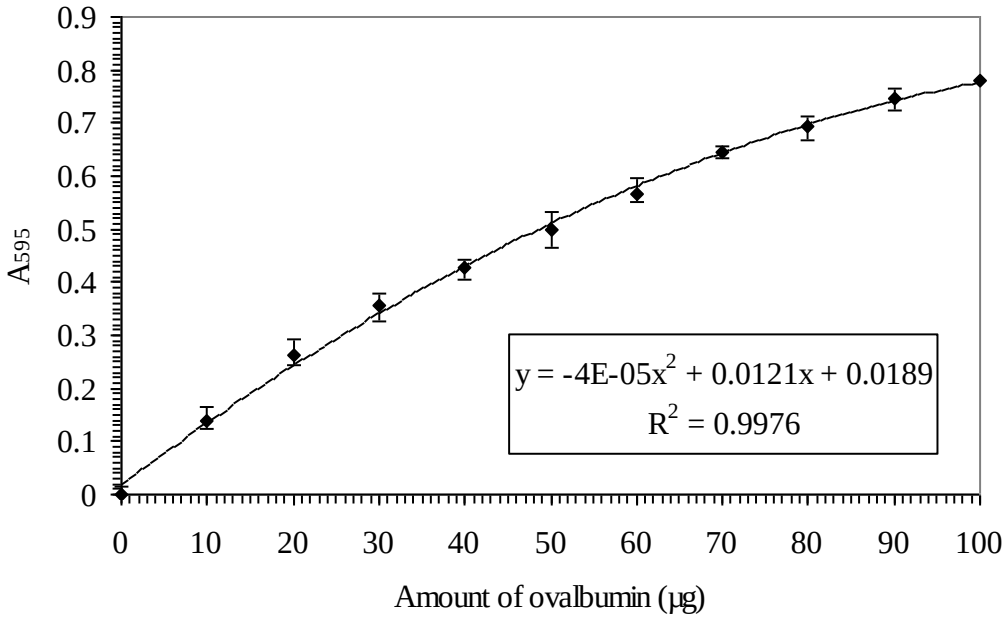


Figure 2.1. Bradford standard curve constructed from ovalbumin.

2.3 Concentration of protein samples

It was often necessary to concentrate dilute protein solutions before analysing them with SDS-PAGE or before continuing with further purification steps. Dilute protein samples were concentrated by dialysis against polyethylene glycol (PEG) 20 000 or SDS-KCl precipitation for proteins requiring further concentration before loading onto sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) gels (Trask and Muller, 1983).

2.3.1 Dialysis against PEG 20 000

PEG 20 000 is a dry hydrophilic polymer that can be used as a simple, cost-effective method of concentrating dilute protein samples. The PEG 20 000 draws water in the protein sample through the dialysis membrane, thereby concentrating the protein sample.

2.3.1.1 Materials

Dialysis tubing (10 000 MW cut-off).

PEG 20 000.

2.3.1.2 Procedure

Gloves were worn throughout the concentration procedure to eliminate contamination of the protein sample with skin keratins. The protein sample to be concentrated was placed into dialysis tubing (10 000 MW cut-off) and sealed with dialysis clips. The dialysis bag was placed into a container and covered with thick layer of PEG 20 000. The container was left at 4˚C until the protein sample had been sufficiently concentrated. The dialysis bag was removed from the container and rinsed with dH_2O to remove any PEG 20 000 that had adhered to the dialysis tubing, before removing the concentrated protein sample.

2.3.2 SDS-KCl precipitation of proteins

Proteins were precipitated with the SDS-KCl method (Trask and Muller, 1983) whenever the volume of protein solutions was too large to load into SDS-PAGE wells or when protein solutions were too dilute to view on an SDS-PAGE gel. The protein becomes coated with SDS, which then complexes to the potassium ion (K^+). This results in an insoluble complex, which is effectively precipitated with centrifugation. The pellet can then be resuspended in a smaller volume, resulting in concentrated protein solution.

2.3.2.1 Reagents

5% (w/v) SDS. SDS (0.5 g) was dissolved in 10 ml dH_20.

3 M KCl. KCl (2.24g) dissolved in 10 ml dH_20.

2.3.2.2 Procedure

10% (v/v) of a 5% (w/v) SDS solution and 10% (v/v) of a 3 M KCl solution were gently mixed with the protein solution. The solution was centrifuged (10 000 *g*, 2 min) in a desktop micro centrifuge. The pellet was resuspended in a reduced volume of solution C (Section 2.4.1). An equivalent volume of reducing sample buffer (Section 2.4.1) was added, and the solution boiled for 2 min before loading onto an SDS-PAGE gel.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method described by Laemmli (1970) to analyse protein samples. The method involves boiling the proteins to be analysed with sodium dodecyl

sulphate (SDS), a strong anionic detergent. Under these conditions, the proteins become denatured and coated with SDS. The SDS masks the proteins with a negative charge thereby allowing the proteins to migrate through the gel matrix, toward the anode, separating, based on their molecular weight (Dennison, 1999).

The gel is made up of chains of polymerized acrylamide, cross-linked by N, N-methylene bis-acrylamide. The cross-linking forms pores for the protein to migrate through and provides the gel with rigidity and strength (Sambrook and Russell, 2001). The proportions of acrylamide and N, N-methylene bis-acrylamide can be varied to give a different pore size, which in turn results in gels capable of different ranges of protein separation.

The protein samples were routinely boiled in the presence of β-mercaptoethanol, in addition to SDS. β-mercaptoethanol is a reducing agent able to cleave disulfide bonds. Cleaving disulfide bonds allows for the separation of protein subunits and polypeptide chains held together by disulfide bonds.

2.4.1 Reagents

Solution A: Monomer solution [30% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide]. Acrylamide (29.9 g) and bis-acrylamide (0.8 g) were dissolved in 100 ml dH_2O and filtered through Whatman no. 1 paper. The solution was stored in an amber, glass bottle at 4˚C.

Solution B: Running gel buffer [1.5 M Tris-HCl, pH 8.8]. Tris (18.148 g) was dissolved in approximately 80 ml of dH_2O and adjusted to pH 8.8 with HCl and the volume made up to 100 ml. The solution was filtered through Whatman no. 1 paper and stored at 4˚C

Solution C: Stacking gel buffer [0.5 M Tris-HCl, pH 6.8]. Tris (1.5 g) was dissolved in 20 ml dH_2O and adjusted the pH to 6.8 with HCl. The volume was made up to 25 ml with dH_2O. The solution was filtered through Whatman no. 1 paper and stored at 4˚C.

Solution D: 10% (w/v) SDS (lauryl sulfate). SDS (10 g) was dissolved in 100 ml dH_2O.

Solution E: 10% (w/v) ammonium persulfate. Ammonium persulfate (0.1 g) made up to 1 ml with dH_2O.

Solution F: Tank buffer [250mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS]. Tris (3 g) and glycine (14.4 g) were dissolved in dH_2O and made up to 1 l. Just prior to use 10% (w/v) SDS (Solution D) was added (1 ml of 10% (w/v) SDS per 100 ml of tank buffer).

0.1% (m/v) Bromophenol blue. Bromophenol blue (0.001 g) was dissolved in 1 ml dH_2O and stored at room temperature.

SDS-PAGE sample buffer. 0.5 M Tris-HCl, pH 6.8 (solution C) (1.2 ml), glycerol (1 ml), 10% (w/v) SDS (solution D) (2 ml), 0.1% (w/v) bromophenol blue (0.5 ml) were mixed together and made up to 9.5 ml with dH_2O. The buffer was stored at 4˚C.

SDS-PAGE reducing sample buffer. Sample buffer (475 µl) was added to β-mercaptoethanol (25 µl) just before use, in a fume hood.

N, N, N',N'-tetramethylenediamine (TEMED).

2.4.2 Procedure

Running and stacking gels were prepared according to Table 2.1 depending on the concentration of acrylamide required. The gels were cast using a Bio-Rad Mini Protean® II vertical slab electrophoresis unit. The running gel was cast first and allowed to set under a small layer of dH_2O. The dH_2O was poured off and the stacking gel layered on top of the running gel. The combs were placed into the stacking gel to form wells and left to set. The gels were assembled in the running tank and covered with tank buffer (Solution F). Protein samples for reducing SDS-PAGE were combined with reducing treatment buffer in a 1: 1 ratio and boiled for 2 min before loading. The β-mercaptoethanol reduces the proteins disulphide bonds, thus disrupting the protein structure for ease of separation on the SDS-PAGE gel. The gels were electrophoresed at 18 mA per gel until the dye front was approximately 0.5 cm from the bottom of the gel. The gels were removed from the electrophoresis apparatus and placed into the appropriate staining solution (Section 2.5) or used for western blotting (Section 2.6).

Molecular weight markers were run alongside protein samples in order for the molecular mass of sample proteins to be determined. A standard curve of the log of the molecular weight marker proteins vs. the motility (Rf value) of the protein standards was constructed. The size of sample protein was calculated from the curve. A standard curve was constructed for each percentage gel and for each type of molecular weight marker.

Table 2.1. Preparation of running gels and stacking gels for Laemmli SDS-PAGE gels

Solution	Volume (ml)					
	Running gel (%)				Stacking gel (%)	
	12.5	10.0	7.5	5.0	4.0	3.0*
A	6.25	5.0	3.75	2.5	0.94	0.71
B	3.75	3.8	3.75	3.75	0	0
C	0	0	0	0	1.75	1.75
D	0.15	0.15	0.15	0.15	0.07	0.07
E	0.075	0.15	0.075	0.075	0.035	0.035
dH_2O	4.75	5.9	7.25	8.5	4.3	4.53
TEMED	0.0075	0.006	0.0075	0.0075	0.015	0.015

*A stacking gel concentration of 3% was used only with a running gel of 5%.

2.5 Staining protein gels

2.5.1 Coomassie staining

Coomassie Blue R-250 dye was routinely used to detect protein bands after SDS-PAGE. It is an aminotriarylmethane dye with the ability to bind to the NH_3^+ groups on proteins by a combination of van der Waals forces and electrostatic interactions (Sambrook and Russell, 2001). This method of staining protein is simpler than silver staining (Section 2.5.2) but is far less sensitive.

2.5.1.1 Reagents

Coomassie stain stock solution [1% (w/v) Coomassie Blue R-250]. Coomassie Blue R-250 (1 g) dissolved in dH_2O (100 ml) with stirring for 1 h at room temperature. The solution was filtered through Whatman No. 1 filter paper.

Coomassie staining solution [0.125% (w/v) Coomassie Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Coomassie stain stock solution (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml). The solution was made up to 500 ml with dH_2O and stored in an airtight bottle.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) mixed with acetic acid (100 ml) and made up to 1 l with dH_2O.

Destaining solution II [5% (v/v) methanol, 7% (v/v) acetic acid]. Methanol (50 ml) mixed with acetic acid (70 ml) and made up to 1 l with dH_2O.

2.5.1.2 Procedure

Immediately following electrophoresis, gels were left in Coomassie staining solution in a sealed container (4 h or overnight). Destaining of the gel was achieved by soaking the gel in destain I (overnight or until the gel had a fairly clear background). This step was followed by soaking the gel in destain II until the background was completely clear. The gel was stored in a Ziploc® plastic bag with a small volume of dH_2O.

2.5.2 Silver staining

Silver staining was used to view small quantities of protein on SDS-PAGE gels. With the method of silver staining used (Blum *et al*., 1987), the gel is placed in silver nitrate, which provides silver ions for reaction with protein. The formaldehyde reduces the silver ions to metallic silver, resulting in the development of the protein bands (Merril, 2002).

2.5.2.1 Reagents

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.0185% (v/v) formaldehyde]. Methanol (50 ml), glacial acetic acid (12 ml), and formaldehyde (50 µl of a 37% solution) were made up to 100 ml with dH_2O.

50% (v/v) Ethanol. Absolute ethanol (50 ml) was made up to 100 ml with dH_2O.

Pretreatment solution [0.02% (w/v) $Na_2S_2O_3.5H_2O$]. $Na_2S_2O_3.5H_2O$ (0.02 g) was dissolved in 100 ml dH_2O.

Silver nitrate impregnation solution [0.2% (w/v) $AgNO_3$, 0.02775% (v/v) formaldehyde]. $AgNO_3$ (0.2 g) was dissolved in 100 ml dH_2O, and formaldehyde (75 µl of a 37% solution) was added.

Developing solution [6% (w/v) Na_2CO_3, 0.0004 % (w/v) $Na_2S_2O_3.5H_2O$, 0.0185% (v/v) formaldehyde]. Na_2CO_3 (6 g) was dissolved in 95 ml of dH_2O, $Na_2S_2O_3.5H_2O$ (2 ml of pretreatment solution) and formaldehyde (50 µl of a 37% solution) were added and the volume was made up to 100 ml.

Stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and glacial acetic acid (12 ml) were made up to 100 ml with dH_2O.
50% (v/v) methanol. Methanol (50 ml) was made up to 100 ml with dH_2O.

2.5.2.2 Procedure

All steps were carried out on a rocker (The Belly Dancer), at room temperature and in glassware that had been thoroughly cleaned. The glassware was cleaned with soap and water, followed by 70% (v/v) ethanol and then rinsed with dH_2O, to avoid background staining. After electrophoresis the gel was left in fixing solution overnight. The gel was washed in 50 % (v/v) ethanol (3 x 20 min), followed by incubation in pretreatment solution (1 min). The gel was washed with dH_2O (3 x 20 s) and then soaked in impregnation solution (20 min). This was followed by washing with dH_2O (3 x 20 s). The gel was then placed in developing solution until the first protein bands started to become visible. The developing solution was immediately replaced with dH_2O and the protein bands were allowed to develop fully. Colour development was stopped by soaking the gel in stopping solution (10 min). The gel was then washed with 50% (v/v) methanol (approximately 20 min), photographed and then stored in a Ziploc® plastic bag.

2.6 Western blotting

Towbin *et al.* (1979) were the first to describe the transfer of proteins from SDS-PAGE to nitrocellulose membranes. The procedure involves transferring protein from a replica SDS-PAGE gel to nitrocellulose electrophoretically. The SDS-PAGE gel is placed with the nitrocellulose in a "sandwich" which is then placed into the blotting apparatus in such an orientation that the gel is positioned on the cathode side and the nitrocellulose is positioned on the anode side. This results in the negatively charged SDS-coated proteins being transferred out of the gel towards the positive anode, and therefore becoming bound to the nitrocellulose. Ponceau S stain was used to determine whether the transfer of protein from the gel to the nitrocellulose was successful before continuing with the blotting procedure. A blocking step was required to block the remaining sites on the nitrocellulose, preventing antibodies from binding to the nitrocellulose non-specifically. In the present study the nitrocellulose was blocked with a solution of 5% (w/v) non-fat milk powder in tris buffered saline. This is a cost-

effective blocking solution that results in low background. The disadvantage of using non-fat milk to block the nitrocellulose is that it can cause the transferred protein to be removed from the nitrocellulose if it is incubated for an exceptionally long period (Harlow and Lane, 1999).

2.6.1 Reagents

Blotting buffer. Tris (6.05 g) and glycine (14.4 g) dissolved in dH_2O (750 ml). Methanol (200 ml) added and volume made up to 1 l with dH_2O. Just prior to use, 10% (w/v) SDS was added (100 µl 10% (w/v) SDS (Section 2.4.1) per 100 ml of blotting buffer).

Tris buffered saline (TBS) [20 mM Tris, 200 mM NaCl, pH 7.4]. Tris (2.42 g) and NaCl (11.69 g) dissolved in dH_2O (approximately 980 ml) and adjusted to pH 7.4 with HCl. The volume was made up to 1 l with dH_2O.

5% (w/v) Fat-free powdered milk. Elite powdered milk (5 g) was dissolved in TBS (100 ml).

0.5% (w/v) Bovine serum albumin (BSA)-TBS. BSA (0.5 g) was dissolved in TBS (100 ml).

4-Chloro-1-naphthol substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2]. 4-chloro-1-naphthol (0.015 g) was dissolved in methanol (5 ml). The volume was made up to 25 ml with TBS and 30% (v/v) H_2O_2 (10 µl) added.

Ponceau S stain [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid]. Ponceau S (0.1g) was dissolved in glacial acetic acid (1 ml) and made up to 100 ml with dH_2O.

2.6.2 Procedure

Gloves were worn throughout the following procedure to prevent skin keratins attaching to the nitrocellulose, which could cause background. Following SDS-PAGE of duplicate gels (Section 2.4), one gel was stained with Coomassie as a reference gel, and the duplicate gel was used to transfer protein onto nitrocellulose. The nitrocellulose, SDS-PAGE gel and blotting sheets were immersed in blotting buffer (5 min). The nitrocellulose was placed on top of a foam pad and three sheets of filter paper. The gel was carefully placed on top of the nitrocellulose, followed by another three sheets of filter paper. A glass rod was used to smooth out any air bubbles and the second foam pad was placed on top of the filter paper. The sandwich was placed into the blotting sandwich cassette. The cassette was then placed into the blotting apparatus with the gel on the anodal side of the nitrocellulose. The blotting

apparatus was placed into the tank and immersed in blotting buffer. Transfer of protein from the gel to the nitrocellulose was achieved at 100 mA for 16 h.

The nitrocellulose was removed from the apparatus and immersed in Ponceau S stain for a few seconds to determine the efficiency of protein transfer. The nitrocellulose was washed with dH_2O to enable viewing of the transferred bands. The molecular weight marker bands were marked with pencil, and if transfer had been successful, the Ponceau S stain was removed by placing the nitrocellulose in dH_2O containing a few drops of NaOH. Once the colour had been removed, the nitrocellulose was washed several times with dH_2O and allowed to air dry. Non-specific binding of antibodies was prevented by blocking with 5% (w/v) non-fat milk powder in TBS (1 h, room temperature). The nitrocellulose was washed with TBS (3 x 5 min) and then incubated with primary antibody in 0.5% (w/v) BSA-TBS (2 h). The nitrocellulose was washed with TBS again (3 x 5 min). An appropriate HRPO-conjugated secondary antibody in 0.5% (w/v) BSA-TBS was incubated with the nitrocellulose (1 h). After washing the nitrocellulose with TBS (3 x 5 min), 4-chloro-1-naphthol substrate solution was added and the blot left to develop in the dark. Once visible bands had developed against a pale background, the nitrocellulose was removed from the substrate, washed several times with dH_2O and allowed to dry between filter paper before photographing.

2.7 Agarose gel electrophoresis of DNA

Agarose is a linear polymer composed of alternating residues of D- and L- galactose. These alternating residues are linked by α- (1-3) and β- (1-4) glycosidic linkages, forming helical fibres that aggregate into supercoiled structures (Sambrook and Russell, 2001). Gelation of agarose results in a three dimensional mesh which acts as a molecular sieve, separating DNA molecules on the basis of their size and conformation (Snustad and Simmons, 2000). The movement of larger molecules is thus hindered to a greater degree than smaller molecules, resulting in the 'sieving' process.

The most common method of viewing DNA in agarose gel is staining the DNA with the fluorescent dye ethidium bromide. Ethidium bromide intercalates between the DNA bases, making the DNA visible under UV light (Sambrook *et al.*, 1989).

2.7.1 Reagents

50X TAE (Tris-acetate-EDTA) buffer. Tris (24.2 g) was dissolved in 50 ml dH_20. Glacial acetic acid (5.7 ml) and 0.5 M EDTA (10 ml) were added and the volume made up to 100 ml. The buffer was stored at room temperature.

1X TAE buffer. 50X TAE (20 ml) was diluted to 1 l.

0.5 M Na_2EDTA. Disodium ethylenediaminetetra-acetate.$2H_2O$ (Na_2EDTA) (18.6 g) was dissolved in dH_2O (80 ml) and adjusted to pH 8.0 with NaOH. The volume was made up to 100 ml with dH_2O and autoclaved.

10 mg/ml Ethidium bromide. Ethidium bromide was used at a final concentration of 0.5 μg/ml.

6x Gel loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol]. Bromophenol blue (0.06 g), xylene cyanol FF (0.06 g) and glycerol (7.5 ml) were made up to 25 ml with dH_2O and thoroughly mixed. The loading dye was stored at 4˚C. The 6x gel loading dye (1 μl) was added per 5 μl of DNA sample.

1% agarose. For mini-gels, agarose (0.25 g) was suspended in 25 ml of 1x TAE. For larger agarose gels, agarose (0.8 g) was suspended in 80 ml 1x TAE. The suspension was heated in a microwave oven in an Erlenmeyer flask, until the agarose had fully melted. During melting, the volume of the suspension would decrease due to evaporation and thus the volume was adjusted to its original volume with dH_2O. The agarose was allowed to cool to approximately 60˚C before adding ethidium bromide solution to a final concentration of 0.5 μg/ml, and pouring the gel. Low melting point agarose gels were prepared as for normal agarose gels except that they were left at 4˚C to solidify rather than at room temperature.

2.7.2 Procedure

The electrophoresis gel-casting tray was sealed with rubberised clasp seals and the comb was placed into the comb grooves on the tray. The casting apparatus was placed on a level surface. Melted agarose containing 0.5 μg/ml ethidium bromide, that had been allowed to cool to approximately 60˚C was then poured into the casting tray to a thickness of approximately 3-5 mm. Air bubbles that formed whilst pouring the gel, were quickly removed with a clean pipette tip, before the gel set. The agarose was left at room temperature to solidify (low melting point agarose gels were left at 4˚C to solidify). Once the agarose gel had set, the comb and seals were removed from the casting tray and the tray was placed into the

electrophoresis tank. The gel was orientated in such a way that the wells were placed at the cathode. The gel was covered with 1x TAE containing 0.5 μg/ml ethidium bromide to approximately 1 mm above the gel. The marker DNA and samples (mixed with 6x gel loading buffer) were loaded into the wells using a micropipette. The lid of the electrophoresis apparatus was securely fitted to the tank and the electrical leads were connected to a power pack so that the DNA would migrate from the negative cathode (black) to the positive anode (red). A voltage of 60-80 V was applied until the loading dye had migrated approximately 1 cm from the end of the gel. The electrical leads were disconnected and the casting tray holding gel was removed from the apparatus. The gel was viewed and photographed under UV light using the VersaDoc^{TM} imaging system and the Quantity One software from Bio-Rad.

The size of DNA fragments was determined from DNA ladders run alongside sample DNA. A standard curve of the log of the size of the DNA standards vs. the motility (Rf value) of the DNA standards was constructed. The size of sample DNA was calculated from the curve.

2.8 Preparation of protease inhibitors

A cocktail of protease inhibitors were included in buffers used for resuspending bacterial cell pellets and sonication. Leupeptin was used to inhibit cysteine and trypsin proteinases. L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) was used to inhibit cysteine proteinases and phenylmethyl-sulfonyl fluoride (PMSF) to inhibit serine proteinases. In addition the protease inhibitors L-I-tosylamide-2-phenyl-ethyl chloromethylketone (TPCK), pepstatin A and disodium ethylenediaminetetra-acetate (Na_2EDTA) were used to inhibit chymotrypsin-like proteinases, aspartic proteinases and metalloproteinases, repectively.

20 mM (10 mg/ml) Leupeptin. Leupeptin (0.01 g) was dissolved in sterile dH_2O (1 ml), aliquoted and stored at –20˚C. Leupeptin was used at a final concentration of 2 μg/ml.

10 mM (1.74 mg/ml) Phenylmethyl-sulfonyl fluoride (PMSF). PMSF (0.0174 g) was dissolved in propan-2-ol (isopropanol) (10 ml), aliquoted and stored at –20˚C. The inhibitor was used at a final concentration of 0.6 mM (100 μg/ml).

3 mg/ml L-I-tosylamide-2-phenyl-ethyl chloromethylketone (TPCK). TPCK (0.03 g) was dissolved in ethanol (10 ml), aliquoted and stored at –20˚C. The inhibitor was used at a final concentration 100 μg/ml.

0.5 M Disodium ethylenediaminetetra-acetate.2H_2O (Na_2EDTA). Na_2EDTA (18.6 g) was dissolved in dH_2O (80 ml) and adjusted to pH 8.0 with NaOH. The volume was made up to 100 ml with dH_2O. EDTA was used a final concentration of 1 mM.

10 mM L-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64). E-64 (0.007 g) was dissolved in sterile dH_2O (2 ml), aliquoted and stored at –20˚C. It was used at a final concentration of 0.02 mM.

1 mM Pepstatin A. Pepstatin A (0.0066 g) was dissolved in DMSO (10 ml), aliquoted and stored at –20˚C. Pepstatin A was used at a final concentration of 1 μM.

2.9 Preparation of bacterial media, antibiotics and isopropyl-β-D-thiogalacto-pyranoside (IPTG)

Antibiotics were used with bacterial expression to ensure that only cells containing plasmids having antibiotic resistance grew. Ampicillin was used when cells transformed with the plasmids pMAL-c2X, pTS822, pGEX4T1, pGEX4T1C18, pKK223-3-PfLDH and pKK223-3-PvLDH were cultured. Ampicillin inhibits the synthesis of the bacterial cell wall by interfering with the formation of peptido-glycan cross-links. Ampicillin-resistant plasmids possess the *bla* gene, which codes for the enzyme β-lactamase. β -lactamase cleaves the β -lactam ring of ampicillin, rendering it inoperable.

Chloramphenicol was used when bacterial cells transformed with the RIG plasmid were grown. Chloramphenicol blocks peptidyl transferase on the bacterial 50S ribosomal subunit, thereby inhibiting translation. Plasmids resistant to chloramphenicol possess the *cat* gene that encodes chloramphenicol acetyltransferase. The enzyme inactivates chloramphenicol by acylation.

Ampicillin (50mg/ml). Ampicillin (0.5g) was dissolved in sterile dH_2O (10 ml) and filter-sterilised through a 0.22 μm acetate filter. Ampicillin was stored in aliquots at –20˚C and used at a final concentration of 50 μg/ml.

Chloramphenicol (34 mg/ml). Chloramphenicol (0.034 g) was dissolved in absolute ethanol (1 ml) and stored at –20˚C. Chloramphenicol was used at a final concentration of 100 µg/ml.

0.1 M isopropyl-β-D-thiogalacto-pyranoside (IPTG). IPTG (0.238 g) was dissolved in 10 ml sterile dH_2O and filter-sterilised through a 0.22 µm acetate filter. Stored in the dark at –20˚C.

Lysogeny broth (LB). Tryptone (10 g), yeast extract (5 g), NaCl (5 g), and glucose (2 g) were dissolved in 1 l dH_2O and sterilised by autoclaving.

Note: Although LB broth (Bertani, 1951) is most commonly referred to as Luria broth, the acronym LB was intended to stand for lysogeny broth (Bertani, 2004).

LB agar plates. Tryptone (10 g), yeast extract (5 g), NaCl (5 g), glucose (2 g) and bacto-agar (15 g) were dissolved in 1 l dH_2O and sterilised by autoclaving before use. The agar was cooled to approximately 50˚C before addition of antibiotics and pouring of plates.

2x Yeast extract, tryptone (2xYT) medium. Tryptone (16 g), yeast extract (10 g), NaCl (5 g) and glucose (20 g) were dissolved in 900 ml dH_20 and the volume made up to 1 l. The media was autoclaved and stored at room temperature until needed.

Super optimal catabolizer (SOC) media. Tryptone (20 g), yeast extract (5 g), NaCl (0.5 g), 1 M KCl (2.5 ml) were dissolved in 900 ml of dH_2O and adjusted to pH 7 with NaOH. The volume was made up to 970 ml and the media was autoclaved. Prior to use, sterile 1 M $MgCl_2$ (10 ml) and filter-sterilised 1 M glucose (20 ml) was added.

Super optimal broth (SOB) media. Tryptone (20 g), yeast extract (5 g), NaCl (0.5 g), 1 M KCl (2.5 ml) were dissolved in 900 ml of dH_2O and adjusted to pH 7 with NaOH. The volume was made up to 990 ml and the media was autoclaved. Prior to use, sterile 1 M $MgCl_2$ (10 ml) was added.

2.10 Polymerase chain reaction

PCR was routinely used as a method of screening bacterial colonies for recombinant clones. Although colony PCR provides a simple and rapid method for screening for recombinant clones, it is possible to generate false-positives, especially if primers designed to be specific for the insert DNA are used (Álvarez *et al*., 2002). Insert DNA that has not ligated into the plasmid vector, is still able to be transformed into competent bacterial cells, along with non-recombinant plasmid. When the bacterial colonies are screened using PCR, the insert specific primers amplify the free insert DNA, giving a positive result for a recombinant clone

(Álvarez *et al*., 2002). To ensure that false-positives are avoided, the primer pair used should consist of one insert-specific primer and one plasmid-specific primer, or as used in the present study, both primers used should be plasmid-specific. The sequences of the plasmid specific primers used are indicated in Table 2.2.

Different annealing temperatures were used for each primer pair (Table 2.4), as the annealing temperature should ideally be 5˚C lower than the lowest melting temperature (T_m) of the primer pair (Innis and Gelfand, 1990).

Table 2.2. The base pair sequence and the amount supplied of the primers used for colony PCR

Name	Sequence	Amount supplied (nMoles)
pMAL (Forward)	5'- GGTCGTCAGACTGTCGATGAAGCC - 3'	45.50
pMAL (Reverse)	5' - CGCCAGGGTTTTCCCAGTCACGAC - 3'	80.20
PGEX4T1 (Forward)	5' - GGGCTGGCAAGCCACGTTTGGTG - 3'	70.00
PGEX4T1 (Reverse)	5'- CCGGGAGCTGCATGTGTCAGAGG - 3'	82.60

2.10.1 Reagents

10X PCR buffer reaction buffer (Mg^{2+} and detergent free) (800 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$, pH 9.4-9.5).

25 mM $MgCl_2$.

FIREPol® DNA Polymerase I.

dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP).

Concentrated TE [10 mM Tris-HCl, 1 mM EDTA, pH 8]. Tris (0.061g) and EDTA (0.019g) was dissolved in dH_2O (40 ml) and adjusted to pH 8.0 with HCl. The volume was made up to 50 ml with dH_2O and sterilized by autoclaving.

100 µM primer stock solutions. Primers were suspended in concentrated TE. The volume used to prepare each primer stock solution was calculated as follows: Volume of TE (µl) = 10 X nanomoles (nMoles) of primer (Table 2.2). Stock solutions were stored in aliquots at –20˚C.

10 µM primer working solutions. 100 µM Primer stock solutions (10 µl) were diluted with sdH_2O (90 µl). The working solution was stored in aliquots at -20˚C.

2.10.2 Procedure

A transformed bacterial colony was picked with a sterile toothpick and transferred to a microcentrifuge tube containing 20-50 µl sterile dH_2O. The cells were completely resuspended by vortexing, boiled for 5 minutes and then centrifuged (8 000 *g*, 2 min). The supernatant (10 µl) was used for PCR. The PCR reagents were pipetted into sterile PCR tubes according to the amounts and order shown in Table 2.3.

Table 2.3. Reaction constituents and conditions for PCR

Order	Reagent	Volume (µl)	Final concentration
1	10X PCR buffer ($MgCl_2$ free) (10X)	5	1X
2	Sterile dH_2O	23.5	-
3	$MgCl_2$ (25 mM)	5	2.5 mM
4	dNTPs (10 mM of each dNTP)	1	0.2 mM
5	Forward primer (10 µM)	2.5	0.5 µM
6	Reverse primer (10 µM)	2.5	0.5 µM
7	DNA	10	-
8	Polymerase (5 U/ µl)	0.5	2.5 U
Final volume		50	

The amplification of DNA was achieved using the following conditions: 94˚C for 5 minutes, followed by 25 cycles of: 94˚C for 30 seconds (denaturation), *χ˚C for 30 seconds (annealing of primers), 60˚C for 2 minutes (extension), and then 72˚C for 7 minutes, with termination at 4˚C.

*The temperature for annealing of primers (χ˚C) was set to 5˚C less than the lowest melting temperature (T_m) of the primer pair (see Table 2.4 for the temperatures used for each primer pair used).

Table 2.4. Annealing temperatures used for each primer pair used in screening for recombinant bacterial clones with PCR

Name	Melting temperature (T_m)	Annealing temperature used for primer pair in PCR
pMAL (Forward)	61.3˚C	56.3
pMAL (Reverse)	64.1˚C	
PGEX4T1 (Forward)	65.7˚C	59˚C
PGEX4T1 (Reverse)	64.0˚C	

2.11 Sequencing

Plasmid DNA isolated by the alkaline lysis method (Section 2.12.1) was sequenced at the SEGOLI Sequencing Unit at the International Livestock Research Institute (Nairobi, Kenya).

2.12 Isolation of plasmid DNA

The alkaline lysis method (Ish-Horowicz and Burke, 1981; Birnboim and Doly, 1979) (Section 2.12.1) was used for routine plasmid isolation. The Wizard® *Plus* SV Minipreps DNA Purification System (Section 2.12.2) was used for isolating plasmid DNA required for cloning purposes.

2.12.1 Midi-preparation of plasmid DNA by alkaline lysis with SDS

The alkaline lysis method (Ish-Horowicz and Burke, 1981; Birnboim and Doly, 1979) of plasmid DNA isolation is a simple, inexpensive procedure that provides reproducible results (Sambrook and Russell, 2001). The method involves exposing bacterial cells to SDS at alkaline pH. This results in the cell wall degrading and releasing the cell contents, including the plasmid DNA, into the supernatant. Chromosomal DNA is completely denatured due to the alkaline solution causing disruption of the base pairing. Provided the exposure to the basic solution is not too severe or for an extended period of time, the two strands of the plasmid DNA will anneal back together correctly when the pH of the solution is neutralised. The denatured bacterial proteins, chromosomal DNA and broken cell wall become combined in large complexes, which are coated with SDS. When the sodium ions are replaced with

potassium ions, the complexes precipitate from the solution (Ish-Horowicz and Burke, 1981). The complexes are easily removed by centrifugation, leaving the isolated plasmid DNA in the supernatant.

2.12.1.1 Reagents

LB media. Section 2.9.

2xYT media. Section 2.9.

Ampicillin. Section 2.9.

Alkaline lysis solution I [25 mM Tris-HCl, 50 mM glucose, 10 mM EDTA, pH 8]. Glucose (0.9 g), tris (0.3 g) and EDTA (0.37 g) were dissolved in dH_2O (90 ml) and adjusted to pH 8. The volume was made up to 100 ml with dH_2O. The buffer was aliquoted and sterilised by autoclaving, before storing at 4˚C.

Alkaline lysis solution II [0.2 M NaOH, 1% (w/v) SDS). NaOH (1.6 g) was dissolved in dH_2O (200 ml). This NaOH solution (9 ml) was combined with 10% (w/v) SDS (Section 2.4.1) (1 ml) just before use.

Alkaline lysis solution III [5 M potassium acetate, glacial acetic acid]. Potassium acetate (29.45 g) was dissolved in glacial acetic acid (11.5 ml) and dH_2O (28.5 ml). The buffer was stored at 4˚C and transferred to ice just before use.

Sodium-Tris-EDTA (STE) buffer [10 mM tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 8]. Tris (0.1211 g), NaCl (0.5844 g) and EDTA (0.0372 g) were dissolved in dH_2O (90 ml). The volume was made up to 100 ml with dH_2O. The buffer was aliquoted and sterilised by autoclaving.

Tris-EDTA (TE) buffer [100 mM Tris-HCl, 10 mM EDTA, pH 8.0]. Tris (1.211 g) and EDTA (0.3722 g) were dissolved in dH_2O (90 ml). The volume was made up to 100 ml with dH_2O. The buffer was aliquoted and sterilised by autoclaving.

Phenol.

Chloroform.

DNAse-free RNAse A (10 mg/ml). RNAse A from bovine pancreas (0.005 g) was dissolved in 0.01 M sodium acetate, pH 5.2 (500 µl) and heated at 100˚C for 15 min. The solution was slowly cooled to room temperature and 1 M Tris-HCl, pH 7.4 (50 µl) was added to adjust the pH. The solution was aliquoted and stored at –20˚C.

0.01 M Sodium acetate, pH 5.2. Sodium acetate (0.164 g) was dissolved in dH_2O (180 ml) and adjusted to pH 5.2 with glacial acetic acid. The volume was made up to 200 ml and the buffer was autoclaved.

1 M Tris-HCl, pH 7.4. Tris (3.0 g) was dissolved in dH_2O (20 ml) and adjusted to pH 7.4 with HCl. The volume was made up to 25 ml and autoclaved.

2.12.1.2 Procedure

LB or 2xYT media (10 ml) (Section 2.9) containing ampicillin (50 µg/ml) (Section 2.9) was inoculated with a single transformed bacterial colony. The culture was allowed to grow overnight at 37˚C with shaking. The bacterial cells were pelleted (2 000 *g*, 10 min, 4˚C) and the media was discarded. The pellet was suspended in ice-cold STE buffer (2.5 ml) and centrifuged (2 000 *g*, 10 min, 4˚C). The STE buffer was removed and the bacterial pellet was suspended in ice-cold alkaline lysis solution I (200 µl). The solution was vortexed vigorously to ensure the bacterial cells were completely suspended in solution and then transferred to a 1.5 ml microcentrifuge tube. Freshly prepared alkaline lysis solution II (400 µl) was added to the microcentrifuge tube and mixed by inverting the microcentrifuge tube rapidly five times. This was placed on ice and ice-cold alkaline lysis solution III (300 µl) was added. The microcentrifuge tube was inverted several times to ensure mixing of the solution before it was left on ice for 5 minutes. The bacterial lysate was centrifuged (10 000 *g*, 5 min, 4˚C). The supernatant (600 µl) was transferred to a fresh 1.5 ml microcentrifuge tube. Phenol: chloroform (1:1) solution (600 µl) was added to the supernatant. The organic and aqueous phases were mixed by vortexing. The emulsion was centrifuged (10 000 *g*, 2 min, 4˚C). The upper aqueous layer was transferred into a fresh 1.5 ml microcentrifuge tube. To precipitate the DNA, propan-2-ol (600 µl) was added to the aqueous solution and vortexed. The solution was left at room temperature for 2 min and then centrifuged (10 000 *g*, 5 min, room temperature) to collect the precipitated DNA. The supernatant was removed by gentle aspiration. The microcentrifuge tube was inverted on paper towelling to allow any remaining supernatant to drain away from the DNA pellet. The pellet was washed with 70% (v/v) ethanol (1 ml) and recovered by centrifugation (10 000 *g*, 2 min, room temperature). The ethanol was removed by gentle aspiration. The microcentrifuge tube was left to stand open at room

temperature for 5 min to allow any residual ethanol to evaporate. The DNA pellet was suspended in TE buffer (100 µl) containing 20 µg/ml DNAse-free RNAse A. The plasmid DNA solution was gently vortexed for 10 s and then stored at –20˚C.

2.12.2 Wizard® *Plus* SV Minipreps DNA Purification System

For cloning and sequencing purposes, plasmid DNA was isolated using the Wizard® *Plus* SV Minipreps DNA Purification System, from Promega (USA), according to the manufacturers' instructions. The kit provides a simple and reliable method of isolating plasmid DNA. Plasmid DNA can immediately be used for other molecular biology applications such as restriction enzyme digests, cloning and sequencing, without further purification. The kit involves lysis of the transformed bacterial cells with an alkaline solution containing SDS. The DNA is then bound to resin in a Minicolumn, washed with ethanol and eluted (Wizard® *Plus* SV Minipreps DNA purification system technical bulletin, Promega).

2.13 Restriction enzyme digestion of DNA

Restriction enzymes that cut DNA molecules internally at specific base pair sequences are type II restriction endonucleases. Digesting two different DNA molecules with the same restriction endonuclease, results in the formation of the same sticky end. This allows new DNA molecules to be created by combining the restricted fragments from each restriction endonuclease digestion. It is for this reason that these enzymes are invaluable tools in molecular cloning.

The restriction enzymes *Bam*HI (5' G↓GATCC 3') and *Eco*RI (5' G↓AATTC 3'), were used in the present study. *Bam*HI and *Eco*RI exhibit the ability to cleave DNA sequences that are similar to their recognition sequence. This phenomenon is known as star activity. Star activity only occurs under conditions of high glycerol concentration, excess enzyme, and low ionic strength and at non-optimal pH (New England Biolabs pMAL™ Protein Fusion and Purification System Instruction Manual).

2.13.1 Reagents

*Bam*HI.

10 X NEBuffer 2 [500 mM NaCl, 100mM Tris-HCl, 100 mM MgCL2, 10 mM dithiothreitol, pH 7.9].

*Eco*RI.

10 × Buffer H (90 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl).

2.13.2 Procedure

The volumes of the reagents used were dependent on the amount of digested DNA required. The following procedure was typically used, although it was scaled-up when necessary: 10X restriction enzyme buffer (4µl), dH_2O (14 µl), plasmid DNA from miniprep (20 µl) and restriction enzyme (2 µl) were pipetted into a sterile 1.5 ml microcentrifuge tube and incubated at 37°C. Omitting the restriction enzyme and adjusting the volume of water accordingly, provided a non-digested control. Each restriction enzyme was used with a specific restriction enzyme buffer (Table 2.5). Digestion of the plasmid was confirmed by viewing the digested and non-digested control DNA on an agarose gel.

Table 2.5. The specific restriction enzymes and buffers used to digest plasmid DNA.

Enzyme	Buffer name	Composition of buffer (1X)
*Bam*HI	NEBuffer 2	50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1mM Dithiothreitol, pH 7.9 at 25 °C
*Eco*RI	Buffer H	90 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, pH 7.5 at 37 °C

2.14 Dephosphorylation

The linearised plasmid has the ability to re-ligate, which would decrease the number of recombinant plasmids obtained upon ligation of the DNA insert into the plasmid vector. To minimize the possibility of re-ligation, the plasmid is dephosphorylated with shrimp alkaline phosphatase. Phosphatase catalyses the dephosphorylation of the 5’ phosphate ends of the

plasmid DNA, thus preventing re-ligation with the 3' end. Shrimp alkaline phosphatase (from arctic shrimp) was used, as it is inactivated after 15 min at 65˚C.

2.14.1 Reagents

Shrimp alkaline phosphatase.

10X phosphatase buffer [0.5 M Tris-HCl, 50 mM $MgCl_2$, pH 8.5].

Sterile dH_2O. dH_2O was autoclaved to sterilise.

Linearised plasmid DNA.

2.14.2 Procedure

The volumes of the reagents used were dependent on the amount of plasmid DNA required to be dephosphorylated. Typically the following procedure was used: plasmid DNA (100 µl), 10X buffer (12 µl) and sterile dH_2O (4 µl) were mixed together in a 1.5 ml microcentrifuge tube by pipetting the contents up and down gently. Shrimp alkaline phosphatase (4 µl) was added and mixed as before. The reaction was left at 37˚C overnight (approximately 16-18 h). The shrimp alkaline phosphatase was inactivated by incubating the microcentrifuge tube in a 65˚C water bath for 15 min.

2.15 Purification of plasmid DNA

2.15.1 Wizard® DNA Clean-up System

The Wizard® DNA Clean-up System was used to remove restriction enzymes, salts and phosphates from plasmid DNA. The DNA binds to a resin, which is then washed with 80% (v/v) propan-2-ol to remove the salts, phosphates or restriction enzymes. The DNA is eluted with sterile dH_2O pre-warmed to 70˚C.

2.15.1.1 Materials and reagents

80% (v/v) Propan-2-ol. Propan-2-ol (16 ml) was made up to 20 ml with sdH_2O.

Wizard® DNA Clean-up resin (Promega).

Wizard® DNA Clean-up minicolumn (Promega).

2.15.1.2 Procedure

Pre-warmed (37˚C) Wizard® DNA Clean-up resin (1 ml) was added to a sterile 1.5 ml microcentrifuge tube. The DNA sample was added and mixed by inverting the microcentrifuge tube several times. The plunger from a 2 ml syringe was removed and the syringe barrel was attached to the Luer-Lok® extension of a minicolumn. The Wizard® DNA Clean-up resin with bound sample DNA was then pipetted into the 2 ml syringe barrel. The syringe plunger was carefully inserted into the syringe barrel and the DNA-resin slurry slowly pushed into the minicolumn, using the plunger. The minicolumn was disconnected from the syringe and the plunger was removed from the syringe barrel. The syringe was then reconnected to the minicolumn and 80% (v/v) propan-2-ol was pippetted into the syringe barrel. The plunger was inserted into the syringe barrel and the propan-2-ol was pushed through the minicolumn. The minicolumn was detached from the syringe and placed into a 1.5 ml microcentrifuge tube. The resin was dried by centrifugation (10 000 *g*, 2 min, room temperature). The minicolumn was transferred to a sterile microcentrifuge tube and pre-warmed (70˚C) sterile dH_2O (25 µl) was pipetted into the minicolumn to elute the DNA. The minicolumn was left for 1 min at room temperature before centrifuging (10 000 *g*, 20 s, room temperature). The elution was repeated once more with a further 25 µl of sterile dH_2O, into a fresh sterile microcentrifuge tube.

2.15.2 Low melting point (LMP) agarose purification of DNA

Excising the completely linearised plasmid DNA from low melting point (LMP) agarose provided a convenient method for obtaining completely digested DNA from a restriction enzyme digest which would often contain a mixture of completely digested linear plasmid DNA and partially digested DNA. This method was also useful for separating DNA insert from the plasmid DNA that it had been dropped out of by restriction enzyme digestion. The excised DNA bands were purified from the LMP agarose using the Wizard® PCR Preps DNA Purification System from Promega (USA). The system works on a method similar to that of the Wizard® DNA Clean-up System.

2.15.2.1 Materials and reagents

Wizard® PCR Preps DNA purification resin (Promega).

Direct purification buffer [50 mM KCl, 10 mM tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100 (Promega).

Wizard® minicolumns (Promega).

2.15.2.2 Procedure

The DNA sample was separated by electrophoresis at 60 V in a TAE agarose gel of the required percentage LMP agarose. The gel was observed under UV light and the required DNA band was excised from the gel using a clean, sterile scalpel blade. The excised band was placed in a sterile 1.5 ml microcentrifuge tube and incubated at 70˚C until the agarose was completely melted. The PCR preps resin was warmed at 37˚C for 10 minutes, to dissolve any crystals and aggregates. Prior to use, the resin was allowed to cool to 30˚C. The PCR preps resin (1 ml) was added to the melted agarose and mixed thoroughly for 20 seconds by inverting the microcentrifuge tube several times. The plunger was removed from a 2 ml disposable syringe and the syringe barrel was attached to the Luer-Lok® extension of a Wizard® minicolumn for each sample to be purified. The resin-DNA slurry was pipetted into the syringe barrel. The plunger was inserted into the syringe barrel and used to slowly push the resin-DNA slurry into the minicolumn. The syringe was detached from the minicolumn and the plunger removed. The syringe barrel was reconnected to the minicolumn and 80% (v/v) propan-2-ol (2 ml) was pipetted into the syringe barrel. The plunger was inserted into the syringe barrel and used to gently push the propan-2-ol through the minicolumn. The minicolumn was removed from the syringe and placed into a 1.5 ml microcentrifuge tube. The minicolumn was centrifuged (10 000 *g*, 2 minutes) to dry the resin. The minicolumn was transferred to a new sterile microcentrifuge tube and sterile dH_2O (50 µl), preheated to 65-70˚C, was added to the minicolumn to elute the DNA. The minicolumn was allowed to stand for 1 minute before centrifuging (10 000 *g*, 20 seconds). The minicolumn was discarded and the eluted DNA stored at –20˚C until required.

2.16 Ligation of plasmid DNA and insert DNA

Insert DNA was ligated into restriction enzyme-digested plasmid DNA with T4 DNA ligase. Ligase catalyses the covalent joining of phosphodiester bonds between 5'-phosphoryl-termini and 3'-hydroxyl-termini of nucleic acid molecules. DNA ligases are very useful for

creating unique combinations of nucleic acids and attaching them to plasmid vectors for molecular cloning.

2.16.1 Reagents

T4 DNA ligase.

2X Ligation buffer.

5X DNA dilution buffer.

2.16.2 Procedure

Plasmid DNA that had been restricted with restriction enzymes, dephosphorylated and purified (2.5 µl) was combined with the insert DNA (5 µl), 1X DNA dilution buffer (2.5 µl) and 2X ligation buffer (10 µl) in a 1.5 ml sterile microfuge tube. This was mixed by gentle pipetting, before adding T4 DNA ligase (1µl). This was mixed by gentle pipetting and incubated at 16˚C overnight. The ligation controls used are displayed in Table 2.6.

Table 2.6. Ligation controls

Control number	BamHI cut?	Dephosphorylated?	Ligase?	Insert DNA?	Plasmid DNA?	Competent cells?
1	Yes	Yes	Yes	Yes	Yes	Yes
2	No	No	No	No	Yes	Yes
3	Yes	No	No	No	Yes	Yes
4	Yes	Yes	No	No	Yes	Yes
5	Yes	No	Yes	No	Yes	Yes
6	Yes	Yes	Yes	No	Yes	Yes
7	No	No	No	No	Yes	Yes

2.17 Concentration and purification of DNA

For cloning procedures, it was often necessary to concentrate DNA samples or change the buffer the DNA was suspended in. A method of precipitating DNA with ethanol and sodium acetate (Wallace, 1987) was frequently used. The procedure involved exposing a DNA sample to salt and ethanol at a low temperature (-20˚C). Precipitated DNA was collected by

centrifugation. The DNA was then resuspended in a smaller volume in order to give a more concentrated DNA solution, or in a buffer more appropriate for the subsequent use of the DNA.

2.17.1 Reagents

3 M Sodium acetate, pH 5.5. Sodium acetate trihydrate (20.4 g) was dissolved in dH_2O (40 ml) and adjusted to pH 5.5 with glacial acetic acid. The volume was made up to 50 ml with dH_2O. The buffer was sterilized by autoclaving.

2.17.2 Procedure

Sodium acetate, pH 5.5 was added to the DNA sample to $1/10^{th}$ of the sample volume and mixed by gentle pipetting. Ethanol (100%) was added (2.5x the sample volume) and mixed by gentle pipetting. The solution was left at –20˚C for at least 1 h before recovering the DNA by centrifugation (14 000 *g*, 25 min, 4˚C). The supernatant was carefully removed without disturbing the pellet. The DNA pellet was washed with 70% (v/v) ethanol (500 µl). The DNA was recovered by centrifugation (12 000 *g*, 5 min, 4˚C). The ethanol was carefully removed without disturbing the pellet. The DNA pellet was left to air dry for 10 min before being dissolved in an appropriate volume of sterile dH_2O.

2.18 Transformation of bacterial cells with DNA

Two different methods of transformation were used in the present study, depending on the type of plasmid DNA being transformed. For supercoiled plasmid DNA, the heat shock method (Section 2.18.2) of transformation was used. For ligation reactions, electroporation (Section 2.18.1) was used to transform bacteria. In order for the plasmid DNA to be taken up by the bacterial cells with each of these methods, the cells first have to be made competent. For transformation by electroporation, cells were made competent using the glycerol method (Dower *et al.*, 1988) (Section 2.18.1). Cells being transformed by the heat shock method were made competent using an adaptation of the $CaCl_2$ method (Section 2.18.2) described by Cohen *et al.* (1972). Table 2.7 displays the *E. coli* cell strains used and the plasmids they were transformed with.

Table 2.7. Genotypes of *E. coli* cells transformed with recombinant plasmids

Name	Genotype	Recombinant plasmid cells were transformed with
E. coli JM103	endA1 glnV44 sbcBC rpsL thi-1 Δ(lac-proAB) F`[traD36 proAB$^+$ lacI^q lacZΔM15]	pTS822; pMAL
E. coli JM109	endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB$^+$ Δ(lac-proAB) glnV44 e14- [F` traD36 pro AB$^+$ lacI^q lacZΔM15] hsdR17($r_k^-m_k^+$)	pGEX4T1; pGEX4T1C18
E. coli BL21	F$^-$ *ompT hsdSB* (r_B^- m_B^-) *gal dcm*	pGEX4T1; pGEX4T1C18
E. coli BL21(DE3)	F$^-$ ompT gal dcm lon hsdS_B($r_B^-m_B^-$)λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	pGEX4T1; pGEX4T1C18
Turbo competent *E. coli* (New England Biolabs)	F` *proA*$^+$*B*$^+$ *lac*I^q Δ*lacZM*15/*fhuA2* Δ(*lac-proAB) glnV zgb-210::Tn10* (Tet^R) *endA1 thi-1* Δ(*hsdS-mcrB*)5	pKK223-3-PfLDH; pKK223-3-PvLDH

2.18.1 Transformation of competent *E.coli* (glycerol method) cells by electroporation

Electroporation is a process allowing bacterial cells to take up free DNA. The mechanism involves exposure of the cells to a quick electric pulse, causing a type of structural rearrangement of the membrane, resulting in the dimpling of the cell membrane (Weaver, 1993). These dimples are thought to then form temporary hydrophobic pores ranging in diameter from 2 nm to 7 nm (Sambrook and Russell, 2001). Whilst the pores are open, ions and water-soluble molecules, including free DNA, are able to cross the membrane into the cytoplasm of the cell (Weaver, 1993).

