Recombinant Expression and Immuno-screening for Vaccine Candidates in *Plasmodium falciparum*

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Declaration

Help in the synthesis of a codon-optimised version of CIDR1 was provided by Dr. Toby Fagin and immunisation of laboratory animals was carried out by Herlan Sera-lab, UK. Subject to these exceptions, I hereby declare that this thesis is of my own composition and describes my own work. It has not been submitted in any other application for a higher degree.

Saad Alkahtani

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Abbreviations

AMA1	Apical merozoite antigen 1
Amp	Ampicillin
AP	Alkaline phosphatase
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl-transferase
cDNA	Complementary DNA
CHO cells	Chinese hamster ovary cells
CIDR	Cysteine-rich interdomain region
CSA	Chondroitin sulphate A
CSP	Circumsporozoite protein
DBL domain	Duffy binding-like domain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ELISA	Enzyme-linked Immunosorbent Assay
FCS	Fetal calf serum
GAG(s)	Glycosaminoglycan(s)

gDNA	Genomic deoxyribonucleic acid
IFA	Indirect immunofluorescence assay
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
iRBC(s)	Infected red blood cell(s)
Kb	kilobase
KDa	kilodalton
LB	Luria Bertani medium
mAb	Monoclonal antibody
MCS	Multiple cloning site
mRNA	Messenger ribonucleic acid
MSP1	Merozoite surface protein-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Parasitised erythrocyte
PfEMP-1	*Plasmodium falciparum* erythrocyte membrane protein-1
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute Medium 1640
SDS	Sodium dodecyl sulphate
Tris	Tris[hydroxymethyl]aminomethane

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Abstract

Plasmodium falciparum is the major cause of human malaria morbidity and mortality. Despite considerable research efforts, no effective vaccine against *P.falciparum* malaria is yet available. There is thus urgent need for basic pre-clinical and clinical research on vaccine development. To obtain antigens in suitable state for initiating vaccine prototype development it will be necessary to scale up production, in optimal expression systems, of recombinant proteins that are correctly folded and which induce appropriate antibody responses.

In the intra-erythrocytic parasite, only mature forms can adhere to the host endothelial surfaces and thus sequester in different organs. This sequestration appears to be mediated by parasite-encoded molecules exported to the erythrocyte surface. The best understood, and probably the most important of these molecules is the *P. falciparum* erythrocyte membrane protein-1 PfEMP-1. Although PfEMP-1 is highly polymorphic it nevertheless has structurally conserved features and these are considered possible vaccine targets. Antibodies directed to these molecules could protect patients from the clinical symptoms by blocking cytoadhesion. Humans make antibody responses to these molecules and particularly agglutinating antibody responses to PfEMP1 have been shown to correlate with the development of acquired immunity in endemic areas.

The main aim of this study was to develop a fast and efficient way of expressing *P. falciparum* antigenic epitopes as conformationally conserved antigenic structures whose expression can be readily 'scaled-up' for clinical testing. The *E. coli* and *P. pastoris* systems were used, both singly and as linked production systems using 'shuttle vectors' that can be dually expressed in both microorganisms. Initially a proof-of-principle experiment to express the CIDR1 domain of PfEMP1 has been conducted. It was expressed and purified in good quantity and used to immunize rabbits. Polyclonal antibodies from rabbit were then assessed with immunoblotting, ELISA studies and immuno-fluorescence. The sequenced 3D7 *P. falciparum* genome was then exploited to construct degenerate primers for several domains of PfEMP1 (namely DBLα, β and γ). These primers were used to amplify targets from FCR3CSA parasites. PCR products were then ligated into the dual expression vector

pPICHOLI1 plasmid. Several erythrocyte surface antigen expression libraries were constructed and shown to be a representative and reliable.

Development and optimisation of microarray and high-throughput screening assays in malaria vaccine development was carried out to accelerate the process of identifying malaria vaccine candidates. Growth of colonies of *P. pastoris* on filters on agar plates for high-throughput screening, a novel procedure, was optimized. These libraries were screened using pooled sera patients from endemic areas in Sudan, as well as sera from male and pregnant women suffering from the pregnancy malaria syndrome from a holoendemic malaria transmission zone, Ghana. Different domains from a gene of particular interest, the NF54 *var*2CSA gene were also amplified for study. The domains of DBL3X and DBL4ε have been produced and purified from mid-scale induction experiments. These recombinant proteins then were then assessed by immunoblotting and ELISA methods.

Chapter 1. Introduction.

1.1 The Malaria Problem.

Malaria is caused by protozoan parasites of the genus *Plasmodium* (Kingdom: *Protista*, Subkingdom: *Protozoa*, Phylum: *Apicomplexa*, Class: *Aconoidasida*, Subclass: *Coccidiasina*, Order: *Haemosporida*, Suborder: *Haemosporidina*) (Smith, T., 2002). About 40% of the world's population lives in areas potentially at risk of malaria, mainly in tropical and subtropical regions (Figure 1.1). This results in 300–500 million clinical cases and 1.5- 2.7 million deaths annually, although the majority of serious cases occur in children under the age of five years (Snow *et al*, 2005, Nahlen *et al*, 2005).

Malaria is a disease that can be cured if the symptoms are recognized and adequate treatment is initiated promptly (WHO, 2005). Unfortunately, in many parts of the world this is not being done and the malaria situation is barely under control. Resurgent malaria is due to the combined effects of global spread of parasite drug resistance, insecticide resistance, civil disturbance, climatic changes, increase in population, increase in mobility, lack of healthcare infrastructure and difficulties in implementing and maintaining vector control programs (Greenwood & Mutabingwa, 2002). There is also a strong relationship between malaria and poverty and there has been essentially no economic growth or improvement of living standards for most people in sub-Saharan Africa in the last three decades (Sachs & Maloney, 2002, Chima *et al*, 2003). The increase in drug resistance of the malaria parasites requires the continuous discovery of new and more potent drugs. Escaping from this burden is highly desirable and underlies much of the current interest in finding a malaria vaccine to aid in the fight against the disease.

The overall aim of the work in this thesis, a small part of the malaria vaccine development effort, is to investigate the problem of identifying malaria-protective antigens and developing such antigens more efficiently into vaccine prototypes that could be used in 'proof-of-principal' clinical trials.

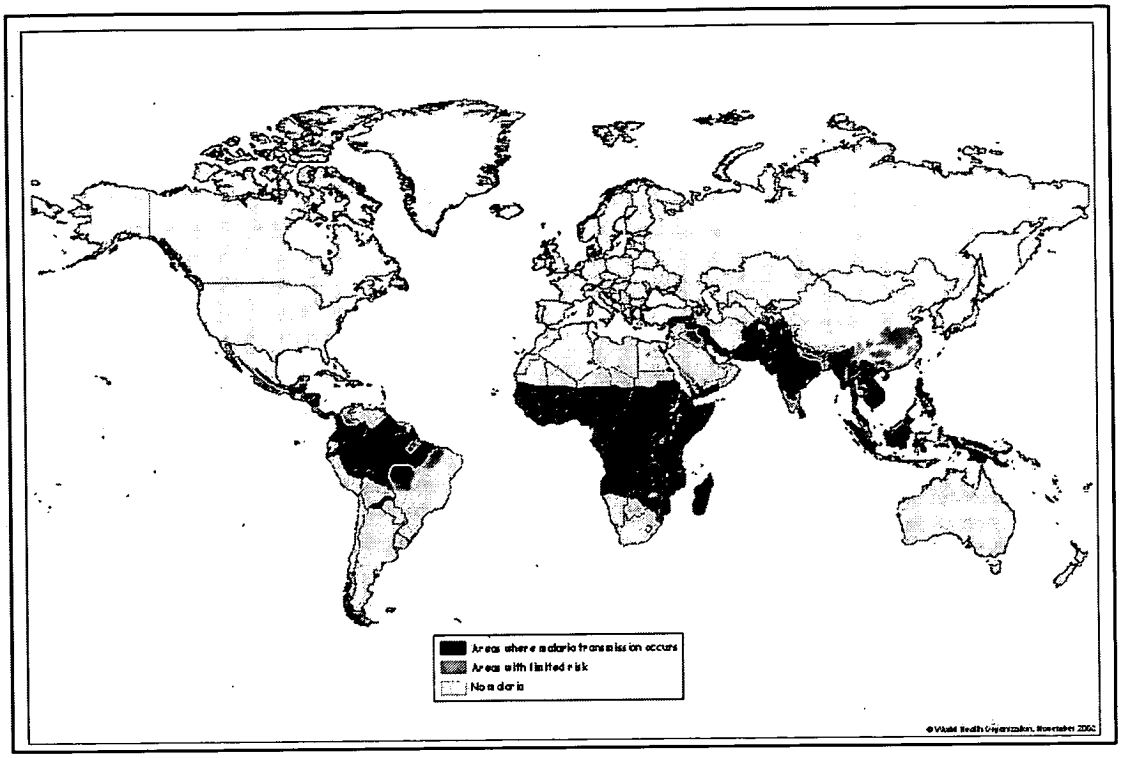


Figure 1.1: Global distribution of malaria (WHO, 2002).

1.2 Life cycle and parasite-host interactions linked to each stages of *P. falciparum.*

Human beings are infected with malaria through the bite of the female mosquitoes of the genus *Anopheles* which have previously been infected with *Plasmodium* (Phillips, 1984, Good *et al,* 2001). Sporozoites (between 10 and 100 sporozoites) are injected into the dermis and blood capillaries (Rosenberg *et al,* 1990, Frevert *et al,* 2005). Sporozoites are initially arrested by binding to stellate cell-derived extracellular matrix proteoglycans. The arrested sporozoites glide along the sinusoidal cell layer until they recognize heparan sulfate and chondroitin sulfate proteoglycans on the surface of Kupffer cells. The sporozoites then actively invade and safely traverse these cells of the liver and eventually invade and develop in hepatocytes (Frevert *et al,* 2005). They make their way to liver and enter the hepatocytes to initiate the DNA replication and growth phase referred to as pre-erythrocytic schizogony (Shin *et al,* 1982). No clinical signs of disease are observed during this stage. The circumsporozoite protein (CSP) and thrombospondin-related

adhesive proteins (TRAP) are both associated with the surface of sporozoites and have been identified as major parasite ligands. Both the heparan sulphate (HS) receptor and the low-density lipoprotein receptor related protein (LRP) have been identified as hepatocyte surface receptors for sprorozoite invasion (Cerami *et al,* 1994, Chitnis, 2001).

After 5 to 6 days, *P. falciparum* exo-erythrocytic schizonts mature and burst, releasing thousands of merozoites into the blood stream where they invade the red blood cells (RBCs) to begin the asexual blood cycle of erythrocytic schizogony. The parasite feeds on the hemoglobin leaving behind a free haem, which is sequestered as a de-toxified crystalline substance called haemozoin (Egan, 2002). During a 48 period the parasite grows from small 'ring stages' to the rapidly metabolizing trophozoite and finally to the schizont stage at which the multiplication of the single invading nucleus to 8-40 daughter merozoites is complete. The parasite bursts the erythrocyte, releasing merozoites into the circulation where they invade fresh erythrocytes and continue to multiply via this asexual cycle (Kirk, 2001).

The erythrocytic cycle is responsible for the development of most of the pathology of the disease (Nagao *et al,* 2003). It is also the period of maximum exposure of the parasite to the human immune system and most of the drugs currently used for malaria treatment target the rapidly metabolising erythrocytic stages. Several proteins such as merozoite surface protein-1 (MSP-1), erythrocyte-binding protein (EBA-175) and apical merozoite antigen-1 (AMA-1) have been characterized and identified as the parasite ligands during this stage (Duraisingh *et al,* 2003a and b). Sialic acid and glycophorin B and C have been identified as *P. falciparum* merozoite receptors on the erythrocyte surface (Dolan, 1994).

Through mechanisms which are still largely unknown, some merozoites in the blood are triggered to develop into sexual forms known as male or female gametocytes. In *P. falciparum* these mature over 12-17 days into elongated or crescent shaped parasitized cells respectively (Carter *et al,* 1984). When a female *Anopheles* mosquito ingests parasitized erythrocytes in a blood meal, these gametocytes are released to become free gametes in the mosquito stomach and after sexual fusion and migration of the resulting ookinete into the gut wall, undergo

further development into mature sporozoites within the oocyst. Sporozoites migrate to the salivary glands and penetrate the acinar cells ready to be injected into skin capillaries when the mosquito again takes a human blood meal (Sinden, 2002) (Figure 1.2).

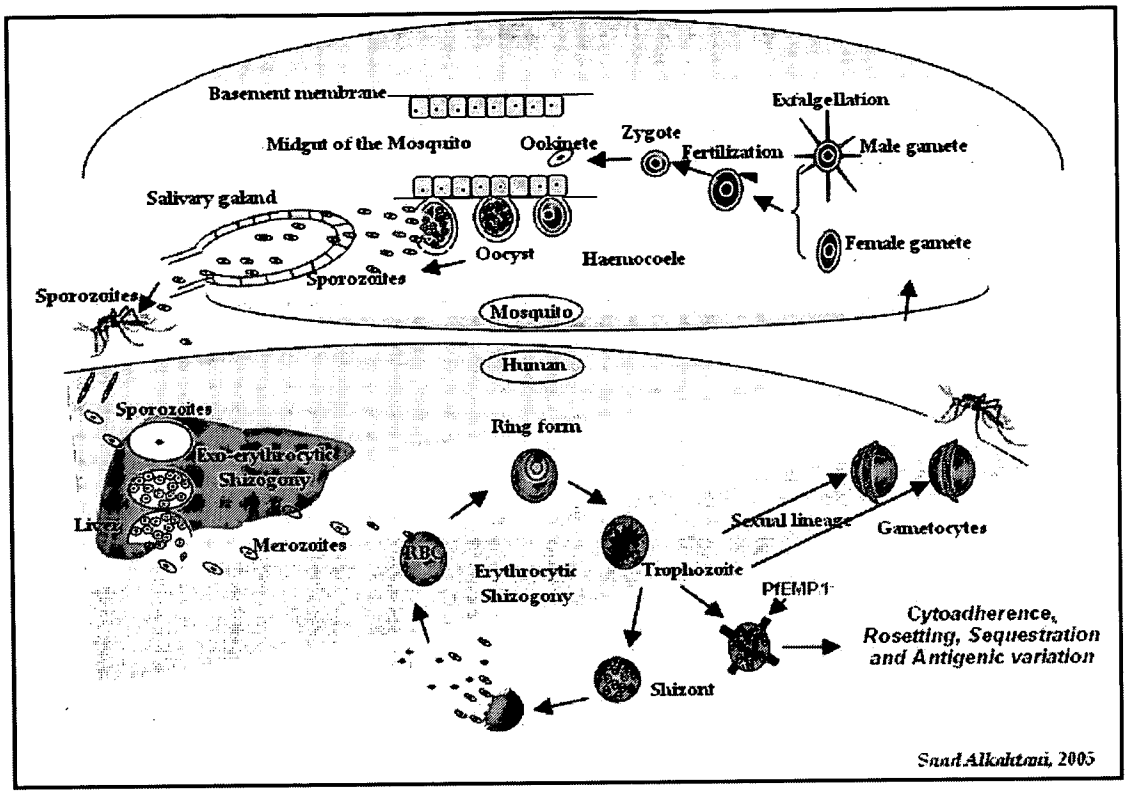


Figure 1.2: Life cycle of *P. falciparum*.

