

**IDENTIFICATION OF TRAFFICKING DETERMINANTS  
NEEDED FOR TARGETING OF APICAL ORGANELLE  
PROTEINS IN *PLASMODIUM FALCIPARUM***

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Molecular Medicine

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## **DECLARATION**

I, Melanie Wepener declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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Signature of candidate

\_\_\_\_\_ day of \_\_\_\_\_ 20\_\_\_\_\_

## **ABSTRACT**

To invade host erythrocytes, the malaria parasite, *Plasmodium falciparum*, produces invasion proteins, which are translocated through the secretory pathway by an N-terminal signal peptide. Additional motifs allow the proteins to be transported to the secretory organelles, the rhoptries and micronemes. Proteins of the Duffy Binding-Like Erythrocyte Binding Protein (DBL-EBP) family possess a C-terminal cysteine-rich domain, proposed to be the motif necessary for targeting. To investigate this hypothesis, mini-gene constructs were created from two DBL-EBPs, MAEBL and JESEBL by overlap extension PCR, and used to transfect 3D7 *P. falciparum* parasites. It was anticipated that only chimaeric proteins containing the domain would be correctly localized to the secretory organelles, while those without the domain would remain within the cytoplasm. Two constructs were produced for MAEBL, containing the signal peptide, transmembrane domain and cytoplasmic tail, with the C-terminal cysteine-rich domain either present, M1 (CCys), or absent, M2 ( $\Delta$ CCys). These were cloned into the pARL-GFP plasmid that had been modified by replacing the *crt* promoter with the *ama-1* promoter for stage-specific expression. A third construct contained the JESEBL signal peptide and C-terminal cysteine-rich domain, J (CCys), which was cloned into the pARL-mCherry/*ama-1* promoter plasmid. Successfully transfected parasites were visible 25-30 days post-transfection and the presence of the episomal plasmids was verified by PCR. Microscopic analysis of live parasites revealed diffuse fluorescence in the cytoplasmic regions for control transfectants carrying the empty pARL2-GFP and pARL-mCherry vectors, as expected. The chimaeric M2 ( $\Delta$ CCys) protein showed a similar pattern to the control GFP, however, chimaeric M1 (CCys) was localized to the parasite apex, indicating secretory organelle localization. These results showed that the C-terminal cysteine-rich domain is sufficient and necessary for apical trafficking of MAEBL. The chimaeric J (CCys) protein was localized throughout the cytoplasmic and apical regions, indicating partial trafficking of the protein to the secretory organelles. This was further confirmed by co-localization studies whereby EBA-175 was used as a micronemal marker. The JESEBL C-terminal cysteine-rich domain is therefore sufficient but not completely adequate for correct protein trafficking. Based on these data and other published studies, two hypothetical models are proposed for the transport of MAEBL and JESEBL to the apical organelles.

## **RESEARCH OUTPUTS**

### **Creation of transgenic *Plasmodium falciparum* parasites expressing chimaeric invasion proteins**

Melanie Wepener and Theresa L. Coetzer

- Oral presented at the 6<sup>th</sup> Multilateral Initiative on Malaria (MIM) Pan-African Malaria Conference, Durban, South Africa, October 2013

### **Overlap extension PCR as a method for creating *Plasmodium falciparum* chimaeric mini-genes**

Melanie Friend (Wepener) and Theresa L. Coetzer

- Poster presented at Wits Research Day, University of the Witwatersrand, Johannesburg, South Africa, September 2012
- Poster presented at The South African Society of Biochemistry and Molecular Biology (SASBMB) Congress, Drakensburg, South Africa, January 2012
- Poster presented at the Molecular Biosciences Research Thrust Research Day, University of the Witwatersrand, Johannesburg, South Africa, December 2011

### **The conserved C-terminal cysteine-rich domain is necessary for trafficking the erythrocyte binding proteins MAEBL and JESEBL**

Melanie Wepener and Theresa L. Coetzer

- Manuscript in preparation



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## **ABBREVIATIONS**

aa- amino acids

AMA-1- apical membrane antigen-1

ARO- Armadillo Repeats-only

bp- base pairs

CBP- CREB-binding protein

CCys- C-terminal cysteine-rich domain

*crt*- chloroquine resistance transporter

CT- Cytoplasmic tail

DB- Duffy blood group

DBL- Duffy binding-like

DBP- DB proteins

*dhfr*- dihydrofolate reductase

EBA-175- erythrocyte binding antigen-175

EBP- Erythrocyte binding protein

*E. coli*- *Escherichia coli*

ER- Endoplasmic reticulum

GPI- Glycosylphosphatidyl inositol

GFP- Green fluorescent protein

Kb- Kilobases

LB- Luria Broth

MAEBL- merozoite adhesive erythrocyte binding-like protein

ms- milliseconds

OD- Optical Density



*P. falciparum* or *Pf- Plasmodium falciparum*

*P. berghei- Plasmodium berghei*

*P. yoelii- Plasmodium yoelii*

*P. vivax- Plasmodium vivax*

PlasmoDB- *Plasmodium* genome database

pRBC- parasitized erythrocytes

PV- Parasitophorous vacuole

RAMA- rhoptry associated membrane antigen

RAP1- rhoptry associated protein 1

RBL- Reticulocyte binding protein-like

RE- Restriction enzyme

SP- Signal peptide

spp- species

*T. gondii* or *Tg- Toxoplasma gondii*

T<sub>m</sub>- Melting temperature

TM- Transmembrane domain

TO- Thiazole Orange

TRAP- Thrombospondin-related anonymous protein

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## **CHAPTER 1**

### **INTRODUCTION**

#### 1.1. Background of malaria infection

The most severe form of human malaria is caused by the *Plasmodium falciparum* (*P. falciparum*) parasite. This parasite belongs to the phylum *Apicomplexa* as it possesses a set of membrane organelles known as the apical complex. The *P. falciparum* genome sequence was published in 2002 (Gardner *et al.*, 2002), providing an expanse of information to aid further research into malaria infection.

Malaria transmission occurs in regions across the equatorial belt, including South America, South-East Asia, the Western Pacific, the Eastern Mediterranean and most severely, sub-Saharan Africa. Up to 3.5 billion people around the world (approximately half of the world's population) are at risk of malaria infection, with ongoing malaria transmission occurring in 97 countries and territories. Latest reports published in December 2013 showed approximately 207 million cases of malaria in 2012, and of this number, it is estimated that malaria claimed the lives of 627 000 people (World Malaria Report 2013, **www.who.int**). More than 90% of malaria deaths occur in Africa, where a child dies from malaria every minute.

The spread of parasite resistance to anti-malaria medications, the lack of an effective vaccine, and the fragility of malaria control programmes have increased the need for new innovations in *Plasmodium* research.

### 1.2. *P. falciparum* life cycle

The parasite lifecycle (reviewed in Cowman *et al.*, 2012 & Cowman and Crabb, 2006) is complex, consisting of sexual and asexual stages (figure 1). The parasite is transmitted via the female *Anopheles* mosquito. When the mosquito takes a blood meal, parasites are injected into the human host in the form of sporozoites. The motile sporozoites travel to the liver, where they infect hepatocytes. Within the hepatocyte, the sporozoite develops and replicates to form thousands of merozoites. The non-motile merozoites are then released into the bloodstream, where they invade erythrocytes. Once within the erythrocyte, the merozoite develops and progresses through the ring, trophozoite and schizont stages, forming up to 32 daughter merozoites. As these daughter merozoites egress from the infected erythrocyte, they contact and invade more erythrocytes, causing high levels of infection and disease symptoms. A few of the intraerythrocytic parasites form male and female gametocytes. These are ingested by the mosquito and consequently develop into gametes which fuse to form a zygote within the insect's gut. The zygote develops into an oocyst, where sporozoites are formed and released. These enter the salivary glands, allowing the parasite to be spread among human hosts.

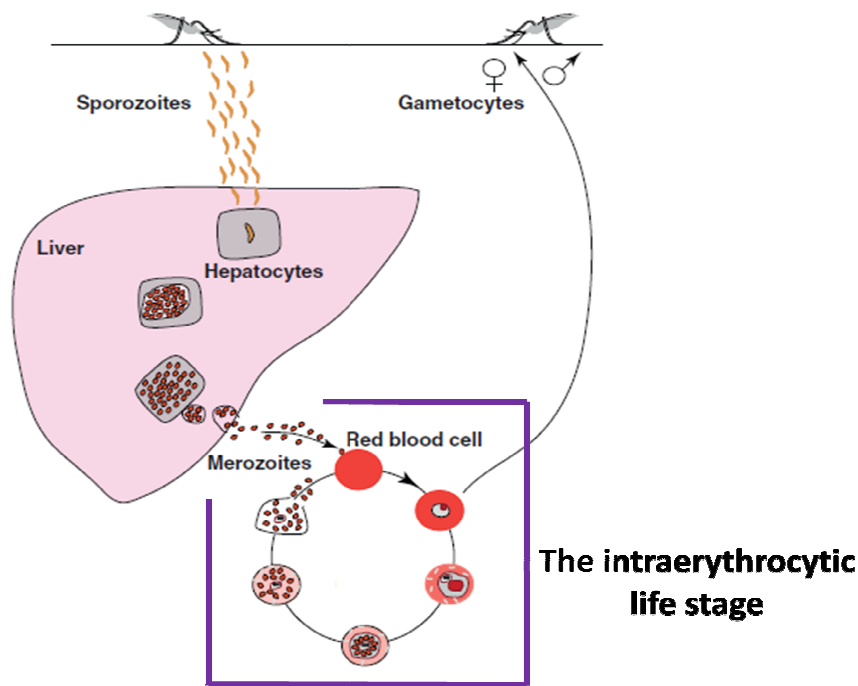


Figure 1. The *P. falciparum* life cycle. The transmission of the parasite between the mosquito and human host, including the asexual intraerythrocytic life stage is shown (Tham *et al.* 2012).

### 1.3. The intraerythrocytic life stage

The merozoite possesses the invasion machinery needed for host erythrocyte entry (reviewed in Cowman *et al.*, 2012 & Cowman and Crabb, 2006). A merozoite is similar to a lemon in shape, with specialised secretory organelles, the rhoptries, micronemes and dense granules, found at the apical end, opposite a large nucleus (Bannister *et al.*, 2000, figure 2). Proteins on the surface of the merozoite interact with receptors on the erythrocyte membrane. The merozoite then re-orientates to form a junction between the apical end of the merozoite and the erythrocyte (Aikawa *et al.*, 1978, Dvorak *et al.*, 1975). Movement of this junction allows the merozoite to enter the erythrocyte, surrounded by a parasitophorous vacuole (PV).



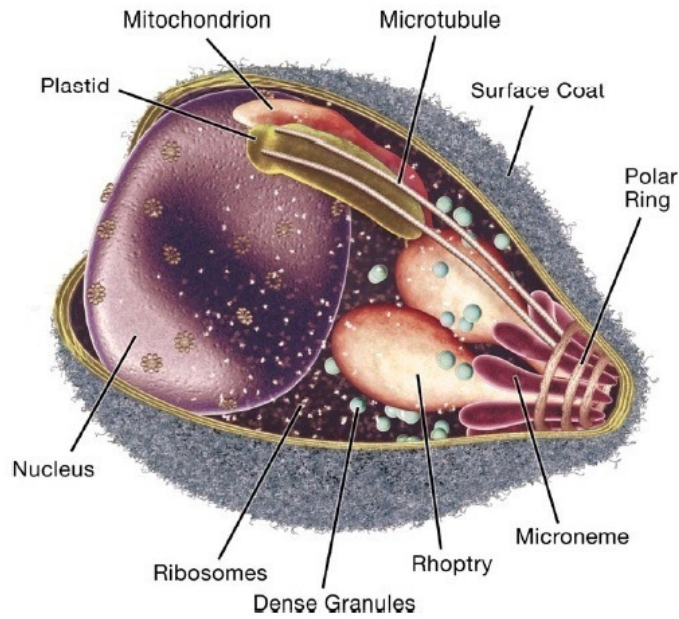


Figure 2. The ultrastructure of a merozoite. The apical region containing the secretory organelles, the micronemes, rhoptries and dense granules is shown. The large nucleus is situated opposite to the apical region (Cowman & Crabb, 2006).

Proteins contained within the secretory organelles are released during merozoite invasion, to aid in attachment to the host cell, and development of the moving junction and PV. This process allows the invasion proteins to be secreted at the time of, or just before the merozoite attaches to the host erythrocyte. Adams *et al.* (1990) suggested that this rapid ‘just in time’ process may aid in evasion of host immune detection, however, advanced imaging studies are still unable to elucidate the exact timing of the secretion of rhoptry or microneme contents (Hanssen *et al.*, 2013). *P. falciparum* parasites are able to utilize various erythrocyte surface receptors, by virtue of the many redundant invasion proteins (Dolan *et al.*, 1990). However, exact functions of many of these proteins are still widely unknown.

#### 1.4. The erythrocyte binding proteins

*Plasmodium* merozoites recognise and bind to receptors on the surface of host cell erythrocytes, including Duffy blood (DB) group antigens and sialic acids on surface glycoporphins. Miller *et al.* (1975) were the first to observe the ability of *Plasmodium spp* (*P. knowlesi* and *P. vivax*) to utilize the DB group determinants for host erythrocyte invasion. The parasite-derived invasion proteins, which bind to these receptors, were identified and grouped as a family of DB proteins (DBPs) (Adams *et al.*, 1990). The DBP domain responsible for binding was found to be an N-terminal cysteine-rich domain, which was named region II (Adams *et al.*, 2001, Chitnis and Miller, 1994).

A *P. falciparum* derived 175kDa antigen, now named erythrocyte binding antigen-175 (EBA-175), utilizes sialic acid on erythrocyte glycoporphins as a binding receptor (Camus and Hadley, 1985) in an essential invasion reaction (Sim *et al.*, 1990). The region of the EBA-175 protein responsible for erythrocyte binding was a cysteine-rich domain, homologous to region II of the DBPs (Sim *et al.*, 1994). Furthermore, Sim *et al.* (1994) elucidated the EBA-175 binding site as glycoporphin A.

Adams *et al.* (1992) deduced that the DBPs and sialic acid-binding EBA-175 belong to a gene family due to similarity in exon/intron gene structure and amino acid homology. The family was named the erythrocyte binding protein (EBP) family. Furthermore, multiple *P. falciparum* genes have been identified to have erythrocyte binding domains related to those of the EBP family, termed Duffy binding-like (DBL) domains (Peterson *et al.*, 1995). Members of the DBL-EBP superfamily have multi-exon structures; conserved boundaries between exons and introns; a conserved N-terminal cysteine-rich domain (DBL domain) and a C-terminal cysteine-rich (CCys) domain (Adams *et al.*, 1992, figure 3). Six *dbl-ebp*

genes have been identified in the *P. falciparum* genome, *eba-175* (Camus and Hadley, 1985), *eba-140/baeb1* (Mayer *et al.*, 2001, Thompson *et al.*, 2001), *eb1-165/pebl* (Triglia *et al.*, 2001), *eb1-1* (Peterson and Welles, 2000), *eba-181/jesebl* (Gilberger *et al.*, 2003b), and the *merozoite adhesive erythrocyte binding-like* protein (*maeb1*) (Kappe *et al.*, 1998). These proteins contain tandem DBL domains named F1 and F2, with the exception of MAEBL which contains the M1/M2 binding domain (see section 1.4.1). EBA-175 and BAEBL are well characterized while *pebl* is predicted to be a pseudogene. Furthermore, the encoded CCys of EBL-1 contains only four of the eight conserved cysteine residues, but the overall DBL-EBP characteristics are present.

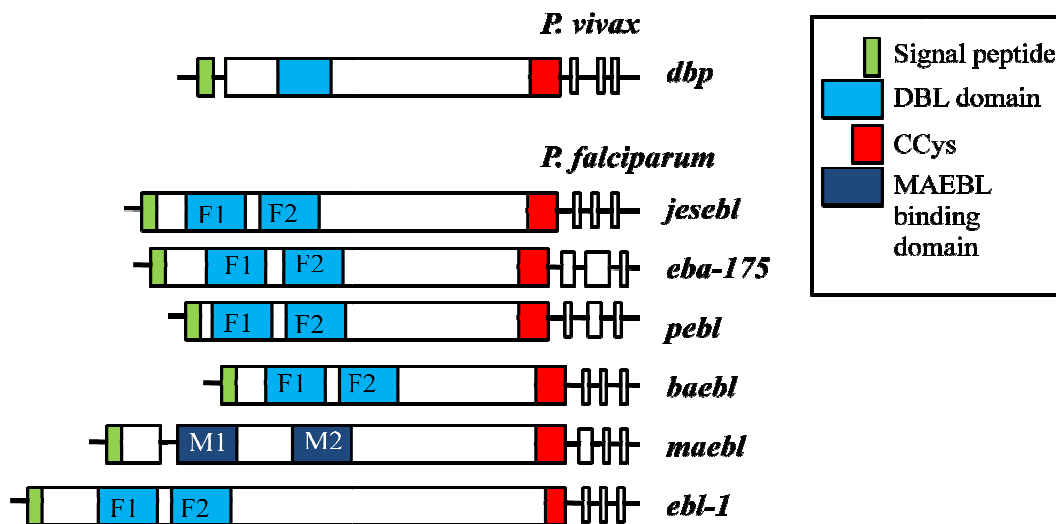


Figure 3. Schematic representations of the *P. falciparum* *dpb-ebp* gene family. Genes are related to the *P. vivax* *dbp* based on amino acid homology and gene structure. Conserved domains (coloured blocks), exons (white blocks) and introns (solid lines) are indicated. *P. falciparum* proteins contain tandem DBL binding domains (F1/F2), with the exception of MAEBL (Adams *et al.*, 2001).

*P. falciparum* is able to switch between invasion pathways (Cortes, 2008, Dolan *et al.*, 1990), and different invasion proteins have been found to function cooperatively (Lopaticki *et al.*, 2011). Therefore, invasion proteins are not ideal drug targets. However, the regulators which allow newly synthesized invasion proteins to be trafficked to the

secretory organelles, may be common between different invasion proteins. Therefore, it is important for new research to span this area of parasite biology.

#### 1.4.1. MAEBL

MAEBL is expressed in both the sporozoite and the erythrocytic stages of the *P. falciparum* life cycle (Ghai *et al.*, 2002). *Maebl* gene structure was first identified in the rodent parasites *Plasmodium yoelii yoelii* and *Plasmodium berghei* (Kappe *et al.*, 1998) and has subsequently been shown to be conserved in *Plasmodium* species, including *P. falciparum* (Blair *et al.*, 2002) and the avian parasite *P. gallinaceum* (Martinez *et al.*, 2013). Furthermore, *maebl* is conserved between *P. falciparum* strains (Blair *et al.*, 2002).

*Maebl* encodes a unique and complex transcript. Firstly, it is suspected to be a bicistronic message as the ~2kb 5' untranslated region contains a multi-exon open reading frame predicted to encode a mitochondrial ATP-synthase subunit (gene ID PF11\_0485) (Balu *et al.*, 2009). Secondly, it has been found that two functional isoforms of *maebl* exist in sporozoite and erythrocytic stages, across *Plasmodium* species, due to alternative splicing of 3' exons (Saenz *et al.*, 2008, Singh *et al.*, 2004).

MAEBL plays a role in erythrocyte invasion, although the exact function is still unknown. However, the highly conserved manner in which *maebl* evolved indicates a possible role in initial interactions with the host erythrocyte (Michon *et al.*, 2002). Fu *et al.* (2005) performed a targeted disruption of the *maebl* gene and proposed that MAEBL may be involved in the processing or control of other invasion-related proteins, or that it may be important when conditions are not optimal. The invasion mechanism utilized by MAEBL is both sialic acid-dependent and trypsin-insensitive, indicating a novel alternative pathway

dissimilar to those used by other invasion proteins (Fu *et al.*, 2005). Preiser *et al.* (2004) further showed the importance of MAEBL in sporozoite infection of hepatocytes, as antibodies against various regions of MAEBL inhibited sporozoites from penetrating hepatocytes and therefore reduced the development of liver-stage parasites.

MAEBL is a unique member of the DBL-EBP family in two ways. Firstly, MAEBL localizes within the rhoptry organelles, unlike other EBPs which localise to the micronemes (Blair *et al.*, 2002). Secondly, *maebl* has an altered functional domain at the N-terminal. Instead of the DBL domain, MAEBL has tandem duplicate cysteine-rich regions M1 and M2 which are similar to the first two domains of a different parasite protein, Apical Membrane Antigen-1 (AMA-1) (Kappe *et al.*, 1998). Phylogenetic analysis has shown that *maebl* evolved as an intact gene before the speciation of *Plasmodium* and is not a result of recombination between *ama-1* and an *ebp* (Michon *et al.*, 2002). However, MAEBL is homologous to DBL-EBPs in the CCys domain, which has an ancient origin in evolutionary history, indicating an important functional role (Michon *et al.*, 2002).

The *Pfmaebl* gene (PF3D7\_1147800.1) is 6762bp long, consisting of five exons (figure 4). The coding sequence is 6168bp, encoding a 2056 amino acid (aa) predicted product (Blair *et al.*, 2002). The *Pf*-MAEBL protein consists of six domains: (1) N-terminal signal peptide (SP); (2) the binding domain, M1 and M2; (3) repeat region; (4) CCys; (5) transmembrane domain (TM) and (6) cytoplasmic tail (CT) (Ghai *et al.*, 2002). Other than the important function in host receptor binding, it has been suggested that residues within the M1 and M2 domains, which are highly conserved in AMA-1 domains I and II, may also infer a important role for the MAEBL protein structure (Chesne-Seck *et al.*, 2005).

The SP directs protein trafficking through the Endoplasmic Reticulum (ER) and Golgi (Noe *et al.*, 2000), while no roles have been reported for the repeat region or the CCys.

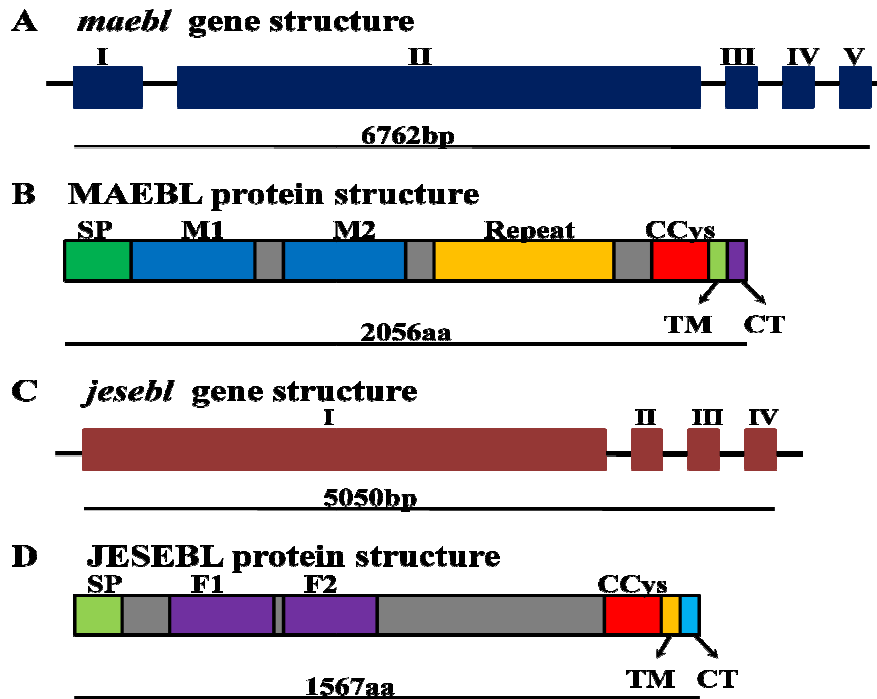


Figure 4. Schematic representations of *maebl* and *jesebl* gene and protein structures. Predicted amino acid and nucleotide sequence lengths are shown. Exons are represented with roman numerals, introns are shown by black lines between exons and protein domains are indicated. Based on information obtained from Kappe *et al.* (1998) and Gilberger *et al.* (2003b).

#### 1.4.2. JESEBL

In 2003, an EBA-175 paralogue, termed EBA-181 or JESEBL, was described (Gilberger *et al.*, 2003b). As is the case with EBA-175, JESEBL localizes to merozoite micronemes. Little is known about this protein or the receptor to which it binds, however, it is known to bind to the human erythrocyte membrane protein 4.1 (Lanzillotti and Coetzer, 2006). This interaction may aid in erythrocyte receptor binding or cause the erythrocyte cytoskeleton to become destabilized, thus assisting parasite invasion.

The *jesebl* gene (PF3D7\_0102500) is 5050bp long, comprising four exons, separated by three introns. The JESEBL protein is 1567aa long, consisting of five regions. The protein structure is very similar to MAEBL (figure 4), other than the two tandem DBL cysteine-rich domains, termed F1 and F2, which are characteristic of DBL-EBPs (Gilberger *et al.*, 2003b). Polymorphisms within the F1/F2 binding domain have been found to change the level of erythrocyte receptor binding without changing the parasite invasion profile (Maier *et al.*, 2009). These changes possibly alter the protein surface structure, thereby changing its binding affinity. Similar findings were recorded for JESEBL polymorphisms expressed in Brazilian patients (Lobo *et al.*, 2004)

JESEBL is an important invasion protein. A study of patients from Papua New Guinea showed that antibody responses to regions of JESEBL were strongly associated with protection from the development of symptomatic malaria (Richards *et al.*, 2010). Furthermore, studies of South American field isolates pointed to JESEBL as a major player in erythrocyte invasion by *P. falciparum* parasites (Lopez-Perez *et al.*, 2012, Villasis *et al.*, 2012).

### 1.5. Protein trafficking

*P. falciparum* parasites require complex transport mechanisms to sort proteins within cellular compartments (secretory organelles, food vacuole or apicoplast) and to export proteins to extracellular compartments such as the PV, the Maurer's clefts or the host cell.

#### 1.5.1. Trafficking of *P. falciparum* proteins to the secretory organelles

Eukaryotes synthesise secretory proteins from RNA molecules using ribosomes. The synthesised proteins are then translocated into the Golgi by means of an N-terminal signal

peptide. This is also true for many *P. falciparum* secretory proteins, including those of the DBL-EBP family. Once these proteins have passed through the ER and Golgi network, further targeting signals should direct translocation into the secretory organelles. However, recent reports agree that conserved motifs driving this mechanism are yet to be discovered (reviewed in Deponte *et al.*, 2012).

N-terminal motifs have been identified to be the trafficking determinants for various invasion proteins. Transfected parasites expressing chimaeric GFP-fusion proteins showed that only the first 20aa of the *P. falciparum* Armadillo Repeats-Only (ARO) protein (Cabrera *et al.*, 2012) and the first 24aa of *PfRhopH2* (Ghoneim *et al.*, 2007) are required for rhoptry localization. An elegant study by Richard *et al.* (2009) gave a detailed account of the rhoptry trafficking signals for rhoptry associated protein 1 (RAP1). It was found that amino acids 22-55 were sufficient and necessary for targeting, refining previous studies that implicated the first 344 N-terminal amino acids in rhoptry localization (Baldi *et al.*, 2000). The site contained within the 22-55aa sequence was found to bind to the C-terminus of rhoptry associated membrane antigen (RAMA), using a pull-down assay. RAMA acts as an escorter via association with lipid rafts (Topolska *et al.*, 2004). The RAP1 rhoptry-targeting signal was compared to other *Plasmodium* rhoptry proteins but the motif was not conserved (Richard *et al.*, 2009).

Motifs contained within the N-terminus are not the only trafficking determinants previously identified in *Plasmodium*. The thrombospondin-related anonymous protein (TRAP) contains a tyrosine motif in the CT which is important for sporozoite microneme localization in *P. berghei* (Bhanot *et al.*, 2003). Similarly, a conserved acidic motif in the CT of *Plasmodium* reticulocyte binding protein-like (RBL) proteins may be important for



trafficking to rhoptries (DeSimone *et al.*, 2009). The CT of merozoite surface sheddase *PfSUB2* does not contribute to microneme trafficking, while the TM was found to be necessary for this role (Child *et al.*, 2013). However, the CT was implicated in further translocation of *PfSUB2* to the merozoite surface. Similarly, it has been found that the CT is not required for microneme trafficking of EBA-175, EBA-140, EBA-181 (Gilberger *et al.*, 2003a, Treeck *et al.*, 2006) or AMA-1 (Treeck *et al.*, 2009).

The dispensability of the CT for these various *Plasmodium* invasion proteins leads one to question whether they need to interact with escorter proteins which contain the required CT signal, to interact with the trafficking machinery. A cargo receptor protein fulfilling this function has been identified in the closely related apicomplexan parasite *Toxoplasma gondii* (Sloves *et al.*, 2012). It was found that the CT of this cargo protein, *TgSORTLR*, is essential for the apical trafficking of various microneme (MIC1 and MIC4) and rhoptry (ROP1, ROP2, ROP4 and ROP5) proteins. It was predicted that the *TgSORTLR* CT physically interacts with the MIC and ROP proteins in the Golgi compartment and then coordinates with cytosolic trafficking proteins such as clathrin, adaptor protein adaptins, Sec23/Sec24 and vacuolar sorting proteins Vsp9, Vps26 and Vsp35.

#### 1.6. The C-terminal cysteine-rich domain

The CCys domain is highly conserved between members of the EBP family, across *Plasmodium* species (Adams *et al.*, 1992, Kappe *et al.*, 1997). The domain contains eight conserved cysteine residues. These residues are highlighted in the related proteins *P. falciparum* EBA-175 and *P. yoelii* EBL (figure 5).

*Pf* EBA-175 NK C ENEISVKY C DHMIHEEIPKLT C TKEKTRNL CC AVSDY C MSYFTYDSEEYYN C TKREFDDPSYT C FR  
*Py* EBL NM C TNDVSSKY C DYMKDKISSGN C SNDEKQL CC SISDY C LNYFDYNSNKYYD C TKKEFSDPLYK C FS

Figure 5. Protein sequence alignment of the EBP family. The alignment of the CCys domains of the *P. falciparum* (EBA-175) and *P. yoelii* (EBL) EBPs show eight highly conserved cysteine amino acids (red). Sequence information was taken from PlasmoDB, gene IDs PF3D7\_0731500 and PY05977.

The CCys, the SP, and accurate timing of expression are sufficient and necessary for trafficking EBA-175 from the Golgi and ER network to the appropriate destination, the micronemes (Treeck *et al.*, 2006). A single amino acid change whereby the second cysteine residue within the CCys domain was substituted with arginine caused the *P. yoelii* EBL protein localization to change from the micronemes to the dense granules (Otsuki *et al.*, 2009). A similar result has been shown for the same cysteine substitution and other amino acid substitutions in *P. falciparum* (Sakura *et al.*, 2013), indicating the importance of individual residues in protein trafficking.

Figure 6 shows the eight conserved cysteine residues in four *P. falciparum* DBL-EBP proteins, EBA-175, BAEBL, JESEBL and MAEBL. The homology between the CCys domains of these proteins leads to the possibility that this domain may be a conserved trafficking determinant for this protein family. To test this theory, the two candidates, MAEBL and JESEBL were chosen for evaluation.

EBA-175 NK C ENEISVKY C DHMIHEEIPKLT C TKEKTRNL CC AVSDY C MSYFTYDSEEYYN C TKREFDDPSYT C FR  
 BAEBL NK C NNEYSMEY C TYSDEKNSSPGP C SREERKKL CC QISDY C LKYFNFYSEIYYN C IKSEIKSPEYK C FK  
 JESEBL NT C SNEHSLKY C QYMERNKDLEET C SEDKRLHL CC EISDY C LKFFNPKSIEYFD C TQKEFDDPTYN C FR  
 MAEBL NM C NNDFFSKY C DYMKDNISSTG C SNEERKSL CC SISDF C LKYFDHNSNKYYD C TKIEFADPLYR C FK

Figure 6. Protein sequence alignment of the DBL-EBP family. The alignment of the CCys domains of the EBL proteins EBA-175, BAEBL (EBA-140), JESEBL and MAEBL show eight highly conserved cysteine amino acids (red). Sequence information was taken from PlasmoDB, gene IDs PF3D7\_0731500, PF3D7\_1301600, PF3D7\_0102500 and PF3D7\_1147800.1 respectively).