Preparation of competent cells for electroporation requires washing the cells with ice-cold water. Washing the cells at low temperature reduces the risk of arcing, which is the uneven transfer of charge across the cell-DNA suspension. Arcing occurs at high temperatures and at high ionic strength, and therefore washing with ice-cold water greatly reduces the number of ions present, in addition to keeping the cells at a reduced temperature. If arcing occurs, the efficiency of transformation is greatly reduced (Sambrook and Russell, 2001).

2.18.1.1 Reagents

2xYT media. Section 2.9.

Sterile ice-cold dH_2O. dH_2O was autoclaved and placed on ice until needed.

10% (v/v) glycerol. Glycerol (10 ml) was made up to 100 ml with dH_2O and mixed thoroughly before autoclaving.

Super optimal broth (SOB) media. Section 2.9.

2.18.1.2 Procedure

An overnight culture (10 ml; 2xYT media) of appropriate *E. coli* cells was grown at 37˚C with shaking. Fresh 2xYT media (99 ml) was inoculated with 1 ml of the overnight culture and incubated at 37˚C with shaking until the culture reached an OD_{600} of 0.9. The culture was left on ice for 30 min before centrifuging (3 600 *g*, 10 min, 0˚C). The supernatant was discarded and the cell pellet was resuspended in ice-cold sterile dH_2O (100 ml). The cells were centrifuged (3 600 *g*, 10 min, 0˚C) and resuspended in ice-cold sterile dH_2O (50 ml). The cells were centrifuged (3 600 *g*, 10 min, 0˚C). The cell pellet was resuspended in ice cold sterile 10% (v/v) glycerol (2 ml) before centrifuging (3 600 *g*, 10 min, 0˚C). The cells were resuspended in ice cold sterile 10% (v/v) glycerol (1 ml). Aliquots (50 µl) were stored in sterile microfuge tubes on ice until required. DNA (1-2 µl) was gently mixed with freshly prepared competent *E. coli* cells (50 µl) and incubated on ice for 30 min. The DNA and cell mixture was pipetted into a pre-chilled, sterile electroporation cuvette (0.2 cm gap). A Bio-Rad Gene Pulser was used to apply one pulse of 25 microfarads, 2.5 kilovolts, and 200 ohms for approximately 4.8 milliseconds. Pre-warmed SOB media (300 µl) was gently mixed with the transformed cells. The cells and media were transferred to a sterile microfuge tube and

incubated at 37°C for 1 h. LB plates containing appropriate antibiotic were spread with the transformed cells (200µl) and incubated at 37°C overnight.

2.18.2 Transformation of competent *E. coli* cells (calcium chloride method) using heat shock

The mechanism behind the uptake of free DNA by cells with heat shock is not understood. The crucial step of the method involves briefly exposing competent cells to an elevated temperature. The temperature and length of exposure need to be accurate otherwise transformation efficiency is reduced (Sambrook and Russell, 2001). Once transformed, the cells are supplemented with a rich media to allow the cells to recover and begin expressing the antibiotic resistance marker, encoded by the plasmid. Competent *E. coli* cells were prepared using the modified method of Cohen *et al.*, (1972). It is a highly effective method, typically yielding 5 X 10^6 – 2 X 10^7 transformed colonies per microgram of supercoiled plasmid (Sambrook and Russell, 2001).

2.18.2.1 Reagents

2xYT media. Section 2.9.

0.1 M $CaCl_2$. $CaCl_2.2H_2O$ (1.47 g) was dissolved in 100 ml dH_2O and sterilised by autoclaving.

LB agar plates. Section 2.9.

Super optimal catabolizer (SOC) media. Section 2.9.

2.18.2.2 Procedure

Media (2xYT, 10 ml) was inoculated with one *E. coli* colony from an LB plate and left to grow at 37˚C with shaking overnight. The overnight culture was added to fresh 2xYT (90 ml) and left at 37˚C with shaking until an OD_{600} of 0.3-0.4. The culture was transferred to ice cold, sterile centrifuge tubes and left on ice for 10 min. The cells were pelleted (4500 *g*, 10 min, 4˚C). The supernatant was discarded and pellet was resuspended in ice-cold sterile 0.1 M $CaCl_2$ (40 ml). The cells were pelleted as before, and resuspended in 0.1 M $CaCl_2$ (2 ml). The cells were aliquoted (20 µl) into sterile 1.5 ml microcentrifuge tubes and stored on ice until use. The cells were used for transformation on the day of preparation.

Super coiled recombinant plasmid DNA (1 µl) was incubated with freshly prepared competent cells (20 µl) on ice for 30 min. Cells were heat-shocked at 42˚C for 90 s, and then immediately placed on ice for 2 minutes. Pre-warmed (37˚C) SOC media (80 µl) was added to the cells and gently mixed. The cells were then incubated for 1 h with shaking at 37˚C. LB agar plates containing appropriate antibiotics were then spread with the cells (50 µl), and then incubated overnight, inverted at 37˚C.

2.19 Preparation of glycerol stocks

Stocks of transformed bacterial clones and bacterial cell lines were frozen at –70˚C in glycerol according to the method described by Sambrook *et al*. (1989).

2.19.1 Reagents

Sterile 50% glycerol. Glycerol (25 ml) was made up to 50 ml with dH_2O and autoclaved.
LB media. Section 2.9.

2.19.2 Procedure

LB media (10 ml) containing relevant antibiotic with a single bacterial colony was left to grow overnight at 37˚C with shaking. Sterile 50% glycerol (150 µl) was added to the bacterial culture (850 µl) in a sterile 1.8 ml cryotube and vortexed gently to mix. The cells were rapidly frozen in liquid nitrogen and then stored at -70˚C.

2.20 Recombinant expression and purification of *Pf*33-MBP and MBP from pMAL-c2X and pTS822 plasmids

2.20.1 Confirmation of the identity of the pMAL-c2X and pTS822 plasmids

The plasmids pMAL-c2X and pTS822 used in this study were from glycerol stocks previously prepared (Smallie, 2003) and it was thus decided to confirm the identity of the insert sequence. Glycerol stocks were streaked onto LB media plates (Section 2.9) and left inverted overnight at 37˚C. LB broth (10 ml) (Section 2.9) containing 100 µg/ml ampicillin (Section 2.9) was inoculated with a single colony from the LB media plates and incubated at 37˚C with shaking overnight. Plasmids were isolated using the midi-preparation of plasmid DNA by alkaline lysis with SDS (Section 2.12.1) and then restricted with *Bam*HI (Section

2.13) to linearise pMAL-c2X, and to drop out the *P. falciparum* DNA insert from pTS822. The restricted plasmid DNA was analysed on a 1.2% (w/v) agarose gel (Section 2.7). In addition, the plasmids were confirmed with PCR (Section 2.10) and sequencing (Section 2.11).

2.20.2 Expression of *Pf*33-MBP or MBP alone

2.20.2.1 Reagents

2xYT media. Section 2.9.

50 mg/ml Ampicillin. Section 2.9.

0.1 M IPTG. Section 2.9.

Column buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM NaN_3]. Tris (2.42 g), NaCl (11.69 g) and EDTA (0.372 g) were dissolved in approximately 980 ml dH_2O and adjusted to pH to 7.4 with HCl. NaN_3 (0.065 g) was added and the volume made up 1 l.

2.20.2.2 Procedure

An overnight culture (100 ml 2xYT or LB) of pTS822 expressing *Pf*33-MBP or of pMAL-c2X expressing MBP, was diluted 1:10 in fresh 2xYT or LB (900 ml). Ampicillin was added to a final concentration of 100 μg/ml and the culture left at 30˚C until an optical density at 600 nm of 0.5-0.6 was reached. IPTG was added to a final concentration of $3x10^{-4}$ M. The culture was then supplemented with additional ampicillin (1 ml of a 50 mg/ml stock) and placed back into the incubator for 4-6 h. The cultures were supplemented with ampicillin (1 ml) every 2 h. The cultures were centrifuged to pellet the cells (4000 *g*, 20 min, 4°C). The media was discarded and the pellets were resuspended in column buffer (50 ml). Lysozyme was added to a final concentration of 1 mg/ml. Protease inhibitors were added (Section 2.8) and the resuspended cells were frozen at -20 ˚C. The cells were thawed and then sonicated (4 x 30 s). The lysate was clarified by centrifugation (9000 *g*, 30 min, 4˚C), followed by filtration through Whatman No. 1 filter paper.

2.20.3 Affinity purification of fusion protein and MBP on amylose resin

2.20.3.1 Reagents

Column buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM NaN_3]. Section 2.20.2.1.

10 mM maltose in column buffer [20 mM Tris-HCl, 10 mM maltose, 200 mM NaCl, 1 mM EDTA, 1 mM NaN_3]. Maltose (0.36 g) was dissolved in column buffer (100 ml).

0.1% (m/v) SDS. SDS (0.1 g) was dissolved in 100 ml of dH_2O.

2.20.3.2 Procedure

Amylose resin was placed into a glass column (internal diameter 1.5 cm, height 5 cm, bed volume 8.8 ml). The resin was washed with 5 column volumes of column buffer (flow rate 10 ml/h). pMAL or pTS822 lysate was diluted (1:5) with column buffer. The lysate was cycled through the column overnight at 4˚C. The column was then washed with column buffer until the absorbance of a fraction (1 ml) was approximately 0.010. MBP or *Pf*33-MBP was eluted with 10 mM maltose. Fractions of 1 ml were collected during elution. The absorbance of the collected fractions was determined (280 nm). Fractions with absorbance readings above 0.3 were pooled and protein concentration determined (Bradford, 1976) (Section 2.2). If necessary the pooled protein was concentrated using PEG 20 000 (Section 2.3.1). The column was regenerated with 3 column volumes of dH_2O, 3 column volumes of 0.1% (m/v) SDS, 1 column volume dH_2O and 3 column volumes of column buffer and stored at 4˚C.

2.20.4 Factor Xa cleavage of fusion protein *Pf*33-MBP

Factor Xa was used to cleave MBP from *Pf*33-MBP fusion protein.

2.20.4.1 Reagents

Cleavage buffer [100 mM NaCl, 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0]. Tris (3 g), NaCl (8.7 g) and $CaCl_2.2H_2O$ (0.29 g) were dissolved in 980 ml dH_20 and titrated to pH 8.0 with HCl. The volume was made up to 1 l.

Factor Xa.

1 mg/ml Soybean trypsin inhibitor. Soybean trypsin inhibitor (0.001 g) was dissolved in sterile dH_2O (1 ml), aliquoted and stored at –20˚C.

100 µg/ml Soybean trypsin inhibitor. Soybean trypsin inhibitor (1 mg/ml) (100 µl) was made up to 1ml with sterile dH_2O.

2.20.4.2 Procedure

Amylose-purified *Pf*33-MBP was dialysed against cleavage buffer (3 x 2h). The fusion protein was then concentrated with PEG 20 000 (Section 2.3.1). The quantity of protein present was determined (Bradford, 1976) (Section 2.2). Factor Xa (200 µg/ml) was added to a final concentration of 1 µg per mg of fusion protein. As a positive control, factor Xa was added to MBP2* paramyosinΔSal (control fusion protein from New England Biolabs) to the same final concentration. As a negative control, an equivalent volume of cleavage buffer was added to an equivalent amount of both the fusion protein and the control fusion protein. The cleavage reactions, including the controls were carried out overnight at room temperature with rotation. Factor Xa activity was inhibited by boiling with reducing treatment buffer (Section 2.4.1) initially for pilot studies, then with soybean trypsin inhibitor at a final concentration equal to that of the final Factor Xa concentration used.

2.20.5 DEAE-Sepharose ion exchange separation of cleaved *Pf*33-MBP

2.20.5.1 Reagents

10 mM Tris-HCl, 25 mM NaCl, pH 8.0. Tris (1.211 g) and NaCl (1.461 g) were dissolved in dH_2O (980 ml). The pH was adjusted to 8.0 with HCl and the volume made up to 1 l.

20 mM Tris-HCl, 25 mM NaCl, pH 8.0. Tris (2.422 g) and NaCl (1.461 g) were dissolved in dH_2O (980 ml). The pH was adjusted to 8.0 with HCl and the volume made up to 1 l.

20 mM Tris-HCl, 500 mM NaCl, pH 8.0. Tris (2.422 g) and NaCl (14.61 g) were dissolved in dH_2O (480 ml). The pH was adjusted to 8.0 with HCl and the volume made up to 500 ml.

DEAE-Sepharose.

2.20.5.2 Procedure

The *Pf*33-MBP Factor Xa cleavage mixture (Section 2.20.4.2) was dialysed against 20 mM Tris-HCl, 25 mM NaCl, pH 8.0. DEAE-Sepharose (6 ml) was poured into a column (approximately 1 x 10 cm) and washed with 10 mM Tris-HCl, 25 mM NaCl, pH 8.0 (approximately 40 ml). The Pf33-MBP Factor Xa cleavage mixture was loaded onto the

column and the flow-through collected in fractions. The column was washed with 10 mM Tris, 25 mM NaCl, pH 8.0 until the absorbance of the fractions at 280 nm reached a reading of zero. A gradient of 25 mM NaCl to 500 mM NaCl (25 ml each) in 20 mM Tris-HCl, pH 8.0 was started. Fractions were collected and monitored with absorbance readings at 280 nm.

2.20.6 'On column' cleavage of *Pf*33-MBP

2.20.6.1 Reagents

Column buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM NaN_3]. Section 2.20.2.1.

Cleavage buffer [100 mM NaCl, 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0]. Section 2.20.4.1.

Factor Xa.

2.20.6.2 Procedure

Amylose resin (1 ml) was placed in a 10 ml polypropylene column and equilibriated with column buffer. Clarified lysate from *Pf*33-MBP expression (Section 2.20.2.2) was cycled through the column overnight. The resin was washed with column buffer to remove any unbound protein. The absorbance of the column buffer was read at 280 nm to determine when all the unbound protein had been washed away. *Pf*33-MBP bound to the amylose resin was equilibriated with cleavage buffer. Cleavage buffer (1 ml) was added to the amylose resin. The column was gently rocked from side to side to resuspend the resin in the cleavage buffer. Factor Xa (20 μg) was added and the column was left to gently rock overnight at room temperature. Fractions (0.5 ml) were collected immediately from the column. Additional cleavage buffer was then added and fractions were collected. The absorbance of the fractions was monitored at 280 nm to determine when all of the *Pf*33 had been washed from the column. The amylose column was then equilibriated with column buffer and the bound MBP was eluted with 10 mM maltose. Fractions were collected and monitored with absorbance readings at 280 nm. The resin was regenerated as described in Section 2.20.3.2.

2.21 Expression of *Pf*33 as a GST fusion protein

2.21.1 Subcloning 822 bp PFC0760c gene sequence into pGEX4T1

The 822 bp portion of the PFC0760c gene sequence was subcloned into the pGEX4T1 expression vector, resulting in a glutatione-*S*-transferase fusion protein (*Pf*33-GST).

The pGEX4T1 and pTS822 plasmids were isolated using the Wizard® Plus SV minipreps DNA purification system (Section 2.12.2) and digested with *Bam*HI (Section 2.13) to linearise the pGEX4T1 plasmid and to release the 822 bp insert from pTS822. Digestion of the plasmids was confirmed by agarose gel electrophoresis (Section 2.7). The digested pGEX4T1 plasmid was dephosphorylated (Section 2.14). Both the insert DNA and the pGEX4T1 plasmid were excised from low melting point agarose gel (Section 2.7), purified (Section 2.15.1) and ligated together (Section 2.16). The pGEX4T1*Pf*33 plasmid was purified by sodium acetate-ethanol precipitation (Section 2.17) and electroporated into competent cells (Section 2.18.1) which were then spread onto LB media plates (Section 2.9) containing ampicillin (Section 2.9). Colonies were assayed for insert by colony PCR (Section 2.10) and restriction enzyme digestion (Section 2.13).

2.21.2 Expression of *Pf33*-GST

Expression of *Pf*33-GST was performed as for *Pf*33-MBP expression, with three modifications. The cells were pelleted by centrifugation (7700 *g*, 10 min, 4°C) and the cell pellet was resuspended in ice cold PBS containing 1% (v/v) triton X-100 (Section 2.21.3.1) (40 ml per litre of culture). Protease inhibitors and lysozyme were added as for *Pf*33-MBP expression (Section 2.20.2.2). After sonication, the supernatant was clarified by centrifugation (12 000 *g*, 10 min, 4°C), followed by filteration through Whatman no. 1 paper. GST was expressed from non-recombinant pGEX4T1 as a control.

2.21.3 Glutathione agarose purification of *Pf33*-GST

2.21.3.1 Reagents

Phosphate buffered saline (PBS), pH 7.2, 0.02% (w/v) NaN_3. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dH_2O (approximately 950 ml) and titrated to pH 7.2 if necessary. NaN_3 (0.2 g) was added and the volume was made up to 1 l with dH_2O.

PBS-Triton [PBS with 1% (v/v) Triton X-100]. Triton X-100 (1 ml) was dissolved in PBS (100 ml).

Glutathione agarose column. Lyophilised glutathione agarose (70 mg) was swollen in dH_2O (14 ml) overnight at 4°C. This resulted in approximately 1 ml of resin which was placed in a Poly-Prep® Chromatography column and equilibrated with 20 column volumes of PBS.

50 mM Tris-HCl buffer, pH 8. Tris (3.03 g) was dissolved in approximately 470 ml dH_2O and adjusted to pH 8 with HCl. The volume was made up to 500 ml with dH_2O.

Elution buffer [10 mM reduced glutathione]. Reduced glutathione (0.092 g) was dissolved in 50 mM tris-HCl buffer, pH 8 (30 ml).

Cleansing buffer 1 [100 mM Sodium borate buffer, pH 8, 500 mM NaCl]. Boric acid (0.6183 g) and NaCl (2.922 g) were dissolved in dH_2O (90 ml). The pH was adjusted to 8.0 with NaOH and the volume made up to 100 ml with dH_2O.

Cleansing buffer 2 [100 mM Sodium acetate buffer, pH 4, 500 mM NaCl]. Sodium acetate (0.82 g) and NaCl (2.9 g) were dissolved in dH_2O (90 ml). The pH was adjusted to 4.0 with glacial acetic acid and the volume made up to 100 ml with dH_2O.

2.21.3.2 Procedure

The glutathione agarose column was equilibriated with PBS-Triton. Sonicated and clarified fusion-protein supernatant (20 ml) (Section 2.21.2) was cycled through the column (overnight, 4°C). The resin was washed with PBS-Triton until the absorbance reading at 280 nm was zero. *Pf*33-GST was eluted with elution buffer. Fractions (1 ml) were collected and analysed by SDS-PAGE (Section 2.4).

Regeneration of the column was achieved by washing with 5 column volumes of cleansing buffer 1, 5 column volumes of dH_2O, 5 column volumes of cleansing buffer 2, followed by 5 column volumes of dH_2O. The resin was stored in PBS supplemented with NaN_3 at a final concentration of 0.02% (w/v) at 4°C.

2.22 Intraperitioneal (I. P.) injection of mice

2.22.1 Materials

Terumo® U-100 insulin 1cc 27 Gauge x ½" syringes.

2.22.2 Procedure

Stabilate was removed from storage in liquid nitrogen and allowed to thaw in water that had been pre-warmed at 37˚C. The stabilate was diluted if necessary with filter-sterilised PBS and then drawn up into a 1cc 27 Gauge x ½" syringe. The mouse to be injected was picked up by grasping the tail with the thumb and forefinger of one hand. Without letting go of the tail, the mouse was placed on top of the wire cage and allowed to pull away. Using the other hand, the fur on the shoulders and back were grasped between the thumb and forefinger and pulled tight, the whole time, still holding onto the tail with other hand. It was necessary to pull the skin tightly so as to prevent the mouse from being able to move its head too much as this would enable it to turn its head and bite. Still holding the mouse with both hands, the mouse was turned so that it was on its back, with its abdomen facing upward. The mouse held at a 45 degree angle (head pointing downwards), to ensure that no organs would accidentally be penetrated during I.P. injection. Using the third or fourth finger of the hand that was pulling the skin tight, the tail was secured between the palm and finger, thereby restraining the mouse with one hand. The syringe was held in the first two fingers of the free hand. The needle was inserted at an angle of approximately 30 degrees to the surface of the skin and approximately 45 degrees to the mouse midline. The needle was carefully pushed into the skin until the needle enters the peritoneum, which was indicated by a slight easing of resistance to the needle. The plunger was depressed with the thumb, to release the required amount of parasitized red blood cells. The needle was removed, and the mouse returned to its cage.

2.23 Preparation of blood films for determining parasitemia in mice

Thin and thick blood films were prepared from blood from the tail midvein of *Plasmodium* infected mice. Thin films were routinely used to monitor the percentage of parasitized red blood cells in infected mice. The disadvantage of thin films is that the parasite is not easily detected at very low levels. The use of thick blood films offer a higher probability of observing parasites when they are at very low levels as a large number of red blood cells are concentrated into a very small area. The preparation of thick and thin blood films and the determination of percentage parasitemia were based on the method of Warhurst and Williams (1996).

2.23.1 Thick blood film

2.23.1.1 Materials

Glass slides.

Blood lancets.

Methanol.

2.23.1.2 Procedure

The mouse was restrained as for I. P. injection (Section 2.22). The tip of the tail was rested on a clean surface top. With a free hand, a sterile lancet was used to prick the tail mid-vein. A drop of blood was collected onto a clean glass slide. With the corner of another glass slide, the blood droplet was spread slightly by moving the second slide gently in a circular motion. The blood smear was allowed to air dry and then fixed for 30 seconds in methanol. After allowing the slides to air dry, they were stained with Giemsa (Section 2.23.3).

2.23.2 Thin blood film

2.23.2.1 Reagents

As for thick blood films (Section 2.23.1.1)

2.23.2.2 Procedure

A drop of mouse blood was collected onto a clean slide as explained for a thick blood film (Section 2.23.1.2). The slide was placed on a level surface and a second clean glass side was touched to the drop of blood. This causes the blood to spread along the length of the second. The second slide was held at an angle of approximately 45 degrees to the first slide pushed firmly along the first slide, ensuring that the slides were in even contact with one another. The blood smear was allowed to air dry and then fixed for 30 seconds in methanol. After allowing the slides to air dry, they were stained with Giemsa (Section 2.23.3).

2.23.3 Giemsa staining of thick and thin blood films

2.23.3.1 Reagents

Methanol.

Giemsa stain, modified.

2.23.3.2 Procedure

Once the films had air dried after fixing in methanol for 30 seconds, they were stained for 30 min with Giemsa. The slides were washed under running water and allowed to air dry.

2.24 Determining the number of parasitized red blood cells from thin blood films

2.24.1 Procedure

The fixed and stained thin blood films were observed under an oil immersion objective (100 X). Suitable fields of view were obtained for counting. These fields had an even spread of red blood cells with no red blood cells overlapping. The total number of red blood cells was counted within such a field. Then the number of those red blood cells, containing parasites, was determined. This was repeated for three to five fields per film. The total number of red blood cells divided by the number of parasitized red blood cells, multiplied by one hundred gave the percentage parasitemia.

2.25 Collection of blood from mice using retro-orbital bleeding

Blood was collected from mice from the retro-orbital plexus. This was performed as a terminal procedure under anaesthetic.

2.26 Preparation of stabilate from infected blood

Stocks of parasitized red blood cells (stabilate) were prepared and stored under liquid nitrogen so that further mice could be infected if necessary.

2.26.1 Reagents

RPMI-1640 medium (as per Sigma instructions). RPMI-1640 (0.624 g) was dissolved in sterile dH_2O (approximately 54 ml). Sodium bicarbonate (0.120 g) was added and stirred until dissolved. The pH was adjusted to pH 7.0 with dilute HCl. The solution was made up to 60 ml with sterile dH_2O and filter-sterilized with a 0.22 μm acetate filter. Aliquots (5 ml) were stored in sterile tubes at 4˚C in the dark. Deterioration of the liquid medium was recognised either by a change in pH, colour or by the presence of precipitates or a cloudy appearance. Aliquots of Media showing signs of deterioration were discarded.

Parasite freezing medium [RPMI-1640 containing 10% glycerol]. Sterile glycerol (1 ml) was added to filter-sterilized RPMI-1640 medium (9 ml) and vortexed briefly to mix. The freezing media was stored at 4˚C, but was discarded if a colour change was observed or if any precipitate was visible.

Sterile glycerol. Glycerol was autoclaved to sterilise.

2.26.2 Procedure

The number of parasitized and non-parasitized red blood cells were determined (Section 2.24). The whole blood collected from the infected mouse (Section 2.25) was transferred from the heparinized vacutainer to a sterile microcentrifuge tube. The volume of whole blood was noted. The blood was centrifuged (1000 *g*, 4 min, 4˚C) to pellet the red blood cells. The volume of the plasma supernatant was determined. The plasma was transferred to a sterile microcentrifuge tube and stored at –20˚C. The volume of packed red blood cells was estimated by subtracting the plasma volume from the whole blood volume. The packed red blood cells were very gently washed with RPMI-1640 medium with a volume equal to the plasma volume and then centrifuged as before (1000 *g*, 4 min, 4˚C). Care was taken to not shear the red blood cells by using pipette tips that had been cut to give a larger opening when resuspending the pellet. The washing was repeated a total of three times. The red blood cells were then diluted to the required number of parasitized red blood cells per 100 µl, with RPMI-1640 containing 10% (v/v) glycerol.

2.27 Determining the number of red blood cells in a mouse blood sample

It was necessary to determine the number of red blood cells present in a sample of mouse blood. This was achieved using a haemocytometer and based on the method described by Hudson and Hay (1989) and Mishell *et al.*, (1980).

2.27.1 Reagents

RPMI-1640. Section 2.26.1.

2.27.2 Procedure

Whole blood was diluted 1:20 in RPMI-1640 (i.e. 10 µl whole blood made up to 200 µl with RPMI-1640). This was further diluted by a factor of 10 to give a final dilution of 1:200 (i.e. 100 µl of the 1:20 dilution was made up to 1 ml with RPMI-1640). A cover slip was placed over the haemocytometer chamber and the 1:200 dilution of whole blood (25 µl) was allowed to run under the cover slip and into the chamber. The haemocytometer chamber was observed under 40x magnification.

The total number of RBC's was calculated as follows:

Total RBC's = (sum of RBC counted in 5 blocks)/5 x total blocks x dilution factor x 10^4

= (sum of RBC counted 5 blocks)/5 x 25 x 200 x 10 000

= number RBC / ml

2.28 Solubilization of parasitized red blood cells

Plasmodium infected red blood cells having 30-40% parasitemia were lysed and used for western blotting purposes. Uninfected red blood cells were lysed and used as controls.

2.28.1 Reagents

Solubilization buffer [20 mM Tris-HCl, pH 7.8, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 0.5% (v/v) triton X-100, 0.05% (w/v) NaN_3]. Tris (0.12 g), NaCl (0.15 g) and EDTA (0.093 g) were dissolved in dH_2O (40 ml) and adjusted to pH 7.8 with HCl. PMSF (5 ml of 10 mM PMSF) was added. NaN_3 (0.025 g) and triton X-100 (250 µl) were added and the volume made up to 50 ml with dH_20.

10 mM (1.74 mg/ml) Phenylmethyl-sulfonyl fluoride (PMSF). PMSF (0.0174 g) was dissolved in propan-2-ol (isopropanol) (10 ml), aliquoted and stored at –20˚C.

2.28.2 Procedure

The number of red blood cells was determined (Section 2.27). The red blood cells were centrifuged (1000 *g*, 4 min, 4 ºC). The serum was removed and the red blood cell packed volume was estimated. The red blood cells were resuspended in an appropriate volume of

solubilization buffer to give a final red blood cell count of 1 X 10^9 RBC/ ml. The red blood cells were left overnight at 4 ºC. The cells were sonicated (10 x 15 s) and the red blood cell debris was removed by centrifugation (10 000 *g*, 1 h, 4 ºC). The supernatant was aliquoted and stored at -70 ºC until needed for running on an SDS-PAGE gel and western blotting.

2.29 Coupling peptides to rabbit albumin using glutaraldehyde

Coupling peptides to proteins with the cross-linking reagent glutaraldehyde is a simple effective method, which retains the native antigenicity of the protein and peptides (Reichlin, 1980). The coupling reaction involves a single free amino group and thus is generally not used for peptides with internal amino groups, as this could result in large multimeric complexes being formed (Thorpe, 1994).

2.29.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dH_2O (approximately 950 ml) and titrated to pH 7.2 if necessary. The volume made up to 1 l with dH_2O.

2% (v/v) Glutaraldehyde solution. 25% glutaraldehyde (80 µl) was made up to 1 ml with dH_2O.

10 mg/ml $NaBH_4$. $NaBH_4$ (0.002 g) dissolved in dH_2O (200 µl).

2.29.2 Procedure

Peptide FKLGSCYLYIINRNLKEI (0.004g, 1.8 x 10^{-6} moles, M_r 2169.5) was dissolved in dimethylsulfoxide (DMSO) (50 µl). Additional DMSO was added in 50 µl increments to dissolve the peptide, if necessary. The dissolved peptide solution was then made up to 500 µl with PBS, pH 7.2. The carrier protein, rabbit albumin (0.0031 g, 4.5 x 10^{-8} moles, M_r 68200) was dissolved in PBS (500 µl). The rabbit albumin and peptide were then combined and 2% (v/v) glutaraldehyde solution (1 ml) was added drop wise with gentle stirring. The solution was then stirred for a further 2h at 4˚C. The reaction was stopped with the addition of 10 mg/ml $NaBH_4$ (200 µl). The solution was stirred for 1h at 4˚C. The rabbit albumin-peptide conjugate was then dialysed against 3 changes of PBS containing 0.02% (w/v) NaN_3 and then stored at –20˚C in four aliquots of equal volume.

2.30 Coupling peptides to rabbit albumin using 3-maleimidobenzoic acid N-hydroxysuccinimide (MBS)

MBS is a hetero-bifunctional coupling reagent developed by Kitagawa and Aikawa (1976). The reagent can be used to link peptides to carrier proteins via cysteine residues and free amino groups (Harlow and Lane, 1988). MBS acylates the amino groups on the carrier protein via the active ester. This reaction is followed by the formation of thioester bonds upon addition of a thiol group to the double bond of the maleimide (Kitagawa and Aikawa, 1976). The cysteine residues of the peptide were reduced with DTT prior to coupling, although this step was not completely necessary. The reduced peptide was separated from the DTT with chromatography. The separation of the reduced peptide from DTT was monitored with 5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB), a reagent commonly referred to as Ellman's reagent, as it was developed by Ellman (1959). DTNB is an aromatic disulfide that enables the detection of thiol groups. It reacts with aliphatic thiols, forming a mixed disulfide and 2-nitro-5-thiobenzoate, which possesses an intense yellow colour (Habeeb, 1972).

2.30.1 Reagents

Sephadex® G10.

Sephadex® G25 fine.

100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 7.0. $NaH_2PO_4.2H_2O$ (15.6 g) was dissolved in approximately 950 ml dH_2O and adjusted to pH 7.0 with concentrated NaOH. NaN_3 (0.2 g) was added and the volume made up to 1 l.

Reducing buffer [100 mM Tris-HCl, 1mM EDTA, 0.02% (w/v) NaN_3, pH 8]. Tris (1.21 g) and EDTA (0.037 g) were dissolved in 90 ml dH_2O and titrated to pH 8 with HCl. NaN_3 (0.02 g) was added and the volume made up to 100 ml.

Ellman's reagent buffer [100 mM Tris-HCl, 10 mM EDTA, 0.1% SDS, pH 8]. Tris (1.21 g) and EDTA (0.37 g) were dissolved in approximately 80 ml dH_2O and titrated to pH 8 with HCl. SDS (0.1 g) was added and the volume made up to 100 ml.

Ellman's reagent (10 mM). Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid) (0.01 g) was dissolved in Ellman's reagent buffer (2.5 ml).

10 mM Dithiothreitol (DTT). DTT (0.0077g) was dissolved in reducing buffer (5 ml) just before use.

2.30.2 Procedure

Reduction of peptides: Peptide CFKLGSCYLYIINRNLKEI (0.004g, 1.8 x 10^{-6} moles, M_r 2270.66) and peptide SDDDNRQIQDFEC (0.004 g, 2.5 x 10^{-6} moles, M_r 1583) were dissolved in DMSO (50 µl). If necessary, additional DMSO was added in 50 µl increments to fully dissolve the peptide. The peptide solution was made up to 1 ml with reducing buffer. DTT (1 ml) was added to the peptide solution and left to incubate at 37˚C for 1.5 h. Molecular exclusion chromatography (MEC) was used to separate the reduced peptide from unreacted DTT. The peptide solution was loaded onto a Sephadex G10 column (15 x 110 mm, 10 ml/h) equilibrated with 100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 7.0. The column was continually washed with 100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 7.0, whilst collecting 500 µl fractions. Each of the fractions was tested for reduced peptide by combining 10 µl of the fraction and 10 µl of Ellman's reagent. The development of a yellow colour indicated the presence of reduced peptide. The fractions of reduced peptide were pooled. Unreacted DTT eluted after reduced peptide, and produced an intense yellow colour when tested with Ellman's reagent. These fractions were discarded.

Activation of carrier: Rabbit albumin (0.003 g, 4.4 x 10^{-8} moles, M_r 68200, for peptide CFKLGSCYLYIINRNLKEI; 0.0043g, 6.3 x 10^{-8} moles, M_r 68200, for peptide SDDDNRQIQDFEC) was dissolved in PBS (1725 µl) to give a molar ratio of carrier to peptide of 1:40. MBS (0.0006 g, 4.7 x 10^{-8} moles, M_r 12570, for peptide CFKLGSCYLYIINRNLKEI; 0.0008 g, 6.4 x 10^{-8}, for peptide SDDDNRQIQDFEC) was dissolved in dimethyl formamide (DMF) (275 µl) to give a molar ratio of carrier protein to MBS of 1:40. The rabbit albumin and MBS were combined and stirred for 30 min at room temperature. The solution was then passed over a Sephadex G25 column (15 x 130 mm, 10 ml/h) equilibrated with 100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 7.0, to separate activated carrier from excess MBS. The fractions were monitored for activated carrier by reading the absorbance of the fractions at 280 nm. Fractions containing activated carrier were combined with pooled reduced peptide fractions and stirred gently for 3 h at room temperature. This was then aliquoted into four equal volumes and stored at –20˚C until needed.

2.31 Immunisation of chickens with antigen and rabbit albumin-conjugated peptides

Anti-peptide antibodies were raised in chickens. Two chickens for each immunogen were immunised intramuscularly at two sites in their breast muscles. The chickens were immunised at week 0, with the rabbit albumin-peptide conjugate (Section 2.29 and 2.30) (100 μg), emulsified with Freunds complete adjuvant in a 1:1 (v/v) ratio. Booster immunisations were performed at weeks 2, 4, and 6 with the same amount of peptide emulsified in Freund's incomplete adjuvant in a 1:1 (v/v) ratio. Eggs were collected prior to the initial immunisation, (week 0) to serve as pre-immune control eggs. After the initial immunisation, the eggs were collected for a period of 12 weeks. The eggs were stored at 4˚C until the immunoglobulin Y (IgY) from the egg yolk was isolated (Section 2.32).

2.32 Isolation of immunoglobulin Y from chicken egg yolk

Immunoglobulin Y (IgY) was isolated from chicken egg yolks (Goldring and Coetzer, 2003). Polson *et al*. (1980; 1985) developed the technique, which involves the use of a hydrophilic polymer, polyethylene glycol (PEG) to precipitate protein. The ability of PEG to fractionally precipitate proteins was recognised by Polson *et al.* in 1964. PEG precipitates proteins by excluding the proteins from water. With increasing concentrations of PEG, the protein is eventually brought to its solubility limit and precipitates out of solution (Dennison, 1999). The procedure for the isolation of IgY involved precipitation steps of increasing PEG concentration. Vitellin and lipids were precipitated first with 3.5% (w/v) PEG. Increasing the PEG concentration to 12% (w/v) precipitates the IgY.

2.32.1 Reagents

Isolation buffer [100 mM $NaH_2PO_4.2H_2O$, 0.02% NaN_3, pH 7.6]. $NaH_2PO_4.2H_2O$ (15.6 g) was dissolved in 950 ml dH_2O and titrated to pH 7.6 with NaOH. NaN_3 (0.2 g) was added and the volume was made up to 1 l.

10% (w/v) Sodium azide (NaN_3). NaN_3 (10 g) was dissolved in dH_20 (100 ml).

2.32.2 Procedure

The egg yolk was separated from the albumin and gently washed under running water. The yolk sac was removed and the volume of yolk determined. A volume of isolation buffer, equal to twice the yolk volume, was then added to the yolk and stirred gently. To this 3.5% (w/v) PEG 6000 was added. This was stirred until the PEG had fully dissolved and the lipids had separated out of solution. The solution was then centrifuged (4 420 *g*, 30 min). The supernatant was filtered through absorbent cotton wool and the volume of resulting filtrate noted. The yellow pellet containing the lipid and vitellin fractions was discarded. To the filtrate, 8.5% (w/v) PEG 6000 was added. This increased the PEG concentration from 3.5% (w/v) to 12% (w/v). The PEG was fully dissolved and the solution was centrifuged (12 000 *g*, 10 min) to pellet the IgY. The supernatant was discarded and the pellet was suspended in a volume of isolation buffer equal to the yolk volume. PEG 6000 was added to 12% (w/v) and dissolved. The solution was centrifuged (12 000 *g*, 10 min). The supernatant was discarded and the pellet was suspended in isolation buffer, in a volume equal to $1/6^{th}$ of the yolk volume. Sodium azide (10% (w/v) solution) was added to a final concentration of 0.1%. The isolated IgY was then stored at 4˚C in sterile bottles.

2.32.3 Determination of IgY concentration

The absorbance of a 1:50 dilution of IgY in isolation bufferr (Section 2.32.1) was read at 280 nm in quartz cuvettes. The concentration of IgY in the undiluted solution was then determined using the extinction coefficient of a 1 mg/ml of IgY at 280 nm of 1.25 (Coetzer, 1993).

2.33 Preparation of affinity matrices

Peptides were coupled to affinity matrices for the purification of antibodies produced against the peptides.

2.33.1 Coupling of peptide or protein to AminoLink®

The AminoLink® coupling gel consists of a 4% cross-linked beaded agarose matrix with aldehyde functional groups. The coupling reaction involves the formation of a labile Schiff base. This occurs due to the primary amine groups on the peptide or protein reacting

with aldehyde groups on the matrix. A stable secondary amine bond is formed between the peptide/protein and the matrix through reduction of the of the Schiff base intermediate with sodium cyanoborohydride ($NaCNBH_3$). Unreacted aldehyde groups on the matrix are quenched with Tris-HCl. Additional $NaCNBH_3$ is added to form a stabile Schiff base. The additional $NaCNBH_3$ reduces the Schiff base intermediate formed between the Tris primary amine and the matrix. $NaCNBH_3$ is the favoured reducing reagent as it is gentle and it is specific toward the Schiff base structure (Hermanson, 1996).

2.33.1.1 Reagents

Coupling buffer [0.1 M NaH_2PO_4, 0.05% (w/v) NaN_3, pH 7-7.5]. $NaH_2PO_4 2H_2O$ (7.8 g) was dissolved in dH_2O (490 ml) and the solution was adjusted to pH 7.4 with NaOH. NaN_3 (0.25 g) was added and the volume made up to 500 ml.

Quenching buffer [1 M Tris-HCl, pH 7.4]. Tris (12.11 g) was dissolved in dH_2O (80 ml) and titrated to pH 7.4 with HCl. The solution was then made up to 100 ml with dH_2O.

Washing buffer [1 M NaCl]. NaCl (29.22 g) was dissolved in dH_2O (500 ml).

Sodium cyanoborohydride solution [1 M $NaCNBH_3$]. $NaCNBH_3$ (0.0314 g) was dissolved in dH_2O (500 µl). This was prepared in a fume hood as $NaCNBH_3$ is toxic.

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dH_2O (approximately 950 ml) and titrated to pH 7.2 if necessary. The volume made up to 1 l with dH_2O.

AminoLink® Coupling Gel.

2.33.1.2 Procedure

Peptide (0.004 g) was dissolved in DMSO (500 µl). This was diluted 1:3 in coupling buffer. To obtain an AminoLink® Coupling Gel bed volume of 2 ml, 4 ml was pipetted into a Poly-Prep® chromatography column and washed three times with coupling buffer (each wash volume equivalent to 10 column volumes). The gel was allowed to settle and the supernatant discarded. The peptide solution was added and the cyanoborohydride solution (25 µl/ml) was added in a fume hood. This was mixed for two hours at room temperature and then left to stand (4 h, room temperature). The gel was then washed once with quenching buffer. A volume of quenching buffer equal to the volume of the gel was then added and

cyanoborohydride solution (50 µl/ml) was added in the fume hood. This was then mixed (end-over-end rotation, 30 min, room temperature). The gel was allowed to settle and was then washed with washing buffer until the absorbance of the eluant at 280 nm reached a zero reading. The gel was then washed with coupling buffer and stored at 4˚C until required.

2.33.2 Coupling of peptides to SulfoLink® Coupling Gel

The SulfoLink® coupling gel contains iodoacetyl groups which allow for the immobilization of ligands possessing sulfhydryl groups. Prior to coupling, it is necessary to reduce the terminal cysteine residues of the peptide to be immobilized with DTT. Ellman's reagent is used in this protocol to detect reduced peptide as before (Section 2.30.1).

2.33.2.1 Reagents

50 mM Tris-HCl, 5 mM EDTA, pH 8.5. Tris (6.057 g) and EDTA (1.86 g) were dissolved in approximately 950 ml of dH_2O and titrated to pH 8.5 with HCl. The volume was then made up to 1 l with dH_2O.

Washing buffer [1 M NaCl]. NaCl (58.44 g) dissolved in approximately 950 ml dH_2O, and the volume made up to 1 l.

Ellman's reagent buffer [100 mM Tris-HCl, 10 mM EDTA, 0.1% SDS, pH 8]. Section 2.30.1.

10 mM Dithiothreitol (DTT). Section 2.30.1.

10 mM Ellman's reagent. Section 2.30.1.

SulfoLink® Coupling Gel.

50 mM L-cysteine. L-cysteine. HCl (0.00878 g) dissolved in general buffer (1 ml). This buffer was prepared just prior to use.

100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 7.0. Section 2.30.1.

2.33.2.2 Procedure

The peptide (0.005 g) was solubilised in DMSO (50 µl). If necessary, additional DMSO was added in 50 µl increments to fully dissolve the peptide. The volume was then made up to 500 µl with 50 mM Tris-HCl, 5 mM EDTA, pH 8.5. The dissolved peptide was reduced with 10 mM DTT (500 µl) (1.5 h, 37˚C). After incubation, the reduced peptide was

separated from unreacted DTT by molecular exclusion chromatography (MEC) as described in Section 2.30.1.

SulfoLink® Coupling gel (2 ml of the 50% (v/v) slurry) was pipetted into a Poly-Prep® chromatography column to give a bed volume of 1 ml. The gel was washed with 6 column volumes of 50 mM Tris-HCl, 5 mM EDTA, pH 8.5. The fractions from the MEC column containing reduced peptide was added to the SulfoLink® coupling gel and mixed (end-over-end rotation, 15 min, room temperature). This was then left to stand (30 min, room temperature). The column was washed with 3 column volumes of 50 mM Tris-HCl, 5 mM EDTA, pH 8.5, before incubation with 50 mM cysteine (1 ml) to block unreacted sites (end-over-end rotation, 15 min, room temperature). This was left to stand (30 min, room temperature). The column was washed with 16 column volumes of 1 M NaCl, followed by 2 column volumes of 0.1 M NaH_2PO_4, 0.05% (w/v) NaN_3, pH 6.5. The resin was stored at 4˚C until required.

2.34 Affinity purification of chicken IgY anti-peptide antibodies

IgY isolated from chicken egg yolk contains a pool of polyclonal antibodies, to a number of different antigens that the chickens have been exposed to, including the immunogen. Antibodies not specific for the peptide they were raised against could interfere with assays and it was therefore necessary to isolate specific anti-peptide antibodies from the IgY pool. The IgY pool was passed over affinity matrices with the respective peptides immobilized to achieve this.

2.34.1 Reagents

Washing buffer [100 mM NaH_2PO_4, 0.05% (w/v) NaN_3, pH 6.5]. $NaH_2PO_4.2H_2O$ (15.6 g) was dissolved in approximately 950 ml dH_2O and titrated to pH 6.5 with NaOH. NaN_3 (0.5 g) was added and the volume made up to 1 l.

Elution buffer [100 mM glycine, 0.02% (w/v) NaN_3, pH 2.8]. Glycine (7.5 g) was dissolved in 950 ml dH_2O and titrated to pH 2.8 with HCl. NaN_3 (0.2 g) was added and the volume made up to 1 l.

Neutralising buffer [1 M NaH_2PO_4, 0.02% (w/v) NaN_3, pH 8.5]. $NaH_2PO_4.2H_2O$ (15.6 g) was dissolved in 90 ml dH_2O and titrated to pH 8.5 with NaOH. NaN_3 (0.02 g) was added and the volume made up to 100 ml.

2.34.2 Procedure

IgY isolated from the eggs collected in weeks 4-8 and 9-12 for each chicken were pooled and filtered through Whatman no.1 filter paper. The affinity columns (Section 2.33.1 and 2.33.2) were equilibrated with washing buffer. The IgY pool was cycled overnight (10 ml/h, RT) through the column to allow for binding of the anti-peptide antibodies to the peptide on the column. The antibodies were cycled backwards through the column to avoid blockages and the column running dry. The column was washed with approximately 12 column volumes of washing buffer to remove any unbound IgY. Bound IgY was eluted with elution buffer. Fractions of 900 µl were collected into 1.5 ml tubes containing neutralising buffer (100 µl). The elution of IgY was monitored spectrophotometrically at 280 nm. Elution buffer (900 µl) and neutralising buffer (100 µl) was used as a blank. Fractions containing purified IgY were pooled and 10% (w/v) NaN_3 was added to a final concentration of 0.1%. The antibodies were stored at 4˚C. The column was washed with approximately 12 column volumes of washing buffer before storing at 4˚C.

2.35 Enzyme linked immunoadsorbent assay (ELISA)

The progress of antibody production in chicken eggs was monitored using the ELISA technique, which was first introduced by Engvall and Perlmann (1971).

2.35.1 Reagents

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dH_2O (approximately 950 ml) and titrated to pH 7.2 if necessary. The volume was made up to 1 l with dH_2O.

Carbonate coating buffer, pH 9.6 [0.159% (w/v) Na_2CO_3 and 0.293% (w/v) $NaHCO_3$]. Na_2CO_3 (0.159 g) and $NaHCO_3$ (0.293 g) were dissolved in approximately 90 ml dH_2O and titrated to pH 9.6 with dilute HCl. The volume was made up to 100 ml with dH_2O.

0.5% Bovine serum albumin (BSA)-PBS. BSA (0.5 g) was dissolved in PBS (100 ml). This was prepared just before use.

0.1% PBS-Tween. Tween 20 (1 ml) was made up to 1 l with PBS.

0.15 M Citrate-phosphate buffer, pH 5.0. A citric acid solution (21 g/l) was titrated to pH 5.0 with a solution of $Na_2HPO_4.2H_2O$ (36.5 g/l).

2, 2′-Azinobis [3-ethyl-2, 3-dihydrobenzthiazole-6-sulfonate (ABTS) substrate solution [0.05 %(w/v) ABTS, 0.0015% (v/v) H_2O_2, in citrate-phosphate buffer]. ABTS (0.0075 g) and 30% H_2O_2 (7.5 µl) dissolved in 0.15 M citrate-phosphate buffer, pH 5.0 (15 ml).

2.35.2 Procedure

Peptides FKLGSCYLYIINRNLKEI, CFKLGSCYLYIINRNLKEI and SDDDNRQIQDFEC were coated at a concentration of 1 µg/ml in PBS (150 µl/well). Recombinant maltose binding protein was coated at a concentration of 1 µg/ml in carbonate coating buffer, pH 9.6 (150 µl/well). The coated plates were left at 4˚C overnight. The ELISA plates were emptied and patted upside down on paper towelling to remove any residual liquid. Non-specific binding of antibodies was prevented by blocking with 0.5% (w/v) BSA-PBS (200 µl/well, 1 h at 37˚C). The plates were then washed three times with PBS-Tween and patted dry as before. IgY from individual weeks was used at a concentration of 25 µg/ml in 0.5% (w/v) BSA-PBS (100 µl/well, 2 h at 37˚C). Affinity purified antibodies were diluted appropriately in 0.5% (w/v) BSA-PBS (100 µl/well, 2 h at 37˚C). The plates were then washed three times with PBS-Tween and patted dry as before. HRPO-conjugated rabbit anti-chicken IgY secondary antibody was diluted 1:20 000 dilution in 0.5% (w/v) BSA-PBS (120 µl/well, 1 h at 37˚C). The plates were then washed three times with PBS-Tween and patted dry as before. ABTS substrate solution (150 µl/well) was added and the colour was left to develop in the dark for 45 min before the A_{405} of each well was measured using a VERSAmax tunable microplate reader.

2.36 Immunofluorescence assays

Slides were coated with *P. yoelii yoelii* parasitized mouse red blood cells according to the method described by Perlmann *et al.* (1984).

The native *P. yoelii yoelii* protein PY05757 and *P. yoelii yoelii* LDH were probed with the anti-peptide antibodies on *P. yoelii yoelii* infected red blood cells. The protocol used was based on a method employed by Rasoloson *et al.* (2004). Anti-chicken antibodies conjugated with fluorescein isothiocyanate (FITC) were used to detect bound anti-peptide antibodies. The slides were counterstained with 4', 6-diamide-2'-phenylindole dihydro chloride (DAPI) to indicate the parasite nuclear material.

2.36.1. Reagents

Coating buffer. Na_2CO_3 (0.0795 g) and $NaHCO_3$ (0.1465 g) were dissolved in approximately 40 ml dH_2O and titrated to pH 9.6 with dilute HCl. NaN_3 (0.01 g) was added and the volume made up to 50 ml.

Isotonic Tris buffer [0.15 M Tris, pH 7.2]. Tris (0.908 g) was dissolved in sterile dH_2O (approximately 40 ml) and adjusted to pH 7.2 with HCl. The solution was then made up to 50 ml with sterile dH_2O. As the solution was usually filter sterilized with a 0.22 µm acetate filter, the pH was adjusted to approximately pH 7.0 due to the tendency of solutions to increase in pH during the filtering process.

0.9% (w/v) NaCl. NaCl (0.9 g) dissolved in sterile dH_2O (100 ml).

Hanks balanced salt solution (HBSS) [Sigma 9.5 g/l]. HBSS (0.95g) was dissolved in sterile dH_2O (approximately 90 ml) by stirring gently on a magnetic stirrer. Sodium bicarbonate was added to a final concentration of 0.35 g/l. The solution was stirred until the sodium bicarbonate dissolved. The solution was then adjusted to pH 6.9 - 7.0 and made up to 100 ml with sterile dH_2O.

Tris buffered Hanks balanced salt solution (TBH). Isotonic Tris buffer, pH 7.2 (10 ml), 0.9% (w/v) NaCl (90 ml) and Hanks balanced salt solution (HBSS) (100 ml) were combined and filter sterilize using a 0.22 µm acetate filter.

1% (v/v) Glutaraldehyde. 25% (v/v) Glutaraldehyde (200 µl) was made up to 5 ml with PBS.

0.05% (v/v) Tween-TBH. Tween-20 (50 µl) was diluted to 100 ml with TBH.

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dH_2O (approximately 950 ml) and titrated to pH 7.2 if necessary. The volume was made up to 1 l with dH_2O.

2% (w/v) BSA- PBS. BSA (0.5 g) was dissolved in PBS (25 ml).