1.3 Clinical manifestation of malaria.

Three *Plasmodium* species cause malaria morbidity, *Plasmodium vivax* (Grassi and Felletti, 1890, Labbac, 1899), *Plasmodium malariae* (Laveran, 1881, Grassi and Felletti, 1890) and *Plasmodium ovale* (Stephens, 1922) but only *Plasmodium falciparum* (Welch, 1897, Schaudinn, 1902) results in both high morbidity and mortality. The most characteristic features of malaria are fever paroxysms occurring at regular intervals. This coincides with the discharge of metabolic waste along with the merozoites after erythrocyte lysis (Beeson *et al,* 2002b). Anaemia is also common, mainly due to parasite destruction of infected red blood cells (iRBC),

decreased erythropoiesis and enhanced clearance of both infected and non-infected RBC, by the spleen and macrophages (Ekvall, 2003). Malaria complications can also include splenomegaly, pulmonary oedma, respiratory distress, hypoglycemia and renal failure (Greenwood *et al,* 2005).

However cerebral malaria is the most serious complication and the most frequent cause of death. Severe headache is the usual presenting symptom, followed by drowsiness, confusion, and coma. This may be due to the occlusion of the blood flow in the brain by infected cells sequestering inside the microvasculature. Host receptors such as the intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) are up-regulated in cerebral malaria. This sequestration of mature stage parasites in the microvasculature is caused by parasite ligands such as PfEMP-1 binding to these host receptors (Miller *et al,* 2002).

In children and adults without exposure to malaria, an increase in parasitaemia correlates with increase in severity of clinical symptoms. However, after a number of malaria episodes, some degree of immunity will develop among people living in areas endemic for the disease. This acquired immunity appears to be lost in the syndrome of pregnancy associated malaria (PAM) (it will be described in detail in section 1.8 of this chapter). Because of the interesting, important and clearly-defined nature of the syndrome, PAM-derived sera from Ghanaian females have been used in this thesis to help distinguish between specific and more generalized antibody-mediated immune responses to malaria.

1.4 The molecular pathology and malaria parasite-host interactions.

1.4.1 Modifications to parasitized erythrocytes (PE).

During the asexual intra-erythrocytic stages, the composition and function of the PE cytosol and plasma membrane are extensively altered, in a programmed pattern that is synchronized with the parasite's cell cycle (Arnot & Gull, 1998). About 12 – 16 hours after invasion of the erythrocytes, as the ring stage begins to transform to trophozoite stage, a tubolvesicular membrane network (TVM) extending from the

parasite vacuole membrane (PVM), that surrounds the parasite, is established. The parasite appears to establish a system within the iRBC cytosol to allow the trafficking of parasite proteins in and out of the infected cell. Several parasite-derived proteins are translocated and sorted to compartments in the IE cytosol and to different locations near or at the plasma membrane where they can be associated with the cytoskeleton as well as anchored in the lipid bi-layer and displayed at the surface.

Membranous structures such as the Maurer's clefts are formed beyond the PVM. Vesicles have also been observed budding off from the parasite and moving through the RBC cytoplasm, perhaps independently of the TVM (Hibbs & Saul, 1994, Hibbs *et al,* 1997). The parasite also modifies the erythrocyte membrane itself so that it changes its permeability and places the parasite-derived proteins in knob like structures at the cell surface. These knobs act as attachment points for the sequestering parasites in the vascular beds (Beeson *et al,* 2002b).

1.4.2 Parasite cytoadherence and sequestration.

Probably to avoid immune clearance by the spleen, erythrocytes infected with the more mature stages of parasites (trophozoites), adhere to endothelial cells and sequester in post-capillary venules of various organs (Baruch *et al,* 2002b). The most important parasite-derived molecule expressed on the surface of infected RBCs involved in mediating these adhesion reactions is PfEMP-1 (*P. falciparum*-infected erythrocyte membrane protein). Around 60 loci encoding different members of this gene family have to date been found in the near complete genome sequence of the 3D7 clone. Specific forms of these variants have been shown to correlate with adhesion to different ligands on the human endothelium (Smith, J. *et al,* 1995, Su *et al,* 1995, Baruch *et al,* 1997).

Host ligands of PfEMP1 include Chondroitin Sulfate A (CSA), intercellular adhesion molecule 1(ICAM-1), CD36, CD31, P- and E-selectin and vascular cell adhesion molecule-1 (VCAM-1). Adhesion to these ligands has in several cases been localized to specific parts of the PfEMP-1 molecule. The contact residues for CD36 binding, for example, have been proposed to be in the CIDR1α domain (Baruch *et al,*

1995, Baruch *et al,* 1999), binding to CSA has been reported to be localized to DBLγ3 (Reeder *et al,* 1999, Buffet *et al,* 1999) and binding of ICAM1 to DBLβ2 (Smith *et al,* 2000a, b) (see Figure: 1.3). CD31 and P-selectin binding parasites are found in patients with malaria, although at lower frequency than CD36 and ICAM-1. Both P-selectin and ICAM-1 are able to mediate rolling adhesion of the (PE) and this may play a role in capturing IEs from the blood flow, working in synergy with other receptors (e.g. CD36) to produce efficient tethering (McCormick *et al,* 1997). VCAM-1 and E-selectin have also been shown to act as receptors *in vitro,* but do not seem to play a major role *in vivo* (Ockenhouse *et al,* 1992).

1.4.3 Rosetting.

Infected RBCs of some parasites can adhere to uninfected RBCs to form 'rosettes' (Udomsangpetch *et al,* 1989). Parasites with the rosetting phenotype are more likely to occur in patients with cerebral disease (Carlson *et al,* 1990), although definitive proof that rosetting causes severe malaria is lacking. It is postulated that rosetting could facilitate invasion of new erythrocytes after schizogony or the iRBCs use the uninfected erythrocytes as 'shields' that help them avoid recognition by the immune system. Rosetting may also play a direct role in malaria pathogenesis by obstructing microvascular blood-flow (Kaul *et al,* 1991).

The only consistent difference between rosetting and non-rosetting clones is the expressed PfEMP1 molecule, although it seems that rosetting is mediated by multiple receptor-ligand interactions (Heddini *et al,* 2001). It therefore seems likely that PfEMP1 is the rosetting ligand in all parasites and the DBL1α domain may be the responsible ligand (Rowe *et al,* 1997, 2002b). A central part of the domain between the fifth and the twelfth cysteine residues has been found to be the functionally important section (Mayor *et al,* 2005). Chen *et al.* raised antibodies against DBL1α that disrupted rosette formation *in vitro* and prevented sequestration *in vivo* (Chen *et al,* 2004). Heparin sulfate (HS) proteoglycans (Chen *et al,* 1998), and complement receptor 1 (CR1) (Rowe *et al,* 1997), ABO blood group sugars (Carlson & Wahlgren, 1992) and CD36 (Handunnetti *et al,* 1992) on the surface of uninfected RBCs are

thought to act as receptors for the adhesion of infected RBCs in the formation of rosettes.

1.5 *P. falciparum* antigens implicated in disease severity.

1.5.1 PfEMP1 and the *var* gene family.

The PfEMP-1 molecule was first characterized in the early 1980s. It was identified as a large parasitized erythrocyte surface molecule varying considerably in size (200 –400kDa) (Leech *et al,* 1984). More than a decade later, the genes encoding PfEMP1, known now as the *var* gene family, were discovered. Most *var* genes are located in the subtelomeric regions on both ends of the parasite's 14 chromosomes, although chromosomes 4,7,12 and 8 contain *var* genes that are centrally located (Gardener *et al,* 2002). Further analysis of *var* gene sequences indicate that most of the family can be divided into three broadly defined classes based on chromosomal location (either telomeric, subtelomeric or in the central part of the chromosome), direction of transcription (either towards or away from the telomere), and type of upstream flanking region, referred to as Ups A, B, C, D and E (Lavstsen *et al,* 2003). *Var* genes have a two-exon structure separated by a 0.17-1.2 kb intron. The first exon is highly polymorphic, encodes the extra-cellular binding region and transmembrane domain and varies in size between 3.5 and 9.0 kb. The second exon is more conserved, encodes an acidic cytoplasmic tail and is between 1.0 and 1.5 kb in size. (Gardner *et al,* 2002) (Figure 1.3.A). Each parasite genotype encodes approximately 60 different *var* loci (Thompson *et al,* 1997, Gardner *et al,* 2002, Bahl *et al,* 2002, www.plasmodb.com), gene repertoires differing extensively between parasite strains (Peterson *et al,* 1995, Kyes *et al,* 1997a, Ward *et al,* 1999, Fowler *et al,* 2002). However, there are *var* genes that appear quite conserved between parasite genotypes and which may play important roles in malaria pathogenesis (Fried and Duffy, 2002, Rowe *et al,* 2002a, Lavstsen *et al,* 2003, Salanti *et al,* 2003, 2004, Winter *et al,* 2003, Gupta *et al,* 1994, Bull *et al,* 1999, Nielsen *et al,* 2002, Deitsch & Hviid, 2004).

The PfEMP1 extracellular framework is predominantly composed of the following basic building blocks: an N- (NTS) region, DBL domains (from α to ε domains),

CIDR domains (α and β) and C2 (Fig 1.3-B). DBL domains have as a unifying feature a conserved structure composed of invariant cysteine residues. The cysteines are unevenly distributed among ten semi-conserved homology blocks (A–J) in which DBL conservation is significantly concentrated. Within the DBL fold is a vast diversity of sequences, with a large proportion of differences occurring in the hypervariable blocks (I–X) that flank the homology blocks. Conserved CIDR characteristics include 13 or more invariant cysteine residues. However, unlike other variant gene families that apparently function only as clonally variant targets of immunity, such as the *Trypanosome* variant surface antigens, *var* genes also encode cytoadherent properties. Thus, the *var* gene repertoire is shaped by selective forces acting to both maintain binding capability and escape immunity (Roberts *et al,* 1993, Smith, J *et al,* 2001).

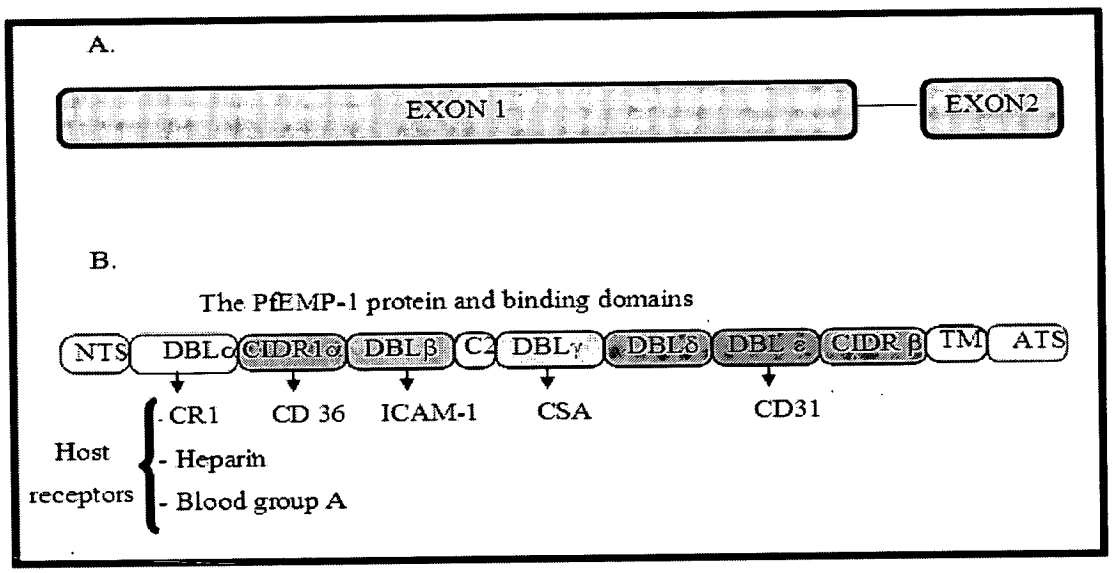


Figure 1.3: Schematic representation of a *var* gene (A) and PfEMP1 protein architecture and binding domains (B). Individual domains involved in binding to several host receptors are indicated. CR1=complement receptor 1, HS=heparan sulfate, CSA=chondroitin sulfate A, ICAM-1=intracellular adhesion molecule 1 and CD36 (after Smith, J. *et al,* 2001).

1.5.1 The *rif*, *stevor* and *clag* gene families.

Other antigenically variable multi-gene families genes have been discovered in Plasmodium such as the *rif* (repetitive interspersed family) genes encoding proteins known as rifins (Kyes *et al,* 1999). These 27-39 kDa antigens comprise a 200 member family, the largest gene family in *P. falciparum. Rif* genes are transcribed in the asexual stages of the parasite and rifins are transported to the surface of the infected red blood cell where they are detectable 14 to 16 hours after parasite invasion (Kyes *et al,* 1999). They can switch transcription and undergo antigenic variation. *Rif* genes have 2 exons, the first of which encodes a putative signal peptide and the second of which encodes an extracellular domain made up of a conserved and a variable region, followed by a transmembrane segment and a short intracellular portion (Cheng *et al,* 1998, Abdel-Latif *et al,* 2002). Unlike *var* genes, several *rif* genes are believed to be concomitantly expressed on the surface of the infected erythrocyte. They are immunogenic and their high copy number, sequence variability and red blood cell surface location indicate a potentially important role for rifin in malaria host-parasite interaction (Gardener *et al,* 2002) although a specific role in parasite adhesion has not yet been determined.

Another multi-gene family called the *stevor* genes (subtelomeric variable open reading frame) which comprise 30-40 genes/haploid genome. These genes encoding 30KDa proteins are called stevors. They may be variant antigens and have sequence similarity to rifins, but are less polymorphic and their function is unknown. It is not clear if they are actually surface exposed antigens. Some of these genes are expressed in gametocytes and the structure of these genes differs from those expressed in asexual parasites (Cheng *et al,* 1998, Gardener *et al,* 2002). The *clag* family (cytoadherence linked asexual gene), another gene family potentially involved in adhesion, although probably not surface expressed, may affect cytoadherence indirectly through PfEMP1 transport rather than interacting directly with a host receptor (Craig, 2000, Craig & Scherf, 2001).