### 1.7. Aims of the project

The aim of this project is to investigate the role of the CCys domain in trafficking of DBL-EBPs. It is aimed to determine if the CCys is necessary for targeting MAEBL to the rhoptries and JESEBL to the micronemes of *P. falciparum* parasites. The specific objectives are as follows:

- Culture 3D7 *P. falciparum* parasites.
- Design primers and perform PCR and overlap-extension PCR to create chimaeric constructs.
- Modify the pARL-GFP and pARL2-mCherry vectors by replacing the *chloroquine resistance transporter (crt)* promoter with the *ama-1* promoter.
- Subclone the chimaeric constructs into the pARL vectors.
- Transfect 3D7 *P. falciparum* parasites with the chimaeric constructs so that they will express fluorescent GFP- and mCherry-fusion proteins.
- Analyse the transfectants by fluorescence microscopy and co-localization studies to determine protein localization.

This project will lay the foundations for future work in protein trafficking signals of DBP-EBPs and aid in the identification of possible *P. falciparum* escorter proteins.

## **CHAPTER 2**

### **MATERIALS AND METHODS**

All commercial reagents and the compositions of non-commercial reagents are listed in appendix A and appendix B respectively.

#### 2.1. Culturing techniques

##### 2.1.1. Culture composition and maintenance

Working stocks of *P. falciparum* (3D7) cultures were kept frozen under liquid nitrogen and thawed when needed. The parasites were cultured in a sterile hood as by the method originally defined by Trager and Jensen (1976), with adjustments. Cultures consisted of parasitized erythrocytes (pRBCs) and washed erythrocytes, kept at a haematocrit of 5%. Cultures were maintained at 37°C in 25cm<sup>3</sup> (small) or 75cm<sup>3</sup> (medium) sterile culture flasks (Nunc, Denmark), in 5ml (small) or 20ml (medium) complete medium. Cultures were gassed with a mixture of 5.5% CO<sub>2</sub>, 2.75% O<sub>2</sub> and 91.25% N<sub>2</sub> (Afrox, South Africa) for 1 minute for small flasks and 3 minutes for medium flasks, and the lids were closed. Complete medium was changed daily and cultures were continually diluted with fresh erythrocytes to maintain a parasitaemia of less than 3% trophozoites and less than 6% rings. Smears were also prepared on a daily basis to allow visualization of the life stage, parasitaemia and general health of the parasite cultures.

##### 2.1.2. Washing and diluting erythrocytes

Blood was collected from volunteers in 6ml acid citrate dextrose tubes (Becton Dickinson Biosciences, USA). These tubes were centrifuged at 1000 x g for 10 minutes at 4°C (Eppendorf Centrifuge 5702R, Germany), allowing the blood to separate into three layers. In a sterile hood, the top plasma and buffy layers were aspirated, leaving only a layer of

erythrocytes in the tube. Two volumes of incomplete medium were added and the contents of the tube were mixed by inversion to wash the erythrocytes. Centrifugation was repeated (1000 x g/10 min/4°C) and the top layer was aspirated. This wash was performed twice. The erythrocytes were then diluted in one volume of incomplete medium and stored at 4°C for up to two weeks.

### 2.1.3. Preparation of culture from frozen stocks

Frozen stocks, contained in cryotubes (TPP, Switzerland), were removed from liquid nitrogen and defrosted at 37°C. Under sterile conditions, the thawed cells were transferred to a 15ml centrifuge tube (Greiner bio-one, Germany). For every millilitre of cells, 0.1ml of 12% sodium chloride was slowly added while stirring. The tube was incubated at room temperature for 5 minutes before the addition of 9 volumes of 1.6% sodium chloride. The suspension was gently mixed and then centrifuged at 400 x g for 5 minutes at room temperature (Eppendorf Centrifuge 5702 R, Germany). After the supernatant was removed, the pellet was resuspended in 9 volumes of sodium chloride/glucose solution (Blomqvist, 2008). Centrifugation was repeated and the pellet was made up to a volume of 0.75ml using freshly washed and diluted erythrocytes (approximately 500µl of 1:1 diluted erythrocytes). The cells were then transferred to a small culture flask and complete medium was added to make a total volume of 5ml. The medium was made up with 1% albumax, instead of the usual 0.5% albumax; the increased protein constituent of the medium aids in parasite growth. The flask was then gassed and incubated as in 2.1.1. Smears were prepared and medium was changed daily (using the 'boosted albumax' medium) until parasitaemia reached approximately 3%. At this point culturing was continued with medium containing the usual 0.5% albumax.

#### 2.1.4. Freezing culture stocks

Cultures high in the parasite ring stage (5% or more) were frozen to make stocks as described by Normark (2008). A 20ml culture was transferred to two 15ml centrifuge tubes, under sterile conditions, and centrifuged at 400 x g for 5 minutes at room temperature. After removal of the supernatant, the pellet was resuspended in freezing solution at a ratio of 1:1. The suspension was then mixed well and transferred to sterile cryotubes (TPP, Switzerland) (0.5 – 1.0 ml per tube). Tubes were incubated at room temperature for 5 minutes before storing in liquid nitrogen.

#### 2.1.5. Smear Preparation

Smears were prepared on a daily basis. Flasks were tilted upward, allowing the culture medium to flow back and exposing the thin layer of erythrocytes at the bottom of the flask. A glass Pasteur pipette (Marienfeld, Germany) was then used to take up  $\pm 10\mu\text{l}$  of erythrocytes by capillary action. These erythrocytes were transferred to a glass microscope slide (Lasec, South Africa) and smeared thinly using the back of a second glass slide. The smear was allowed to air dry inside the flow hood and then stained using the Rapidiff Stain Set (Clinical Sciences Diagnostics CC, South Africa). Slides were viewed at 1000X magnification under oil immersion (Zeiss Axiostar, Germany).

#### 2.1.6. Calculating parasitaemia

##### 2.1.6.1. Calculating parasitaemia manually

The slides were viewed in five fields. In each field the percentage parasitaemia was estimated using the following calculation:

$$\% \text{ Parasitaemia} = \frac{\text{Number of infected RBCs}}{\text{Total number of RBCs}} \times 100$$

The average of the five calculations was taken as the estimated parasitaemia in each case.

#### 2.1.6.2. Calculating parasitaemia via Flow Cytometry

The DNA-binding dye thiazole orange (TO) was used to measure parasitaemia by flow cytometry using the method described by Engelbrecht and Coetzer (2013). Sorenson's phosphate buffer (0.9ml) was used to dilute 10 $\mu$ l of *P. falciparum* culture and TO was added to a final concentration of 1mM (0.1ml of 10mM stock in methanol) in a 1.5ml microcentrifuge tube (Eppendorf, Germany). The tube was incubated in the dark at room temperature for 20 minutes before the suspension was analysed using a Beckman Coulter Gallios flow cytometer (Beckman Coulter Inc., USA). To control for background, an identical procedure was carried out whereby *P. falciparum* culture was exchanged for uninfected erythrocytes. Excitation was by 488 nm blue laser and emission was measured at  $525 \pm 20$  nm (FL1 channel, 545/40BP filter). Optical alignment was monitored with Beckman Coulter Flow Check fluorspheres (Beckman Coulter Inc., USA). Erythrocytes were gated on a forward- versus side-scatter dot plot and analysed on a FL1 integral (log) histogram, with regions for uninfected, ring-infected and trophozoite-infected erythrocytes delineated. Approximately 50 000 events in the erythrocyte gate were counted. Post-acquisition analyses were performed with Beckman Coulter Kaluza (v1.1) software.

#### 2.1.7. Feeding and diluting stock cultures

Cultures were diluted when smears revealed the percentage of trophozoites in the culture to be 3% or more. For cultures with lower parasitaemia, the medium was merely changed by tilting the flask up and aspirating the old medium with a glass Pasteur pipette and pump system. Freshly made complete medium was then added in the following volumes: 5ml for a small flask and 20ml for a medium flask. To dilute 5ml cultures in 1:2, 1:5 or 1:10 ratios, 2.5ml, 1ml or 0.5ml respectively of the culture was transferred to a sterile flask. Washed and diluted erythrocytes (see section 2.1.2) were then added to maintain a haematocrit of

5% in the new culture (250µl, 400µl and 450µl respectively) and complete medium was added to make a final volume of 5ml. Flasks were then gassed and incubated as in 2.1.1.

#### 2.1.8. Synchronisation of cultures

High ring stage parasite cultures (5% or more) were synchronized using D-Sorbitol, as described by Lambros and Vanderberg (1979). Cultures (5ml) were transferred to sterile 15ml centrifuge tubes and centrifuged at 1000 x g for 5 minutes at room temperature (Eppendorf Centrifuge 5702 R, Germany). The supernatant was aspirated and the pellet was resuspended in 10 volumes of 5% D-Sorbitol (approximately 2.5ml) and incubated for 7-10 minutes at 37°C. The sorbitol treatment causes lysis of the late-stage parasites (trophozoites and schizonts), leaving only the ring-stage parasites intact. The contents of the tube were mixed slowly by inversion before a second centrifugation. The supernatant was aspirated and the pellet was washed with complete medium. Washed and diluted erythrocytes (approximately 100µl) and complete medium (approximately 5ml) were added to the final erythrocyte pellet and the culture was returned to a flask and gassed as in 2.1.1. Smears confirmed synchronisation of cultures.

### 2.2. Preparation of nucleic acids

#### 2.2.1. RNA extraction

Synchronised 5ml cultures containing ~5% late trophozoite to schizont stage parasites were used for RNA extraction. The experiment was carried out in sterile conditions and all instruments and surfaces were cleaned with RNaseZap<sup>TM</sup> (Ambion), to prevent RNase contamination. Cultures were transferred to sterile 15ml tubes and centrifuged at 1000 x g for 5 minutes at 4°C (Eppendorf Centrifuge 5702 R, Germany). The supernatant was aspirated and the pellet was resuspended in 1980µl PBS and 20µl 5% saponin (final



concentration of 0.05% saponin). Incubation at room temperature for five minutes allowed erythrocytes to be lysed (Schlichtherle and Wahlgren, 2008). This was followed by a second centrifugation with the same conditions. The supernatant was aspirated and the pellet was washed with PBS until the supernatant was clear, resulting in a pellet composed of pure parasites.

Total RNA was extracted from the parasites using Tri Reagent® (Sigma-Aldrich, USA), following the recommended protocol. TRI Reagent lyses parasites, releasing endogenous proteins and nucleic acids. Addition of chloroform and subsequent centrifugation separates the solution into three phases, a lower red organic phase containing the proteins, an upper colourless aqueous phase where the RNA is suspended, and a DNA interphase. It was then possible to precipitate and purify the RNA from the aqueous phase. The RNA pellet was dissolved in 20-30µl of nuclease-free water. Heating the sample to 60°C for 15 minutes aided in this process. The concentration and purity of RNA was determined spectrophotometrically using a Nanodrop 1000 (Thermospecific, USA). An  $A_{260}/A_{280}$  value greater than 2 indicates pure RNA. RNA integrity was visualized by electrophoresis (as per section 2.3) and the solution was stored at -70°C.

### 2.3. Agarose Gel Electrophoresis

Standard 1% agarose gels (50ml) were electrophoresed in 1X TAE buffer. By this method, DNA fragments may be separated based on size. RNA fragments were also separated in this way to check RNA integrity, however, size readings for RNA fragments were not accurate using this method. For visualization, 2.5µl of 10mg/ml ethidium bromide was added to a 50ml gel. This allows nucleic acid fragments to be seen under UV light using the Syngene gel doc and GeneSnap version 6.05 imaging software (Syngene, UK). For

every gel, 5µl of DNA ladder (MassRuler™ DNA Ladder Mix ready-to-use, Fermentas, Europe) was loaded into the first well, as a size standard. Loading dye (10X FastDigest® Green Buffer, Fermentas, Europe) was added to samples in a 1 dye: 5 sample ratio before loading into adjacent wells. The power source was set to provide maximum current at a voltage of 100V for electrophoresis. Current was applied for approximately 1 hour for adequate fragment separation. The concentrations of the separated products were calculated by Gene Tools imaging software (SynGene, UK).

## 2.4. Plasmid preparation

### 2.4.1. Preparation of the pARL2-GFP and pARL-mCherry plasmids for cloning

The pARL2-GFP and pARL-mCherry plasmids were kindly provided by Dr Jude M. Przyborski (Marburg, Germany). Plasmid extractions were carried out using an alkaline extraction method, as described by Birnboim *et al.* (1979). Sterile Luria Broth (LB) (20ml), containing 100µg/ml ampicillin (20µl of 100mg/ml ampicillin stock), was inoculated with *E. coli* plasmid glycerol stock (10-200µl), in an aseptic environment. The culture was incubated at 37°C in a shaking incubator (225-250 rpm) for 16-18 hours. A Thermo Biomate 5 Spectrophotometer (Merk, Germany), was used to measure the optical density (OD) of the culture, at 600nm, to check for bacterial growth. When OD<sub>600</sub> readings were found to be higher than 2.0, cells were harvested. If readings were lower than 2.0, cultures were returned for further incubation. Bacterial cells were harvested in 15ml centrifuge tubes (Greiner bio-one, Germany) by centrifugation at 3000 x g for 10 minutes at 4°C (Eppendorf Centrifuge 5702R, Germany). The pellets were then re-suspended in 0.05 volumes of ice cold re-suspension solution (1ml). After 5 minutes incubation at room temperature, 0.1 volumes of lysis solution (2ml) was added to the suspension, and mixed by gentle inversion. The solution was then neutralized by the addition of 0.075 volumes of

ice cold neutralizing solution (1.5ml), followed by 5 minutes incubation on ice. The released plasmid DNA was then separated from the lysed cells and denatured material by a further 20 minute centrifugation. The supernatant, containing the plasmid DNA, was transferred to sterile 2ml microcentrifuge tubes (Eppendorf, Germany) and 0.2 volumes of 1:1 phenol:chloroform (1ml for every tube containing 1ml supernatant) was added, to aid in plasmid purification. The solution was separated by 5 minutes centrifugation, 3000 x g at 4°C (Eppendorf Centrifuge 5415 R, Germany). The supernatant, containing the plasmid DNA, was transferred to fresh 1.5ml microcentrifuge tubes (Eppendorf, Germany), before adding 3 volumes of ice cold 100% ethanol (1.2ml for every tube containing 400µl supernatant). The solution was incubated at room temperature for 5 minutes, followed by 10 minutes centrifugation. The supernatant was then decanted, and the plasmid-containing pellet was air dried. The pellet was re-suspended in 800µl TE buffer, containing 100µg/ml RNase A (8µl of 10mg/ml stock), and incubated at 37°C for 30 minutes, allowing contaminating RNA to be removed. The solution was neutralized by the addition of 80µl cold neutralizing solution. One millilitre of 1:1 phenol:chloroform was added and the phases were separated by 5 minutes centrifugation at 3000 x g at 4°C. The aqueous phase, devoid of RNase A and degraded RNA, was transferred to sterile 2ml microcentrifuge tubes. To precipitate the plasmid DNA, 0.1 volumes of 3M sodium acetate and 2.5 volumes of ice cold 100% ethanol were added and the solution was incubated at -70°C for a minimum of 15 minutes. Upon removal from the freezer, DNA was pelleted at 3000 x g at 4°C for 30 minutes. The supernatant was decanted and the pellet was washed with 1ml 70% ethanol (5 minutes centrifugation at max speed) before being air dried. Final re-suspension was in ~100µl of nuclease free water, followed by 15 minutes incubation at room temperature to aid in re-suspension of the DNA. The resulting products were analysed by electrophoresis as per 2.3 and stored at -20°C.

#### 2.4.2. Plasmid preparation for transfections

Large amounts of purified plasmid (>100µg) are required to transfect *P. falciparum* parasites. To achieve this, the NucleoBond® Xtra Maxi kit for Plasmid DNA Purification (MN, Germany) was used. This preparation relies on the principle of anion-exchange. The NucleoBond® Xtra anion-exchange columns and column filters are designed to allow solutions to be passed through by gravity flow. Starter cultures of 5ml sterile LB, containing 100µg/ml ampicillin (5µl of 100mg/ml ampicillin stock), were inoculated with a scraping of plasmid glycerol stocks. The culture was allowed to grow for 8 hours at 37°C, shaking at 225- 250 rpm. Bacterial growth was then measured as in 2.4.1. OD<sub>600</sub> readings of 0.3 or more indicated that bacteria were in exponential growth phase and therefore ready for re-inoculation. Larger LB (containing 100µg/ml ampicillin) volumes of 250ml were then inoculated with 1ml of starter culture. The larger cultures were allowed to grow for no longer than 17 hours under the same conditions as the starter cultures. The ODs of the cultures were again measured as in 2.4.1, to ensure sufficient bacterial growth (OD<sub>600</sub> higher than 2). Cultures were transferred to 250ml centrifuge tubes (Beckman, USA) and bacterial cells were harvested by centrifugation at 6000 x g at 4°C for 15 minutes, using an Avanti® J-E Centrifuge (Beckman Coulter, USA). The kit's protocol for low-copy plasmid purification was followed, as pARL plasmids are low-copy number plasmids. Volumes of the Resuspension, Lysis, Neutralization and Elution buffers used in this procedure, were determined by the formula:  $Vol = culture\ vol \times OD_{600} / 50$ . To desalinate and concentrate the eluted DNA, NucleoBond® Finalizers were used. This system allows the solution to be filtered by means of a silica membrane. The final product was eluted from the Finalizer membrane with 1ml of TE Buffer, and passed through the filter for a second time to increase purity. The plasmid DNA was stored at -20°C.

## 2.5. Primer design

### 2.5.1. Primer specifications and parameters

Sequences of the relevant *P. falciparum* genes, *jesebl* (PF3D7\_0102500), *maebl* (PF3D7\_1147800.1) and *apical membrane antigen-1* (*ama-1*) (PF3D7\_1133400) were obtained from the *Plasmodium* genome database, PlasmoDB version 8.2 (<http://plasmodb.org/plasmo/>). Primers were designed using Integrated DNA Technologies SciTools OligoAnalyzer ([www.idtdna.com](http://www.idtdna.com)). A list of all primer sequences may be found in appendix C. Recognition sites for the restriction enzymes (REs) *Xho*I, *Avr*II and *Not*I were included in the design of the primers, to allow ligation of the amplified products into the pARL2-GFP and pARL-mCherry vectors. For overlap-extension PCR, overlapping sequences were added to the ends of primers, thereby allowing the resulting products to be connected to one another. This principal is represented in figure 7. The criteria used to design the primers included minimal hair-pin and primer-dimer formation, and melting temperatures of no less than 45°C for the *P. falciparum*-specific template. Primer sequences were also checked against the *Plasmodium* genome database by means of a BLAST search, to ensure minimal non-specific binding. Primer synthesis was out-sourced to Inqaba Biotech (South Africa). Primers were received in lyophilised form and dissolved in the recommended amounts of nuclease-free water, to make a final concentration of 100µM. Primer dilutions of 10µM and 2µM were made from the 100µM stocks and all were stored at -20°C.

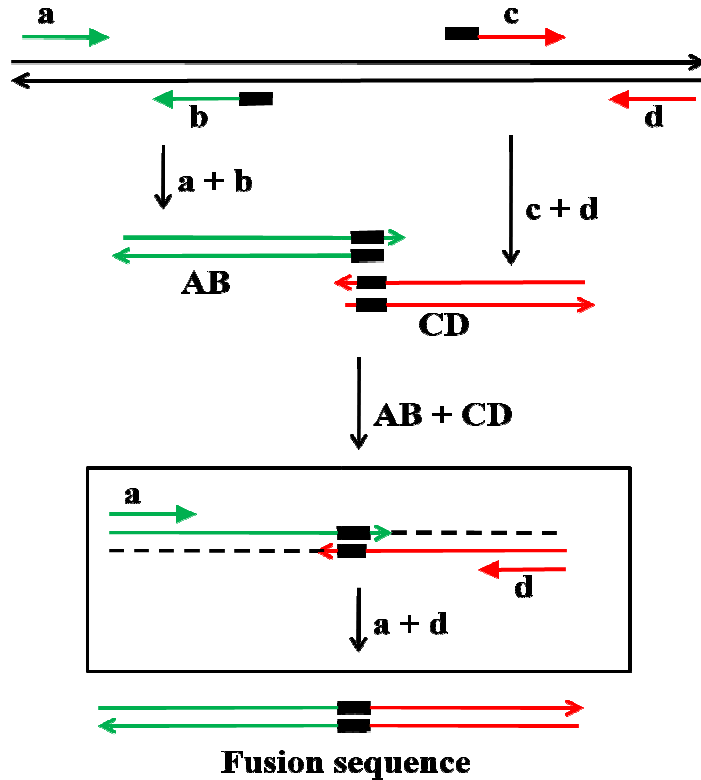


Figure 7. Schematic diagram representing overlap extension PCR using primers a,b,c & d. Overlapping ends are shown by solid black rectangles. PCR products are represented by capital letters. The open rectangular box shows the PCR reaction whereby the two regions are joined together.

## 2.5.2. Regions chosen for amplification

### 2.5.2.1. *maebl*

The *maebl* SP (1-174) and TM (5959-6030) sequences are available on the PlasmDB (figure 8). The CT starts directly after the TM and its sequence ends at the end of the *maebl* coding sequence (6031-6168). Adams *et al.* (1992) described the CCys region to span the last 104 residues of the second exon and immediately precede the TM. This sequence information and comparison of the *maebl* coding sequence to the *eba-175* CCys coding sequence (Treeck *et al.*, 2006) were used to define this region as 5619-5958. Importantly, the chosen CCys region contained all eight conserved cysteine amino acids.

### ***maebl* coding sequence**

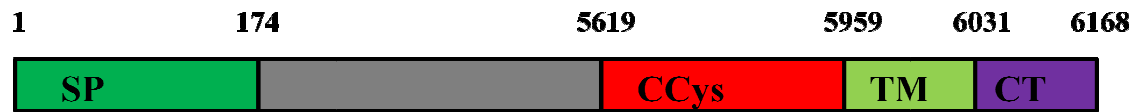


Figure 8. Schematic representation of the *maebl* coding sequence. Domains and their relevant base pair positions are shown.

#### 2.5.2.2. *jesebl*

The *jesebl* SP sequence (1-108) is available on PlasmoDB. The *jesebl* CCys coding sequence (4179-4461) was designed by comparison with the *eba-175* CCys coding sequence, so as to contain the eight conserved cysteine amino acids (figure 9).

### ***jesebl* coding sequence**



Figure 9. Schematic representation of the *jesebl* coding sequence. Domains and their relevant base pair positions are shown.

#### 2.5.2.3. The *ama-1* promoter region

Primers used to amplify the *ama-1* promoter region were designed to amplify 1455bp of genomic sequence directly upstream of the start of the *ama-1* coding sequence (Triglia *et al.*, 2000). The *ama-1* genomic and coding sequences are available on PlasmoDB.

### 2.6. cDNA synthesis

First-strand cDNA was synthesized from 5µg total parasite RNA in a reverse-transcription PCR reaction using SuperScript<sup>TM</sup> III Reverse Transcriptase (Invitrogen), following the recommended protocol. The 5µg of total RNA was transferred to a sterile, nuclease-free

0.6ml microcentrifuge tube (QSP, USA). To the tube the following was added: 2 pmol of gene-specific antisense primer (1µl of 2µM stock), 1µl of 10mM dNTP Mix and nuclease-free water up to 13µl. The reaction was then heated to 65°C for 5 minutes, using a Mastercycler Gradient thermocycler (Eppendorf, Germany), and then incubated on ice for 1 minute. This incubation allows the primer to bind to the RNA. To complete the reverse transcription reaction, 4µl of 5X First-Strand Buffer (1X final concentration), 1µl of 0.1M DTT (5mM final concentration), 40 units of the RNase inhibitor, RiboLock (1µl of 40U/µl stock), and 200-400 units of SuperScript<sup>TM</sup> III RT enzyme (1-2µl of 200U/µl stock) were added to the reaction, to a final volume of 20µl. The tube was incubated at 55°C for 1 hour, followed by an inactivation step of 15 minutes at 70°C. One microlitre of the cDNA was used per subsequent PCR and the remainder was stored at -20°C.

## 2.7. PCR

All PCR reactions were carried out using a Mastercycler Gradient Thermocycler (Eppendorf, Germany). Reactions were always started at a temperature of 94°C. Resulting PCR products were analysed spectrophotometrically using a Nanodrop 1000 (Thermospecific, USA), and visualised as per 2.3.

Reactions were performed using High Fidelity PCR Enzyme Mix (Fermentas, Europe) or Go Taq® Green Master Mix (Promega, USA). The High Fidelity enzyme mix was used because it contains a blend of DNA polymerases with high proofreading activity, and it is able to amplify products up to 10kb in length. These products may then be used in further reactions. To the template DNA, in a 0.6ml nuclease-free microcentrifuge tube (QSP, USA), the following was added: 5µl of 10X High Fidelity PCR Buffer (containing 15mM MgCl<sub>2</sub>), 5µl of 10mM dNTP mix, 0.5 or 1µM of gene-specific antisense primer (2.5µl or



5µl of 10µM stock), 0.5 or 1µM of gene-specific sense primer (2.5µl or 5µl of 10µM stock), 1.25 or 2.5 units of High Fidelity PCR Enzyme Mix (0.25µl or 0.5µl of 5U/µl stock, depending on the length of the desired product) and nuclease-free water up to 50µl.

Go Taq® Green Master Mix was used in PCRs when it was only required to visualize the resulting products. The Go Taq® enzyme allows erroneous bases to be incorporated into amplicons rendering the product inappropriate for use in further experiments. Reactions were carried out in 0.6ml nuclease-free microcentrifuge tubes as follows: 12.5µl Go Taq® Green Master Mix, 0.8 or 1µM of gene-specific primers (2µl or 2.5µl of 10µM stocks), 200-300ng template DNA and nuclease-free water up to a final reaction volume of 25µl.

The PCR thermal cycling conditions varied according to the template DNA and primers used. As per the High Fidelity PCR Enzyme Mix recommendations, all protocols included an initial denaturation at 94°C for 2 minutes and 25-35 cycles. Thermal cycles included three temperature changes for denaturation, annealing and extension. Denaturation temperature and time were based on manufacturer recommendations and annealing temperatures were based on primer melting temperatures, calculated using Integrated DNA Technologies SciTools OligoAnalyzer ([www.idtdna.com](http://www.idtdna.com)). It is recommended to use an extension temperature of 72°C however, the *P. falciparum* genome is very AT-rich, making the lower extension temperature of 68°C more suitable. Extension times were based on length of the product, 1 minute per kilobase.

#### 2.7.1. PCR to amplify *maebl* and *jesebl* gene domains

For primers containing DNA sequence overhangs, two annealing temperatures were used. These temperatures were based on the melting temperature of the whole primer and that of

the template-specific portion of the primer only. The first annealing temperature allows the template-specific portion of the primer to bind to the template. Once the added bases have been incorporated into the PCR product, the second annealing temperature allows the whole primer to bind to the desired product. Primers without added overhangs required only one annealing temperature. Optimal PCR thermal cycling conditions were set as follows:

Amplification of the *maebl* SP from cDNA, using primers M-S1 and M-AS2 or M-S1 and M-AS3 (figure 10):

Initial denaturation: 94°C for 2 minutes

Denaturation: 94°C for 30 seconds

Primer annealing: 52°C for 1 minute, 5 cycles and then 58°C for 1 minute, 27 cycles

Extension: 68°C for 1 minute

Final extension: 68°C for 10 minutes

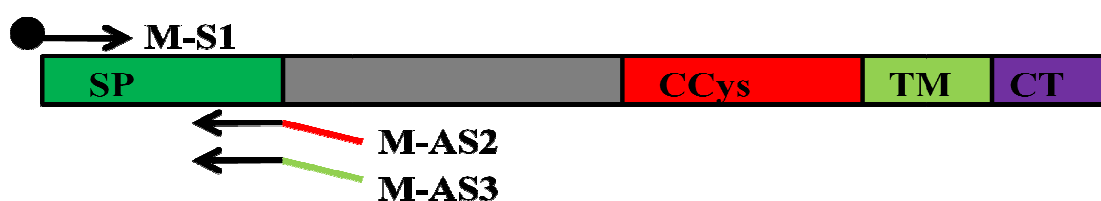


Figure 10. The *maebl* coding sequence showing SP specific primers. Forward primer: M-S1 with *Xho*I restriction site (black circle). Reverse primers: M-AS2 & M-AS3 with overhangs (red and green lines).

Amplification of the *maebl* CCys, TM and CT from cDNA, using primers M-S2 and M-AS1 and *maebl* TM-CT only from cDNA, using primers M-S3 and M-AS1 (figures 11 and 12):

Initial denaturation: 94°C for 2 minutes

Denaturation: 94°C for 30 seconds

Primer annealing: 45°C for 1 minute, 30 cycles

Extension: 68°C for 1 minute

Final extension: 68°C for 10 minutes

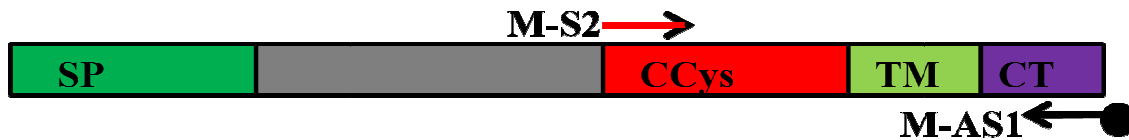


Figure 11. The *maeb1* coding sequence showing CCys-TM-CT specific primers. Forward primer: M-S2 (complementary to M-AS2 overhang). Reverse primer: M-AS1 with *AvrII* restriction site (black circle).

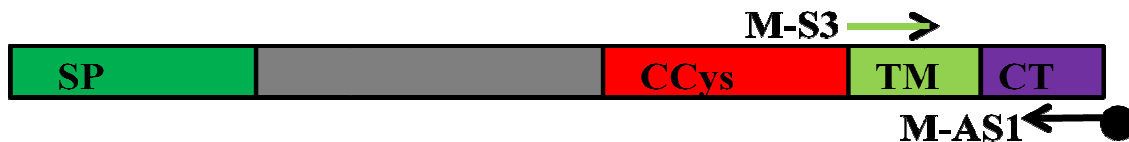


Figure 12. The *maeb1* coding sequence showing TM-CT specific primers. Forward primer: M-S3 (complementary to M-AS3 overhang). Reverse primer: M-AS1 with *AvrII* restriction site (black circle).

Amplification of the *jesebl* SP from cDNA, using primers J-S1 and J-AS3 (figure 13):

Initial denaturation: 94°C for 2 minutes

Denaturation: 94°C for 30 seconds

Primer annealing: 49°C for 1 minute, 5 cycles and then 58°C for 1 minute, 27 cycles

Extension: 68°C for 1 minute

Final extension: 68°C for 10 minutes



Figure 13. The *jesebl* coding sequence showing SP specific primers. Forward primer: J-S1 with *XhoI* restriction site (black circle). Reverse primer: J-AS3 with overhang (red line).