2.36.2. Procedure

BALB/c mice were infected with *P. yoelii yoelii* parasitised mouse red blood cells by I.P. injection (Section 2.22). Blood was collected from the infected mice by retro orbital bleeding (Section 2.25) when the percentage of parasitized red blood cells in the mice was determined to be approximately 30% (Section 2.24). The blood was collected into Heparin coated Vacutainer^{TM} tubes and kept on ice. The volume of whole blood was determined and then the red blood cells were centrifuged (1 000 x *g*, 1 min). The plasma volume was determined in order to calculate the red blood cell packed volume. The red blood cells were suspended in a volume of TBH approximately equal to the whole blood volume and centrifuged as before to pellet the red blood cells. The supernatant was discarded and the packed red blood cells were suspended to 1% (v/v) haematocrit in TBH. The wells of 8 mm diameter, 8-welled slides were pre-treated with one drop of coating buffer per well (approximately 20 µl per well) for 30 minutes at room temperature. The coating buffer was then aspirated. One drop (approximately 20 µl) of the 1% (v/v) haematocrit was placed in each of the pre-treated wells and left for 30 minutes in order to allow the red blood cells to settle and adhere to the surface of the slide. Unbound red blood cells were washed off the slides by immersing them upside down in TBH and gently shaking them. The red blood cells were then fixed to the wet slides by covering them in a solution of 1% (v/v) glutaraldehyde in PBS. After 10 seconds the glutaraldehyde solution was allowed to run off the slides and the fixation procedure repeated. The slides were washed with sterile dH_2O and allowed to air dry. The slides were stored at –20˚C until needed.

The wells of the slide coated with parasitized mouse red blood cells were blocked with 2% (w/v) BSA-PBS (20 µl per well, 1 h, RT). The blocking solution was aspirated and the wells washed with PBS (20 µl/well, 2 x 3 min). The PBS was aspirated and the wells were incubated with primary antibody (pre-immune IgY, anti-PfLDH IgY (controls) and anti-peptide FKLGSCYLYIINRNLKEI, CFKLGSCYLYIINRNLKEI and SDDDNRQIQDFEC IgY, 10 µg/ml in 2% (w/v) BSA-PBS, 1 h, RT). The primary antibody was aspirated and the wells were washed with PBS (20 µl/well, 3 x 5 min). The remainder of the protocol was carried out in the dark. Each well was incubated with secondary antibody (donkey anti-chicken-FITC conjugate diluted 1:100 in 2% (w/v) BSA-PBS, 20 µl/well, 1 h, RT). The wells

were washed with PBS (20 µl/well, 2 x 5 min) and stained with DAPI (5 µg/ml in PBS) (15 min, RT). The excess stain was rinsed off with PBS and the slides were stored overnight in the dark in a vacuum desiccator. Slides were viewed at a magnification of 1 000x using an Olympus AX70 fluorescent microscope. A green filter was used for FITC and a UV filter was used for DAPI. Digital photographs of the slides were taken with the CC12 Soft Imaging System™ colour camera and viewed with the analySIS™ software. Z-stack images were performed using the Confocal Zeiss LSM 710 microscope and ZEN light imaging software.

CHAPTER 3
Bioinformatic studies

3.1 Introduction

Bioinformatics is the study of genes, genomes and proteins at the sequence, structural and functional level (Xiong, 2006). The goal of using bioinformatics is to have a better understanding of how a gene or protein operates at a molecular level, without necessarily having the protein or gene to physically work with. Provided that the sequence is available for that protein or gene of interest, it is possible to make predictions about the gene and of the proteins structure, function, or location within the cell or organism. However, it must be remembered when using bioinformatics that the predictions made are not confirmed facts: bioinformatics has limitations and therefore must always be followed up with wet lab experiments.

In the present study, a number of bioinformatics tools were used to find information about a hypothetical *Plasmodium* protein. The protein sequence of a *P. chabaudi adami* protein *Pca* 96, was used as a query sequence in BLAST searches to find proteins with similar sequences. The sequences were also used to search for signal sequences, *Plasmodium* export element (PEXEL) motifs and conserved domains. In addition, predictions were made about the sub-cellular locations of the protein.

3.2 Bioinformatic methods

3.2.1 Basic Local Alignment Search Tool (BLAST) searches and CLUSTALW sequence alignments

The BLAST program (Altschul *et al.*, 1990) is a useful program allowing for a query protein or DNA sequence to be compared to other protein or DNA sequences within databases. Sequences determined to have sequence similarity to the query sequence are reported with an expectation value (E-value). The E-value describes the number of sequences matched to the query sequence one might expect to see by chance in a database of a particular size. The lower the E-value, the more statistically significant is the sequence match.

The amino acid sequence of *Pca* 96 was used as a query sequence in a BLASTP (protein to protein database) and TBLASTN (protein to translated nucleic acid database) search at PlasmoDB (http://www.plasmodb.org/).

The nucleotide sequences from the TBLASTN search that did not have protein sequences available, were downloaded from PlasmoDB (http://www.plasmodb.org/) and translated into amino acid sequences at Expasy (http://www.expasy.org/tools/dna.html). The amino acid sequence of *Pca* 96 was aligned with the amino acid sequences and translated amino acid sequences producing matches with the BLAST searches. The sequence alignment was performed using the program CLUSTALW (Chenna *et al.*, 2003), at the European Bioinformatics Institute (EBI) website (http://www.ebi.ac.uk/clustalw/).

3.2.2 Expression profiles

High-density oligonucleotide arrays were used to create mRNA expression profiles of genes for all of the stages of the *P. falciparum* life cycle (Le Roch *et al*, 2003). In the present study, the data from Le Roch *et al*. (2003) was used to construct graphs of the mRNA expression levels for the gene encoding the *P. falciparum* protein PFC0760c. This was done to determine at what stage of the parasite life cycle the expression increased, as this could possibly provide insight into the role PFC0760c has within the parasite life cycle, as it is assumed that the mRNA expression levels could possibly reflect the protein expression level, although it is understood that this may not necessarily always be the case. *P. falciparum* lactate dehydrogenase (*Pf*LDH) (PF13_0141) was used as a comparison for levels of mRNA expression.

3.2.3 *Plasmodium* export element (PEXEL) motif searches and signal sequence predictions

Secreted *Plasmodium* protein families such as *P. falciparum* erythrocyte membrane protein (*Pf*EMP1), repetitive interspersed family (RIFIN) and subtelomeric variable open reading frame (STEVOR) are exported out of the parasitophorous vacuole and into the host erythrocyte, where they alter the host red blood cell to maximize the parasites survival. Exported proteins generally have a sequence located at the N-terminus that directs the protein to the endoplasmic reticulum (Wickham *et al.*, 2001; Haldar *et al.*, 2002). The proteins are

then transported through the parasite membrane via the golgi apparatus, to the parasitophorous vacuole. In order for the proteins to be transported from the parasitophorous vacuole into the host red blood cell, it appears necessary for the proteins to have both a signal sequence and a motif that targets the protein to the host red blood cell. The motif, the *Plasmodium* export element (PEXEL) (Marti *et al*., 2004) or the vacuolar transport signal (VTS) (Hiller *et al*., 2004) as it is also known, consists of a short sequence of alternating charged, hydrophobic amino acids separated by non-charged amino acid residues (Marti *et al*., 2005). The sequence R/KxLxQ/E is typically located 15-20 amino acids downstream of the signal sequence (Marti *et al*., 2005). The presence of a PEXEL motif in a protein sequence can aid with predictions of where a protein is located within a cell and can essentially aid with predictions of the proteins function. All of the different combinations of the motif R/KxLxQ/E were searched for in the amino acid sequences of PFC0760c and the *P. yoelii yoelii* sequence PY05757 which was found to have high sequence similarity to PFC0760c and *Pca* 96 (Section 3.2.1).

The programs SIG-Pred (http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.html), and Sigfind 2.1 (http://www.stepc.gr/~synaptic/sigfind.html) were used to determine if the sequences possessed hydrophobic N-terminals signal sequences.

3.2.4 Transmembrane domain prediction

Transmembrane proteins are proteins having one or more sections of their structure crossing a biological membrane. These sections have amino acids that are predominantly hydrophobic in nature, suiting the hydrophobic environment of a lipid membrane bi-layer. These sections are composed of twenty or more amino acid residues (Westhead *et al*., 2002). The sections of the protein that are on either side of the membrane tend to be hydrophilic due to the aqueous environment. The membrane spanning regions of proteins often adopt the structure of an alpha helix.

One method of predicting membrane-spanning regions within proteins is with the program TMHMM. It is a program based on a hidden Markov model and is able to predict the presence of a transmembrane domain and the orientation, with respect to which sections of the protein are inside the membrane, and which are outside the membrane.

The amino acid sequences of *Pca* 96, PFC0760c and PY05757 were assessed for membrane spanning regions using TMHMM (http://www.cbs.dtu.dk/Services/TMHMM/).

3.2.5 Subcellular localization predictions

Prediction of a proteins sub cellular localisation can give insight to the proteins role in the cell. Due to so little being known about *Pca* 96, PFC0760c and PY05757, it was decided attempt to predict their subcellular location, using the web-based programs SubLoc and pTARGET.

3.2.5.1 pTARGET subcellular localization prediction

pTARGET (http://bioapps.rit.albany.edu/pTARGET/) has the ability to predict the subcellualr location of eukaryotic proteins. The program predicts proteins targeted to the cytoplasm, endoplasmic reticulum, golgi, lysozyme, mitochondria, nucleus, plasma membrane, peroxisome and whether they are secreted extracellularly (Guda, 2006). The prediction algorithm calculates two scores which are then summed to give the final prediction of the proteins subcellular localization. One score is based on the relative amino acid weights which are calculated from the amino acid composition of the protein sequence. The other score is based on the presence or absence of Pfam domains that are in a specific location within the protein sequence (Guda and Subramaniam, 2005). pTARGET has been shown to accurately predict protein subcellular localization to 96-99% (Guda and Subramaniam, 2006). A confidence level is given with each of the predictions which indicate the trustworthiness of that prediction. The confidence value is expressed as a percentage of the ratio of the calculated score to the total score required for making a true prediction.

3.2.5.2 Prediction of Subcellular Localization by SubLoc

SubLocv1.0 (http://www.bioinfo.tsinghua.edu.cn/SubLoc/) has the ability to predict the subcellular localization of prokaryotic and eukaryotic proteins, based on their amino acid composition (Hua and Sun, 2001). For eukaryotes, SubLoc can predict proteins targeted to the nucleus, cytoplasm, mitochondria and extra cellular matrix. SubLoc has a prediction accuracy of 79.4% for eukaryotic proteins, and 91.4% for prokaryotic proteins (Hua and Sun, 2001).

3.2.6 Conserved domains

Conserved domains are compact structural or functional parts of a protein that have been used and are still being used throughout the process of evolution (Wheeler *et al*., 2001). Searching for conserved domains in a protein of unknown structure and function could give insight into possible functions or structures of the protein (Wheeler *et al*., 2001). The conserved domain search (CD-Search) at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), allows conserved domains to be searched for on a protein query. CD-Search uses the Conserved Domain Database (CDD). The CDD contains data from Simple Modular Architecture Research Tool (SMART), Protein families (Pfam) and Clusters of Orthologous Groups of proteins (COGs) databases.

3.2.7 Helical wheel plot

A helical plot shows an end-on view of an alpha helix. The angle of rotation between adjacent amino acid residues is 100˚. In the arrangement of alpha helices, the hydrophobic amino acid residues are typically located on one side of the alpha helix (Mount, 2001). The conserved peptide sequence was used to plot a helical wheel plot using the programs Helixator (http://www.tcdb.org/progs/helical_wheel.php) and EMBOSS Pepwheel (http://www.tcdb.org/progs/pepwheel.php). The plot was performed to deduce if the sequence could possibly be an alpha helix. The programs utilised use different algorithms and thus will provide greater confidence in the results if both algorithms provide a similar result.

3.2.8 Structure prediction

A protein with a known 3 dimensional structure, having significant sequence similarity to *Pca* 96, PFC0760c or PY05757 was searched for using a sequence search at the Protein Data Bank (PDB) (http://pdb.org/pdb/search/advSearch.do?st=SequenceQuery). A significant sequence similarity of 40-50% would allow the amino acid of the query sequence to be superimposed onto the PDB entry, resulting in a sufficiently accurate model of the proteins structure (Mount, 2001; Lesk, 2002). In addition, the sequences of the proteins were used as query sequences with the program Phyre (www.sbg.bio.ic.ac.uk/phyre/) (Kelley and Sternberg, 2009), to determine which protein folds may be present in the proteins.

3.2.9 T-cell epitope prediction

SYFPEITHI (Rammensee *et al*., 1999) is a database allowing for the prediction of T-cell epitopes. The database consists of peptide sequences known to bind MHC class I and II molecules. The SYFPEITHI program (http://www.syfpeithi.de/) was used to determine if T-cell epitopes were present in the *Pca* 96 protein sequence. The epitopes predicted for *Pca* 96 were searched for in the amino acid sequence of PFC0760c.

3.2.10 Protein interaction

A version of the yeast-two hybrid assay has been used to determine *P falciparum* protein-protein interactions (LaCount *et al*., 2005). The plasmoMAP project at PlasmoDB uses the query gene entered to determine functional interactions with other *P. falciparum* genes. Each gene determined to have a functional interaction with the query gene is given a likelihood score. The higher the likelihood score the greater the confidence of the functional interaction. A likelihood score of 10 or above is a fairly good indication of the functional interaction being true. A second program called PlasmoPredict was used for predicting protein interactions for PFC0760c (www.bioinformatics.leeds.ac.uk/~bio5pmrt/PlasmoPredict/PlasmoPredict.html).

3.3 Results

3.3.1 BLAST searches and sequence alignments

In order to find other *Plasmodium* proteins with sequence similarity to *Pca* 96, the amino acid sequence of *Pca* 96 was used to perform a BLASTP search in all of the *Plasmodium* organisms available at PlasmoDB. The first ten results are displayed in Table 3.1. Proteins with high sequence similarity to *Pca* 96 were detected for *P. chabaudi chabaudi*, *P. yoelii yoelii*, *P. berghei*, *P. falciparum*, *P. vivax*, and *P. knowlesi*. Sequence alignments that accompany the BLAST results (not shown) revealed a sequence of amino acids that appeared to be present in five of the first six proteins listed in Table 3.1: *P. chabaudi chabaudi* PCAS_080770, *P. yoelii yoelii* PY05757, *P. falciparum* PFC0760c, *P. vivax* PVX_095365, and *P. knowlesi* PKH_082410. The *P. berghei* protein PB000626.03.0 did not appear to contain the sequence. The amino acid sequence of *Pca* 96 was used to perform a TBLASTN at PlasmoDB to determine if the protein had high sequence identity to translated DNA sequences

not available for BLASTP searches (Table 3.2). The genes encoding the proteins listed in Table 3.1 are observed in the TBLASTN results (Table 3.2). In addition, a number of *P. gallinaceum* and *P. reichenowi* sequences were observed to have sequence similarity to *Pca* 96. The alignments accompanying the TBLASTN results revealed regions containing the same sequence of amino acids identified in the BLASTP search in a translated *P. reichenowi* gene (Pr_3502696.c000127860.Contig1) and a translated *P. gallinaceum* gene (Pg_Contig89) (results no shown). All of the sequences containing this common amino acid sequence were downloaded from PlasmoDB. No protein sequences were available for the *P. reichenowi* and *P. gallinaceum* genes and therefore the nucleotide sequences were obtained from PlasmoDB and were translated into amino acid sequences at Expasy. The sequences were aligned with *Pca* 96, using CLUSTALW (Figure 3.1). The sequence alignment revealed the amino acid sequence FKL(G/S)SCYLYIINRN(L/M)KEI that appeared to be highly conserved across all the *Plasmodium* species used (Figure 3.1, text highlighted in grey).

The data from the alignment in Figure 3.1 was used to construct a basic schematic diagram to show how the sequences matched up with respect to one another based on their amino acid sequences, and where the consensus sequence FKLGSCYLYIINRNLKEI was located on each sequence (Figure 3.2).

Table 3.1. Results obtained from a BLASTP* search with *Pca* 96 at PlasmoDB

Name	Location	E-value
PCAS_080770 *P. chabaudi chabaudi*	chab08:354177-362783(+)	3.7×10^{-281}
PY05757 *P. yoelii yoelii* str. 17XNL	AABL01001868:46-4620(+)	3.0×10^{-151}
PB000626.03.0 *P.berghei* str. ANKA	PB_RP3514:1-1014(+)	2.7×10^{-86}
PFC0760c *P. falciparum* 3D7	Pf3D7_03:696215-706399(-)	9.0×10^{-47}
PKH_082410 *P. knowlesi* strain H	PK4.chr08:1136597-1145422(+)	7.5×10^{-34}
PVX_095365 *P. vivax* SaI-1	CM000449:1047817-1056702(+)	5.4×10^{-31}
PF14_0631 *P. falciparum* 3D7	Pf3D7_14:2694749-2703985(-)	2.0×10^{-07}
PFE0465c *P. falciparum* 3D7	Pf3D7_05:389914-398997(-)	3.0×10^{-07}
MAL13P1.295 *P. falciparum* 3D7	Pf3D7_13:2368215-2374292(+)	4.0×10^{-07}
PB000726.00.0 *P.berghei* str. ANKA	PB_RP0629:2391-4853(+)	1.2×10^{-06}

*Only the first ten results are shown

Table 3.2. Results obtained from a TBLASTN* search with *Pca* 96 at PlasmoDB

Name	Reading frame	E-Value
chab08 *P. chabaudi chabaudi*	3	1.10×10^{-294}
PB_RP3514 *P. berghei* str ANKA	1	4.70×10^{-179}
AABL01001868 *P. yoelii yoelii* str 17XNL....	1	1.00×10^{-159}
Pg_Contig89 *P. gallinaceum*	-3	9.50×10^{-45}
Pf3D7_03 *P. falciparum* 3D7	-1	1.30×10^{-37}
Pr_3502696.c000127860.Contig1 *P. reichenowi*	2	3.50×10^{-36}
PK4.chr08 *P. knowlesi* strain H	2	1.40×10^{-26}
CM000449 *P. vivax* SaI-1	1	6.40×10^{-22}
Pf3D7_14 *P. falciparum* 3D7	-1	3.10×10^{-07}
Pg_2265551.c000128678.Contig1 *P.gallinaceum*	-1	5.80×10^{-07}

*Only the first ten results are shown

```
Pca 96                -----------------------FSVSSDHITLIKCNILKMFKLGSCYLYIINRNLKEIKIL 39
P. chabaudi chabaudi  -----AESD-NNNLTPSDDREFSVSSDHITLIKCNILKMFKLGSCYLYIINRNLKEIKIL 1949
P. yoelii yoelii      -----VENDDNNNLNSSKNQEFSVNSDHITLIKCNILKMFKLGSCYLYIINRNLKEIKIL 586
P. falciparum         NNINNVKNIVDINNYLVNNLQLNKDNDNIIIIKFNILKLFKLGSCYLYIINRNLKEIQML 2406
P. vivax              ----------------------SPSNGSEHIVIIKCNILKLFKLGSCYLYIINRNMKEIQIL 1839
P. reichenowi         NNVNNVNNIVDINNYLVNNLQLNKDNDNIIIIKFNILKLFKLGSCYLYIINRNLKEIQML 1407
P. knowlesi           ----------------------SPLNGSEHIVIIKCNILKLFKLGSCYLYIINRNLKEIQVL 1822
P. gallinaceum        --------------------LSLNKDSDNIIIIKCNILKLFKLSSCYLYIINRNLKEIQNL 528
                                          ..::* :** ****:***.**********:***: *

Pca 96                FDKINSLEENIQSLNLFINNLKDQNKNNEVIKINNEEQILQLKNSLQNNENCINNLNDNL 99
P. chabaudi chabaudi  KDKINSLEDNIQSLNLFINNLKDQNKNNEVIKINNEEQILQLKNSLQNNENCINNLNDNL 2009
P. yoelii yoelii      KDKIHSFEENIQSLNLFINNLKDQHKNNEMIKINNEEQIVELKNNLKNNENCINNLNDNL 646
P. falciparum         KNQILSLEESIKSLNEFINNLKNENEKNELIKINNFEEILKLKNNLQDNESCIQNLNNYL 2466
P. vivax              KNQVSSLKQSIQTQNAFIQSLKKENKKNEVIQVNSIDEIAQLKGDLQTNESSLHSLRNNL 1899
P. reichenowi         KNQILSLEESIKSLNEFINNLKNENEKNELIKINNFEEILKLKNNLQDNESCIQNLNNYL 1467
P. knowlesi           KNEVSSLKQSIQTQNEFIQSLKTKNEKDRVIQVSNMDEIAQLKDNLQTNESSIQCLRTNL 1882
P. gallinaceum        KNQVSCLEQSIESLNKFIDNLKDENNKNELIKVNNLEEILKLKNNIQNNESFIKNLKDNL 588
                       ::: .:::.*:: * **:.** ::::::.:*::.. ::* :**..:: **. :: *.  *

Pca 96                KQKDEMNNSNIKNLIKYKNFIINLVHQTNVFLHIFKTMSTQQVIQNSEYNQLTQLLRKEL 159
P. chabaudi chabaudi  KQKDEMNNSNIKNLIKYKNFIINLVHQTNVFLHIFKTMNTQQVIQNSEYNQLTQLR-KEL 2068
P. yoelii yoelii      KQKDEMNNSNIQNLIKYKSFIINLVYQSNIFFHIFKNINKQKVIQNSIFNQLTQLR-KEL 705
P. falciparum         KKNEELNKINVKNIFKYKGYIIHLIQQSNVFCKIFKHFNENKIIDQSIINKLLYLK-KSF 2525
P. vivax              KNTEQMNKLHEMNLGKCKSFIMHLLQENIIICNIFKMVNSRKRVEASIINQLRGLK-KAF 1958
P. reichenowi         KKNEELNKINVKNIFKYKGYIIHLIQQSNVFCKIFKHFNENKIIDQTIINKLLYLR-KSF 1526
P. knowlesi           KNTEEMNKLHALNLGKYKNFIGHLIQQNIVICDIFKIVNSRRRVEDSILNQLKSLK-KAF 1941
P. gallinaceum        KKNEDINEKNLKNIYKYKSFIVHLIEQSTILCEIFKNISSKKKIDASILNQLSNLR-KSF 647
                      *:.:::*: :  *: * *.:* :*: :. :: .*** .. .: :: :  *:*  *  * :

Pca 96                DPYLNDSIMISELESK--EKSDEANANNDLLNIPSTFSYENYEHIQIFTNKYNLIIERGQ 217
P. chabaudi chabaudi  DPYLNDSIMISELESK--EKSDEANANNDLLNIASTFSYENYEHIQIFTNKYNLIIERGQ 2126
P. yoelii yoelii      DFYLNDSIMISELENK--EKSNLNNFENNLLNIVSTFSCENYEHIQIFTNKFNLIIERGQ 763
P. falciparum         DFYMYDSVIQEIR------------ENKNIIINQDFLTDEYFKHIQTFTKTCNVLIQRG- 2572
P. vivax              HLYMNDSLVEELQRGGGCDDPILAKQEMRWTNGNCNVVEEVLQNVERFAHSFNSMVGEGR 2018
P. reichenowi         DFYMYDSVIQEIR------------ENKNILINQDFLTEEYFKHIQTFTKTCNVLIQRG- 1573
P. knowlesi           HMFMYDTIMDDLQGNVGYDDSTIVKQKGKWTNVNYEIVEEVFQNVQRFANNFNYMIENGK 2001
P. gallinaceum        NYYMYDSVVEELK--------------KKNLNINNEFNEECLQNIKLFAENYNLKIEKGE 693
                      . :: *::: .                          .  *  :::: *:.. *  : .*
```

Figure 3.1. A portion of an alignment of protein sequences and translated nucleotide sequences. Sequences producing significant scores with BLAST analysis with *Pca* 96 and containing the sequence FKL(G/S)SCYLYIINRN(L/M)KEI (highlighted in grey) were aligned with *Pca* 96 using CLUSTALW (http://ebi.ac.uk/clustalw/). This sequence appeared to be highly conserved across *Plasmodium* species. An asterisk (*) indicates that all the amino acid residues are identical in all sequences in the alignment at that position. A colon (:) is an indication that conserved substitutions are present at the position within the sequences in the alignment, and a full stop (.) means that semi-conserved substitutions have been observed at that position within the sequences in the alignment.

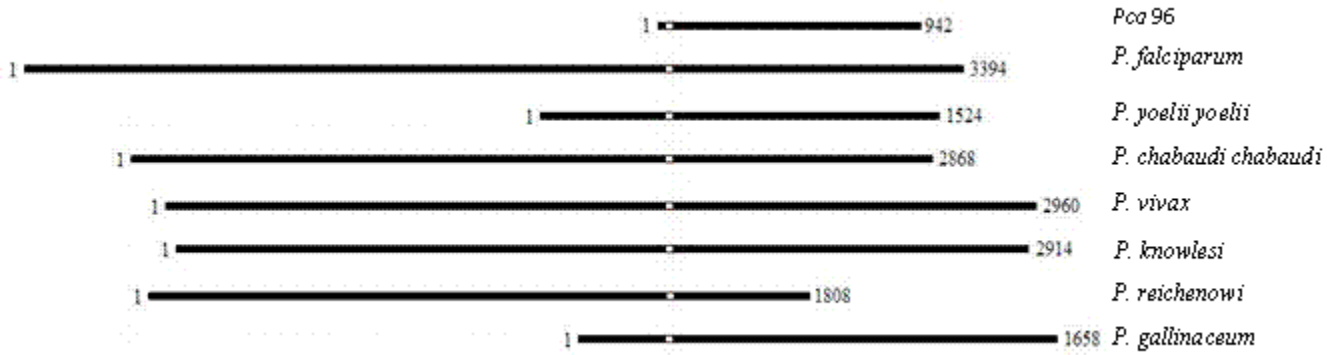


Figure 3.2. Position of *Plasmodium* proteins with high sequence similarity to *Pca* 96 and with the conserved sequence FKL(G/S)SCYLYIINRN(L/M)KEI in relation to one another. The position of the conserved sequence (white) is shown for *Pca* 96, *P. falciparum* PFCO760c, *P. yoelii yoelii* PY05757, *P. chabaudi chabaudi* PCAAS_080770, *P.vivax* PVX_95365, *P. knowlesi* PKH_082410 and the translated DNA sequences of *P. reichenowi* Pr_3502696.c000127860.Contig1 and *P. gallinaceum* Pg_Contig89.

3.3.2 Expression levels

Expression levels were determined for all *P. falciparum* genes by high-density oligonucleotide arrays of RNA from merozoites and intracellular asexual blood stages of malaria parasites (Le Roch *et al*, 2003). The data from Le Roch *et al.* (2003) was used to construct graphs of the mRNA expression levels of PFC0760c, and compare them to graphs for the data for *P. falciparum* lactate dehydrogenase (*Pf*LDH) (PF13_0141). *Pf*LDH is expressed at high levels in all stages of the parasite life cycle, with the highest expression of mRNA observed during the early trophozoite through to the early schizont stages of the parasite life cycle with absolute expression levels ranging from 3800 to 5400 (Figure 3.3, A and B). PFC0760c appears to be expressed at considerably lower levels in comparison to *Pf*LDH (Figure 3.3), with the highest levels ranging from 88 to 175, during the early trophozoite to late schizont stages of the life cycle (Figure 3.3 A and B). Different expression levels are obtained for the two different synchronising methods (Figure 3.3 A and B), but a similar pattern of expression is observed in both. Comparison of mRNA expression levels between the gametocyte and the

sporozoite stages of the life cycle indicates that *Pf*LDH is expressed at a considerably higher level at the sporozoite stage (1266) than at the gametocyte stage (183) (Figure 3.3 C). PFC0760c is expressed at a marginally higher level at the sporozoite stage (60) than at the gametocyte stage (45) (Figure 3.3, C).

The expression data from Le Roch *et al*. (2003) was used to plot graphs of the genes listed in Table 3.3. The proteins encoded by these genes have been identified to be involved in metabolism, cell division, cell growth, DNA synthesis, protein synthesis and transcription (Bowman *et al*., 1999). It was thought that by comparing the patterns of mRNA expression for PFC0760c to the mRNA expression patterns for these genes, inferences could possibly be made about the role of PFC0760c in the parasite, if the expression data of PFCO760c was similar to that of the genes in any of the categories in Table 3.3. Unfortunately, no significant pattern was evident with regards to what stages of the life cycle these genes were expressed (results not shown) and therefore no inferences could be made about PFC0760c from this data.

Table 3.3. *P. falciparum* genes and their putative roles in the *Plasmodium* parasite (Bowman *et al*., 1999)

Role	Genes
Metabolism	PFC0050c (acyl-coA synthase PfACS2)
	PFC0170c (dihydrolipoamide acyltransferase, putative)
	PFC0275w (FAD-dependent glycerol-3-phosphate dehydrogenase, putative)
	PFC0395w (asparagines synthase , putative)
	PFC0710w (inorganic phosphate putative)
	PFC0830w (trophozoite stage antigen)
	PFC0935c (N-(acetylglucosamine-1-phosphate transferase, putative)
	PFC0950c (peptidase, putative)
	PFC0995c (acyl coA: diacylglycerol acyltransferase, putative)
Cell growth, division and DNA synthesis	PFC0305w (EB1 homology, putative)
	PFC0340w (DNA polymerase epsilon subunit B, putative)
	PFC0385c (serine/threonine protein kinase, putative)
	PFC0525c (glycogen synthase kinase 3)
	PFC0595c (serine/threonine protein phosphatase, putative)
	PFC0755c (protein kinase, putative)
	PFC0770c (kinesin-like protein, putative)
	PFC0860w (kinesin, putative)
Transcription and post-transcriptional modification	PFC0155c (DNA-directed RNA polymerase subunit I, putative)
	PFC0805w (DNA-directed RNA polymerase II, putative)
	PFC0825c (cleavage and polyadenylation specificity factor protein, putative)
Protein synthesis	PFC0200w (60S ribosomal protein L44, putative)
	PFC0225c (elongation factor (EF-TS), putative)
	PFC0290w (40S ribosomal protein S23, putative)
	PFC0295c (40S ribosomal protein S12, putative)
	PFC0300c (60S ribosomal protein L7, putative)
	PFC0400w (60S acidic ribosomal protein P2, putative)
	PFC0470w (valine-tRNA ligase, putative)
	PFC0535w (60S ribosomal protein L26, putative)

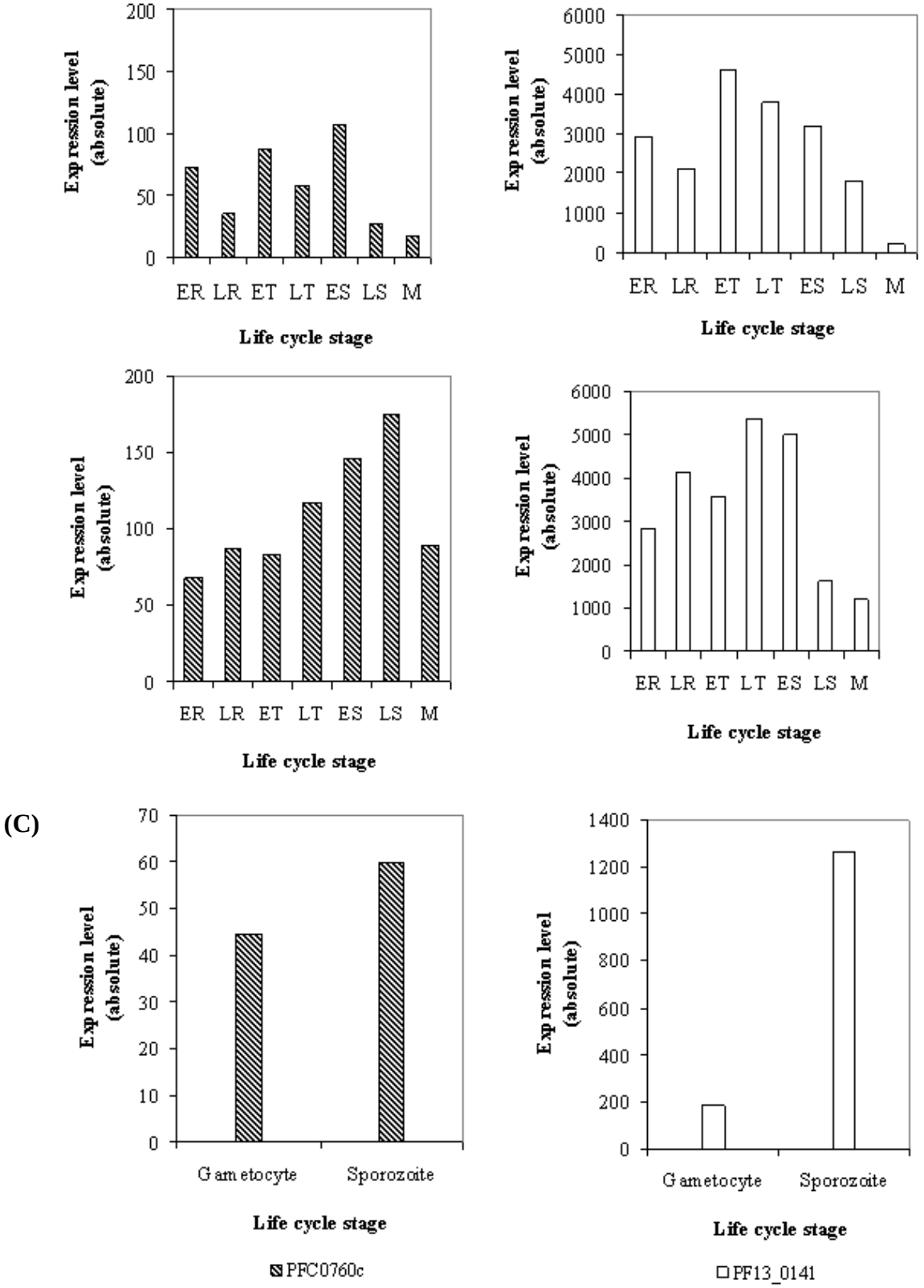


Figure 3.3. Expression profiles of *P. falciparum* proteins. The expression levels of the hypothetical protein PFC0760c (hatched columns) compared to *P. falciparum* lactate dehydrogenase (PF13_0141) (open columns) as a control. The data is from high-density oligonucleotide arrays of RNA from merozoites and intracellular asexual blood stages of parasites: (A) synchronised by means of a 5% D-sorbitol treatment, (B) synchronised by means of a temperature cycling incubator and (C) in the gametocyte and sporozoites stages of the life cycle (Le Roch *et al.,* 2003). ER - early ring stage; LR - late ring stage; ET - early trophozoite stage; LT - late trophozoite stage; ES - early schizont stage; LS - late schizont stage; M - merozoite stage.

3.3.3 *Plasmodium* export element (PEXEL) motif searches and signal sequence predictions

Due to very little being known about PFC0760c and PY05757, it was decided to determine if the proteins were exported from the parasite to the red blood cell. Thus, the amino acid sequences of both proteins were checked for signal sequences and PEXEL motifs.

The motif R/KxLxQ/E was searched for in the amino acid sequences of PFC0760c and PY05757. A number of motifs matching the sequence R/KxLxQ/E were detected for PFC0760c (result not shown). However, all of these motifs were typically 60 amino acids or more downstream from the N-terminus. True PEXEL motifs are accompanied with a signal peptide and tend to be 15-20 amino acids downstream from the hydrophobic signal sequence located at the N-terminus of the protein (Hiller *et al*., 2004).

PY05757 had three possible PEXEL motifs. The first motif KKLTD (amino acids 51-55) was unlikely to be a true PEXEL motif as the second amino acid is charged instead of being non-charged. In addition, the motif is located too far downstream from the N-terminus to be a true PEXEL motif, as are the second, KVLNE (amino acids 69-73) and the third, KYLSE (amino acids 83-87) possible PEXEL motifs. Analysis of the sequences using the web-based programs Sig-pred and Sigfind 2.1 revealed no signal peptides for either of the proteins (result not shown), thus making it unlikely that the proteins were exported. The proteins PFC0760c and PY05757 are hydrophilic at their N-terminus (Chapter 5, Figure 5.1 and 5.2), which supports the findings of the signal peptide programs, as proteins with signal peptides usually have approximately twenty hydrophobic amino acids at the N-terminus.

3.3.4 Transmembrane domain prediction

The program TMHMM 2.0 was used to predict the presence of transmembrane domains in the proteins *Pca* 96, PFC0760c and PY05757. PFC0760c and PY05757 were predicted to not have a transmembrane domain by TMHMM 2.0. A transmembrane domain was predicted on the C-terminus of *Pca* 96 (Figure 3.4). From the TMHMM prediction it appears that the C-terminus of *Pca* 96 is located within a membrane and the N-terminus located outside of the membrane.

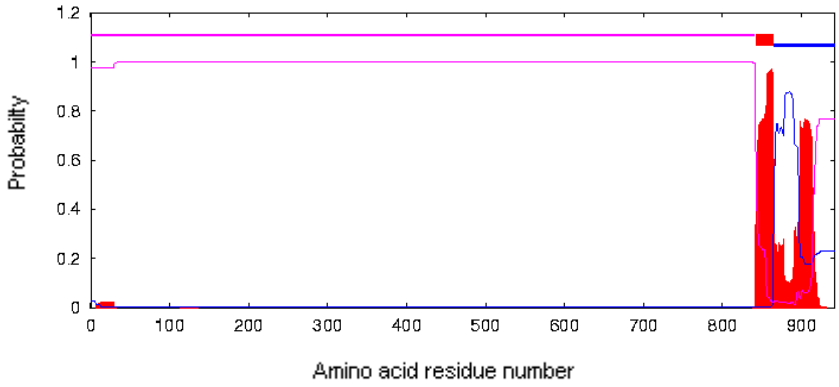


Figure 3.4. Results for the prediction of transmembrane domains in *Pca* 96. The program TMHMM 2.0 (www.cbs.dtu.dk/services/TMHMM/) was used to predict the presence of transmembrane helices for the protein *Pca* 96. Regions predicted to be transmembrane domains are depicted in red, areas predicted to be inside the membrane are indicated in blue and areas predicted to be outside the membrane are depicted in pink.

3.3.5 Subcellular location prediction

The possible subcellular locations of *Plasmodium* proteins were evaluated using pTARGET and SubLoc. *Pca* 96, PFC0760c, PY05757 and PCAS_080770 were all predicted to be cytoplasmic proteins by pTARGET and nuclear proteins by SubLoc (Table 3.4). Both programs had very high confidence levels and reliability indexes for these predictions. The subcellular location was predicted for control proteins lipoamide acyltransferase (PFC0170c), elongation factor TS (PFC0225c) and glycerol-3-phosphate dehydrogenase (PFC0275w), as they have an implied biological function suggesting mitochondrial import (Bowman *et al.*, 1999). pTARGET accurately predicted PFC0170c and PFC0225c as proteins located to the mitochondria and predicted PFC0275w to be located to the golgi. SubLoc predicted PFC0170c to be nuclear and accurately predicted PFC0225c and PFC0275w to be located in the mitochondria. Although SubLoc predicted the location of PFC0170c incorrectly and pTARGET predicted the location of PFC0275w incorrectly according to Bowman *et al.* (1999), the confidence levels, reliability indexes and expected accuracies for these two predictions were lower in comparison to those predicted correctly. This could have given an indication that the prediction was not accurate, if the actual location of the protein had not been known beforehand.

Table 3.4. Subcellular prediction of proteins using pTARGET and SubLoc

	pTARGET prediction		SubLoc prediction		
Protein	Predicted location	Confidence	Predicted location	Reliability index	Expected accuracy
Pca 96	Cytoplasm	81.4%	Nuclear	10	~100%
PFC0760c (conserved protein, function unknown)	Cytoplasm	100.0%	Nuclear	7	95%
PY05757 (hypothetical protein)	Cytoplasm	100.0%	Nuclear	10	~100%
PCAS_080770 (conserved protein, function unknown)	Cytoplasm	100%	Nuclear	6	97%
PFC0170c*(dihydrolipoamide acyl transferase, putative)	Mitochondria	87.6%	Nuclear	1	56%
PFC0225c*(elongation factor (EF-TS), putative)	Mitochondria	100%	Mitochondria	2	74%
PFC0275w*(FAD-dependent glycerol-3-phosphate dehydrogenase, putative)	Golgi	75.1%	Mitochondria	2	74%

*Control: mitochondrial proteins (Bowman *et al.*, 1999)

3.3.6 Conserved domains

CD Search in the CDD (Conserved domains database) was used to determine the presence of predicted conserved domains within the amino acid sequences of *Pca* 96, PFC0760c, PY05757 and PCAS_080770. No conserved domains were detected for *Pca* 96. Conserved domains were detected for PFC0706c, PY05757 and PCAS_080770 (Figure 3.5 and Table 3.5). The predominant domain observed in each of the proteins was a SMC (structural maintenance of chromosomes) protein domain (SMC_prok_A, SMC_prok_B, SMC_N and SMC). A myosin class II heavy chain protein domain (KOG0161) was also identified in PY05757 and PCAS_080770. The C-terminal end of the predicted conserved domains for the amino acid sequences of PY05757 and PCAS_080770 is located approximately 100 amino acid residues downstream of the conserved FKLGSCYLYIINRNLKEI sequence (Figure 3.5, arrows), thus supporting the idea that the sequence is likely to be of biological importance.

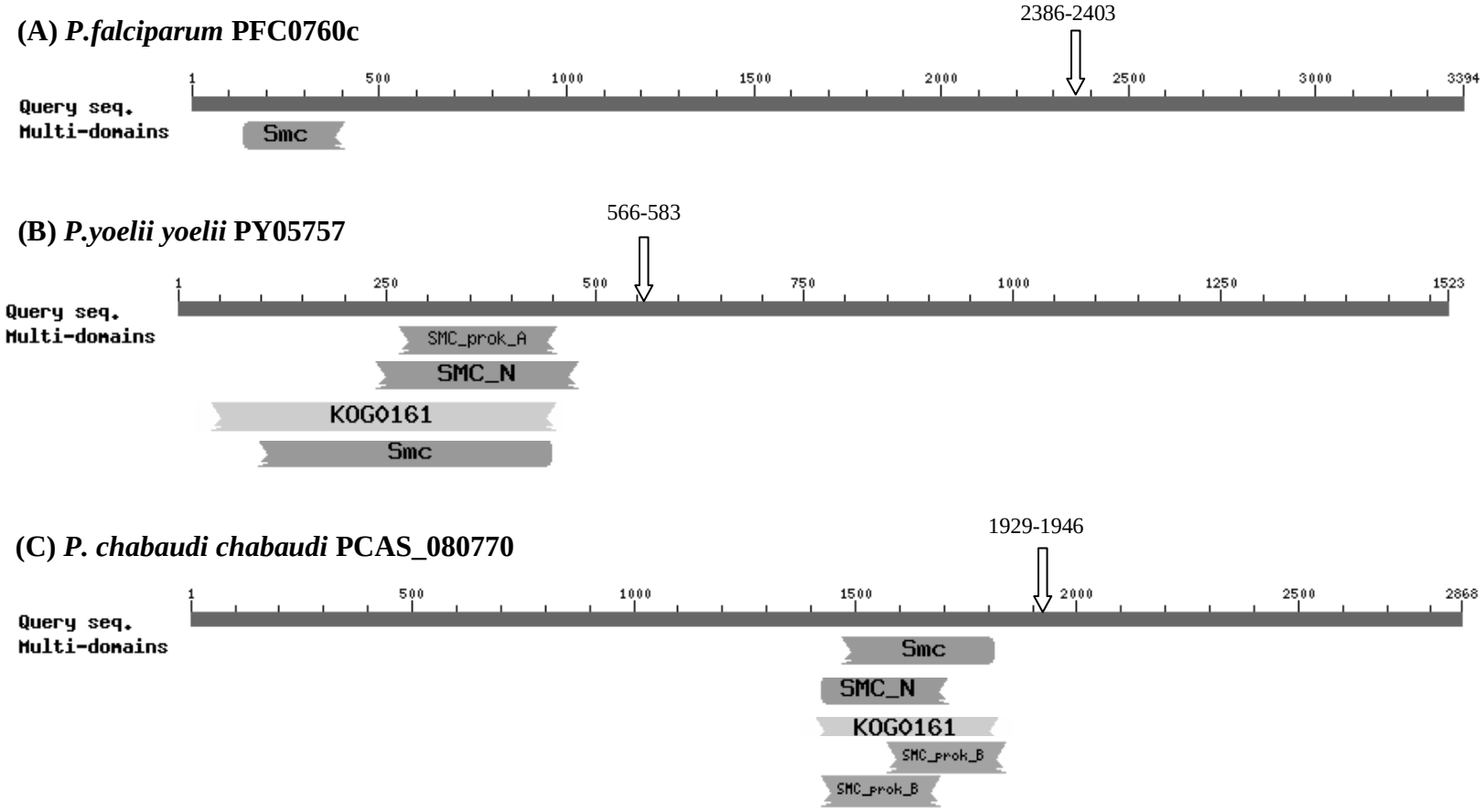


Figure 3.5. The location of conserved domains identified in *.P. falciparum, P. yoelii yoelii* and *P. chabaudi chabaudi* proteins. (A) PFC0760c, (B) PY05757 and (C) PCAS_080770. Variations of the SMC (structural maintenance of chromosomes) protein domain (SMC_prok_A, SMC_prok_B, SMC_N and SMC) were identified using the conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). A myosin class II heavy chain protein domain (KOG0161) was also identified. The location of the conserved sequence is indicated by arrows.

Table 3.5. Results of a conserved domain search in the proteins PFC0760c, PY0575 and PCAS_080770

Protein query	Database	Domain*	PSSM-ID	E-value
PFC0760c	CDD__31608 PSSMs	COG1196: Smc, Chromosome segregation ATPases (Cell division and chromosome partitioning)	31389	$7x10^{-5}$
PY05757	CDD__31608 PSSMs	TIGR02169:, SMC_prok_A, chromosome segregation protein SMC, primarily archaeal type	131224	$5x10^{-5}$
PY05757	Pfam__10340 PSSMs	Pfam02463, SMC_N, RecF/RecN/SMC N terminal domain	111369	$1x10^{-4}$
PY05757	KOG_ _4825	KOG0161, Myosin class II heavy chain (Cytoskeleton)	35383	$2x10^{-9}$
PY05757	COG_ _4873	COG1196, Smc, Chromosome segregation ATPases (Cell division and chromosome partitioning)	31389	0.002
PY05757	TIGR_ _3603	TIGR02169, SMC_prok_A, chromosome segregation protein SMC, primarily archaeal type	131224	$5x10^{-5}$
PCAS_080770	CDD__31608 PSSMs	COG1196 Smc	31389	$3x10^{-8}$
PCAS_080770	Pfam__10340 PSSMs	Pfam 02463,SMC_N, RecF?RecN/SMC N terminal domain	111369	$1x10^{-7}$
PCAS_080770	KOG_ _4825	KOG0161, Myosin class II heavy chain [Cytoskeleton	35383	$3x10^{-13}$
PCAS_080770	COG_ _4873	COG1196, Smc, Chromosome segregation ATPases (Cell division and chromosome partitioning)	31389	$3x10^{-8}$
PCAS_080770	TIGR_ _3603	TIGR02168, SMC_prok_B, chromosome segregation protein SMC, common bacterial type	131223	$9x10^{-7}$
PCAS_080770	TIGR_ _3603	TIGR02168, SMC_prok_B, chromosome segregation protein SMC, common bacterial type	131223	0.001

*None of the domains are assigned to a superfamily

3.3.7. Helical wheel plot of the conserved peptide sequence FKLGSCYLYIINRNLKEI

The results obtained from Pepwheel and Helixator (Figure 3.6) display the same pattern: one side of the helix has predominantly hydrophobic amino acid residues and the other side is composed predominantly of polar and charged amino acids. This is a typical alpha helix composition, thereby suggesting that the conserved peptide sequence FKLGSCYLYIINRNLKEI could have the structure of an alpha helix.

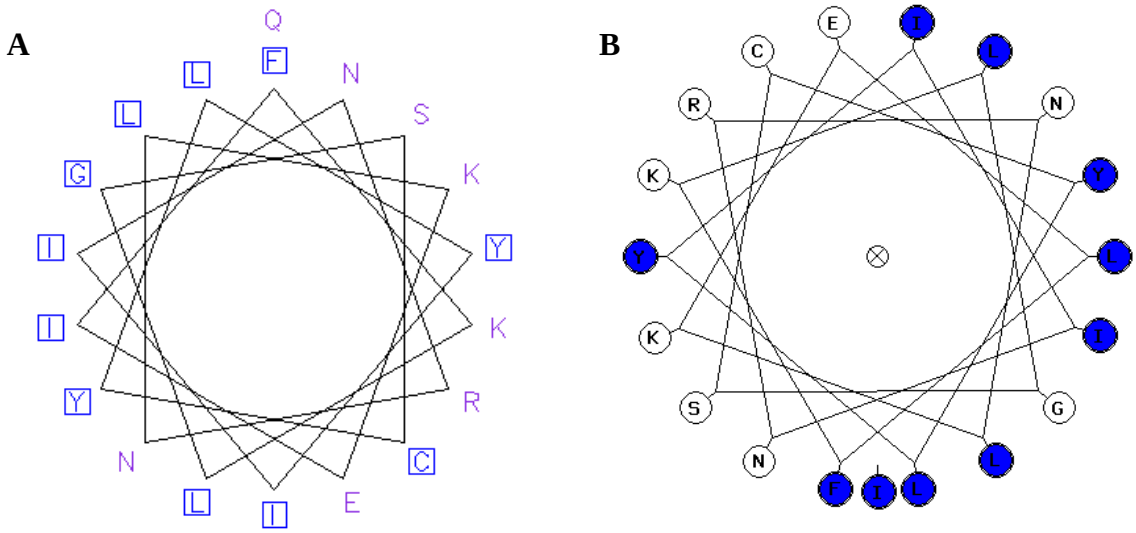


Figure 3.6. Helical wheel plot of conserved peptide sequence. Peptide sequence FKLGSCYLYIINRNLKEI was used with the programs EMBOSS Pepwheel (http://www.tcdb.org/progs/pepwheel.php) (A) and Helixator (http://www.tcdb.org/progs/helical_wheel.php) (B), to predict alpha helices. Hydrophobic amino acids are shown in blue squares in (A) and blue circles in (B).

3.3.8. Structure predictions

A sequence search at the PDB with *Pca* 96 and PFC0760c produced no results as no proteins in the PDB were determined to have sequence identity to either of the proteins. For this reason the sequences were used at the Phyre homology modelling server to determine which known protein folds were possibly present. A number of folds were identified for both protein sequences (Tables 3.6 – 3.8). The following protein folds were identified for both *Pca* 96 and PFC0760c: contractile, transport, signalling, apoptosis, cell adhesion membrane, transmembrane and *de novo* protein folds. An additional four folds were identified for PFC0760c that were not identified in *Pca* 96: triple-helix coiled coil, transcription, cell invasion and beta and beta prime subunits of DNA dependent RNA polymerase.

Table 3.6. Phyre fold prediction results for *Pca* 96

SCOP Code	Percentage identity	E-value	Estimated Precision	Fold/PDB descriptor	Superfamily
c1i84V_ (length:1184)	3%	2.8×10^{-09}	100 %	PDB header: contractile protein	Chain: V: PDB Molecule:smooth muscle myosin heavy chain;
c1dg3A_ (length:592)	4%	4.4×10^{-07}	100 %	PDB header: signaling protein	Chain: A: PDB Molecule:protein (interferon-induced guanylate-binding
c1c1gC_ (length:284).	11%	2.5×10^{-06}	95 %	PDB header: contractile protein	Chain: C: PDB Molecule:tropomyosin;
c2dfsM_ (length:1080)	2%	3.4×10^{-06}	95 %	PDB header: contractile protein/transport protein	Chain: M: PDB Molecule:myosin-5a;
c2r03A_ (length:697)	4%	3×10^{-05}	95 %	PDB header: apoptosis	Chain: A: PDB Molecule: programmed cell death 6-interacting protein;
c2i1kA_ (length:575)	4%	0.00017	95 %	PDB header: cell adhesion, membrane protein	Chain: A: PDB Molecule:moesin;
c1sjjB_ (length:863)	2%	0.00023	95 %	PDB header: contractile protein	Chain: B: PDB Molecule:actinin;
c1ciiA_ (length:602)	4%	0.00024	95 %	PDB header: transmembrane protein	Chain: A: PDB Molecule:colicin ia;
c2fkjC_ (length:366)	7%	0.00055	95 %	PDB header: *de novo* protein	Chain: C: PDB Molecule:outer surface protein a;

Table 3.7. Phyre fold prediction results for amino acid residues 1-1347 of PFC0760c

SCOP Code	Percentage identity	E-value	Estimated Precision	Fold/PDB descriptor	Superfamily
c1i84V_ (length:1184)	5%	7.2 x 10-25	100 %	PDB header: contractile protein	Chain: V: PDB Molecule: smooth muscle myosin heavy chain;
c1ciiA_ (length:602)	6%	2.6 x 10-23	100 %	PDB header: transmembrane protein	Chain: A: PDB Molecule: colicin ia;
c1c1gC_ (length:284)	12%	3.7 x 10-22	100 %	PDB header: contractile protein	Chain: C: PDB Molecule: tropomyosin;
c1sjjB_ (length:863)	5%	1.1 x 10-21	100 %	PDB header: contractile protein	Chain: B: PDB Molecule: actinin;
c2r03A_ (length:697)	4%	2.3 x 10-20	100 %	PDB header: apoptosis	Chain: A: PDB Molecule: programmed cell death 6-interacting protein;
c1dg3A_ (length:592)	5%	8.5 x 10-20	100 %	PDB header: signaling protein	Chain: A: PDB Molecule: protein (interferon-induced guanylate-binding
c2i1kA_ (length:575)	5%	2.2 x 10-19	100 %	PDB header: cell adhesion, membrane protein	Chain: A: PDB Molecule: moesin;
c1hciB_ (length:476)	9%	8.5 x 10-18	100 %	PDB header: triple-helix coiled coil	Chain: B: PDB Molecule: alpha-actinin 2;
c2dfsM_ (length:1080)	3%	1.6 x 10-15	100 %	PDB header: contractile protein/transport protein	Chain: M: PDB Molecule:myosin-5a;
c2oexB_ (length:351)	10%	3.5 x 10-14	100 %	PDB header: protein transport	Chain: B: PDB Molecule: programmed cell death 6-interacting protein;

.