1.6 Immunity to malaria: the general situation.

Infection with malaria parasites can induce a specific immunity, which may bring about clinical cure, but does not usually lead to complete elimination of parasites from the body (Bull & Marsh, 2002, Moorthy & Hill, 2002). Whereas immunity to many pathogens, particularly viruses, is long lived after a single infection/clinical episode, immunity to *P. falciparum* is incomplete, even in adults who have lived in high transmission areas all their lives. The main burden of disease falls on young children (Baird, 1995, 1998, Bull & Marsh, 2002). Anti-malaria immunity develops slowly and its precise mechanisms are unclear. Nor have unambiguously 'protective' antigens of the parasite been identified. Both the extreme antigenic diversity of the parasite and the existence of mechanisms of immune evasion may explain the difficulty in clearly identifying such protective antigens and acquired immunity is clearly not an absolute defence against re-infection (Warrell & Gilles, 2002). This state of disease resistance in an infected host associated with continuous parasitic infection is sometimes called premonition (Smith, T. *et al,* 1998). The host is resistant to new clinical episodes, as long as the pre-existing infection continues but once the infection is eradicated, immunity does not persist for long and cannot prevent re-infection (Smith, T. *et al,* 1999a, b and c).

Where malaria transmission is stable and holo-endemic, transmission occurs for at least 6 months in a year and is intense. Children suffer repeated attacks from the age of around 6 months. Those who do not die, have a substantial degree of immunity by the age of five or six years (Gupta *et al,* 1999). When immunity is established, patients may still suffer attacks of malaria, but these are comparatively mild and last for only a few days. Older people are little affected (Moore *et al,* 2002). In areas where malaria transmission is unstable, there are changes in transmission from one season to another, and from one year to the next. The transmission season is short, and infection of any one individual is comparatively infrequent, so that immunity does not reach a high level (Giha *et al,* 2000). When an outbreak of malaria occurs, usually following explosive breeding of mosquitoes, it does so in the form of an epidemic with people of all ages being susceptible, although there are indications of age-dependent diminution of incidence (Creasey *et al,* 2004).

Individuals living in endemic areas exposed to repeated infection, ultimately develop partial resistance or tolerance to the disease (Good, 2001). In such cases, even if the individual develops relatively high parasitemia, they show mild symptoms. Persons with this type of immunity can however be reservoirs for infection, because, though they show little or no symptoms, they carry the parasites. There is good evidence of trans-placental transfer of antibodies to a child born of an immune mother. This gives passive immunity to the child, but only during the first 6 - 9 months of life (Miller & Smith, 1998).

1.7 Immune mechanisms against red blood stages.

A Innate immunity.

Non-specific innate immune mechanisms are clearly important in the acquisition of protection against malaria. Theoretically, following infection with a single *P. falciparum* sporozoite infection in humans, one would expect all erythrocytes to be infected within about 16-18 days after the rupture of the initial liver schizont. However, in practice, parasitemia peaks much lower than this, at only 0.1% on average, suggesting that non–specific defense mechanisms may effectively control parasite growth even before strain antigen-specific immunity develops. Neutrophil, macrophage, and natural killer (NK) cells have been shown to be involved in this type of immunity. NK cells have been thought primarily to secret cytokines rather than have direct cytotoxicity (Sherman, 1998). Nevertheless, because of their ability to lyse target cells in non-MHC restricted fashion, NK cells participate in innate immunity mechanisms against malaria. Neutrophils also produce IFNγ that activates macrophages thus linking innate and acquired responses.

Neutrophil-mediated phagocytosis of *P. falciparum* has been observed in infected individuals, as has killing of intra-erythrocytic, asexual forms of *P. falciparum.* Cytokines, such as IFNγ and TNFα and TNFβ appear to augment the anti-parasitic effect of neutrophils (Kumaratilake , 1990, Kumaratilake *et al,* 1991). Macrophages can also detect, 'screen out' and destroy abnormal and deformed iRBCs in the spleen during malaria infection (Weiss, 1990).

B Humoral Immunity.

That this arm of immunity operates has been confirmed experimentally using the IgG fraction of sera from immune adults to transfer protective immunity. Transfer of purified, pooled hyper-immune IgG from African adults to Thai patients acted to reduce the parasite load, although this treatment did not induce sterile immunity (Bouharoun-Tayoun *et al,* 1990). In terms of the mechanisms involved in antibody-mediated protection, cytophilic antibodies (IgG1, IgG3) have been proposed to exert an anti–parasitic effect by their participation in the so-called ADCI reaction (Antibody-Dependent Cellular Inhibition) (Brown *et al,* 1986, Bouharoun-Tayoun *et al,* 1995). Opsonizing antibodies promoting merozoite phagocytosis have been also observed (Warrell & Gilles, 2002). Antibodies have been found to be important in monocyte/macrophage and neutrophil mediated inhibition of *P. falciparum* replication *in vitro* (Kumaratilake *et al,* 1991). Antibodies capable of binding to the parasitized erythrocyte surface may also prevent binding and sequestration of these cells to the capillary endothelium and enhance removal of these cells by the spleen and other tissues of the reticulo-endothelial system (Urban *et al,* 1999).

In the newborn child of an immune mother, the transfer of IgG across the placenta and possibly other factors protects the child during the early months of life (Riley *et al,* 2001). Antibody-mediated protective responses could be directed against surface proteins of the merozoite leading them to agglutinate, thereby prevent the invasion of RBCs, in which case they act either by blocking key steps in the invasion process (Green, 1981), or by rendering the merozoite susceptible to secondary effects, such as phagocytosis or complement-mediated damage (Taylor *et al,* 2001). There is experimental evidence for all these reactions, and it is difficult to determine which are important. There are likely to be several distinct mechanisms whereby the host inhibits parasite replication. Where the destruction of parasites occurs is not known, but it does not seem to be in the circulating blood. The major site is probably the spleen, and evidence for this comes from experimental models and of course the classical observations on splenic enlargement in disease endemic populations.

The flow of blood is slowed down in the spleen and infected RBCs come in close contact with the macrophages lining the blood vessels, causing the release of radical

oxygen intermediates (ROI), Nitric oxide (NO), and TNFα that damage the intra-enythrocytic parasites, while infected cells and free merozoites are phagocytosed (Jacobs *et al,*. 1995). Auto-antibodies against normal RBC produced during human malaria may play a role in the pathogenesis of hemolysis and anemia of malaria (Daniel-Ribeiro, 2000).

C Cellular immunity.

$CD8^+$ T cells operate via the major histocombatibility complex (MHC) restricted mechanisms to destroy hepatocytes infected by pre-erythrocyte stage parasites. However direct, contact-mediated cytotoxicity is not effective against infected erythrocytes, probably because of lack of expression of Class 1 MHC on the erythrocyte surface (Hoffman, 1989 a and b). However as the parasite matures, it induces a series of morphological and antigenic changes in the host erythrocyte membrane. Some changes are because of alteration of the host constituents, perhaps by partial disruption or digestion of integral membrane proteins, but others result from the parasite inserting its own molecules into the host cell membrane. These antigens are potentially targets for immunity (Chen *et al,* 2000 a and b, Malaguarnera & Musumeci, 2002).

The major involvement of T cells in immunity to the erythrocyte stage of the parasite seems to be in providing help for the production of antibodies and in activation of macrophages and NK cells through $CD4^+$ Th2 cells. The malaria antigens are captured by antigen presenting cells (APC) and presented to Th2 T cells that are activated by interleukin-4 (IL-4), Th2 in turn secretes IL-4, 5, 6, and 13 that activates B cells to proliferate and differentiate into antibody secreting cells. Antibody binding to the surface of the merozoite and to the proteins that are externalized from the apical complex of organelles involved in erythrocyte recognition and invasion, may have an important role in immunity to asexual blood stages. Such antibody could neutralize the parasite, e.g. by blocking invasion of RBC, or lead to immunoglobulin constant region (FC) dependent mechanisms of parasite killing by macrophages, agglutinate the merozoites and/or possibly contribute to antibody-dependent cellular inhibition (ADCI) (Taylor-Robinson, 1995 a and b, Garraud *et al,* 2003).

The intracellular parasite and its antigens activate $CD4^+$ T cells through interleukin-12 that in turn induces secretion of IFNγ that promotes activation of macrophages and NK cells. These cells secrete low levels of TNFα that work together with IFNγ to induce production of NO and reactive oxygen intermediates (Taylor-Robinson, 1995 a and b). The intracellular parasites are thought to be susceptible to cytokines produced by these immune cells. One possibility is that there would be initial parasite clearance by phagocytic cells in the reticule-endothelial system, leading to the recruitment of antigen–specific lymphocytes and the production of cytokines. Parasites would then be damaged whenever they passed through organs such as the spleen that are acting as parasite-killing 'cytokine factories'. High levels of circulating of INFγ and TNFα are more often found in patients with severe malaria than uncomplicated cases (Kwiatkowski, 1990) Animal models and indirect evidence from clinical observations suggest phagocytosis of infected red blood cells by splenic macrophage are important potential components of host defence mechanisms against blood-stage parasites (Wyler *et al,* 1981).

Red blood cells lack nuclei and nucleic acid synthesis and thus cannot support viral replication, and express little or no MHC molecules, particularly in humans (mouse RBCs have higher levels of residual MHC). The absence of MHC classe I and II molecules makes the interior of erythrocytes a privileged site where infections can escape T cell surveillance. Different individuals in a population differ in the MHC molecules they express and will present different sets of peptides from pathogens. All individuals in a population will not be equally susceptible to any given pathogen, thereby limiting its spread. That pathogens can exert selective pressure on frequencies of particular MHC alleles is suggested by the finding that HLA-B53 alleles are significantly associated with survival from lethal malaria. This allele is common in individuals living in West Africa, where malaria is endemic (Hill, T. *et al,* 1991, 1992 a and b).

1.8 An immunological and parasitological puzzle: the syndrome of pregnancy- associated malaria (PAM).

Tens of thousands of pregnant women are infected annually with *P. falciparum* resulting in an estimated 75,000 – 200,000 infant deaths directly attributable to PAM (Steketee *et al,* 1996b and c, Steketee *et al,* 2001). *P. falciparum* infection in adults living in holo-endemic areas rarely causes severe malaria. Pregnant women are an exception. Both immature and mature stage parasites appear to be associated with the syncytio-trophoblast surface (Figure: 1.4). It has been suggested that parasites responsible for PAM express a distinct set of molecules mediating adhesion to receptors located only in placental tissue (Staalsoe *et al,* 2004, Cox *et al,* 2005).

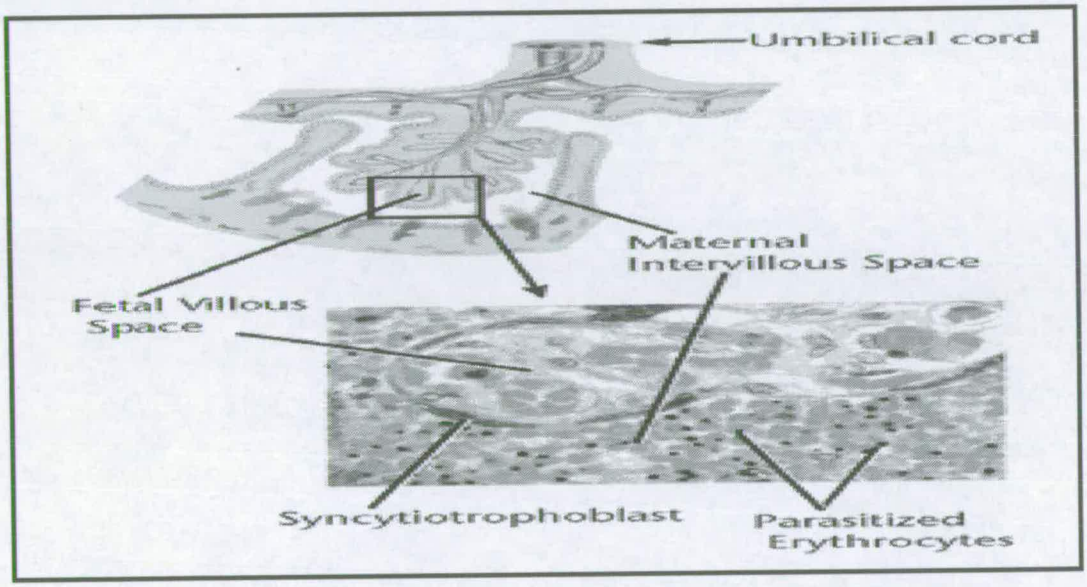


Figure 1.4: Placenta containing *P. falciparum*-infected erythrocytes. Infected erythrocytes recovered from placenta bind CSA and adhere to syncytiotrophoblasts. Immunity to PAM has been shown to be parity-dependent; therefore multiparous women have fewer placental parasites when compared to primiparous women. Women who have antibodies that block infected erythrocyte adherence to CSA have fewer parasites in the placenta at delivery when compared to women without these blocking antibodies. (Staalsoe *et al,* 2004).

1.8.1 Epidemiology of pregnancy associated malaria.

In all malarious areas, the frequency and severity of this infection are greater in pregnant women than in the same women before pregnancy and in non-pregnant adult women (Gilles, 1986). The risk of infection is also higher for women in their first pregnancies (primigravidis) (Rasti *et al,* 2004).

In areas where malaria transmission is low, the degree of the acquired immunity present in women prior to pregnancy is likely to be low, rendering both the mother and her foetus susceptible to the most severe form of malaria. In contrast, in areas of high malaria endemicity, women have acquired a significant protective immunity and the effects on the mother and her foetus are less severe (Steketee *et al,* 1996a). Primigravidae are always the most at risk in all areas (Okoko *et al,* 2002). However, in meso-endemic area where exposure to malaria is low, secundigravidae have been documented to suffer similar effects of infection to those of primigravidae (Nosten *et al,* 1991). The increased risk of malaria is highest in the first half of the pregnancy and decreases progressively until delivery (Menendez, 1994).

Adhesion of *P. falciparum* to placental receptors causes sequestration of mature infected erythrocytes in the placental blood spaces, allowing these parasites to grow and multiply. This leads to inflammatory responses and deposition of fibrinoid material in the placental wall (Brabin *et al,* 2004). Foetal and postnatal consequences include spontaneous abortion, stillbirth and intrauterine growth retardation (Kasumba *et al,* 2000), low birth weight and premature delivery (Menendez *et al,* 2000). It is uncertain how placental infection contributes to foetal morbidity, but it could impair exchange of nutrients and gases, and allow the foetus to be exposed to maternal immune cells of pro-inflammatory cytokines such as TNFα and IFNγ (Warrell & Gilles, 2002).

1.8.2 Host-parasite interactions in placenta.

The *var* genes called *var*1CSA, CS2*var* and *var*2CSA have all, at various times been implicated as the key genes encoding the parasite receptor that is both capable of binding placental receptors such as CSA and the target of protective, parity dependent antibodies (Ricke *et al,* 2000).