Amplification of the *jesebl* CCys from cDNA, using primers J-S2 and J-AS4 (figure 14):

Initial denaturation: 94°C for 2 minutes

Denaturation: 94°C for 30 seconds

Primer annealing: 45°C for 1 minute, 30 cycles

Extension: 68°C for 1 minute

Final extension: 68°C for 10 minutes

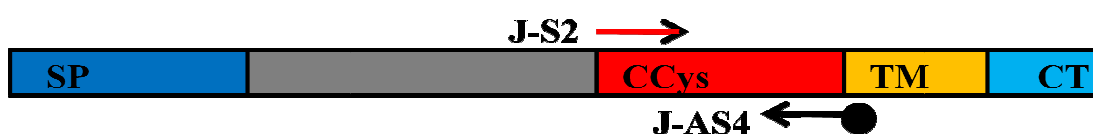


Figure 14. The *jesebl* coding sequence showing CCys specific primers. Forward primer: J-S2 (complementary to J-AS3 overhang). Reverse primer: J-AS4 with *AvrII* restriction site (black circle).

#### 2.7.2. Overlap extension PCR

Overlap extension PCR is used to join to PCR fragments together and re-amplify the resulting product (Ho *et al.*, 1989). The template for the reaction consisted of 2µl of each of the PCR products to be joined together. Two different annealing temperatures were incorporated into the PCR conditions. The first annealing temperature allows the two overlapping ends to anneal to each other while the second annealing temperature is optimal for primer annealing and, therefore, amplification of the product. The PCR conditions were set as follows:

To join the *maebl* SP to the *maebl* CCys-TM-CT using primers M-S1 and M-AS1; and to join *maebl* SP to the *maebl* TM-CT using primers M-S1 and M-AS1 (figures 15 and 16):

Initial denaturation: 94°C for 2 minutes

Denaturation: 94°C for 30 seconds

Primer annealing: 45°C for 1 minute, 5 cycles and then 58°C for 1 minute, 25 cycles

Extension: 68°C for 1 minute

Final extension: 68°C for 10 minutes

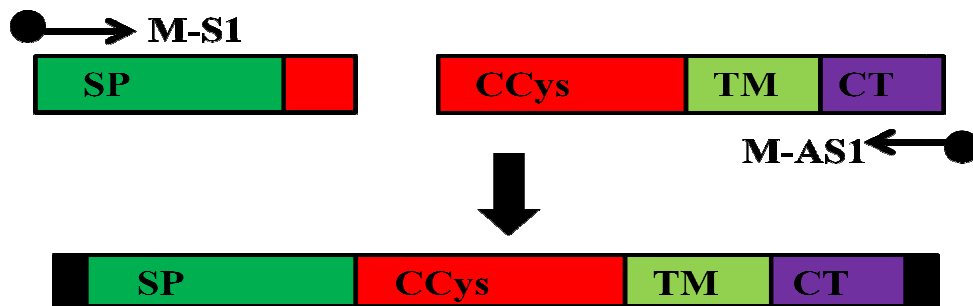


Figure 15. Overlap extension PCR to join *maebl* SP to *maebl* CCys-TM-CT. Forward primer: M-S1 with *Xho*I restriction site (black circle). Reverse primer: M-AS1 with *Avr*II restriction site (black circle). Restriction sites are incorporated into the resulting product (black rectangles).

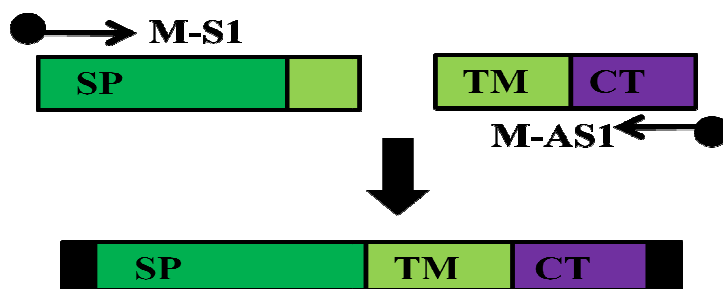


Figure 16. Overlap extension PCR to join *maebl* SP to *maebl* TM-CT. Forward primer: M-S1 with *Xho*I restriction site (black circle). Reverse primer: M-AS1 with *Avr*II restriction site (black circle). Restriction sites are incorporated into the resulting product (black rectangles).

To join the *jesebl* SP to the *jesebl* CCys using primers J-S1 and J-AS4 (figure 17):

Initial denaturation: 94°C for 2 minutes

Denaturation: 94°C for 30 seconds

Primer annealing: 45°C for 1 minute, 5 cycles and then 60°C for 1 minute, 25 cycles

Extension: 68°C for 1 minute

Final extension: 68°C for 10 minutes

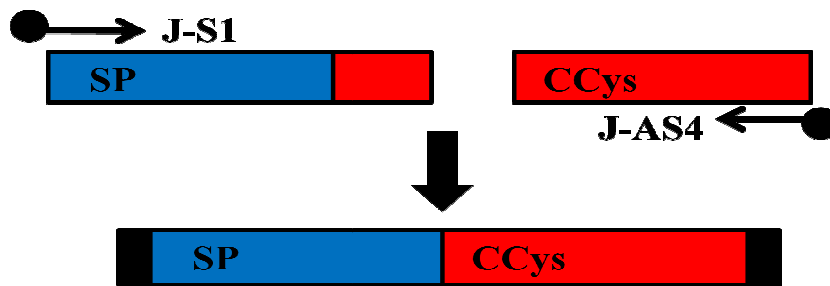


Figure 17. Overlap extension PCR to join *jesebl* SP to *jesebl* CCys. Forward primer: J-S1 with *XhoI* restriction site (black circle). Reverse primer: J-AS4 with *AvrII* restriction site (black circle). Restriction sites are incorporated into the resulting product (black rectangles).

### 2.7.3. PCR to amplify the *ama-1* promoter region

The endogenous *crt* promoter was exchanged with the *ama-1* promoter in the pARL2-GFP and pARL-mCherry vectors, to ensure stage-specific expression of chimaeric proteins. The *ama-1* promoter region is exceptionally AT-rich (86%) which made amplification problematic. The amplification reaction was optimised using Go Taq® Green Master Mix (Promega, USA) and varying conditions. The final product was amplified with High Fidelity PCR Enzyme Mix (Fermentas, Europe) using the optimised conditions.

#### 2.7.3.1. Reactions employing Go Taq® Green Master Mix (Promega, USA)

The *ama-1* promoter region was amplified from 200-500ng of *P. falciparum* genomic DNA (provided by Warren A. Vieira, department of Molecular Medicine and Haematology, the University of the Witwatersrand), using primers AMA-S and AMA-AS.

The PCR conditions were as follows:

Initial denaturation: 94°C for 2 minutes

Denaturation: 94°C for 1 minute

Primer annealing: 39.3°C-43.0°C for 1 minute, 5 cycles and then 56°C-58°C for 1 minute, 24-30 cycles

Extension: 62°C, 63°C, 64°C or 68°C for 2 minutes

Final extension: 62°C, 63°C, 64°C or 68°C for 10 minutes

#### 2.7.3.2. Reactions employing High Fidelity PCR Enzyme Mix (Fermentas, Europe)

The *ama-1* promoter region was amplified from 200ng of *P. falciparum* genomic DNA (provided by Warren A. Vieira), using primers AMA-S and AMA-AS. Reactions using an extension temperature of 68°C were unsuccessful due to the extremely AT-rich nature of this region, therefore, 64°C was used. The PCR conditions were as follows:

Initial denaturation: 94°C for 2 minutes

Denaturation: 94°C for 1 minute

Primer annealing: 42.6°C for 1 minute, 5 cycles and then 58°C for 1 minute, 24 cycles

Extension: 64°C for 2 minutes

Final extension: 64°C for 10 minutes

#### 2.8. PCR product and plasmid purification

All PCR products containing only the desired amplicon were purified using a QIAquick<sup>®</sup> PCR Purification Kit (Qiagen, UK). This process was necessary as High Fidelity components interfere with the action of REs, in subsequent reactions. Products were also purified post RE and plasmid dephosphorylation reactions, so as to remove the enzymes and other reagents before ligation. The protocol was followed as per the manufacturer's instructions. The QIAquick columns contain an anion-exchange resin which allows DNA to bind at high salt concentrations and low pH conditions. A pH-indicator showed the alkalinity of the sample to be purified. A sample with a pH greater than 7.5 turned violet in the presence of the indicator. In this case, 10µl of 3M Sodium acetate (pH 5) was added to reactions to decrease the pH to 7.5 or less, changing the indicator yellow and, therefore, allowing the DNA to bind to the resin. After impurities were washed away, the DNA was

eluted in a more alkaline medium. For this purpose, 20-60µl of Buffer EB (Tris Buffer, pH 8.5) or nuclease free water was used. Eluted DNA was stored at -20°C.

## 2.9. Gel Extraction

When PCR reactions resulted in non-specific amplicons, the product of interest was extracted from a standard 1% or 0.8% agarose gel using the QIAquick® Gel Extraction Kit (Qiagen, UK). The kit makes use of the same principal as the QIAquick® PCR Purification Kit (Qiagen, UK) mentioned in section 2.8. This kit was used as it allows a high recovery of purified DNA. The fragment of interest was visualized using a UV Transilluminator (UVP LLC, UK). It was then excised from the gel under UV light, using a clean, sharp scalpel and weighed in a colourless microcentrifuge tube (Eppendorf, Germany). Excision was carried out in the shortest possible time, as the UV light damages nucleic acids. DNA was purified from the gel slice according to the manufacturer's recommendations. For the elution, 30µl of nuclease-free water was used. Samples were visualised by gel electrophoresis (refer to 2.3) before storage at -20°C.

## 2.10. Cloning

### 2.10.1. Enzyme Digestion

Gene Tools imaging software (SynGene, UK) was used to quantify PCR products separated by agarose gel electrophoresis. The products, to be inserted into the pARL2-GFP and pARL-mCherry vectors, were digested with the FastDigest® REs (Fermentas, Europe) in 30µl reaction volumes (table 100). One microlitre of each of these REs is able to digest up to 1µg of DNA in minimal time, with no star activity, using a universal buffer. Reactions were set up in 0.6ml microcentrifuge tubes (QSP, USA) as follows: 500-1900ng PCR product DNA, 2µl 10X FastDigest® Buffer, 1µl F FastDigest® RE #1, 1µl FastDigest®



RE #2 and nuclease-free water up to 30µl. The reactions were incubated at 37°C for 15- 20 minutes, to allow the enzymes to digest the PCR products at their terminal restriction sites. Plasmid DNA was digested in a 20µl reaction as follows: ±1µg Plasmid DNA, 2µl 10X FastDigest® Buffer, 1µl FastDigest® RE #1, 1µl FastDigest® RE #2, 1µl FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas, Europe) and nuclease-free water up to 20µl (table 1). These reactions were incubated at 37°C for 3.5-4 hours. Even though incubation time is recommended to be 5 minutes, longer times were used to ensure optimal digestion and dephosphorylation. All digestion products were then purified as per 2.8 and eluted in 20- 30µl nuclease-free water before storage at -20°C.

**Table 1. Templates and enzymes employed in enzyme digestions.**

| Template                            | RE #1       | RE #2        | FastAP |
|-------------------------------------|-------------|--------------|--------|
| <b>PCR product</b>                  |             |              |        |
| <i>ama-1</i> promoter               | <i>XhoI</i> | <i>NotI</i>  | No     |
| M1                                  | <i>XhoI</i> | <i>AvrII</i> | No     |
| M2                                  | <i>XhoI</i> | <i>AvrII</i> | No     |
| J                                   | <i>XhoI</i> | <i>AvrII</i> | No     |
| <b>Plasmid</b>                      |             |              |        |
| pARL-mCherry                        | <i>XhoI</i> | <i>NotI</i>  | Yes    |
| pARL2-GFP                           | <i>XhoI</i> | <i>NotI</i>  | Yes    |
| pARL-mCherry/ <i>ama-1</i> promoter | <i>XhoI</i> | <i>AvrII</i> | Yes    |
| pARL2-GFP/ <i>ama-1</i> promoter    | <i>XhoI</i> | <i>AvrII</i> | Yes    |

RE- Restriction enzyme

FastAP- Thermosensitive Alkaline Phosphatase (Fermentas, Europe)

### 2.10.2. Ligation

Purified RE-digested PCR products and plasmid DNA were separated on the same agarose gel, to be quantified with Gene Tools imaging software (SynGene, UK). The ligation molar ratio of insert DNA to vector DNA was calculated at 3:1, with approximately 200ng total DNA. To perform these calculations, the In-Fusion® Molar Ratio Calculator

(<http://bioinfo.clontech.com/infusion/molarRatio.do>) was used. The Rapid DNA Ligation Kit (Roche Applied Science, Germany) was used to perform ligations. The vector and insert DNA were added to 2µl 5x DNA Dilution Buffer and nuclease-free water up to a volume of 10µl. T4 DNA Ligation Buffer (10µl) and 1µl T4 DNA Ligase were then added to the reaction and mixed. To allow ligation, the reaction was incubated at 16°C for 0.5-3 hours. Controls were included for each ligation reaction, whereby the insert DNA was replaced by nuclease-free water. Colonies present on control plates indicated that plasmid DNA was either incompletely digested by REs, leaving plasmid in circular form, or dephosphorylation by alkaline phosphatase was incomplete, allowing linear plasmid to re-ligate into circular form.

### 2.10.3. Transformation

The ligation products were used to transform *E. coli* XL10-Gold Competent Cells (Agilent Technologies, South Africa). Growth tubes (14ml, BD Falcon, USA) were chilled on ice before adding 50-100µl competent cells and 2-4µl β-Mercaptoethanol. The manufacturer's protocol recommends 100µl, however, it was found that transformations were adequate using half the reaction volume. The tubes were incubated on ice for 10 minutes with gentle swirling every 2 minutes before adding 6µl ligation mix. Tubes were incubated on ice for a further 30 minutes before heat-pulsing for 30 seconds at 42°C, followed by incubating on ice for 2 minutes. LB was pre-heated to 42°C and 400µl of this was added to each growth tube to make a final volume of ~500µl. One hour incubation at 37°C, shaking at 225-250 rpm, allowed transformed cells to propagate before they were plated (in a sterile environment) on agar plates containing 100µg/ml ampicillin. For each transformation, 50µl of cells were spread on one plate and the remaining 450µl on a second plate, and both were incubated at 37°C, upside-down for 18- 24 hours. It was expected that the plate containing

50µl of transformed cells would show a moderate amount of bacterial colonies, and the other plate would show bacterial growth of approximately nine times that of the 50µl plate. If this was not the case, it showed a low level of transformation efficiency. Furthermore, it was expected to observe no or very little growth on control plates.

## 2.11. Verification of transformation results

### 2.11.1. Colony PCR

Sterile 10µl plastic pipette tips were used to pick 5-10 single colonies from each of the plates containing transformed colonies. For each control plate, 2 colonies were picked. The colonies were each resuspended in 10µl of nuclease-free water. Half of each resuspension (5µl) was added to a PCR reaction and the other half was used for plasmid preparation (see 2.11.2). In a 0.6ml microcentrifuge tube (QSP, USA), the following reagents were added to the 5µl suspension: 12.5µl Go Taq<sup>®</sup> Green Master Mix (Promega, USA), 0.8µM of each of the appropriate forward and reverse primers (2µl of 10µM stocks, table 2), and nuclease-free water to make up 25µl. The PCR reactions were performed using a Mastercycler Gradient Thermocycler (Eppendorf, Germany), and the appropriate protocol. The resulting PCR products were analysed by gel electrophoresis, as per 2.3, to inspect the presence or absence of the desired products.

**Table 2. Primers used in Colony PCR reactions.**

| <b>Plasmid used in transformation</b> | <b>Forward primer</b> | <b>Reverse primer</b> |
|---------------------------------------|-----------------------|-----------------------|
| pARL-mCherry/ <i>ama-1</i> promoter   | AMA-S                 | AMA-AS                |
| pARL-mCherry/ <b>J</b>                | J-S1                  | J-AS4                 |
| pARL2-GFP/ <b>M1</b>                  | M-S1                  | M-AS1                 |
| pARL2-GFP/ <b>M2</b>                  | M-S1                  | M-AS1                 |

### 2.11.2. Plasmid preparation and enzyme digestion

Plasmid DNA was extracted from transformed colonies that were found to be positive by the PCR screen (2.11.1). Single colony resuspensions (5µl) were added to 3ml sterile LB containing 100µg/ml ampicillin (3µl of 100mg/µl stock) in 25ml glass flasks, and incubated at 37°C for 18-24 hours, shaking at 225-250 rpm. Of the 3ml colony culture, 1.5ml was made into stocks and DNA was extracted from the other 1.5ml. To create stocks, 500µl of the culture was added to 500µl 60% glycerol and frozen at -70°C. Plasmid preparations were carried out using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, USA), following the manufacturer's instructions. The kit makes use of an alkaline-SDS lysis procedure followed by silica-binding technology to produce pure plasmid preparations. The plasmid DNA was eluted in 50µl of nuclease-free water and used directly for RE digestions, to determine if the desired insert was present. The enzyme reactions were set up in 0.6ml microcentrifuge tubes (QSP, USA) containing 5µl purified plasmid, 2µl of FastDigest® Buffer, 1µl of each of the FastDigest® REs (Fermentas, Europe) and 11µl nuclease-free water. Reactions were incubated at 37°C for 15 minutes to 1 hour, to ensure sufficient digestion. The resulting DNA fragments were visualized by electrophoresis (refer to 2.3).

### 2.11.3. Sequencing

Plasmid preparations found to contain ligated inserts (as determined by 2.11.1 and 2.11.2) were chosen for sequencing. Agar plates (containing 100µg/ml ampicillin) were streaked with the corresponding glycerol stock, and incubated at 37°C for 18-24 hours. The plates were then sent to Inqaba Biotech for sequencing. Sequencing primers were designed and produced by Inqaba Biotech to recognise sequence segments within plasmid backbones and within the inserts, as required. The sequences of these primers remain the intellectual

property of Inqaba Biotech. Results were provided in Finch TV format and sequences were manually compared to the desired sequence to screen for nucleotide changes, insertions or deletions.

## 2.12. Transfections of *P. falciparum* erythrocyte-stage parasites

### 2.12.1. Preparation of parasites for transfection

Cultures consisting of 5% ring-stage parasites were used for transfections. To achieve this, two rounds of D-Sorbitol synchronization were carried out 48 hours apart, as per 2.1.8. The 5ml culture was transferred to a 15ml centrifuge tube (Greiner bio-one, Germany) and centrifuged at 1400 x g for 5 minutes at 4°C (Eppendorf Centrifuge 5702 R, Germany). The pellet, containing the parasitized erythrocytes, was then used immediately for the transfection.

### 2.12.2. Preparation of plasmid for transfection

Plasmids were extracted using the NucleoBond® Xtra Maxi kit for Plasmid DNA Purification (MN, Germany) and eluted in 1ml TE buffer as per 2.4.2. A 1:10 dilution of the preparation was made in nuclease free water, and 2µl of the dilution was linearized with a RE. In a microcentrifuge tube (Eppendorf, Germany), the following were added to the plasmid DNA: 2µl 10X FD Green Buffer, 1µl FD RE, and nuclease-free water up to 20µl. Digestion was carried out at 37°C for 10-15 minutes. The whole 20µl reaction was loaded into a 1% agarose gel and electrophoresed (refer to 2.3) for the resulting product to be quantified by Gene Tools imaging software (SynGene, UK). Quantification indicated total yields ranging from 100µg to 500µg of purified plasmid per 1ml concentrated eluate.

Plasmid preparations of 100µg to 300µg were concentrated, to be used for transfections. To achieve this, 1 volume of 1:1 phenol:chloroform was added to and mixed with the plasmid eluate. The mixture was centrifuged at 3000 x g for 5 minutes at 4°C (Eppendorf Centrifuge 5415 R, Germany) and the upper aqueous phase, containing the plasmid DNA, was transferred to a fresh 1.5ml microcentrifuge tube. An equal volume of chloroform was then added to and mixed with the solution and centrifugation was repeated. The upper aqueous phase was again transferred to a fresh tube and to it 1/10 volume 3M sodium acetate and 2.5 volumes 100% ethanol were added to precipitate the DNA. The tube was then stored at -70°C for a minimum of 15 minutes, to aid in the precipitation. Centrifugation was carried out at maximum speed (16100 x g) for 30 minutes at 4°C to ensure all DNA was pelleted in the tube. The supernatant was removed and the pellet was washed with 70% ethanol, air-dried, resuspended in 30µl TE buffer and used immediately for the transfection.

#### 2.12.3. Preparation of erythrocytes for transfection

Fresh whole blood collected from a volunteer in a 6ml acid citrate dextrose tube (Becton Dickinson Biosciences, USA), was centrifuged at 1000 x g for 10 minutes at 4°C. The top buffy and plasma layers were aspirated and the packed erythrocytes were used immediately for transfections.

#### 2.12.4. Transfection procedure

Transfections were carried out in a sterile hood. The 30µl of concentrated plasmid, 200µl of packed erythrocytes and 370µl of pre-warmed (at 37°C) cytomix were mixed and added to a 2mm sterile cuvette (BioRad, USA). The cuvette content was electroporated using a Gene Pulser Xcell<sup>TM</sup> (BioRad, USA) electroporator set to 310V and 950µF. Time and

actual voltage were recorded. Electroporation time should range between 12 and 15 milliseconds (ms). The content of the cuvette was then transferred to a small (25cm<sup>3</sup>) culture flask (Nunc, Denmark), using complete medium to wash out the cuvette and aid transfer. To the flask, parasites were added to make a final concentration of ~1% parasitaemia and complete medium to make a final volume of 5ml before gassing as per 2.1.1. A smear was prepared and the medium was changed the day following the transfection. On the second day post-transfection, medium was changed, slides were prepared and the selective drug WR99210 (kindly provided by Dr Jude M. Przyborski, Marburg, Germany) was added to a final concentration of 2nM (1µl of 10µM stock). Parasites that had taken up the episomal plasmid gained resistance to the WR99210 drug as the plasmid contains the dihydrofolate reductase (dhfr) gene. Transfected cultures were kept under continual drug pressure with tri-weekly medium changes and smear preparations. In addition, fresh packed erythrocytes were added to the culture weekly (100µl), to replenish haematocrit to 5% while parasitaemia was too low to be visualized. It took 25-30 days for parasitaemia to be high enough for parasites to be visualized on smears.

### 2.13. Verification of transfection by DNA extraction and PCR analysis

DNA was extracted from a 5ml culture of transfected parasites, using the hypotonic lysis protocol (Cowman *et al.*, 2008). The culture was transferred to a 15ml centrifuge tube (Greiner bio-one, Germany) and centrifuged at 1000 x g for 5 minutes at 4°C. After removal of the supernatant, the pellet was resuspended in 14ml PBS. The centrifugation was repeated and the pellet was then resuspended in 4 volumes (approximately 1ml) of hypotonic solution, allowing the erythrocytes to be lysed. To remove proteins, 1 volume (approximately 250µl) of 18% SDS was added to the suspension and vortexed before

incubation at room temperature for 2-3 minutes. A phenol:chloroform purification was performed to remove salts and contaminants, whereby 8 volumes (approximately 2ml) of 1:1 phenol:chloroform was added and the suspension was centrifuged at 3000 x g for 10 minutes at 4°C. The upper aqueous phase, containing the parasite DNA (including episomal plasmids), was transferred to a 1.5ml microcentrifuge tube (Eppendorf, Germany) and DNA was precipitated as per 2.12.2. The resulting pellet was dissolved in 600µl TE buffer, with the addition of 10µg RNase A (1µl of 10mg/ml stock) and incubated at 37°C for 30 minutes, to remove contaminating RNA from the sample. DNA was again extracted by a phenol:chloroform purification, using 1 volume (600µl) of 1:1 phenol:chloroform. One volume (600µl) of chloroform was added to the aqueous phase and the sample was centrifuged at maximum speed (16100 x g) for 5 minutes at 4°C (Eppendorf Centrifuge 5415 R, Germany). As before, the upper DNA-containing aqueous phase was transferred to a fresh microcentrifuge tube and DNA was again precipitated as per 2.12.2. The final pellet, consisting of pure parasite genomic and episomal DNA, was dissolved in 50µl TE buffer, incubated at room temperature for 15 minutes and then used for PCR or stored at 4°C.

To test for the presence of the appropriate plasmid within the transfected parasites, a PCR was performed. Approximately 300ng DNA was amplified in a 25µl reaction volume with Go Taq<sup>®</sup> Green Master Mix (Promega). A forward vector-specific primer and a reverse insert-specific primer were used at a final concentration of 1µM (2.5µl of 10µM stocks), and nuclease-free water was used to make up the reaction volume. Appropriate positive (plasmid purified from bacterial stocks) and negative (3D7 parasite DNA obtained from Warren A. Vieira) controls were included to verify PCR results (table 3). The reaction was carried out using a Mastercycler Gradient thermocycler (Eppendorf, Germany), set at 94°C



for 2 minutes, followed by 35 cycles of 94°C, 55°C and 68°C for 1minute each, and a final extension period of 10 minutes at 68°C. Gel electrophoresis was used to visualize PCR products as per 2.3.

**Table 3. Templates and primers used for PCR verification of transfectants.**

| Transfectants          | Templates                           | Primers        |
|------------------------|-------------------------------------|----------------|
| pARL-mCherry control   | DNA extracted from parasites        | pARL-S/ mC-AS  |
|                        | Purified plasmid (positive control) |                |
|                        | 3D7 DNA (negative control)          |                |
| pARL2-GFP control      | DNA extracted from parasites        | pARL-S/ GFP-AS |
|                        | Purified plasmid (positive control) |                |
|                        | 3D7 DNA (negative control)          |                |
| pARL-mCherry/ <b>J</b> | DNA extracted from parasites        | pARL-S/ J-AS4  |
|                        | Purified plasmid (positive control) |                |
|                        | 3D7 DNA (negative control)          |                |
| pARL2-GFP/ <b>M1</b>   | DNA extracted from parasites        | pARL-S/ M-AS1  |
|                        | Purified plasmid (positive control) |                |
|                        | 3D7 DNA (negative control)          |                |
| pARL2-GFP/ <b>M2</b>   | DNA extracted from parasites        | pARL-S/ M-AS1  |
|                        | Purified plasmid (positive control) |                |
|                        | 3D7 DNA (negative control)          |                |

## 2.14. Microscopy studies

### 2.14.1. Live imaging

To view the fluorescently labelled live parasites, 70µl of incomplete medium was added to 30µl of whole culture. To this mixture, 4µl of 1:100 diluted DAPI (blue nuclear stain) was added and incubated in the dark for 5 minutes due to the light sensitivity of the stain. The erythrocytes were pelleted by centrifugation at 1000 x g for 3 minutes at room temperature (Eppendorf Centrifuge 5415 R, Germany), and washed with 1ml incomplete medium. The final pellet was diluted with 200µl incomplete medium and used directly for viewing.

A 5µl aliquot of the stained suspension was used to spot a glass microscope slide (Lasec, South Africa), which was then covered by a glass cover slip. The cells were viewed under 63x magnification on an Olympus BX63 fluorescence microscope with a UPLSAPO 100x/1.4 objective lens. Multi-channel mirror cubes U-FDICT, U-FBNA, U-FYW and U-FUNA were used to view DIC Grey (550nm) and GFP (510nm), mCherry (610) and DAPI (455nm) fluorescence respectively (table 4). A DP80 (1x1 Binning) camera (Olympus, Japan) with 0.63 adapter magnification was used in conjunction with CellSense Dimension software for photographing and editing the fluorescent images.

**Table 4. Microscope specifications for live imaging.**

| Sample                      | Channel  |             |               |             |
|-----------------------------|----------|-------------|---------------|-------------|
|                             | DIC Grey | GFP (green) | mCherry (red) | DAPI (blue) |
| <b>pARL2-GFP control</b>    |          |             |               |             |
| Exposure time               | 2ms      | 1.7s        | /             | 31.3ms      |
| Gain                        | 1x       | 1x          | /             | 1x          |
| <b>pARL2-GFP/M1</b>         |          |             |               |             |
| Exposure time               | 5.7ms    | 190.5ms     | /             | 24.4ms      |
| Gain                        | 1x       | 2x          | /             | 1x          |
| <b>pARL2-GFP/M2</b>         |          |             |               |             |
| Exposure time               | 5.7ms    | 344.8ms     | /             | 12.7ms      |
| Gain                        | 1x       | 2x          | /             | 1x          |
| <b>pARL-mCherry control</b> |          |             |               |             |
| Exposure time               | 3.8ms    | /           | 285.7ms       | 11.4ms      |
| Gain                        | 1x       | /           | 2x            | 1x          |
| <b>pARL-mCherry/J</b>       |          |             |               |             |
| Exposure time               | 3.8ms    | /           | 344.8ms       | 11.4ms      |
| Gain                        | 1x       | /           | 2x            | 1x          |

#### 2.14.2. Co-localization studies

The location of the fluorescently labelled protein within the parasite is more accurately determined by co-localization studies. This procedure makes use of a labelled antibody against a known protein, whose location can be compared to the intrinsic fluorescent

protein. For microneme localization, a primary antibody against the well characterized micronemal protein EBA-175 was used. The green Alexa Fluor®488 goat anti-mouse secondary antibody was used for fluorescent labelling. Antibodies against known rhoptry proteins were unfortunately unavailable.

When performing co-localization studies, cells have to be fixed before being made permeable for the uptake of antibodies. Erythrocytes were fixed using methods described by Tonkin *et al.* (2004). Two and a half millilitres of whole culture was first pelleted and washed with 1ml PBS (1000 x g, 3min, room temperature). Cells were then resuspended in 1ml of 4% formaldehyde/0.0075% glutaraldehyde and fixed for 30 minutes by constant mixing using an intelli-mixer (ELMI Ltd, Latvia) at 12 rpm. Cells were pelleted by centrifugation at 3000 x g for 3 minutes at room temperature (Eppendorf Centrifuge 5415 R, Germany), and washed with 1ml PBS. The pellet was resuspended in 1ml 0.1% Triton X-100 and mixed for 10 minutes, to permeabilize the cells before they were pelleted and washed with 1ml PBS with centrifugation at maximum speed (16100 x g, 3min, room temperature). To reduce free aldehydes remaining from the fixing step, the pellet was resuspended in 1ml 0.1mg/ml sodium borohydride (NaBH<sub>4</sub>) and mixed for 10 minutes. Cells were washed before resuspension in 1ml 3% BSA in PBS, and mixed for 1 hour. This agent blocks globular proteins to prevent non-specific binding to the antibody. The cells were pelleted and resuspended in 1ml 1:1000 primary antibody (diluted in 3% BSA in PBS) by mixing for 1 hour. The cells were then washed with 1ml PBS three times, with 10 minutes of mixing for each wash. The secondary antibody was added to a final concentration of 5µg/ml (1.25µl diluted in 500µl of 3% BSA), and 2µl of 1:100 diluted DAPI was added to the suspension and mixed for 1 hour. Cells were again washed 3 times with 1ml PBS and resuspended in 50µl PBS. As per section 2.14.1, some of the cell

suspension was mounted for viewing at 63x magnification using the Olympus BX63 system (table 5).

**Table 5. Microscope specifications for co-localization imaging.**

| Sample                      | Channel  |             |               |             |
|-----------------------------|----------|-------------|---------------|-------------|
|                             | DIC Grey | GFP (green) | mCherry (red) | DAPI (blue) |
| <b>3D7</b>                  |          |             |               |             |
| Exposure time               | 7.1ms    | 15.2ms      | 400ms         | 7.3ms       |
| Gain                        | 1x       | 1x          | 1x            | 1x          |
| <b>pARL-mCherry control</b> |          |             |               |             |
| Exposure time               | 7.1ms    | 15.2ms      | 142.9ms       | 3ms         |
| Gain                        | 1x       | 1x          | 1x            | 1x          |
| <b>pARL-mCherry/J</b>       |          |             |               |             |
| Exposure time               | 7.1ms    | 15.2ms      | 400ms         | 7.3ms       |
| Gain                        | 1x       | 1x          | 1x            | 1x          |

## **CHAPTER 3**

### **RESULTS**

#### 3.1. *P. falciparum* growth rate

The physiology of the *P. falciparum* parasite is such that growth in the intraerythrocytic life stage is exponential. When culturing these parasites in laboratory conditions, it is essential to understand the parasite growth rate in order to keep cultures healthy. The parasite growth rate may be accurately determined by flow cytometry. Counts were conducted on duplicate Pf3D7 cultures using this method over 4 days (figure 18). Starting cultures contained mixed ring and trophozoite stage parasites with very low parasitaemia (<1% in both flasks). The low starting parasitaemia contributed to the very small increase in growth over the first 24 hours. However, after 48 hours, parasitaemia increased approximately four-fold each day as the parasites developed through the 48 hour life cycle (figure 18B). Raw cytometric data showed this progression as trophozoites developed into schizonts, producing multiple merozoites which infected erythrocytes, producing an increase in ring-stage parasites after 24 hours, which developed into trophozoites after 48 hours, completing the life cycle (figure 18A). These flow cytometry counts showed that manual counts were slightly underestimated. Furthermore, these results indicated that cultures were well maintained.