Table 3.8. Phyre fold prediction results for amino acid residues 1347- 3394 of PFC0760c

SCOP Code	Percentage identity	E-value	Estimated Precision	Fold/PDB descriptor	Superfamily
c1i84V_ (length:1184)	3%	1.3×10^{-05}	95 %	PDB header: contractile protein	Chain: V: PDB Molecule: smooth muscle myosin heavy chain;
c2dfsM_ (length:1080)	3%	0.0022	95 %	PDB header: contractile protein/transport protein	Chain: M: PDB Molecule:myosin-5a;
c2fkjC_ (length:366)	7%	0.0029	95 %	PDB header: *de novo* protein	Chain: C: PDB Molecule: outer surface protein a;
c1dg3A_ (length:592)	4%	0.11	90 %	PDB header: signaling protein	Chain: A: PDB Molecule: protein (interferon-induced guanylate-binding
c2r03A_ (length:697)	4%	0.6	80 %	PDB header: apoptosis	Chain: A: PDB Molecule: programmed cell death 6-interacting protein;
d1twfa_ (length:1449)	3%	1	75 %	beta and beta-prime subunits of DNA dependent RNA-polymerase	beta and beta-prime subunits of DNA dependent RNA-polymerase
c1twgA_ (length:1733)	3%	1.3	70 %	PDB header: transcription	Chain: A: PDB Molecule: dna-directed rna polymerase ii largest subunit;
c1c1gC_ (length:284)	6%	1.9	60 %	PDB header: contractile protein	Chain: C: PDB Molecule: tropomyosin;
c1zroA_ (length:602)	7%	1.9	60 %	PDB header: cell invasion	Chain: A: PDB Molecule: erythrocyte binding antigen region ii;

3.3.9 T-cell epitope predictions

Approximately 100 epitopes were predicted for *Pca* 96 (result not shown). Many of these epitopes overlapped in sequence due to the SYFPEITHI program searching for decamers, nonamers or 15-mers within each HLA-type available. Each of these epitopes was searched for in the amino acid sequence of the *P. falciparum* protein PFC0760c. Two of these epitopes, CYLYIINRNL and GSCYLYIINRNLKEI, were found within the PFC0760c sequence (Table 3.9). It is interesting to note that these epitopes are part of the conserved peptide sequence identified (Figure 3.1).

Table 3.9. T-cell epitopes present in *Pca* 96 and PFC0760c

Epitope type	Epitope sequence
H2-Kd	CYLYIINRNL
HLA-DRB1*1101;HLA-DRB1*0401(Dr4Dw4)	GSCYLYIINRNLKEI

3.3.10 Protein interaction

Seven proteins were suggested to have a functional interaction with PFC0760c by the plasmoMAP project at PlasmoDB (Table 3.10). The predicted protein interactions with PFD0855c and MAL7P1.87 are unlikely to occur due to their likelihood scores being 2. Only likelihood scores of 10 or above are deemed to be likely predictions. The remaining five predicted interactions with the proteins PF10_0024, PFF0595c, PF11_0249, PFL2520w and PFE1255w have likelihood scores ranging from 24 to 33, making them likely interactions. It is unfortunate that the majority of these proteins are hypothetical and therefore have no known function, as their functions could give insight into the possible role of PFC0760c. PFF0595c is leucine-rich repeat protein and PFL2520w is a reticulocyte binding-like protein.

Results from PlasmoPredict (Table 3.11) protein-protein interaction revealed a further 7 different genes with unassigned functions that are likely to interact with PFC0760c. The most predominant feature noticed about the proteins predicted to interact with PFC0760c here are that the majority of the proteins are involved in binding, of RNA, DNA, ATP, proteins or chromatin. A second feature that was noticed is that many of the proteins are nucleus or chromatin-bound.

Table 3.10. Proteins likely to interact with PFC0760c (plasmoMAP project at PlasmoDB)

Gene	Genomic Location	Current annotation	Likelihood Scores
PF10_0024	MAL10: 103,119 - 104,684 (-)	Hypothetical protein	33
PFF0595c	MAL6: 512,967 - 519,543 (-)	Leucine-rich repeat protein 5	33
PF11_0249	MAL11: 940,526 - 944,332 (+)	Hypothetical protein	24
PFL2520w	MAL12: 2,136,038 - 2,144,859 (+)	*Plasmodium falciparum* reticulocyte binding-like protein, homolog 3	24
PFE1255w	MAL5: 1,045,458 - 1,051,249 (+)	Hypothetical protein, conserved	24
PFD0855c	MAL4: 787,231 - 790,227 (-)	Hypothetical protein, conserved	2
MAL7P1.87	MAL7: 814,096 - 818,253 (-)	Hypothetical protein, conserved	2

Table 3.11. Proteins likely to interact with PFC0760c (PlasmoPredict)

Gene	Confidence	Current annotation
PFI0975c	0.743548	Conserved protein, unknown function
PFE0385w	0.0523973	Conserved protein, unknown function
PF14_0079	0.477289	Transcription factor, putative. Molecular function: transcription factor activity. Cellular component: nucleus.
PF14_0170	0.0170835	NOT family protein, putative. Cellular component: CCR4-NOT complex
PF13_0315	0.0437991	RNA binding protein, putative. Molecular function: protein binding; nucleic acid binding; RNA binding.
PFE1335c	0.403624	Conserved protein, unknown function
PF13_0073	0.197272	*Plasmodium* exported protein (hyp12), unknown function
PF07_0053	0.823403	Conserved protein, unknown function
PF11780w	0.325308	*Plasmodium* exported protein (PHISTc), unknown function
PF10_0232	0.819349	Chromodomain-helicase-DNA-binding protein 1 homolog, putative. Cellular component: chromatin; nucleus. Molecular function: nucleic acid binding; DNA binding; chromatin binding; helicase activity; ATP binding. Biological process: chromatin assembly or disassembly
PFL0350c	0.620328	Conserved protein, unknown function
PFF0880c	0.061322	Conserved protein, unknown function
PF11_0524	0.725247	Ism4 homologue, putative
MAL13P1.206	0.0658551	Sodium-dependent phosphate transporter. Biological process: phosphate transport. Cellular component: integral to plasma membrane; membrane. Molecular function: inorganic phosphate transmembrane transporter activity.
MAL13P1.310	0.0462875	Calpain. Biological process: proteolysis. Cellular component: intracellular. Molecular function: calcium-dependent cysteine-type endopeptidase activity
PF14_0678	0.602822	Exported protein 2. Cellular compartment: parasitophorous vacuolar membrane

3.4 Discussion

In an attempt to gain some insight into the possible structure and function(s) of PFC0760c, a 'guilt by association' strategy (Tedder, *et al*., 2010) has been employed in the present study, using bioinformatics to attempt to find proteins of known function with similar expression profiles to PFC0760c, sub-cellular location of PFC0760c and predicted protein-protein interactions, among other parameters.

The BLASTP, TBLASTN and CLUSTALW sequence alignments revealed a sequence of amino acids FKLGSCYLYIINRKEI that appeared conserved across *P. falciparum, P. yoelii yoelii, P. chabaudi chabaudi, P. vivax, P. knowlesi, P. gallinaceum* and *P. reichenowi* sequences in addition to *Pca* 96. This sequence being conserved, suggests that the sequence is of importance, whether it be of structural or immunological importance. The BLAST results and alignments also indicated that PFC0760c is likely the *P. falciparum* equivalent of *Pca* 96.

The role of PFC0760c in the parasite life cycle is currently unknown. Discovering the role of PFC0760c within the parasite life cycle would provide valuable information about the protein. For this reason the mRNA expression levels (Le Roch *et al*., 2003) of PFC0760c were examined. It was assumed that the mRNA expression levels from Le Roch *et al*. (2003) would be representative of the corresponding protein expression levels, and could therefore be used to infer the level of protein expression at the different stages of the parasite life cycle, although it is understood that this may not necessarily be the case. PFC0760c mRNA expression levels are significantly lower than that of PF13_0141 (*Pf*LDH), and it was therefore assumed that the protein expression levels for PFC0760c would be significantly lower than the protein expression levels for *Pf*LDH. mRNA expression profiles are not available for *Pca* 96 or PY05757. The low levels of mRNA expression may result in difficulties arising in detecting the native proteins with western blotting or immunofluorescence. Comparing the mRNA levels of PFC0760c to those of *Pf*LDH suggests that PFC0760c is unlikely to be a metabolic protein, as one would expect the mRNA expression levels to be much greater that what is observed for PFC0760c.

PEXEL domains were searched for within PFC0760c and PY05757 to determine if the proteins were exported out of the parasitophorous vacuole. Although PFC0760c and PY05757 do not appear to have true PEXEL domains, there could still be a possibility of

the proteins being exported out of the parasite, as not all exported proteins have a specific motif. *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), has a PEXEL motif, but no signal sequence, yet it is exported to the host erythrocyte (Wiser 2007). A second *P. falciparum* antigen (EXP-1) possessing a signal sequence but no PEXEL motif, is exported from the parasite to the host erythrocyte (Wiser 2007). A Mauer's cleft protein, skeleton-binding protein 1 (SBP1), has no signal sequence or PEXEL motif (Wiser 2007). There appear to be a number of different ways for proteins to be exported out of the parasite without PEXEL domains and signal sequences and therefore no assumptions should be made before wet lab experiments are done to determine for definite whether the proteins are exported out of the parasite or not. The proteins could possibly be fused to green or yellow fluorescent reporter proteins and monitored as Marti *et al*. (2004) did with KAHRP, GBP130, Rifin and *Pf*EMP1 to determine whether the proteins are exported or not.

Although the proteins *Pca* 96, PFC0760c and PY05757 are similar in sequence, only *Pca* 96 was predicted to have a transmembrane domain. This result is confirmed by the fact that the protein was found to be associated with the membranes of red blood cells infected with *P. chabaudi adami* (Wanidworanun *et al*., 1987). It is possible that transmembrane domains do exist for PFC0760c and PY05757 because even though analysis of transmembrane prediction programs showed TMHMM to have the best overall performance, it does have a tendency to under predict (Möller *et al*., 2001).

The proteins *Pca* 96, PFC0760c and PY05757 were found to have structural maintenance of chromosomes (SMC) domains within their amino acid structures. SMC proteins are a family of chromosomal ATPases (Gillespie and Hirano, 2002). The proteins consist of polypeptides of 1000 –1500 amino acid residues. They form dimers consisting of two anti-parallel coiled-coil arms, which are connected via a flexible hinge. An ATP binding domain is located at the end of each of the arms. The dimer forms a functional complex with a specific set of non-SMC subunits. These functional complexes have been associated with forming the core of a cohesion complex that is responsible for chromatid cohesion; forming part of the condensing complex which is vital during mitotic chromosome condensation; DNA repair, metabolism and recombination and organising and separating chromosomes. It is therefore possible that PFC0760c, PCAS_080770 and

PY05757 are involved with chromosome maintenance. It would be necessary to confirm this with experiments and data first before any definite conclusions could be made.

The subcellular location was predicted for the proteins *Pca* 96, PFC0760c, PY05757 and PCAS_080770, in addition to control proteins of known location. One program predicted *Pca* 96, PFC0760c, PY05757 and PCAS_080770 to be located in the nucleus, and the other program predicted them to be located within the cytoplasm. This result emphasises the need to back bioinformatics predictions with experiments to confirm the results. Immunofluorescence confocal microscopy with antibodies targeting the protein could be one method used to confirm the predicted locations of these predictions. One of the scores that pTARGET uses to predict the location of the proteins is based on the absence or presence of Pfam domains. CD Search uses Pfam domains (Marchler-Bauer and Bryant, 2004) and detected domains in PFC0760c, PY05757 and PCAS_080770 involved in chromosome segregation. This could explain perhaps why pTARGET predicted PFC0760c, PY05757 and PCAS_080770 to be nuclear proteins.

Alpha helices, with beta sheets, form the majority of the proteins core structure. The amino acid sequences of protein cores tend to remain consistent, due to space and environmental constrictions. Loop regions are not confined and have no effect on the structures within the protein core. In addition, loop regions tend to have more deletions, substitutions and insertions of amino acid residues (Mount, 2001). If the conserved sequence FKLGSCYLYIINRNLKEI is in fact an alpha helix as suggested by the programs Pepwheel and Helixator, then this may be an explanation of why the sequence appears so highly conserved: if the sequence is an alpha helix, the helix will be in the core of the protein and therefore unlikely to experience changes in amino acid sequence. The sequence KEIQMLKNQILSLEESIKSLNEFINNLKN from PFC0760c, was found to be an alpha helical coiled coil domain (Villard *et al*. 2007). This sequence is located directly after the conserved sequence FKLGSCYLYIINRNLKEI in the amino acid sequence of PFC0760c, and includes the last three amino acid residues of the conserved sequence, thus reinforcing the alpha helix prediction results for the conserved sequence.

Prediction of the structural folds present in PFC0760c and *Pca* 96 revealed multiple folds predicted to be part of the proteins structure. It would be necessary to go to greater in-depth investigation to determine the possible overall structure of the proteins. The most

accurate way to determine the proteins structure would be to subject the native protein to X-ray crystallography or a nuclear magnetic resonance (NMR) study. However, large quantities of pure native protein are required to do this, and obtaining large quantities of purified native protein is not always practical or possible as was the case here.

Prediction of T-cell epitopes within the amino acid sequences of *Pca* 96 and PFC0760c produced an interesting result. The two predicted T-cell epitopes that were present in both the *Pca* 96 and PFC0760c amino acid sequences were both part of the conserved sequence FLKGSCYLYIINRNLKEI. This result supports the suggestion that the conserved sequence is of biological importance.

Protein-protein interactions based on the yeast–two hybrid technology (LaCount *et al*., 2005) can provide very useful insight into protein function (Birkholtz *et al*., 2008) because proteins found in the same sub-cellular location, or involved in the same biological pathways, are often found to interact with one another (Tedder *et al*., 2010). Yeast-two hybrid technology is however not completely fail-safe, as artefacts and limited coverage can cause problems (Tedder *et al*., 2010). PFC0760c was predicted to functionally interact with 23 *P. falciparum* proteins (Table 3.10 and 3.11). Unfortunately, many of the proteins that PFC0760c were predicted to interact with are yet to be assigned a function. This result is not surprising, considering that more than 50% of *P. falciparum* genes have no assigned function and thus remain hypothetical genes (Tedder *et al*., 2010). Hopefully as more genes are assigned functions, the clearer the role of PFC0760c will become. However, there were a number of proteins involved in binding of DNA, RNA, proteins, ATP or chromatin. This result, in conjunction with the conserved domain result strongly suggests that PFC0760c plays a role in chromosome maintenance, involving the binding of nucleic acids.

An additional method for finding out the possible function of PFC0760c, is the location of the PFC0760c gene on chromosome 3 as well as PFC0760c gene neighbours could be investigated as this may give an idea of the genes possible role in the parasite. The *var* genes, encoding *Pf*EMP1 are located at the sub-telomeres on chromosomes, are involved in producing variable antigens on the surface of infected red blood cells and contributing to the parasites pathogenesis. *Stevor* and *rif* genes are also located at the sub-telomeres and also involved in variant antigen expression on infected red blood cells (Cheng *et al*., 1998), thereby suggesting that genes of similar function are associated

together on the chromosomes, and that different regions of the chromosome have regions containing genes responsible for different functions.

In conclusion, the results from this chapter suggest that PFC0760c is a nuclear protein both in terms of its location and possible mode of action.

CHAPTER 4

Recombinant expression of the *Plasmodium falciparum* protein PFC0760C

4.1 Introduction

There are many different systems for recombinant protein expression, including yeast cells (*Pichia. pastoris* and *Saccharomyces cerevisiae*), insect cells (baculovirus), plant cells and bacterial cells (*Escherichia coli*). *E. coli* is possibly the most widely used as the organism grows quickly with inexpensive substrates and there are a wide range of cloning and expression vectors and host strains available (Baneyx, 1999; Flick *et al*., 2004).

Recombinant production of proteins provides an attractive means for obtaining large quantities of a protein for characterisation and vaccine development (Birkholtz *et al*., 2008). However, not all proteins will be expressed at favourable yields and in their correct conformation. A number of factors need to be optimised and considered to obtain high yields of correctly folded, active recombinant protein. This is especially true for recombinant *Plasmodium* proteins. *Plasmodium* genes have a high adenine /thymine content. The *P. falciparum* genome has an AT content of 80.6% (Gardner *et al*., 2002) and the *P. yoelii yoelii* genome, 77.4% AT (Carlton *et al*., 2002). The resulting proteins have large numbers of lysine and arginine residues in addition to many low complexity regions (Flick *et al*., 2004). *Plasmodium* parasites favour different codons for amino acids in comparison to the *E. coli* expression hosts (Zhou *et al*., 2004). These factors, among others result in generally very low levels of expression, truncated proteins or proteins that are predominantly expressed in inclusion bodies.

This chapter describes the expression of an 822 bp region of the gene coding the *P. falciparum* hypothetical protein PFC0760c. The 822 bp region was previously cloned into the expression vector pMAL-c2X (plasmid pTS822) (Smallie, 2003). In the present study, the recombinant protein (*Pf*33-MBP) was expressed and purified and expression of *Pf*33 as a glutathione *S*-transferase (GST) fusion protein (*Pf*33-GST) was performed by sub cloning into the pGEX4T1 vector.

4.2 pMAL-c2X expression vector

The polylinker insertion site in the pMAL-c2X vector is positioned downstream from the *mal*E gene, which is derived from *E. coli* K-12 (di Guan *et al.*, 1988). The *mal*E gene codes for maltose-binding protein (MBP) and therefore the recombinant protein expressed from the pMAL-c2X plasmid is an MBP-fusion protein. The system is under the control of the tac promoter. The vector also carries the *lacI*^{q} gene, coding for the *lac* repressor. In the absence of IPTG, the *lac* repressor keeps expression from the tac promoter low, therefore minimizing expression of the recombinant fusion protein. The signal sequence of the *mal*E gene has been deleted and therefore the expressed fusion protein remains in the cytoplasm.

An advantage of using this system is that purification of the fusion protein can be easily achieved in a non-denaturing, single affinity purification step (di Guan *et al.*, 1988; Maina *et al*., 1988). MBP binds to cross-linked amylose resin with high affinity and can be eluted with 10 mM maltose (di Guan *et al.*, 1988; Maina *et al*., 1988). The pMAL vector includes a sequence that codes for the amino acid sequence Ile-Glu-Gly-Arg, which is a specific cleavage sequence for the enzyme Factor Xa. The sequence is positioned between the *mal*E gene and the polylinker site (Appendix Figure A1), allowing cleavage of the protein of interest from MBP, with a few vector derived amino acid residues remaining attached to the protein of interest.

4.3 Results

4.3.1 Confirmation of the identity of the pMAL-c2X and pTS822 plasmids

To confirm the identity of the pMAL-c2X (control) and pTS822 plasmids before pursuing recombinant expression, each plasmid was digested with *Bam*HI and the digests were analysed on an agarose gel (Figure 4.1 A and B). pMAL-c2X yielded a product of approximately 6400 bp, which is close to the reported size of 6648 bp (New England Biolabs pMAL^{TM} Protein Fusion and Purification System Manual) (Figure 4.1 A, lane 3). *Bam*HI digestion of pTS822 resulted in a band of approximately 6400 bp and one of approximately 870 bp (Figure 4.1 B, arrow lane 3). The band at 870 bp corresponds to the insert DNA, although the expected size is 820 bp. PCR of non-recombinant pMAL-c2X with plasmid-specific primers resulted in a PCR product of approximately 230 bp (Figure

4.1 C, lane 2), which is close to the expected size of 200 bp. A PCR product of approximately 1020 bp was obtained for pTS822, as expected (Figure 4.1 C, lane 3).

The insert of the plasmid pTS822 was sequenced (JV) and compared to the sequence obtained previously by Smallie (2003) (TS) and the PFC0760c sequence using CLUSTALW (Section 3.2.1) (Figure 4.2). The sequences are identical except for one base pair at position 102 of TS sequence (Cytosine instead of Adenine) and 801 of the TS sequence (Thymine instead of Guanine). These differences are possibly due to sequencing anomalies in the TS sequence, as the JV sequence obtained in the present study has 100% sequence identity to the PFC0760c sequence. Therefore the pMAL-c2X and pTS822 plasmids were used for recombinant expression.

4.3.2 Optimisation of expression of the recombinant *P. falciparum* MBP fusion protein (*Pf*33-MBP)

An attempt was made to optimise the expression of the *Pf*33-MBP fusion protein from the pTS822 vector. Expression at two different temperatures (30˚C and 37˚C) was performed (Figure 4.3) for pTS822 and pMAL-c2X as an expression control. No significant difference in expression of MBP or fusion protein appeared evident from observation of the gel (Figure 4.3). This could be due to possible discrepancies with loading concentration, although attempts were made to ensure the same loading concentration for each well. The protein bands at 30 kDa and 40 kDa appear to be reasonably consistent with one another, providing confidence that the loading concentrations are reasonably uniform. However, duplicate cultures should have been used to confirm the consistency of the loading concentration.

The SDS-PAGE gel of *Pf*33-MBP expression also confirmed that the pTS822 plasmid was expressing a fusion protein, as the MBP protein band is not visible in the lanes containing pTS822 induced samples (Figure 4.3).

pTS822 was also induced for different time intervals. The yield of recombinant protein increased slightly with increasing length of induction; however the yields of lower molecular weight *Pf*33-MBP proteins increased. Induction for 1 h (Figure 4.4 B, lane 3) and 3 h (Figure 4.4 B, lane 4) revealed only two bands (79 kDa; 60 kDa) with western blotting using anti-MBP serum. With increasing length of induction (5-11 h), four bands

(79 kDa; 60 kDa; 45 kDa; 37 kDa) were detected. These lower molecular weight proteins still had the MBP tag as the anti-MBP serum detected the protein bands.

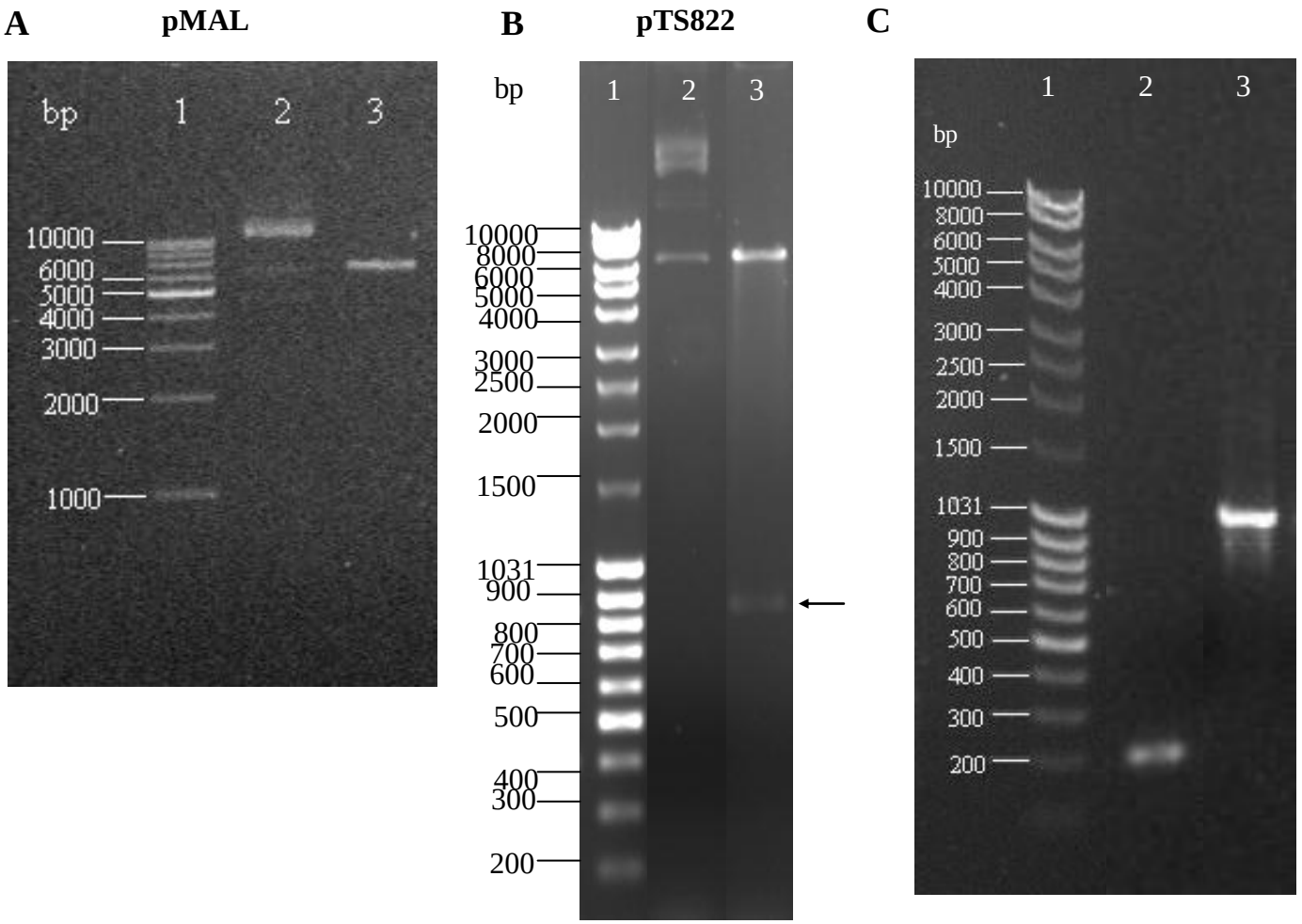


Figure 4.1. Analysis of PCR products and *Bam*HI restriction enzyme digest of the plasmids pMAL-c2X and pTS822. pMAL-c2X (A, lane 3) and pTS822 (B, lane 3) digested with *Bam*HI. Non-digested pMAL–c2X(A, lane 2) and non-digested pTS822 (B, lane 2) controls. Samples were analysed on a 1.2 % (m/v) agarose gel, stained with ethidium bromide. PCR products of the pMAL-c2X (C, lane 2) and pTS822 (C, lane 3) plasmids, analysed on a 1 % (m/v) agarose gel with DNA ladder (lane 1) and stained with ethidium bromide.

```
TS              ----------------------------------------------------TCCAAC 6
JV              ---------------------------------------------------GGATCCAAC 9
PFC0760c        AACAATATAAACAATGTTAACAATATAAACAATGTTAAAAACATTGTAGATATTAACAAC 7080
                                                                      ****

TS              TATCTTGTTAATAATCTTCAATTAAATAAAGACAATGATAATATTATTATTATTAAATTT 66
JV              TATCTTGTTAATAATCTTCAATTAAATAAAGACAATGATAATATTATTATTATTAAATTT 69
PFC0760c        TATCTTGTTAATAATCTTCAATTAAATAAAGACAATGATAATATTATTATTATTAAATTT 7140
                ************************************************************

TS              AATATTTTAAAACTATTCAAATTAGGTTCATGATATTTATATATTATTAATCGTAATTTA 126
JV              AATATTTTAAAACTATTCAAATTAGGTTCATGCTATTTATATATTATTAATCGTAATTTA 129
PFC0760c        AATATTTTAAAACTATTCAAATTAGGTTCATGCTATTTATATATTATTAATCGTAATTTA 7200
                ****************************** ***************************

TS              AAAGAAATCCAAATGTTGAAAAATCAAATCCTTTCCTTAGAAGAAAGCATTAAAAGCTTA 186
JV              AAAGAAATCCAAATGTTGAAAAATCAAATCCTTTCCTTAGAAGAAAGCATTAAAAGCTTA 189
PFC0760c        AAAGAAATCCAAATGTTGAAAAATCAAATCCTTTCCTTAGAAGAAAGCATTAAAAGCTTA 7260
                ************************************************************

TS              AATGAATTCATTAATAATCTAAAAAACGAAAATGAAAAAAATGAATTAATTAAAATAAAT 246
JV              AATGAATTCATTAATAATCTAAAAAACGAAAATGAAAAAAATGAATTAATTAAAATAAAT 249
PFC0760c        AATGAATTCATTAATAATCTAAAAAACGAAAATGAAAAAAATGAATTAATTAAAATAAAT 7320
                ************************************************************

TS              AATTTTGAAGAAATACTCAAATTAAAAAATAATCTACAAGATAATGAAAGTTGTATACAA 306
JV              AATTTTGAAGAAATACTCAAATTAAAAAATAATCTACAAGATAATGAAAGTTGTATACAA 309
PFC0760c        AATTTTGAAGAAATACTCAAATTAAAAAATAATCTACAAGATAATGAAAGTTGTATACAA 7380
                ************************************************************

TS              AACTTAAATAATTATTTAAAAAAAAAATGAAGAATTAAATAAAATTAATGTAAAAAATATT 366
JV              AACTTAAATAATTATTTAAAAAAAAAATGAAGAATTAAATAAAATTAATGTAAAAAATATT 369
PFC0760c        AACTTAAATAATTATTTAAAAAAAAAATGAAGAATTAAATAAAATTAATGTAAAAAATATT 7440
                ************************************************************

TS              TTCAAATATAAAGGATATATAATTCATTTAATACAACAAAGTAATGTCTTTTGTAAAATT 426
JV              TTCAAATATAAAGGATATATAATTCATTTAATACAACAAAGTAATGTCTTTTGTAAAATT 429
PFC0760c        TTCAAATATAAAGGATATATAATTCATTTAATACAACAAAGTAATGTCTTTTGTAAAATT 7500
                ************************************************************

TS              TTTAAACATTTTAATGAAAATAAAATTATTGATCAAAGTATTATAAACAAATTACTTTAT 486
JV              TTTAAACATTTTAATGAAAATAAAATTATTGATCAAAGTATTATAAACAAATTACTTTAT 489
PFC0760c        TTTAAACATTTTAATGAAAATAAAATTATTGATCAAAGTATTATAAACAAATTACTTTAT 7560
                ************************************************************

TS              TTAAAAAAATCCTTTGATTTTTATATGTATGATTCGGTTATACAAGAAATAAGAGAAAAT 546
JV              TTAAAAAAATCCTTTGATTTTTATATGTATGATTCGGTTATACAAGAAATAAGAGAAAAT 549
PFC0760c        TTAAAAAAATCCTTTGATTTTTATATGTATGATTCGGTTATACAAGAAATAAGAGAAAAT 7620
                ************************************************************

TS              AAAAATATAATAATAAATCAAGATTTTTTAACAGATGAATATTTTAAACATATACAAACC 606
JV              AAAAATATAATAATAAATCAAGATTTTTTAACAGATGAATATTTTAAACATATACAAACC 609
PFC0760c        AAAAATATAATAATAAATCAAGATTTTTTAACAGATGAATATTTTAAACATATACAAACC 7680
                ************************************************************

TS              TTTACCAAAACATGTAATGTATTAATTCAAAGGGGATATCTCAGCATCTTAAAAGATACA 666
JV              TTTACCAAAACATGTAATGTATTAATTCAAAGGGGATATCTCAGCATCTTAAAAGATACA 669
PFC0760c        TTTACCAAAACATGTAATGTATTAATTCAAAGGGGATATCTCAGCATCTTAAAAGATACA 7740
                ************************************************************

TS              AACAATGATTTCTTTATACAAAACAAACAAAGTAATCAACAAGGAAATCAAAATGGTAAC 726
JV              AACAATGATTTCTTTATACAAAACAAACAAAGTAATCAACAAGGAAATCAAAATGGTAAC 729
PFC0760c        AACAATGATTTCTTTATACAAAACAAACAAAGTAATCAACAAGGAAATCAAAATGGTAAC 7800
                ************************************************************

TS              CATATAAATATGTGTAACATATATCCAGATGATGAAATCAATGTAACTGCTGATCAACAA 786
JV              CATATAAATATGTGTAACATATATCCAGATGATGAAATCAATGTAACTGCTGATCAACAA 789
PFC0760c        CATATAAATATGTGTAACATATATCCAGATGATGAAATCAATGTAACTGCTGATCAACAA 7860
                ************************************************************

TS              ATTTTTGATGGGACGGAAAACGTACCACATCGGA-------------------------- 820
JV              ATTTTTGATGGTACGGAAAACGT------------------------------------ 812
PFC0760c        ATTTTTGATGGTACGGAAAACGTACAACAGTCCTTACAAAATGAGGAAGATTATGTAAAT 7920
                *********** ***********
```

Figure 4.2. Alignment of the sequence compiled from the forward and reverse sequencing results of the pTS822 DNA insert (JV) with PFC0760c and the sequence obtained by Smallie (2003) (TS). Base pairs that are different from the reference sequence are highlighted in gray at positions 102 and 801.

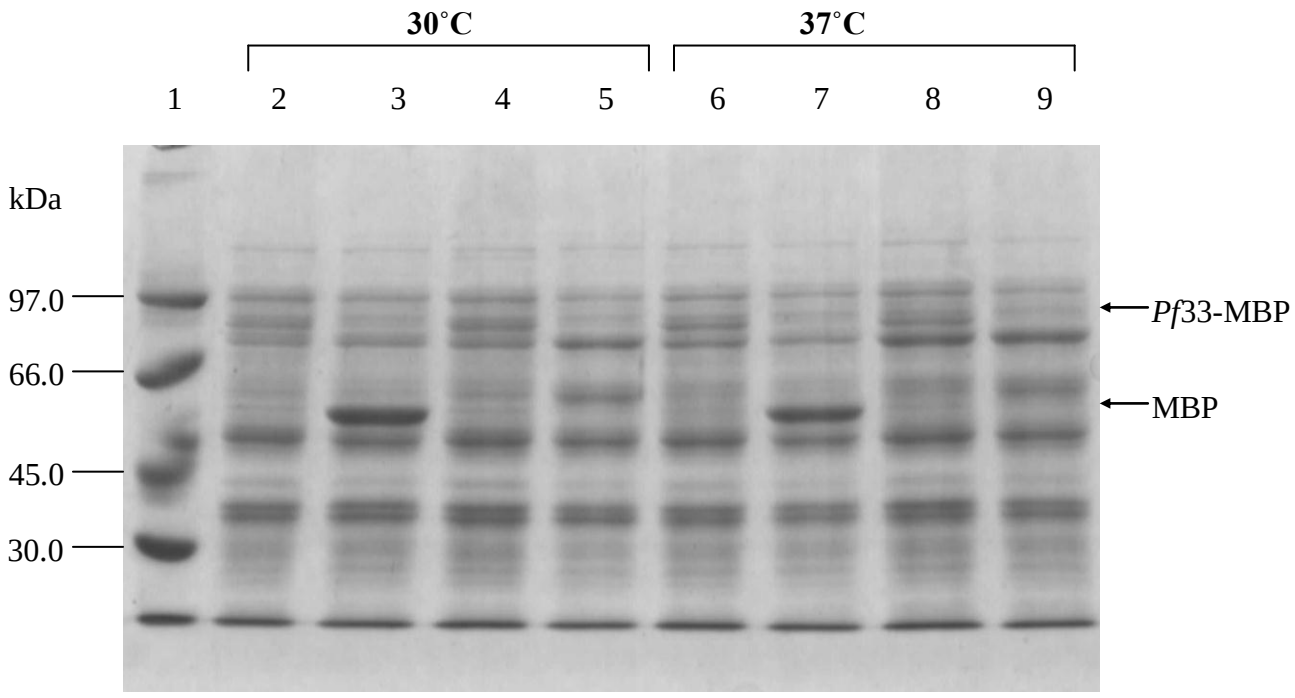


Figure 4.3. Comparison of *Pf*33-MBP and MBP expression at different temperatures. Expression of *Pf*33-MBP and MBP was conducted at 30˚C (lanes 2-5) and 37˚C (lanes 6-9). Lane 1, molecular weight marker; lane 2, pMAL uninduced; lane 3, pMAL induced; lane 4, pTS822 uninduced; lane 5, pTS822 induced; lane 6, pMAL uninduced; lane 7, pMAL induced; lane 8, pTS822 uninduced; lane 9, pTS822 induced.

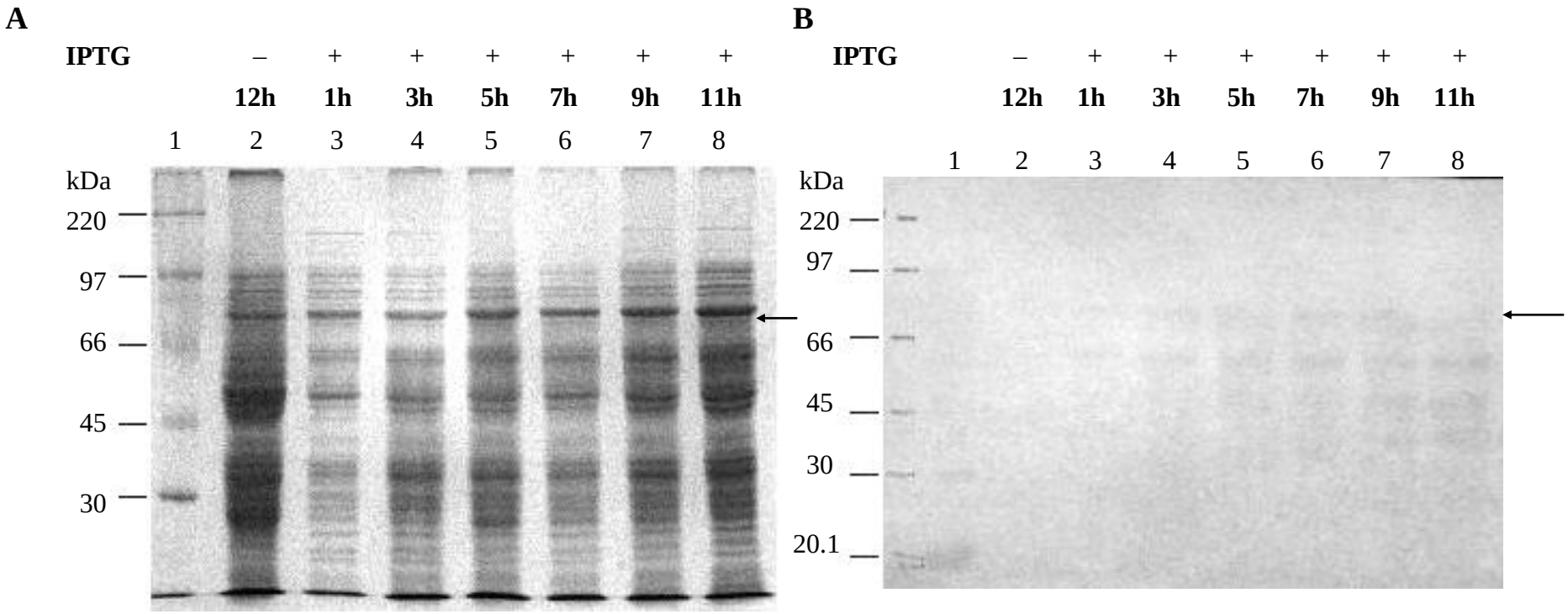


Figure 4.4. Analysis of *Pf*33-MBP expression samples from pTS822 induced for different time intervals. (A) 10% SDS-PAGE gel, (B) Western blot analysis of the MBP-fusion protein *Pf*33-MBP with rabbit anti-MBP serum (New England Biolabs). Lane 1, Amersham High-Range Rainbow Molecular Weight Marker; lane 2, uninduced pTS822 lysate (12h); lanes 3-8, pTS822 lysates induced with IPTG for different time intervals of 1h, 3h, 5h, 7h, 9h, and 11h respectively. The arrow in (A) shows the position of the 79 kDa band of the fusion protein that is not easily visible on the gel.

To rule out the possibility of protease degradation of the protein during lysis of the host cells, protease inhibitors were always used. However, an ‘in house’ preparation of individually prepared protease inhibitors was routinely used. It was thought possible that some of the inhibitors may not be fully active in the preparation. It was thus decided to compare the banding pattern on SDS-PAGE of cells lysed in the presence of the commercially prepared protease inhibitor cocktail to the ‘in house’ protease inhibitor preparation routinely prepared in the laboratory (Figure 4.5) No difference in banding pattern was observed between the use of the two different inhibitor cocktails. Four protein bands recognised by anti-MBP antibodies (approximately 79 kDa, 60 kDa, 45 kDa and 37 kDa) were observed for both samples (Figure 4.5, arrows lanes 5 and 7).

A low level of *Pf*33-MBP expression was observed from pTS822 and thus the plasmids pKK223-3-*Pf*LDH (expressing *P. falciparum* lactate dehydrogenase) and pKK223-3-*Pv*LDH (expressing *P. vivax* lactate dehydrogenase) were used as additional expression controls (Figure 4.6). A protein band of approximately 34 kDa, corresponding to LDH was detected by anti-LDH IgY (Figure 4.6 B). The same protein bands are clearly visible in the SDS-PAGE analysis (Figure 4.6 A), indicating successful expression of both *Pf*LDH and *Pv*LDH.

A number of factors are thought to cause low yields and difficulties with expression: percentage AT base pair composition (Baca and Hol, 2000; Matambo *et al.*, 2004), molecular weight and pI (Mehlin *et al*, 2006; Vedadi *et al*., 2007), and protein disorder (Linding *et al*., 2003). The theoretical molecular weight and pI, base pair composition and disorder of the proteins were therefore investigated.

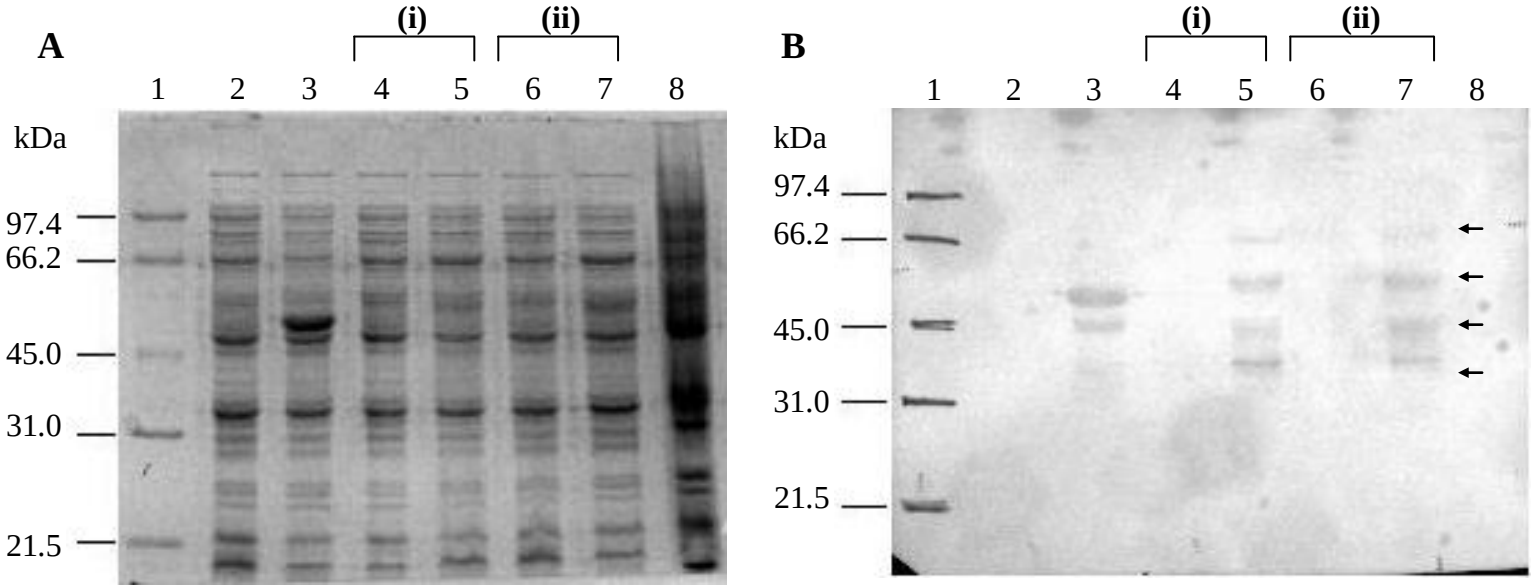


Figure 4.5. Comparison of protein expression when different protease inhibitors are used during isolation of *Pf*33-MBP from pTS822. (A) 10 % (m/v) acrylamide gel, (B) western blot analysis with rabbit anti-MBP serum. *E. coli* cells containing the pTS822 plasmid expressing recombinant *Pf*33-MBP were lysed open in the presence of a protease inhibitor cocktail consisting of individual inhibitor stocks prepared in the lab (i) (lanes 4 and 5) and in the presence of a protease inhibitor cocktail from Sigma (ii) (lanes 6 and 7). Lane 1, Bio-Rad low molecular weight marker; lane 2, uninduced pMAL-c2X lysate; lane 3, IPTG-induced pMAL-c2X lysate expressing recombinant MBP; lanes 4 and 6, uninduced lysates from expression of recombinant *Pf*33-MBP; lanes 5 and 7, IPTG-induced lysates from expression of recombinant *Pf*33-MBP; lane 8, *E. coli* lysate (with no plasmid).

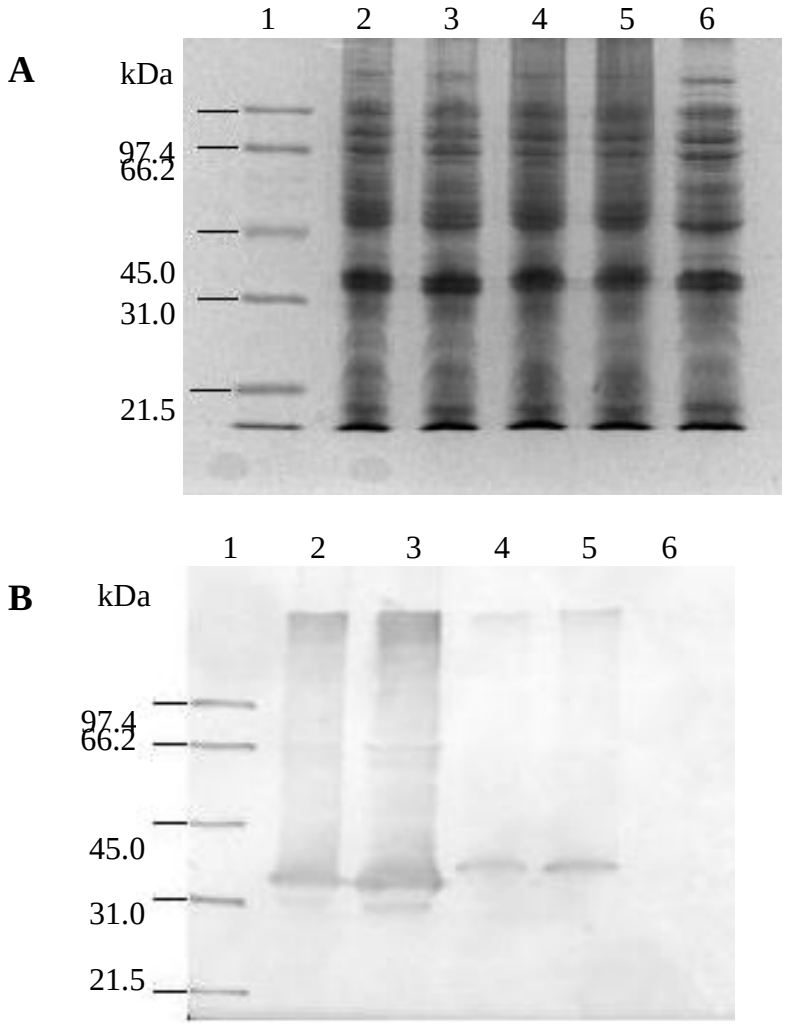


Figure 4.6. *P. falciparum* lactate dehydrogenase (*Pf*LDH) and *P.vivax* lactate dehydrogenase (*Pv*LDH) expression lysates. Lysates were analysed by SDS-PAGE (10% acrylamide) (A) and western blotting with anti-LDH IgY at 1 µg/ml (B). Lane 1, Bio-Rad low molecular weight marker; lane 2, uninduced *Pf*LDH; lane 3, IPTG-induced *Pf*LDH; lane 4, uninduced *Pv*LDH; lane 5, IPTG-induced *Pv*LDH; lane 6, lysate of turbo competent *E. coli* host cells (New England Biolabs).

4.3.3 Theoretical molecular weight and pI of proteins

Mehlin *et al.* (2006) examined if the molecular weight and pI of a protein could hamper the soluble recombinant expression of that protein. Mehlin *et al*. (2006) found that proteins having a pI above 9.5 would be unlikely to be solubly expressed. Only 0.4% of all the *Plasmodium* proteins with a pI above 9.5 tested by Mehlin *et al.* (2006) were solubly expressed. In addition, only 1.6% of proteins with a molecular weight over 56 kDa were solubly expressed.

Therefore the theoretical molecular weights and pI for each protein in the present study were determined from their amino acid sequences (www.expasy.org) (Table 4.1). All of the proteins investigated (Table 4.1) had a pI below 9.5 with the highest pI being recorded at 7.63 for *Pv*LDH, followed by a pI of 7.11 for *Pf*33. Recombinantly expressed *Pf*33-MBP was determined to have a theoretical molecular mass of 79 kDa and MBP expressed alone was determined to have a molecular mass of 54 kDa.

4.3.4 Base pair composition of *Plasmodium* proteins

The base pair composition of *Plasmodium* proteins and recombinant proteins was determined using the sequence utilities at the DNA Learning Centre (DNALC) (Table 4.2) (http://www.dnalc.org/bioinformatics/dnalc_nucleotide_analyzer.htm). The base pair composition for *P. falciparum* lactate dehydrogenase (*Pf*LDH) and *P. vivax* lactate dehydrogenase (*Pv*LDH) were analysed as controls. All DNA sequences coding for the *Plasmodium* proteins of interest were calculated to be AT rich, with percentages of A and T base pairs as high as 79% for PFC0760c. The least AT rich *Plasmodium* gene examined was that for Pv116630 (*Pv*LDH) with an AT base pair composition of 54% (Table 4.2). The base pair compositions of *Pf*33-MBP and MBP were determined to be 62% and 48% respectively.

Table 4.1. Theoretical molecular weight and pI of *Plasmodium* proteins and the MBP-fusion (*Pf*33-MBP) and MBP and *Pf*33 after Factor Xa cleavage.

Protein	Theoretical molecular weight (kDa)	Theoretical pI
*Pf*33-MBP	79.07	5.59
*Pf*33 (cleaved from *Pf*33-MBP)	33.28	7.11
MBP (cleaved from *Pf*33-MBP)	45.8	5.09
MBP (pMAL-c2X expressed)*	54.33	5.28
PF13_0141 (*Pf*LDH)*	34.108	7.63
Pv116630 (*Pv*LDH)*	32.915	6.67
PFC0760c	402.956	4.43

*Expression control proteins

Table 4.2. Base pair composition and sizes of the genes encoding *Plasmodium* proteins

Name	Size (bp)	AT composition
*Pf*33-MBP	1999	62.28%
*Pf*33	826	80.39%
MBP*	1386	48.34%
PF13_0141 (*Pf*LDH)*	951	66.98%
Pv116630 (*Pv*LDH)*	912	54.06%
Pca 96	3288	70.53%
PFC0760c	10185	79.03%
PY05757	4575	75.69%

* Expression control proteins

4.3.5 Protein disorder prediction

Proteins can be ordered or disordered. Ordered proteins will experience small, thermally driven movements around the atoms making up the backbone of the protein. In contrast, intrinsically disordered proteins will experience more dynamic movements of the atoms and position of the protein backbone (Vucetic *et al*., 2005). Proteins intrinsically disordered at their N or C-termini, or even in regions within the protein tend to lead to difficulties in protein expression, purification and crystallization (Linding *et al*., 2003). It was for this reason that the PFC0760c and the *Pf*33-MBP fusion protein sequences were analysed for protein disorder probability using the VSL2 predictor of intrinsically disordered regions at Disprot (www.disprot.org/predictors.php). The prediction data was processed into a graphical format (Figure 4.7). MBP and *P. falciparum* LDH (*Pf*LDH) were

included as controls (Figure 4.7 B and D) as recombinant expression of *Pf*LDH (Turgut-Balik *et al*., 2001) and MBP (present study, Figure 4.3, lane 3) has been successful.