The recombinant derived fragments of the *var*1CSA gene DBL3γ domain were the first molecules to be shown to have some capacity to bind CSA and their transcription seemed to be concomitantly selected with CSA binding (Buffet *et al,* 1999). However it has since been found that this gene is relatively rarely transcribed in parasites from placental infections (Rowe *et al,* 2002 a) and transcribed equally in parasites from placental infections and infections of children (Fried & Duffy, 1996, 2002) as well as being transcribed by non-CSA binding parasites (Kyes *et al,* 2003, Salanti *et al,* 2003). An alternative candidate for the role of encoding the key protective immunogen is CS2*var* (Reeder *et al,* 1999). It has been identified that both the CIDR1α and DBLγ domains of this gene bind CSA (Reeder *et al,* 2000). Antibodies raised to DBLγ showed specific inhibition of CS2 parasite to CSA (Reeder *et al,* 1999). However Rowe and colleagues showed that this gene is not well conserved among different isolates (Rowe *et al,* 2002 a and b) and it appears that gene has no direct involvement in placental adhesion, thus seeming to eliminate its candidacy as the key protective immunogen for PAM (Rowe & Kyes, 2004).

Salanti and colleagues have identified a third candidate CSA-binding ligand , the *var*2CSA gene (Salanti *et al,* 2003, 2004). It is well conserved among different parasite isolates and its transcription is up regulated following CSA-selection. Unlike the first two genes, the transcription of this gene is consistent with the pattern that would be expected for a placental parasite CSA-binding ligand (Rowe & Kyes, 2004). A number of recent publications have shown that placental *P. falciparum* isolates transcribed *var*2csa but not *var*1csa (Salanti *et al,* 2004, Jensen *et al,* 2004, Tuikue Ndam *et al,* 2005). The PfEMP1 proteins expressed on the surface of these parasite contains CIDR regions but do not bind to CD36 (Buffet *et al,* 1999, Reeder *et al,* 1999). This suggests that the DBL3 and CIDR1 region may combine to form the CSA binding site (Craig & Scherf, 2001). It has been proposed that the CIDR1 from CSA binding parasites may be able to mediate adhesion to CSA (Degen *et al,* 2000). It is possible that this domain represents a cryptic binding domain *in vivo* (Craig & Scherf, 2001).

Different host receptors have been identified as possible targets for placental parasite sequestration (Bessin *et al,* 2002b). The most studied is CSA, a glycosaminoglycan (Fried and Duffy, 1996). The minimal binding motif was

identified as a dodecasaccharide with two to three sulphate groups (Wahlgren & Spillman, 2000). While low sulphated CSA appears to be the main receptor for parasite cytoadhesion in the placenta, other adhesive host/parasite receptor interactions probably contribute to placental sequestration. CD36 and ICAM-1 have been suggested to contribute the cytoadhesion to placenta (Andrews & Lanzer, 2002). Another member of the GAG family, hyaluronic acid (HA), has been identified as a potential receptor for PE adhesion in the placenta (Besson *et al,* 2000, 2002b). It has also been shown that natural IgM antibodies bind to CSA-selected parasite clones, potentially enhancing placental sequestration (Creasey *et al,* 2003) and the nonimmune IgG (Flick *et al,* 2001). Sharling and colleagues also showed that the placental parasites that are differentially recognized by males and females from malaria endemic regions recognize a trypsin-resistant entity (Sharling *et al,* 2004).

1.8.3 Antibody-mediated Protection from PAM.

Antibodies that develop after multiple pregnancies are associated with lower levels of PE's in the placenta and these have the capacity to block the CSA binding of *P. falciparum* isolated from various different parts of the world. When a woman is infected with *P. falciparum* in her first or second pregnancy, antibodies develop against the parasite variants that adhere in the placenta. These antibodies then contribute to protection from malaria in subsequent pregnancies (Rowe *et al,* 2002a). The broad specificity of this protection implies that a vaccine which can reproduce this effect is feasible.

One of this study aims is develop effective techniques of identifying iRBC surface molecules that may play a role in eliciting a strong immune response. This would be useful for vaccine prototypes by applying these techniques to the specific phenomenon of PAM. The principles of this strategy however could be applied to other specific types of malaria.

1.9 Vaccine development in the post–genomic era.

About 5300 *P. falciparum* antigens have been identified after whole-genome sequencing (Gardner *et al,* 2002). The genome database can be used for identifying hundreds of candidates for vaccination. Researchers have now sequenced the genomes of 140 bacteria, 1,600 viruses and 9 parasites (Rappuoli & Covacci, 2003). Genome sequences and supercomputers are thus directing vaccine science researchers through the genome sequences of microbes to develop potential vaccines for a variety of infectious diseases. Looking at homology in surface proteins across species may help scientists make better predictions of which genes might code for proteins that would make promising vaccine candidates. However to move from databases of microbe genomes then to vaccines, it is necessary to insert pathogen genes into recombinant expression systems to produce experimental antigens. Cloning of the gene coding for the antigen is usually necessary in order to produce the antigen (Capecchi *et al,* 2004). However, while straightforward in theory, this approach has difficulties where disease agents do not have obvious immuno-dominant protective antigens to form the basis for the experimental vaccine.

1.10 Is a malaria vaccine feasible?

There are several thousand proteins, carbohydrates and lipids made by malaria parasites during human infection. Antibody and/or cellular immune responses are made to most of these molecules or antigens. An additional and serious obstacle to vaccine development is the fact that many antigenic proteins vary between different individuals of the malaria parasite population. Malaria parasite populations are not composed of identical organisms but are mixtures of genetically different and antigenically polymorphic individual clones.

Malaria parasites can switch production of infected red blood cell surface protein antigens probably to evade the host's antibody-mediated immune response against surface-exposed molecules. Thus each member of the diverse and polymorphic population has an additional capacity for immune system evasion by clonal antigenic variation. In many areas of Africa where conditions sustain huge populations of mosquitoes, humans receive one or more infected bites each night and every bite

may introduce 10-100 sporozoites belonging to half a dozen, or more, different clonal lines. Malaria clearly poses a formidable challenge to the human immune system. Where it not for the fact that humans living with malaria transmission become partially immune to malaria, albeit after heavy childhood morbidity and mortality, it would be difficult to imagine a successful malaria vaccine being invented.

The biological evidence that suggests that a malaria vaccine is possible includes,

1. The observation that people living in endemic areas who have been repeatedly exposed to malaria eventually develop immunity against severe disease (Baird, 1995).

2. The demonstration that protection against malaria can be induced by immunising volunteers with irradiated malaria sporozoites (Clyde *et al,* 1973, Hoffman *et al,* 2002).

3. The demonstration that antibodies purified from life-long residents of endemic areas can be transferred into other individuals and confer some protection against the effects of malaria infection (Cohen *et al,* 1961).

4. The demonstration that experimental vaccines already in hand can provide significant protection against malaria infection in humans. Anti-sporozoite vaccines such RTS, S have provided consistent, if relatively low-level, short duration protection in humans (Kester *et al,* 2001, Alonso *et al,* 2004).

1.11 Vaccine bio-technology: Protein expression.

Heterologous expression systems are based on the insertion of a gene of interest into a host cell for translation and expression into protein. There are several protein expression systems in prokaryotes and eukaryotes. They include: bacteria - e.g. *E. coli,* yeast (e.g. *P. pastoris* and *S. cerevisiae*), cultured insect cells and cultured mammalian cells. The advent of recombinant DNA technology makes it technically feasible to produce any protein of interest in a number of different organisms. However technical feasibility does not guarantee production success. Factors affecting expression include initiation of transcription, RNA elongation, RNA

stability, initiation of translation, translational elongation, polypeptide folding and post-translational processing including amino-terminal modification of polypeptide, disulphide bond formation, proteolytic cleavage of precursor forms, glycosylation and modification of amino acids within proteins. Production is also affected by the rate of degradation (Rai & Padh, 2001)

The prokaryotic *E. coli* expression system is the most frequently used. Although it has many advantages, recombinant production often leads to inactive and insoluble proteins in inclusion bodies. Although methods have been developed to obtain correctly folded proteins from these inclusion bodies, the process of refolding cannot be successfully applied to all proteins (Pandey *et al,* 2002). In addition, *E. coli* does not readily produce proteins with disulfide bonds and it produces all its proteins without glycosylation due to its lacking enzymes that carry out eukaryotic-type post-translational modifications. Yeasts are simple eukaryotes and the most frequently used alternative to bacteria. The main species used are *P. pastoris* and *S. cerevisiae.* The main advantages of yeast cells are that they perform many eukaryotic and thus more human type post-translational modifications. The main disadvantage of yeast cells is the presence of highly active proteases that degrade foreign proteins. Insect cell expression systems using baculovirus and mammalian cells can produce correctly folded proteins (Pizarro *et al,* 2003). However, so far only a few proteins have been successfully produced on an industrial scale using these systems mainly because these systems achieve very limited yields, thus making large-scale production uneconomical.

1.12 Malaria gene expression.

High A/T content and consequences of frequent asparagine and lysine-rich repeats in the *P. falciparum* genome are thought to be the main reason for the frequent problem of early termination in the mRNA translation process. The genetic code is degenerate and not all of the 61 codons that encode amino acids are used equally. Which of these 61 codons are the most commonly used depends strongly on the organism and, in fact, its genomic base composition. Usually, the frequency of the codon usage reflects the abundance of their cognate tRNAs. Thus, in a situation

where the codon usage of the pathogen protein of interest differs significantly from the common codon of the expression host, this can and does cause problems during expression. The majority of *P. falciparum* derived recombinant proteins are expressed only as truncated forms or as insoluble inclusion bodies within the bacterial cells and the expression of *P. falciparum* derived proteins, especially membrane-bound proteins, is still a considerable challenge (Flick *et al,* 2004).

Three main methods are available to increase the expression levels of gene sequences whose codon usage is different from that of the host cell; site-directed mutagenesis, co-expression of the sequence with genes which encode rare tRNAs and re-synthesis of the whole gene to be expressed to make it more consistent with the codon usage of the host expression system. Such methods have been used to express the entire EBA 175 protein (Yadava & Ockenhouse, 2003). X-ray crystallography has since been used to determine the atomic structure of a key portion of the EBA-175 (Duraisingh *et al,* 2003 a and b). For expression in *P. pastoris*, the codons of *P. falciparum* antigens also need to be optimized and potential glycosylation sites have to be removed. So far, this system is perhaps the most promising one for GMP (industrial scale and guaranteed quality) productions. *P. pastoris* has been used to express several malaria proteins such as CSP, $MSP\text{-}1_{19}$ and MSP-1-AMA-1 hybrids for malaria vaccine studies (Kocken *et al,* 2002, Zhang and Pan 2005). The more conventional yeast expression system *S. cerevisiae* has been used to express the Pvs25 (Miles *et al,* 2002).

1.13 Current *P. falciparum* vaccine development projects.

A genetically engineered, recombinant-produced malaria vaccine appears to offer the greatest hope of adding to the armoury of weapons for malaria control and significantly improving global child and public health. There are four general targets of possible vaccine candidate antigens derived from the different life-cycle stages of the parasite (Figure 1.5).

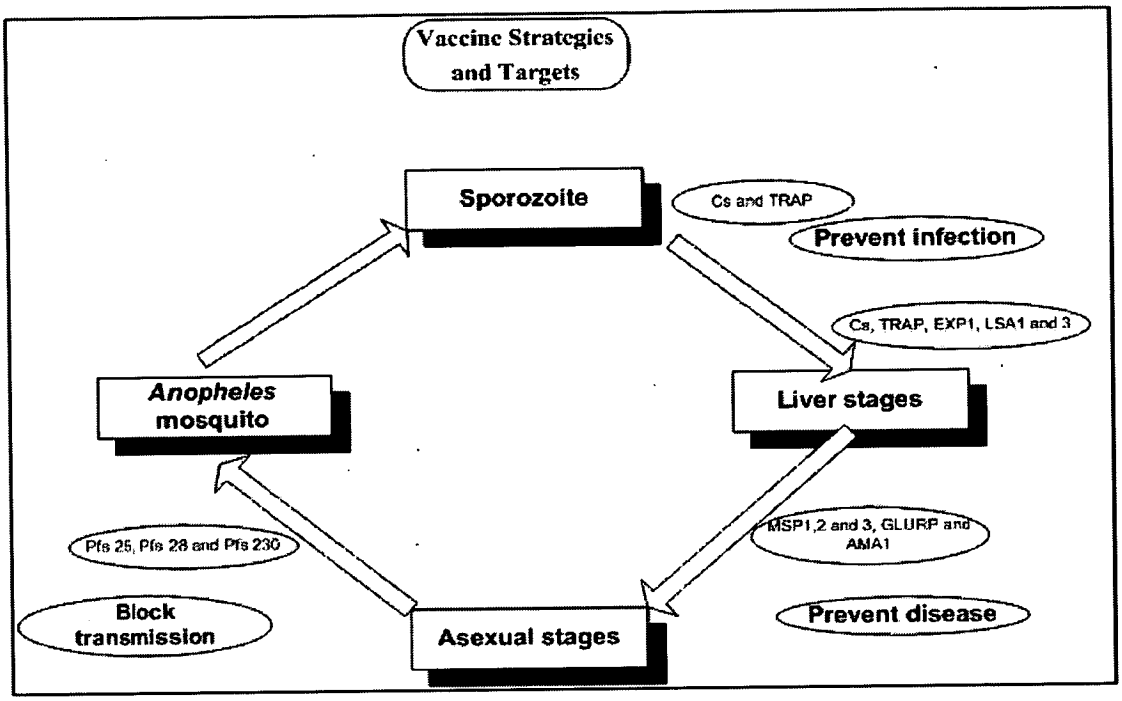


Figure 1.5: Malaria life cycle and long established vaccine targets. CS = circumsporozoite, TRAP = thrombospondin-related adhesive protein, EXP-1 = exported antigen 1, LSA1 = liver stage antigen 1, MSP-1 = merozoite stage protein 1, GLURP = glutamate-rich protein, AMA-1 = apical membrane antigen 1.

1.13.1 The pre-erythrocytic stage malaria vaccines.

An ideal malaria vaccine would induce high titres of functional antibodies against sporozoites to prevent parasites entering the liver stage and induce potent cytotoxic T -lymphocyte immunogenicity to kill infected hepatocytes. The circumsporozoite protein (CSP) is the predominant antigen on the surface of sporozoites, and many vaccine constructs including CSP are in various stages of studies and trials in Africa, the USA, and Europe (Milich, 2001, Moorthy & Hill, 2002, Nardin *et al,* 2004, Oliveira *et al,* 2005). However the CSP-based malaria vaccine for which clinical development is most advanced is the well-known candidate RTSS/AS02A.

RTSS/AS02A is a hybrid recombinant polypeptide produced by *S. cerevisiae* and consists of two components: RTS is a polypeptide chain consisting of part (2/3) of the circumsporozoite protein fused to HBsAg and S is the hepatitis B surface antigen

polypeptide alone. RTS,S with adjuvant SBAS02 confers sterile immunity in approximately 50% of volunteers when administered on a two- or three-dose schedule. (Kester *et al,* 2001, Alonso *et al,* 2004). The adjuvant, SBAS2, is an oil-in-water emulsion of *Salmonella enterica* cell wall-derived monophoryl lipid A and a saponin-type detergent adjuvant (QS-21). This adjuvant is essential for the protective effect seen with this vaccine (Lalvani *et al,* 1999). A variation on this type of CS/virus like particle vaccine is the ICC-1132 CS/hepatitis B core particle. It contains B cell epitopes and a universal T cell epitope in combination with the highly immunogenic hepatitis B core antigen (HBcAg) as a delivery vector. It has been shown that antibodies neutralize these epitopes and appears to be safe and well tolerated (Milich, 2001).