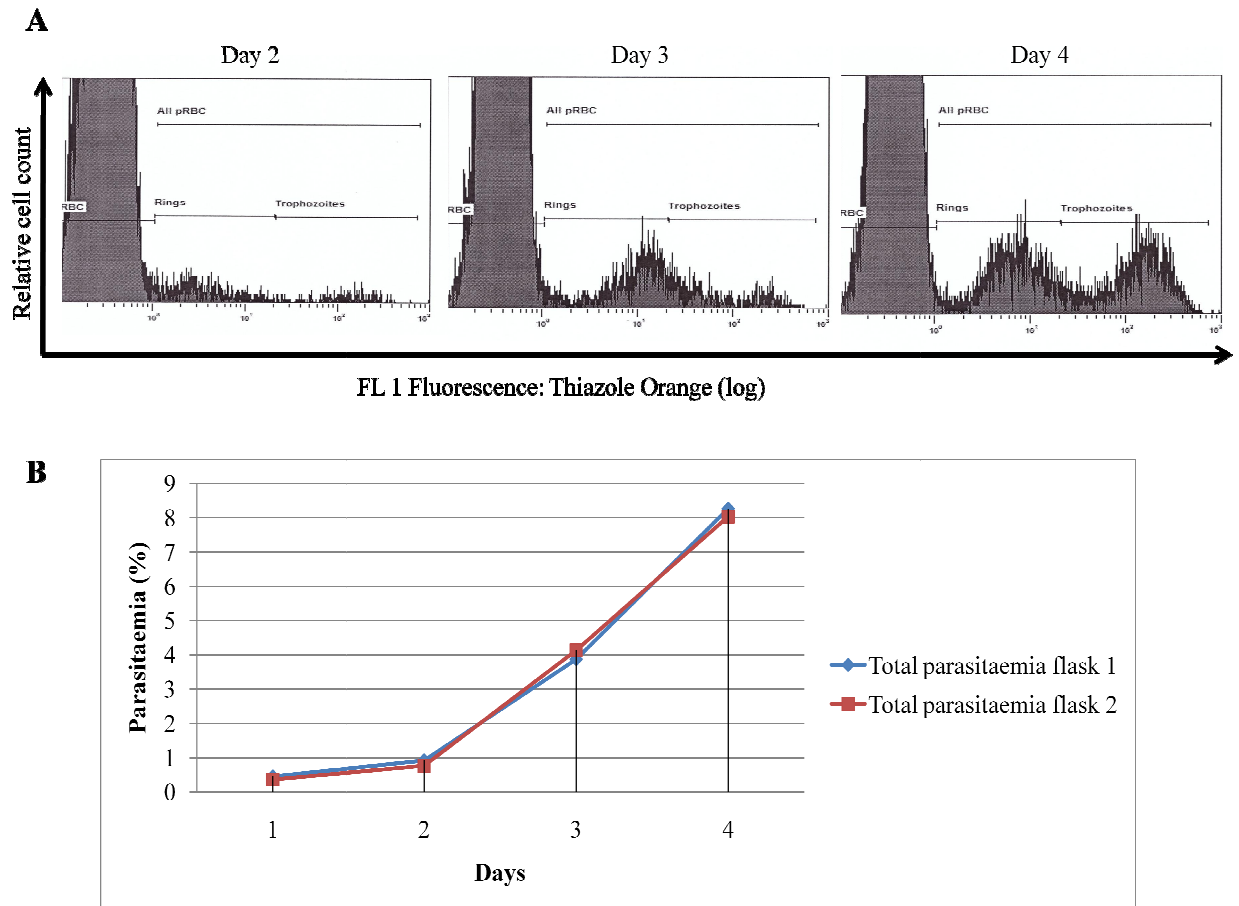


Figure 18. *P. falciparum* growth rate measured by flow cytometry. A. Raw flow cytometer data showing low levels of rings and trophozoites on day 2, followed by an increase in rings on day 3 as trophozoites develop over 24 hours, followed by an exponential increase in parasitaemia after 48 hours (day 4). B. Total parasitaemia increased from less than 1% on the first day, to 8% on day 4.

### 3.2. pARL-mCherry and pARL2-GFP vectors

The pARL2-GFP and pARL-mCherry vectors contain the endogenous *crt* promoter which allows expression throughout the intra-erythrocytic life stages of *P. falciparum*. MAEBL and JESEBL are both stage-specific proteins expressed only in the schizont life stage. For this reason, the *crt* promoter was exchanged with the *ama-1* promoter, which allows the proteins to be expressed in schizonts (Triglia *et al.*, 2000, figure 19).

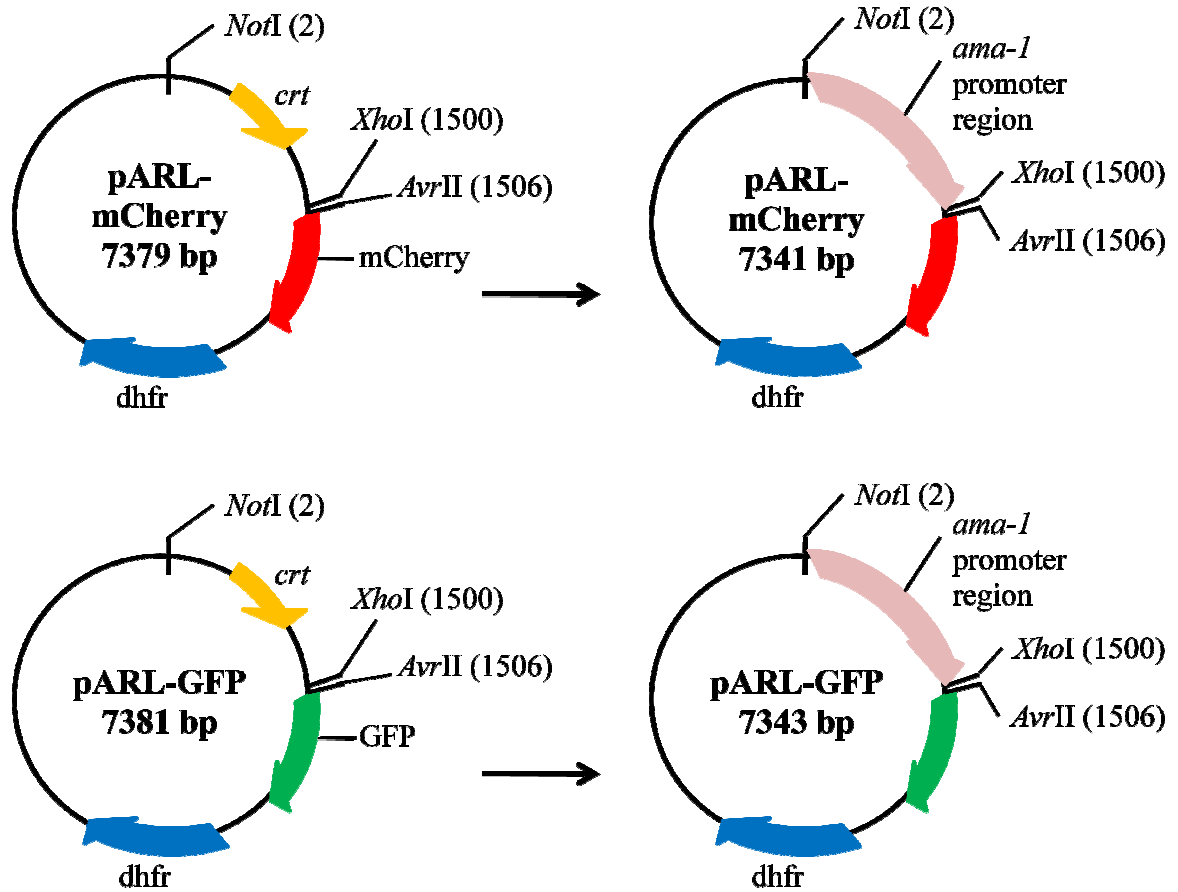


Figure 19. Vector maps showing pARL-mCherry and pARL2-GFP. Restriction enzyme sites (*NotI*, *AvrII* and *XhoI*) and the dihydrofolate reductase (*dhfr*) selectable markers are shown for both vectors. The region containing the *crt* promoter was exchanged with the *ama-1* promoter region for both vectors.

### 3.3. Purification of the *ama-1* promoter

Triglia *et al.* (2000) defined the *ama-1* promoter to be contained within the 1455bp sequence upstream of the *ama-1* coding sequence. This region was amplified from *P. falciparum* 3D7 genomic DNA using primers AMA-S and AMA-AS (table 6, appendix C).

**Table 6. Parameters for primers used to amplify the *ama-1* promoter region.**

|                  | Template-specific portion |         | Whole primer |         |              |
|------------------|---------------------------|---------|--------------|---------|--------------|
| Name             | Length (bp)               | Tm (°C) | Length (bp)  | Tm (°C) | RE site      |
| Sense primer     |                           |         |              |         |              |
| AMA-S            | 22                        | 50.1    | 32           | 65.4    | <i>Xho</i> I |
| Antisense primer |                           |         |              |         |              |
| AMA-AS           | 20                        | 53.6    | 32           | 67.2    | <i>Not</i> I |

RE- Restriction enzyme

Tm- Melting temperature, calculated by Integrated DNA Technologies SciTools

OligoAnalyzer, 0.25µM oligo, 50mM Na<sup>+</sup>, 1.5mM Mg<sup>++</sup>, 1mM dNTP.

Two primer annealing temperatures were incorporated into the PCR to allow annealing of the template-specific portion, and the whole primer (with added RE sites) to the template DNA respectively. PCR parameters were optimized as described below.

The initial reaction was designed to amplify 300ng of template DNA with Go Taq® using a gradient of annealing temperatures. The first annealing temperatures used were 40°C, 41°C and 42°C for 5 cycles, with corresponding second annealing temperatures of 56°C, 57°C and 58°C respectively, for 30 cycles. The extension temperature was set at 68°C (due to the high AT content) for 2 minutes. Visualization of the products showed only a very faint banding pattern, so the PCR was continued for a further 10 cycles using the second annealing temperature. The expected size product of 1455bp was absent and only non-specific amplicons were visualized for each annealing temperature combination (Figure 20). To optimize, a more diverse primer annealing temperature gradient was used. Both 200ng and 500ng of template DNA were amplified using an initial annealing temperature range of 39.3°C to 43°C for 5 cycles and the second annealing temperature set at 58°C for 30 cycles. Extension temperature was changed from 68°C to 64°C.



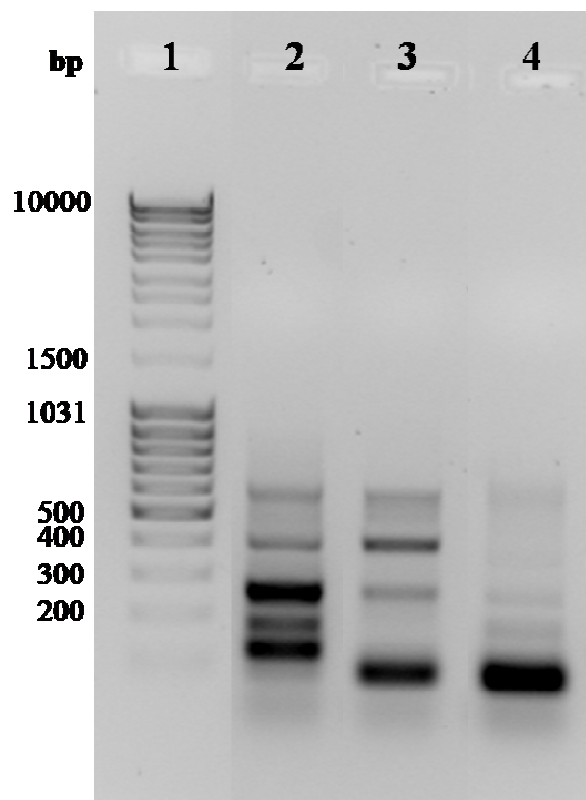


Figure 20. Amplification of the *ama-1* promoter region shown by agarose gel. 1: DNA size standard. *Pf ama-1* amplification products resulting from primer annealing temperature combinations of 40°C and 56°C (2) 41°C and 57°C (3) 42°C and 58°C (4).

As shown in figure 21, the amplification resulted in products of the expected size (1455bp) and non-specific amplicons for all reactions employing 200ng template. A concentrated amount of template DNA (500ng) inhibits amplification, probably due to an increased amount of contaminants, such as haem, remaining from the DNA preparation. Strong products were present in reactions employing initial annealing temperatures of 39.3°C and 42.6°C. Annealing temperatures below 45°C are not ideal for amplification by PCR as these low temperatures promote non-specific primer annealing. Therefore, the higher initial annealing temperature of 42.6°C, an extension temperature of 64°C and an amount of 200ng DNA template were chosen for subsequent reactions.

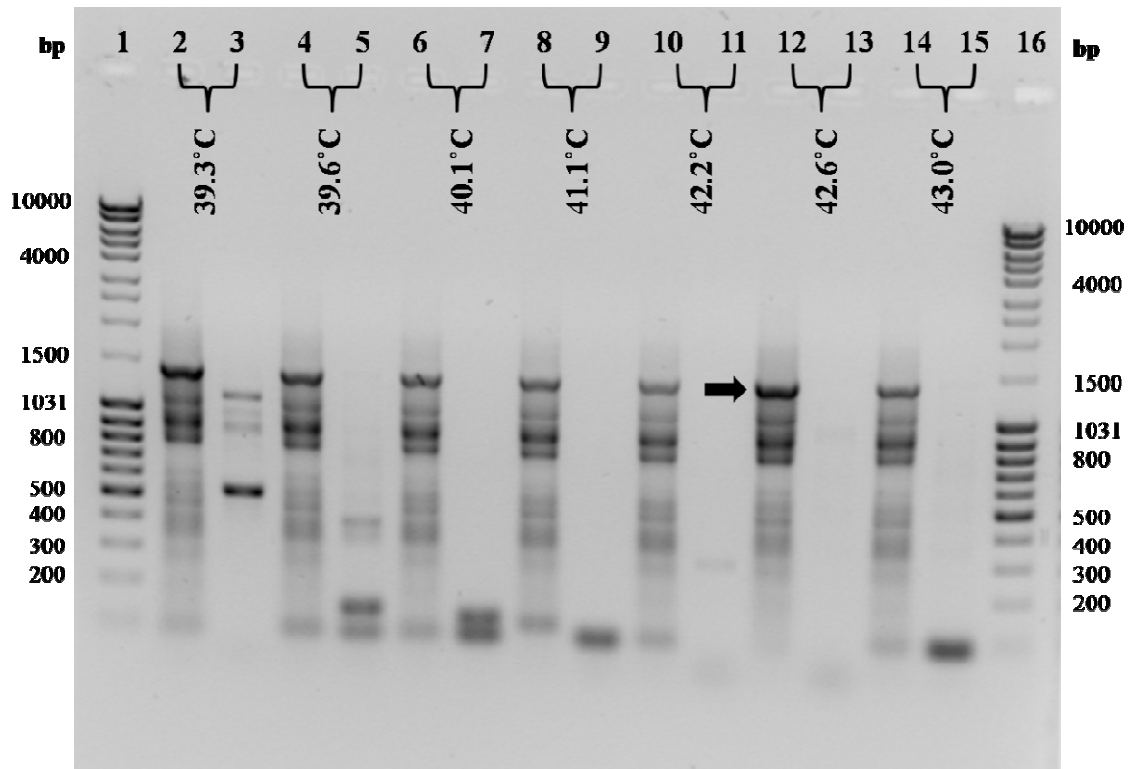


Figure 21. Optimization of the *ama-1* promoter region amplification shown by agarose gel. 1&16: DNA size standard. Even lanes: Reactions employing 200ng template DNA. Odd lanes: Reactions employing 500ng template DNA. Initial primer annealing temperatures are shown, while the second annealing temperature was constant at 58°C. The black arrow indicates the desired 1455bp product amplified with the initial annealing temperature of 42.6°C.

Figure 22 represents the products obtained by duplicate reactions where High Fidelity PCR Enzyme Mix was used to amplify of 200ng of template DNA with annealing temperatures 42.6°C and 58°C for 5 and 30 cycles respectively, and an extension temperature of 64°C. As expected, both reactions showed multiple amplicons, including the desired product.

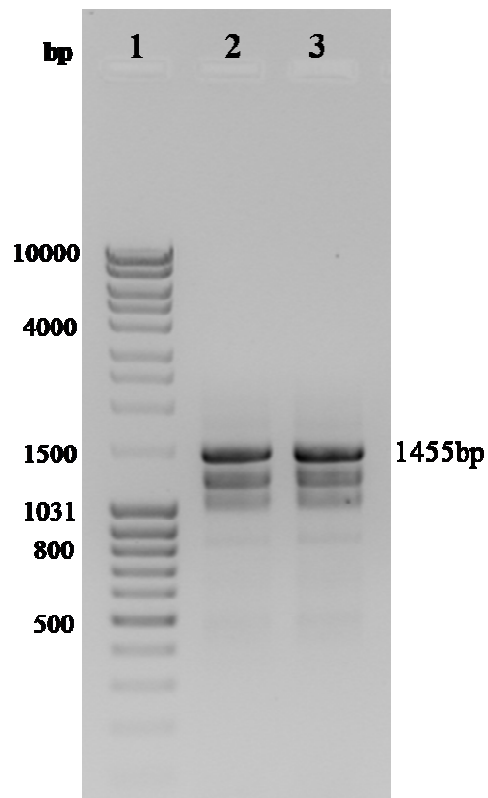


Figure 22. Amplified *ama-1* promoter region shown by agarose gel. 1: DNA size standard. 2&3: *Pf ama-1* products amplified using High Fidelity PCR Enzyme Mix.

The *Pf ama-1* products amplified with High Fidelity PCR Enzyme Mix were pooled and separated on a 0.8% agarose gel at 80V for 1.5hours. The 1455bp product of interest was extracted and purified in a final volume of 30 $\mu$ l of nuclease-free water. The concentration of the resulting product was 27ng/ $\mu$ l (figure 23).

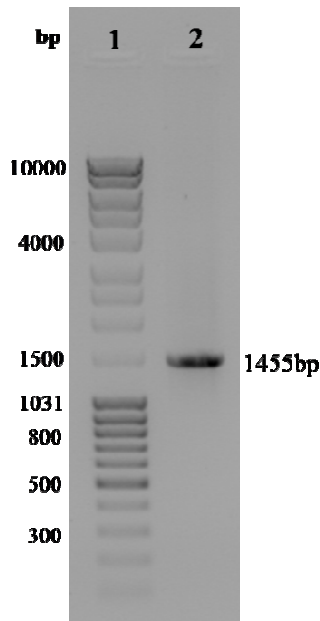


Figure 23. Purified *Pf ama-1* promoter region. 1: DNA size standard. 2: 1455bp *ama-1* promoter region.

#### 3.4. Exchanging the pARL-mCherry *crt* promoter region with the *ama-1* promoter region

The pARL-mCherry plasmid was purified from *E. coli* glycerol stocks in a final volume of 30µl nuclease free water. The plasmid DNA was linearized by digestion with *Xho*I and then visualised by gel electrophoresis, revealing a concentration of 70ng/µl.

The linear pARL-mCherry plasmid was digested with *Not*I, to separate the 1493bp region containing the *crt* promoter from the plasmid backbone, and the resulting ‘sticky ends’ were dephosphorylated to prevent reinsertion of the 1493bp region. The RE sites incorporated into the *ama-1* promoter region PCR product were also digested with *Not*I and *Xho*I, for directional insertion into the plasmid. Both the plasmid and the insert were purified and visualised by gel electrophoresis. The concentrations of the *ama-1* promoter insert and the 5886bp plasmid backbone were 17.8ng/µl and 50ng/µl respectively (figure 24).

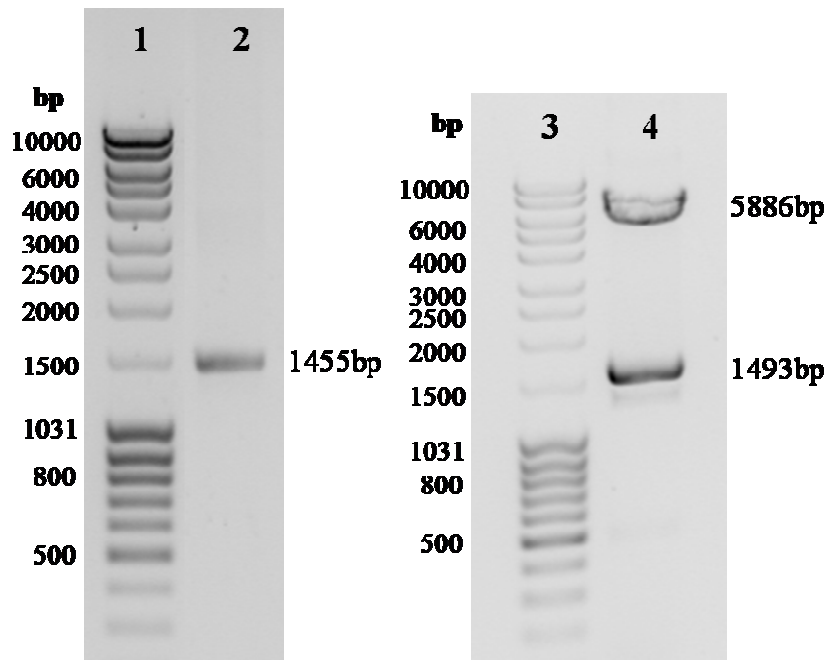


Figure 24. Agarose gels of digested and purified pARL-mCherry and *ama-1* promoter insert. 1&3: DNA size standard. 2: *Pf ama-1* promoter region (1455bp). 4: pARL-mCherry with 1493bp *crt* promoter region and 5886bp plasmid backbone.

The ligation reaction, which was used for transformation, contained 115ng of the digested and purified pARL-mCherry plasmid (2.3µl) and 85.4ng of the digested and purified *ama-1* promoter insert (4.8µl). Transformations were successful as bacterial colonies grew on the agar plates. However, the negative control also showed colony growth, although at a much lower level, indicating a degree of plasmid recirculization. To identify colonies with the pARL-mCherry plasmid containing the *ama-1* promoter insert, 10 individual colonies were picked from the experimental plate for further analysis.

### 3.4.1. Verification of the pARL-mCherry/*ama-1* promoter construct

#### 3.4.1.1. Colony PCR

PCRs were performed using each of the 10 chosen colonies as templates with the AMA-S and AMA-AS primers. An annealing temperature of 58°C and an extension temperature of

64°C were employed for 30 cycles. Figure 25 shows that all colonies produced a product of 1455bp in size, indicating the presence of the pARL-mCherry/*ama-1* promoter plasmid.

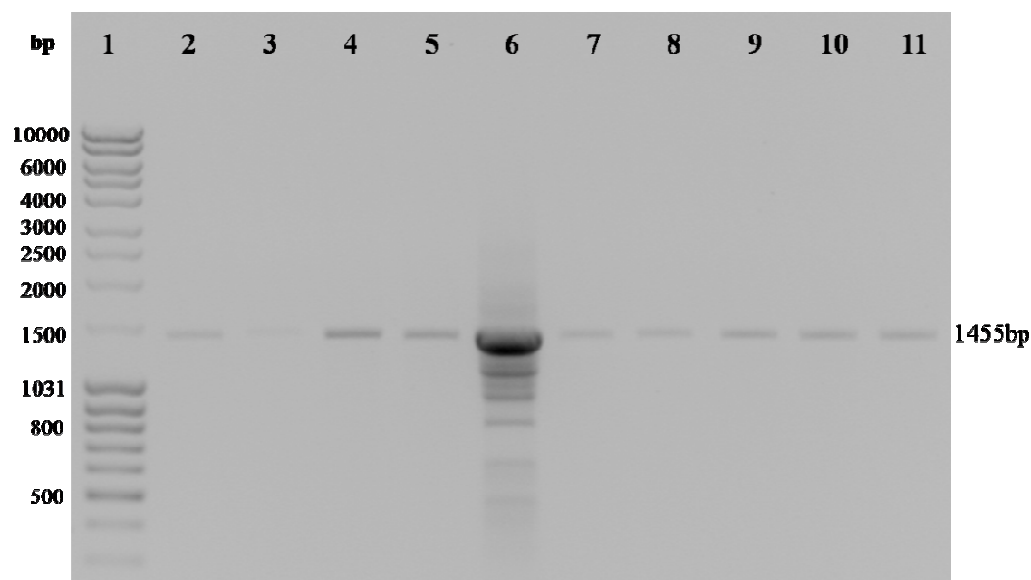


Figure 25. Colony PCR results of *E. coli* transformed with the pARL-mCherry/*ama-1* promoter plasmid. 1: DNA size standard. 2-11: PCR products amplified from 10 individual colonies. The expected product of 1455bp is present in all colonies. The strongest amplicon was produced in colony 5 (lane 6) with non-specific amplicons at approximately 1300bp, 950bp, 800bp, 600bp and 450bp.

#### 3.4.1.2. Restriction enzyme digestion and sequencing

Plasmid DNA was purified from colony 5 and digested with *NotI/XhoI*, and *XhoI/EcoRI* to determine banding patterns (figure 26). The original pARL-mCherry/*crt* plasmid showed three products of 3537bp, 3190bp and 652bp when digested with *XhoI/EcoRI*. However, exchanging the promoter region changes the *XhoI/EcoRI* digestion profile to give two products of 4151bp and 3190bp. Digestion of colony 5 plasmid DNA with *NotI/XhoI* showed the 5886bp plasmid backbone with the 1455bp *ama-1* promoter insert. These banding patterns strongly suggested that the original pARL-mCherry *crt* promoter was successfully exchanged with the desired *ama-1* promoter region. Sequence results

confirmed the removal of the original 1493bp region and the insertion of the 1455bp *ama-1* promoter region in the correct orientation with no mutations (plasmid map in appendix D).

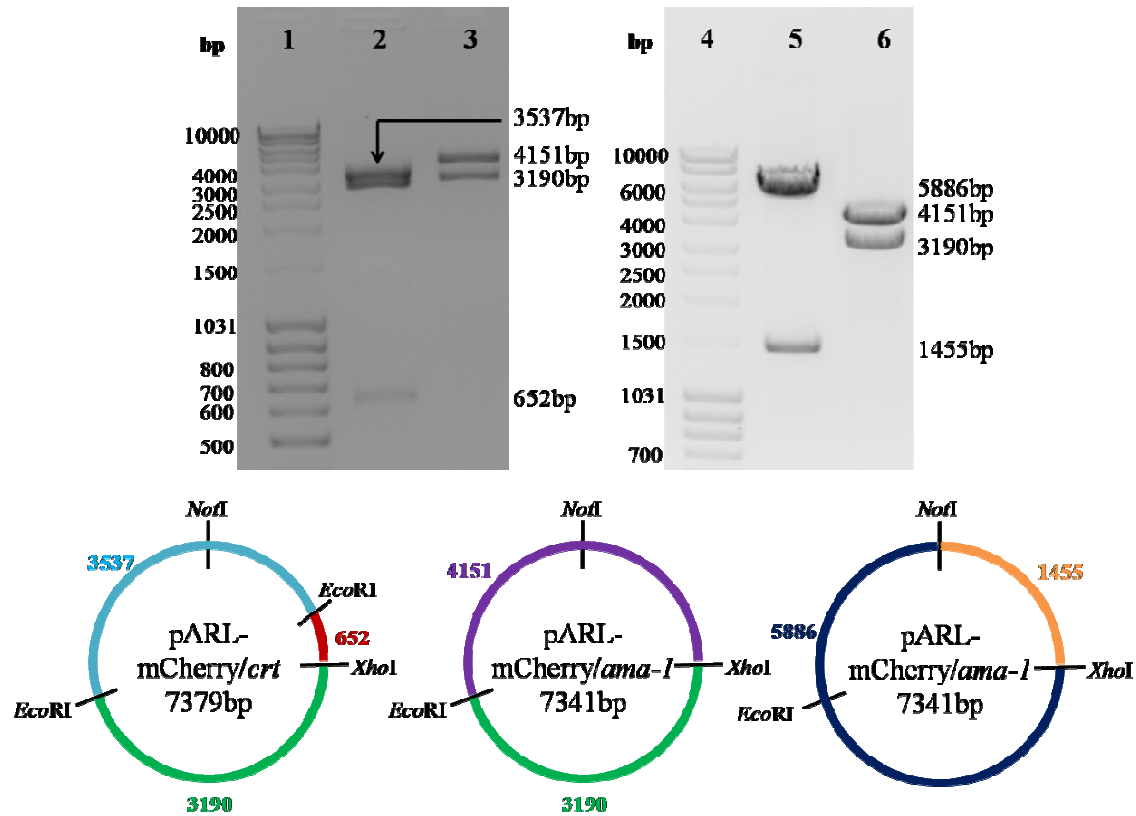


Figure 26. Restriction enzyme digestion of pARL-mCherry/*ama-1* and pARL-mCherry/*crt* plasmids. 1&4: DNA size standards. 2: Original pARL-mCherry/*crt* digested with *XhoI*/*EcoRI*. 3&6: Engineered pARL-mCherry/*ama-1* digested with *XhoI*/*EcoRI*. 5: pARL-mCherry/*ama-1* digested with *NotI*/*XhoI*.

### 3.5. Exchanging the pARL2-GFP *crt* promoter region with the *ama-1* promoter region

The 1455bp *ama-1* promoter region was excised from pARL-mCherry/*ama-1* promoter plasmid DNA by digestion with *NotI* and *XhoI*. The region was then gel purified and inserted into the pARL2-GFP plasmid as described in section 3.4 (plasmid map in appendix E). This work was carried out by Alisje Steyn, department of Molecular Medicine and Haematology, the University of the Witwatersrand.