Proteins are considered disordered if they have one or more disordered regions (Vucetic *et al*., 2005). A disordered region is considered so if its probability prediction value is above 0.5 (Peng *et al*., 2006) (red line, Figure 4.7). Multiple regions were predicted disordered for protein PFC0760c (Figure 4.7 C). In contrast, only four very short regions of *P. falciparum* LDH were predicted to have a disorder probability above 0.5, resulting in the majority of the protein predicted to be ordered (Figure 4.7 D). Large sections of the fusion protein *Pf*33-MBP (Figure 4.7 A) were predicted disordered, as was MBP (Figure 4.7 B). From these results, protein disorder prediction does not appear to be a reliable prediction of difficulty in protein expression in the present study.

4.3.6 Purification of *Pf*33-MBP

Lysates from pTS882 and pMAL-c2X expression were passed over separate amylose columns to purify *Pf*33-MBP and MBP, respectively. *Pf*33-MBP and MBP were eluted from the amylose with 10 mM maltose in 20 mM tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4. The absorbance at 280 nm of the collected fractions was determined (Figure 4.8).

*Pf*33-MBP eluted after 6-13 ml of 10 mM maltose (Figure 4.8 A). The average yield of *Pf*33-MBP obtained from amylose purification was 3 mg. This consisted of the full length *Pf*33-MBP (79 kDa band), in addition to the lower molecular weight *Pf*33-MBP proteins that also bound to the amylose column. SDS-PAGE analysis of amylose purified *Pf*33-MBP (Figure 4.9, lane 2), revealed three major bands (approximately 79 kDa, 60 kDa and 45 kDa) and one minor band (approximately 33 kDa), in addition to a couple of minor bands below 21.5 kDa and above 97.4 kDa. The protein bands at 79 kDa, 60 kDa, 45 kDa and 33 kDa are the same protein bands detected by the anti MBP antibodies in Figures 4.4 and 4.5. MBP eluted in four fractions after 2-3 ml of 10 mM maltose had passed through the column (Figure 4.8 B). Maximum absorbance values were obtained for fractions four and five. SDS-PAGE analysis of the fractions revealed the MBP to be considerably pure (result not shown).

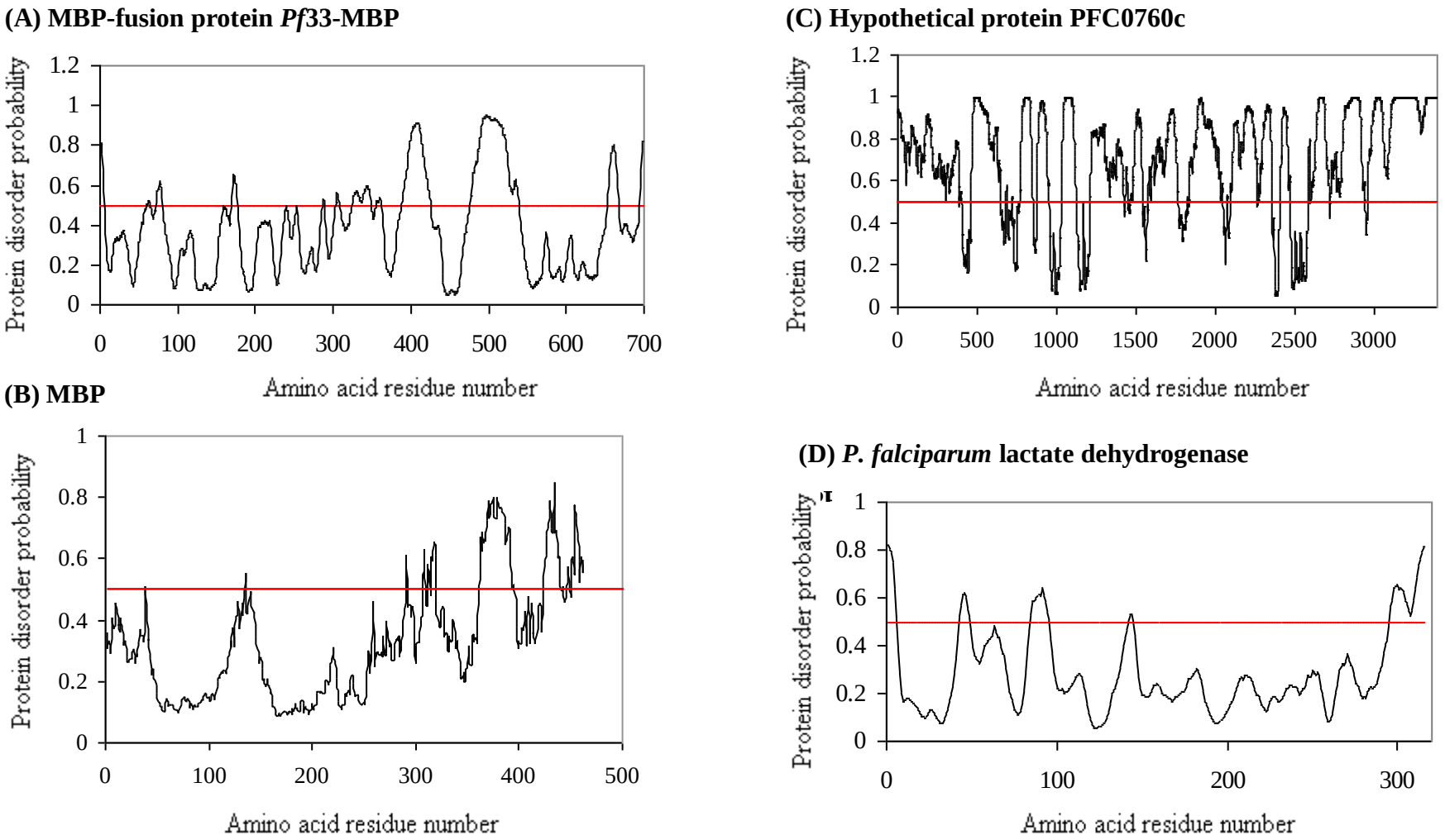


Figure 4.7. Protein disorder predictions for *Plasmodium* proteins. Protein disorder predictions were performed for maltose-binding (MBP) fusion protein *Pf*33-MBP (A), MBP (control) (B), *P. falciparum* hypothetical protein PFC0760c (C) and *P. falciparum* lactate dehydrogenase (PF13_0141) (control) (D), using the VSL2 predictor of intrinsically disordered regions at Disprot (www.disprot.org/predictors.php) (Vucetic *et al.*, 2005). Proteins are considered disordered at probabilities over 0.5 (red line).

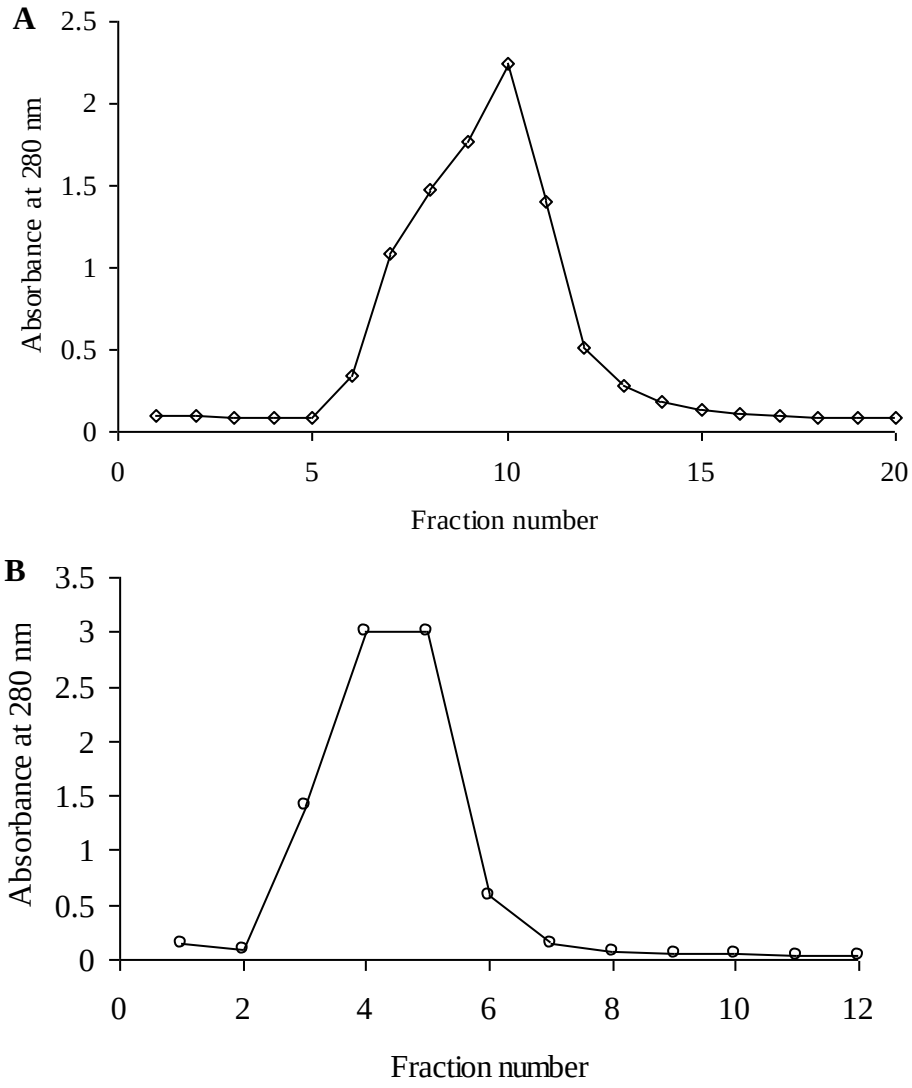


Figure 4.8. Amylose purification of *Pf*33-MBP and MBP. Lysate from pTS822 expression (A) and pMAL-c2X expression (B) was passed over an amylose column. The *Pf*33-MBP and MBP proteins were eluted with 10 mM maltose in 20 mM tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4. Fractions (1 ml) were collected and the absorbance at 280 nm determined.

4.3.7 Factor Xa cleavage of *Pf*33-MBP

To separate *Pf*33 from MBP, amylose-purified *Pf*33-MBP was incubated with Factor Xa at different concentrations ranging from 10 µg/ml to 0.05 µg/ml, at room temperature and at 4˚C. The samples were left to cleave for 46 h and were immediately boiled in the presence of reducing SDS-PAGE buffer and analysed on an SDS-PAGE gel (Figure 4.9). *Pf*33 was almost completely cleaved from MBP with 10 µg/ml Factor Xa, incubated at room temperature for 46 h. Protein bands of approximately 79 kDa, 63 kDa, 43 kDa and 34 kDa were observed (Figure 4.9 A, lane 3). The protein bands at 79 kDa, 63 kDa and 34 kDa appear as minor bands, having low intensity. With increasing Factor Xa concentration, these bands become virtually invisible on the gel, whereas the prominent protein band at 43 kDa, becomes more intense.

The protein band at 34 kDa is close to the expected size of cleaved *Pf*33 (33 kDa). However, this band appears to be too low in intensity to represent *Pf*33 The dominant protein band observed at 43 kDa is close to the expected size of cleaved MBP (45kDa). As there is only one dominant band after complete cleavage, it was assumed that *Pf*33 and MBP are possibly the same size after Factor Xa cleavage, and thus appear together as one dominant band at 43 kDa. Western blotting analysis and probing with 2 different antibodies such as an anti-peptide antibody raised against MBP and an anti-peptide antibody raised against *Pf*33, could have answered this.

4.3.8 Diethylaminoethyl (DEAE)-Sepharose^{TM} ion exchange chromatography to separate *Pf*33 from MBP after Factor Xa cleavage

The cleaved *Pf*33-MBP sample was passed over DEAE Sepharose to separate *Pf*33 from MBP. Proteins with a net negative charge at the pH being used become bound to the positively charged resin. At pH 8.0, both *Pf*33 and MBP are predicted to have a net negative charge, as the pH of the buffer is above their pI of 7.11 and 5.09, respectively. Thus they would both bind to the resin and be eluted with a NaCl gradient. *Pf*33 is likely to elute at a lower NaCl concentration than MBP.

The absorbance of the fractions collected was monitored at 280 nm for the presence of protein (Figure 4.10 A). Four absorbance peaks were observed. The first peak would have

been excess *Pf*33-MBP cleavage mixture. The second peak was observed after the NaCl gradient was started. The protein eluted from the column at a NaCl concentration of approximately 100 mM. This should correspond to *Pf*33 as it was expected to elute first due to a lower nett negative charge. The second peak of protein was observed to elute at a concentration of approximately 370 mM NaCl, which is expected to be MBP. The last absorbance peak was observed at a NaCl concentration of approximately 400 mM. This peak could be due to small amounts of un-cleaved *Pf*33-MBP. This peak could also be due to Factor Xa as it elutes at a NaCl concentration in the region of 400 mM (New England Biolabs pMAL^{TM} Protein Fusion and Purification System Manual). Cleaved MBP is reported to elute as a sharp peak at 100-150 mM NaCl (New England Biolabs pMAL^{TM} Protein Fusion and Purification System Manual), however this doesn't appear to be the case here.

MBP expressed from non-recombinant pMAL-c2X was passed over the DEAE Sepharose column as a control (Figure 4.10 B). MBP eluted at a NaCl concentration of approximately 275 mM. MBP expressed from pMAL-c2X has a different pI (5.28) and molecular mass (54 kDa), in comparison to cleaved-off MBP (pI/of 5.09; molecular mass of 45.8) from *Pf*33-MBP and would therefore be expected to elute at different NaCl concentrations. MBP expressed from pMAL-c2X would be expected to elute at a lower salt concentration in comparison to cleaved-off MBP and therefore MBP expressed from pMAL-c2X was not an adequate control.

The eluted fractions (filled diamonds, Figure 4.10 A) from the DEAE purification of cleaved *Pf*33-MBP was analysed on an SDS-PAGE gel (Figure 4.11) to determine which fractions contained *Pf*33 and which contained MBP. Protein bands of approximately 43 kDa (Figure 4.11 A lanes 5-8 and lane 13; B lanes 9-10), 45 kDa and 50 kDa (Figure 4.11 lane 11) were observed. The protein bands of 43 kDa correspond to the same molecular mass observed for the predominant band in the cleaved *Pf*33-MBP sample (Figure 4.9, A, lane 3). Unfortunately antibodies were not used to confirm the identity of the proteins.

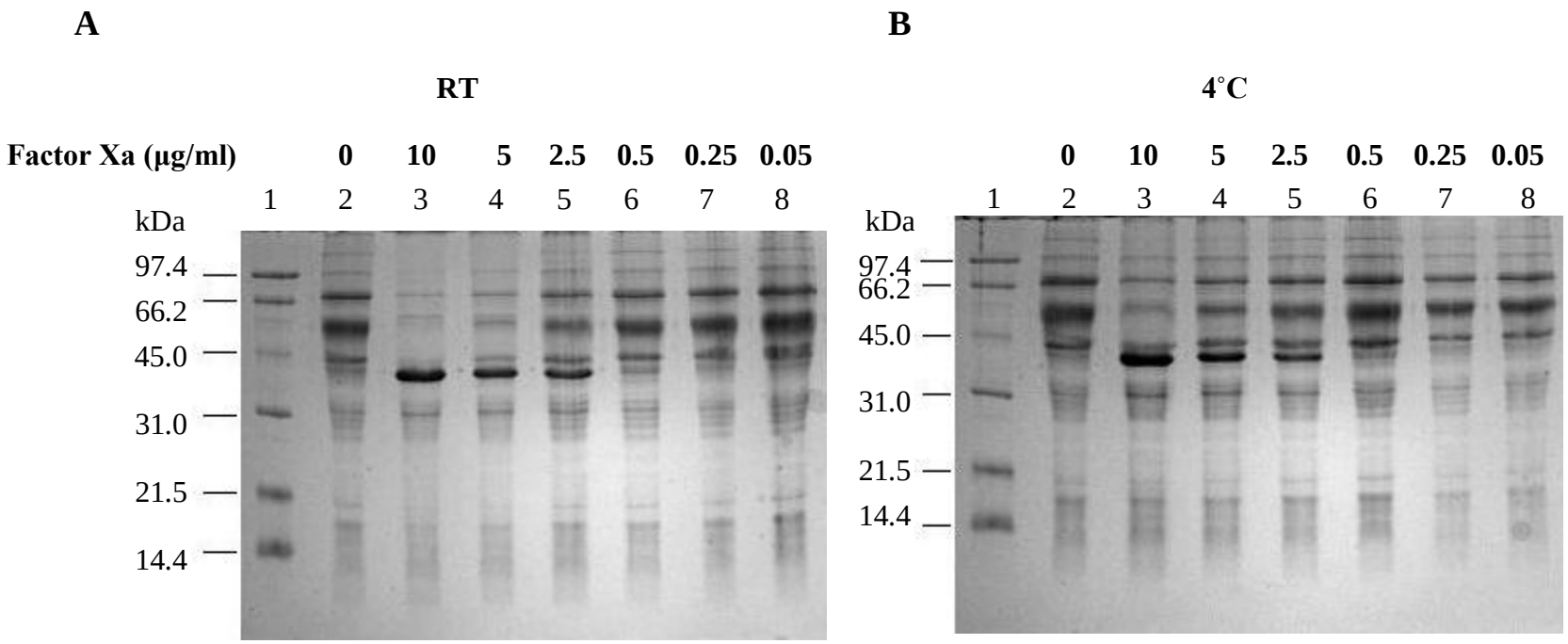


Figure 4.9. Analysis of protease cleavage of amylose-purified *Pf*33-MBP with Factor Xa on a 12.5% acrylamide gel. (A) Samples from incubation with factor Xa at room temperature for 46 h, (B) Samples from incubation with factor Xa at 4˚C for 46 h. Factor Xa was used at a final concentration of 10 µg/ml (lane 3), 5 µg/ml (lane 4), 2.5 µg/ml (lane 5), 500 ng/ml (lane 6), 250 ng/ml (lane 7) and 50 ng/ml (lane 8). *Pf*33-MBP incubated with buffer, as a non-cleaved control (lane 2) and a protein molecular weight marker (lane 1) were included.

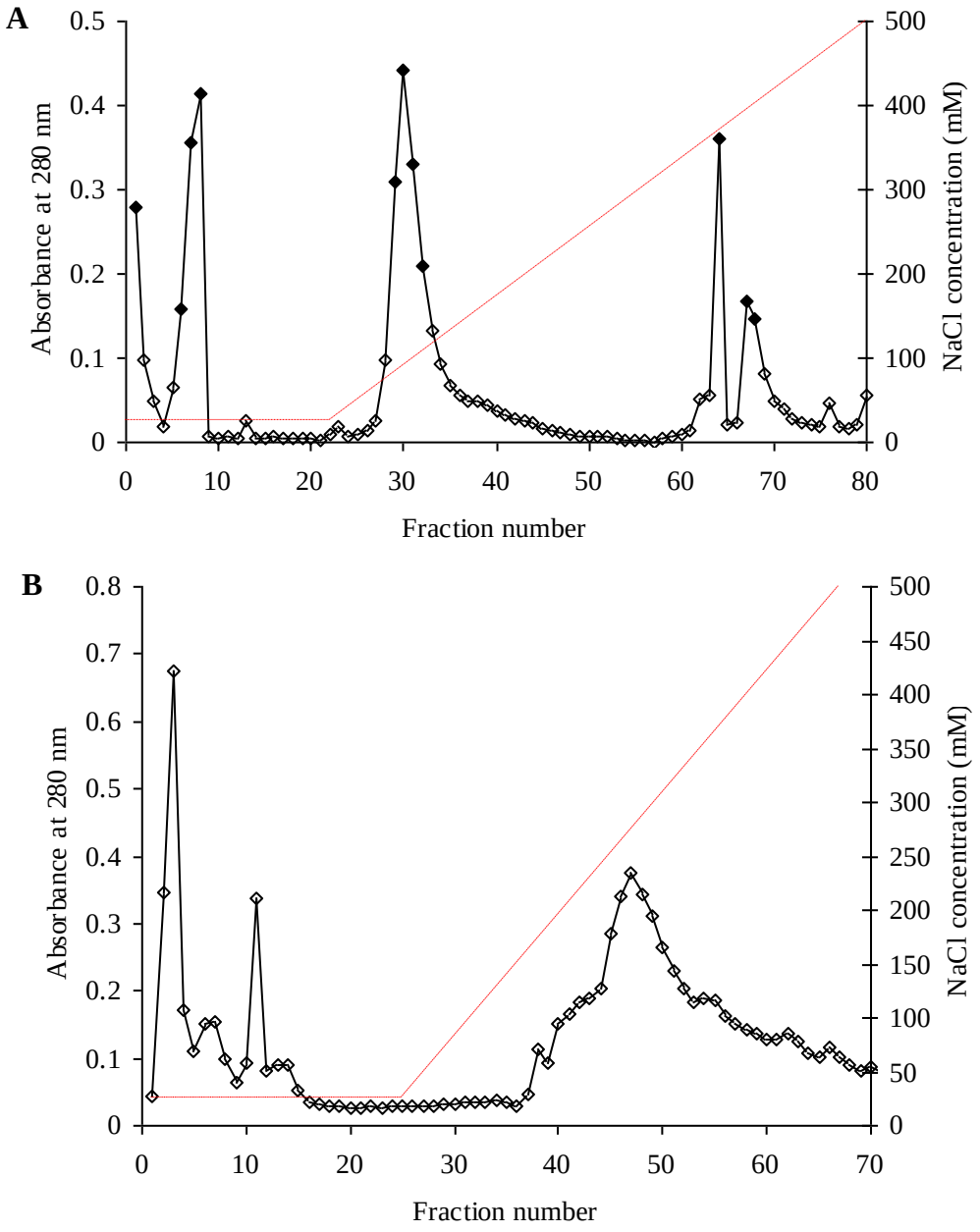


Figure 4.10. DEAE Sepharose purification of cleaved *Pf*33 and MBP. Factor Xa-treated *Pf*33-MBP (A) was separated with ion exchange chromatography. Amylose-purified MBP expressed from pMAL-c2X was used as a control (B). The absorbance of each fraction (1 ml) was read at 280 nm. A NaCl gradient of 25 mM – 500 mM NaCl was used (dashed line) to elute bound protein. Filled diamonds (♦) indicate the fractions analysed by SDS-PAGE (Figure 4.11).

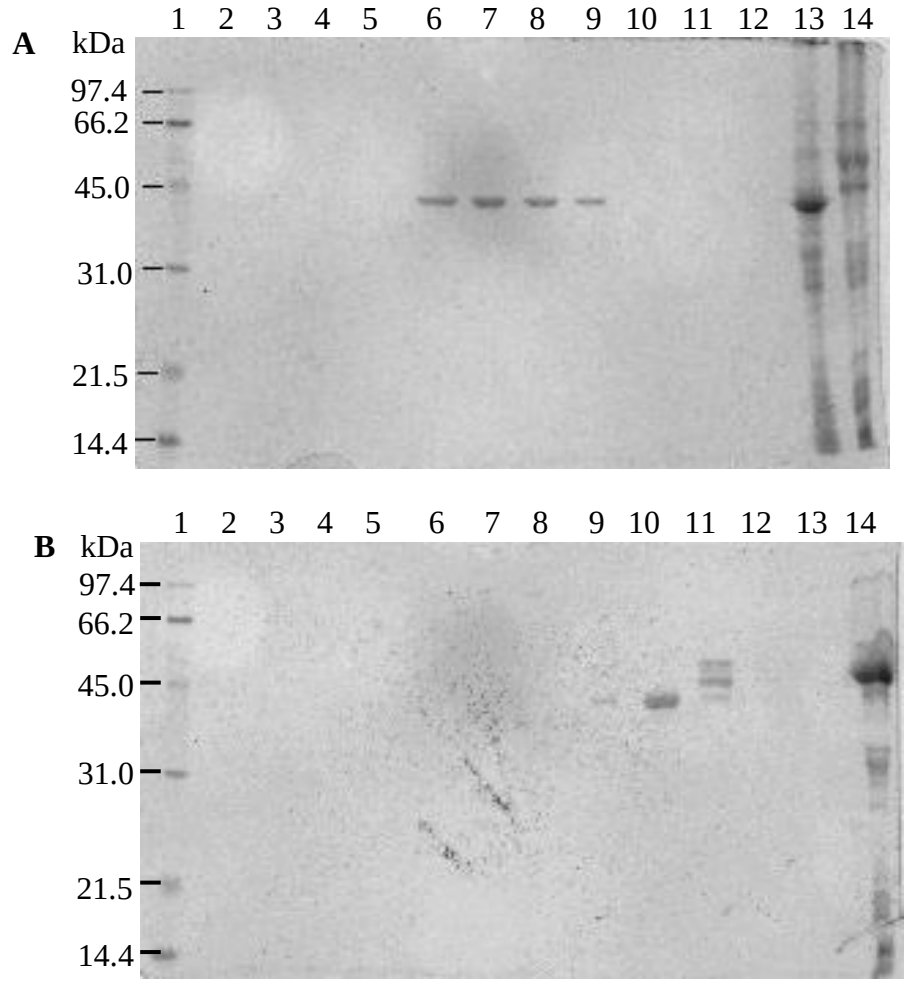


Figure 4.11. Analysis of fractions obtained from DEAE Sepharose purification of cleaved *Pf*33-MBP. (A) Fractions from DEAE Sepharose purification of cleaved *Pf*33-MBP. Lane 1, Bio-Rad low molecular weight marker; lane 2 fraction 1; lane 3, fraction 6; lane 4, fraction 7; lane 5, fraction 8; lane 6, fraction 29; lane 7, fraction 30; lane 8, fraction 31; lane 9, fraction 32; lane 10, fraction 64; lane 11, fraction 67; lane 12, fraction 68; lane 13, amylose-purified factor Xa-cleaved *Pf*33-MBP; lane 14, amylose-purified, non-cleaved *Pf*33-MBP. (B) Fractions from DEAE Sepharose purification of cleaved *Pf*33-MBP. Lane 1, Bio-Rad low molecular weight marker; lane 2 fraction 5; lane 3, pooled fractions 14-26; lane 4, fraction 36; lane 5, fraction 41; lane 6, fraction 42; lane 7, fraction 43; lane 8, fraction 45; lane 9, fraction 53; lane 10, fraction 54; lane 11, fraction 59; lane 12, fraction 64; lane 13, fraction 67; lane 14, amylose-purified MBP. Fractions were run on a 12.5% SDS-PAGE gel and silver-stained.

4.3.9 'On-column' Factor Xa cleavage of *Pf*33-MBP

'On-column' cleavage of *Pf*33-MBP was attempted as an alternative method for separating MBP and *Pf*33. *Pf*33-MBP was cycled over an amylose resin column, to allow binding of the MBP fusion to amylose. The *Pf*33-MBP bound to the amylose resin was then incubated with Factor Xa to cleave off *Pf*33, leaving MBP bound to the amylose. The MBP was later eluted with 10 mM maltose. The absorbances of the fractions collected were determined at 280 nm to determine the presence of protein (Figure 4.12). An SDS-PAGE gel and western blot of the 'on-column' cleavage fractions was run (Figure 4.13). The bound MBP fraction (Figure 4.13, lane 8) has a banding pattern almost identical to the cleaved off *Pf*33 (Figure 4.13, lane 7), except that there is more of the 43 kDa band in lane 7 than in lane 8. However, when probed with anti-MBP antiserum (Figure 4.13 B) the banding pattern is identical, suggesting that all of the 3 bands observed in each lane are MBP or have MBP as part of their sequence.

It was thought possible that *Pf*33 was being recognised by anti-MBP antibodies as perhaps MBP and *Pf*33 had some similar epitopes. To ensure that there were no linear epitopes, an alignment of their amino acid sequences was done using the ClutalW program (Section 3.2.1). No sequence similarity exists between the two sequences (Result not shown) and therefore the anti-MBP antibodies are detecting MBP in Figure 4.13 B. The possibility also existed that there was perhaps a second Factor Xa recognition site that perhaps resulted in some of the MBP still being attached to *Pf*33. Factor Xa cleaves at the sequence IEGR and sometimes IDGR. Only one IEGR was observed in the MBP-fusion amino acid sequence, at a position corresponding to the plasmid derived Factor Xa site (result not shown).

A further possibility to explain the results could be that after cleavage, some of the MBP protein was not tightly bound and therefore eluted with *Pf*33 before elution with maltose.

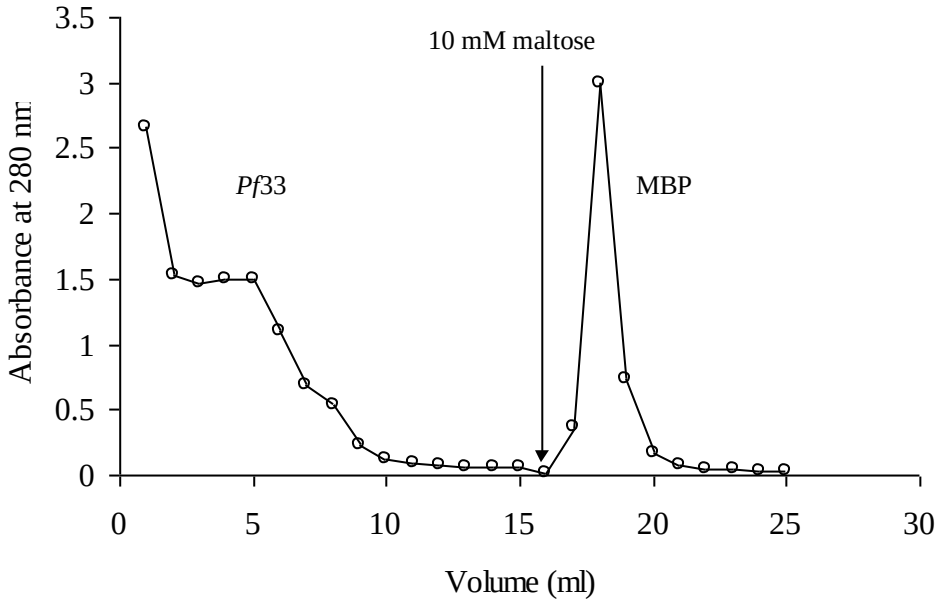


Figure 4.12. Absorbance readings of fractions collected from 'on-column' cleavage of *Pf*33-MBP. Fractions (1 ml) were read at 280 nm.

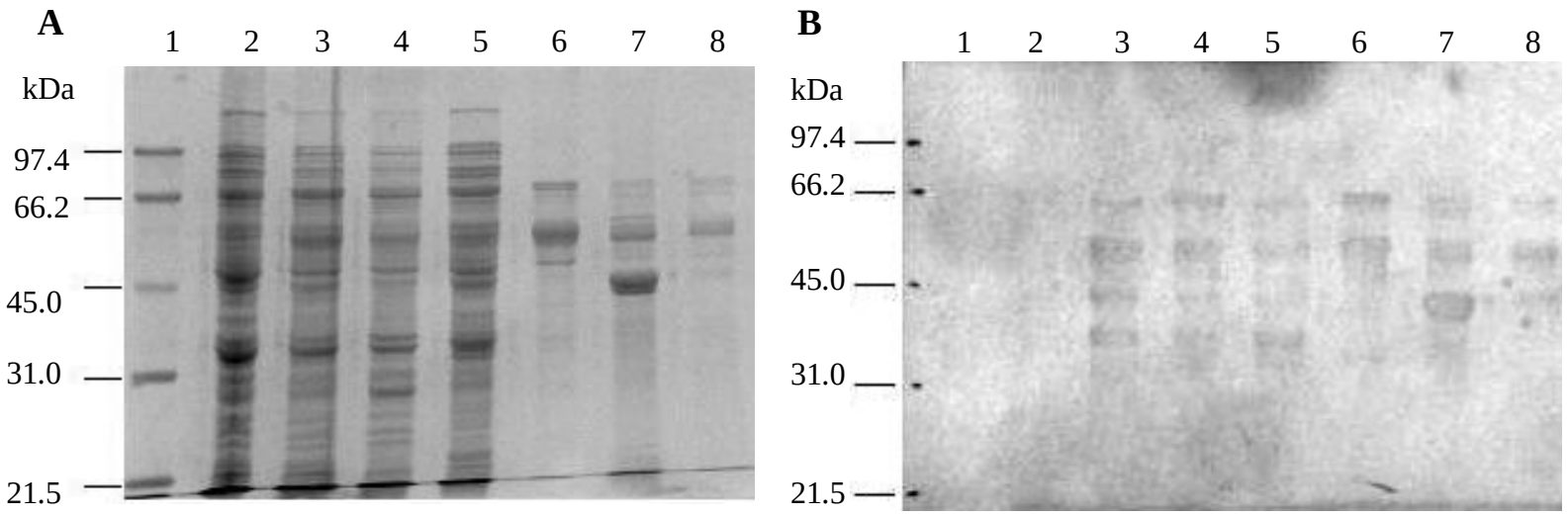


Figure 4.13. Separation of *Pf*33 from MBP using 'On-column' cleavage. (A) 10 % (m/v) acrylamide gel, (B) western blot analysis with rabbit anti-MBP serum. Lane1, Bio-Rad low molecular weight marker; lane 2, uninduced pTS822 lysate; lane 3, IPTG-induced pTS822 lysate; lane 4, pellet from centrifugation of sonicated, IPTG-induced pTS822 lysate; lane 5, supernatant from centrifugation of sonicated, IPTG-induced pTS822 lysate; lane 6, *Pf*33-MBP purified from IPTG-induced pTS822 supernatant on amylose resin; lane 7, product from 'on-column' cleavage of *Pf*33-MBP with Factor Xa; lane 8, bound fraction from 'on-column' cleavage of *Pf*33-MBP, eluted with 10 mM maltose.

4.4 Expression of *Pf*33 as a glutathione *S*-transferase fusion protein

4.4.1 Sub-cloning of the *P. falciparum* insert into pGEX4T1

It was decided to attempt expression of the recombinant *P. falciparum* protein in a second vector, pGEX4T1, to determine if better yields of *Pf*33 could be obtained.

The plasmid pGEX4T1 has a polylinker region downstream from the gene expressing a 26 kDa glutathione *S*-transferase (GST) protein from *Schistosoma japonicum* (Smith and Johnson, 1988). When a gene of interest is cloned into the polylinker, the resulting expressed protein is a GST fusion, with GST at the N-terminus of the protein, and the protein of interest at the C-terminus. GST fusions can be affinity purified on a glutathione agarose column. Elution of the fusion protein is under non-denaturing conditions, using reduced glutathione (Smith and Johnson, 1988). The GST can be removed by cleavage with thrombin, as a thrombin cleavage site is positioned between the gene expressing GST and the polylinker (Appendix Figure A2).

The *P. falciparum* DNA insert was excised from pTS822 with *Bam*HI (Figure 4.1 B, arrow) and ligated into *Bam*HI-digested pGEX4T1. Originally the insert was to be excised by double digestion with *Eco*RI and *Sal*I, to ensure that the insert was ligated into the vector in the correct orientation. However, restriction enzyme site analysis of the insert DNA with restriction enzyme mapper software, revealed an *Eco*RI site within the insert sequence (Figure 4.14). Digesting with *Xmn*I and *Sal*I was not an option either, as the pGEX4T1 vector does not possess an *Xmn*I site in the poylinker region (Appendix Figure A2). The insert could be amplified with primers having suitable restriction enzyme sites, but it was decided to first attempt cloning with the insert restricted with *Bam*HI.

pGEX4T1 was linearised with *Bam*HI and analysed by agarose gel electrophoresis (Figure 4.15). The expected size of 4 900 bp for pGEX4T1 was observed (Figure 4.15). The insert from pTS822 (Figure 4.1 B) was ligated into linear pGEX4T1 and transformed into *E. coli* cells. Transformed colonies were screened using colony PCR. The PCR products were analysed on an agarose gel (Figure 4.16 A and B). Five of the fifteen colonies screened had a PCR product of approximately 1 000 bp, indicating the presence of the DNA insert. The remaining ten colonies had a PCR product of approximately 200 bp,

which matched the size obtained for the non-recombinant pGEX4T1 control that was PCR amplified (Figure 4.16 A and B, lane 3).

GAGGATTTCA**GAATTC**GGATCCAACTATCTTGTTAATAATCTTCAATTAAATAAAGA
CAATGATAATATTATTATTATTAAATTTAATATTTTAAAACTATTCAAATTAGGTTC
ATGATATTTATATATTATTAATCGTAATTTAAAAGAAATCCAAATGTTGAAAAATCA
AATCCTTTCCTTAGAAGAAAGCATTAAAAGCTTAAAT**GAATTC**ATTAATAATCTAAA
AAACGAAAATGAAAAAAATGAATTAATTAAAATAAATAATTTTGAAGAAATACTCAA
ATTAAAAAATAATCTACAAGATAATGAAAGTTGTATACAAAACTTAAATAATTATTT
AAAAAAAAATGAAGAATTAAATAAAATTAATGTAAAAAATATTTTCAAATATAAAGG
ATATATAATTCATTTAATACAACAAAGTAATGTCTTTTGTAAAATTTTTAAACATTT
TAATGAAAATAAAATTATTGATCAAAGTATTATAAACAAATTACTTTATTTAAAAAA
ATCCTTTGATTTTTATATGTATGATTCGGTTATACAAGAAATAAGAGAAAATAAAAA
TATAATAATAAATCAAGATTTTTTAACAGATGAATATTTTAAACATATACAAACCTT
TACCAAAACATGTAATGTATTAATTCAAAGGGGATATCTCAGCATCTTAAAAGATAC
AAACAATGATTTCTTTATACAAAACAAACAAAGTAATCAACAAGGAAATCAAAATGG
TAACCATATAAATATGTGTAACATATATCCAGATGATGAAATCAATGTAACTGCTGA
TCAACAAATTTTTGATGGGACGGAAAACGTACCACATC**GGATCCTCTAGAGTCGACC**
TGCAGG

Figure 4.14. Restriction enzyme map of the *P. falciparum* insert DNA. Underlined portions are part of the pMAL-c2X vector polylinker region. An internal *Eco*RI site, GAATTC (**red**, boldface) and *Hind*III site, AAGCTT (grey, boldface) were predicted using a restriction enzyme mapper program (www.restrictionmapper.org). *Xmn*I, GAGGATTT (**brown** boldface); *Bam*HI, GGATCC (**blue** boldface); *Xba*I, TCTAGA (**purple,** boldface); *Sal*I, GTCGAC (**orange** boldface) and *Pst*I, CTGCAG (**green** boldface) sites derived from the vector were also predicted.

Due to the insert DNA being able to ligate into the vector in two different orientations, it was necessary to confirm the orientation of the insert. In pGEX4T1, an *Eco*RI site is located 3 base pairs downstream from the *Bam*HI site (Appendix Figure A2), into which the DNA insert was ligated. An *Eco*RI site is also present in the insert DNA, approximately 195 bp away from the *Bam*HI site at the 5' end of the insert (Figure 4.14). It was thought possible to use the *Eco*RI sites to determine the orientation of the ligated insert DNA. The PCR products were digested with *Eco*RI and analysed by agarose gel electrophoresis (Figure 4.16 C). The PCR product of a recombinant plasmid having the DNA insert in the correct orientation would yield products of 636 bp, 100 bp and 259 bp upon digestion with *Eco*RI (Figure 4.17). A DNA insert in the incorrect orientation in a recombinant plasmid amplified by PCR and digested with *Eco*RI would yield products of

201 bp, 100 bp, and 694 bp (Figure 4.17). On inspection of the agarose gel, it was very difficult to determine which of the inserts were in the correct orientation as the products yielded by digestion of inserts in the incorrect orientation would have very similar sizes to those of correct orientation. In addition, the DNA ladder used did not have sufficiently small DNA fragments to obtain accurate sizes for the digested PCR products. PCR products from colony 18 appeared to have the closest match to the theoretical sizes of an *Eco*RI digestion of the PCR product from an insert in the correct orientation. Plasmid (pGEX4T1*Pf*33) was isolated from colony 18 and sequenced. The results from sequencing pGEX4T1*Pf*33 was aligned with the PFC0760c DNA sequence, and a sequence which was constructed from the DNA sequencing results obtained previously for the insert DNA in pTS822 (Section 4.3.1 and Figure 4.2), and the pGEX4T1 vector DNA sequence (http://seq.yeastgenome.org/vectordb/vector_descrip/COMPLLETE/PGEX4T1.SEQ.html) (result not shown). The sequence is identical to the PFC0760c sequence and the sequence constructed from the previous sequencing results and the pGEX4T1 sequence.

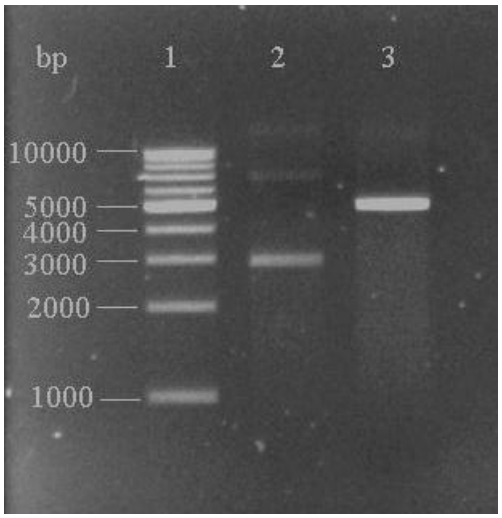


Figure 4.15. *Bam*HI restriction enzyme digest of pGEX4T1. Lane 1, DNA ladder; lane 2, non-digested pGEX4T1 (control); lane 3, *Bam*HI-digested pGEX4T1. Samples were analysed on a 1% (m/v) agarose gel stained with ethidium bromide.

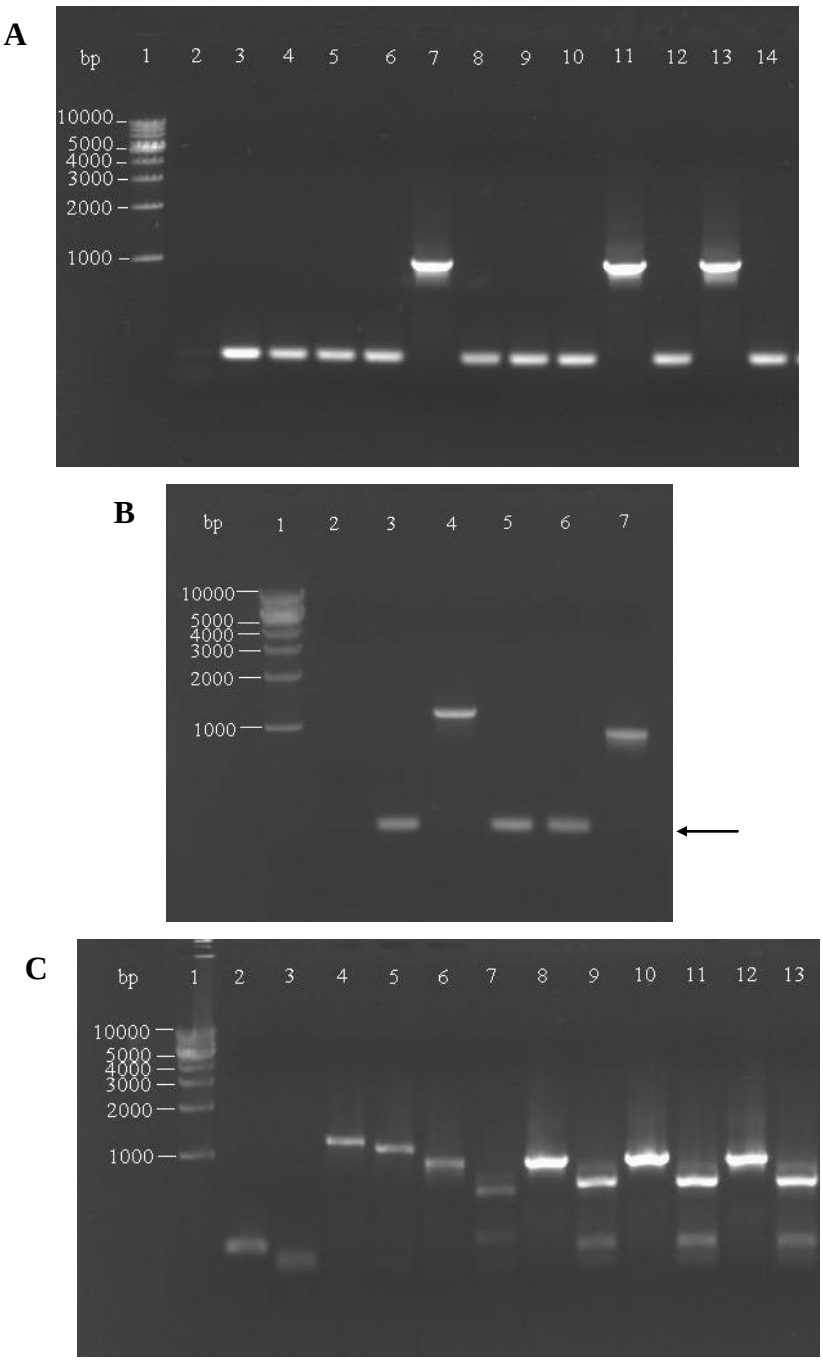
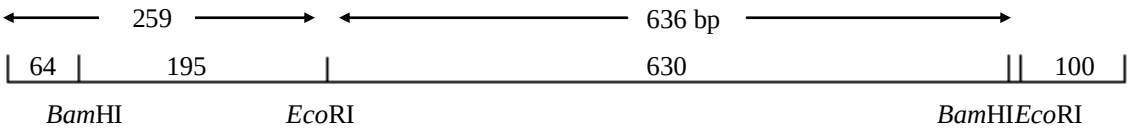


Figure 4.16. Analysis of *E. coli* colonies transformed the 822 bp *P. falciparum* DNA insert ligated into pGEX4T1. The transformed colonies were analysed by colony PCR. (A) Colony 22 (lane 7), colony 26 (lane 11) and colony 28 (lane 13) all appear to have an insert. (B) Colony 15 (lane 4), and colony 18 (lane 7) appear to have an insert. Non-recombinant pGEX4T1 was included as a control (lane 3). PCR products corresponding to the size of the non-recombinant pGEX4T1 (200 bp) did not possess the DNA insert (arrow). The PCR products indicating the presence of insert DNA were digested with *Eco*RI. (C) *Eco*RI digested PCR products from colonies 15 (lane 5), 18 (lane 7), 22 (lane 9), 26 (lane 11) and 28 (lane 13) and non-digested controls for colonies 15 (lane 4), 18 (lane 6), 22 (lane 8), 26 (lane 10) and 28 (lane 12) were analysed on a 1% (w/v) agarose gel. Non-recombinant pGEX4T1 digested with *Eco*RI (lane 3) and non-digested non-recombinant pGEX4T1 (lane 2) controls were included. Lane 1 contains the DNA ladder.

(A) Correct orientation



(B) Incorrect orientation

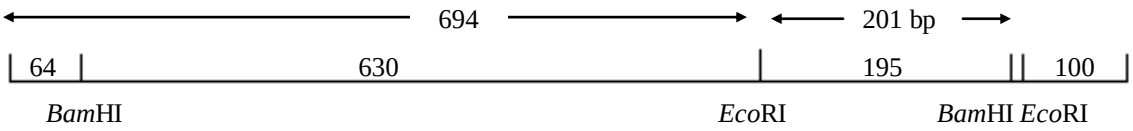


Figure 4.17. Schematic representation of the PCR products that would result from pGEX4T1 recombinants ligated with the insert DNA. Two possible orientations could result from ligation of the insert into pGEX4T1: the correct orientation (A) and the incorrect orientation (B). *Eco*RI digestion of PCR products from recombinant plasmids with the insert in the correct orientation would yield DNA fragments of 259 bp, 636 bp and 100 bp. *Eco*RI digestion of PCR products from recombinant plasmids with the insert in the incorrect orientation would yield DNA fragments of 694 bp, 195 bp and 100 bp. The *Eco*RI and *Bam*HI restriction enzyme sites are shown.

4.4.2 Recombinant expression of GST and the GST-fusion protein (*Pf*33-GST)

As experienced with the pMAL system, the expression level of the GST-fusion protein (*Pf*33-GST) from the pGEX4T*Pf*33 plasmid was low. No fusion-protein band was clearly evident on the gel (Figure 4.18 A, lane 5). Expression of the GST control was clearly visible on with SDS-PAGE analysis, with a dominant protein band observed at approximately 26 kDa (Figure 4.18 A, lane 3). Detection of *Pf*33-GST with monoclonal anti-GST antibodies produced a very faint signal (Figure 4.18 B, lane 5 arrow) at a molecular mass of 59 kDa. This compares favourably to the predicted molecular weight of approximately 59.5 kDa of *Pf*33-GST. However this size is also very close to the dimer molecular weight for GST of 58.5 kDa (GST Gene Fusion System Handbook, Amersham Biosciences), but a dimer was not observed in the control.

The anti-GST antibodies detected another protein of approximately 22 kDa in all of the lanes (Figure 4.18 B). As no *E. coli* lysate was included in this experiment as a control, it could not be determined if the bands being detected were possibly *E. coli* proteins. It is possible the protein band at 22 kDa is *E. coli* biotin carboxyl carrier protein (BCCP). BCCP

is a small subunit of *E. coli* acetyl-CoA carboxylase that migrates on a reducing SDS-PAGE gel at 22.5 kDa (Li and Cronan, 1992). The anti-GST antibody is a biotinylated antibody and requires streptavidin for detection. Thus BCCP would produce a signal on the nitrocellulose upon addition of streptavidin.

An attempt was made to improve expression of *Pf*33-GST using the RIG plasmid which expresses rare tRNAs. The addition of the RIG plasmid should result in increased expression of *Pf*33-GST if the reason for low expression is due to insufficient rare tRNAs. Expression of *Pf*33-GST was attempted with *E. coli* cells co-transformed with pGEX4T1*Pf*33 and the RIG plasmid. The presence of the RIG plasmid did not appear to increase expression levels of *Pf*33-GST (result not shown).

4.4.3 Purification of GST and *Pf*33-GST

*Pf*33-GST and GST were purified using a glutathione-agarose affinity column. The fractions were analysed by SDS-PAGE and western blotting (Figure 4.19). SDS-PAGE analysis of the fractions collected from purification of *Pf*33-GST revealed protein bands at approximately 59 kDa, 53 kDa and 46 kDa in addition to minor protein bands below a molecular mass of 45 kDa (Figure 4.19 A). Western blotting with monoclonal anti-GST antibodies only detected the protein band at 59 kDa (Figure 4.19 B).

SDS-PAGE analysis of glutathione agarose purified GST revealed a major protein band at approximately 26 kDa (Figure 4.19 C), corresponding to GST. In addition, a number of minor protein bands were observed between 45 kDa and 66.2 kDa, and at approximately 20 kDa. These bands were not detected by the anti-GST antibodies in the western blot (Figure 4.19 D), which suggests that they are contaminating *E. coli* proteins that were not removed with glutathione agarose purification. However, a protein of approximately 59 kDa was also detected by anti-GST antibodies, in the fractions collected from GST purification. This is the same size observed for *Pf*33-GST. It is unclear if the 59 kDa band observed in both gels is simply an anomaly or *Pf*33-GST.

Further expression of *Pf*33 as a GST fusion protein was abandoned as it was thought to not be any better than expression as an MBP fusion.