Other synthetic peptide CS-based vaccines include the Pf CS 282–383 long synthetic peptide vaccine, shown to elicit $CD8^+$ and $CD4^+$ T lymphocyte responses in humans. This vaccine was well tolerated and strong sporozoite-specific antibodies were elicited in a Phase 1 clinical trial (Lopez *et al,* 2001). Among other pre-erythrocytic stage candidates are the thrombospondin-related adhesive protein (TRAP, also known as SSP2) and several liver stage antigens expressed on the hepatocytes containing exo-erythrocytic schizonts, including LSA-1, LSA-3, and exported antigen 1 (EXP-1). LSA-3 is a conserved 200-kilodalton protein. In two chimpanzees (*Pan troglodytes*) immunization with LSA-3 induced protection against successive heterologous challenges with large numbers of *P. falciparum* sporozoites. (Daubersies *et al,* 2000).

1.13.2 Asexual or blood stage vaccines.

Parasites antigens are expressed on the surface of infected erythrocytes and also on free merozoites. These antigens are being studied in many of the current attempts to manufacture malaria vaccines. The goal of anti-erythrocytic vaccines is to dramatically improve upon naturally acquired immunity. Such vaccines would be designed for the community to reduce the total burden of the disease without necessarily eliminating the infection.

A Merozoite vaccine candidates.

The majority of candidate blood stage vaccines in advanced development and early stage clinical trials are proteins identified as being on, or closely associated with, the surface of the merozoites. The most advanced production prototypes are merozoite surface protein 1 (MSP-1) (C-terminal domains MSP-1_{19} or MSP-1_{42}) (Angov *et al,* 2003) and apical membrane antigen 1 (AMA-1). MSP-1 has been produced as C-terminal lyophilized recombinant antigen expressed in *E. coli,* consisting of the 42-kD C-terminal end combined with the adjuvant AS02A. It has been found to be safe and immunogenic in mice, rabbits, and rhesus macaques and induced antibodies as measured by ELISA and against native parasite MSP-1 on infected red blood cells by IFA assay and invasion inhibition assay (Angov *et al,* 2003, Polley *et al,* 2005). Vaccine candidates based on the smaller 19-kDa C-terminal fragment of MSP-1 are also being developed independently as candidate vaccines (Garraud *et al,* 1999).

The apical membrane protein 1 molecule of *P. falciparum* (PfAMA-1) is synthesized as an 83-kDa precursor, from which an N-terminal pro-domain is cleaved. The mature 66-kDa form of PfAMA-1 is found in merozoite micronemes. Further processing results in the shedding of fragments of 44 or 48 kDa and occurs in association with the relocation of the molecule to the merozoite surface (Narum *et al,* 1994). Recombinant AMA-1 has been shown to protect animal models (rodents and primates) against parasite challenge and this protection appears to be antibody-dependent (Anders *et al,* 1998). Both rabbit and human anti-AMA1 antibodies were found to be strongly inhibitory to the invasion of erythrocytes by merozoites (Saul *et al,* 2005). *P. falciparum* AMA-1, produced in *P. pastoris,* has also been tested in challenge experiments by vaccinating *Aotus vociferans* monkeys and then challenging them with *P. falciparum* parasites. Significant protection from an otherwise lethal challenge with *P. falciparum* was observed and the protection induced by AMA1 was superior to that obtained with a form of MSP1 used in the same trial. However the protection induced by a combination vaccine of AMA1 and MSP1 was not superior to the protection obtained with AMA1 alone, although the immunity (i.e. antibody responses) generated appeared to operate against both vaccine components (Stowers *et al,* 2002). While AMA-1 is an attractive candidate

antigen, there is considerable antigenic polymorphism in this molecule. This could affect the degree of protection afforded by a monomorphic variant.

Other merozoite proteins (e.g. MSPs 2-7) are at an earlier stage of development although MSP-2 has been used in two Phase I trials in test of the immunogenicity and safety of a vaccine containing three recombinant malaria antigens (fragments from MSP-1, MSP-2 and RESA) from the asexual stage of *P. falciparum*. Human antibody responses observed with MSP2 in Montanide ISA720 were similar those obtained in an earlier trial which used MSP2 with alum as the adjuvant (Saul *et al,* 1999). Merozoite surface protein 3 (MSP-3) is a vaccine candidate antigen of molecular mass of 48 KDa. Purified anti-MSP-3 IgG from naturally immune adults subjects have been shown to be capable of inducing antibody-dependent cellular inhibition reaction (ADCI) in an assay that mimics cooperation between monocytes and cytophilic parasite-specific antibodies (Oeuvray *et al,* 1994). Glutamate-rich protein (GLURP) derived long synthetic peptides have also been identified as targets of human antibodies that are potentialy effective in ADCI. It has been proposed that GLURP may also play a role in the induction of protective immunity against *P. falciparum* malaria. (Oeuvray *et al,* 2000).

The rhoptry-associated protein 1 and 2 (RAP1/RAP2) are located in the rhoptry internal organelles of merozoites. It has been reported that the anti-RAP1 mAb are able to inhibit merozoite invasion *in vitro*. Additionally, successful immunization with parasite-derived or recombinant RAP1 and RAP2 of *P. falciparum* in monkeys has been demonstrated (Collins *et al,* 2000). Another vaccine candidate antigen, the ring-infected erythrocyte surface antigen (RESA) is present in the apical region of merozoites. This antigen is released into the parasitophorous vacuole during merozoite invasion and translocated to the inner surface of the RBC membrane. Ring-infected erythrocyte surface antigen elicits both humoral and cellular immune responses in *P. falciparum*-primed donors (Wipasa *et al,* 2002).

Most vaccine clinical trials during the 1990s were carried out with the chemically synthesized multi-epitope peptide vaccine SPf66, by the Pattaroyo group in Colombia (Moreno & Pattarroyo, 1989). This vaccine is a multi-antigen multi-stage vaccine comprising chiefly of peptides 35.1, 55.1 and 83.1. Peptide 83.1 corresponds

to the N-terminal sequences of MSA-1 while peptides 35.1 and 55.1 are partial sequences from unidentified blood stage proteins (Facer & Tanner, 1997). The construct also includes two linking NANP sequences from the circumsporozoite protein. Although there have been numerous controversies about this vaccine (e.g. see Herrera *et al,* 1990, Acosta *et al,* 1999, Schellenberg *et al,* 1999) using very large numbers of Amazonian monkeys, the Pattaroyo group claimed to have been able to identify a multi-epitope synthetic vaccine that initially showed great promise in *Aotus* monkey challenge experiments and in early human clinical trials in South America (Perlaza *et al,* 1998). However further field trials in Africa and S.E Asia failed to repeat this early success and this candidate has now been largely dropped from the global vaccine development effort.

B Malaria vaccines based on infected erythrocyte surface proteins.

In addition to antigens on the surface of merozoites, parasite proteins exposed on the surface of infected RBC are regarded as potential vaccine candidates. Antibodies can be directed against such exposed antigens to enhance opsonization, complement mediated killing and to block the infected erythrocytes from adhering to the lining of blood vessels. A potentially serious obstacle to malaria vaccine development is the fact that many antigenic proteins, and particularly those present on the surface of the parasitized red blood cell, vary between different individuals of the *Plasmodium* population. As mentioned above, malaria parasite populations are not identical clones but are genetically diverse and antigenically polymorphic.

Furthermore, individual malaria parasites can switch their production of infected red blood cell surface protein antigens in order to evade the host's antibody-mediated immune response against these vulnerable blood-stage surface-exposed molecules. Thus each member of the genetically diverse and antigenically polymorphic population has the additional capacity for individual immune system evasion by clonal antigenic variation. *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) is the major family of variant surface proteins expressed on the surface of infected RBC. PfEMP1 proteins contain a conserved head structure composed of 300-400 amino acid long Duffy binding like domains (DBL domains), named after

their homology to the *Plasmodium vivax* merozoite protein that binds to the Duffy antigen on red blood cells).

DBL domains are often separated by the so-called cysteine residue rich inter-domain regions (CIDR domains). The CIDR domains are also known to be responsible for binding to several host receptors and have been used as the immunization antigen in *Aotus* monkey challenge experiments with *P. falciparum.* After immunization with a particular CIDR1 domain (Malayan camp isolate), the monkeys controlled the primary infection (Baruch *et al,* 2002a). It has also been found that immunization with PfEMP1-DBL1 domains can generate antibodies that will disrupt the 'rosettes' formed by the binding of uninfected red blood cells to infected erythrocytes. Antibodies against PfEMP-1 have been generated in a variety of ways including immunization with hybrid PfEMP-1 Semliki Forest Virus like particles RNA particles and boosting with recombinant PfEMP-1 protein (Chen *et al,* 2004) and using recombinant DBL-fusion proteins expressed in *E. coli* to generate antibodies in rats. Five out of ten recombinant DBLα fusion proteins were immunogenic and induced antibodies that reacted with conserved peptides derived from PfEMP1 (Oguariri *et al,* 2003).

1.13.3 Anti-toxin vaccines.

A number of the parasite antigens expressed on the surface of the infected red blood cell are linked to the surface through a glycophosphatidyl inositol (GPI) anchor. The concept of an antitoxic vaccine is based on the observation that individuals who are frequently exposed to malaria suffer few symptoms, although they remain susceptible to infection. An effective GPI vaccine may prevent or reduce the severity of disease. Proof of principle experiments carried out in rodent models indicate that the observed levels of morbidity and mortality are greatly reduced in mice immunised with GPI preparations (Schofield *et al,* 2002). The role of GPIs in the establishment of malaria in the human host remains to be unraveled. People living in malaria endemic areas express naturally elicited anti-GPI Ab. Sera from adults who are resistant to clinical malaria contain high levels of persistent anti-GPI Ab, whereas susceptible children lack or have low levels of short-lived Ab. Anti-GPI

Ab responses are correlated with protection against malaria-related febrile illness and haemoglobin loss (Naik *et al,* 2000).

1.13.4 Transmission Blocking Vaccines.

Transmission-blocking vaccines aim to stimulate the production of human antibodies to molecules present on the surface of the sexual stage gametes. When the gametocytes are ingested by the mosquito, antibodies in the blood meal will have access to the released gametes in the mosquito mid-gut and inhibit the development of the parasite through the mosquito phase of the life cycle thus halting transmission. Transmission blocking vaccines on their own, if developed, will not confer protection against the development of the disease in at risk populations and will need wide-spread and long periods of sustained vaccination in order to significantly cut down on transmission levels (Ballou *et al,* 2004). Pfs25, a 25-kDa surface antigen of zygotes and ookinetes and Pfs28, a 28-kDa surface antigen of late ookinetes, are two of the lead vaccine candidates. Both comprise four tandem epidermal growth factors (EGF)-like domains that anchored to the parasite surface by glycosylphosphatidylinositol. Produced as recombinant proteins in yeast, both of these antigens have been shown to induce transmission-blocking antibodies in experimental laboratory animals (Barr *et al,* 1991).

Two vaccine candidates, Pfs25 and Pfs28, were produced as single recombinant fusion proteins. The 39-kDa chimeric proteins, having a C-terminal His6 tag, were secreted by *S. cerevisiae,* using the prepro-factor leader sequence. Pfs25-28 fusion proteins were more potent than either Pfs25 or Pfs28 alone in eliciting antibodies in mice that blocked oocyst development in *Anopheles* mosquitoes. Complete inhibition of oocyst development in the mosquito midgut was achieved with fewer vaccinations, at a lower dose, and for a longer duration than with either Pfs25 or Pfs28 alone (Gozar *et al,* 1998).

Pfs25 is a dominant protein on the surface of *P. falciparum* zygotes and a transmission blocking vaccine based on this is currently in clinical trials. Pfs25 was discovered by a monoclonal antibody which when fed along with a parasitized blood meal to a mosquito resulted in a blockage in the infectivity of that mosquito. A number of Pfs25 - like compounds have also been discovered in other species of

Plasmodium. Their structure all shares four tandem EGF-like domains (Duffy & Kaslow, 1997). Duffy and his co-workers discovered Pfs28, a similar but immunologically distinct antigen to Pfs25. Pfs230 another sexual-stage *P. falciparum* surface antigen can also elicit antibodies which block the infectivity of gametes to mosquitoes (Duffy & Kaslow, 1997).

1.14 Thesis aims and methodology.

To obtain antigens in a suitable state for initiating vaccine prototype development it is necessary to scale up production in optimal expression systems. The recombinant proteins needtobe correctly folded and which induce appropriate antibody responses. The overall aim of this study is to find faster and more effective ways of identifying and developing vaccine candidate antigens that can be used to elicit immunity to the erythrocytic stage in the human host and produce biologically active proteins in quantities that can be easily scaled up for vaccine test batch production. Methods chosen for analysis were based upon the yeast expression system *P. pastoris* and *E. coli.* A specific aim of this study was to develop a fast and efficient way of expressing *P. falciparum* antigenic epitopes as conformationally conserved antigenic structures whose expression can be readily 'scaled-up' for clinical testing. The *E. coli* and *Pichia pastoris* systems were used both singly and as linked production systems using 'shuttle vectors' that can be dually expressed in both microorganisms.

Initially a proof-of-principle type experiment to express and purify in good quantity the recodoned CIDR1 domain of PfEMP1 of 3D7 parasite in *P. pastoris* (strain X33) was conducted. The plasmid pPICZαA was used as the expression vector. The recombinant protein was used to immunize rabbits. The Polyclonal rabbit serum was assessed using immuno-blotting and immuno-fluorescence assays.

The sequenced 3D7 *P. falciparum* genome was exploited to construct degenerate primers for several domains of PfEMP1 (namely DBLα, DBLβ and DBLγ). These primers were used to amplify targets from FCR3CSA parasites. PCR products were ligated into the dual expression pPICHOLI1 vector. Six different *P. falciparum* gDNA DBL expression libraries were successfully produced in *E.coli* and in *P. pastoris.* Experiments were conducted to optimise the growth, lysis and immunoscreening of colonies on filters on agar plates for high-throughput screening.

Different domains from a gene of particular interest, the *var*2CSA gene were also amplified for expression and screening studies. These recombinant proteins were screened to investigate differential recognition by pools of serum from selected

groups living in malaria endemic zones, notably adult males and females who had experienced malaria during pregnancy.