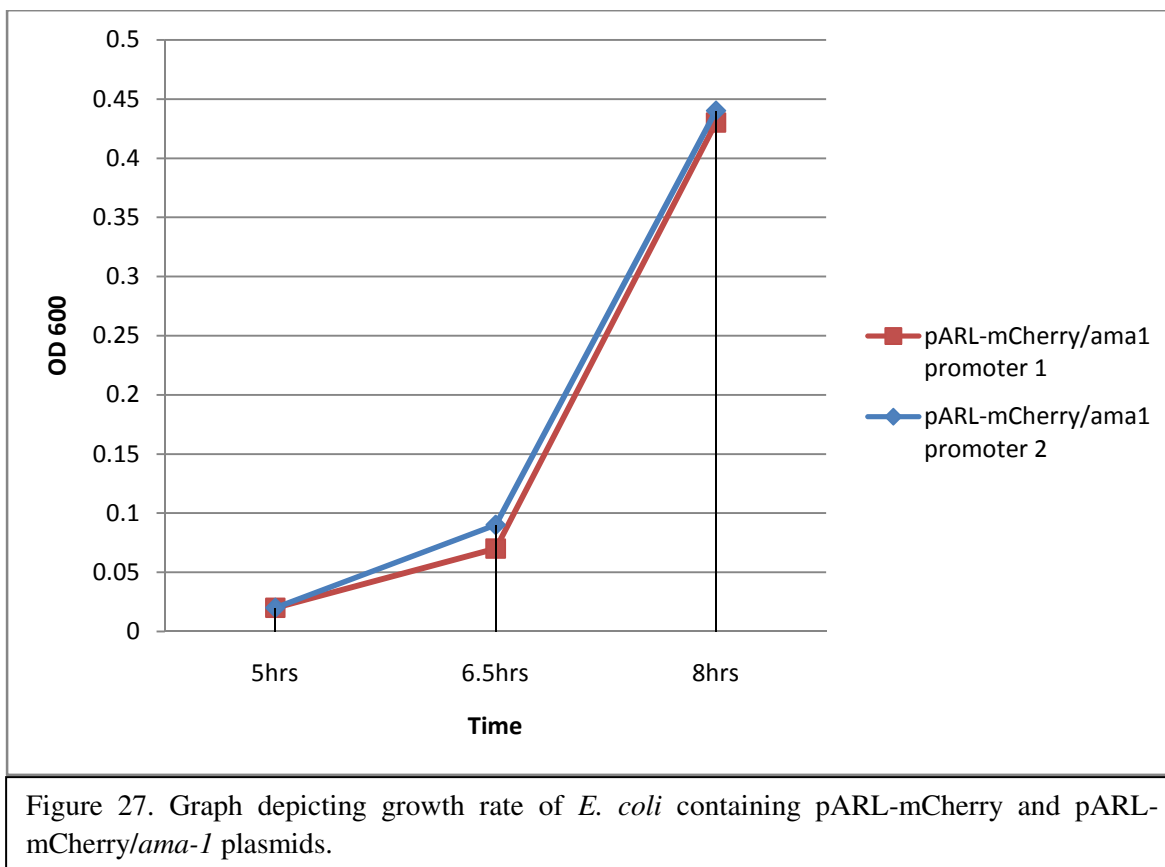
### 3.6. Optimization of large-scale plasmid DNA preparations

To obtain large amounts of plasmid DNA (250-500µg) from a frozen *E. coli* glycerol stock, the bacteria had to be grown in large volumes. A small amount of the stock must be introduced into a small volume starter culture and be allowed to grow and reach exponential growth before inoculating a larger culture volume. The volumes of cultures and inoculants, and the growth times of starter and large cultures were optimized.

#### 3.6.1. Growth time for starter cultures

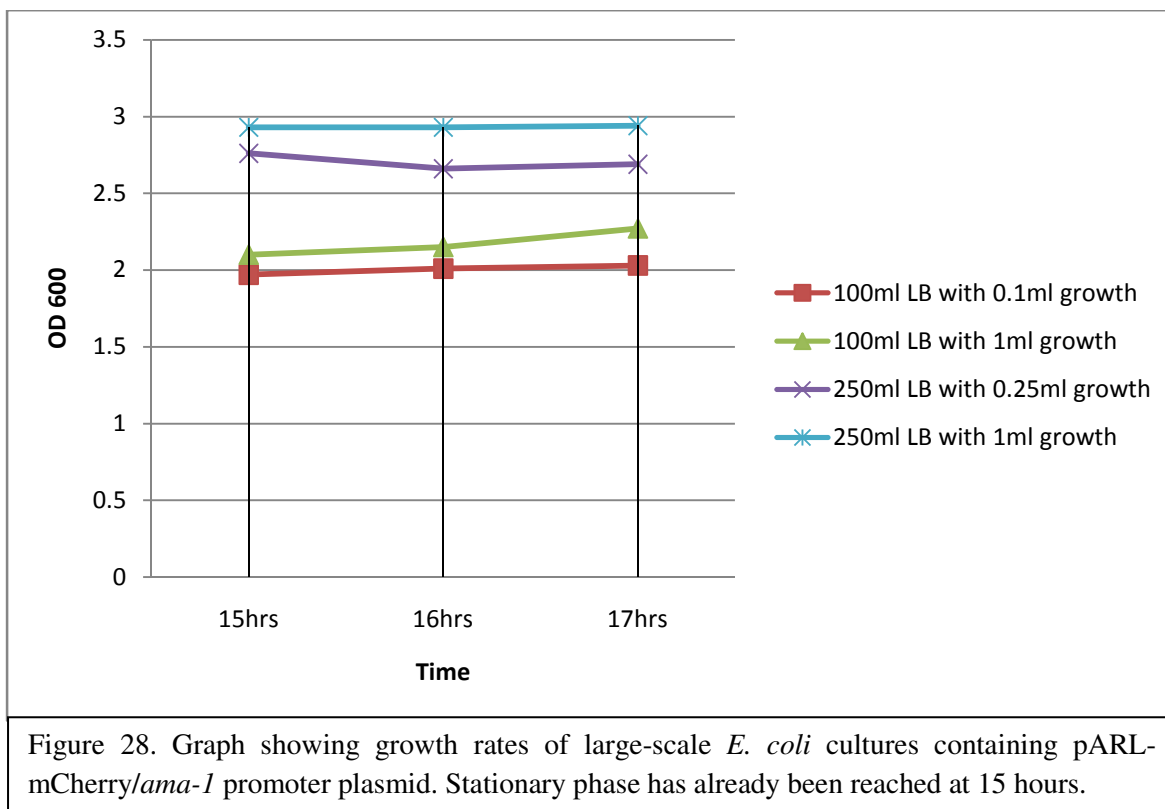
Two pARL-mCherry/*ama-1* promoter plasmid glycerol stocks were used to inoculate 5ml LB starter cultures. OD<sub>600</sub> measurements showed very low growth at 5 hours, with little change after 6.5 hours, indicating the lag phase. However, after 8 hours of growth, the tested cultures showed OD<sub>600</sub> measurements greater than 0.4 (figure 27). According to Sezonov *et al.* (2007), exponential growth is reached at an OD<sub>600</sub> of 0.3. Therefore, growth was stopped at 8 hours and these cultures were used to inoculate the large volume LB cultures.





### 3.6.2. Volumes of large cultures and inoculants

Starter cultures were used to inoculate volumes of 250ml, 100ml and 5ml LB. Inoculants were used at a volume of 1ml or 1/1000 of the larger volume (0.25ml, 0.1ml and 5 $\mu$ l). At 15 hours, OD<sub>600</sub> readings were approximately 2.0 for all cultures and there was very little change in readings at 16 and 17 hours (figure 28). These results indicated that the bacteria had already reached stationary phase. The 250ml cultures showed better growth than the 100ml cultures, probably due to better aeration within the larger flasks. Similar growth was measured for cultures inoculated with 1ml versus 1/1000 volume.



In conclusion, for optimal large-scale plasmid preparation, 5ml starter cultures were allowed to grow for no more than 8 hours, 1ml of the starter culture was used to inoculate a larger volume of 250ml and this larger culture was allowed to grow for ~17 hours.

### 3.7. Generation of chimaeric *maebl* and *jesebl* constructs

To evaluate the role of the CCys domain in trafficking, three *P. falciparum* chimaeric constructs were created from *maebl* and *jesebl* coding sequences. The first *maebl* construct, **M1**, was designed to contain the SP joined to the CCys, TM and CT while the second *maebl* construct, **M2**, lacked the CCys domain. The third construct, **J**, was designed to be produced from *jesebl* coding sequence and contained the SP joined to the CCys. The SP was included for all constructs to allow for correct trafficking of the chimaeric proteins into the ER.

### 3.7.1. Primer design

The *Pf*-MAEBL (PF3D7\_1147800.1) protein consists of six domains: putative N-terminal SP; two tandem cysteine-rich regions; repeat region; CCys; TM and CT (Ghai *et al.*, 2002). Primers were designed to amplify the 174bp SP sequence; the 546bp sequence spanning the CCys, TM and CT; and the 207bp sequence spanning the TM and CT only (figure 29). The sense primer for SP amplification was labelled M-S1, and included an *Xho*I RE site for directional cloning into the pARL2-GFP/*ama-1* promoter plasmid. Two antisense primers were designed for this domain. Both contained the same template recognition sequence, however, M-AS2 included an overhanging sequence to allow the SP to be joined to the CCys, and M-AS3 included an overhanging sequence to allow joining to the TM. The sense primer to amplify the sequence spanning the CCys-TM-CT region was labelled M-S2 and the sense primer to amplify the sequence spanning the TM-CT region was labelled M-S3. A single antisense primer, M-AS1, was designed for the amplification of both these sequences. Primer M-AS1 included an *Avr*II RE site for cloning.

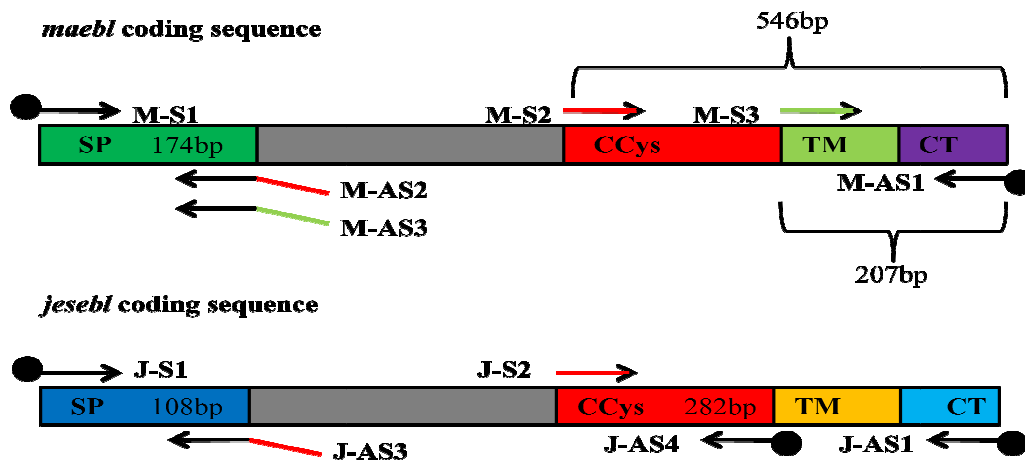


Figure 29. The *maeb1* and *jesebl* coding sequences. Primers are indicated by arrows. Corresponding overlapping primer sequences are shown in green and red. Solid black circles represent incorporated restriction enzyme sites.

The protein structure of *Pf*-JESEBL (PF3D7\_0102500) is very similar to MAEBL. Two tandem DBL cysteine-rich domains, which are characteristic of EBPs, replace the MAEBL

tandem cysteine-rich regions and the repeat region is absent (Gilberger *et al.*, 2003b). Primers were designed to amplify the SP and the CCys (figure 29). The sense and antisense primers designed for the amplification of the SP (108bp) were labelled J-S1 and J-AS3 respectively. As was the case with the MAEBL primers, J-S1 included a *XhoI* site for cloning into the pARL-mCherry/*ama-1* promoter plasmid, and J-AS3 contained an overhanging sequence to allow the SP to be joined to the CCys. The primer pair designed to amplify the CCys (282bp) were designated J-S2 and J-AS4, with an *AvrII* site included on J-AS4. J-AS1 was designed to reverse transcribe *jesebl* RNA. Primer parameters are shown in table 7. Primer sequences are recorded in appendix C.

**Table 7. Parameters for primers used to amplify regions of *maebl* and *jesebl*.**

| Name              | Template-specific portion |           | Whole primer |         | RE site       |
|-------------------|---------------------------|-----------|--------------|---------|---------------|
|                   | Length (bp)               | Tm (°C)   | Length (bp)  | Tm (°C) |               |
| <i>maebl</i>      |                           |           |              |         |               |
| Sense primers     |                           |           |              |         |               |
| M-S1              | 27                        | 60.4      | 37           | 66.4    | <i>Xho</i> I  |
| M-S2              | 21                        | 52.2      | 21           | 52.2    |               |
| M-S3              | 21                        | 55.9      | 21           | 55.9    |               |
| Antisense primers |                           |           |              |         |               |
| M-AS1             | 21                        | 53.1      | 31           | 67.5    | <i>Avr</i> II |
| M-AS2             | 21/28                     | 52.2/60.4 | 49           | 69.4    |               |
| M-AS3             | 21/28                     | 55.9/60.4 | 49           | 70.6    |               |
| <i>jesebl</i>     |                           |           |              |         |               |
| Sense primers     |                           |           |              |         |               |
| J-S1              | 24                        | 56.8      | 34           | 68.8    | <i>Xho</i> I  |
| J-S2              | 27                        | 53.8      | 27           | 53.8    |               |
| Antisense primers |                           |           |              |         |               |
| J-AS1             | 23                        | 57.1      | 33           | 70.2    | <i>Avr</i> II |
| J-AS3             | 27/24                     | 53.8/60.6 | 51           | 69.8    |               |
| J-AS4             | 24                        | 53.7      | 34           | 66.7    | <i>Avr</i> II |

\* Where a forward slash is present, values are for overhanging sequence/template recognition sequence.

Tm- Melting temperature, calculated by Integrated DNA Technologies SciTools OligoAnalyzer, 0.25µM oligo, 50mM Na<sup>+</sup>, 1.5mM Mg<sup>++</sup>, 1mM dNTP.

### 3.7.2. *P. falciparum* RNA isolation and production of cDNA

A 5ml *P. falciparum* culture was synchronized by two D-sorbitol treatments, 48 hours apart. Approximately 27 hours post synchronization, when smears showed the presence of schizont-stage parasites, RNA was extracted. Schizont-stage parasites were used, specifically, because *maebl* and *jesebl* are only transcribed during this stage. Spectrophotometric readings showed RNA concentration to be 430ng/μl, with an  $A_{260}/A_{280}$  reading of 2.11. The sample showed good integrity, as visualized by gel electrophoresis, with clear products visible for ribosomal RNA subunits and a smear representing the mRNA fraction (figure 30).

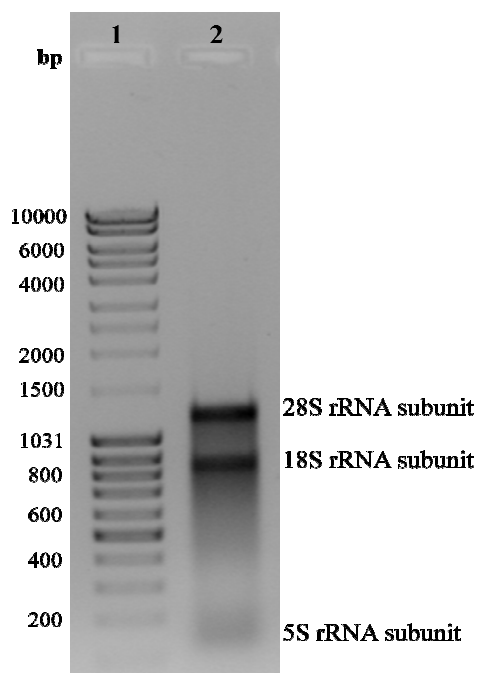


Figure 30. Agarose gel indicating sample integrity of RNA isolated from *P. falciparum*. 1: DNA size standard. 2: *Pf*RNA sample

RNA was then reverse transcribed in two separate reactions to produce *maebl* and *jesebl* single stranded cDNA. For this purpose, the antisense primers M-AS1 and J-AS1 were used to reverse transcribe the whole *maebl* and *jesebl* mRNA sequences. Both of these

products were then amplified in PCR reactions employing both sense and antisense primers to create double stranded cDNA products.

### 3.7.3. Amplification of different regions of *maebl* and *jesebl* using PCR

**Table 8. Primers used to amplify *maebl* and *jesebl* domains.**

| Primer pair | Domain                  | Domain size (bp) | Expected PCR product size (bp) |
|-------------|-------------------------|------------------|--------------------------------|
| M-S1/M-AS2  | <i>maebl</i> SP 1       | 174              | 205                            |
| M-S1/M-AS3  | <i>maebl</i> SP 2       | 174              | 205                            |
| M-S2/M-AS1  | <i>maebl</i> CCys-TM-CT | 546              | 556                            |
| M-S3/M-AS1  | <i>maebl</i> TM-CT      | 207              | 217                            |
| J-S1/J-AS3  | <i>jesebl</i> SP        | 108              | 145                            |
| J-S2/J-AS4  | <i>jesebl</i> CCys      | 282              | 292                            |

The 174bp *maebl* SP was amplified in two reactions using (1) M-S1 and M-AS2 and (2) M-S1 and M-AS3 and the 108bp *jesebl* SP was amplified with J-S1 and J-AS3 (table 8, figure 31). The M-S1 primer includes an added 10bp for RE recognition and both M-AS2 and M-AS3 are extended by 21bp overhangs. The resulting PCR products are therefore expected to be 205bp in length. Similarly, additional bases on the J-S1 and J-AS3 primers result in an expected length of 145bp for *jesebl* SP.

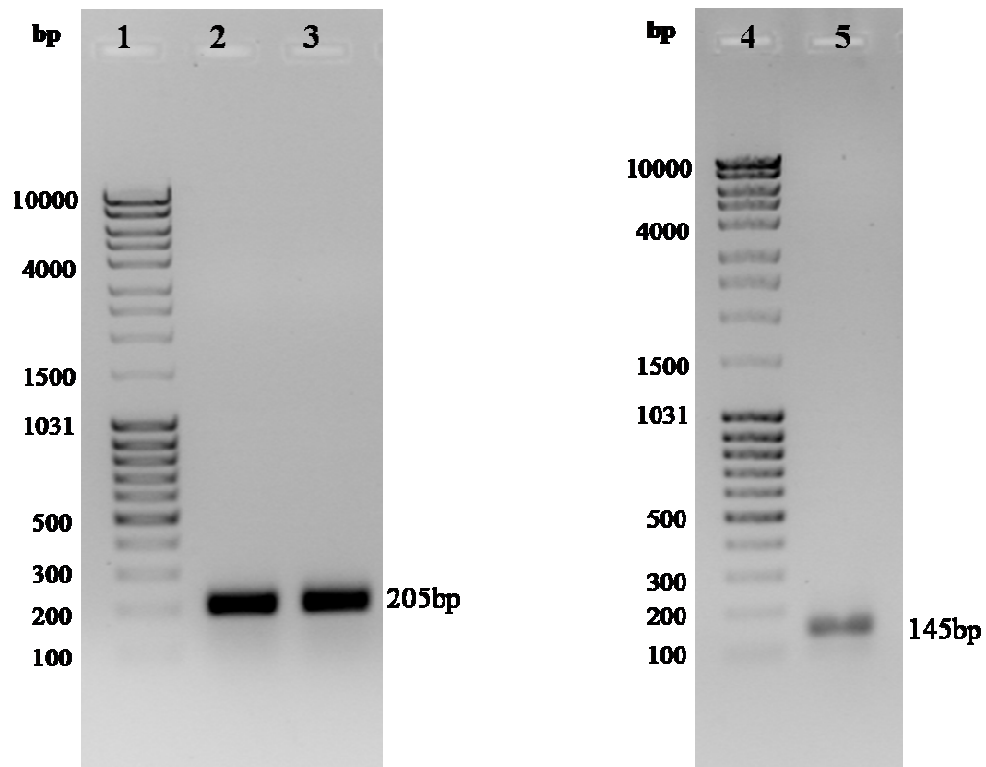


Figure 31. PCR results of amplified *maekl* SP and *jesebl* SP shown by agarose gel. 1&4: DNA size standards. 2: *maekl* SP 1 amplified with M-S1 and M-AS2. 3: *maekl* SP 2 amplified with M-S1 and M-AS3. 5: *jesebl* SP amplified with J-S1 and J-AS3.

The *maekl* CCys-TM-CT sequence was amplified using M-S2 and M-AS1, and M-S3 and M-AS1 were used to amplify the *maekl* TM-CT sequence. These two sequences are 546bp and 207bp respectively. With the additional 10bp added on to M-AS1, for RE recognition, the expected products are 556bp and 217bp respectively. Primers J-S2 and J-AS4 amplified the *jesebl* CCys, with the expected size of 292bp (table 8, figure 32).

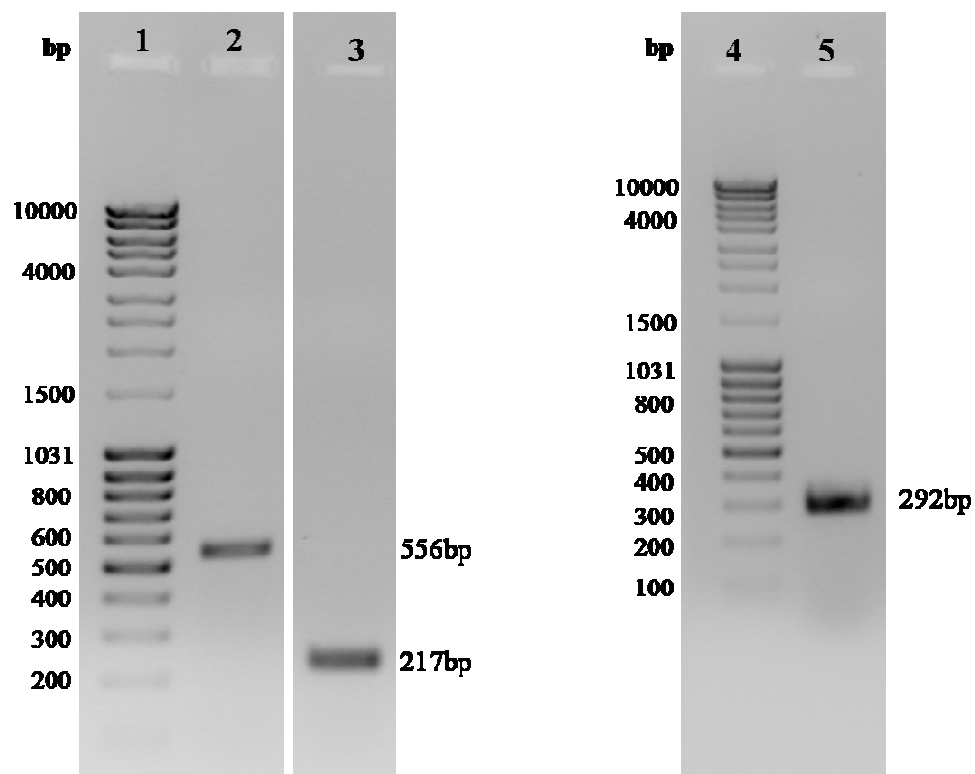


Figure 32. PCR results of amplified *maeb1* CCys-TM-CT, *maeb1* TM-CT and *jesebl* CCys shown by agarose gel. 1&4: DNA size standards. 2: *maeb1* CCys-TM-CT amplified with M-S2 and M-AS1. 3: *maeb1* TM-CT amplified with M-S3 and M-AS1. 5: *jesebl* CCys amplified with J-S2 and J-AS4.

#### 3.7.4. Overlap extension PCR

The *maeb1* and *jesebl* PCR products were joined together in overlap-extension PCRs to create constructs **M1**, **M2** and **J** as represented in figure 33. The expected sizes of **M1**, **M2** and **J** were 740bp, 401bp and 410bp respectively.



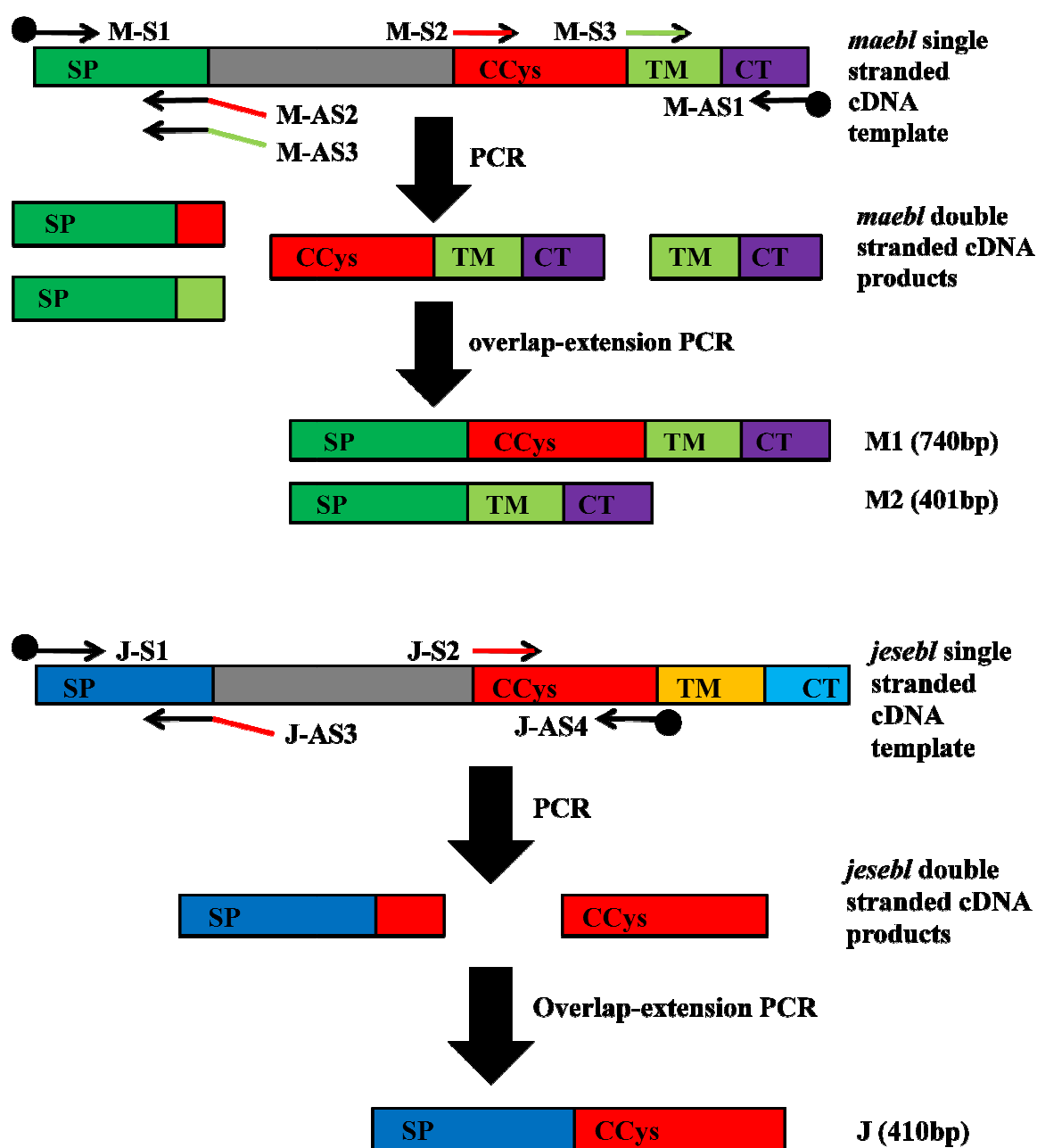


Figure 33. Schematic representation of overlap extension PCR to create chimaeric constructs M1, M2 and J.

In an overlap extension PCR, the two template sequences are added to a single reaction mixture, containing appropriate primers, which joins and simultaneously amplifies the resulting product. The components necessary to produce constructs M1, M2 and J are listed in table 9.

**Table 9. Reaction components for production of M1, M2 and J using overlap extension PCR.**

| <b>Construct</b>        | <b>M1</b>               | <b>M2</b>          | <b>J</b>           |
|-------------------------|-------------------------|--------------------|--------------------|
| <b>Template 1</b>       | <i>maebl</i> SP 1       | <i>maebl</i> SP 2  | <i>jesebl</i> SP   |
| <b>Template 2</b>       | <i>maebl</i> CCys-TM-CT | <i>maebl</i> TM-CT | <i>jesebl</i> CCys |
| <b>Sense primer</b>     | M-S1                    | M-S1               | J-S1               |
| <b>Antisense primer</b> | M-AS1                   | M-AS1              | J-AS4              |

Overlap extension PCRs were successful, as amplicons of the expected sizes were obtained by agarose gel electrophoresis (figure 34). The amplicon expected for **M1** (740bp) was clearly visible, however two smaller products of approximately 275bp and 350bp were also present. These products are probably due to non-specific binding of the primer pair. PCR temperature adjustments were employed in an attempt to eliminate these non-specific amplicons, however, these attempts were unsuccessful. The PCR product of interest was instead extracted by gel purification. Construct **M2** was amplified at the expected size of 401bp. Very faint non-specific amplicons were observed at 500bp and 700bp, however, the very low amounts of these amplicons rendered them insignificant in comparison to the fragment of interest. The expected amplicon size of 410bp was produced for construct **J** and a second insignificant non-specific product of approximately 375bp was also present. Both **M2** and **J** PCR products were purified before subsequent enzyme digestions.

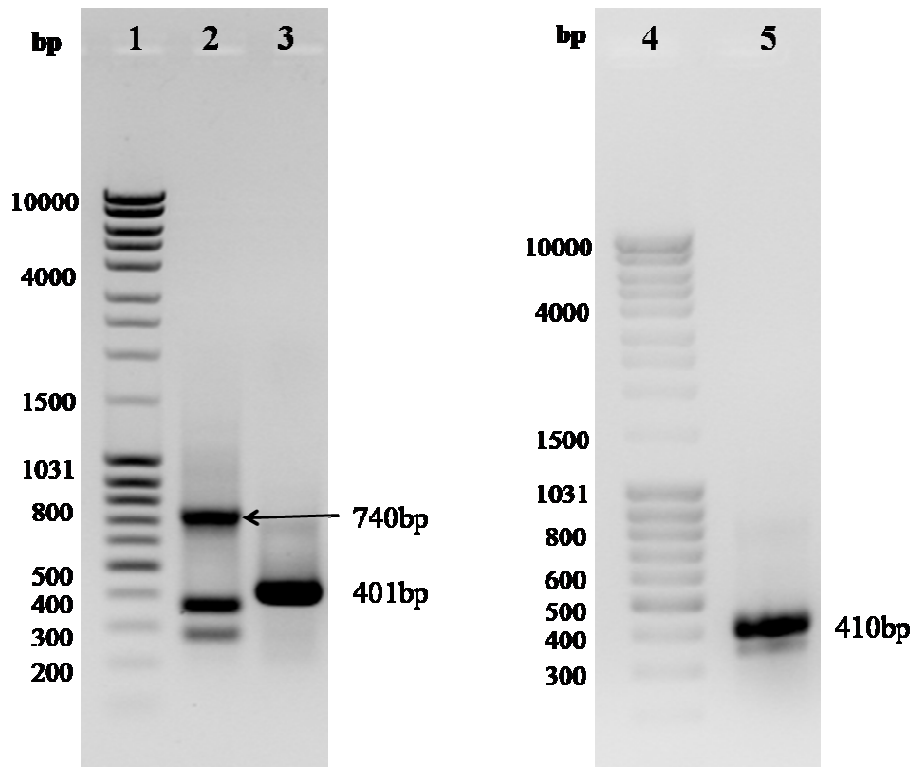


Figure 34. Overlap extension PCR of **M1**, **M2** and **J** shown by agarose gel. 1&4: DNA size standards. 2: **M1** at expected size of 740bp. 3: **M2** at expected size of 401bp. 5: **J** at expected size of 410bp.

### 3.8. Insertion of construct **J** into pARL-mCherry/*ama-1* promoter plasmid

#### 3.8.1. Ligation and transformation

Both the plasmid (1µg) and insert (500ng) DNA were digested with *Xho*I and *Avr*II and the plasmid was dephosphorylated with alkaline phosphatase (See appendix D for plasmid map). Digestion of the insert decreases its size from 410bp to 402bp. The plasmid and insert digestions were then purified and quantified at 49ng/µl and 42ng/µl respectively (figure 35). A ligation reaction was carried out containing ~29ng insert DNA (0.7µl) and ~165ng vector DNA (3.4µl) and used to transform competent *E. coli* cells. Experimental plates showed four times more growth than control plates. Five colonies were picked from the experimental plates and 2 were picked from the control plate for further analysis.