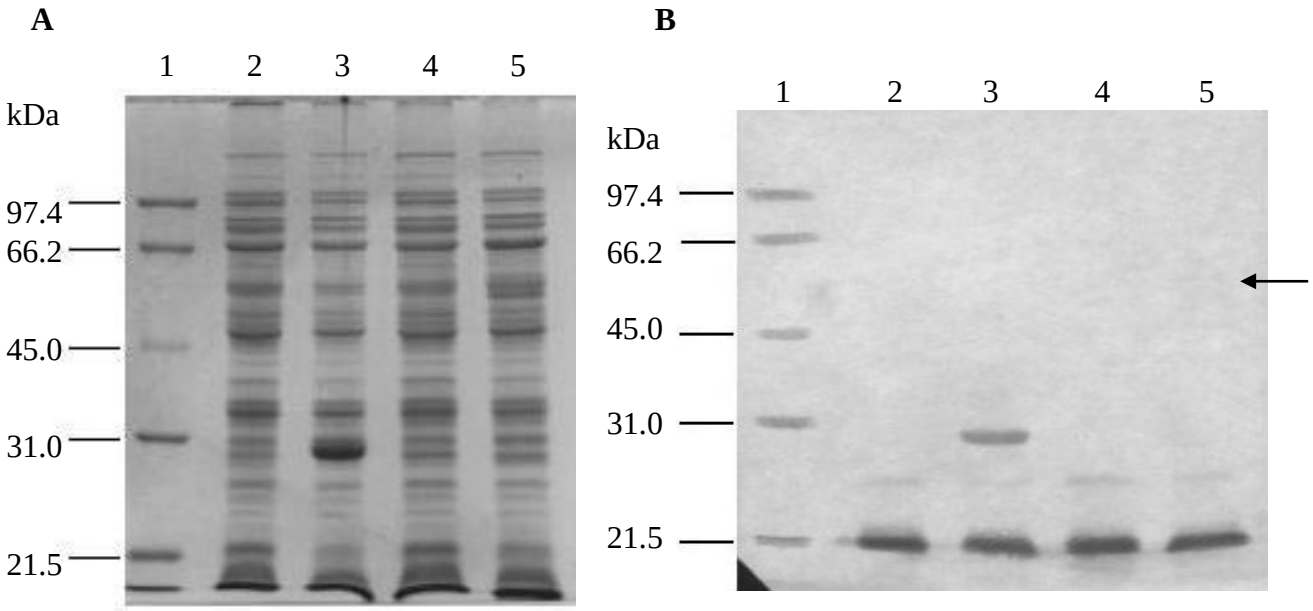


Figure 4.18. Analysis of expression of the GST-fusion protein, *Pf*33-GST, by SDS-PAGE and western blotting. (A) 10% SDS-PAGE, (B) western blot with monoclonal anti-GST tag antibodies. Lane 1, Bio-Rad low molecular weight marker; lane 2, un-induced non-recombinant pGEX4T1; lane 3, IPTG-induced non-recombinant pGEX4T1 expressing GST; lane 4, un-induced recombinant pGEX4T1C18; lane 5, IPTG-induced recombinant pGEX4T1C18 expressing *Pf*33-GST. The arrow indicates the position where a faint band, corresponding to *Pf*33-GST, was observed in the original western blot.

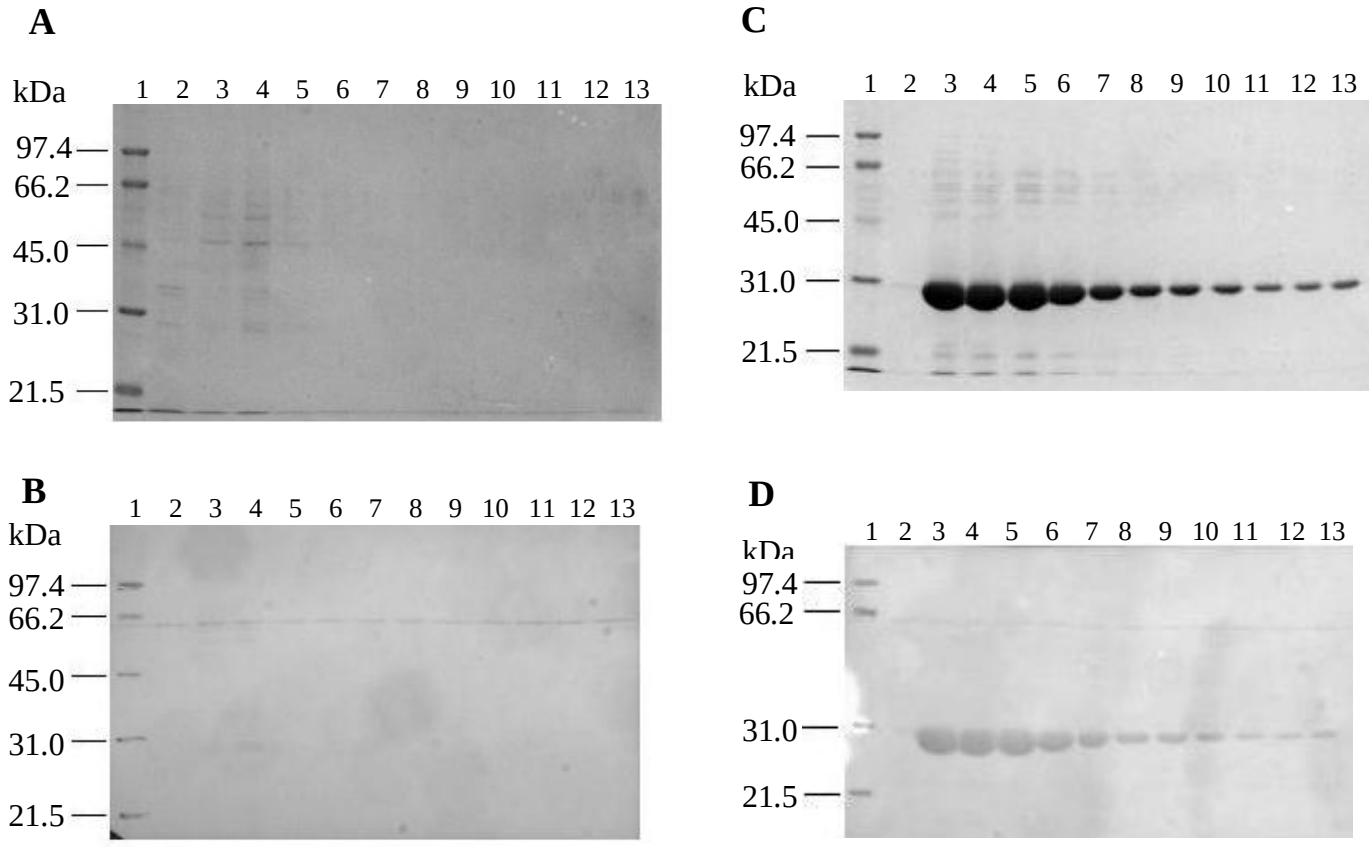


Figure 4.19. SDS-PAGE gel analysis and western blotting of glutathione-agarose-purified GST fusion protein, *Pf*33-GST from expression of pGEX4T1*Pf*33 and recombinant GST. (A) 10 % (m/v) acrylamide gel of *Pf*33-GST expression. (B) Western blot analysis of *Pf*33-GST expression with biotinylated monoclonal anti-GST antibodies (1 µg/ml). Lane 1, Bio-Rad low molecular weight marker; Lanes 2-13, fractions 1-12 from the affinity gel. (C) 10 % (m/v) acrylamide gel of glutathione-agarose-purified recombinant glutathione-*S*-transferase (GST) from expression in pGEX4T1. (D) Western blot analysis with biotinylated monoclonal anti-GST antibodies (1 µg/ml). Lane 1, Bio-Rad low molecular weight marker; Lanes 2-13, fractions 1-12 from the affinity gel.

4.5 Discussion

The pMAL-c2X and pTS822 plasmid identities were established by restriction enzyme digests, PCR and sequencing (Figures 4.1 and 4.2). The determined experimental plasmid sizes of the plasmids were similar to the theoretical sizes expected. The sequencing result for pTS822 was identical to the PFC0760c sequence and it was therefore confirmed that the plasmid was the correct one.

Different temperatures (Figure 4.3) and periods of IPTG induction (Figure 4.4) were used to optimise expression of *Pf*33-MBP. It was determined that inducing for long periods of time resulted in a larger yield of lower molecular weight *Pf*33-MBP proteins. The observation of lower molecular weight proteins with the pMAL system has been experienced by others using the pMAL plasmids to produce recombinant proteins. Lower molecular weight bands were observed by di Guan *et al*. (1988) with expression of MBP-β-galactosidase hybrid protein and MBP *Pst*I hybrid protein. These lower molecular weight proteins were assumed to be degradation products. Protease inhibitors were used during harvest of the *Pf*33-MBP protein and therefore it was thought unlikely that the lower molecular weight proteins were due to protease cleavage post-expression. Transforming the pTS822 plasmid into a protease deficient *E. coli* host strain, such as *E. coli* BL21 (DE3), may be beneficial but was not explored. It is also possible that the lower molecular weight *Pf*33-MBP proteins are due to cryptic start sites, which are not unusual for *Plasmodium* genes (Amante *et al*., 1997; Rathore *et al*., 2005; Bharadwaj *et al*., 1998). These cryptic start sites result in truncated products when expressed in *E. coli*.

Recombinant protein expression at 30˚C instead of the conventional temperature of 37˚C did not appear to increase protein expression. An alternative temperature for expression could be utilised. A temperature of 25˚C for expression has resulted in successful, soluble, full-length expression of falcipain 1 from *P. falciparum* (Goh *et al*., 2005). Cho *et al*. (2008) experienced a 1.2 fold increase in total expressed protein when their MBP fusion protein was expressed at 22˚C as opposed to 37˚C. It is likely that particular temperatures for expression will be more beneficial for some proteins than others and it may be necessary to experiment with a range of temperatures to obtain predominantly full-length *Pf*33-MBP protein.

Successful expression has been achieved with inducing protein expression at post-log phase (A_{600} of 2.0), rather than at mid-log phase (A_{600} of 0.6) (Flick *et al.*, 2004). The protein from induction at mid-log phase resulted in a truncated protein, with a small yield of the overall protein being the full-length intact protein, as is the case with *Pf*33-MBP. Induction at post-log phase resulted in intact, full-length protein. Inducing expression at post-log phase needs to be tested to determine if this will result in a higher yield of full-length *Pf*33-MBP.

The base pair composition of the recombinant proteins was determined, as high adenine/thymine content has been argued to cause problems with recombinant protein expression (Kane, 1995; Baca and Hol, 2000; Matambo *et al.*, 2004). The DNA sequence for *Pf*33 has an adenine/thymine content of 80%, but when combined with the MBP sequence (adenine/thymine content of 48%), to form *Pf*33-MBP, the adenine/thymine content is 62%. *Pf*LDH has an adenine/thymine content of 67%, however it expressed more efficiently than *Pf*33-MBP, which had greater adenine/thymine content. Mehlin *et al.*, (2006) found that codon usage and base pair composition did not appear to be contributing factors towards poor recombinant protein expression. It appears that base pair composition is possibly not a contributing factor to the poor *Pf*33-MBP expression either, although *Pf*LDH and *Pf*33-MBP were expressed from different plasmids which may have had a part to play.

All of the proteins expressed had predicted disorder within their protein sequences (Figure 4.7). *Pf*33-MBP appeared to have approximately eleven regions within the protein sequence that were predicted to be disordered. *Pf*LDH was predicted to have four disordered regions and MBP was predicted to have approximately eight disordered regions. Both of these proteins were successfully expressed, which suggests that protein disorder does not appear to have a significant impact on the expression of *Pf*LDH and MBP.

Expression of *Pf*33 was attempted as a GST fusion protein to try to increase protein expression, but proved to be less successful. Comparisons of recombinant protein expression as an MBP-fusion versus a GST-fusion has previously been done and the MBP constructs produced superior recombinant fusion proteins in comparison to the GST constructs (Wang *et al.*, 1999; Cho *et al.*, 2008). It is possible that recombinant expression of *Pf*33-GST could have been improved, by altering the growth temperature to between

20°C and 30°C, inducing at a higher cell density for a shorter period, increasing aeration of the cells or using a different *E. coli* strain (Saluta and Bell, 1998). Further expression with the GST fusion construct pGEX4T1*Pf*33 was not continued.

*Pf*33-MBP was not expressed as successfully as MBP, *Pf*LDH and *Pv*LDH. For this reason the pI, and molecular mass and number of rare codons was determined, as these factors have been implicated to play a role in poor recombinant protein expression (Mehlin *et al*, 2006; Vedadi *et al*., 2007; Saul and Battistutta 1988; Kane, 1995; Sayers and Price, 1995; Baca and Hol, 2000; Yadava and Ockenhouse, 2003; McNulty *et al.*, 2003; Matambo *et al.*, 2004; Zhou *et al.*, 2004). The pI's of the proteins were all below 9.5, and should therefore not impact protein expression negatively (Mehlin *et al*., 2006). MBP (54 kDa), *Pf*LDH (34 kDa) and *Pv*LDH (33 kDa) all have a molecular mass below 56 kDa, and *Pf*33-MBP has a molecular mass greater than this (79 kDa). This correlates well with the findings of Mehlin *et al*, (2006) as all the proteins with molecular mass below 56 kDa were successfully expressed. The one protein with a molecular mass above this, *Pf*33-MBP, was poorly expressed.

The presence of the lower molecular weight *Pf*33-MBP proteins was thought to possibly be due to *Plasmodium* using codons that are very rarely used in *E. coli*. Recombinantly expressing a gene having these rare *E. coli* codons in an *E. coli* host, often results in translational errors, frame shifts and decreases in protein expression (Saul and Battistutta 1988; Kane, 1995; Sayers and Price, 1995; Baca and Hol, 2000; Yadava and Ockenhouse, 2003; McNulty *et al.*, 2003; Matambo *et al.*, 2004; Zhou *et al.*, 2004). For this reason, the number of rare codons was determined for the *Pf*33 DNA and for *Plasmodium* LDH DNA sequences (Table 4.3). Expression of *Plasmodium* LDH's were used as controls for expression conditions throughout the present study (Figure 4.6). Eleven percent of the codons (30/275 codons) in the *Pf*33 sequence are rare codons for *E. coli*. *Pf*LDH and *Pv*LDH were found to have very similar percentages of rare codons: 12% (38/317 codons) and 10.5% (32/304 codons) respectively. *Pf*LDH and *Pv*LDH expressed more successfully than *Pf*33-MBP, even though their codon usage was similar to that of *Pf*33. This suggests that codon usage is perhaps not a major contributing factor for poor expression in this study.

Table 4.3. Rare *E. coli* codons and their frequency within the *Plasmodium* proteins being recombinantly expressed.

Codon	*Pf*33 insert DNA sequence	*Pf*LDH DNA sequence	*Pv*LDH DNA sequence
CGG (Arginine)	1	0	0
AGA (Arginine)	1	6	2
AGG (Arginine)	1	0	2
ATA (Isoleucine)	15	5	3
GGA (Glycine)	4	10	11
ACA (Threonine)	3	5	3
CCA (Proline)	2	9	5
GGG (Glycine)	1	0	4
ACC (Threonine)	2	3	2

The RIG plasmid (Baca and Hol, 2000) codes for three tRNAs (ArgU, IleX and GlyT) cloned from *E. coli* (Appendix Figure A3). These genes enable the expression of tRNAs recognising the codons AGA/AGG (arginine), ATA (isoleucine) and GGA (glycine) (Baca and Hol, 2000). Due to cloning artefacts, the RIG plasmid also carries genes for tRNAs recognising the codons TAC (tyrosine) and ACA/ACC (threonine) (Baca and Hol, 2000). It was thought that high levels of these tRNAs could possibly aid *E. coli* translation of *Plasmodium* mRNAs that are abundant in these codons, and thus potentially increase the yield of the recombinant protein. Success with expressing full-length *P. falciparum* proteins and reducing the quantity of lower molecular weight proteins with co-transformation of this plasmid has been observed (Matambo *et al.*, 2004). However, no improvement in recombinant protein expression was observed with utilisation of the RIG plasmid in the present study. Zhou *et al*. (2004) also did not experience any improvement in expression of a *Plasmodium* vaccine candidate with the RIG plasmid. However, altering the codons to match those most commonly used by *E. coli*, without changing the amino acid sequence, resulted in an intense increase in protein expression (Yadava and Ockenhouse, 2003; Zhou *et al*., 2004). It is therefore possible that if the *Pf*33 sequence was optimised for an *E. coli*

host, a larger yield of full-length *Pf*33-fusion protein may be obtained and fewer truncated proteins expressed.

Truncated proteins could be due to priming from an internal Shine-Dalgarno sequence (Shine and Dalgarno, 1974), upstream of an internal methionine residue as Turgut-Balik *et al*. (2001) and Berwal *et al*. (2008) experienced with the expression of *P. falciparum* LDH. However, no Shine-Dalgarno sequence was found to be present in this sequence (result not shown).

*Pf*33-MBP was purified with amylose affinity chromatography. Analysis of the purified protein by SDS-PAGE revealed three major bands (approximately 79 kDa, 60 kDa and 45 kDa) and one minor band (33 kDa), in addition to a couple of minor bands below 21.5 kDa and above 97.4 kDa. Maina *et al*. (1988) obtained similar results: multiple protein bands were observed after amylose purification of their MBP-paramyosin fusion protein. It was proposed that the multiple protein bands were due to early termination of the fusion protein or due to digestion by *E. coli* proteases.

*Pf*33-MBP fusion protein was cleaved by Factor Xa at a concentration of 10 μg/ml. DEAE Sepharose™ was used to separate *Pf*33 from MBP after Factor Xa cleavage. Analysis of the fractions off the column by SDS-PAGE revealed protein bands of approximately 43 kDa for the fractions assumed to contain *Pf*33, and the fractions assumed to contain MBP. Un-cleaved *Pf*33-MBP should have been passed over the DEAE Sepharose column as a control as the sample loaded would inevitably contain at least a small proportion of un-cleaved *Pf*33-MBP. It is also possible that the MBP and *Pf*33 remained associated even after Factor Xa cleavage and therefore eluted together, as experienced by Ko *et al*. (1993). Unfortunately, the fractions were not subjected to western blotting in addition to SDS-PAGE analysis.

An alternative strategy could be to pass the cleavage mixture over the amylose column for a second time in place of using ion exchange chromatography, even though others had experienced no success with this method (Dr Alain Boulangé, University of KwaZulu-Natal, South Africa, personal communication). This method should remove the cleaved off MBP, as performed by Maina *et al*. (1988). Maina *et al*. (1988) did however experience a small quantity of MBP that would not bind to column with this method.

Factor Xa cleavage followed by ion exchange chromatography, did not appear to be successful at separating *Pf*33 from MBP. 'On column' cleavage was attempted as an alternative as this method had been successful with a glutathione-*S*-transferase (GST) fusion protein (Huson, 2006). 'On column' cleavage of the GST fusion protein resulted in a highly purified protein, free of GST. 'On column' cleavage of *Pf*33-MBP however, did not appear to result in purified *Pf*33. SDS-PAGE analysis and western blotting of the fraction that should have contained *Pf*33 looked similar to the fraction that should have contained MBP (Figure 4.14). It is possible that the Factor Xa cleaved non-specifically as experienced by others (Ko *et al*., 1993) and therefore *Pf*33 would possibly still have had a portion of MBP attached. It is possible that MBP and *Pf*33 formed a tight association even after Factor Xa cleavage as experienced by Ko *et al*. (1993), as described earlier. This tight association, in addition to the possibility of MBP being weakly bound to the amylose, may provide some explanation for the results observed in Figure 4.14.

The way forward with expression of *Pf*33 may be to involve a different system such as the one used by Cho *et al*. (2008) and Pryor and Leiting (1997). They used a dual tag system of MBP and His-tag, thereby increasing the purification options available after Factor Xa cleavage. A second option could be to use a system constructed by Muench *et al*., (2003) after they had numerous problems expressing and purifying an enoyl reductase-MBP fusion protein. They added a tobacco etch virus protease cleavage site into the pMAL-c2X plasmid linker region, between the region coding for MBP and the region coding for enoyl reductase. Following the tobacco etch virus cleavage site they inserted a histidine tag. The cells containing this new modified plasmid were co-transformed with a plasmid expressing the tobacco etch virus protease. As the fusion protein was expressed, the MBP was cleaved off and the enoyl reductase was purified using the histidine tag. A system like this could possibly solve many of the problems experienced with the purification of *Pf*33.

Despite the problems associated with recombinantly expressing *Plasmodium* proteins, it appears that successful expression is possible. Successful recombinant expression of *Plasmodium* proteins include falcipain-2 MBP-fusion (Goh *et al.*, 2003); falcipain 1 cysteine protease (Goh *et al*., 2005); enoyl reductase (Muench *et al*., 2003); LDH (Turgut-Balik *et al*., 2001; Turgut-Balik *et al*., 2004; Brown *et al*., 2004); orotidine-

5’-monophosphate decarboxylase (Cinquin *et al*., 2001); erythrocyte membrane protein 1 (Flick *et al.*, 2004); F2 domain of erythrocyte binding antigen-175 (Yadava and Ockenhouse, 2003); heat shock protein 70 (Matambo *et al*., 2004); FALVAC-1 (Zhou *et al*., 2004); in addition to 63 *P. falciparum* proteins (Mehlin *et al*., 2006). It appears to just be a matter of finding the optimal conditions and the appropriate vector.

CHAPTER 5

Immunological studies

5.1 Introduction

Antibodies were produced in chickens against two *Plasmodium* proteins. Chickens were chosen for the production of the antibodies as they yield high concentrations of specific antibodies (Krief *et al.*, 2002; Gassmann *et al.*, 1990; Gruber and Hartung, 2004; Chui *et al.*, 2004). In addition, antigens injected into chickens are often more immunogenic than the same antigen injected into mammals due to the chickens phylogenetic distance from mammals (Gassmann *et al.*, 1990). Large amounts of antibodies are maternally transferred to the egg yolk (Patterson *et al.*, 1962) providing immunity for the developing chick. Therefore the eggs of immunized chickens are collected and the IgY isolated from the egg yolks using polyethylene glycol (PEG) precipitation (Polson *et al.*, 1980; 1985; Goldring and Coetzer, 2003). This provides a non-invasive method of harvesting antibodies, thus minimizing distress to the animals (Gassmann *et al.*, 1990; Krief *et al.*, 2002; Gruber and Hartung, 2004; Tini *et al.*, 2002).

Chicken anti-peptide antibodies were raised against peptides from *P. falciparum* PFC0760c protein and the *P. yoelii yoelii* protein PY05757 (http://www.plasmodb.org). Using peptides as antigens is advantageous, especially when the target protein is not easily obtained or available in sufficient quantities for immunisation. Provided that the anti-peptide antibodies produced were able to react with the native protein, those antibodies would prove invaluable for isolating and characterizing the native protein. The anti-peptide antibodies produced in this study were used to probe for the native proteins in an immunofluorescence assay and in western blotting of *Plasmodium* infected red blood cells. In addition, the peptides were coupled to affinity matrices and used to purify human anti-malaria antibodies able to bind to these peptides, from an antibody pool prepared from plasma isolates from Malawi (Goldring *et al.*, 1992; Taylor *et al.*, 1992).

5.2 Production of antibodies in chickens against synthetic peptides

5.2.1 Selection of peptides

The amino acid sequences of the *Plasmodium falciparum* protein, PFC0760c and the *Plasmodium yoelii yoelii* protein, PY05757, were analysed for regions predicted to be immunogenic using the Predict 7 program (Cármenes *et al.*, 1989). The algorithms predicting hydrophilicity (Hopp and Woods, 1981; Kyte and Doolittle, 1982), flexibility (Karplus and Shulz, 1985), surface probability (Janin *et al*., 1978) and antigenicity (Welling *et al*., 1985) were used for the entire amino acid sequences of PY05757 (Figure 5.1 A) and PFC0760c (Figure 5.2 A). Regions of 12 or more consecutive amino acids having values above zero for hydrophilicity, flexibility, and surface probability were considered sequences possible for peptide synthesis. A number of sequences were predicted to be favourable for peptide synthesis for both proteins. However, many of these sequences produced significant matches during BLAST searches, to other *P. yoelii yoelii* proteins, mouse (*Mus musculus*) and chicken (*Gallus gallus*) proteins (results not shown). It was necessary to select peptide sequences that would be sufficiently foreign to chickens, in order to raise chicken anti-peptide antibodies. It was also essential for the peptide sequence selected for PY05757 to not have sequence similarity with mouse proteins, or other *P. yoelii yoelii* proteins, as the antibodies raised would be required to detect the native PY05757 protein in *P. yoelii yoelii* infected mouse red blood cells. *Plasmodium* proteins tend to be lysine rich (Flick *et al.*, 2004; Vedadi *et al.*, 2007) and therefore any sequences having multiple lysine residues were also not further considered for synthesis.

The peptide sequence SDDDNRQIQDFE, corresponding to amino acid residues 1341-1352 of PY05757 was identified as a candidate for raising antibodies. The plots of hydrophilicity, flexibility and surface probability were all above zero (Figure 5.1 B). In addition, a BLAST search at NCBI with the "search for short nearly exact matches" option (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) produced a significant match only for the hypothetical protein PY05757 (Table 5.1). No *Gallus gallus* (chicken) or *Mus musculus* (mouse) proteins produced significant matches, suggesting that the peptide sequence is likely to be foreign and hence immunogenic in chickens and mice

Due to the small molecular mass of the peptide, it was necessary to couple the peptide to a carrier protein, rabbit albumin, to increase the antibody production. The peptide SDDDNRQIQDFE was synthesized with a cysteine residue at the C-terminus, allowing coupling to the rabbit albumin carrier with 3-maleimidobenzoic acid N-hydroxysuccinimide (MBS) via the sulfhydryl group (Section 2.30). The cysteine residue was synthesised on the C-terminus of the peptide, as Predict 7 predicted the amino acids at the C-terminus to be slightly less immunogenic than those at the N-terminus (Figure 5.1 B).

In addition, peptides FKLGSCYLYIINRNLKEI and CFKLGSCYLYIINRNLKEI were synthesized (GenScript Corporation, USA). These peptides were identical in sequence, except that one was synthesized with a cysteine residue at the N-terminus for coupling to rabbit albumin with MBS (Section 2.30). The positioning of the cysteine residue was due to the C-terminus predicted to be more immunogenic than the N-terminus with Predict 7 (Figure 5.2 B). Peptide FKLGSCYLYIINRNLKEI was coupled to rabbit albumin using glutaraldehye (Section 2.29). The peptides were synthesised with substitution of the internal cysteine residue with an alpha amino-butyric acid.

Peptide FKLGSCYLYIINRNLKEI corresponds to amino acid residues 567-584 of PY05757 (Figure 5.2 A, between solid black lines) and amino acid residues 2386-2403 of PFC0760c (Figure 5.2 A, between red dotted lines). The peptide was chosen for synthesis, as an alignment of the amino acid sequences of *Plasmodium* proteins having high sequence identity to *Pca* 96, revealed the sequence to be highly conserved across eight *Plasmodium* species (Chapter 3).

Peptide FKLGSCYLYIINRNLKEI did not appear to be a sequence that would be particularly immunogenic according to the predictions of Predict 7 (Figure 5.2 B), but it was decided to attempt to raise antibodies against it nevertheless, as it was thought useful to have a peptide that could potentially recognize the protein of interest in multiple *Plasmodium* species.

The peptide sequence was used to perform a BLAST search using the “search for short nearly exact matches” option at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), to ensure that no sequence similarity existed between the peptide sequence and chicken proteins. Hypothetical proteins from *P. falciparum*, *P. yoelii yoelii* and *P. chabaudi*

chabaudi returned significant matches (Table 5.2), corresponding to proteins having high sequence similarity to *Pca* 96.

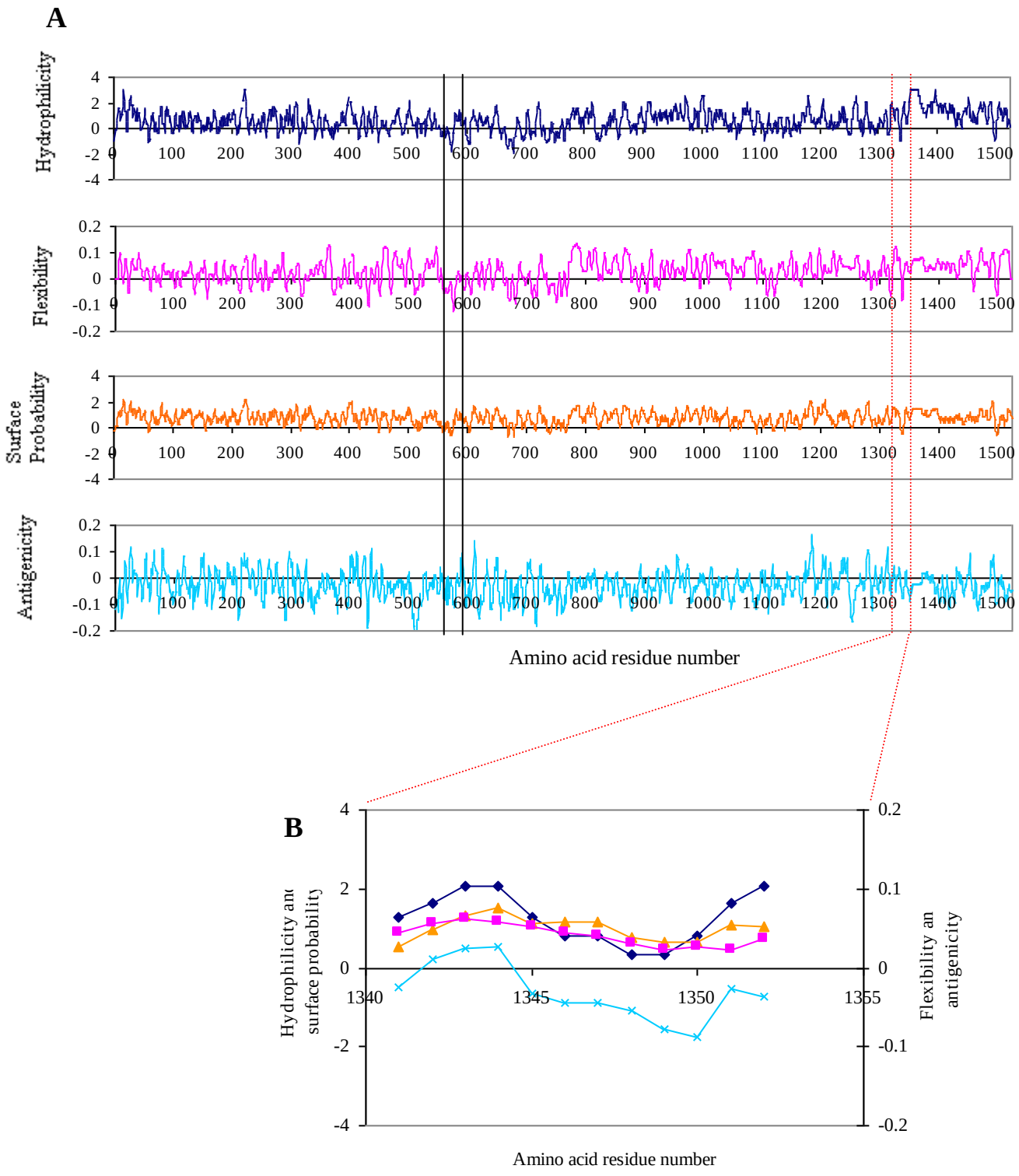


Figure 5.1. Hydrophilicity, surface probability, flexibility and antigenicity predictions of the full length *P. yoelii yoelii* protein PY05757. Prediction of the hydrophilicity (♦), surface probability (▲), flexibility (■) and antigenicity (x) of protein PY05757 with Predict 7 (A). The position of the conserved sequence FKLGSCYLYIINRNLKEI is located between the solid black lines, and the position of peptide SDDDNRQIQDFE is located between the red dotted lines. An enlargement of the section where peptide SDDDNRQIQDFE is located is shown in (B).

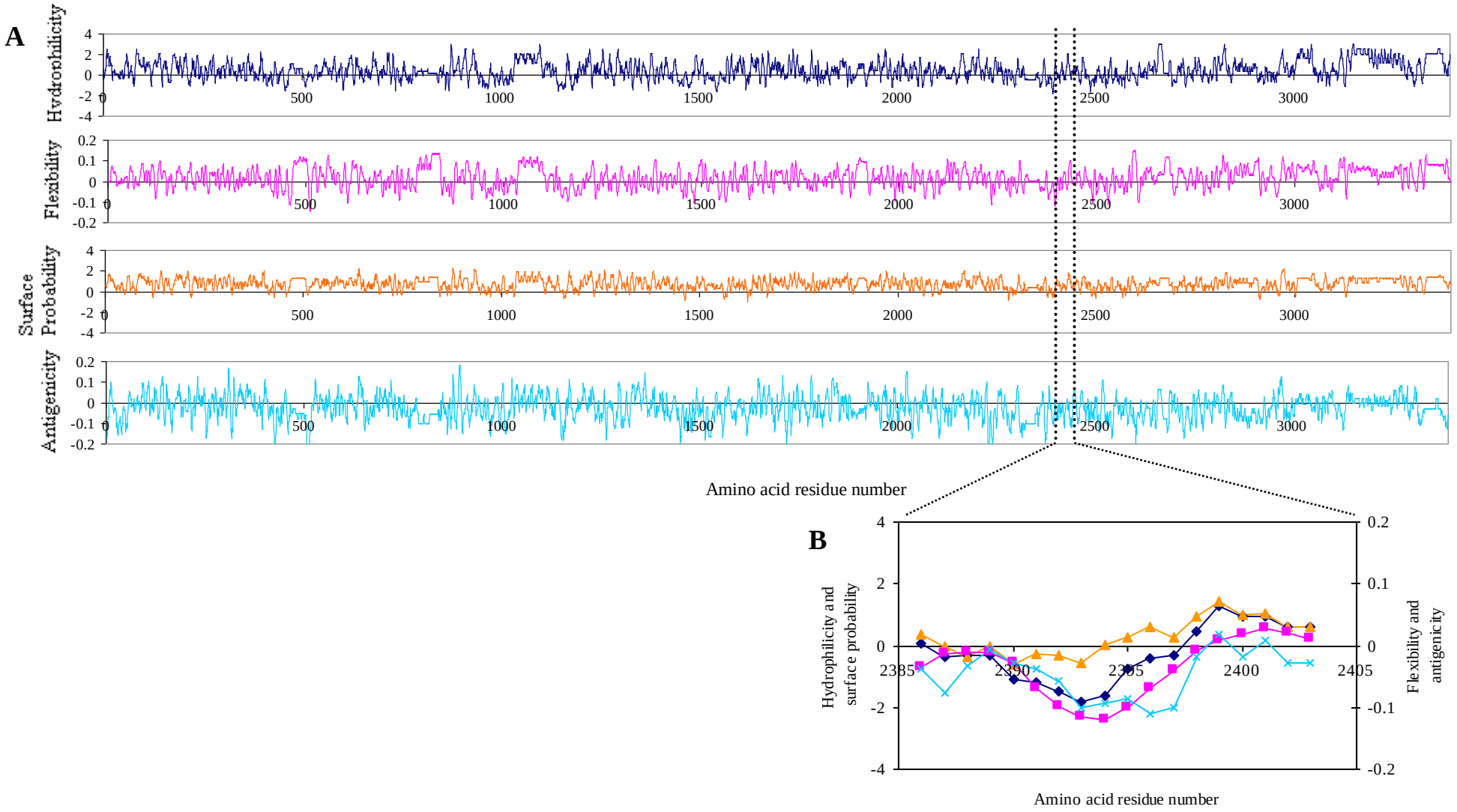


Figure 5.2. Hydrophilicity, surface probability, flexibility and antigenicity predictions of the full length *P. falciparum* protein PFC0760c. Prediction of the hydrophilicity (♦), surface probability (▲), flexibility (■) and antigenicity (x) of protein PFC0760c using Predict 7 (A). The position of the conserved sequence FKLGSCYLYIINRNLKEI is located between the dotted black lines. An enlargement the section where peptide FKLGSCYLYIINRNLKEI is located, is shown in (B).

Table 5.1. Significant matches returned by a BLAST search at NCBI* with the SDDDNRQIQDFE peptide

gi Number	Name	Strain	E-value	Identity (%)
gi|82596448|ref|XP_726266.1| gi|23481602|gb|EAA17831.1|	Hypothetical protein PY05757	*P. yoelii yoelii* str. 17XNL	0.001	100

* BLAST search was performed using the 'search for short nearly exact matches' option at NCBI.

Table 5.2. Significant matches returned by a BLAST search at NCBI* with the FKLGSCYLYIINRNLKEI peptide

gi Number	Name	Strain	E-value	Identity (%)
gi|3758855|emb|CAB11140.1| gi|16805257|ref|NP_473285.1|	Hypothetical protein	*P. falciparum* 3D7	$1x10^{-09}$	100
gi|82596448|ref|XP_726266.1| gi|23481602|gb|EAA17831.1|	Hypothetical protein PY05757	*P. yoelii yoelii* 17XNL	$1x10^{-09}$	100
gi|70941771|ref|XP_741132.1| gi|56519318|emb|CAH81368.1|	Hypothetical protein	*P. chabaudi chabaudi*	$1x10^{-09}$	100
gi|23508927|ref|NP_701595.1| gi|23496764|gb|AAN36319.1|	Hypothetical protein PFL1165w	*P. falciparum* 3D7	0.64	61%

* BLAST search was performed using the 'search for short nearly exact matches' option at NCBI.

5.3 Results

5.3.1 Production of chicken anti-peptide antibodies against *P. falciparum* protein PFC0760c and *P. yoelii yoelii* protein PY05757

5.3.1.1 Measuring anti-peptide antibody titres in chickens

The peptides FKLGSCYLYIINRNLKEI, CFKLGSCYLYIINRNLKEI and SDDDNRQIQDFE were coupled to rabbit albumin and injected into two chickens as described in Sections 2.29, 2.30 and 2.31. The production of chicken anti-peptide antibodies was monitored using ELISA. High levels of antibodies detecting peptide FKLGSCYLYIINRNLKEI were observed between weeks three and nine for one chicken immunized with the peptide (Figure 5.3 A). The second chicken immunized with peptide FKLGSCYLYIINRNLKEI did not produce antibodies to the peptide. Chickens immunized with peptide CFKLGSCYLYIINRNLKEI produced high levels of antibodies against the peptide FKLGSCYLYIINRNLKEI (Figure 5.3 A). Production of antibodies was evident between weeks four and twelve (chicken 1) and weeks seven and ten (chicken 2) (Figure 5.3 A). Antibodies produced against peptide SDDDNRQIQDFE were observed at high levels from weeks two to six (chicken 2) and six and eight (chicken 1), before the antibody levels started to go down (Figure 5.3 B).

The production of IgY against the carrier protein, rabbit albumin, was also monitored with ELISA (Figure 5.4). All chickens immunized with rabbit albumin peptide conjugates produced high levels of anti-rabbit albumin IgY, rising around week 3 and reaching a plateau at week 12. One chicken immunized with peptide FKLGSCYLYIINRNLKEI conjugate (Figure 5.4 A) produced lower levels of antibodies to rabbit albumin in comparison to its counterparts.

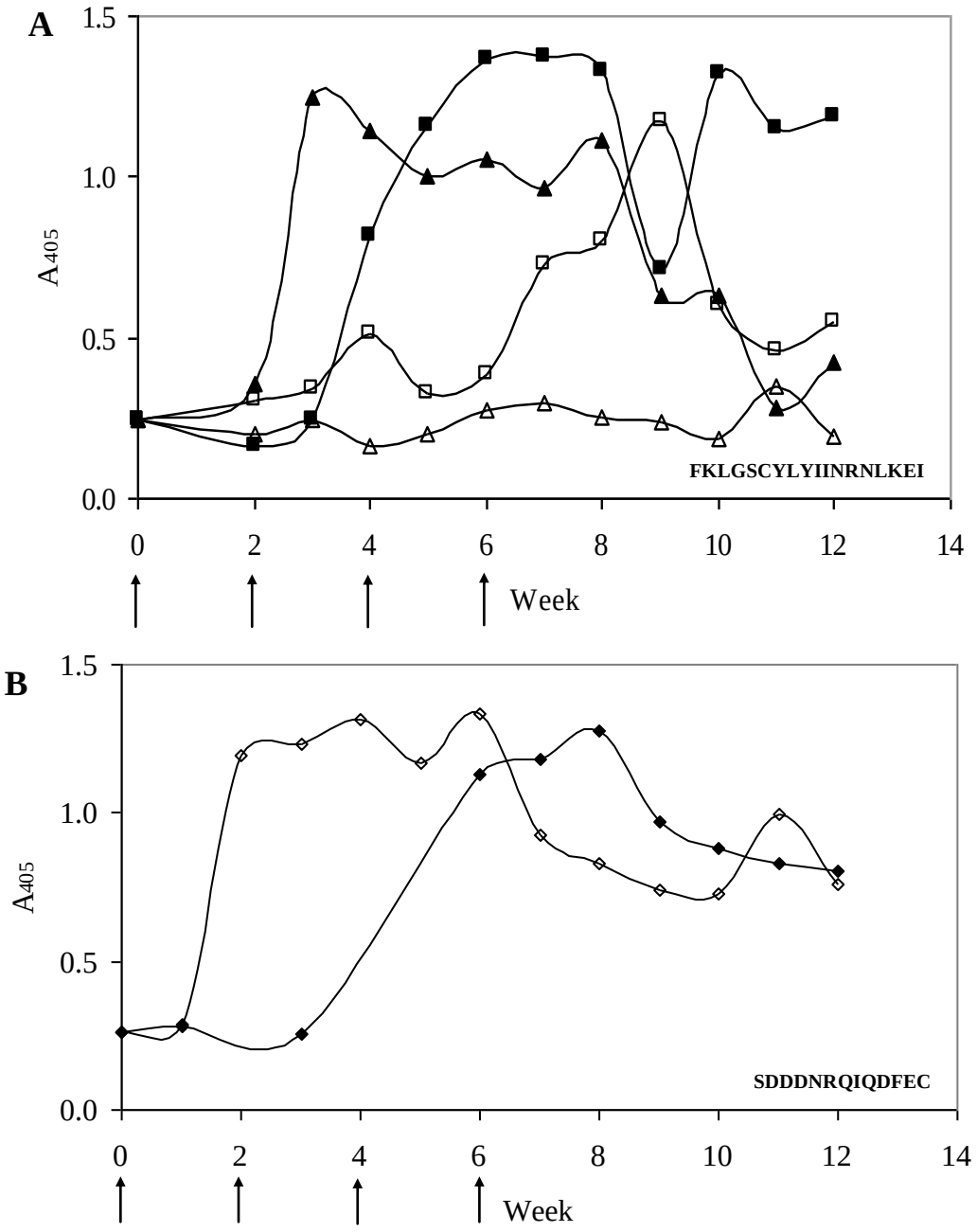


Figure 5.3. ELISA analysis of chicken anti-peptide antibody (IgY) production against peptide FKLGSCYLYIINRNLKEI and SDDDNRQIQDFEC. The peptides were coated at 1 µg/ml in PBS. The anti-peptide IgY produced against peptide FKLGSCYLYIINRNLKEI (▲;Δ), peptide CFKLGSCYLYIINRNLKEI (■;□) (A) and peptide SDDDNRQIQDFEC (♦;◊) (B) were used at a final concentration of 25 µg/ml. IgY from chicken 1 is represented by closed symbols and IgY from chicken 2 is represented by open symbols. Chickens were immunized with the peptide conjugate emulsified in Freund's complete adjuvant at week 0, and in Freund's incomplete adjuvant at weeks 2, 4, and 6 (arrows). The absorbance readings at 405 nm represent the average of duplicate experiments.

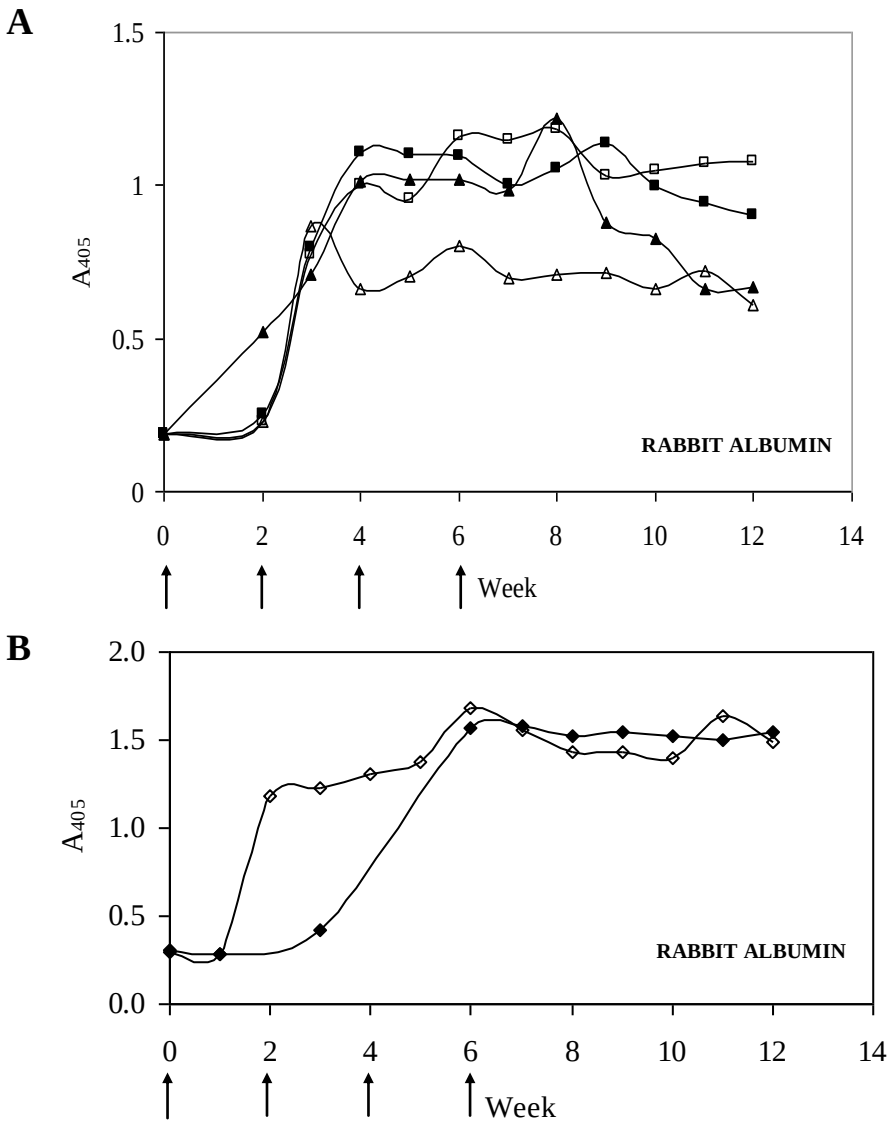


Figure 5.4. ELISA of antibody production in chickens against the carrier protein rabbit albumin. (A) Anti-peptide FKLGSCYLYIINRNLKEI (▲;Δ) and CFKLGSCYLYIINRNLKEI (■;□) IgY detecting rabbit albumin. (B) Anti-peptide SDDDNRQIQDFEC (♦;◊) IgY detecting rabbit albumin. Rabbit albumin was coated at 1 µg/ml and the anti-peptide antibodies were used at 25 µg/ml. Chickens were immunized with the peptide conjugate emulsified in Freund's complete adjuvant at week 0, and in Freund's incomplete adjuvant at weeks 2, 4, and 6 (arrows). The absorbance readings at 405 nm represent the average of duplicate experiments.

5.3.1.2 Affinity purification of anti-peptide antibodies

The anti-peptide FKLGSCYLYIINRNLKEI antibodies isolated from each week were pooled and affinity purified on an AminoLink® column coupled with peptide FKLGSCYLYIINRNLKEI (Figure 5.5 A) (Section 2.33.1 and 2.34). The yield of purified anti-peptide FKLGSCYLYIINRNLKEI IgY from multiple purifications was 6 mg. The anti-peptide CFKLGSCYLYIINRNLKEI antibodies from each week were pooled and affinity purified on a SulfoLink® column coupled with peptide CFKLGSCYLYIINRNLKEI (Figure 5.5 B) (Section 2.33.2 and 2.34). The yield of purified anti-peptide CFKLGSCYLYIINRNLKEI IgY from multiple purifications was 12 mg. The anti-peptide SDDDNRQIQDFEC antibodies were pooled and affinity purified on a SulfoLink® column coupled with peptide SDDDNRQIQDFEC (Figure 5.5 C) (Section 2.33.2 and 2.34). The yield of affinity purified anti-peptide SDDDNRQIQDFEC IgY from multiple purifications was 11 mg. The anti-peptide FKLGSCYLYIINRNLKEI and CFKLGSCYLYIINRNLKEI antibodies typically eluted after 5-6 ml of 100 mM glycine, pH 2.8 (Figure 5.5 A and B). The anti-peptide SDDDNRQIQDFEC antibodies eluted earlier, generally after 4-5 ml of 100 mM glycine, pH 2.8 (Figure 5.5 C).

5.3.1.3 Evaluation of purified anti-peptide antibodies with ELISA

The affinity purified anti-peptide antibodies were tested for their specificity for peptide using ELISA. Purified anti-peptide FKLGSCYLYIINRNLKEI antibodies and purified anti-peptide CFKLGSCYLYIINRNLKEI antibodies recognized both peptides with similar specificity (Figure 5.7 A and B). All of the affinity purified antibodies had superior recognition of the peptides in comparison to the non-purified antibodies, as they produced high signals at low concentrations (Figure 5.6 A and B; Figure 5.7).

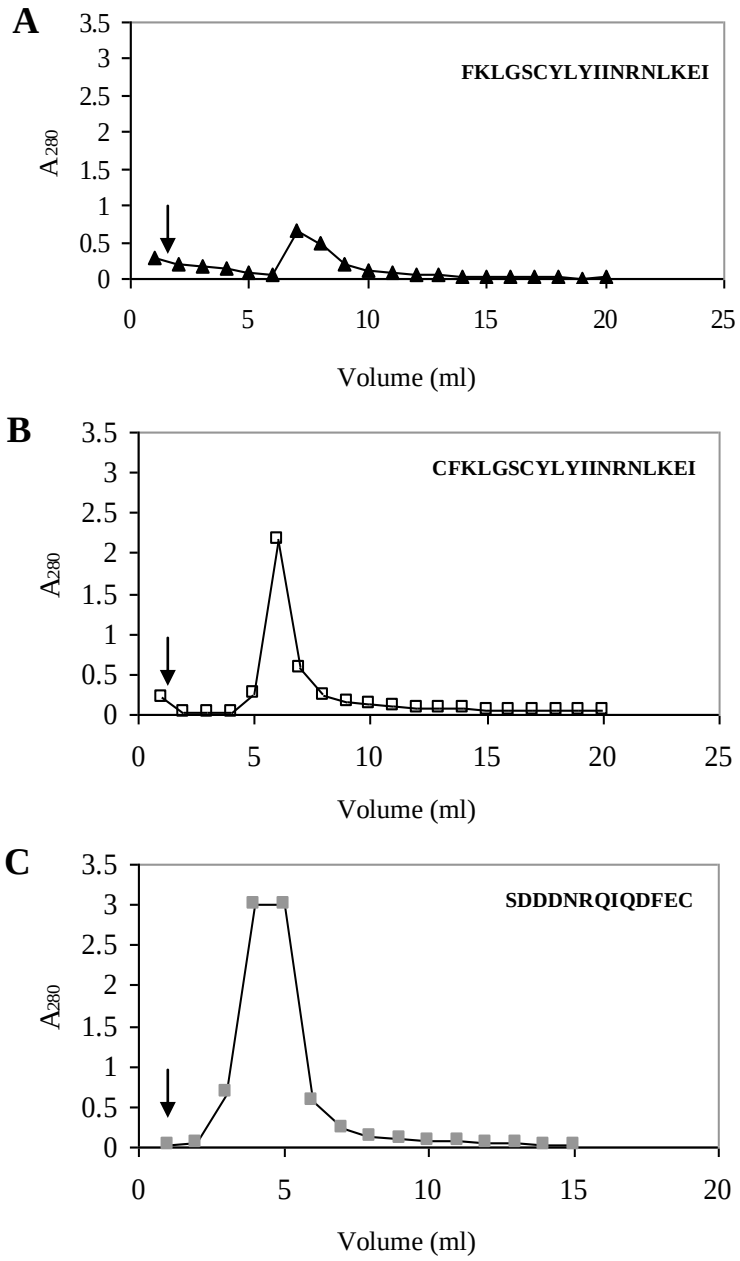


Figure 5.5. Elution of affinity purified chicken anti-peptide antibodies from the peptide affinity matrix. (A) Anti-peptide FKLGSCYLYIINRNLKEI IgY (chicken 1, weeks 9-12) (▲), (B) anti-peptide CFKLGSCYLYIINRNLKEI IgY (chicken 1, weeks 9-12) (□) and (C) anti-peptide SDDDNRQIQDFEC IgY (chicken 2, weeks 4-7) (■). Anti-peptide FKLGSCYLYIINRNLKEI IgY was purified on AminoLink® resin coupled with peptide FKLGSCYLYIINRNLKEI. Anti-peptide CFKLGSCYLYIINRNLKEI IgY was purified on SulfoLink® resin coupled with peptide CFKLGSCYLYIINRNLKEI. Anti-peptide SDDDNRQIQDFEC IgY was purified on SulfoLink® resin coupled with peptide SDDDNRQIQDFEC. The anti-peptide antibodies were eluted at low pH with 100 mM glycine, pH 2.8 (arrow).