Chapter 2. Heterologous expression of PfEMP-1 fragments in *Pichia pastoris.*

2.1. Aims.

The aim of this study was to investigate the utility of a heterologous expression system, the yeast *P. pastoris*, for expression of one of the major structural domains of the modular and variant protein PfEMP1. A Cysteine-Rich Interdomain Region (CIDR) domain of PfEMP1 was chosen as the first domain-type structure to be expressed. The reasons underlying this were that the CIDR region has been reported to encode a particular binding property, particularly that of binding to CD36 (Baruch *et al,* 1995, Newbold *et al,* 1997). Immunization of *Aotus* monkeys with the 'M2 region', a conserved motif of CIDR1 has elicited high titre anti-sera that were found to be primarily variant-specific. (Baruch *et al,* 2003, Miller *et al,* 2002). There are also reports that some variants of this region can mediate binding to CSA on the surface CHO cells (Degen *et al,* 2000). The sequences of the CIDR1 domain have thus been considered to be potential vaccine candidates – a part of the so-called 'conserved head structure' of PfEMP-1 molecules (Gamain *et al,* 2001).

The CIDR1 domain used in this study was derived from the 3D7 strain of *P. falciparum.* The PfEMP1 *var* gene containing this particular CIDR1 was classified as a type 5 *var* gene organised into DBLα, CIDRα, DBLβ, C2, DBLγ and CIDR-nonα domains (Gardener *et al,* 2002) (Figure 2.1). A similar but somewhat differently organised classification scheme has been proposed by other workers (Lavstsen *et al,* 2003). Previously, the CIDR was re-codoned in the laboratory (T. Fagin and P. Bruce) to match the codon usage of *P. pastoris* and cloned into the pPICZαA expression vector (Figure 2.2). The objective was to explore whether the *Pichia* system can be used to produce large quantities of correctly folded recombinant CIDR1. Such conformationally correct antigens are needed in order to raise specific antibodies to this antigen and to develop assays to assess its immunogenicity. The choice of this domain was basically due to its ability fro binding to CD36 receptors, and if we managed of express this domain, we will be able to carry out host /parasite adhesion inhibition assays. Later on, it was found that the gene contains this domain was categorised in the group A (comprises telomeric genes transcribed towards the

telomere encoding PfEMP1s with complex domain structures) that consists of ten genes consistently identified as a distinct group by sequence analysis of Sequence of *var* 5' regions (Lavstsen *et al,* 2003). Interestingly, recombinant CIDR domains based on the group A sequences do not bind CD36, by contrast to CIDR domains produced on the basis of groups B and C. Group A *var* genes mainly encode large PfEMP1s with complex multi-domain structure (Lavstsen *et al,* 2003).

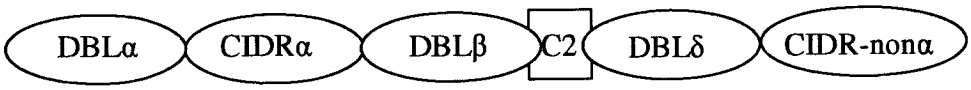


Figure 2.1. PfEMP1 protein architecture of type 5 var genes that includes the CIDR1α investigated in this study (Gardner *et al*, 2002).

2.2. A+T richness, codon usage bias and re-codonisation.

Different organisms often show strong preference for one of the several codons that encode the same amino acid in the 'degenerate' genetic code. Why such preferences arise is debated but in practical biotechnological terms, if the tRNA pool sizes do not correspond to the preferred codon usage, translation will not be optimal. Expression in heterologous systems is usually up-regulated and driven by a strong promoter and this increases the likelihood of 'draining the tRNA pool' for uncommon tRNAs. This could lead to premature chain termination and affect protein folding as the polypeptide chain is progressively synthesized by the ribosome moving along the mRNA. In *S. cerevisiae* and *P. pastoris,* it is known that adenosine rich stretches of sequence, prevalent in *P.falciparum* genes, can act as polyadenylation and/or transcription termination signals, resulting in low-level gene expression or truncated mRNA (Sreekrishna *et al,* 1997, Sorensen *et al,* 1989, Pizzi & Frontali 2001). The *P. falciparum* genome is one of the most A/T rich of any genome known (typically 80% A/T rich genes and up to 85% A+T in its intragenic regions). In general, taxonomically close organisms use similar codons for their protein synthesis whereas taxonomically distant organisms tend to utilize different codons (Ikemura, 1982). All heterologous expression systems thus experimented with, apart from *Dictyostelium* (Howard *et al,* 1988) and *Tetrahymena* (Peterson *et*

al, 2002), have more G+C rich codon-usage than *P. falciparum*. Notably, the entire CS protein of *P. falciparum* has been expressed and localized to the cell surface in the free-living ciliate *Tetrahymena thermophila*. (Peterson *et al*, 2002). The codon bias problem, as it affects *E. coli* expression, has been addressed by Stratagene who have engineered novel bacterial expression lines (BL21-CodonPlus® RPIL line). This *E.coli* strain contains a plasmid encoding extra copies of tRNA that recognize rare *E. coli* codons. This has had success in allowing over-expression of A+T codon rich *Plasmodium* genes (Makoff *et al*, 1989, 1994). However this has not removed the need to synthesise a codon-optimized version of the sequence of interest (Yadava & Ockenhouse, 2003).

2.3. *Pichia pastoris* expression system.

P. pastoris (Guillermand) Phaff 1956; is a methylotrophic budding yeast (Phylum: *Ascomycota*, Class: *Hemiacomycetes*, Order: *Saccharomycetules*, Family: *Saccharomycetaceae*). It is considered as non-conventional yeast, compared with the conventional baker's yeast *S. cerevisiae* (Cereghino & Cregg, 1999). This system has a number of advantages for protein expression. *Pichia* can be grown to higher densities than *S. cerevisiae* and it has the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation, and proteolytic processing. The post-translational modifications made by *P. pastoris* may be more suitable for protein production for use in humans. It is specialised for growth on methanol as a sole carbon and energy source, and has an extremely powerful regulated promoter for alcohol oxidase. Alcohol oxidase expression is induced by methanol and when fully induced this enzyme can comprise 30% of total cell protein. The system is relatively easy to scale up for bulk production, particularly if the product can be secreted by the yeast cell (Cregg *et al*, 1993, 2000, Sreekrishna, 1997, Bretthauer & Castellino, 1999, Cereghino & Cregg, 1999, 2000). However, whether *P.pastoris* is the system of choice for *P. falciparum* gene expression remains to be established. The *Pichia* expression vector pPICZαA has been designed for heterologous expression and secretion of recombinant proteins. This expression vector carries a C-terminal tag sequence to allow purification and detection of fusion proteins. The 'tag' consists of six histidine residues to allow purification on nickel

affinity resins and the c-myc epitope sequence for rapid detection by antibodies. Other features of this plasmid include; an inducible AOX1 promoter, α-factor secretion signal for transport/secretion of proteins and the zeocin resistance gene for selection in *E. coli* and yeast. After initial ligation of an insert into the plasmid vector, the construct is amplified in and then extracted from *E.coli* cells of the XL-10 Gold type (Stratagene). PCR-screening was used to confirm the presence of inserts, using primers hybridising to sequences in the yeast alcohol oxidase gene (*AOX3`* and *AOX5`)*. The structure of this vector is shown in Figure 2.2.

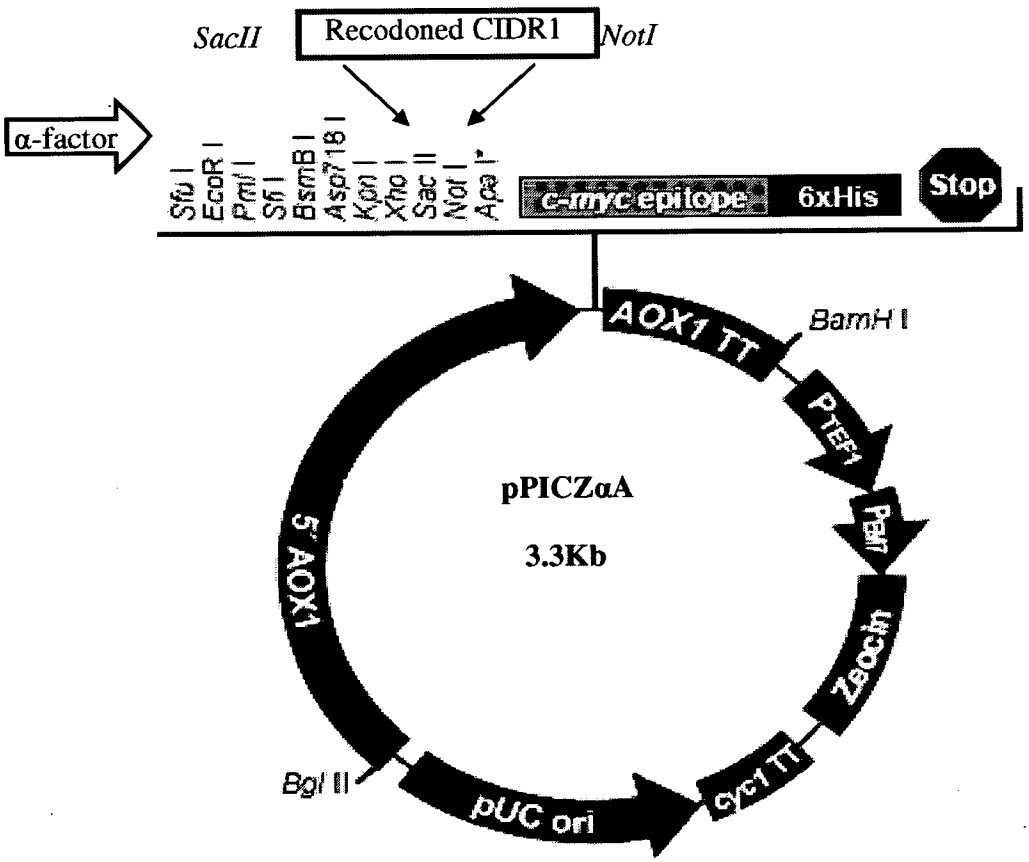


Figure 2.2. The pPICZαA construct used for expression of the secreted version of the synthetic CIDR1. pPICZαA makes use of the AOX1 promoter and it has α-factor signal sequence for secretion of the expressed protein. It carries Zeocin resistance for selection of both yeast and *E.coli* in antibiotic media.

2.4. A Codon-Optimised Version of CIDR1.

A synthetic CIDR1 gene was designed based on (*P. falciparum* 3D7 *var* gene located on chromosome no.6, acc.No. 103738432) to decrease the A/T richness of the sequence and replace rare codons with more commonly used codons in methylotrophic yeast systems. Potential N-glycosylation sites were also mutated as *P. falciparum* lacks the N-glycosylation(Diechmann-Schuppert *et al,* 1992) . Re-synthesis also involved removal of restriction sites that may interfere with downstream cloning techniques. Predicted N-glycoslyation sites (N- X- S/T- N) in the amino acid sequence were identified using the web-based program 'NetNGlyc' at http://www.cbs.dtu.dk/services/NetNGlyc/. To modify these sites, the serine (S) or threonine (T) in the motif was replaced with an alanine (A), as it is the smallest amino acid, predicted to have the minimum effect on secondary structure. It was considered appropriate to remove the N-glycosylation sites as *P. pastoris* is known to glycosylate (and sometimes hyper-glycosylate) at asparagine residues and this can interfere with correct folding and tertiary structure of the protein (Bretthauer & Castellino 1999).

Zeocin resistant clones were sequenced to confirm they were in-frame with the 5' α-factor signal sequence and 3` tag sequences. Resulting recombinant plasmids were linearised by *sal 1* before transfection by electroporation. The resulting recombinant insert was sequenced. The native and recodoned sequences are compared in Figure 2.3(See Appendix 1 for the amino acids alignment).

```
CIDR1-native          ---------------GAAAAACAAAAAATTATGCCCTTTGATGCATTTTTTTTCCTTTGG
CIDR1-Recodoned       CCGCTCGAGAAAAGAGAGAAGCAGAAGATCATGCCATTCGACGCCTTCTTTTCCTGTGG
                                    ** ** ** ** ** ***** ** ** ** ** ******* ***

CIDR1-native          CTAACACAAATGTTAGATGATTCTATAGAATGGAGAAAAAAACTTAAAACCTGTATAAAT
CIDR1-Recodoned       CTGACTCAGATGCTGGACGACTCCATCGAGTGGCGTAAGAAGCTTAAGACCTGCATCAAC
                      ** ** ** *** * ** ** ** ** ** *** * ** ** ***** ***** ** **

CIDR1-native          AATGAAAAACCAACTAATTFTATACGGGGTTGTAAAAAGCCCTGCGAATGTTTTGAAAGA
CIDR1-Recodoned       AACGAGAAGCCAACCAACTGTATCCGTGGTTGTAAGAAGCCTTGCGAGTGTTTCGAGAGA
                      ** ** ** ***** ** * *** ** ******** ***** ***** ***** ** ***

CIDR1-native          TGGGTTGAACAAAAAGAAGAAGAATGGATTTCAATTGAAAAACATTTTGACAAACAAAGA
CIDR1-Recodoned       TGGGTTGAACAGAAGGAGCAGGAGTGGATCTCCATTGAGAAGCACTTTGACAAGCAGAGA
                      ********** ** **  * ** ***** ** ***** ** ** ******** ** ***

CIDR1-native          GATATATCAGAAGAAGAACGTTATATAACACTTGAATATATTTTGAATGAATTTTTTATG
CIDR1-Recodoned       GACATCTCCGAGGAAGAGCGTTACATCACCCTGGAGTACATCCTGAACGAGTTCTTCATC
                      ** ** ** ** ***** ***** ** ** ** ** ** **  **** ** ** ** **

CIDR1-native          GATAAAATTGAAAAAGCTTATGGAATAGAAAAATCAAAAGAATTAAAGGAGAAATTAAAA
CIDR1-Recodoned       GACAAGATCGAGAAGGCTTACGGAATTGAGAAGTCCAAGGAGTTGAAGGAGAAGCTGAAG
                      ** ** ** ** ** ***** ***** ** ** ** ** ** ** ********  * **

CIDR1-native          TCAAATAAAGGTCACGGAATTATAAGAGATACAGAACATTCACAGGATGCAATAAAAATA
CIDR1-Recodoned       TCCAACAAGGGTCACGGAATCATCCGTGACACTGAACACTCCCAAGACGCCATCAAGATC
                      ** ** ** ********** **  * ** ** ***** ** ** ** ** ** ** **

CIDR1-native          TTGCTAGAACATGAATTAGAAGATGCAAAAAAATGCACAGAAACCCATAATGATGAAAAA
CIDR1-Recodoned       TTGCTGGAGCACGAATTGGAGGACGCTAAGAAGTGCACCGAAACCCACAACGACGAGAAG
                      **** * ** ** ***** ** ** ** ** ** ***** ******** ** ** ** **

CIDR1-native          TGTAAAGAACAAGAAGGAGAGCGGAGGTCGCTCTTTAAACCCGGATCCAGAATCCGACGA
CIDR1-Recodoned       TGTAAGGAGCAGGAGGGAGTCCGGTGGTAGATCCCTGAACCCTGATCCAGAATCCGACGA
                      ***** ** ** ** ****  *** *** * **  * ***** ****************

CIDR1-native          CGAAGAAGAAACGGATAATGTAAAAGAGAACCCGTGTGCTGTAGGGAAAAAACTCACTAA
CIDR1-Recodoned       CGAAGAGGAGACTGACAACGTCAAGGAAAACCCATGTGCTGTCGGAAAGAAGCTGACCAA
                      ****** ** ** ** ** ** ** ** ** ***** ******** ** ** ** ** ** **

CIDR1-native          AACTGTGAAACAAATCGCTAGACAGATGCATCAAGCGGCAAAGAAACAATTGGGTAGTAG
CIDR1-Recodoned       GACCGTCAAGCAAATCGCTAGACAAATGCACCAAGCTGCCAAGAAGCAGTTGGGTTCCTC
                       ** ** ** ************** ***** ***** ** ***** ** ******

CIDR1-native          TAGTAGTAGGGCATTGAAGGCTCAT----------------------------
CIDR1-Recodoned       TTCCTCTAGAGCCTTGAAGGCTCACCACCATCACCATCATTAAGCGGCCGCCGC
                      *      *** ** ***********
```

Figure 2.3. Nucleotide Sequence alignment of native and codon-optimised CIDR1. * indicates unchanged nucleotides while gaps indicate where substitutions have been made. The A+T content was reduced from 67.6% to 48.6%.