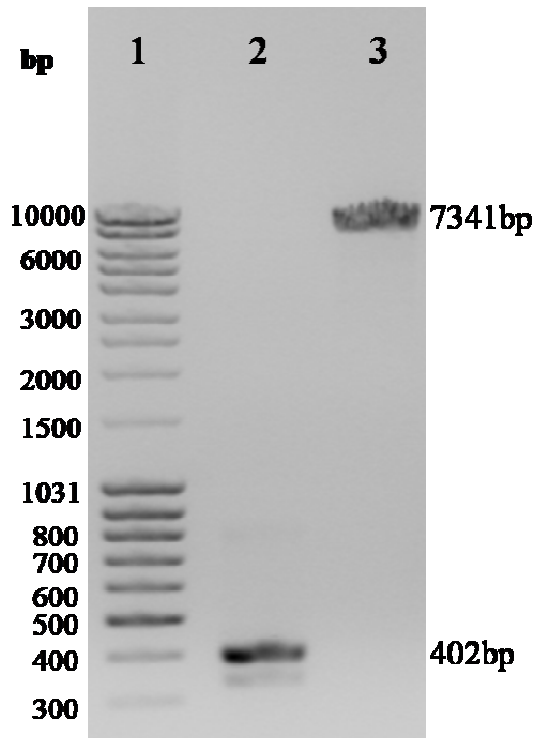


Figure 35. Quantification of pARL-mCherry plasmid and construct **J** by agarose gel electrophoresis. 1: DNA size standard. 2: Digested and purified **J** at expected size of 402bp with a concentration of 42ng/μl. A non-specific product is still present at ~375bp. 3: Digested and purified pARL-mCherry plasmid (containing *ama-1* promoter) at expected size of 7341bp, with a concentration of 49ng/μl.

### 3.8.2. Analysis of colonies transformed with pARL-mCherry/**J**

#### 3.8.2.1. Colony PCR

Each of the 7 chosen colonies was amplified using the insert-specific primers J-S1 and J-AS4. An annealing temperature of 55°C and an extension temperature of 68°C were employed for 30 cycles. The expected product (410bp) was present in all 5 colonies, indicating successful transformation. There was no amplification for either of the control colonies (figure 36).

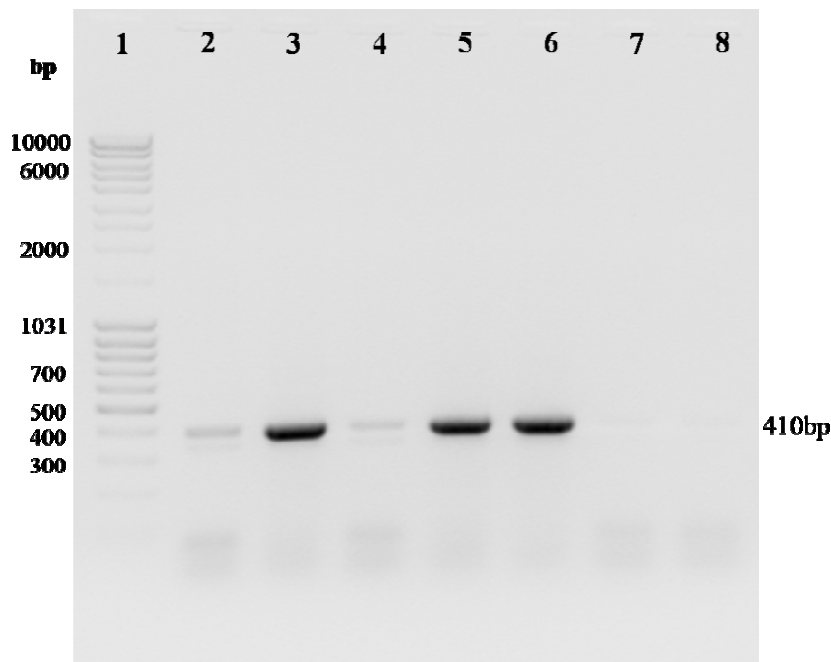


Figure 36. Colony PCR results of *E. coli* transformed with the pARL-mCherry/**J** plasmid. 1: DNA size standard. 2-6: PCR products amplified from 5 individual experimental colonies. The expected 410bp product is present in all colonies, with the strongest products produced by colonies 2, 4&5 (lanes 3, 5&6). There was no amplification for either of the control colonies (lanes 7&8).

#### 3.8.2.2. Restriction enzyme digestion and sequencing

Plasmid DNA was purified from experimental colonies 2 and 4 (figure 37, lanes 3 and 5). The purified plasmids were digested with *XhoI*/*AvrII* and *HindIII*/*HincII*. This restriction analysis suggested that both of the colonies had been transformed with the pARL-mCherry/**J** plasmid as they showed the expected three products of 3613bp, 2126bp and 1992bp for the *HindIII*/*HincII* digests and the expected 7341bp and 402bp products for the *XhoI*/*AvrII* digests (figure 37). If construct **J** had not been inserted into the plasmid, the 2126bp fragment would be decreased to 1736bp in the *HindIII*/*HincII* digestions, and the 402bp product would be absent from the *XhoI*/*AvrII* digestions. Sequence results confirmed the insertion of construct **J** into the pARL-mCherry plasmid, in the correct orientation with no mutations.

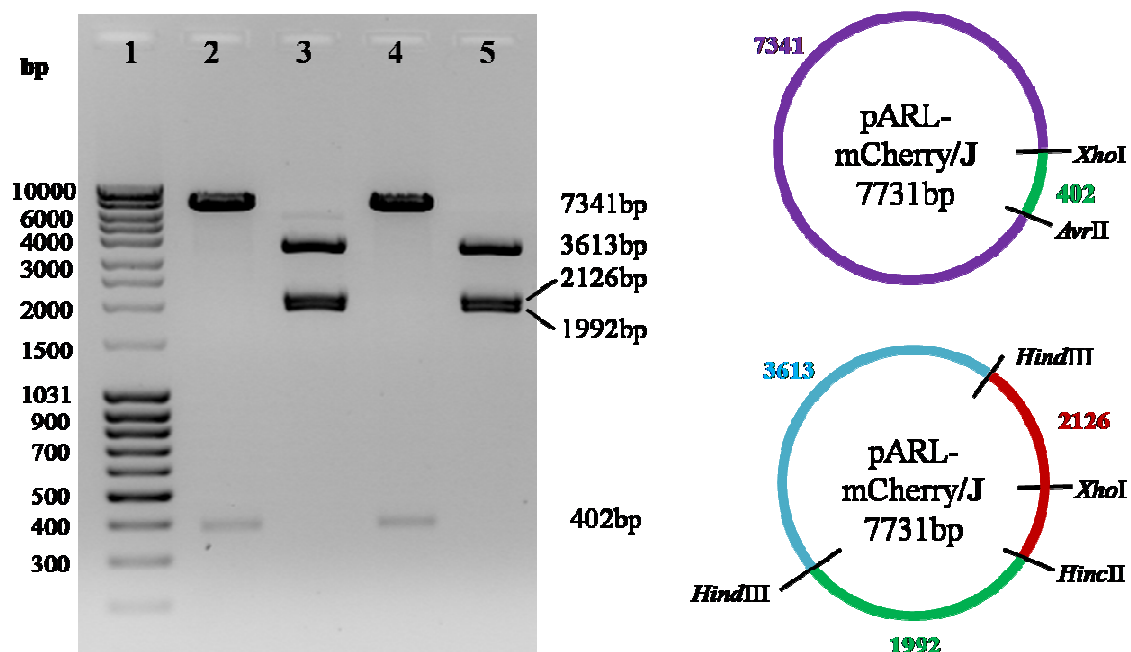


Figure 37. RE digestion of pARL-mCherry/J plasmid DNA extracted from transformed *E. coli*. 1: DNA size standard. 2&4: Plasmid from colonies 2 and 4 digested with *XhoI*/*AvrII*. 3&5: Plasmid from colonies 2 and 4 digested with *HindIII*/*HincII*.

### 3.9. Insertion of constructs **M1** and **M2** into pARL2-GFP/*ama-1* promoter plasmids

#### 3.9.1. Ligation and transformation

As per section 3.8.1, both the pARL2-GFP plasmid and **M1** and **M2** inserts were enzyme-digested, purified and quantified (figure 38, see appendix E for plasmid map). Plasmid concentration was 70ng/μl, **M1** concentration was 24ng/μl and **M2** concentration was 39ng/μl. Ligation reactions were carried out as per table 10, and used to transform competent *E. coli* cells.

**Table 10. Insert and vector amounts for ligations of inserts M1 and M2 into pARL2-GFP plasmids.**

|                   | M1            | M2            |
|-------------------|---------------|---------------|
| Amount of insert  | 47ng (2μl)    | 27ng (0.7μl)  |
| Amount of plasmid | 154ng (2.2μl) | 168ng (2.4μl) |

Transformations using pARL2-GFP/**M1** and pARL2-GFP/**M2** showed 2-3 times more growth than control plates. Ten colonies were picked from each of the experimental plates and 2 were picked from the control plate for further analysis.

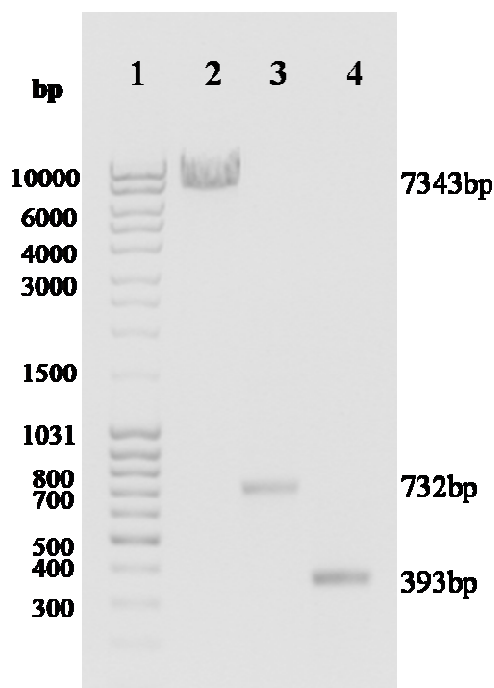


Figure 38. Quantification of pARL2-GFP plasmid and constructs **M1** and **M2** by agarose gel electrophoresis. 1: DNA size standard. 2: Digested and purified pARL2-GFP plasmid (containing *ama-1* promoter) at expected size of 7343bp, with a concentration of 70ng/ $\mu$ l. 3: Digested and purified **M1** at expected size of 732bp with a concentration of 24ng/ $\mu$ l. Digested and purified **M2** at expected size of 393bp with a concentration of 39ng/ $\mu$ l.

### 3.9.2. Analysis of colonies transformed with pARL2-GFP/**M1** and pARL2-GFP/**M2**

#### 3.9.2.1. Colony PCR

Each of the 22 chosen colonies was amplified using the insert-specific primers M-S1 and M-AS1. Annealing and extension temperatures of 58°C and 68°C were used, for 30 cycles. For insert **M1**, eight colonies produced the expected product. The other two colonies gave amplified products of unexpected sizes (~390bp and ~300bp). These products were very similar in size to the non-specific amplicons produced in the **M1** overlap extension PCR (figure 34). It is possible that a small amount of these non-specific products were present in the purified sample and then would have transformed *E. coli*, and thus were present in the

bacterial colonies. There were nine positives for **M2**, and one unexpected product of ~300bp. There was no amplification for either of the control colonies (figure 39).

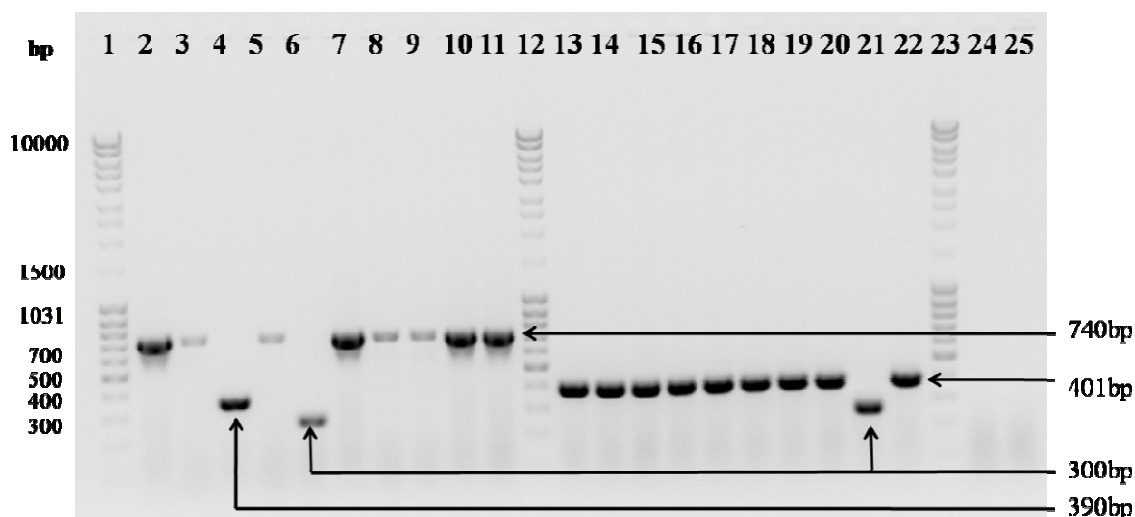


Figure 39. Colony PCR results of *E. coli* transformed with the pARL2-GFP/**M1** and pARL2-GFP/**M2** plasmids. 1,12&23: DNA size standards. 2-11: PCR products amplified from 10 individual pARL2-GFP/**M1** colonies. The expected product was present in 8 of the 10 colonies, with the strongest products produced by colonies 1, 6, 9&10 (lanes 2, 7, 10&11). 13-22: PCR products amplified from 10 individual pARL2-GFP/**M2** colonies. Strong expected products were present in 9 of the 10 colonies. 24&25: Control colonies showing no PCR products.

### 3.9.2.2. Restriction enzyme digestion and sequencing

Plasmid DNA was purified from two positive colonies from each of the pARL2-GFP/**M1** and pARL2-GFP/**M2** transformations. The purified plasmids were digested with *XhoI*/*AvrII*, to excise the inserts, with results suggesting successful transformation, as per section 3.8.2.2. Figure 40 shows the expected product sizes of 7343bp and 732bp for pARL2-GFP/**M1** and 7343bp and 393bp for pARL2-GFP/**M2**. Sequence results confirmed the insertions of the **M1** and **M2** constructs into the pARL2-GFP plasmids, in the correct orientations with no mutations.



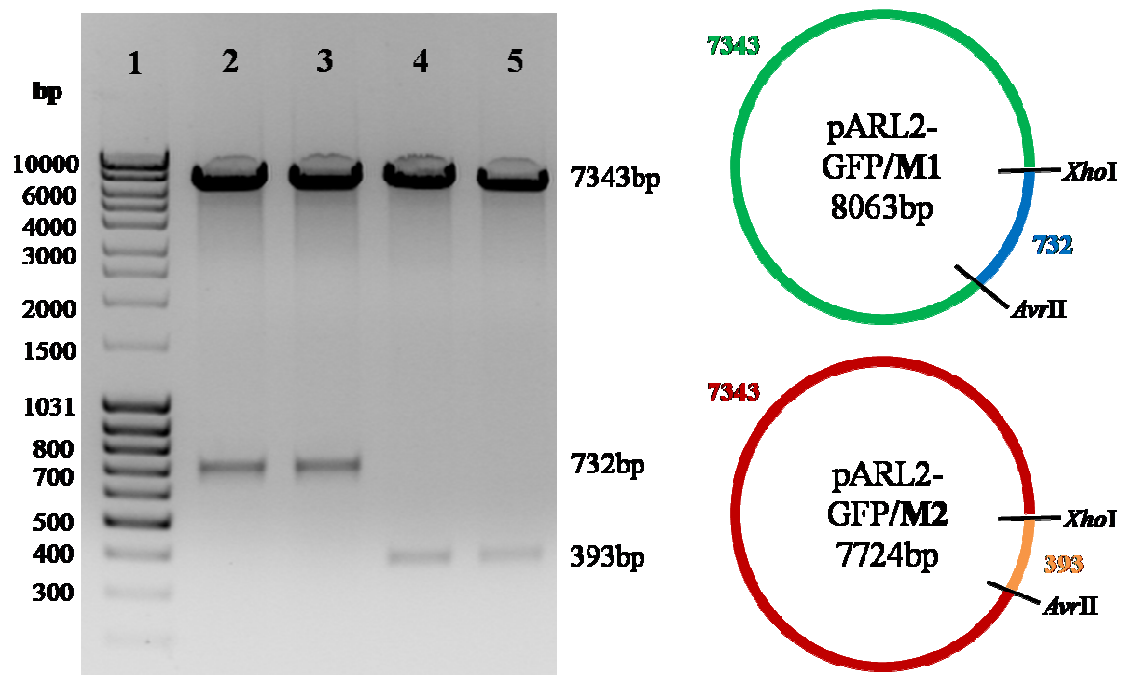


Figure 40. RE digestion of pARL2-GFP/M1 and pARL2-GFP/M2 plasmids extracted from transformed *E. coli*. 1: DNA size standard. 2&3: Plasmid from 2 pARL2-GFP/M1 colonies digested with *XhoI*/*AvrII*. 4&5: Plasmid from 2 pARL2-GFP/M2 colonies digested with *XhoI*/*AvrII*.

### 3.10. Transfection of *P. falciparum* parasites

#### 3.10.1. Control pARL-mCherry and pARL2-GFP plasmids

The pARL-mCherry and pARL2-GFP plasmids, which had been engineered to contain the *ama-1* promoter, were used in transfections to provide controls for the chimaeric constructs **M1**, **M2** and **J**. These plasmids were prepared by large-scale plasmid purification as high concentrations of plasmid (> 100µg) are needed for transfection. Plasmid amounts used, electroporation times and actual voltage reached for each electroporation procedure are recorded in table 11. The electroporation time of 20.1ms, recorded for both of the control plasmids, is much higher than the expected time of 12-15ms. This was probably due to an incorrect salt concentration within the cytomix reaction mixture. Fresh cytomix was therefore prepared for use in subsequent transfection reactions.

**Table 11. Plasmid amounts, electroporation times and recorded voltages for transfections.**

| Plasmid                | Amount used for transfection (µg) | Electroporation time (ms) | Actual voltage (V) |
|------------------------|-----------------------------------|---------------------------|--------------------|
| pARL-mCherry control   | ~250                              | 20.1                      | 307                |
| pARL2-GFP control      | ~100                              | 20.1                      | 309                |
| pARL-mCherry/ <b>J</b> | ~300                              | 12.7                      | 309                |
| pARL2-GFP/ <b>M1</b>   | ~300                              | 15.9                      | 307                |
| pARL2-GFP/ <b>M2</b>   | ~325                              | 12                        | 308                |

Despite the incorrect salt concentrations of the reactions, the electroporated erythrocytes took up both the control pARL-mCherry/*ama-1* promoter and the control pARL2-GFP/*ama-1* promoter plasmids. Erythrocytes containing each of the control plasmids were infected with *P. falciparum* parasites and kept under selective WR99210 drug pressure. Parasites that have successfully taken up the introduced plasmid will become resistant to the drug as the plasmid contains the *dhfr* gene. Therefore, these parasites will grow under constant drug pressure, while those that have not taken up the plasmid will die. pARL-mCherry and pARL2-GFP transfectants were positive for parasite growth at 25 and 27 days post transfection, respectively.

### 3.10.2. Experimental plasmids pARL-mCherry/**J**, pARL2-GFP/**M1** and pARL2-GFP/**M2**

As per section 3.10.1, plasmid amounts used, electroporation times and actual voltage reached for each electroporation procedure are recorded in table 11. Contrary to the high electroporation times recorded for the control plasmids, the times for the experimental plasmids pARL-mCherry/**J**, pARL2-GFP/**M1** and pARL2-GFP/**M2** were within the expected range, at 12.7ms, 15.9ms and 12ms respectively. These results indicated a correct salt concentration within each reaction.

As was the case with the control plasmids, transfections were successful as cultures were positive for parasite growth at 23, 24 and 24 days post transfection for pARL2-GFP/**M1**, pARL-mCherry/**J** and pARL2-GFP/**M2** respectively.

### 3.11. Transfection verification using PCR

DNA was extracted from each of the 5 transfected parasite cultures and used in PCRs. This was carried out to verify the presence of the introduced plasmid DNA in each of the transfectants. Positives controls, employing previously purified plasmid DNA as the template, and negative controls, employing DNA extracted from 3D7 *P. falciparum* parasites as the template were included for each PCR. An annealing temperature of 55°C and an extension temperature of 68°C were used for 30 cycles.

#### 3.11.1. Verification of control pARL-mCherry and pARL2-GFP transfectants

For the control transfectants, containing pARL-mCherry/*ama-1* promoter and pARL2-GFP/*ama-1* promoter, plasmid-specific primers were used. As shown in figure 41, amplification of the extracted parasite DNA and the positive plasmid control resulted in the expected product of ~530bp for both transfectants, verifying the presence of the plasmids. A clear non-specific amplicon of approximately 450bp was also present, among other faint non-specific amplicons. The 450bp product was most probably amplified due to non-specific binding of the forward plasmid-specific primer, as its recognition sequence was within the AT-rich *ama-1* promoter region. This was also the case when the *ama-1* promoter region was amplified for insertion into the pARL plasmids (figure 22). As expected, no amplification took place within the negative controls, only primer dimers were present.

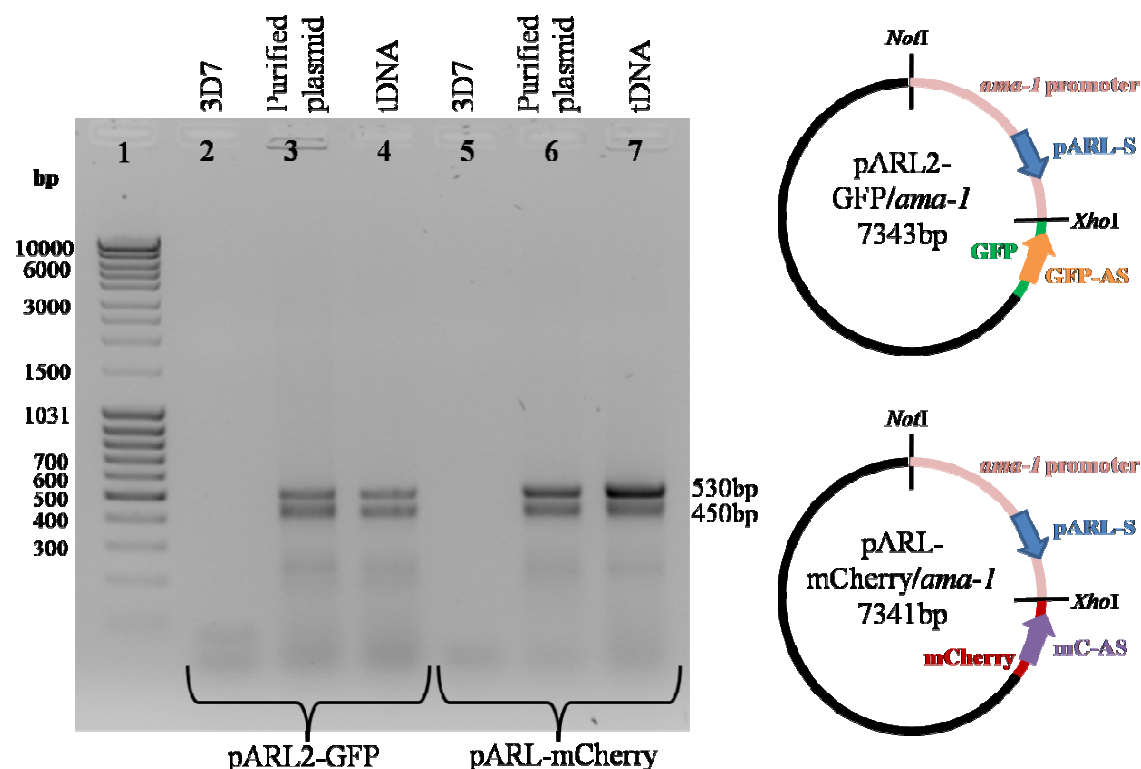


Figure 41. PCR of DNA extracted from *P. falciparum* parasites transfected with pARL-mCherry and pARL2-GFP control plasmids. 1: DNA size standard. 2-4: DNA extracted from pARL2-GFP transfectants (tDNA) and controls (3D7 and purified plasmid). 5-7: DNA extracted from pARL-mCherry transfectants (tDNA) and controls. Non-specific amplicons (~450bp) and the expected ~530bp products are present in both transfectants and both positive controls. Vector maps indicate the positions of the plasmid-specific primers used (pARL-S, GFP-AS & mC-AS).

### 3.11.2. Verification of pARL2-GFP/M1, pARL-GFP/M2 and pARL-mCherry/J transfectants

Plasmid-specific forward primers and insert-specific reverse primers were used in the PCR reactions. As was the case for the control transfectants, the expected amplicons of 1189bp, 850bp and 859bp were observed for the pARL2-GFP/M1, pARL-GFP/M2 and pARL-mCherry/J transfectants and their respective positive controls (figure 42). These results showed that the plasmids had been successfully taken up by the parasites.

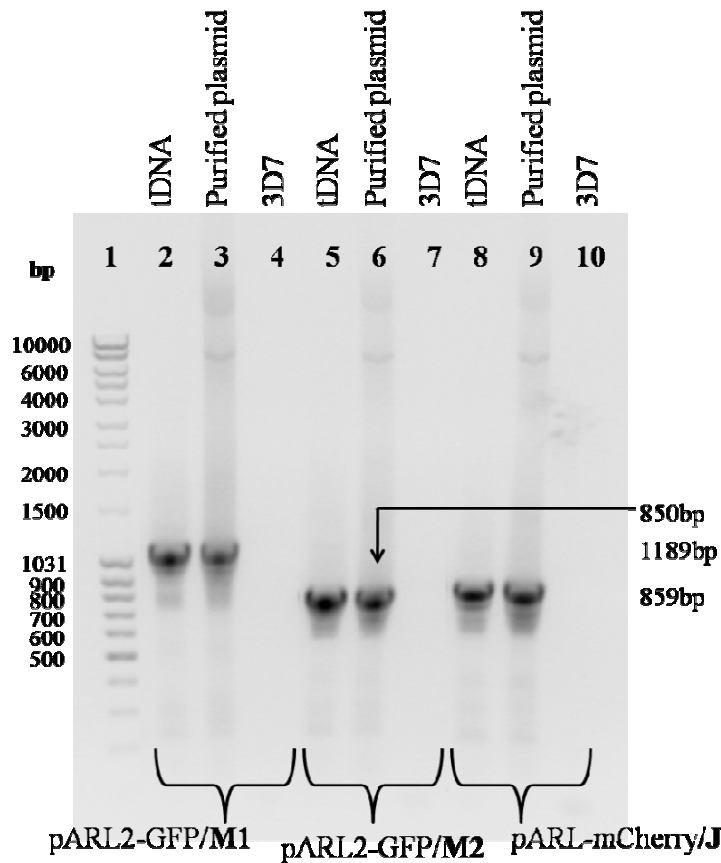


Figure 42. PCR of DNA extracted from *P. falciparum* parasites transfected with pARL2-GFP/M1, pARL2-GFP/M2 & pARL-mCherry/J. 1: DNA size standard. 2-4: DNA extracted from pARL2-GFP/M1 (tDNA) and controls (3D7 and purified plasmid). 5-7: DNA extracted from pARL2-GFP/M2 (tDNA) and controls. 8-10: DNA extracted from pARL-mCherry/J (tDNA) and controls. Products of the expected sizes (1189bp, 850bp & 859bp) are present for all transfectants and their positive controls respectively.

### 3.12. Visualization of transgenic parasites by fluorescence microscopy

#### 3.12.1. Live imaging

##### 3.12.1.1. MAEBL chimaeric proteins, **M1** and **M2**

Live *P. falciparum* parasites containing the pARL2-GFP/*ama-1* promoter construct, the pARL2-GFP/M1 construct, the pARL-GFP/M2 construct, or no construct at all, were stained with the blue nuclear stain, DAPI, and visualized by fluorescence microscopy. Late schizont stage parasites and free merozoites were observed. Unstained control 3D7 parasites containing no construct showed no auto-fluorescence under any of the fluorescent

filters. DAPI stained control parasites showed blue fluorescence in the nuclear regions, with no visible green fluorescence, as expected, indicating the absence of fluorescence bleeding (figure 43, top panel). All three transgenic parasite samples showed both blue and green fluorescence (figure 43). The parasites expressing the pARL2-GFP/*ama-1* promoter control construct showed dispersed green fluorescence surrounding and overlapping the blue nuclear area, suggesting that the GFP was trapped within the cytosol (figure 43, second panel). Parasites expressing the pARL-GFP/**M2**( $\Delta$ CCys) construct showed a similar expression pattern to the control (figure 43, third panel), while parasites expressing the pARL2-GFP/**M1**(CCys) construct showed localized green fluorescence separate and distinct from the blue nuclear region, with no overlap (figure 43, bottom panel). This observation indicated that the GFP-labelled chimaeric **M1** protein was properly transported from the ER, away from the nuclear region, to the apical end of the merozoites.

The chimaeric **M1** (CCys) and **M2** ( $\Delta$ CCys) proteins differed only in the presence or absence of the CCys domain. These data, therefore, strongly suggest that the *maebl* CCys domain is essential and sufficient for transporting the protein to the apical secretory organelles. Without organelle markers, the exact localization could not be shown, however, **M1** was presumably localized to the rhoptries as they are the known destination for MAEBL.

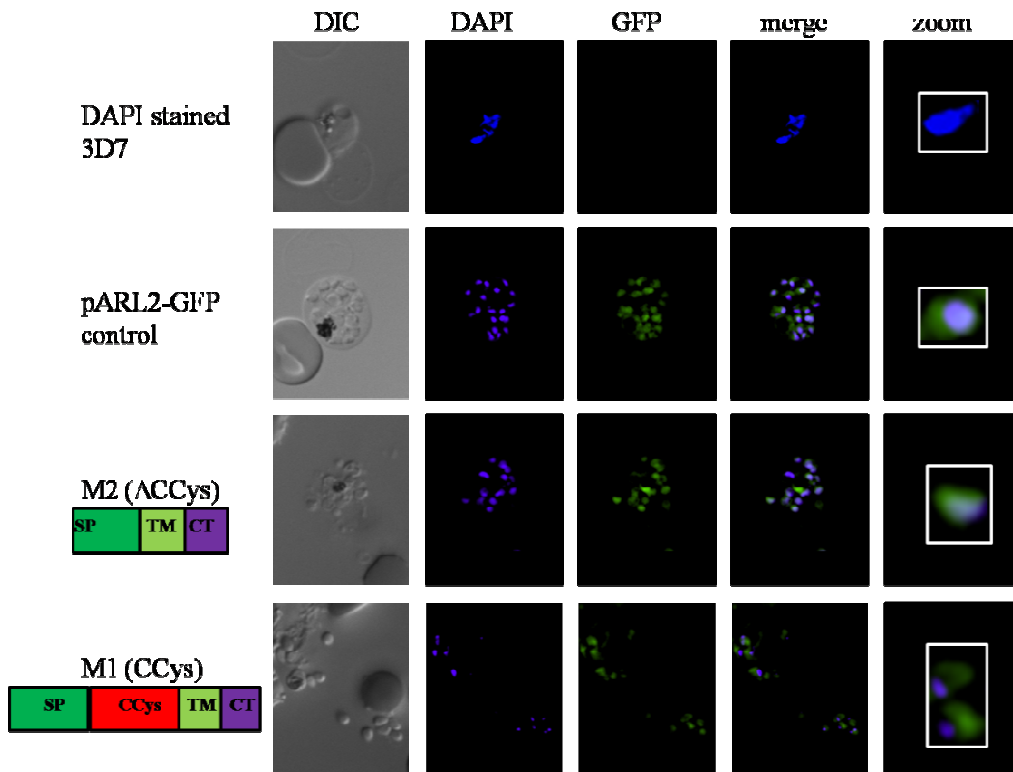


Figure 43. Fluorescence microscopy images of 3D7 parasites, parasites expressing pARL2-GFP/*ama-1* promoter (control), pARL2-GFP/**M1**, or pARL2-GFP/**M2**. The DAPI stained 3D7 parasites show no green auto-fluorescence. The control parasites and parasites expressing chimaeric **M2** protein show diffuse, overlapping blue and green fluorescence, indicating cytoplasmic localization of the GFPs. Parasites expressing the chimaeric **M1** protein showed defined blue and green fluorescent regions, indicating apical localization of **M1**. DIC: differential interference contrast microscopy.