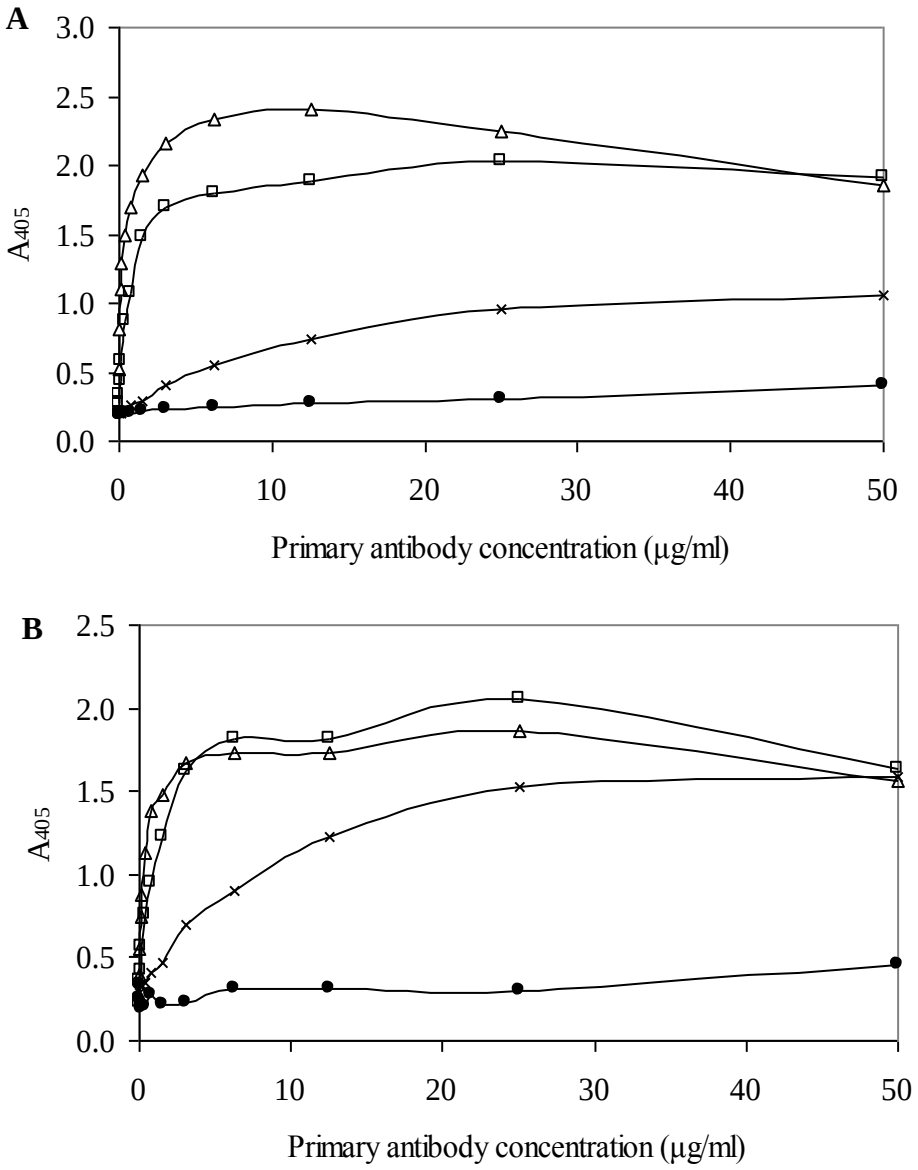


Figure 5.6. Analysis of anti-peptide FKLGSCYLYIINRNLKEI antibodies before and after affinity purification. ELISA plates were coated with peptide FKLGSCYLYIINRNLKEI (A) and CFKLGSCYLYIINRNLKEI (B) at 1 µg/ml. Affinity purified anti-peptide FKL IgY (Δ), affinity purified anti-peptide KEI IgY (□), non-purified anti-peptide FKL IgY and non-purified anti-peptide KEI IgY (x) and non-immune control IgY (●) were diluted from 50 µg/ml to 0.048 µg/ml with serial doubling dilutions. The absorbance readings at 405 nm represent the average of duplicate experiments.

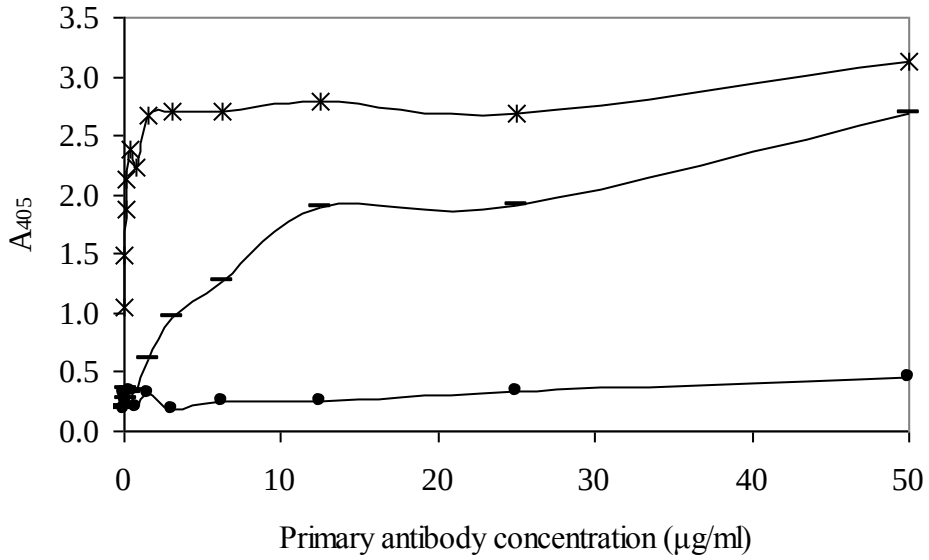


Figure 5.7. Analysis of anti-peptide SDDDNRQIQDFEC antibodies before and after affinity purification. ELISA plates were coated with peptide SDDDNRQIQDFEC at 1 µg/ml. Affinity purified anti-peptide SDDDNRQIQDFEC IgY (ж), non-purified anti-peptide SDDDNRQIQDFEC IgY (▬) and pre-immune control IgY (●) were diluted from 50 µg/ml by serial doubling dilutions to 0.048 µg/ml. The absorbance readings at 405 nm represent the average of duplicate experiments.

5.3.2 Anti-*Plasmodium* lactate dehydrogenase antibodies

Antibodies against *Plasmodium* lactate dehydrogenase (*Pf*LDH) were raised in chickens (Achilonu, 2008; Hurdayal *et al*., 2010). Chickens were immunized with peptide APGKSDKEWNRDDLC coupled to rabbit albumin. The peptide sequence corresponds to a amino acid residues 85-98 of *P. falciparum* lactate dehydrogenase (LDH) (UniProtKB/Swiss-Prot primary accession Q27743). This peptide sequence appears conserved in all *Plasmodium* LDH's, thus making the anti-peptide antibodies produced against it, a useful control, irrespective of the strain of *Plasmodium* being used. *Pf*LDH appears to be expressed at much higher levels than PFC0760c (Chapter 3, Le Roch *et al*., 2003), and therefore it was decided to use *Pf*LDH as a positive control for immunological studies performed with the anti-peptide FKLGSCYLYIINRNLKEI, CFKLGSCYLYIINRNLKEI and SDDDNRQIQDFEC antibodies.

5.3.3 Localisation of native *P. yoelii yoelii* protein, PY05757 and LDH with immunofluorescence

The affinity purified anti-peptide antibodies were used in an immunofluorescence assay to probe for the native PY05757 protein in mouse red blood cells infected with *P. yoelii yoelii* parasites (Figure 5.8 A-D). As a control, native *P. yoelii yoelii* LDH was probed for with anti-*Pf*LDH antibodies (Figure 5.8 D). Green fluorescence is a result of the fluorescein isothiocyanate (FITC) and blue fluorescence due to the staining of parasite nuclear material with 4, 6-diamidino-2-phenylindole (DAPI). The antibodies produced to detect PY0575, and those produced to detect *Plasmodium* LDH, both appeared to detect proteins associated with the parasite. Fluorescence resulting from the antibody produced against PY05757 was seen predominantly in parasites that appear to be in the ring, trophozoite and schizont stages of the parasite life cycle. This observation compares well to the mRNA expression values obtained by Le Roch *et al*. (2003) for PFC0760c, which has high sequence similarity to PY05757. The mRNA for PFC0760c appears to be expressed at its highest levels during the trophozoite and schizont stages of the life cyle (Figure 3.3) (Le Roch *et al*., 2003). The anti-LDH antibodies detected a protein that appeared to be associated with the parasite cytoplasm (Figure 5.8 D) as an even distribution of green fluorescence was observed. Although the fluorescence resulting from the anti-peptide CFKLGSCYLYIINRNLKEI, FKLGSCYLYIINRNLKEI and SDDDNRQIQDFEC IgY's also produced an even green fluorescence throughout the cytoplasm, the fluorescence appeared to be more intense in the nucleus of the parasite.

Slides probed with anti-peptide CFKLGSCYLYIINRNLKEI IgY's were observed with confocal microscopy (Figure 5.9) to obtain a better insight into the possible location of the protein being detected with the antibodies. As observed before, an incessant fluorescent signal was observed throughout the cytoplasm in addition to the intense signal observed on the nucleus of the parasite. These results suggest that the protein is associated with both the parasite nuclear material and the parasite cytoplasm. This compares favourably with the sub cellular location predictions performed (Section 3.3.5). However, co-localisation studies would be required before any definite conclusions can be drawn.

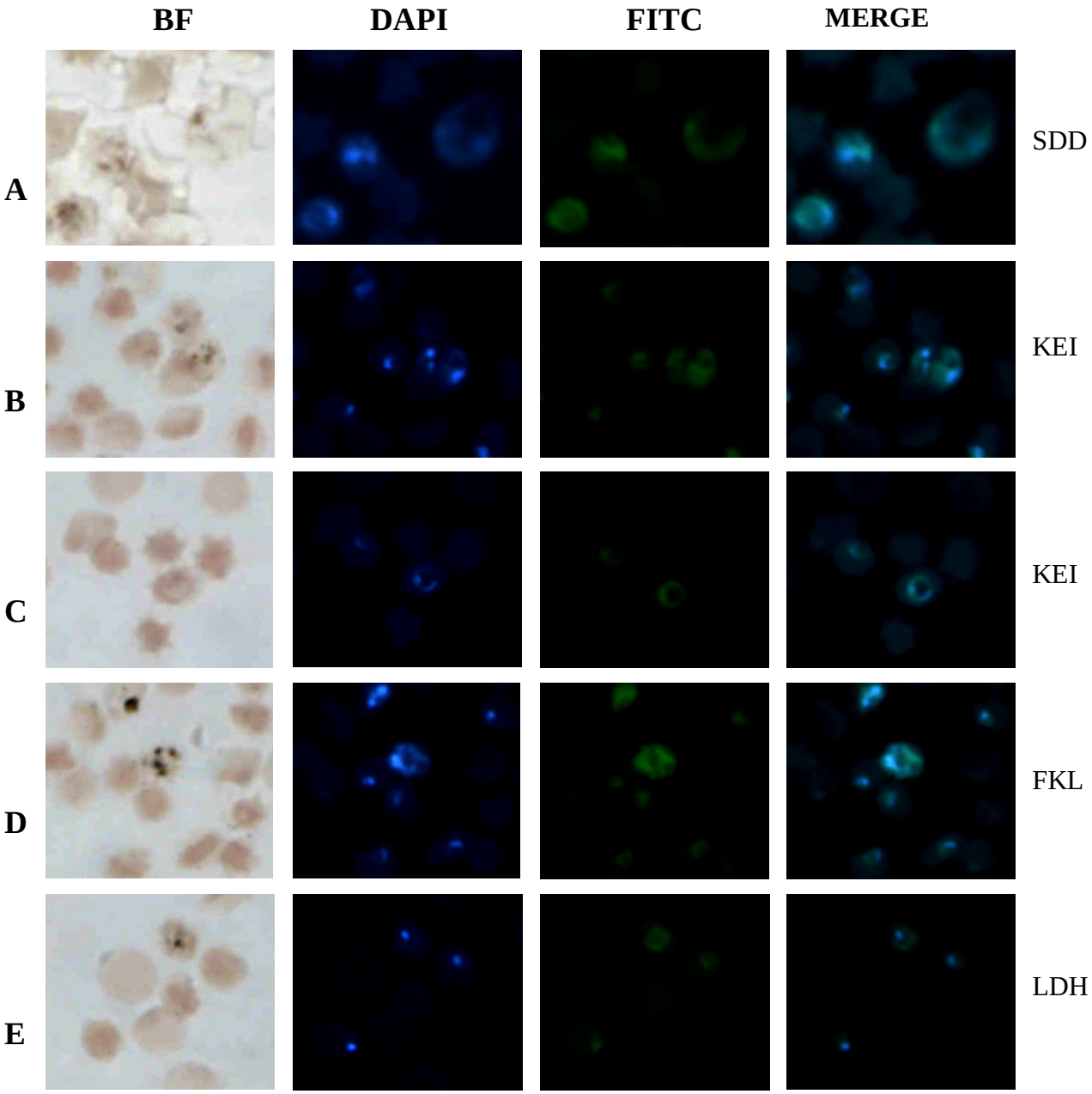


Figure 5.8. Immunofluorescence images of the native *P. yoelii yoelii* protein, PY05757 and *P. yoelii yoelii* lactate dehydrogenase in *P. yoelii yoelii* parasitized red blood cells. *P. yoelii yoelii* infected mouse red blood cells were probed with anti-peptide SDDDNRQIQDFEC IgY (SDD) (A), anti-peptide CFKLGSCYLYIINRNLKEI IgY (KEI) (B and C) and anti-peptide FKLGSCYLYIINRNLKEI IgY (FKL) (D) to detect native PY05757, and with anti-peptide APGKSDKEWNRDDLC IgY (LDH) (E) to detect native *P. yoelii yoelii* lactate dehydrogenase. The anti-peptide antibodies were detected with a donkey anti-chicken antibody conjugated to fluorescein isothiocyanate (FITC). Parasite DNA was stained with 4, 6-diamidino-2-phenylindole (DAPI) (5 µg/ml).

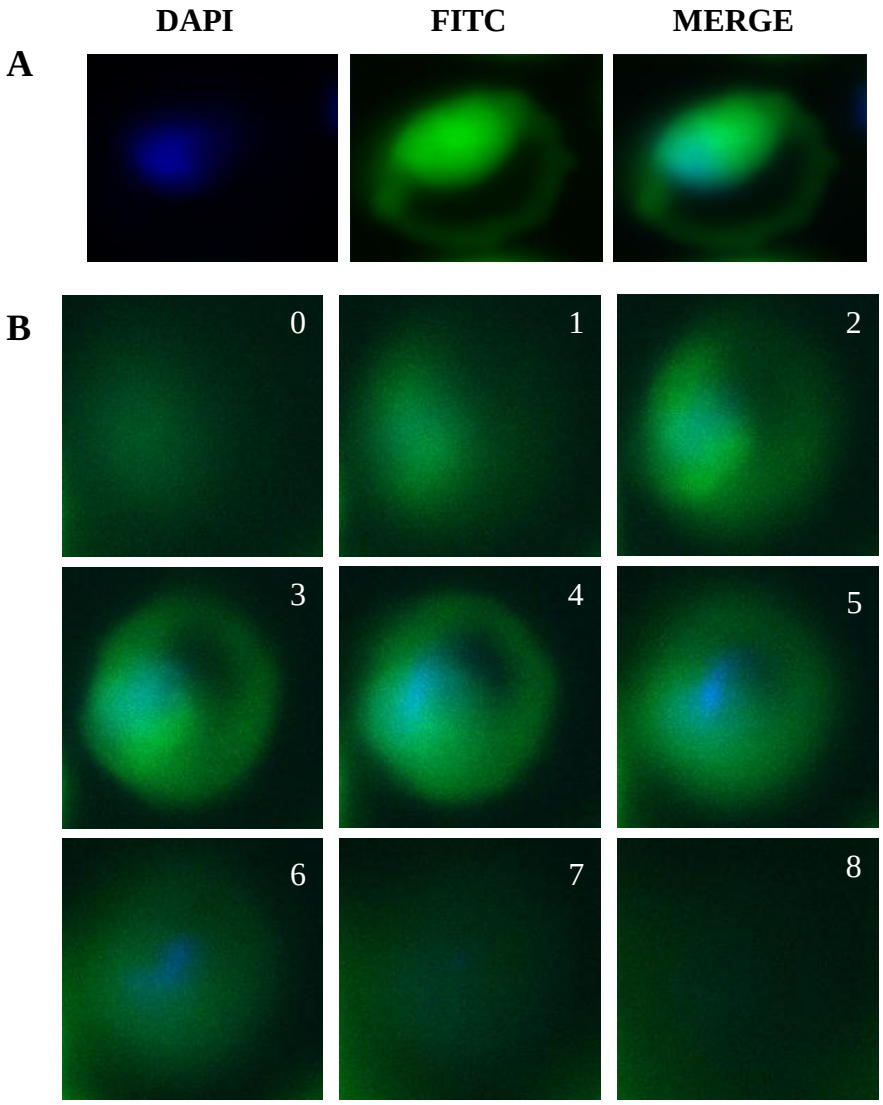


Figure 5.9. Confocal immunofluorescence microscopy of a *P. yoelii yoelii* infected red blood cell to show the location of the protein PY05757. (A) Confocal image of an infected red blood cell probed with anti-peptide CFKLGSCYLYIINRNLKEI chicken antibodies (10 μg/ml). (B) Merged images of a Z-stack progression 0 to 8 through an infected red blood cell, probed with anti-peptide CFKLGSCYLYIINRNLKEI chicken antibodies (10 μg/ml). The anti-peptide antibodies were detected with a donkey anti-chicken antibody conjugated to fluorescein isothiocyanate (FITC). Parasite DNA was stained with 4, 6-diamidino-2-phenylindole (DAPI) (5 μg/ml).

5.3.4 Western blotting of parasite material

Mouse red blood cells infected with mouse strains of *Plasmodium* (*P. yoelii yoelii*; *P. yoelii yoelii* chloroquinine resistant; *P. chabaudi chabaudi*; *P. berghei*) were lysed open (Section 2.28) and the material was analysed on SDS-PAGE and transferred electrophoretically to nitrocellulose (Section 2.4 and 2.6).

The nitrocellulose was probed with anti-*Pf*LDH antibodies (Figure 5.10). Detecting *Plasmodium* proteins on the gel proved difficult as the banding pattern of the proteins observed on the gel for the uninfected control blood sample appeared identical to the banding pattern of the blood samples infected with mouse strains of *Plasmodium* (Figure 5.10 A). The anti-*Pf*LDH antibodies detected a protein band in both *P. yoelii yoelii* samples (arrow, Figure 5.10 B) in the region of 37 kDa, which is close to the predicted size for *P. yoelii yoelii* LDH of 34 kDa (http://www.plasmodb.org/). No LDH was detected in samples infected with *P. berghei* or *P. chabaudi chabaudi*, or in the uninfected control sample. A second protein band was detected at 26 kDa with the anti-*Pf*LDH antibodies. This band was also evident in the uninfected sample. The anti-peptide FKLGSCYLYIINRNLKEI and CFKLGSCYLYIINRNLKEI antibodies did not detect any *Plasmodium* proteins in western blot analysis, even when the antibody concentration was increased as high as 25 μg/ml and 50 μg/ml (result not shown).

The anti-peptide SDDDNRQIQDFEC antibodies were used to probe for *P. yoelii yoelii* protein PY05757 (Figure 5.11 B) on a western blot of *P. yoelii yoelii* infected red blood cells. The anti-peptide SDDDNRQIQDFEC antibodies detected multiple protein bands. The predicted size of protein PY05757 is 178 kDa (http://www.plasmodb.org/). The antibodies detected a protein of 178 kDa in the soluble fraction of the *P. yoelii yoelii* infected (lane 3, Figure 5.11 B) red blood cells, and not in the membrane bound fraction (lane 4). This results correlates well with the transmembrane domain prediction result, which predicted that PY05757 was not membrane bound (Chapter 3). The anti-peptide SDDDNRQIQDFEC antibodies detected protein bands of 250 kDa (lanes 3 and 4), 218 kDa (lane 3), 201 kDa (lane 3), 153 kDa (lanes 2, 3 and 4), and 85 kDa (lanes 3 and 4).

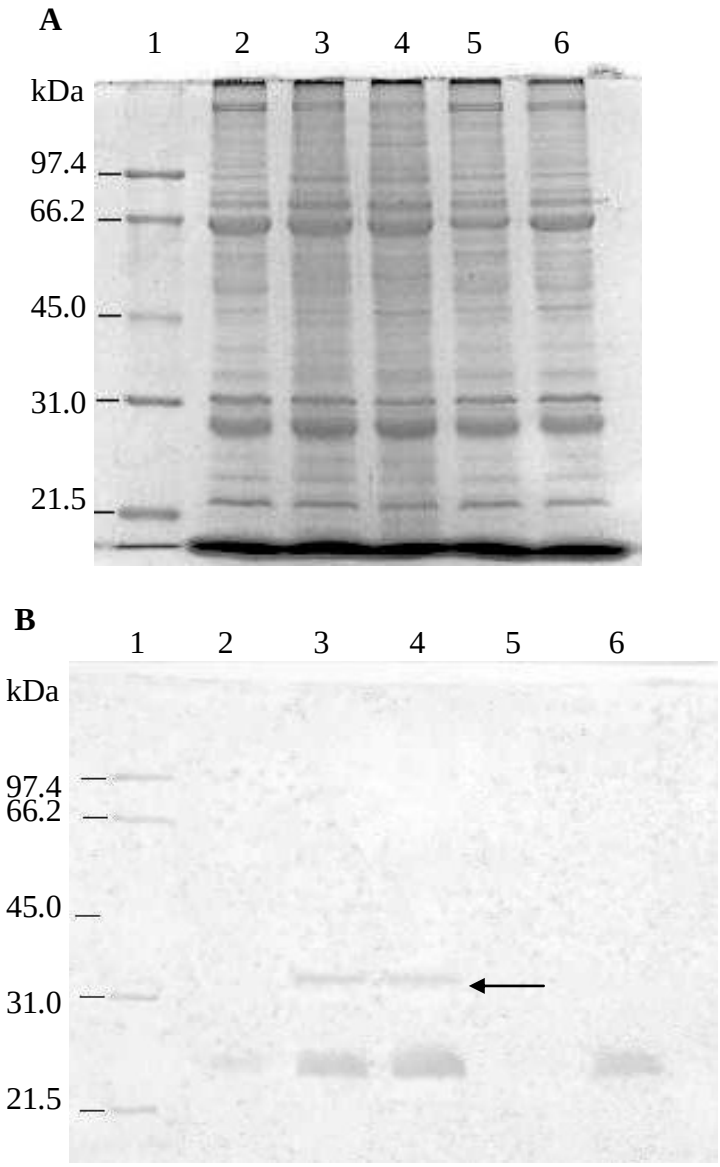


Figure 5.10. Western blotting of *P. yoelii yoelii* LDH. *Plasmodium*-infected mouse red blood cells were analysed on a 10% SDS-PAGE gel (A), and probed with anti-LDH IgY at 10 μg/ml with western blotting (B). Lane 1, Bio-Rad low molecular weight marker; lane 2, Control (uninfected mouse blood); lane 3, *P. yoelii yoelii* infected blood; lane 4, *P. yoelii yoelii* (chloroquinine resistant strain) infected blood; lane 5, *P. berghei* infected blood; lane 6, *P. chabaudi chabaudi* infected blood. The arrow indicates the bands corresponding to *P. yoelii yoelii* LDH.

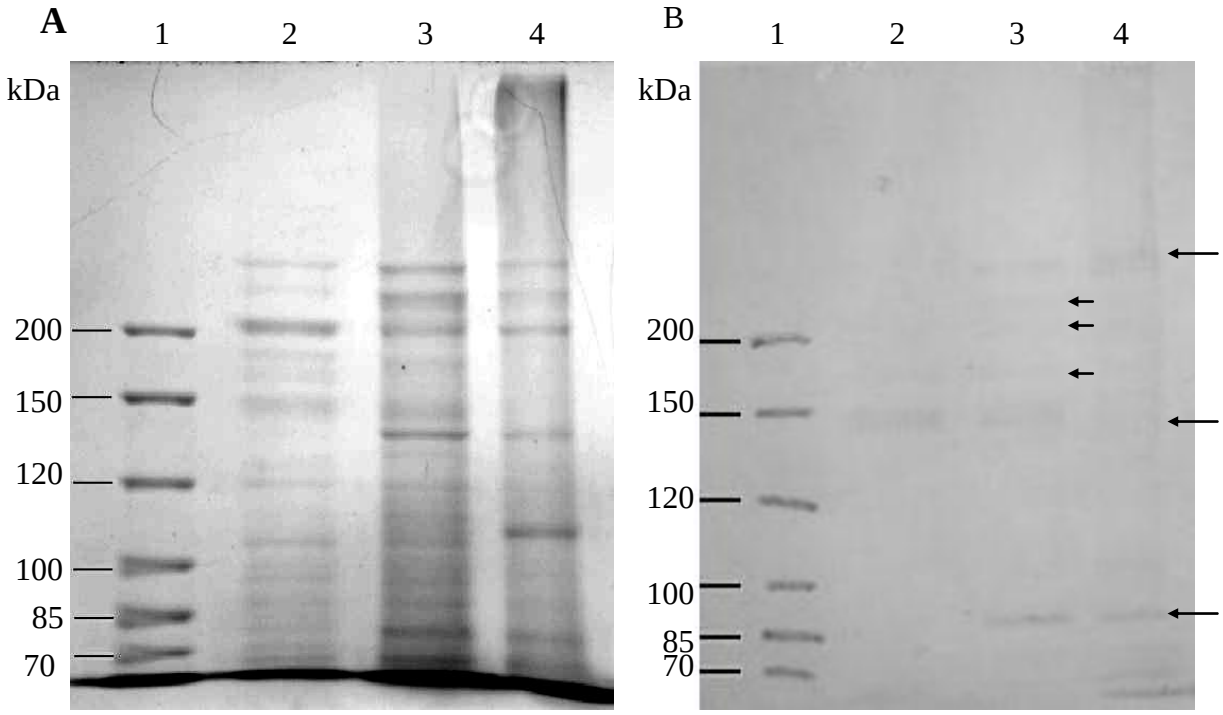


Figure 5.11. Western blotting of *P. yoelii yoelii* protein PY05757. Lysed blood samples from *P. yoelii yoelii* infected mice were analysed on a 5% SDS-PAGE gel (A) and probed with purified anti-peptide SDDDNRQIQDFEC IgY at 10 µg/ml in a western blot (B). Lane 1, Fermentas PageRulerTM Unstained Protein Ladder, lane 2, control (uninfected mouse blood), lane 3, *P. yoelii yoelii* infected blood (soluble fraction), lane 4, *P. yoelii yoelii* infected blood (membrane fraction). The arrows indicate positions on the nitrocellulose where protein bands were detected by the anti-peptide SDDDNRQIQDFEC IgY.

5.3.5 Purification of human anti-malaria antibodies

A pool of human anti-malaria IgG's was passed through an affinity matrix coupled with the peptide for the *P. falciparum* protein, PFC0760c (peptide FKLGSCYLYIINRNLKEI) (Figure 5.12). A low yield (445 µg) of human anti-peptide FKLGSCYLYIINRNLKEI antibodies eluted after 7-8 ml of 100 mM glycine, pH 2.8. The same pool of human anti-malaria IgG's was passed through an affinity matrix coupled with a peptide derived from *P. falciparum* lactate dehydrogenase (*Pf*LDH) (peptide APGKSDKEWNRDDLC). Elution from this matrix yielded 841 µg/ml of human anti-peptide APGKSDKEWNRDDLC antibodies after 6-8 ml of 100 mM glycine, pH 2.8.

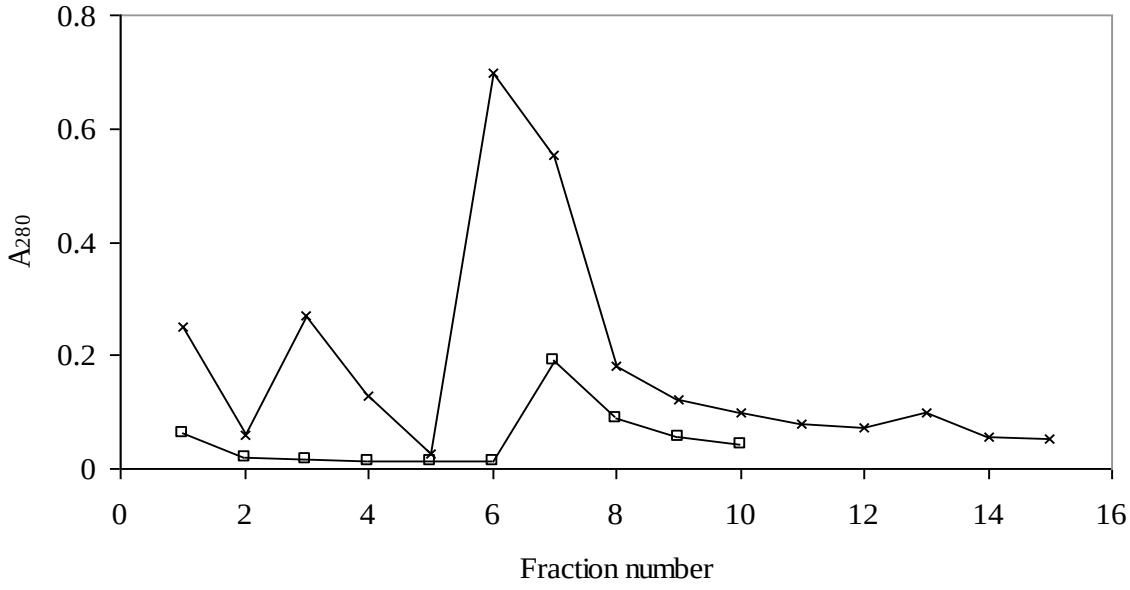


Figure 5.12. Affinity purification of anti-peptide antibodies from a pool of sera from malaria patients. Human antibodies recognising peptide CFKLGSCYLYIINRNLKEI (*P. falciparum* protein, PFC0760c) (□) and peptide APGKSDKEWNRDDLC (*P. falciparum* LDH) (x), from a pool of antibodies from human malaria patients.

5.4 Discussion

Choosing a peptide for raising antibodies can be problematic as *Plasmodium* proteins are lysine rich (Flick *et al.*, 2004; Vedadi *et al.*, 2007). A peptide with many lysine residues would more than likely result in non-specific antibodies that react with multiple *Plasmodium* proteins due to the recognition of lysine residues. It was therefore difficult to choose peptide sequences using the parameters from Predict 7 as the peptide regions that were predicted to have high antigenicity, almost always contained multiple lysine residues. One peptide selected contained two lysine residues in the peptide sequence (FKLGSCYLIINRNLKEI) (Figure 5.2), although Predict 7 did not predict the peptide to be particularly antigenic. A second peptide was selected without any lysine residues (SDDDNRQIQDFE) (Figure 5.1). Anti-peptide antibodies were successfully raised against both peptides in this study.

The ELISA results indicated that the chickens produced antibodies to all of the peptides and the carrier proteins immunised (Figures 5.3 and 5.4). One chicken immunized with the peptide FKLGSCYLYIINRNLKEI-rabbit albumin conjugate, did not produce antibodies against the peptide. The same chicken produced anti-rabbit albumin antibodies,

although there were less antibodies produced in comparison to the antibodies from the three other chickens. Both chickens immunised with the FKLGSCYLIINRNLKEI peptide were immunised from the same rabbit albumin peptide conjugate, therefore it is unlikely that the difference in antibody production between the two chickens was due to the immunogen. This result emphasized the need to immunize more than one chicken during immunization schedules, as it is often observed that several animals immunized with the same dose of immunogen and under the same conditions can produce antiserum with completely different characteristics (Harlow and Lane, 1988; van Regenmortel *et al.*, 1989).

The anti-peptide antibodies produced were pooled and affinity purified (Figure 5.5). The highest yield of purified antibodies (12 mg) was obtained with the CFKLGSCYLIINRNLKEI peptide column and respective antibody pool. The lowest yield of purified antibodies (6 mg) was obtained with the FKLGSCYLIINRNLKEI peptide column and the respective antibody pool. This could possibly be due to better coupling being achieved with the methods used for the CFKLGSCYLIINRNLKEI peptide. The methods used to couple the peptide FKLGSCYLIINRNLKEI would not have been as successful due to the internal lysine residue. The peptide would have been coupled via either or both of the lysine residues. It may have been beneficial to pass the anti-peptide FKLGSCYLIINRNLKEI antibody pool over the CFKLGSCYLIINRNLKEI peptide column, and vice versa to determine if any further antibodies could be purified.

The purified antibodies were compared to non-purified antibodies, and in all three cases, the affinity purified antibodies had a higher specificity for the peptide at a lower concentration of antibody in the ELISAs (Figure 5.6 and Figure 5.7). This would be due to the affinity purification having removed non-specific antibodies, and concentrated the specific anti-peptide antibodies.

Anti-peptide antibodies raised against *Plasmodium* proteins in chickens, have successfully been used to detect native *Plasmodium* proteins in western blots (Merckx *et al.*, 2003; Dorin *et al.*, 2005; Reininger *et al.*, 2005), immunofluorescence microscopy (Dorin *et al.*, 2005; Nunes *et al.*, 2007) and have been used to immunoprecipitate native *Plasmodium* proteins (Merckx *et al.*, 2003; Nunes *et al.*, 2007). The purified antibodies produced in this study were used to detect the native *P. yoelii yoelii* protein PY05757 with immunofluorescence (Figure 5.8 and Figure 5.9) and western blotting (Figure 5.11).

Antibodies produced against *Plasmodium* LDH were used as a positive control in both the immunofluorescence assay (Figure 5.8 and Figure 5.9) and for the western blotting (Figure 5.10). All the antibodies detected protein in the immunofluorescence assay. This provides evidence that a native *P. yoelii yoelii* protein containing the epitopes FKLGSCYLYIINRNLKEI and SDDDNRQIQDFEC is expressed in the *P. yoelii yoelii* life cycle. The protein detected by the anti-peptide antibodies appeared to be associated with the parasite cytoplasm and nuclear material, although this could not be confirmed in the present study. Co-localisation studies would need to be undertaken with antibodies against proteins known to be associated with the parasite nucleus and cytoplasm to confirm this. The protein appeared to be detected predominantly in parasites in the ring and schizont stages of the parasite life cycle. This correlates well with the predicted expression profile of the *P. falciparum* protein PFC0760c, which has high sequence similarity to PY05757 (Chapter 3, Le Roch *et al.*, 2003). However, the slides were prepared from blood with mixed stages of the life cycle, therefore it would be necessary to synchronise parasites and repeat the immunofluorescence assay for each stage of the life cycle to confirm the observation made.

The FITC fluorescent signal observed was weak and thus alternative fluorophore conjugates could be used to improve the immunofluorescence images. FITC photobleaches rapidly, making the capture of images of low-abundance proteins difficult, as these samples require a longer exposure time. FITC also tends to quench after only a few fluorophores have been attached to the antibody, resulting in a weak signal. Alexa Fluor® 488 dye from Invitrogen (http://www.invitrogen.com/site/us/en/home.html, 2010) produces a much brighter signal that is far more photostable than FITC. During conjugation of the Alexa Fluor® 488 dye to antibody, a far greater number of fluorophores attach before quenching, thereby creating a brighter conjugate that requires far less to be used to obtain optimal results. The stability of the conjugate also allows for longer observation and image capture time, which is beneficial for low abundance proteins.

The anti-peptide SDDDNRQIQDFEC antibodies in the western blot detected multiple protein bands (Figure 5.11). One band of particular interest was detected at 177 kDa. This corresponds very closely to the predicted molecular mass of PY05757 of 178 kDa (http://www.plasmodb.org). The anti-peptide FKLGSCYLYIINRNLKEI and

CFKLGSCYLYIINRNLKEI antibodies did not detect any protein bands on the western blot. It is possible that the epitope FKLGSCYLYIINRNLKEI was not available to interact with the antibodies. The peptide was predicted to be located at the surface of the protein and hydrophilic only at the C-terminus of the peptide. Very little is known of the 3-dimensional structure of the protein and it is therefore possible that the epitope could be unavailable for interacting with the antibodies during reducing SDS-PAGE and electrophoretic transfer to nitrocellulose.

Antibodies from a pool of human anti-malarial antibodies bound to peptide CFKLGSCYLYIINRNLKEI when the pool was passed over peptide-bound SulfoLink® column (Figure 5.12). This result suggests that the hypothetical protein PFC0760c is expressed within the *P. falciparum* proteome. It is possible that the antibodies purified on the peptide CFKLGSCYLYIINRNLKEI column could be from the *P. vivax* protein Pv095365, as this protein had a sequence of amino acid residues that had 61% identity to the epitope FKLGSCYLYIINRNLKEI (Table 5.2). The antibody pool was from a number of infections and so therefore it is likely that there are antibodies to *P. falciparum*, *P. vivax*, and *P. ovale* among others (Goldring *et al*., 1992; Taylor *et al*., 1992). Greater yields of human antibodies binding to the LDH peptide column were purified in comparison to the peptide FKLGSCYLYIINRNLKEI column. This suggests that *Plasmodium* LDH is possibly a more abundant and a more immunogenic protein than PFC0760c and its homologues, thus more antibodies were purified from the same sample on the peptide APGKSDKEWNRDDLC affinity matrix than on the peptide FKLGSCYLYIINRNLKEI affinity matrix. This provides practical evidence of what was observed from the data of Le Roch *et al.* (2003), and is in agreement with the data (Chapter 3). These human antibodies could be used in western blots or immunofluorescence microscopy to determine if results similar to those observed with chicken antibodies are obtained.

In future, the anti-peptide antibodies could be used to immunoprecipitate the native proteins so that the native proteins could be further characterised. A *Plasmodium* lysate could be passed over an affinity matrix coupled with the anti-peptide antibodies, to obtain the native protein.

CHAPTER 6

General Discussion

Malaria is a serious disease caused by infection with *Plasmodium* parasites. There were an estimated 863 thousand deaths as a result of *Plasmodium* infections in 2008, with the majority of those deaths being in Africa (World Health Organisation, 2009). Measures to effectively control malaria infection are desperately needed. A vaccine against malaria would be the ideal way to prevent the devastating effects of the disease. With sequencing of the genomes of *P. falciparum* (Gardener *et al.*, 2002) and *P. yoelii yoelii* (Carlton *et al.*, 2002) and the development of the field of bioinformatics, it is possible to identify likely targets for vaccine candidates and to putatively characterise them. It is also possible to clone and recombinantly express vaccine targets allowing for their further study.

A 96 kDa *P. chabaudi adami* protein (*Pca* 96) has shown to produce a protective immune response in challenged mice (Wanidworanun *et al*., 1987), implying that *Pca* 96 might be a viable vaccine candidate. A human malaria orthologue to *Pca* 96 needs to be found in the proteomes of other *Plasmodium* species, to be considered as a human vaccine candidate.

In this study, the sequence of *Pca* 96 was used as a probe to find a *P. yoelli yoelli* and a *P. falciparum* protein equivalent to *Pca* 96. The PY05757 and PFC0760c genes appeared to be good candidates. BLAST searches and sequence alignments with *Pca* 96 revealed a sequence of 18 amino acids conserved (88% identity) across seven *Plasmodium* species. T-cell epitope predictions for both *Pca* 96 and PFC0760c, revealed possible T-cell epitopes, located within the highly conserved amino acid sequence. A helical wheel plot of the sequence indicated the sequence formed an alpha-helical structure. These results support the sequence to be of structural importance. As PFC0706c is of unknown function (Bowman and Horrocks, 2000) further characterisation of PFC0760c was deemed necessary. Bioinformatics data predicted PFC0760c to be a nuclear associated protein. This result was supported by the predicted presence of a protein domain usually found in proteins involved with the structural maintenance of chromosomes. This result suggests that PFC0760c could be involved with chromosome maintenance. In addition, PFC0760c was predicted to interact with a number of proteins involved in binding of DNA, RNA, chromatin, ATP or proteins with yeast-two hybrid predictions. Immunofluorescence studies

with anti-peptide antibodies specific for the conserved amino acid sequence, produced an intense fluorescent signal within the nucleus of *P. yoelii yoelii* infected mouse red blood cells, supporting a nuclear associated protein. However, immunofluorescence microscopy with *P. falciparum* infected human red blood cells needs to be performed to determine if a similar pattern is observed to that seen for *P. yoelli yoelii* infected red blood cells to confirm the result. Immunofluorescence microscopy with synchronized parasites would also provide practical evidence of the stages of the life cycle where PFC0760c is expressed and possibly even give a comparative indication of the levels at which it is expressed. Electron microscopy would provide an additional technique to further characterize the location of PFC0760c and PY05757 within *P. falciparum* and *P. yoelii yoelii* parasites respectively, however this was outside the scope of the present study.

In the present study, a number of proteins with known function were compared to PFC0760c, with respect to their mRNA expression levels (Le Roch *et al*., 2003) with the hope of this data giving a further indication of the role PFC0760c plays within the parasite life cycle. No obvious patterns for the selected proteins were detected. A computer algorithm to detect emerging patterns would be useful to help with this analysis however this was beyond the scope of the present study. An algorithm capable of aiding in the comparative analysis of mRNA expression levels would also allow the mRNA expression levels for PFC0760c to be compared to the mRNA expression levels of other *P. falciparum* genes that are expressed at low levels, to determine if there is any commonality. There is evidence of genes such as the *Rifin*, *Stevor* and *Var* genes being located together in particular areas of chromosomes and sharing similar functions (Cheng, *et al*., 1998). Thus, examining the genes neighbouring PFC0760c and their location on the chromosome may give further insight to the gene. A brief investigation into PFC0760c gene neighbours did not give any clues to the function of the gene.

An 822 bp region of PFC0760c was previously cloned into the pMALc-2X vector (Smallie, 2003). In the present study, the resulting 33 kDa protein was expressed as a MBP fusion protein (*Pf*33-MBP) and affinity purified. The next step that needs to be taken is to immunise mice with *Pf*33-MBP and MBP as a control and then challenge the mice with parasites. However, *Pf*33 is of *P. falciparum* origin and therefore may not result in protection against rodent malaria. The *P. chabaudi chabaudi* (PCAS_080770) or *P. yoelii*

yoelii (PY05757) genes that are the proposed equivalents of PFC0760c would need to be cloned and expressed. These proteins could then be used to immunise mice which would then be challenged with *P. chabaudi chabaudi* or *P. yoelii yoelii* parasites. Alternatively, the conserved peptide could be coupled to a carrier protein and used to immunised mice. The choice of adjuvant would be very important as different adjuvants can give very different protection results (Stoute *et al*., 1997). The CpG oligodeoxynucleotide adjuvant (Pinzon-Charry *et al*., 2010) might be one adjuvant that could be used. Challenge of these mice with parasites would provide answers to whether the conserved sequence is of immunological importance.

Currently there are two studies suggesting that PFC0760c could be of immunological importance. Scorza *et al* (2005) used a multi-epitope DNA vaccine, derived from a genomic *P. chabaudi adami* DNA expression library of 30 000 plasmids. The vaccine induced immunity in mice challenged with *P. chabaudi adami* DK. Vaccine sub-pools were identified and subjected to bioinformatic analysis, which revealed that many of the open reading frames were orthologues of predicted *P. falciparum* proteins. Many were also found to be conserved across three or more *Plasmodium* species. One of these open reading frames was for PFC0760c reinforcing the idea that PFC0760c could have a potential role in protecting against malaria. PFC0760c also made an appearance in the Villard *et al*. (2007) investigation to find vaccine candidates based on an alpha-helical coiled coil protein motif, again indicating the possibility of PFC0760c possibly having a role in protection against malaria

The *Pf*33-MBP could also be useful for coupling to a resin, over which parasite lysate could be passed. This experiment would give an indication of which parasite proteins interact with *Pf*33-MBP. This could also be repeated using the peptide coupled to resin. Characterisation of the proteins bound on the column due to interaction with *Pf*33-MBP or the peptide, by mass spectrometry or N-terminal sequencing, could give further insight to the possible role of PFC0760c, to confirm predicted protein-protein interactions.

Antibodies were successfully produced against the conserved peptide and shown to recognise native *P. yoelii yoelii* protein in immunofluorescence microscopy. These antibodies could provide useful for affinity purifying native PFC0760c from parasite lysate by passing lysate over a column coupled with purified anti-peptide antibodies. Having

access to the native protein would be invaluable for aiding in characterisation of the protein, with regards to structure, function and immunological importance. The native protein could also be used to raise antibodies in chickens, thereby providing polyclonal antibodies to the whole protein, which may be useful for detecting the protein in western blotting, and for further microscopy work.

REFERENCES

Achilonu, I. A. (2008). *Plasmodium yoelii* acetyl-CoA carboxylase: detection and characterisation of the recombinant biotinyl domain. PhD thesis. University of KwaZulu Natal, Pietermaritzburg.

Acosta, C. J., Galindo, C.M., Schellenberg, D., Aponte, J. J., Kahigwa, E., Urassa, H., Armstrong Schellenberg, J. R. M., Masanja, H., Hayes, R., Kitua, A.Y., Lwilla, F., Mshinda, H., Menendez, C., Tanner, M. and Alonso, P. L. (1999). Evaluation of the SPf66 vaccine for malaria control when delivered through the EPI scheme in Tanzania. *Tropical Medicine and International Health* 4, 368–376.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. And Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* 215, 403-410.

Álvarez, N., González, V. M. and Jiménez-Ruiz, A. (2002). Be careful with primers when screening your clones by polymerase chain reaction. *Analytical Biochemistry* 308, 189-191.

Amante, F. H., Crewther, P. E., Anders R. F. and Good M. F. (1997). A cryptic T cell epitope on the apical membrane antigen 1 of *Plasmodium chabaudi adami* can prime for an anamnestic antibody response: implications for malaria vaccine design. *The Journal of Immunology* 159, 5535-5544.

Baca, A. M. and Hol, W. G. J. (2000). Overcoming codon bias: a method for high-level overexpression of *Plasmodium* and other AT-rich parasite genes in *Escherichia coli*. *International Journal for Parasitology* 30, 113-118.

Baird, J. K., (2005). Effectiveness of antimalarial drugs. *The New England Journal of Medicine* 352 (15), 1565-1577.

Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. *Current Opinions in Biotechnology* 10, 411-421.

Bannister, L., and Mitchell, G. (2003). The ins, outs, and roundabouts of malaria. *Trends in Parasitology* 19, 209–213.

Bejon, P., Lusingu, J., Olotu, A., Leach, A., Lievens, M., Vekemans, J., Mshamu, S., Lang, T., Gould, J., Dubois, M., Demoitié, M., Stallaert, J., Vansadia, P., Carter, T., Njuguna, P., Awuondo, K. O., Malabeja, A., Abdul, O., Gesase, S., Mturi, N., Drakeley, C. J, Savarese, B., Villafana, T, Ballou, W. R., Cohen, J., Riley, E. M., Lemnge, M. M., Marsh, K., and von Seidlein, L. (2008). Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. *The New England Journal of Medicine* 359, 2521-2532.

Bertani, G. (1951). Studies on lysogenesis I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* 62, 293-300.

Bertani, G. (2004). Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. *Journal of Bacteriology* 186, 595-600.

Berwal, R., Gopalan, N., Chandel, K., Prasad, G. B. K. S. and Prakash, S. (2008). *Plasmodium falciparum*: enhanced soluble expression, purification and biochemical characterization of lactate dehydrogenase. *Experimental Parasitology* 120, 135-141.

Bharadwaj, A., Sharma, P., Joshi, S. K., Singh, B. and Chauhan, V. S. (1998). Induction of protective immune responses by immunization with linear multiepitope peptides based on conserved sequences from P*lasmodium falciparum* antigens. *Infection and Immunity* 66, 3232–3241.

Birkholtz, L., van Brummelen, A. C., Clark, K., Niemand, J., Maréchal, E., Llinás, M. and Louw, A. I. (2008). Exploring the functional genomics for drug target and therapeutics discovery in *Plasmodia*. *Acta Tropica* 105, 113-123.

Birnboim, H. C. and Doly, J. (1979). A rapid alkaline procedure for screening recombinant plasmid DNA. *Nucleic Acid Research* 7, 1513-1523.

Blum, H., Beier, H., and Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 8, 93-99.

Bowman, S. and Horrocks, P. (2000). Assessing the impact of *Plasmodium falciparum* genome sequencing. *Microbes and Infection* 2, 1479-1487.

Bowman, S., Lawson, D., Basham, D., Brown, D., Chillingworth, T., Churcher, C. M., Craig, A., Davies, R.M., Devlin, K., Feltwell, T., Gentles, S., Gwilliam, R., Hamlin, N., Harris, D., Holroyd, S., Hornsby, T., Horrocks, P., Jagels, K., Jassal, B., Kyes, S., McLean, J., Moule, S., Mungall, K., Murphy, L., Oliver, K., Quail, M. A., Rajandream, M., Rutter, S., Skelton, J., Squares, R., Squares, S., Sulston, J. E., Whitehead, S., Woodward, J. R., Newbold, C. and Barrell, B. G. (1999). The complete nucleotide sequence of chromosome 3 of *Plasmodium falciparum*. *Nature* 400, 532-538.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Brake, D. A., Long, C. A. and Weidanz, W. P. (1988). Adoptive protection against *Plasmodium chabaudi adami* malaria in athymic nude mice by a cloned T cell line. *The Journal of Immunology* 140, 1989-1993.

Brown, W. M., Yowell, C. A., Hoard, A., Vander Jagt, T. A., Hunsaker, L. A., Deck, L. M., Royer, R. E., Piper, R. C., Dame, J. B., Makler, M. T. and Vander Jagt, D. L. (2004). Comparative structural analysis and kinetic properties of lactate dehydrogenases from the four species of human malaria parasites. *Biochemistry* 43, 6219-6229.

Carlton, J. M., Angiuoli, S. V., Suh, B. B., Kooij, T. W., Pertea, M., Silva, J. C., Ermolaeva, M. D., Allen, J. E., Selengut, J. D., Koo, H. L., Peterson, J. D., Pop, M., Kosack, D. S., Shumway, M. F., Bidwell, S. L., Shallom, S. J., van Aken, S. E., Riedmuller, S. B., Feldblyum, T. V., Cho, J. K., Quackenbush, J., Sedegah, M., Shoaibi, A., Cummings, L. M., Florens, L., Yates, J. R., Raine, J. D., Sinden, R. E., Harris, M. A., Cunningham, D. A., Preiser, P. R., Bergman, L. W., Vaidya, A. B., van Lin, L. H., Janse, C. J., Waters, A. P., Smith, H. O., White, O. R., Salzberg, S. L., Venter, J. C., Fraser, C. M., Hoffman, S. L., Gardner, M. J. and Carucci, D. J. (2002). Genome sequence and comparative analysis of the model rodent malaria parasite *Plasmodium yoelii yoelii*. *Nature* 419, 512-519.

Cármenes, R. S., Freije, J. P., Molina, M. M., and Martin, J. M. (1989). Predict7, a program for protein structure prediction. *Biochemical and Biophysical Research Communications* 159, 687-693.

Casares, S. and Richie, T. L. (2009). Immune evasion by malaria parasites: a challenge for vaccine development. *Current Opinions in Immunology* 21, 1-10. doi:10.1016/j.coi.2009.05.015.

Cavacini, L. A., Long, C. A. and Weidanz, W. P. (1986). T-cell immunity in murine malaria: adoptive transfer of resistance to *Plasmodium chabaudi adami* in nude mice with splenic T cells. *Infection and Immunity* 52, 637-643.

Cavacini, L. A., Parke, L. A. and Weidanz, W. P. (1990). Resolution of acute malarial infections by T-cell-dependent non-antibody-mediated mechanisms of immunity. *Infection and Immunity* 58, 2946-2950.