2.5. Induction and immunodetection experiments.

Small-scale and mid-scale expression of CIDR1 in *P. pastoris* resulted in secretion of protein of the expected size showing little degradation (Figure 2.4). To investigate

whether the construct CIDR1-pPICZαA is secreting the altered CIDR1 protein in large quantities, small-scale induction experiments were used to screen selected drug resistant clones for secretion/ production. These cultures were grown for 2 days at 30°C in 10 ml of BMGY in 50-ml Falcon tubes with vigorous shaking. Cells were harvested by low-speed centrifugation, resuspended in 100 ml of BMMY, and cultured for an additional 3 days. Cells were harvested and culture supernatants analyzed for the presence of secreted CIDR1 on a 15% SDS gel. Gels were stained with Coomassie Brilliant Blue to visualise the protein bands (Figure 2.4).

The hexa-histidine tagged protein was detected using an antibody specific for the histidine tag sequence in the expression vector. The positive control used in Western blot analysis of the *Pichia* derived recombinant protein was a His–tagged protein, serpin, a ~55KD *Brugia malayi* serine protease inhibitor expressed in *E. coli*. When the induction was scaled up, a secreted CIDR1 band of the expected molecular weight, 28 KDa was detected by Western blotting. For mid-scale production of CIDR1, recombinant *P. pastoris* was initially cultured in 2-liter baffled flasks (1000 ml of BMGY per flask) for 48 h at 30°C with vigorous shaking (300rpm). Cells were harvested and the pellet from this one litre culture was re-suspended in 120mls of BMMY in a one litre baffled flask then incubated for 72 hours at 30°C with vigorous shaking (300rpm). Methanol was added to a final concentration of 0.5% every 24 hours (although continuous infusion of methanol is preferable to facilitate constant induction by the alcohol oxidase promoter). Culture supernatant samples (15microlitres) were incubated in an equal volume of loading buffer, heated for 10 minutes at 95°C and run on two SDS gels. Following electrophoresis, one gel was stained and the other was transferred to a PVDF membrane using semi wet method for immuno-detection. After overnight blocking in 5% non-fat milk/PBS, membranes were probed with His-tag-HRP antibody (Qiagen). The blot was visualised using an ECL plus detection kit (Amersham Biosciences) (Figure 2.4).

Unfortunately attempts to obtain large quantities (~10 mg/L) of the protein in basic laboratory cultures and shakers failed, probably because of the lack of control of optimal levels of key metabolites such as methanol. Therefore attempts were made to scale up production using the more controlled conditions available with more

sophisticated fermentation technology, available to us at the Edinburgh University in ICMB.

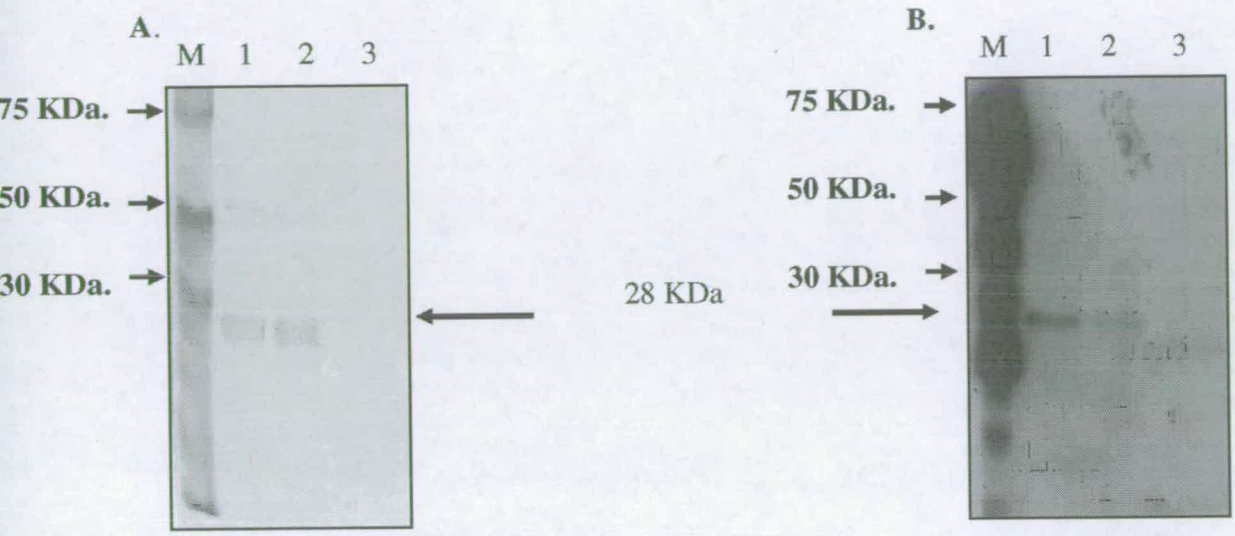


Figure 2.4. Analysis of Pichia supernatants from small-scale cultures. A. Coomassie blue stained polyacrylamide gel. **M.** Molecular weight marker. **1.** *P. pastoris* culture supernatant, 24 hours after methanol induction. **2.** Culture supernatant after 48 hours of induction. **3.** Culture supernatant prior to induction. **B.** Duplicate gel analysed by Western blot using the anti- His tag antibody.

2.6. *Pichia* expression using a fermentor.

The fermentation run consisted of the following stages:

- Glycerol Batch Phase – 20 hours.
- Glycerol Feed Batch phase – 2 hours.
- Starvation phase – 1 hour.
- Methanol Feed Batch – 70 hours.
- Harvest.

Expression was optimized for production using a New Brunswick BioFlo5000 fermentor. One millilitre of frozen glycerol culture (50 mls of the culture obtained after 24 hours of growth of a single colony in YPD plus zeocin, made 80% with glycerol and then stored in aliquots) was used to inoculate 600 ml of BMGY medium

and grown until the culture reached an optical density of 10 at 600 nm. The fermentor, containing 9 litres of bulk fermentation medium was then inoculated with this seed preparation and grown until the glycerol carbon source in the medium is completely consumed (20 hours). The culture is then supplemented with 50% glycerol in the 'glycerol feed phase'. The culture consumes this glycerol in about after 2 hours and a further 1 hour of carbon source starvation is carried out before the production phase of the culture starts. The production phase is started by the addition of 12ml/litre of PTM (a trace salt medium) dissolved in methanol, the alcohol oxidase inducer and carbon source, for three hours. Then after the initial addition a constant feed inoculation was set at 7.3 mls/litre/hour for 2 hours. The methanol feed rate was changed to 11 ml/l/h for 34 hours (giving a total run length of 65 hours). The pH was controlled with 14% ammonium hydroxide. Other fermentation parameters were as follows: the dissolved-oxygen level was maintained by airflow of 90 litres per minute, the air supplemented with oxygen. The pH was maintained at 5.0 and the temperature at 30°C. The culture was subjected to an agitation cascade from 360 to 550 rpm over the 65 hours of the run. Controlled fermentation is the most effective way for the recombinant protein to be expressed in the *Pichia* system because the parameters for maximally effective expression can be met. These include control of the temperature, dissolved oxygen, constant pH, agitation and aeration and methanol concentration. A time dependent product (CIDR1 recombinant) of the expected MW (~28kDa) was consistently increasing in quantity after 24 hours (Figure 2.5).

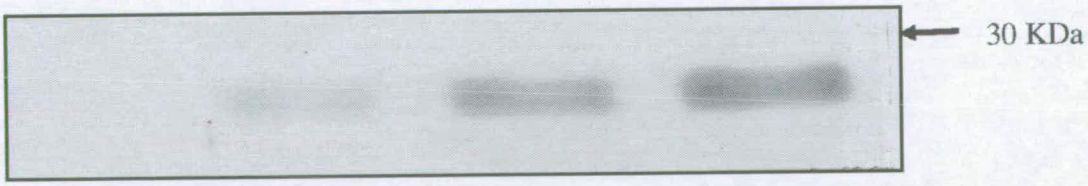


Figure 2.5. Analysis of supernatants from a CIDR1α-pPICZαA-transformed Pichia after fermentor expression. Supernatants were concentrated by centrifugation using a Vivaspin 6 (5,000 MW cut-off), reduced and separated by SDS-PAGE on 4-12% polyacrylamide gels. Proteins were stained using Coomassie blue. Time-dependent expression of a protein of 28kDa is observed; most clearly at 72 hours post induction. MW markers are indicated in kDa.

2.7. Purification of His-Tagged Protein.

After bulk fermentation runs the supernatant was precipitated with ammonium sulfate, and the proteinaceous pellet re-suspended in PBS and dialysied. A 1 ml Ni-NTA Hi Trap chelating column and then a gel filtration column were used for the purification of the recombinant CIDR1. The column was pre-washed with H_2O, loaded with 100mM $NiSO_4$ and then washed again with H_2O. Prior to loading onto the column, the protein sample was dialysed against the column binding buffer (20 mM sodium phosphate, 0.5M NaCl, pH 7.4). The column was then equilibrated and washed with binding buffer. The dialysed protein intended for column purification was spun at 1000g for 30 minutes at room temperature to remove debris.

The cleared supernatant was loaded on the column at a flow rate of 1.2-1.5 ml/min nickel resin-bound; His tagged protein was eluted with 20 mM sodium phosphate, 0.5M NaCl, 0.5M imidazole pH 7.4. Samples were then analysed by SDS-PAGE and quantified by reading the absorbance at 280nm of the fractions after the BioRad DC protein assay had been carried out. Those samples with the greatest concentration of protein were pooled and the elution buffer exchanged by dialysis against PBS. Using SDS-PAGE to analyse the purified product, a dense band of the expected size was detected in addition to a very faint band ($\approx$ 13KDa). The smaller band, of unknown origin, can be eliminated by passage over a gel filtration column (Sephadex G-50). For the gel filtration, the matrix was pre-swollen in 1M Tris, 20 mM NaCl. The PBS dissolved sample was loaded onto the colum and then washed through in the Tris/NaCl buffer. The leading fractions collected contain the larger molecular weight proteins. Figure 2.6 shows the quality of this product.

The results show that the majority of the gel filtered sample is monomeric (80%) with about the expected size (30 KDa). Estimated protein yields were 50 mg/litre. These results confirm our expectation that the controlled fermentation is a much more effective method for the expression of the recombinant protein than induction in flasks.

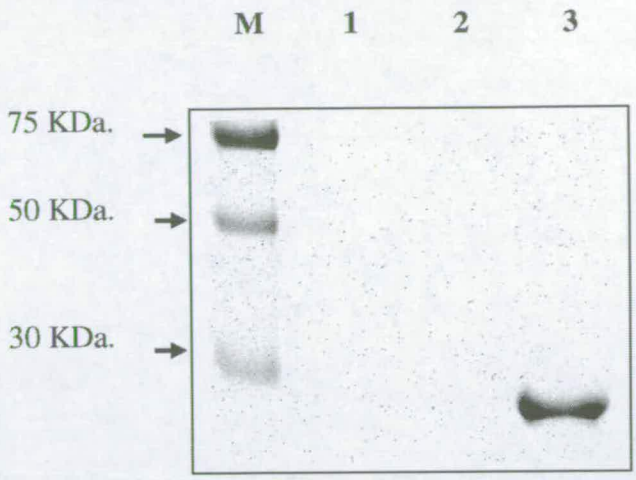


Figure 2.6: Coomassie stained polyacrylamide gel showing the results obtained after two-step purification of the recombinant CIDR1. Lane M is MW marker (Bio-Rad). Lanes 1 and 2 are fractions after washing with the washing buffer and lane 3 is the pooled eluted protein after elution.

2.8. Glycosylation Testing.

In designing the synthetic CIDR1 DNA coding sequence, mutations have been introduced to the sequence to remove sites that are generally N-glycosylated by eukaryotic expression systems. The rationale for this is that glycosylation, rare or absent from native *P.falciparum* antigens, may interfere with the correct folding and sero-recognition of the epitopes. Protein over-glycosylation frequently results in highly heterogeneous products which may be difficult to reproducibly purify to acceptable standards under cGMP. Hyper-glycosylation can also cause antigen hyper-immunogenicity, which can be lethal in experimental animals.

To test for glycosylation, the Pro-Q Fuchsia Glycoprotein Gel Stain Kit (Molecular Probes) was used. This depends on the Periodic acid-Schiff (PAS) reaction. After fixing the gel with 500 mls of 50% methanol, it was washed with ddH_2O and incubated in oxidizing solution (20% Periodic acid). In the dark, the gel was washed in Pro-Q Fuchsia reagent, before being incubated in the metabisulphite reducing agent. After staining with the glycoprotein staining kit, the recombinant CIDR1 expressed in *Pichia* is shown in Figure 2.7. No glycosylation of the 28-kD

wasdetected although some glycosylated high molecular weight material appears to be present in the supernatant.

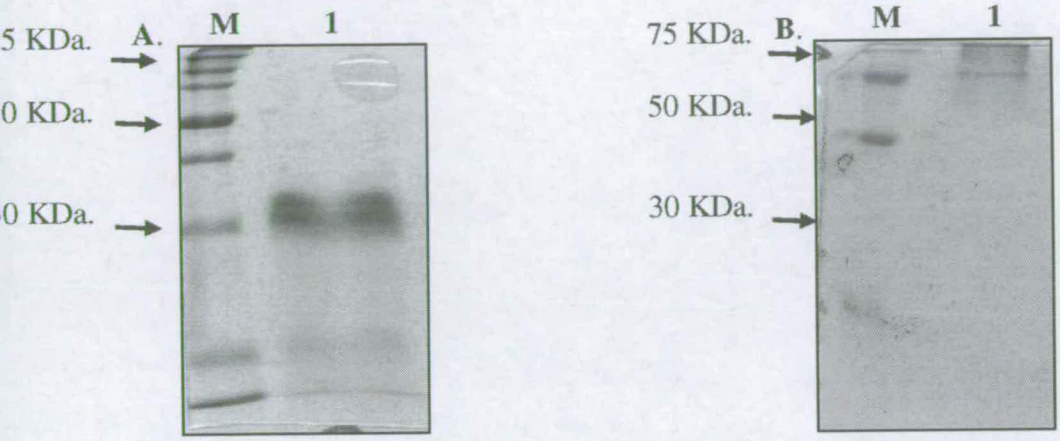


Figure 2.7. A 15 % SDS-PAGE Coomassie stained gel of recombinant CIDR1 (A) and its duplicate gel (B) after glycoprotein staining.