#### 3.12.1.2. JESEBL chimaeric protein **J**

As per section 3.12.1.1., parasites containing the pARL-mCherry/*ama-1* promoter control construct, the pARL-mCherry/**J** construct, and no construct at all, were visualized by fluorescence microscopy. Similarly, 3D7 control parasites showed no auto-fluorescence and no fluorescence bleeding. The parasites expressing the pARL-mCherry/*ama-1* promoter control construct showed dispersed red fluorescence surrounding and overlapping the blue nuclear area, suggesting that the mCherry protein was trapped within the cytosol, as expected (figure 44, top panel). A similar result to that observed for chimaeric protein **M1** (CCys) was expected for chimaeric protein **J** (CCys). However,

mCherry-labelled protein **J** did not show distinct, localized red fluorescence but instead showed dispersed red fluorescence partially overlapping the blue nuclei and partially spread over the apex of the merozoites (figure 44, bottom panel). This suggests that some of chimaeric protein **J** was correctly trafficked to the secretory organelles, while the rest of the protein remained within the cytosol. It is possible that the **J** CCys domain is recognised by the trafficking machinery, but at a lower affinity than for the endogenous, full-length JESEBL.

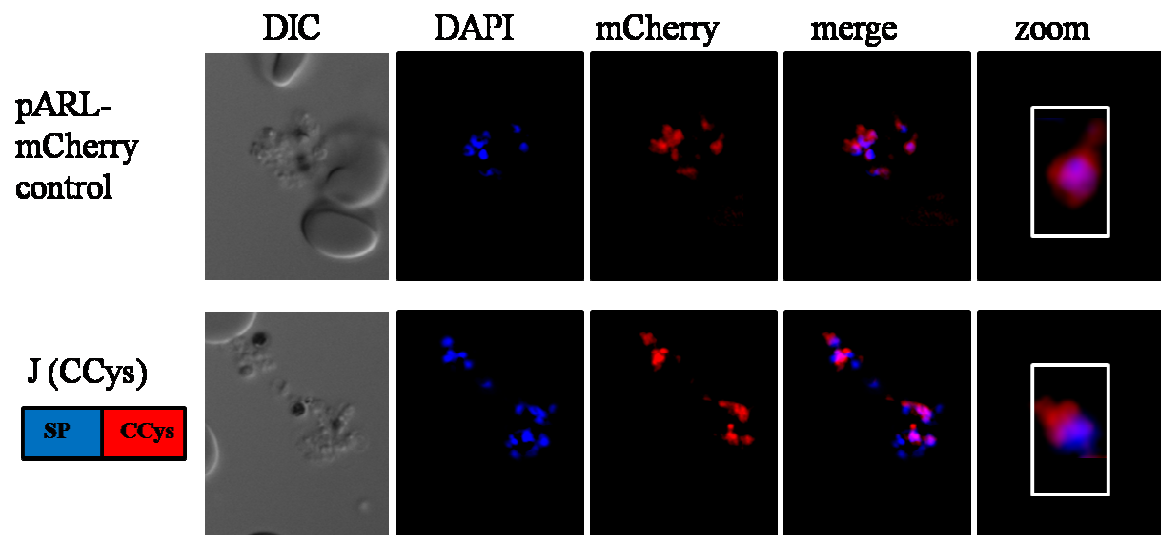


Figure 44. Fluorescence microscopy images of parasites expressing pARL-mCherry/*ama-1* promoter (control) or pARL-mCherry/**J**. The control parasites showed diffuse blue and red fluorescence, indicating cytoplasmic localization of the mCherry protein. Parasites expressing chimaeric **J** protein (red) showed partial overlap with the blue nuclei, as well as apical distribution. DIC: differential interference contrast microscopy.

### 3.12.2. Co-localization

Parasites containing the pARL-mCherry/**J** construct, the empty plasmid or no construct at all were used in co-localization studies. The micronemes were marked green using a primary antibody against the known protein, EBA-175, and a fluorochrome-labelled secondary antibody. Control 3D7 parasites containing no construct showed no auto-



fluorescence and no fluorescence bleeding. Furthermore, addition of the secondary antibody only, showed no non-specific binding (not shown). Control parasites (figure 45, top panel) were stained blue with DAPI and green with the EBA-175 marker. As expected, no red fluorescence was observed and the merged image showed apical (micronemal) localization of EBA-175.

For both the pARL-mCherry/*ama-1* promoter line and the pARL-mCherry/**J** line, there was no fluorescence bleeding observed. For control pARL-mCherry/*ama-1* promoter parasites, the red mCherry protein was dispersed throughout the cytosol and did not co-localize with the microneme marker (figure 45, middle panel). In the pARL-mCherry/**J(CCys)** line there was co-localization of green EBA-175 and red mCherry fluorescence giving a yellow signal, indicating micronemal localization of **J (CCys)**, however, distinct green and red proteins were also visible. This indicated that some of the **J (CCys)** protein was correctly transported to the micronemes, while the rest remained trapped within the cytosol (figure 45, bottom panel). These results agreed with live imaging results.

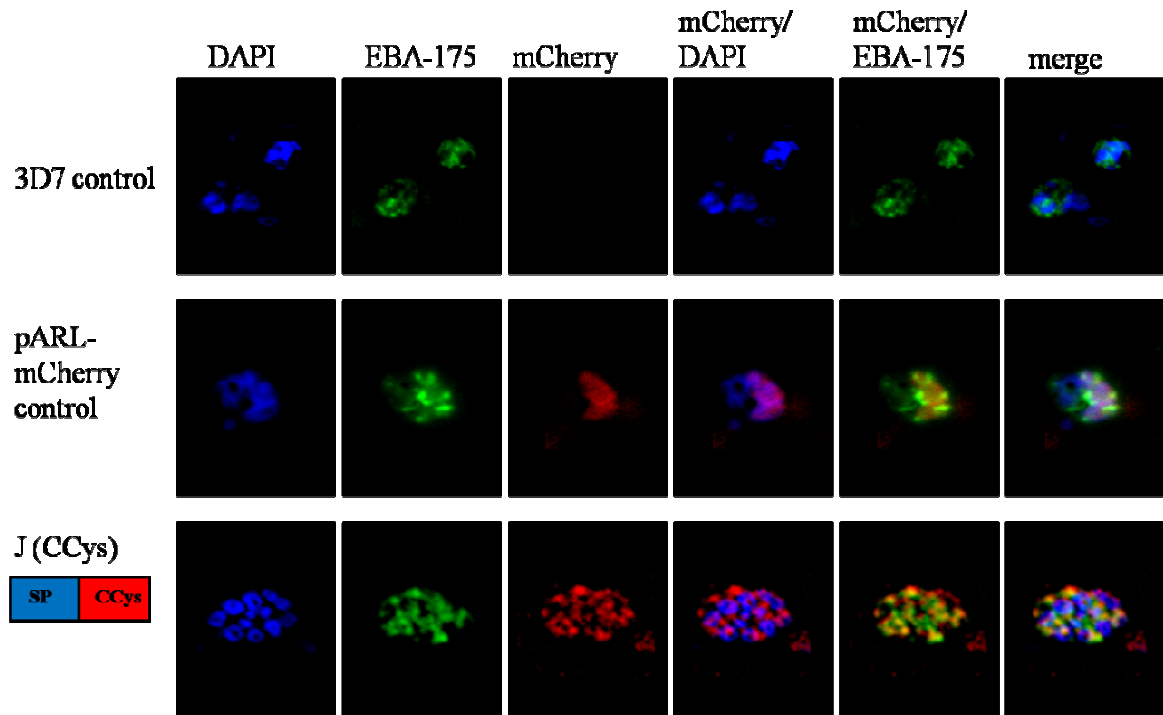


Figure 45. Protein co-localization fluorescence imaging. 3D7 parasites (top panel) and transgenic parasites expressing pARL-mCherry/*ama-1* promoter (middle panel) or pARL-mCherry/J(CCys) (bottom panel) stained with DAPI (blue) and the micronemal marker EBA-175 (green). The control pARL-mCherry/*ama-1* parasites showed distinct green and red fluorescence, indicating cytoplasmic localization of the mCherry protein. Parasites expressing chimaeric J (CCys) protein (red) partially overlapped with EBA-175 (yellow).

## **CHAPTER 4**

### **DISCUSSION**

The *P. falciparum* parasite is a complex eukaryotic organism with an extensive proteome (Lasonder *et al.*, 2002, Pease *et al.*, 2013). The parasite expresses thousands of proteins during the intraerythrocytic life stage which permit host cell invasion and consequent development. These proteins have a variety of final destinations both within cellular compartments and to extracellular targets such as the PV, the Maurer's clefts or the host cell. The trafficking mechanisms employed to sort these proteins are therefore important biological processes. Protein export to the erythrocyte has been well characterized (Marti *et al.*, 2004, Deonte *et al.*, 2012). However, transporting mechanisms within cellular compartments are still widely unknown.

A significant subset of the *P. falciparum* proteome is composed of specialized proteins that are specifically needed for parasite entry into host erythrocytes. These invasion proteins are trafficked to the parasite apical secretory organelles (micronemes and rhoptries), from where they are released at the time of invasion. A multitude of invasion proteins are produced, allowing the parasite a variety of pathways to enter the host cell. The parasite's ability to switch between pathways renders these invasion proteins poor drug targets (Cortes 2008), as evidenced by the fact that many of these proteins have been shown to be nonessential by genetic disruptions. However, the mechanisms which allow various proteins to be transported to the apical organelles may be common. If this were the case, this mechanism may be a potential drug target as it would prevent multiple invasion pathways, and it would most probably be parasite-specific.

A group of invasion proteins, the DBL-EBP family, possesses an N-terminal cysteine-rich DBL domain for recognition of and binding to the erythrocyte surface. These proteins also contain a highly conserved C-terminal cysteine rich domain (Adams *et al.*, 2001). This CCys, together with the SP and correct timing of expression was found to be necessary and sufficient for trafficking of the well known DBL-EBP, EBA-175, to the micronemes (Treeck *et al.*, 2006). The conservation of this domain led to the hypothesis that it may be involved in trafficking of multiple DBL-EBPs to their apical locations.

#### 4.1. The C-terminal cysteine-rich domain is necessary for trafficking of MAEBL but not adequate for trafficking of JESEBL

Two DBL-EBPs were chosen for evaluation, MAEBL and JESEBL. Chimaeric *maebl* and *jesebl* constructs were produced and episomally expressed in 3D7 *P. falciparum* parasites. Two chimaera were produced for MAEBL, **M1** (CCys) and **M2** ( $\Delta$ CCys) (figure 46). Both proteins contained the SP (for transport of the proteins through the ER and Golgi), the TM and the CT. The CCys was included in **M1** and not in **M2**. Based on the findings of Treeck *et al.* (2006), it was hypothesized that chimaeric protein **M1** would be correctly targeted to the secretory organelles and **M2** would remain localized within the cytosol, which was found to be the case. The finding that **M1** was apically localized implies that the protein was trafficked to the known destination of the MAEBL protein, the rhoptries. However, higher resolution microscopy combined with co-localization studies employing a rhoptry marker would confirm the organelle localization of the protein. If both **M1** and **M2** had remained within the cytosol, it would have indicated that other MAEBL domains (the tandem DBL domains or the repeat region) were required for correct protein trafficking. If both **M1** and **M2** had been correctly localized, it would have indicated that the common domains (TM or CT) contributed to trafficking.

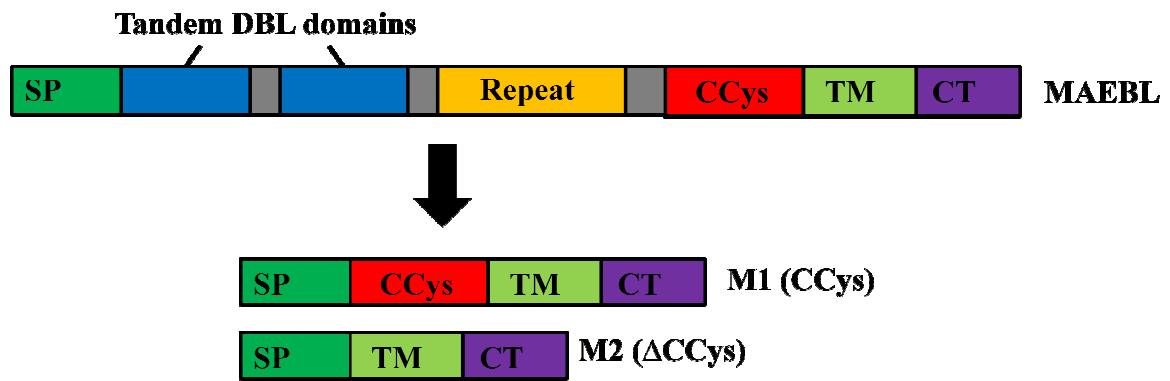


Figure 46. Schematic representation of the chimaeric proteins, **M1 (CCys)** and **M2 (ΔCCys)**, produced from MAEBL.

One construct was produced for JESEBL, construct **J (CCys)**. This construct was made up of the JESEBL CCys and the JESEBL SP (figure 47). Only these two domains were included in the construct as it had already been shown that the cytoplasmic domain did not contribute to correct protein trafficking (Treeck *et al.*, 2006). Based on their observations, it was hypothesised in this study that the JESEBL CCys was necessary and sufficient for correct trafficking to the micronemes. However, **J (CCys)** was only partially correctly localized to the micronemes, the known destination of JESEBL. A large amount of the chimaeric protein remained within the cytoplasmic region while some co-localized with the EBA-175 microneme marker as expected. This indicated that the JESEBL CCys alone was sufficient but not completely adequate for trafficking. It is possible that trafficking machinery was able to recognise the domain but with low affinity. A domain with a larger upstream region than the CCys alone may therefore be necessary for improved binding and subsequent localization. However, improvements on the resolution of the microscopy images generated from the co-localization study would further discern the extent to which protein J is correctly trafficked to the micronemes.

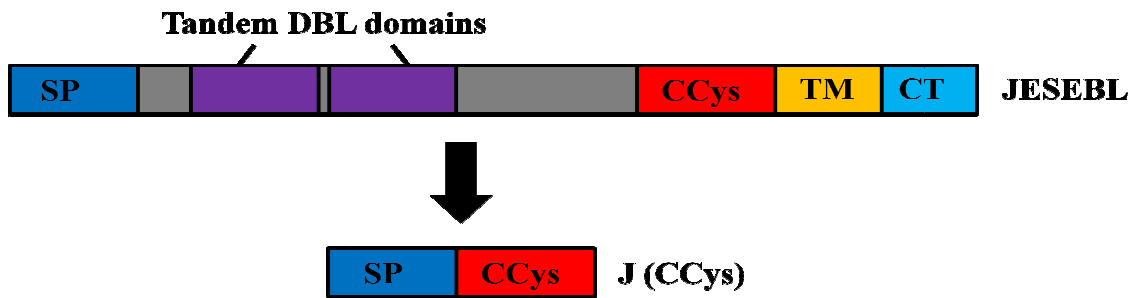


Figure 47. Schematic representation of the chimaeric protein **J (CCys)**, produced from JESEBL.

#### 4.2. Why are the trafficking requirements different for MAEBL and JESEBL?

##### 4.2.1. Differences in the regions specified as the C-terminal cysteine-rich domains

An analysis of the positions of the primers employed by Treeck *et al.* (2006) specified a 282bp region as the CCys of *eba-175*, encoding a 94aa predicted peptide (figure 48). This region included 22 amino acids upstream of the first conserved cysteine residue. In this study, the corresponding 282bp region, based on BLAST sequence alignment, was designated as the CCys in *jesebl*. A slightly larger region of 339bp, encoding an 113aa predicted peptide, was specified for the *maebl* CCys, based on predictions by Adams *et al.* (1992). This domain included 42 amino acids upstream of the initial cysteine residue.

**EBA-175 CCys protein sequence (94aa) as defined by Treeck *et al.* (2006)**

← 22 amino acids →  
LYENRNDSTTKELIKKLAEINKCENEISVKYCDHMIHEEIPKTKTEKTRNLCCA<sup>C</sup>AVSDY<sup>C</sup>  
MSYFTYDSEEY<sup>N</sup>CTKREFDDPSYTCFRKE AFS

**JESEBL CCys protein sequence (94aa)**

← 22 amino acids →  
QYNNRDASKDREEILNRSNTNTCSNEHSLKYCQYMERNKD<sup>L</sup>LET<sup>C</sup>SEDKRLHLCCEIS  
DYCLKFFNP<sup>K</sup>SIEYFDCTQKEFDDPTYNCFRKQRFT

**MAEBL CCys protein sequence (113aa)**

← 42 amino acids →  
SNNNMKGNNNDI<sup>I</sup>YTKLDVEEYKKRDVNETREKIIKISKKNM<sup>C</sup>NNDFSSKYCDYMKDNIS  
SGTCSNEERKSLCCSISDFCLKYFDHNSNKYYDCTKIEFADPLYRCFKKKEFS

**EBA-175 essential trafficking protein sequence as defined by Sakura *et al.* (2013)**

← 48 amino acids →  
KDIRNEENERHLTNQ<sup>N</sup>INISQERDLQKHGFHTMNNLHGDGV<sup>S</sup>ERSQIN<sup>H</sup>SHHG<sup>N</sup>RQDR  
GGNSGNVLNMR<sup>S</sup>NNNNFNNIPSRYNLYDKKLDLDLYENRNDSTTKELIKKLAEINK<sup>C</sup>EN  
EISVKYCDHMIHEEIPKTKTEKTRNLCCA<sup>V</sup>SDY<sup>C</sup>MSYFTYDSEEY<sup>N</sup>CTKREFDDPS  
YT<sup>C</sup>FRKEAFS

Figure 48. Trafficking determinants for EBA-175, JESEBL and MAEBL. Sequences shown: The C-terminal cysteine-rich regions (CCys) of EBA-175 (specified by Treeck *et al.*, 2006), JESEBL and MAEBL (as specified in this study), and the protein sequence necessary for trafficking of EBA-175 as specified by Sakura *et al.* (2013), including the CCys and a 48aa upstream region (green), with the essential arginine residue in purple. The eight conserved cysteine residues are shown in red.

Sakura *et al.* (2013) carried out a study with results contradicting those of Treeck *et al.* (2006). The Japanese group showed that the SP and CCys alone were not sufficient for microneme trafficking of EBA-175. An extra region of 48aa, 66aa upstream of the first conserved cysteine residue, was necessary for correct protein localization (figure 48). An arginine residue within this region was essential for trafficking, as replacement of this residue with alanine caused the protein to be mislocalized. It was suggested that the 48aa sequence was required in conjunction with the CCys domain for correct protein folding and subsequent trafficking.

Similarly, it is possible that **M1 (CCys)** was correctly localized to the secretory organelles because it included essential information within the 42aa sequence upstream of the first conserved cysteine residue (figure 48). However, chimaeric protein **J (CCys)** was not completely trafficked to the micronemes, presumably since it contained only 22aa upstream of the first cysteine residue, in contrast to **M1**. Trafficking machinery may still have been able to recognise the cysteine-rich region in **J** but with low affinity. Additional upstream sequence information may therefore be required for proper folding and optimal recognition and transport of the protein.

#### 4.2.2. Promoter control of gene expression

The pARL plasmids used for transfection contained the constitutive *crt* promoter for continuous expression throughout the *P. falciparum* life cycle. Based on the study by Treeck *et al.* (2006), the *crt* promoter was replaced with the *ama-1* promoter to allow stage specific expression of the **M1 (CCys)**, **M2 ( $\Delta$ CCys)** and **J (CCys)** constructs. Although the endogenous promoter is always best for precise timing of expression, a promoter with an expression profile similar to that of the protein under evaluation is adequate. Two different proteins were evaluated in this study, making a single *ama-1* promoter replacement more efficient than using both the *maebl* and *jesebl* endogenous promoters.

Child *et al.* (2013) proposed that the *ama-1* promoter was not sufficient for the correct timing of expression of *PfSUB2*. Transgenic *P. falciparum* lines were created whereby episomal *PfSUB2* was expressed under the control of either the *ama-1* promoter or the *Pfsub2* promoter. An indirect immunofluorescence assay showed that trafficking of *PfSUB2* to the micronemes was incomplete under the *ama-1* promoter, while higher levels of co-localization with the microneme marker were achieved when *PfSUB2* was under



control of the endogenous promoter. In a similar way, it is possible that the *ama-1* promoter allowed correct timing of expression of construct **M1**, leading to correct localization, but not for construct **J**, leading to partial localization. However, the gene expression profiles of *maebl*, *jesebl*, *ama-1*, *eba-175*, and *Pfsub2* (figure 49) are very similar, indicating that all of these genes should be correctly expressed under the control of the *ama-1* promoter. Differences in timing of protein expression would likely be due to differences in the translation of the different transcripts.

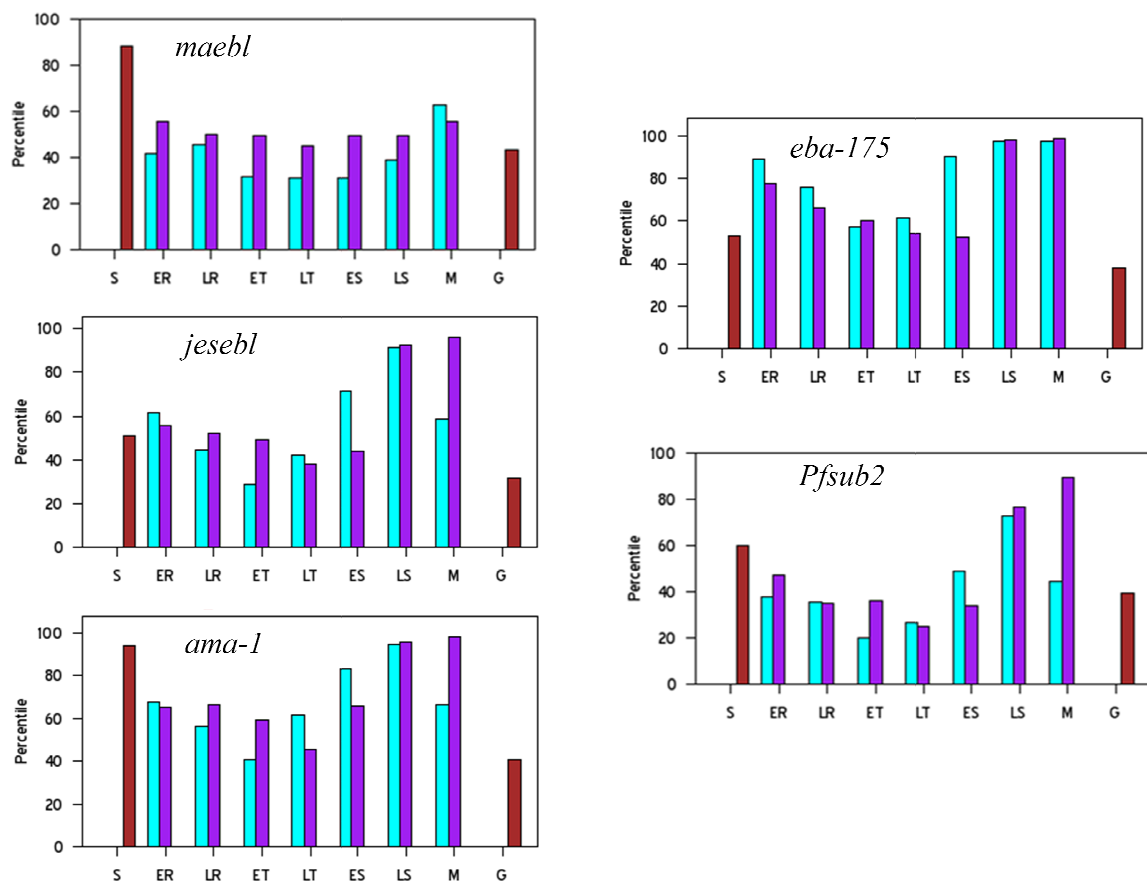


Figure 49. *P. falciparum* gene expression profiles for *maebl*, *jesebl*, *ama-1*, *eba-175* and *Pfsub2*. The x-axes show each of the parasite developmental stages- sporozoites (S), early rings (ER), late rings (LR), early trophozoites (ER), late trophozoites (LT), early schizonts (ES), late schizonts (LS), merozoites (M) and gametocytes (G). The y-axes show gene expression as a percentile relative to all other genes, when parasites are synchronized by **sorbitol** or **temperature** (Le Roch *et al.*, 2003, available on PlasmoDB).

#### 4.3. The role of the C-terminal cysteine-rich domain

Escorter proteins may interact with trafficking machinery via the CCys in order to transport MAEBL to the rhoptries and JESEBL to the micronemes. The high-resolution X-ray crystal structure of the EBA-175 CCys domain revealed a novel fold forming a homodimer whereby the two subunits, consisting of five  $\alpha$ -helices each, are stabilized by four disulfide bridges (Withers-Martinez *et al.*, 2008, figure 50). Three-dimensional structure predictions of the JESEBL and MAEBL CCys domains, performed with Phyre2 (Kelley and Sternberg 2009), confirmed the presence of the same five  $\alpha$ -helices and similar folding as the EBA-175 CCys domain (figure 50). The high conservation of this region among DBL-EBPs indicates that this fold is likely similar for all proteins in the family, conferring a conserved function.

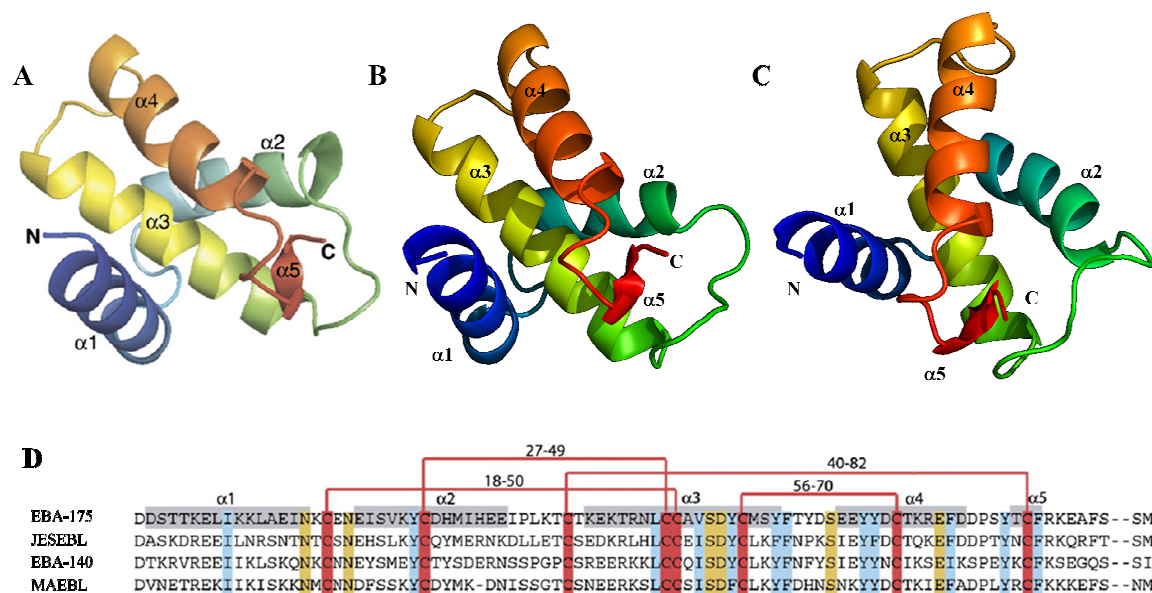


Figure 50. The crystal structure and the amino acid sequence making up the five  $\alpha$ -helices of the C-terminal cysteine-rich domain. Crystal structure of EBA-175 CCys produced by Withers-Martinez *et al.* (2008) (A), MAEBL CCys (B) and JESEBL CCys (C) predicted by Phyre2. All structures show the N and C termini and the 5  $\alpha$ -helices. D. Grey: EBA-175 sequence making up the five helices. Blue & yellow: residues involved in stabilizing the protein fold, which are highly conserved in the DBL-EBPs. Red: Conserved cysteine residues connected by disulfide bonds (red lines) (Withers-Martinez *et al.*, 2008).

The structure of the EBA-175 CCys domain is conformationally similar to KIX domain of the coactivator CREB-binding protein (CBP) (Withers-Martinez *et al.*, 2008), indicating the CCys as a potential binding domain with a mechanism similar to that of the KIX domain. Furthermore, the KIX domain of CBP can bind two different proteins simultaneously (De Guzman *et al.*, 2006). It is therefore possible that the CCys domains of MAEBL and JESEBL are responsible for transport to the rhoptries and micronemes respectively, by interacting with escorter protein complexes. Other *P. falciparum* secretory proteins are known to be transported in complexes. The rhoptry proteins RAP1, RAP2 and RAP3 interact to form a low molecular weight rhoptry complex that is transported via the escorter protein RAMA (Topolska *et al.*, 2004, Richard *et al.*, 2009). Furthermore, the high molecular weight rhoptry protein complex, consisting of RhopH1, RhopH2 and RhopH3 may be transported by the same or a similar escorter protein (Ghoneim *et al.*, 2007).

#### 4.4. Hypothetical trafficking models

MAEBL and JESEBL, like many *P. falciparum* invasion proteins, possess N-terminal signal sequences which direct the newly synthesised proteins through the ER and Golgi. Once within the Golgi, MAEBL and JESEBL are most likely sorted and packaged into transport vesicles destined for their final destinations, the rhoptries and micronemes, respectively (Kats *et al.*, 2008). Treeck *et al.* (2006) identified the EBA-175 CCys domain as the probable recognition site, or 'sorting signal' for protein escorters. Because the cytoplasmic domain of EBA-175 is not needed for microneme localization (Gilberger *et al.*, 2003a, Treeck *et al.*, 2006), these escorters are probably transmembrane proteins containing the cytoplasmic signal needed for recognition by trafficking machinery.

Many *P. falciparum* protein-protein interactions have been identified using the yeast two-hybrid assay (LaCount *et al.*, 2005). Currently, there is no yeast two-hybrid assay data available for MAEBL, however, multiple interactions have been found for JESEBL and EBA-175 using this system (PlasmoDB, table 12). Unfortunately, these data show no common interacting proteins for both JESEBL and EBA-175. Furthermore, none of the interacting proteins are likely candidates for trafficking elements. It was, however, interesting to note that both the JESEBL and EBA-175 micronemal proteins interact with the known rhoptry proteins RhopH2 and RAP, respectively. Additionally, the functions of many of the interacting proteins are yet to be defined and other interactions may also be found. Therefore, a possible common escorter protein may yet be identified.