Chattopadhyay, R., Conteh, S., Li, M., James, E. R., Epstein, J. E. and Hoffman, S. L. (2009). The effects of radiation on the safety and protective efficacy of an attenuated *Plasmodium yoelii* sporozoite malaria vaccine. *Vaccine* 27, 3675-3680.

Cheng, Q., Cloonan, N., Fischer, K., Thompson, J., Waine, G., Lanzer, M. and Saul, A. (1998). s*tevor* and *rif* are *Plasmodium falciparum* multicopy gene families which potentially encode variant antigens. *Molecular and Biochemical Parasitology* 97, 161-176.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G. and Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic acid Research* 31, 3497-3500.

Cho, H., Lee, Y., Chang, R. S., Hahm, M., Kim, M., Kim, Y. B., and Oh, Y. (2008). Maltose binding protein facilitates high-level expression and functional purification of the chemokines RANTES and SDF-1α from *Escherichia coli*. *Protein Expression and Purification* 60, 37-45.

Chowdhury, D. R., Angov, E. Kariuki, T. and Kumar, N. (2009). A potent malaria transmission blocking vaccine based on codon harmonized full length Pfs48/45 expressed in *Escherichia coli*. *PLoS ONE* 4, e6352 1-10.

Chui, L. W., King, R., Chow, E. Y. W. and Sim, J. (2004). Immunological response to *Mycobacterium avium* subsp. *paratuberculosis* in chickens. *The Canadian Journal of Veterinary Research* 68, 302–308.

Cinquin, O., Christopher, R. I. and Menz, R.I. (2001). A hybrid plasmid for expression of toxic malarial proteins in *Escherichia coli*. *Molecular and Biochemical Parasitology* 117, 245-247.

Coetzer, T. H. T. (1993). Type IV collagenase and cathepsins L and H: proteinases involved in tumour invasion, pp. 29. PhD thesis. University of Natal, Pietermaritzburg.

Cohen, S.T., Chang, A. C. Y. and Hsu, L. (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-Factor DNA. *Proceedings of the National Academy of Sciences* 69, 2110-2114.

Compton, S. J., and Jones, C. G. (1985). Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry* 151, 369-374.

Cortes, A. (2008). Switching *Plasmodium falciparum* genes on and off for erythrocyte invasion. *Trends in Parasitology* 24, 517-524.

de Koning-Ward, T. F., O'Donnell, R. A., Drew, D. R., Thomson, R., Speed, T. P. and Crabb, B. S. (2003). A new rodent model to assess blood stage immunity to the *Plasmodium falciparum* antigen merozoite surface protein 1_{19} reveals a protective role for invasion inhibitory antibodies. *The Journal of Experimental Medicine* 198, 869-875.

Dennison, C. (1999). *A guide to protein isolation*. Kluwer Publishers, Dordrecht.

di Guan C., Li P., Riggs P. D. and Inouye H. (1988). Vectors that facilitate the expression and purification of foreign peptides in *Escherichia coli* by fusion to maltose-binding protein. *Gene* 67, 21-30.

Dodoo, D., Aikins, A., Kusi, K. A., Lamptey, H., Remarque, E., Milligan, P., Bosomprah, S., Chilengi R., Osei, Y. D., Akanmori, B. D. and Theisen, M. (2008). Cohort study of the association of antibody levels to AMA 1, MSP 1_{19}, MSP 3 and GLURP with protection from clinical malaria in Ghanaian children. *Malaria Journal* 7, 142-152. doi:10.1186/1475-2875-7-142.

Dorin, D., Semblat, J-P., Poullet, P., Alano, P., Goldring, J. P. D., Whittle, C., Patterson, S., Chakrabarti, D. and Doerig, C. (2005). PfPK7, an atypical MEK-related protein kinase, reflects the absence of classical three-component MAPK pathways in the human malaria parasite *Plasmodium falciparum*. *Molecular Microbiology* 55, 184–196.

Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Research* 16, 6127–6145.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics* 82, 70-77.

Engvall, E., and Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA): Quantitative assay of immunoglobulin G. *Immunocytochemistry* 8, 871-874.

Flick, K., Ahuja, S., Chene, A., Bejarano, M. T. and Chen, Q. (2004). Optimized expression of *Plasmodium falciparum* erythrocyte membrane protein I domains in *Escherichia coli*. *Malaria Journal* 3, 50-58.

Flück, C., Smith, T., Beck, H., Irion, A., Betuela, I., Alpers, M. P., Anders, R., Saul, A., Genton, B. and Felger, I. (2004). Strain-specific humoral response to a polymorphic malaria vaccine. *Infection and Immunity* 72, 6300-6305.

Flueck, C., Frank, G., Smith, T., Jafarshad, A., Nebie, I., Sirima, S. B., Olugbile, S., Alonso, P., Tanner, M., Druilhe, P., Felger, I. and Corradin, G. (2009). Evaluation of two long synthetic merozoite surface protein 2 peptides as malaria vaccine candidates. *Vaccine* 27, 2653-2661.

Frevert, U. and Nardin, E. (2005). Arrest in the liver – a genetically defined malaria vaccine. *New England Journal of Medicine* 352, 1600-1602.

Gao, X., Yeo, K. P., Aw, S. S., Kuss, C., Iyer, J. K., Genesan, S., Rajamanonmani, R., Lescar, J., Bozdech, Z. and Preiser, P. R. (2008). Antibodies targeting the *Pf*RH1 binding domain inhibit invasion of *Plasmodium falciparum* merozoites. *PLoS Pathogens* 4, e1000104. doi:10.1371/journal.ppat.1000104.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S., Paulsen, I. T., James, K., Eisen, J. A., Rutherford, K., Salzberg, S. L., Craig, A., Kyes, S., Chan, M., Nene, V., Shallom, S. J., Suh, B., Peterson,

J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M. W., Vaidya, A. B., Martin, D. M. A., Fairlamb, A. H., Fraunholz, M. J., Roos, D. S., Ralph, S. A., McFadden, G. I., Cummings, L. M., Subramanian, G. M., Mungall, C., Venter, J. C., Carucci, D. J., Hoffman, S. L., Newbold, C., Davis, R. W., Fraser, C. M. and Barrell, B. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 419, 498-511.

Gassmann, M., Thömmes, P., Weiser, T. and Hübscher, U. (1990). Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. *The FASEB Journal* 4, 2528-2532.

Genton, B., Betuela, I., Felger, I., Al-Yaman, F., Anders, R. F., Saul, A., Rare, L., Baisor, M., Lorry, K., Brown, G. V., Pye, D., Irving, D. O., Smith, T. A., Beck, H. P. and Alpers, M. P. (2002). A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. *The Journal of Infectious Diseases* 185, 820-827.

Gillespie, P. J. and Hirano, T. (2002). SMC proteins. *Current Biology* 12, R513.

Girard, M. P., Reed, Z. H., Friede, M. and Kieny, M. P. (2007). A review of human vaccine research and development: Malaria. *Vaccine* 25, 1567-1580.

Goh, L. L., Loke, P., Singh, M. and Sim, T. S. (2003). Soluble expression of a functionally active *Plasmodium falciparum* falcipain fused to maltose-binding protein in *Escherichia coli*. *Protein Expression and Purification* 32, 194-201.

Goh, S. L., Goh, L. L. and Sim, T. S. (2005). Cysteine protease *falcipain* 1 in *Plasmodium falciparum* is biochemically distinct from its isozymes. *Parasitology Research* 97, 295-301.

Goldring, J. P. D., Brake, D. A., Cavacini, L. A., Long, C. A. and Weidanz, W. P. (1989). Cloned T cells provide help for malaria-specific polyclonal antibody responses. *European Journal of Immunology* 19, 559-561.

Goldring, J. P. D. and Coetzet, T. H. T. (2003). Isolation of chicken immunoglobulins (IgY) from egg yolk. *Biochemistry and Molecular Biology Education* 31, 185-187.

Goldring, J. D., Molyneux, M. E., Taylor, T., Wirima, J. and Hommel, M. (1992). *Plasmodium falciparum*: diversity of isolates from Malawi in their cytoadherence to melanoma cells and monocytes *in vitro*. *British Journal of Haematology* 81, 413-418.

Graves, P. M. and Gelband, H. (2006). Vaccines for preventing malaria (SPf66). *Cochrane Database of Systematic Reviews*. doi: 10.1002/14651858.CD005966.

Gregson, A. L., Oliveira, G., Othoro, C., Calvo-Calle, J. M., Thorton, G. B., Nardin, E. and Edelman, R. (2008). Phase I trial of an alhydrogel adjuvanted hepatitis b core virus-like particle containing epitopes of *Plasmodium falciparum* circumsporozoite protein. *PLoS ONE* 3, e1556.

Gruber, F. P. and Hartung, T. (2004). Alternatives to animal experimentation in basic research. *ALTEX* 21, Supplement 1, 3-31.

Grun, J. L. and Weidanz, W. P. (1981). Immunity to *Plasmodium chabaudi adami* in the B cell deficient mouse. *Nature* 290, 143-145.

Grun, J. L., Long, C. A. and Weidanz, W. P. (1985). Effects of splenectomy on antibody-independent immunity to *Plasmodium chabaudi adami* malaria. *Infection and Immunity* 48, 853-858.

Guda, C. (2006). pTARGET: a web server for predicting protein subcellular localization. *Nucleic Acids Research* 34, W210-W213.

Guda, C. and Subramaniam, S. (2005) TARGET: a new method for predicting protein subcellular localization in eukaryotes. *Bioinformatics* 21, 3963-3969.

Habeeb, A. F. S. A. (1972). Reaction of protein sulfhydryl groups with Ellman's reagent. *Methods in Enzymology* 25, 457-464.

Haldar, K., Mohandas, N., Samuel, B.U., Harrison, T., Hiller, N. L., Akompong, T. and Cheresh, P. (2002). Protein and lipid trafficking induced in erythrocytes infected by malaria parasites. *Cellular Microbiology* 4, 383-395.

Harlow, E., and Lane, D. (1988). *Antibodies: a Laboratory Manual*. Cold Spring Harbour, New York.

Harlow, E., and Lane, D. (1999). *Using Antibodies: a Laboratory Manual*. Cold Spring Harbour, New York.

Hay, S. I., Okiro, E. A., Gething, P. W., Patil, A. P., Tatem, A. J., Guerra, C. A. and Snow, R. W. (2010). Estimating the global clinical burden of *Plasmodium falciparum* malaria in 2007. *PLoS Medicine* 7, e1000290. doi:10.1371/journal.pmed.1000290.

Hermanson, G. T. (1996). *Bioconjugate Techniques*. (Hermanson, G. T., Ed.) Academic Press, San Diego.

Hermsen, C. C., Verhage, D. F., Telgt, D. S. C., Teelen, K., Bousema, J. T., Roestenberg, M., Bolad, A., Berzins, K., Corradin, G., Leroy, O., Theisen, M. and Sauerwein, R. W. (2007). Glutamate-rich protein (GLURP) induces antibodies that inhibit *in vitro* growth of *Plasmodium falciparum* in a phase 1 malaria vaccine trial. *Vaccine* 25, 2930–2940.

Hiller, N. L., Bhattacharjee, S., van Ooij, C., Liolios, K., Harrison, T., Lopez-Estrano, C. and Haldar, K. (2004). A host-targeting signal in virulence proteins reveals a secretome in malarial infection. *Science* 306, 1934-1937.

Hisaeda, H., Stowers, A. W., Tsuboi, T., Collins, W. E., Sattabongkot, J. S., Suwanabun, N., Torii, M. and Kaslow, D. C. (2000). Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes. *Infection and Immunity* 68, 6618-6623.

Hoff, J. (2000). Methods of blood collection in the mouse. *Lab Animal* 29, 47-53.

Hoffman, S. L., Goh, L. M. L., Luke, T. C., Schneider, I., Le, T. P., Doolan, D. L., Sacci, J., de la Vega, P., Dowler, M., Paul, C., Gordon, D. M., Stoute, J. A., Church, L. W. P., Sedegah, M., Heppener, D. G., Ballou, W. R and Richie, T. L. (2002). Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *The Journal of Infectious Diseases* 185, 1155-1164.

Hopp, T. P., and Woods, K. R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proceedings of the National Academy of Sciences* 78, 3824-3828.

Hua, S. and Sun, Z. (2001). Support vector machine approach for protein subcellular localization prediction. *Bioinformatics* 17, 721-728.

Hudson, L. and Hay, F. C. (1989). *Practical immunology*. Blackwell Scientific Publications, London.

Hurdayal, R., Achilonu, I., Choveaux, D., Coetzer, T. H. T. and Goldring, J. P. D. (2010). Anti-peptide antibodies differentiate between plasmodial lactate dehydrogenases. *Peptides* 31, 525-532.

Huson, L. (2006). Antibody-mediated inhibition of proteases of African trypanosomes. PhD thesis. University of KwaZulu Natal, Pietermaritzburg.

Hviid, L. (2005). Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. *Acta Tropica* 95, 270-275.

Innis, M. A., Gelfand, D. H. (1990). Optimization of PCRs. In *PCR protocols: a guide to methods and applications*. (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 3-12. Academic Press Inc, San Diego.

Invitrogen. http://www.invitrogen.com/site/us/en/home.html. April 2010.

Ish-Horowicz, D. and Burke, J. F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acid Research* 9, 2989-2998.

Ivory, C. and Chadee, K. (2004). DNA vaccines: designing strategies against parasitic infections. *Genetic Vaccines and Therapy* 2, 17-24.

Janin, J., Wodak, S., Levitt, M., and Maigret, M. (1978). The conformation of amino acid side chains in proteins. *Journal of Molecular Biology* 125, 357-386.

Kaba, S. A., Price, A., Zhou, Z., Sundaram, V., Schnake, P., Goldman, I. F., Lal, A. A., Udhayakumar, V. and Todd, C. W. (2008). Immune responses of mice with different genetic backgrounds to improved multiepitope, multitarget malaria vaccine candidate antigen FALVAC-1A. *Clinical and Vaccine Immunology* 15, 1674-1683.

Kane, J. F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Current Opinion in Biotechnology* 6, 494-500.

Karplus, P. A., and Schulz, G. E. (1985). Prediction of chain flexibility in proteins. *Naturwissenschaften* 72, 212-213.

Kelley, L. A. and Sternberg, M. J. E. (2009). Protein structure prediction on the web: a case study using the Phyre server. *Nature Protocols* 4, 363-371.

Kester, K. E., Cummings, J. F., Ofori-Anyinam, O., Ockenhouse, C. F., Krzych, U., Moris, P., Schwenk, R., Nielsen, R. A., Debebe, Z., Pinelis, E., Juompam, L., Williams, J., Dowler, M., Stewart, V. A., Wirtz, R. A., Dubois, M., Lievens, M., Cohen, J., Ballou, W. R., Heppner, D. G. and the RTS,S Vaccine Evaluation Group. (2009). Randomized, double-blind, phase 2a trial of Falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naïve adults: safety, efficacy, and immunologic associates of protection. *The Journal of Infectious Diseases* 200, 337-346.

Kinyanjui, S. M., Mwangi, T., Bull, P. C., Newbold, C. I. and Marsh, K. (2004). Protection against clinical malaria by heterologous immunoglobulin G antibodies against malaria-infected erythrocyte variant surface antigens requires interaction with asymptomatic infections. *The Journal of Infectious Diseases* 190,1527–1533.

Kitagawa, T., and Aikawa, T. (1976). Enzyme coupled immunoassay of insulin using a novel coupling reagent. *Journal of Biochemistry* 79, 233-236.

Ko, Y. H., Thomas, P. J., Delannoy, M. R. and Pedersens, P. L. (1993). The cystic fibrosis transmembrane conductance regulator: overexpression, purification, and characterization of wild type and Δ508 mutant forms of the first nucleotide binding fold in fusion with the maltose binding protein. *The Journal of Biological Chemistry* 268, 24330-24338.

Krief, A., Letesson, J-J. and Billen, D. (2002). Comparison between 'IgY technology' from chickens and 'IgG technology' from mice production of tailor-made antibodies. *Tetrahedron Letters* 43, 1843-1846.

Kyte, J. and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 157, 105-132.

Labaied, M., Harupa, A., Dumpit, R. F., Coppens, I., Mikolajczak, S. A. and Kappe, S. H. I. (2007). *Plasmodium yoelii* sporozoites with simultaneous deletion of P52 and P36 are

completely attenuated and confer sterile immunity against infection. *Infection and Immunity* 75, 3758-3768.

LaCount, D. J., Vignali, M., Chettier, R., Phansalkar, A., Bell, R., Hesselberth, J. R., Schoenfeld, L. W., Ota, I., Sahasrabudhe, S., Kurschner, C., Fields, S. and Hughes, R. E. (2005). A protein interaction network of the malaria parasite *Plasmodium falciparum*. *Nature* 438, 103-107.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277, 680-685.

Langhorne, J., Ndungu, F. M., Sponaas, A. and Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nature Immunology* 9, 725-732.

Lavazec, C. and Bourgouin, C. (2008). Mosquito-based transmission blocking vaccines for interrupting *Plasmodium* development. *Microbes and Infection* 10, 845-849.

Le Roch, K. G., Zhou, Y., Blair, P. L., Grainger, M., Moch, J. K., Haynes, J. D., De la Vega, P., Holder, A. A., Batalov, S., Carucci, D. J. and Winzeler, E. A. (2003). Discovery of gene function by expression profiling of the malaria parasite life cycle. *Science* 301, 1503-1508.

Le, T. P., Coonan, K. M., Hedstrom, R. C., Charoenvit, Y., Sedegah, M., Epstein, J. E., Kumar, S., Wang, R., Doolan, D. L., Maguire, J. D., Parker, S. E., Hobart, P., Norman, J. and Hoffman, S. L. (2000). Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. *Vaccine* 18, 1893-1901.

Lesk, A. M. (2002). Introduction to bioinformatics. Oxford University Press, Oxford, United Kingdom.

Li, S. and Cronan, J. E. (1992). The gene encoding the biotin carboxylase subunit of *Escherichia coli a*cetyl-coA carboxylase. *The Journal of Biological Chemistry* 267, 855-863

Linding, R., Jensen, L. J., Diella, F., Bork, P., Gibson, T. J. and Russell, R. B. (2003). Protein disorder prediction: implications for structural proteomics. *Structure* 11, 1453-1459.

Luke, T. C. and Hoffman, S. L. (2003). Rationale and plans for developing a non-replicating, metabolically active, radiation-attenuated *Plasmodium falciparum* sporozoite vaccine. *The Journal of Experimental Biology* 206, 3803-3808.

Lusingu, J. P. A., Gesase, S., Msham, S., Francis, F., Lemnge, M., Seth, M., Sembuche, S., Rutta, A., Minja, D., Segeja, M. D., Bosomprah, S., Cousens, S., Noor, R., Chilengi, R. and Druilhe, P. (2009). Satisfactory safety and immunogenicity of MSP3 malaria vaccine candidate in Tanzanian children aged 12-24 months. *Malaria Journal* 8, 163-175.

Maina, C. V., Riggs, P. D., Grandea, A. G. III, Slatko, B. E., Moran, L. S., Tagliamonte, J. A., McReynolds, L. A. and di Guan, C. (1988). A vector to express and purify foreign proteins in *Escherichia coli* by fusion to, and separation from, maltose binding protein. *Gene* 74, 365-373.

Makobongo, M. O., Riding, G., Xu, H., Hirunpetcharat, C., Keough, D., de Jersey, J., Willadsen, P. and Good, M. F. (2003). The purine salvage enzyme hypoxanthine guanine xanthine phosphoribosyl transferase is a major target antigen for cell-mediated immunity to malaria. *Proceedings of the National Academy of Sciences* 100, 2628-2633.

Malkin, E. M., Durbin, A. P., Diemert, D. J., Sattabongkot, J., Wu, Y., Miura, K., Long, C. A., Lambert, L., Miles, A. P., Wang, J., Stowers, A., Miller, L. H. and Saul, A. (2005). Phase 1 vaccine trial of *Pvs*25H: a transmission blocking vaccine for *Plasmodium vivax* malaria. *Vaccine* 23, 3131-3138.

Marchler-Bauer, A. and Bryant, S. H. (2004). CD-Search: protein domain annotations on the fly. Nucleic Acids Research 32, Web Server issue W327–W331.

Marti, M., Baum, J., Rug, M., Tilley, L. and Cowman, A. F. (2005). Signal-mediated export of proteins from the malaria parasite to the host erythrocyte. *Journal of Cell Biology* 171, 587-592.

Marti, M., Good, R. T., Rug, M., Knuepfer, E. and Cowman, A. F. (2004). Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science* 306, 1930-1933.

Matambo, T. S., Odunuga, O. O., Boshoff, A. and Blatch, G. (2004). Overproduction, purification and characterization of the *Plasmodium falciparum* heat shock protein 70. *Protein Expression and Purification* 33, 214-222.

McNulty, D. E., Claffee, B. A., Huddleston, M. J. and Kane, J. F. (2003). Mistranslational errors associated with the rare arginine codon CGG in *Escherichia coli*. *Protein Expression and Purification* 27, 365-374.

Mehlin, C., Boni, E., Buckner, F. S., Engel, L. Feist, T., Gelb, M. H, Haji, L., Kim, D., Liu, C., Mueller, N., Myler, P. J., Reddy, J. T., Sampson, J. N., Subramanian, E., Van Voorhis, W. C., Worthey, E., Zucker, F. and Hol, W. G. J. (2006). Heterologous expression of proteins from *Plasmodium falciparum*: results from 1000 genes. *Molecular and Biochemical Parasitology* 148, 144-60.

Merckx, A., Le Roch, K., Nivez, M.P., Dorin, D., Alano, P., Gutierrez, G. J., Nebreda, A. R., Goldring, D., Whittle, C., Patterson, S., Chakrabarti, D., and Doerig, C. (2003). Identification and initial characterization of three novel cyclin-related proteins of the human malaria parasite *Plasmodium falciparum*. *The Journal of Biological Chemistry* 278, 39839–39850.

Merril, C. R. (2002). Gel staining techniques. In *Encyclopedia of life sciences*, volume 7. (S. Robertson, Ed.), pp. 584-588. Nature Publishing Group, London.

Mishell, B. B., Shiigi, S. M., Henry, C., Chan, E. L., North, J., Gallily, R., Slomich, M., Miller, K., Marbrook, J., Parks, D. and Good, A. H. (1980). Preparation of mouse cell suspensions. In *Selected methods in cellular immunology*. (B. B. Mishell, and S. M. Shiigi, Eds.), pp. 3-27.W. H. Freeman and Company, New York.

Möller, S., Croning, M. D. R. and Apweiler, R. (2001). Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics* 17, 646-653.

Mount, D. W. (2001). Bioinformatics: sequence and genome analysis. Cold Spring Harbor Laboratory Press, New York.

Mueller, A., Camargo, N., Kaiser, K., Andorfer, C., Frevert, U. and Matuschewski, K. (2005 a). *Plasmodium* liver stage developmental depletion of a protein at the parasite-host interface. *Proceedings of the National Academy of Sciences* 102, 3022-3027.

Mueller, A., Labaied, M., Kappe, S. H. I. and Matuschewski, K. (2005 b). Genetically modified *Plasmodium* parasites as a protective experimental malaria vaccine. *Nature* 433, 164-167.

Muench, S. P., Rafferty, J. B., Mcleod, R., Rice, D. W. and Prigge, S. T. (2003). Expression, purification and crystallization of the *Plasmodium falciparum* enoyl reductase. *Acta Crystallographica* D59, 1246-1248.

Mutabingwa, T. K. (2005). Artemisinin-based combination therapies (ACTs): best hope for malaria treatment but inaccessible to the needy! *Acta Tropica* 95, 305-315.

Nardin, E. H., Oliveira, G. A., Calvo-Calle, J. M., Wetzel, K., Maier, C., Birkett, A. J., Sarpotdar, P., Corado, M. L., Thornton, G. B. and Schmidt, A. (2004). Phase I testing of a

malaria vaccine composed of Hepatitis B virus core particles expressing *Plasmodium falciparum* circumsporozoite epitopes. *Infection and Immunity* 72, 6519–6527.

New England Biolabs pMAL™ Protein Fusion and Purification System Instruction Manual. http://www.neb.com/nebecomm/ManualFiles/manualE8000.pdf.

Nunes, M. C., Goldring, J. P. D., Doerig, C. and Scherf. A. (2007). A novel protein kinase family in *Plasmodium falciparum* is differentially transcribed and secreted to various cellular compartments of the host cell. *Molecular Microbiology* 63, 391–403.

Ockenhouse, C. F., Sun, P., Lanar, D. E., Wellde, B. T., Hall, B. T, Kester, K., Stoute, J. A., Magill, A., Kryzych, U., Farley, L., Wirtz, R. A., Sadoff, J. C., Kaslow, D. C., Kumar, S., Church, L. W. P., Crutcher, J. M., Wizel, B., Hoffman, S., Lalvani, A., Hill, A. V. S, Tine, J. A., Guito, K. P., de Taisne, C., Anders, R., Horii, T., Paoletti, E. and Ballou, W. R. (1998). Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-*Pf*7, a pox-vectored, multiantigen, multistage vaccine candidate for *Pasmodium falciparum* malaria. *The Journal of Infectious Diseases* 177, 1664-1673.

Ogutu, B. R., Apollo, O. J., McKinney, D., Okoth, W., Siangla, J., , Dubovsky, F., Tucker, K., Waitumbi1, J. N., Diggs, C., Wittes, J., Malkin, E., Leach, A., Soisson, L.A., Milman, J. B., Otieno, L., Holland, C. A., Polhemus, M., Remich, S. A., Ockenhouse, C. F., Cohen, J., Ballou, W. R., Martin, S. K., Angov, E., Stewart, V. A., Lyon, J. A., Heppner, D. G., Withers, M. R., for the MSP-1 Malaria Vaccine Working Group .(2009). Blood stage malaria vaccine eliciting high antigen-specific antibody concentrations confers no protection to young children in Western Kenya. *PLoS ONE* 4: e4708. doi:10.1371/journal.pone.0004708.

Oliveira, G. A., Wetzel, K., Calvo-Calle, J. M., Nussenzweig, R., Schmidt, A., Birkett, A., Dubovsky, F., Tierney, E., Gleiter, C. H., Boehmer, G., Luty, A. J. F., Ramharter, M., Thornton, G. B., Kremsner, P. G. and Nardin, E. H. (2005). Safety and enhanced immunogenicity of a Hepatitis B core particle *Plasmodium falciparum* malaria vaccine

formulated in adjuvant Montanide ISA 720 in a phase I trial. *Infection and Immunity* 73, 3587–3597

Outchkourov, N. S., Roeffen, W., Kaan, A., Jansen, J., Luty, A., Schuiffel, D., van Gemert, J. G., van de Vegte-Bolmer, M., Sauerwein, R. W. and Stunnenberg, H. G. (2008). Correctly folded Pfs48/45 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in mice. *Proceedings of the National Academy of Sciences* 105, 4301-4305.

Parker, S. E., Monteith, D., Horton, H., Hof, R., Hermandez, P., Vilalta, A., Hartikka, J., Hobart, P., Bentley, C. E., Chang, A., Hedstrom, R., Rogers, W. O., Kumar, S., Hoffman, S. L. and Norman, J. A. (2001). Safety of a GM-CSF adjuvant-plasmid DNA malaria vaccine. *Gene Therapy* 8, 1011-1023.

Patterson, R., Younger, J. S., Weigle, W. O. and Dixon, F. J. (1962). Antibody production and transfer to egg yolk in chickens. *The Journal of Immunology* 89, 272-278.

Peng, K., Radivojac, P., Vucetic, S., Dunker A. K. and Obradovic, Z. (2006). Length-dependent prediction of protein intrinsic disorder. *BMC Bioinformatics* 7, 208-224.

Perlmann, H., Berzins, K., Wahlgren, M., Carlsson, J., Björkman, A., Patarroyo, M. E. and Perlmann, P. (1984). Antibodies in malarial sera to parasite antigens in the membrane of erythrocytes infected with early asexual stages of *Plasmodium falciparum. Journal of Experimental Medicine* 159, 1686-1704.

Perlmann, P. and Troye-Blomberg, M. (2000). Malaria blood stage infection and its control by the immune system. *Folia Biologica* 46, 210-218.

Pinzon-Charry, A., McPhun, V., Kienzle, V., Hirunpetcharat, C., Engwerda, C., McCarthy, J. and Good, M. F. (2010). Low doses of killed parasite in CpG elicit vigorous $CD4^+$ T cell

responses against blood-stage malaria in mice. *The Journal of Clinical Investigation*, doi:10.1172/JCI39222.

Polson, A., Coetzer, T., Kruger, J., von Maltzahn, E., and van der Merwe, K. J. (1985). Improvements in the isolation of IgY from the yolks of eggs laid by immunised hens. *Immunological Investigations* 14, 323-327.

Polson, A., Potgieter, G. M., Largier, J. F., Mears, E. G. F., and Joubert, F. J. (1964). The fractionation of protein mixtures by linear polymers of high molecular weight. *Biochimica et Biophysica Acta* 82, 463-475.

Polson, A., von Wechmar, M. B., and Fazakerley, G. (1980). Antibodies to proteins from the yolk of eggs. *Immunological Communications* 9, 495-514.

Prato, S., Maxwell, T., Pinzon-Charry, A., Schmidt, C. W., Corradin, G. and Lopez, J. A. (2005). MHC class I-restricted exogenous presentation of a synthetic 102-mer malaria vaccine polypeptide. *European Journal of Immunology* 35, 681-689.

Pryor, K. D. and Leiting, B. (1997). High-level expression of soluble protein in *Escherichia coli* using a His6-tag and maltose-binding-protein double-affinity fusion system. *Protein Expression and Purification* 10, 309-319.

Purcell, L. A., Wong, K. A., Yanow, S. K., Lee, M., Spithill, T. W. and Rodriguez, A. (2008). Chemically attenuated *Plasmodium* sporozoites induce specific immune responses, sterile immunity and cross-protection against heterologous challenge. *Vaccine* 26, 4880-4884.

Rammensee, H., Bachmann, J., Emmerich, N. N., Bachor, O. A. and Stevanovic, S. (1999). SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50, 213-219.

Rasoloson, D., Lirong, S., Chong, C.R., Kafsack, B.F. and Sullivan, Jr., D.J. (2004). Copper pathways in *Plasmodium falciparum* infected erythrocytes indicate an efflux role for copper P-ATPase. *The Biochemical Journal* 381, 803-811.

Rathore, D., Nagarkatti, R., Jani, D., Chattopadhyay, R., de la Vega, P., Kumar, S. and McCutchan, T. F. (2005). An immunologically cryptic epitope of *Plasmodium falciparum* circumsporozoite protein facilitates liver cell recognition and induces protective antibodies that block liver cell invasion. *The Journal of Biological Chemistry* 280, 20524–20529.

Ravi, G., Ella, K. and Lakshmi Narasu, M. (2008). Development of pilot scale production process and characterization of a recombinant multiepitope malarial vaccine candidate FALVAC-1A expressed in *Escherichia coli*. *Protein Expression and Purification* 61, 57-64.

Read, S. M., and Northcote, D. H. (1981). Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Analytical Biochemistry* 116, 53-64.

Reichlin, M. (1980). Use of glutaraldehyde as a coupling agent for proteins and peptides. *Methods in Enzymology* 70, 159-165.

Reininger, L., Billker, O., Tewari, R., Mukhopadhyay, A., Fennell, C., Dorin-Semblat, D., Doerig, C., Goldring, D., Harmse, L., Ranford-Cartwright, L., Packer, J. and Doerig, C. (2005). A NIMA-related protein kinase is essential for completion of the sexual cycle of malaria parasites. *The Journal of Biological Chemistry* 280, 31957–31964.

Sacarlal, J., Aide, P., Aponte, J. J., Renom, M., Leach, A., Mandomando, I., Lievens, M., Bassat, Q., Lafuente, S., Macete, E., Vekemans, J., Guinovart, C., Sigaúque, B., Sillman, M., Milman, J., Dubois, M., Demoitié, M., Thonnard, J., Menéndez, C., Ballou, W. R., Cohen, J. and Alonso, P. L. (2009). Long-term safety and efficacy of the RTS,S/AS02A malaria vaccine in Mozambican children. *The Journal of Infectious Diseases* 200, 329-336.

Saluta, M. and Bell, P. A. (1998). Troubleshooting GST fusion protein expression in *E. coli*. *Life Sciences News* 1, 1-3. Amersham Pharmacia Biotech.

Sambrook, J. and Russell, D. W. (2001). *Molecular cloning. A laboratory manual*, third edition. (Sambrook, J. and Russell, D. W., Eds.). Cold Spring Harbor Laboratory Press, New York.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning. A laboratory manual*. (Sambrook, J., Fritsch, E. F. and Maniatis, T., Eds.). Cold Spring Harbor Laboratory Press, New York.

Saul, A. and Battistutta, D. (1988). Codon usage in *Plasmodium falciparum*. *Molecular and Biochemical Parisitology* 27, 35-42.

Sayers, J. R. and Price, H. P. (1995). AGA/AGG Codon usage in parasites: implications for gene expression in *Escherichia coli*. *Parisitology Today* 11, 345-346.

Schofield, L., Hewitt, M. C., Evans, K., Siomos, M. A. and Seeberger, P. H. (2002). Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature* 418, 785-789.

Schofield, L., Vivas, L., Hackett, F., Gerold, P., Schwarz, R. T. and Tachado, S. (1993). Neutralizing monoclonal antibodies to glycosylphosphatidylinositol, the dominant TNF-alpha-inducing toxin of *Plasmodium falciparum*: prospects for the immunotherapy of severe malaria. *Annals of Tropical Medicine and Parasitology* 87, 617-626.

Scopes, R. K. (1994). *Protein purification: Principles and practice*, third edition. pp. 350. Springer-Verlag, New York.

Scorza, T., Grubb, K., Smooker, P., Rainczuk, A., Proll, D. and Spithill, T. W. (2005). Induction of strain-transcending immunity against *Plasmodium chabaudi adami* malaria with a multiepitope DNA vaccine. *Infection and Immunity* 73, 2974-2985.

Sedegah, M., Jones, T. R., Kaur, M., Hedstrom, R., Hobart, P., Tine, J. A. and Hoffman, S. (1998). Boosting with recombinant vaccinia increases immunogenicity and protective efficacy of malaria DNA vaccine. *Proceedings of the National Academy of Sciences* 95, 7648-7653.

Sedegah, M., Weiss, W. W., and Hoffman, S. L. (2007). Cross-protection between attenuated *Plasmodium berghei* and *P. yoelii* sporozoites. *Parasite Immunology* 29, 559-565.

Shine, J. and Dalgarno, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proceedings of the National Academy of Sciences* 71, 1342-1346.

Smallie, T. I. (2003). Cloning and recombinant expression of a 822bp region of a Pf403 *Plasmodium falciparum* gene. MSc thesis. University of Natal, Pietermaritzburg.

Smith, D. B. and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene* 67, 31-40.

Snustad, D. P. and Simmons, M. J. (2000). Principles of genetics, second edition. pp. 536-537. John Wiley and Sons, Inc., New York.

Spring, M. D., Cummings, J. F., Ockenhouse, C. F., Dutta, S., Reidler, R., Angov, E., Bergmann-Leitner, E., Stewart, V. A., Bittner, S., Juompan, L., Kortepeter, M. G., Nielsen, R., Krzych, U., Tierney, E., Ware, L. A., Dowler, M., Hermsen, C. C., Sauerwein, R. W., de Vlas, S. J., Ofori-Anyinam, O., Lanar, D. E., Williams, J. L., Kester, K. E., Tucker, K., Shi, M., Malkin, E., Long, C., Diggs, C. L., Soisson, L., Dubois, M., Ballou, W. R., Cohen,

J., Heppner, D. G. (2009) Phase 1/2a study of the malaria vaccine candidate apical membrane antigen-1 (AMA-1) administered in adjuvant system AS01B or AS02A. *PLoS ONE* 4, e5254. doi:10.1371/journal.pone.0005254.

Stanley, S. L. (1998). Malaria vaccines: are seven antigens better than one? *The Lancet* 352,1163-1164.

Stoute, J. A., Slaoui, M., Heppner, D. G., Momin, P., Kester, K. E., Desmons, P., Wellde, B. T., Garçon, N., Krzych, U., Marchand, M., Ballou, W. R. and Cohen, J. D. (1997). A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. *The New England Journal of Medicine* 336, 86-91.

Stubbs, J., Simpson, K. M., Triglia, T., Plouffe, D., Tonkin, C. J., Duraisingh, M. T., Maier, A. G., Winzeler, E. A. and Cowman, A. F. (2005). Molecular mechanisms for switching of *P. falciparum* invasion pathways into human erythrocytes. *Science* 309, 1384-1387.

Sun, P., Schwenk, R., White, K., Stoute, J. A., Cohen, J., Ballou, W. R., Voss, G., Kester, K. E., Heppner, D. G. and Krzych, U. (2003). Protective immunity induced with malaria vaccine, RTS,S, is linked to *Plasmodium falciparum* circumsporozoite protein-specific $CD4^+$ and $CD8^+$ T cells producing IFN-γ. *The Journal of Immunology* 171, 6961-6967.

Sutherland, C. J. (2009). Surface antigens of *Plasmodium falciparum* gametocytes – a new class of transmission-blocking vaccine targets? *Molecular and Biochemical Parasitology* 166, 93-98.

Taylor, T. E., Molyneux, M. E., Wirima, J. J., Borgstein, A., Goldring, J. D. and Hommel, M. (1992). Intravenous immunoglobulins in the treatment of paediatric cerebral malaria. *Clinical and Experimental Immunology* 90, 357-362.

Tedder, P. M R., Bradford, J. R., McConkey, G. A., Bulpitt, A. J. and Westhead, D. R. (2010). PlasmoPredict: a gene function prediction website for *Plasmodium falciparum*. *Trends in Parasitology* 26, 107-110.

The national department of health malaria statistics. Accessed 13 May 2010. (http://www.doh.gov.za/issues/malaria/statistics.html)

Thorpe, R. (1994). Producing antibodies. In *Immunochemistry. LAB FAX* (M. A. Kerr, and R. Thorpe, Eds.), pp. 63-81. BIOS, Scientific publisher, UK.

Tine, J. A., Lanar, D. E., Smith, D. M., Wellde, B. T., Schultheiss, P., Ware, L. A., Kauffman, E. B., Wirtz, R. A., De Taisne, C., Hui, G. S. N., Chang, S. P., Church, P., Hollingdale, M. R., Kaslow, D. C., Hoffman, S., Guito, K. P., Ballou, W. R., Sadoff, J. C. and Paoletti, E. (1996). NYVAC-*Pf*7: a poxvirus-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *Infection and Immunity* 64, 3833-3844.

Tini, M., Jewell, U. R., Camenisch, G., Chilov, D. and Gassmann, M. (2002). Generation and application of chicken egg-yolk antibodies. *Comparative Biochemistry and Physiology Part A* 131, 569-574.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences* 76, 4350-4354.

Trask, D. K. and Muller, M. T. (1983) Biochemical characterization of topoisomerase I purified from avian erythrocytes. *Nucleic Acids Research* 11, 2779-2787.

Turgut-Balik, D., Akbulut, E., Shoemark, D. K., Celik, V., Moreton, K. M., Sessions, R. B., Holbrook, J. J. and Brady, L. (2004). Cloning, sequence and expression of the lactate dehydrogenase gene from the human malaria parasite, *Plasmodium vivax*. *Biotechnology letters* 26, 1051-1055.

Turgut-Balik, D., Shoemark, D. K., Moreton, K. M., Sessions, R. B. and Holbrook, J. J. (2001). Overproduction of lactate dehydrogenase from *Plasmodium falciparum* opens a route to new antimalarials. *Biotechnology Letters* 23, 917-921.

VanBuskirk, K. M., O' Neill, M. T., De La Vega, P., Maier, A. G., Krzych, Williams, J., Dowler, M. G., Sacci, J. B., Kangwanrangsan, N., Tsuboi, T., Kneteman, N. M., Heppner, D. G., Murdock, B. A., Mikolajczak, S. A., Aly, A. S. I., Cowman, A. F., Kappe, S. H. I. (2009). Preerythrocytic, live-attenuated *Plasmodium falciparum* vaccine candidates by design. *Proceedings of the National Academy of Sciences* 106, 13004-13009.

van der Heyde, H. C., Huszar, D., Woodhouse, C., Manning, D. D. and Weidanz, W. P. (1994). The resolution of acute malaria in a definitive model of B-cell deficiency, the JHD mouse. *The Journal of Immunology* 152, 4557-4562.

Van Regenmortel, M. H. (1989). Structural and functional approaches to the study of protein antigenicity. *Immunology Today* 10, 266-272.

Vedadi, M., Lew, J., Artz, J., Amani, M., Zhao, Y., Dong, A., Wasney, G. A., Gao, M., Hills, T., Brokx, S., Qiu, W., Sharma, S., Diassiti, A., Alam, Z., Melone, M., Mulichak, A., Wernimont, A., Bray, J., Loppnau, P., Plotnikova, O., Newberry, K., Sundararajan, E., Houston, S., Walker, J., Tempel, W., Bochkarev, A., Kozieradzki, I., Edwards, A., Arrowsmith, C., Roos, D., Kain, K. and Hui, R. (2007). Genome-scale protein expression and structural biology of *Plasmodium falciparum* and related Apicomplexan organisms. *Molecular and Biochemical Parasitology* 151, 100-110.

Villard, V., Agak, G. W., Frank, G., Jafarshad, A., Servis, C., Nébie, I., Sirima, S. B., Felger, I., Arevalo-Herrera, M., Herrera, S., Heitz, F., Bäcker, V., Druilhe, P., Kajava, A. V. and Corradin, G. (2007). Rapid identification of malaria vaccine candidates based on α-helical coiled coil protein motif. *PLoS ONE* 7, e645. doi:10.1371/journal.pone.0000645.

Vucetic, S., Obradovic, Z., Vacic, V., Radivojac, P., Peng, K., Iakoucheva, L. M., Cortese, M. S., Lawson, J. D., Brown, C. J., Sikes, J. G., Newton, C. D. and Dunker, A. K. (2005). Disprot: a database of protein disorder. *Bioinformatics* 21, 137-140.

Wallace, D. M. (1987). Precipitation of nucleic acids. *Methods in Enzymology* 152, 41-48.

Walther, M., Dunachie, S., Keating, S., Vuola, J. M., Berthoud, T., Schmidt, A., Maier, C., Andrews, L., Andersen, R. F., Gilbert, S., Poulton, I., Webster, D., Dubovsky, F., Tierney, E., Sarpotdar, P., Correa, S., Huntcooke, A., Butcher, G., Williams, J., Sinden, R. E., Thornton, G. B., Hill, A. V. (2005). Safety, immunogenicity and efficacy of a pre-erythrocytic malaria candidate vaccine, ICC-1132 formulated in Seppic ISA 720. *Vaccine* 23, 857-864.

Wanidworanun, C., Barnwell, J. W. And Shear, H. L. (1987). Protective antigen in the membranes of mouse erythrocytes infected with *Plasmodium chabaudi*. *Molecular and Biochemical Parasitology* 25, 195-201.

Wang, C., Castro, A. F., Wilkes, D. M. and Altenberg, G. A. (1999). Expression and purification of the first nucleotide-binding domain and linker region of human multidrug resistance gene product: comparison of fusions to glutathione *S*-transferase, thioredoxin and maltose-binding protein. *Biochemical Journal* 338, 77-81.

Warhurst, D. C. and Williams, J. E. (1996). Laboratory diagnosis of malaria. *Journal of Clinical Pathology* 49, 533-538.

Weaver, J. C. (1993). Electroporation: a general phenomenon for manipulating cells and tissues. *Journal of Cellular Biochemistry* 51, 426-435.

Weinbaum, F. I., Evans, C. B. And Tigelaar, R. E. (1976). Immunity to *Plasmodium berghei yoelii* in mice. I. The course of infection in T cell and B cell deficient mice. *The Journal of Immunology* 117, 1999-2005.

Welling, G. W., Weijer, W. J., van der Zee, R., and Welling-Wester, S. (1985). Prediction of sequential antigenic regions in proteins. *FEBS Letters* 188, 215-218.

Westhead, D. R., Parish, J. H. and Twyman, R. M. (2002). Instant notes: bioinformatics. BIOS Scientific Publishers Limited, Oxford, United Kingdom.

Wheeler, D. L., Church, D. M., Lash, A. E., Leipe, D. D., Madden, T. L., Pontius, J. U., Schuler, G. D., Schriml, L. M., Tatusova, T. A., Wagner, L. and Rapp, B. A. (2001). Database resources of the National Centre for Biotechnology Information. *Nucleic Acids Research* 29, 11-16.

Wickham, M. E., Rug, M., Ralph, S. A., Klonis, N., McFadden, G. I., Tilley, L. and Cowman, A. F. (2001). Trafficking and assembly of the cytoadherence complex in *Plasmodium falciparum*-infected human erythrocytes. *The EMBO Journal* 20, 5636-5649.

Wiser, M. F. (2007). Export and trafficking of P*lasmodium* proteins within the host erythrocyte. *Acta Biologica Colombiana* 12, 3-18.

World Health Organisation. (2008). World malaria report 2008. http://whqlibdoc.who.int/publications/2008/9789241563697_eng.pdf.

World Health Organisation. (2009). World malaria report 2009. http://whqlibdoc.who.int/publications/2009/9789241563901_eng.pdf.

Wu, Y., Ellis, R. D., Shaffer, D., Fontes, E., Malkin, E. M., Mahanty, S., Fay, M. P., Narum, D., Rausch, K., Miles, A. P., Aebig, J., Orcutt, A., Muratova, O., Song, G., Lambert, L., Zhu, D., Miura, K., Long, C., Saul, A., Miller, L. H., Durbin, A. P. (2008). Phase 1 Trial of malaria transmission blocking vaccine candidates *Pf*s25 and *Pv*s25 formulated with Montanide ISA 51. *PLoS ONE* 3, 1-9 (e2636).

Xiong, J. (2006). Essential bioinformatics. Cambridge University Press, Cambridge.

Yadava, A. and Ockenhouse, C. F. (2003). Effect of codon optimization on expression levels of a functionally folded malaria vaccine candidate in prokaryotic and eukaryotic expression systems. *Infection and Immunity* 71, 4961-4969.

Zhou, Z., Schnake, P., Xiao, L. and Lal, A. A. (2004). Enhanced expression of a recombinant malaria candidate vaccine in *Escherichia coli* by codon optimisation. *Protein Expression and Purification* 34, 87-94.

Zhou, Z., Todd, C. W., Wohlhueter, R. M., Price, A., Xiao, L., Schnake, P., Bonner, P. C., Martin, A. M., Goldman, I. F., De La Vega, P., Udhayakumar, V. and Lal, A. A. (2006). Development, characterization and immunogenicity of a multi-stage, multivalent *Plasmodium falciparum* vaccine antigen (FALVAC-1A) expressed in *Escherichia coli*. *Human Vaccines* 2, 14-23.

APPENDIX

Plasmid maps

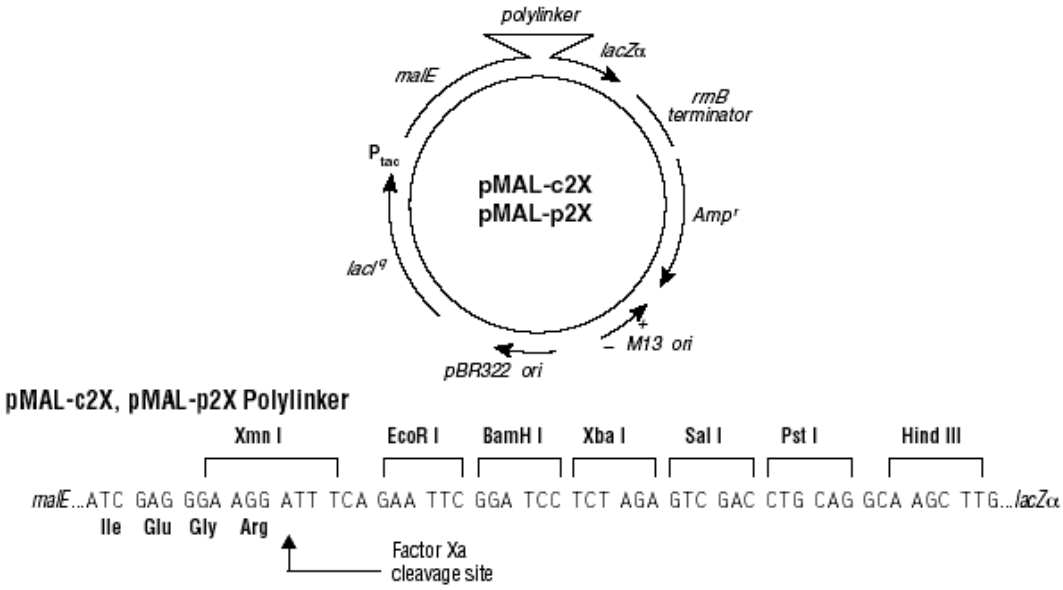


Figure A1. Map of plasmid pMAL-c2X indicating the restriction enzyme sites and Factor Xa protease recognition sequence in the polylinker site. (New England Biolabs).

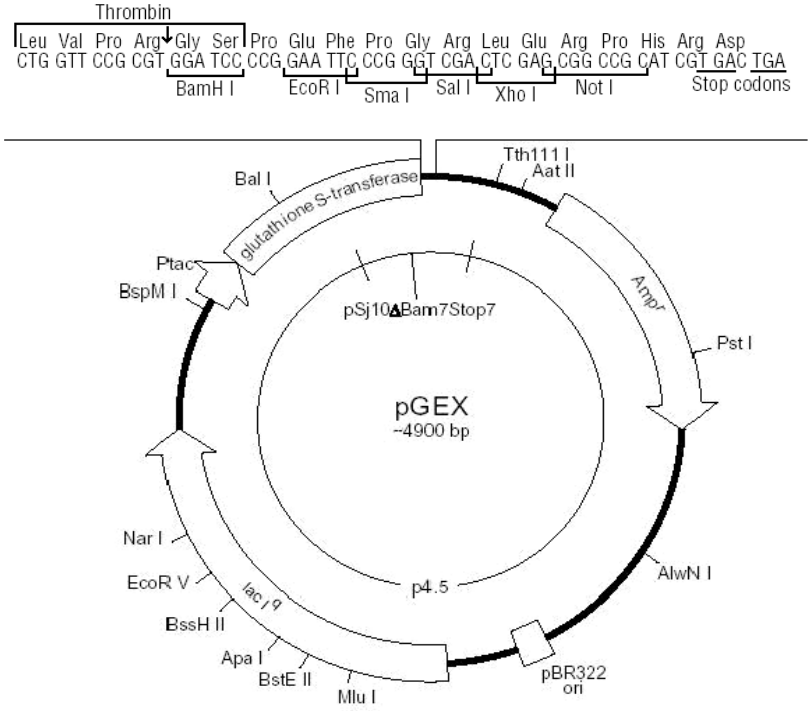


Figure A2. Map of plasmid pGEX4T1 indicating the restriction enzyme sites and thrombin protease recognition sequence, in the polylinker site. (Promega).

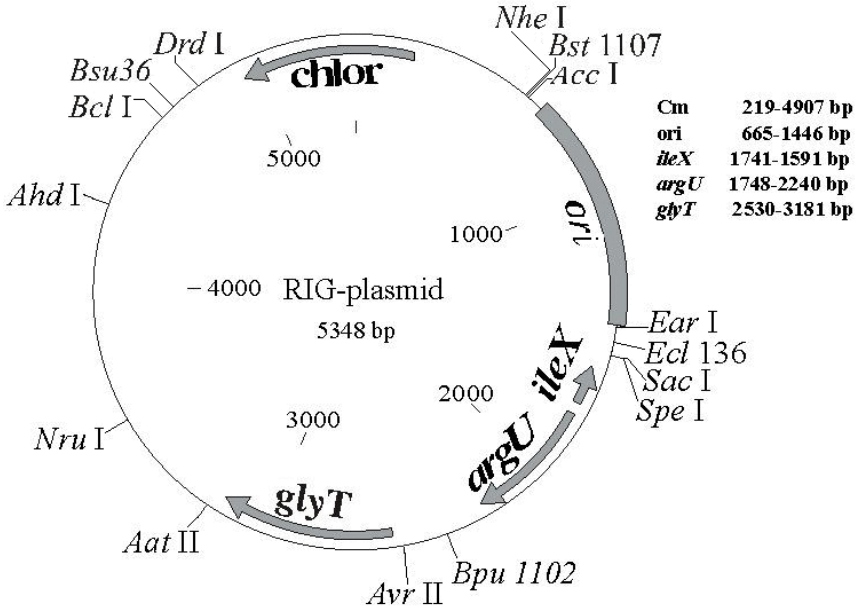


Figure A3. The RIG plasmid.