**Table 12. Protein-protein interactions with JESEBL and EBA-175 determined by yeast two-hybrid assay (LaCount *et al.*, 2005).**

| Proteins interacting with JESEBL<br>(PF3D7_0102500) |                                                                   | Proteins interacting with EBA-175<br>(PF3D7_0731500) |                                                       |
|-----------------------------------------------------|-------------------------------------------------------------------|------------------------------------------------------|-------------------------------------------------------|
| Gene ID                                             | Annotation                                                        | Gene ID                                              | Annotation                                            |
| PF3D7_0929400                                       | RhopH2                                                            | PF3D7_1226500                                        | RAP protein, putative                                 |
| PF3D7_1342000                                       | 40S Ribosomal protein S6, putative                                | PF3D7_0903900                                        | 60S Ribosomal protein L32, putative                   |
| PF3D7_1217200                                       | snoRNA-associated small subunit rRNA processing protein, putative | PF3D7_1216900                                        | DNA-binding chaperone, putative                       |
| PF3D7_1008400                                       | 26S proteasome regulatory subunit 4, putative                     | PF3D7_0708800                                        | Heat shock protein 110                                |
| PF3D7_0919900                                       | Regulator of chromosome condensation, putative                    | PF3D7_1035500                                        | Merozoite surface protein 6                           |
| PF3D7_0823300                                       | Histone acetyltransferase GCN5                                    | PF3D7_0102200                                        | Ring-infected erythrocyte surface antigen             |
| PF3D7_1426100                                       | Basic transcription factor 3b, putative                           | PF3D7_0108700                                        | Secreted ookinete protein, putative                   |
| PF3D7_0311100                                       | Pre-mRNA splicing factor, putative                                | PF3D7_0207600                                        | Serine repeat antigen 5                               |
| PF3D7_0922200                                       | S-adenosylmethionine synthetase                                   | PF3D7_1452400                                        | Conserved <i>Plasmodium</i> protein, unknown function |
| PF3D7_1410200                                       | Cytidine triphosphate synthetase                                  | PF3D7_1324000                                        | Conserved <i>Plasmodium</i> protein, unknown function |
| PF3D7_1126700                                       | Conserved <i>Plasmodium</i> protein, unknown function             | PF3D7_1312800                                        | Conserved <i>Plasmodium</i> protein, unknown function |
| PF3D7_1248700                                       | Conserved <i>Plasmodium</i> protein, unknown function             | PF3D7_0515400                                        | Conserved <i>Plasmodium</i> protein, unknown function |
|                                                     |                                                                   | PF3D7_1021900                                        | Conserved <i>Plasmodium</i> protein, unknown function |
|                                                     |                                                                   | PF3D7_1249800                                        | Conserved <i>Plasmodium</i> protein, unknown function |
|                                                     |                                                                   | PF3D7_0713900                                        | Conserved <i>Plasmodium</i> protein, unknown function |

Based on past studies and the results from the current study, two models are proposed for trafficking of MAEBL and JESEBL to the apical organelles.

#### 4.4.1. The 'escorter' model

The results of the current study showed that the highly conserved CCys domain served as the recognition site in the partial localization of JESEBL to the micronemes. A hypothetical model is represented in figure 51, whereby a common sorting protein recognises the CCys domain in EBA-175 and JESEBL and brings the invasion proteins into proximity with transmembrane escorter proteins which in turn recruit vesicle coat proteins and interact with microneme trafficking machinery such as adaptor complexes and clathrin (Kats *et al.*, 2008, Deponte *et al.*, 2012).

Furthermore, the current study indicates the CCys domain as the trafficking determinant needed for transporting MAEBL to the rhoptries. Richard *et al.* (2009) proposed a rhoptry targeting model for RAP1 via the glycosylphosphatidyl inositol (GPI)-anchored escorter protein RAMA. Based on this model, MAEBL may be packaged into rhoptry-destined vesicles by clustering in lipid rafts via an interaction with a GPI-anchored protein. Furthermore, interactions between the MAEBL CCys domain and a common sorting protein will bring MAEBL into contact with a transmembrane escorter which interacts with rhoptry trafficking machinery (figure 51).

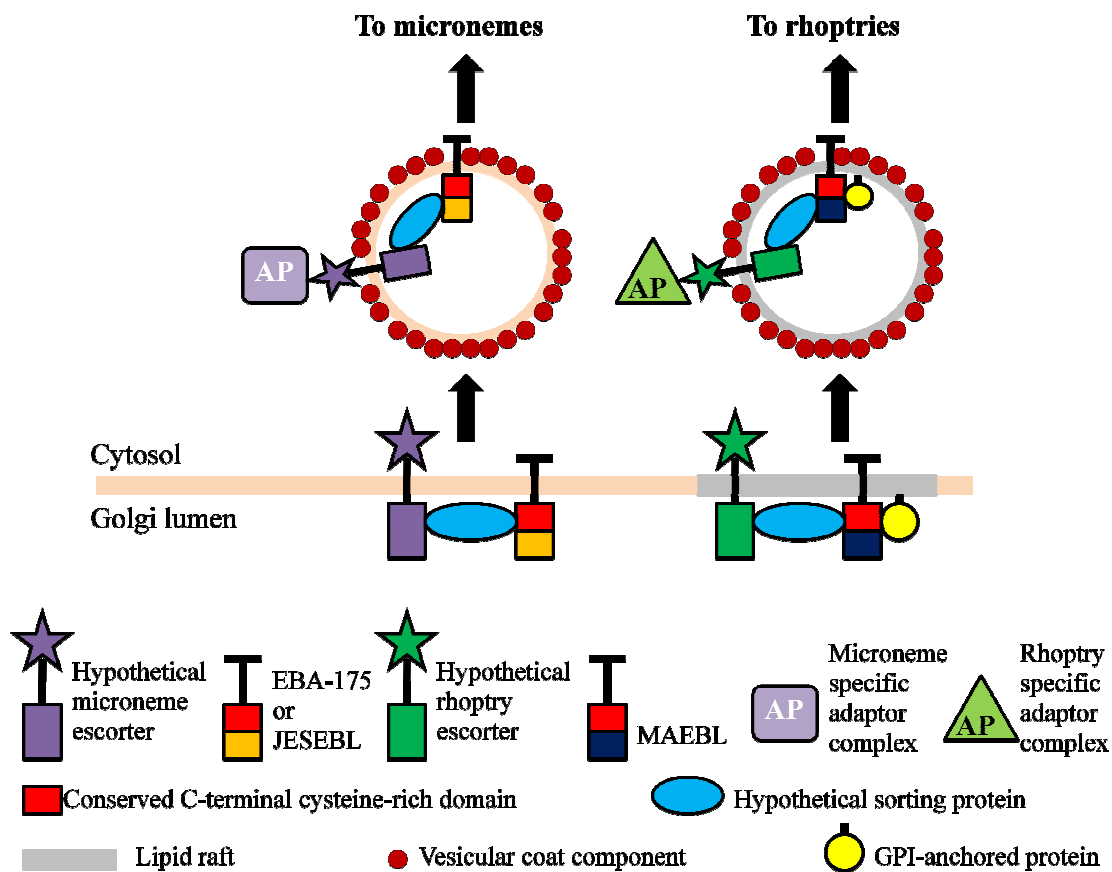


Figure 51. Hypothetical ‘escorter’ model for the transport of EBA-175, JESEBL & MAEBL to the secretory organelles. The hypothetical sorting protein (blue oval) is proposed to be common for all pathways by interacting with the conserved C-terminal cysteine-rich domain present in EBA-175, JESEBL & MAEBL.

#### 4.4.2. The ‘bus-stop’ model

Sloves *et al.* (2012) have shown that simple eukaryotes, such as the apicomplexan parasites, contain common trafficking systems for many proteins. It was shown in *T. gondii* that *TgSORTLR* is a major cargo receptor that physically interacts with multiple microneme and rhoptry proteins and guides them through the Golgi, where they can be packaged into vesicles. This mechanism may be conserved throughout apicomplexans, as a putative sortilin-encoding gene exists in *P. falciparum* (PF3D7\_1451800).

It is therefore possible that a common escorter protein similar to *Tg*SORTLR is responsible for trafficking various microneme and rhoptry proteins to their final destinations simultaneously. This hypothesis, represented in figure 52, is supported by the fact that the known micronemal proteins, EBA-175 and JESEBL, interact with known rhoptry proteins RAP and RhopH2, respectively (table 12). It is hypothesised that the *P. falciparum* SORTLR-type protein binds to both microneme (EBA-175/ JESEBL) and rhoptry (MAEBL) proteins within the Golgi lumen, where the complex is packaged into a vesicle. The cytoplasmic tail of the *Pf*SORTLR protein then recruits the appropriate apical trafficking machinery and vesicular coat components. The microneme and rhoptry proteins are then delivered en-route, in a fashion similar to bus-stop terminals. Specific signals at each organelle allow the release of the organelle-specific proteins. Thereafter, the vesicular coat components and trafficking determinants are recycled for trafficking of the following vesicle.



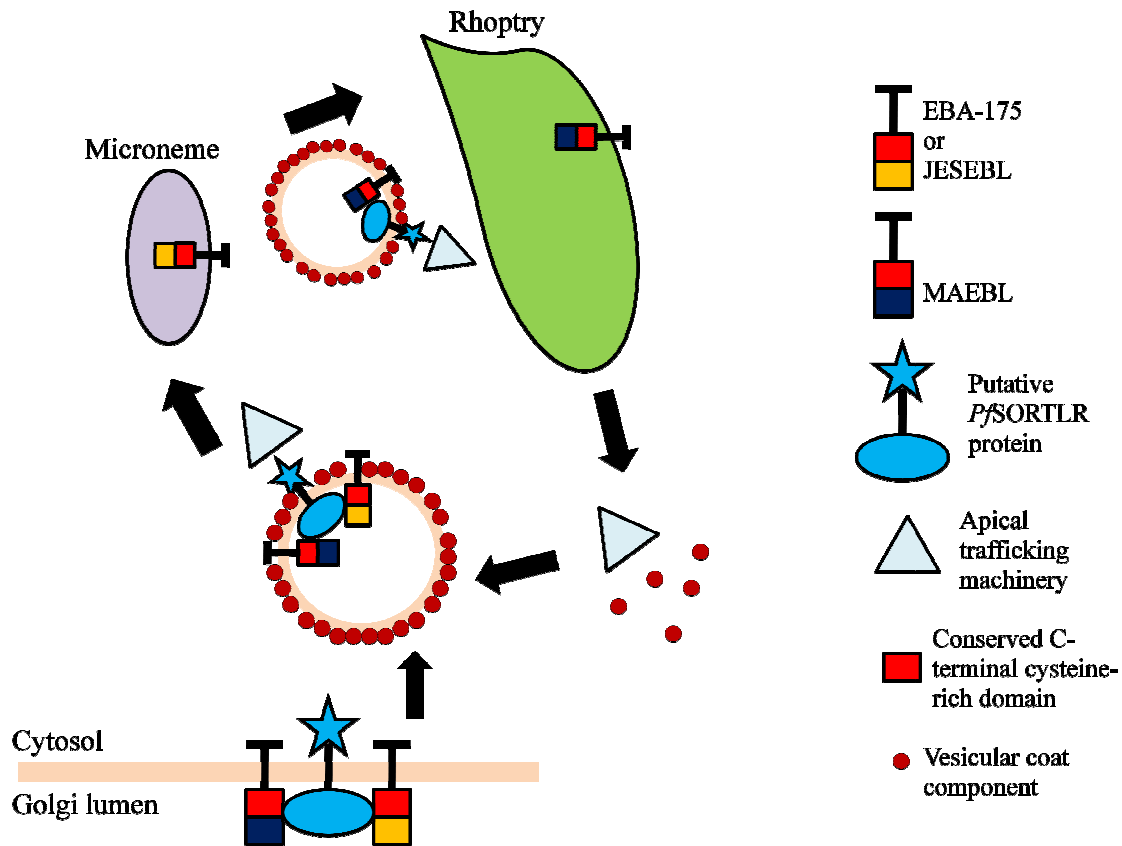


Figure 52. Hypothetical ‘bus-stop’ model for the transport of EBA-175, JESEBL & MAEBL to the secretory organelles. The putative *Pf*SORTLR protein is proposed to bind EBA-175, JESEBL and MAEBL by interacting with the conserved C-terminal cysteine-rich domains, and permit vesicular trafficking of the proteins to the micronemes and rhoptries.

#### 4.5. Conclusion

This study has identified the CCys domain, which is conserved among members of the DBL-EBP family, to be necessary for trafficking of JESEBL and MAEBL to the micronemes and rhoptries, respectively. The *P. falciparum* machinery used for transporting EBA-175, MAEBL and JESEBL to the secretory organelles may be common for all DBL-EBP invasion proteins. If this were the case, this machinery would be a potential drug target.

#### 4.6. Future studies

Future studies should focus on the identification of the potential common EBA-175, JESEBL and MAEBL trafficking determinants. A pull-down assay could be used to identify the interaction between the common EBA-175/JESEBL/MAEBL CCys and potential escorter proteins. This system has previously been used to identify the interaction between RAP1 and the protein escorter RAMA (Richard *et al.*, 2009). Alternatively, a novel protein-protein interaction system, which has been used with success, is that of the *P. falciparum* phage display library (Lauterbach *et al.*, 2003 & Lanzillotti and Coetzer, 2006). The phage display libraries were biopanned against the purified ‘bait’ proteins, human erythrocyte spectrin and protein 4.1, and revealed multiple *P. falciparum* proteins with significant binding specificity. This system could be implemented with the purified EBA-175, MAEBL or JESEBL CCys domain used as ‘bait’.

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## Appendix A

### Commercial chemical reagents

All reagents were stored at room temperature unless otherwise stated.

Acetic acid 17.5M (SMM Instruments, South Africa)

Agar Bacteriological (Merck, Germany)

Agarose (SeaKem® LEAgarose) (Lonza, USA)

Albumax (Gibco, UK) Stored at 4°C

Alexa Fluor®488 goat anti-mouse IgG 2mg/ml (Molecular Probes, USA) Stored at 4°C

Ampicillin (Roche, Germany) Stored at 4°C

Bovine Serum Albumin Fraction V (BSA) (Roche, Germany) Stored at 4°C

Calcium Chloride (CaCl<sub>2</sub>) (Merck, Germany)

Chloroform (Merck, Germany)

DAPI 20mg/ml (Sigma, South Africa) Stored at 4°C

dNTP mix (10mM) (Inqaba, South Africa) Stored at -20°C

D-sorbitol, SigmaUltra (Sigma, South Africa)

EDTA (Ethylenediaminetetraacetic acid) (Merck, Germany)

EGTA (Ethyleneglycolbis-aminoethylether-tetra-acetic acid) (BDH, UK)

Ethanol (BDH, UK)

Ethidium Bromide (Roche, Germany)

Formaldehyde solution (HCHO) (Saarchem, South Africa)

Gentamicin Sulfate (Sigma, South Africa) Stored at 4°C

Glucose (C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) (Saarchem, South Africa)



Glutaraldehyde solution ( $\text{CH}_2(\text{CH}_2\text{CHO})_2$ ) (Saarchem, South Africa)

Glycerol (Merck, Germany)

Hydrochloric acid (HCL) (Merck, Germany)

Hepes (Sigma, South Africa)

Hypoxanthine (Sigma, South Africa)

Magnesium chloride ( $\text{MgCl}_2$ ) (Merck, Germany)

Methanol (Merck, Germany)

Mouse anti-EBA-175 (Provided by WEHI, Melbourne, Australia)

Nuclease-free water (Inqaba, South Africa)

Phenol (Sigma, South Africa)

Phosphate Buffered Saline (PBS) tablets (Sigma, South Africa)

Potassium acetate ( $\text{CH}_3\text{CO}_2\text{K}$ ) (Saarchem, South Africa)

Potassium chloride (KCl) (Saarchem, South Africa)

Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) (Saarchem, South Africa)

Potassium hydroxide (KOH) (Sigma-Aldrich, South Africa)

Potassium phosphate ( $\text{K}_2\text{HPO}_4$ ) (BDH, UK)

RiboLock (Fermentas, Europe)

RNase A (Fermentas, Europe) Stored at  $-20^\circ\text{C}$

RPMI medium 1640 with L-Glutamine and 25mM HEPES (Gibco, UK) Stored at  $4^\circ\text{C}$

Saponin (USA, UK)

Sodium acetate ( $\text{CH}_3\text{COONa}$ ) (Hopkin & Williams, United Kingdom)

Sodium bicarbonate ( $\text{NaHCO}_3$ ) (Saarchem, South Africa)

Sodium borohydride ( $\text{NaBH}_4$ ) (Saarchem, South Africa)

Sodium chloride (NaCl) (Saarchem, South Africa)

Sodium dodecyl sulfate (SDS) (Merck, Germany)

di-Sodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) (Saarchem, South Africa)

Sodium hydroxide (NaOH) (Merck, Germany)

Thiazole Orange (Sigma-Aldrich, South Africa)

Trisaminomethane (Tris) (Roche, Germany)

Triton X-100 (BDH, UK)

Tryptone (BDH, UK)

WR99210 (10mM, in DMSO) (provided by Dr Jude M. Przyborski, Marburg, Germany)

Yeast Extract (Oxoid, UK)

## **Appendix B**

### **Composition of non-commercial reagents**

All reagents used were made up with purified water produced by the MilliQ™ Water System (Millipore Corporation, USA), unless otherwise stated. Heat sterilizations and filter sterilizations, where specified, were carried out in a Speedy Autoclave (Gemmy Industrial Corp, USA) or using Acrodisc® PF Syringe Filters (Pall Corporation, UK) respectively. pH measurements were adjusted using the Beckman® 70 pH meter (Beckman, USA). All reagents were stored at room temperature, unless otherwise indicated.

#### **Acetic acid (5M)**

14.3ml concentrated 17.5M acetic acid

Make up to 50ml with milliQ water

#### **Agar plates**

3g Agar Bacteriological dissolved in 200ml Luria Broth

Autoclave

When cooled (<60°C) add 200µl of 1mg/ml ampicillin

Pour into plates (±14) and allow to cool

#### **Agarose Gel (1%)**

5g agarose dissolved in 50ml 1X TAE buffer (by microwave heating)

Allow to cool to 60°C before adding 2.5µl of 10mg/ml ethidium bromide

Pour into electrophoresis plate with comb in place and allow to set

#### **Albumax (5%)**

2.5g albumax dissolved in 50ml incomplete medium

Filter sterilize

Store at 4°C

#### **Ampicillin**

1g ampicillin dissolved in 10ml milliQ water

1ml aliquots stored at -20°C

**BSA (3%)**

0.45g BSA

Make up to 15ml with PBS

**Calcium chloride (2M)**

11.1g anhydrous  $\text{CaCl}_2$  dissolved in 50ml milliQ water

**Complete medium [0.5% albumax and 0.2% sodium bicarbonate]**

5 ml 5% albumax

2.9 ml 3.6% sodium bicarbonate

Make up to 50 ml with incomplete medium

Store at 4°C

**Cytomix**

6ml 2M KCl

7.5µl 2M  $\text{CaCl}_2$

1ml 1M potassium phosphate buffer (pH 7.6)

10ml 250mM Hepes/ 20mM EGTA

500µl 1M  $\text{MgCl}_2$

Make up to 90ml with milliQ water

Adjust pH to 7.6 using 1M KOH

Make up final volume to 100ml with milliQ water

Filter sterilize

Store at 4°C

**DAPI (Dilute)**

495µl Incomplete medium

5µl 20mg/ml DAPI

**D-sorbitol (5%)**

5g D-sorbitol dissolved in 100ml milliQ water

Filter sterilize

Store at 4°C

**EDTA (800mM)**

3g EDTA

Make up to 85ml with milliQ water

Adjust pH to 8 with NaOH

Make up final volume to 100ml with milliQ water

**Ethanol (75%)**

75ml 100% Ethanol

25ml milliQ water

**Ethanol (70%)**

70ml 100% Ethanol

30ml milliQ water

**Ethidium bromide**

100mg ethidium bromide dissolved in 10ml milliQ water

Store at 4°C

**Formaldehyde (4%)/Glutaraldehyde (0.075%)**

600µl formaldehyde solution

1.13µl glutaraldehyde solution

Make up to 15ml with PBS

**Freezing solution (28% glycerol)**

72ml PBS for freezing

28ml glycerol

Filter sterilize

Store at 4°C

**Glycerol (60%)**

30ml glycerol

20ml milliQ water

**Hepes/EGTA (250mM/ 20mM)**

3g Hepes

0.4g EGTA

Make up to 40ml with milliQ water

Adjust pH to 7.6 using 10M KOH

Make up final volume to 50ml with milliQ water

**Hypotonic solution (5mM Potassium phosphate buffer)**

0.22g  $K_2HPO_4$  dissolved in 200ml milliQ water

Adjust pH to 7.4 using 1M  $KH_2PO_4$

Make up final volume to 250ml with milliQ water

**Incomplete medium**

50 mg Gentamicin Sulfate

50 mg Hypoxanthine

1 tube RPMI medium 1640 with L-Glutamine and 25mM HEPES (15.89g)

Make up to 900ml with autoclaved milliQ water

Mix for at least 1.5 hours

Adjust pH to 6.72 using 1M NaOH

Make up final volume to 1L (1kg) with autoclaved milliQ water

Filter sterilize

Store at 4°C

**Luria Broth (LB)**

10g tryptone

10g NaCl

5g yeast

25ml of 400mM Tris-HCl (pH 8) [10mM]

Make up to 1L with milliQ water

Autoclave

**Lysis solution**

500µl stock 0.4M NaOH [0.2M]

55.6µl 18% SDS [1%]

Make up final volume to 1ml with milliQ water

**Magnesium chloride (1M)**

10.2g anhydrous  $\text{MgCl}_2$  dissolved in 50ml milliQ water

**Neutralization solution**

1ml 5M stock potassium acetate

2ml 5M acetic acid

**Phenol:Chloroform**

Mix equal volumes of phenol and chloroform

**Phosphate Buffered Saline (PBS, pH 7.4)**

Dissolve 1 PBS tablet in 200ml autoclaved milliQ water

Autoclave

Store at 4°C

**PBS for freezing**

7.2g  $\text{NaCl}$  [120mM]

14.8g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  [83mM]

4.4g  $\text{KH}_2\text{PO}_4$  [320mM]

Make up to final volume of 1L with milliQ water

Store at 4°C

**Potassium acetate (5M)**

245.4g  $\text{CH}_3\text{CO}_2\text{K}$  dissolved in 50ml milliQ water

**Potassium chloride (2M)**

7.5g  $\text{KCl}$  dissolved in 50ml milliQ water

**Potassium dihydrogen orthophosphate (1M)**

6.8g  $\text{KH}_2\text{PO}_4$  dissolved in 50ml milliQ water

**Potassium hydroxide (10M)**

5.6g KOH dissolved in 10ml milliQ water

**Potassium hydroxide (1M)**

1ml 10M KOH

9ml milliQ water

**Potassium phosphate (1M)**

8.7g  $\text{K}_2\text{HPO}_4$  dissolved in 50ml milliQ water

**Potassium phosphate buffer (1M, pH 7.6)**

8.7ml 1M  $\text{K}_2\text{HPO}_4$

1.3ml 1M  $\text{KH}_2\text{PO}_4$

**Re-suspension solution**

0.5g Glucose [50mM]

0.6ml of stock 800mM EDTA (pH 8) [10mM]

3.1ml of stock 400mM Tris-HCl (pH 8) [25mM]

Make up to final volume of 50ml with milliQ water

**Saponin (5%)**

0.5g Saponin dissolved in 10ml milliQ water

**SDS (18%)**

18g SDS dissolved in 100ml milliQ water

**Sodium acetate (3M)**

24.6g  $\text{CH}_3\text{COONa}$

Add 90ml MilliQ water

Adjust pH to 5 using acetic acid

Make up to final volume of 100ml with milliQ water

**Sodium bicarbonate (3.6%)**

1.8 g  $\text{NaHCO}_3$  dissolved in 50 ml autoclaved milliQ water



Filter sterilize

Store at 4°C

**Sodium borohydride (0.1g/ml)**

0.005g NaBH<sub>4</sub>

Make up to 50ml with PBS

**Sodium chloride (12%)**

12g NaCl dissolved in 100ml milliQ water

Filter sterilize

**Sodium chloride (1.6%)**

1.6g NaCl dissolved in 100ml milliQ water

Filter sterilize

**Sodium chloride/glucose solution**

0.9g NaCl

0.2g glucose

Make up to 100ml with milliQ water

Filter sterilize

**Sodium hydroxide (1M)**

1g NaOH dissolved in 50ml milliQ water

**Sodium hydroxide (0.4M)**

0.8g NaOH dissolved in 50ml milliQ water

**Sorenson's phosphate buffer (pH 7.2)**

0.42g Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O [47mM]

0.14g KH<sub>2</sub>PO<sub>4</sub> [20mM]

Make up to 50ml with milliQ water

Store at 4°C

**TAE (Tris base, acetic acid and EDTA) buffer, pH8 (50X)**

242g Tris [2M]

5.7ml of 17.5M Acetic Acid [0.1M]

62.5ml of 800mM EDTA (pH 8) [50mM]

Add 900ml MilliQ water

Make up to a final volume of 1000ml with MilliQ water

**TAE buffer (1X)**

20ml of 50X TAE Buffer

980ml MilliQ water

**Tris-HCl (400mM)**

4.8g Tris

Add 85ml MilliQ water

Adjust pH to 8 using diluted HCl

Make up to a final volume of 100ml with MilliQ water

**Tris EDTA (TE) buffer**

2.5ml 400mM Tris-HCl (pH 8) [0.8 mM]

0.1ml 800mM EDTA (pH 8) [1 mM]

Make up to a final volume of 100ml with MilliQ water

**Triton X-100 (0.1%)**

15µl Triton X-100

Make up to 15ml with PBS

**WR99210 (10µM)**

1µl 10mM (in DMSO) in 1ml incomplete medium

Store at 4°C for 1 month

## Appendix C

### Primer sequences

Red bases indicate the restriction enzyme sites with cleavage site shown by an arrow. Bold bases indicate the extra bases added to facilitate enzyme recognition. Overlapping sequence bases are shown in blue.

#### *maebl* Sense primers

M-S1 (with *Xho*I restriction site)

5'- ATAT**C**↓**TCGAG**ATGGGGGTATTGAAACATTTTTCTTT -3'

M-S2

5'- TCGAATAATAACATGAAAGGA -3'

M-S3

5'- AACATGGTTTATTTTGCTGGA -3'

#### *maebl* Anti-sense primers

M-AS1 (with *Avr*II restriction site)

5'- GCGC**C**↓**CTAGG**TTTGATTATGTTTTCTTCCGA -3'

M-AS2 (with C-terminal cysteine-rich domain overlap)

5'- **TCCTTTCATGTTATTATTCG**AAATTCATATTGAATTTCCCCTTTCCA -3'

M-AS3 (with Transmembrane/ cytoplasmic domain overlap)

5'- **TCCAGCAA**AA**ATAA**ACC**ATGTT**AATTCATATTGAATTTCCCCTTTCCA -3'

#### *jesebl* Sense primers

J-S1 (with *Xho*I restriction site)

5'- GCGC**C**↓**TCGAG**ATGAAAGGGAAAATGAATATGTGT -3'

J-S2

5'- CAATATAATAATAGAGATGCTTCTAAA -3'

#### *jesebl* Anti-sense primers

J-AS1 (with *Avr*II restriction site)

5'- GCGC**C**↓**CTAGG**CAAATGTCGTTGTGTCATCATAA -3'

J-AS3 (with C-terminal cysteine-rich domain overlap)

5'- **TTTAGAAGCATCTCTATTATTATATTG**GGGCCTTTCCTTCAAATACTCTTC -3'

J-AS4 (with *Avr*II restriction site)

5' - GCGCC~~↓~~CTAGGTGTAAATCTTTGTTTTCTAAAACA -3'

***ama-1* promoter region Sense primer**

AMA-S (with *Xho*I restriction site)

5' - GCGCC~~↓~~TCGAGTTTTGTACAATTTATAACAAGT -3'

***ama-1* promoter region Anti-sense primer**

AMA-AS (with *Not*I restriction site)

5' - ATATGC~~↓~~GGCCGCTTTAAGTATTAATGAGGTGT -3'

**Plasmid-specific primers**

pARL2-GFP and pARL-mCherry Sense primer (pARL-S)

5' - ACACAAACGTTATACGTACACAGGTT -3'

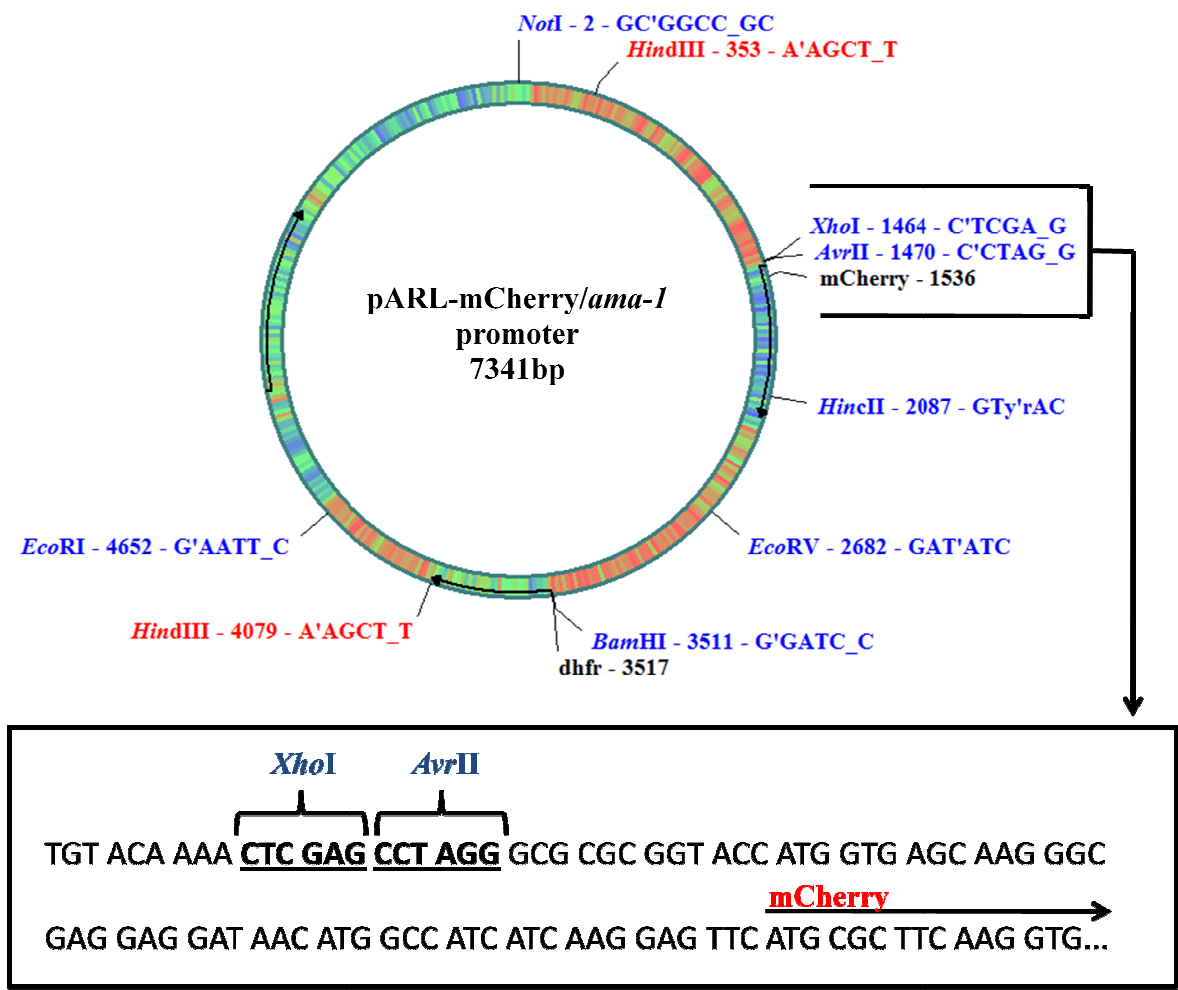
pARL2-GFP Anti-sense primer (GFP-AS)

5' - CCATCTAATTCAACAAGAATTGGGACAAC -3'

pARL-mCherry Anti-sense primer (mC-AS)

5' - CCTTGAAGCGCATGAACTCCTTGA -3'

Appendix D



Appendix E