Lane M. Molecular weight marker. **Lane 1**. Purified proteins stained by Coomassie stain (**A**) and its duplicate stained with glycoprotein staining kit (**B**) to detect both N-linked and O-linked oligosaccharides. The positive controls in the marker in gel (**B**) stained with the Pro-Q Fuschia reagent.

2.9. Reactivity of malaria infection serum with the *Pichia* produced CIDR1.

To investigate whether humans mount an immune response following malaria infection to CIDR1, an ELISA assay was set up using plasma samples collected in Sudan. Serum antibodies to the recombinant CIDR1 were measured using enzyme-linked immunosorbent assay (ELISA). Plate wells were coated with 0.5, 1 and 5 µg/ml of the recombinant CIDR1 in 100µl of coating buffer (15mM Na_2CO_3, 35 mM $NaCO_3$ pH 9.3). The wells were then washed three times in washing buffer (0.05% Tween 20 in PBS). The adherent antigens were blocked to prevent non specific proteinaceous binding with blocking buffer (1% skimmed milk powder in washing buffer). Human plasma diluted 1:500 in blocking buffer was added and incubated

overnight at 4°C. After 3 more washes with the blocking buffer, the wells were incubated for 3 hours at room temperature with horseradish peroxidase-conjugated rabbit anti-human IgG (Dako, diluted 1:5000 in washing buffer). The wells were then washed again 3 times with the washing buffer and incubated for 15 min at room temperature with the substrate (Sigma)(0.1 mg ml^{-1} O-Phenylendiamine +0.012% H_2O_2) in developing buffer (24.5mM citric acid monohydrate and 52 mM Na_2HPO_4, pH 5). The reaction was stopped by addition of 25µl 2M H_2SO_4 and OD measured at 492nm.

The human plasma samples were collected by Dr. Nasreddin Abdulahi from adolescents and adults of both sexes as they presented with malaria symptoms at clinics in and around Gedaref, a large town in Eastern Sudan, during 1989. These samples were part of a batch that Dr. Abdulahi brought to Dr. Arnot's laboratory for analysis during a research visit in 1990. In 1999 Dr. Arnot provided this collection to Dr. Cavanagh for use as a 'bulk source' of anti-malaria antibodies and Dr. Cavanagh identified a subset of this collection (around 20% of the samples) that had moderate-to-high levels of antibodies that recognised C-terminal recombinant fragments of MSP-1. Their known reactivity to merozoite surface antigens made them a useful and available (but not particularly 'valuable') sample set for initial investigation of possible antibody responses to erythrocyte surface antigens.

$MSP1_{19}$ was used as positive control and measure of that individual serum's reactivity to a defined *P.falciparum* merozoite antigen (the GST-fusion protein containing $MSP\text{-}1_{19}$ was a gift from Dr David Cavanagh). Because a pooled malaria naïve serum was used a negative control, and no positive control serum or monoclonal antibody was applicable to this assay, it is not possible to create a standard curve for either positive or negative control sera. Nonetheless with these sera and controls it is certainly possible to detect a fairly strong level of reactivity to at least one recombinant antigens with the majority of Sudanese infection serum. No such malaria antigen reactive antibodies are present in the Scottish malaria naïve serum pools (Figure 2.8).

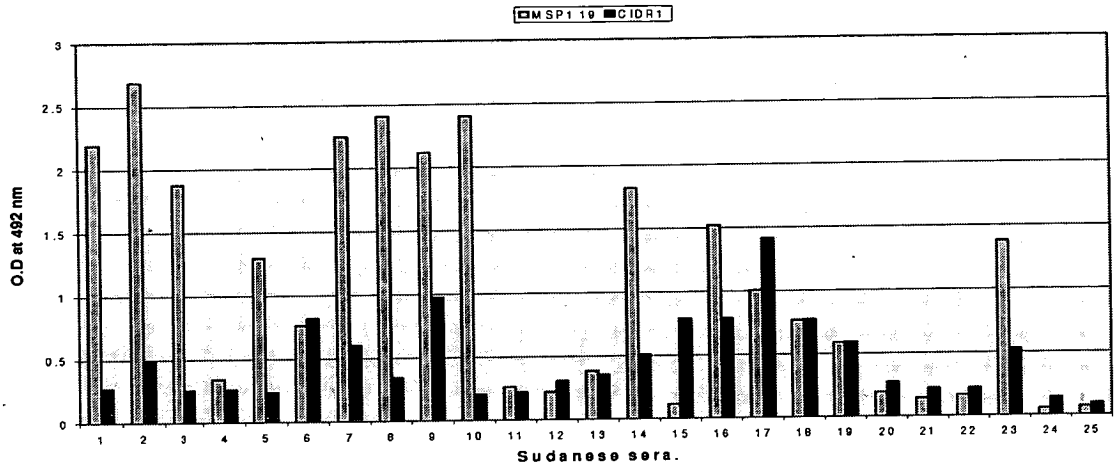


Figure 2.8. Detection of recombinant CIDR1 and MSP-119 by individual Sudanese acute infection sera. Columns 1-24 show individual Sudanese sera reacted with the two malaria antigens coated to microtitre plate wells. Column 25 is the Scottish malaria naïve serum pool reaction with these antigens.

These results show there were different levels of reactive antibodies in different individuals. As $MSP1_{19}$ is both less polymorphic and more abundant than CIDR1, this probably explains why its reactivity was generally higher than that observed to the CIDR. In fact these 'Gedaref clinic' sera were pre-selected because they were known to be 'mid-high-responder' anti-MSP-1 samples, with antibody levels similar to those found in many Sudanese infection and post-infection samples from the nearby village of Daraweesh (Cavanagh *et al,* 1998). Few or none of the donors of these original blood samples were pregnant; many were certainly males and children. Low, but usually above negative control responses were made by most of these Sudanese individuals to the recombinant CIDR1. In one or two cases (9 and 17) moderately high antibody responses to the CIDR1 antigen are made. There is no absolute correlation between responses to $MSP1_{19}$ and the PfEMP1 derived CIDR domain but where there is a clear response to the CIDR there tends to be a medium to strong response to the $MSP1_{19}$ antigen. 7/25 samples gave very low or negative responses to the two recombinant antigens (serum 4, 11, 12, 13, 20, 21 and 22). In 6 positive samples (out of 25 tested samples), responses to CIDR1were stronger than those to the $MSP1_{19}$ antigen. To further test the antigenicity of the purified yeast

recombinant protein, the CIDR1 protein was tested against pools of antisera from a more highly endemic malaria transmission zone. The serum consisted of a 'male serum pool' of 20 serum samples from men living in a malaria endemic region of Ghana. The 'pregnant female serum pool' consists of pooled serum from blood stream of 15 Ghanaian women, collected from the peripheral blood. This pool included 5 primigravidae, nine secundigravidae and one multigravid woman. Six Scottish malaria naïve individuals donated sera to create the 'malaria negative pool' serum. The bacterially produced $MSP1_{19}$ GST fusion recombinant protein was again included as a positive control. The Ghanaian sera were a gift from Dr. Mike Ofori and Dr Trine Staalsoe, University of Copenhagen.

When the PfEMP-1 CIDR1 derived antigens were probed with both the male and the pregnant female serum pools sera, there was strong reaction with both sera, again stronger to MSP-1, but no gender specific reactivity was observed. This was not in any case expected, as neither protein has been implicated in placental adhesion (Figure 2.9). This finding is not exclusive as if we are to conclude a clear gender specific recognition, reasonable number of individual male and pregnant sera should be tested , then a statically conclusion could be reveal whither an antigen is recognised in gender specific manner.

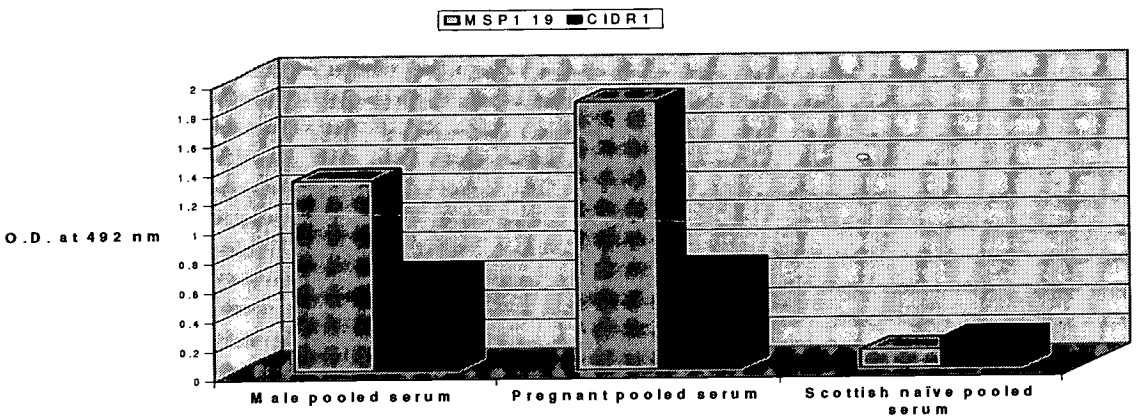


Figure 2.9. ELISA readings from male, pregnancy and Scottish serum pools with the purified CIDR1 and MSP-1 proteins. All sera were used in a dilution of (1:1000).

2.10. Rabbit immunization to produce anti-CIDR1 antibodies.

Immunisations experiments using the CIDR recombinant product were carried out by the Harlan Bio-Sera lab Company according to their rabbit immunisation protocols (see Material and Methods). The antigen concentrations required for each immunization was 50 - 200μg administered with complete Freund's adjuvant to ensure a high quality/quantity response. This was followed by three booster injections of 100 μg at days 14, 28, and 56 with Freund's incomplete adjuvant. Both ELISA and IFA assays tested antisera obtained 4 weeks after the last injection of adjuvanted protein for reactivity. Antibodies were purified on protein A columns. In ELISA, affinity purified rabbit anti-CIDR1 immunoglobulin reacted strongly with the *Pichia* product, as they did in Western blot (the pre-immune rabbit was the negative control).

2.11. IFA assays using anti-CIDR1 antibody.

Rabbit sera from the immunization study were tested for reactivity to *P. falciparum* 3D7 parasite(from which the *var* gene that contains the CIDR1 domain were isolated) infected RBCs. IFA was carried out on acetone-fixed iRBCs, infected with the 3D7 parasite. The corresponding rabbit pre-immune sera as used as a negative control. Panels of well-characterised monoclonal antibodies to the late trophozoites and MSP1 on the surface of the merozoites (Dr. Jana McBride, Edinburgh University) were used as positive controls. Whilst the monoclonal antibody controls strongly recognized acetone-fixed RBCs infected with late stage parasites, the immunised rabbit sera showed either very faint or no recognition at all. Thus the immunization regime failed to produce antibodies that recognize any antigen on the surface of continuously cultured 3D7 parasite. As the *var* gene containing the CIDR1 domain has Ups A sequence, it most likely that gene will not be detected in the transcriptional level and then will not be translated.

2.12. Conclusions.

These results indicate that the *Pichia* expression system expresses recombinant CIDR1 protein, although controlled fermentation is necessary to obtain good quantities of material for functional and structural analysis. Using this system, a time dependent product of the expected MW (~28kDa) was consistently produced. This product was confirmed as CIDR1 in Western Blot using antibodies to the polyhistidine tag. Detection of CIDRI during fermentation peaked and then decreased with time, suggesting that some degradation was taking place. Measures were taken to protect and maintain the integrity of the *Pichia* expressed protein; protease inhibitors were added to supernatants to prevent degradation and concentrated supernatants were re-suspended in or dialysed against 15 mM Tris/500mM NaCl, pH7.5, to protect against aggregation. Some evidence of aggregation was observed in case of the *Pichia* supernatants analysed here, which quickly took on a cloudy appearance. Aggregates have been common in the production of other PfEMP-1 domains and this makes their handling and analyses problematic (Dr. Graham Bentley, Institute Pasteur, personal communication).

The affinity purified CIDR1 protein was used in ELISA using sera from malaria endemic areas and comparing this with the reaction obtained with malaria naive sera using an $MSP1_{19}$ control. There were different levels of reactivity; the MSP-1 was almost always recognised more strongly than the PfEMP-1 derived product. Following rabbit immunizations an anti-CIDR antiserum was also tested for reactivity to *P. falciparum* infected RBCs. A panel of previously characterised cross-reactive monoclonal antibodies to iRBC surface (Dr Jana McBride and Dr David Cavanagh, Edinburgh University) was used as a positive control. Whilst the monoclonal antibody controls were seen to strongly recognize acetone-fixed RBCs infected with late stage parasites, none of rabbit sera from this immunization study was shown to recognize RBCs. This finding was somewhat disappointing although probably to be expected as it is commonly observed that raising polyclonal antibodies to the iRBC surface antigens is difficult and has been achieved by few groups (Chen *et al,* 2004; Gamain *et al,* 2004).

The fact that the antisera did not recognize 3D7-infected red blood cells indicates that we have no reagent which reacts with this specific CIDR domain on the infected erythrocyte surface. This may be due to the fact that we have no way of selecting for an enriched population of infected red blood cells specifically expressing the CIDR domain that we have raised the antisera against or that the CIDR1 is not expressed on the surface. Thus there is no direct evidence that the PfEMP-1 molecule containing this CIDR antigen is on the surface of a significant proportion, or indeed any, of the continuously antigenically varying parasites in our unselected cultures.

The methylotrophic eukaryotic expression host, *P. pastoris*, has been successfully developed as an alternative to the *E.coli* system (Hollenberg & Gellessien 1997: Cereghino & Cregg 2000). For *Plasmodium* proteins, the *P. pastoris* system is already being successfully used in the production of a recombinant form of Pfs25H, a cysteine rich 25kDa *P. falciparum* surface antigen of zygotes and ookinetes and a candidate for a transmission –blocking malaria vaccine (Zou *et al.*, 2003). Protein expressed in *P. pastoris* has been shown to be superior to that produced in *E. coli* based on its ability to bind to red blood cells. The antibodies generated against the *Pichia*-produced protein prevented the binding of recombinant EBA to red blood cells. These antibodies recognize EBA-175 present on merozoites (Yadava & Ockenhouse, 2003). Codon optimization has proven successful for the expression of other malaria genes (Nagata et al, 1999; Narum *et al.* 2001: The production of recombinant *P. falciparum* proteins in *Pichia* have been shown in MSP-1 (Morgan et al, 1999) and AMA-1 (Kocken *et al*, 2002).

The recombinant CIDR1, Produced during this part of thesis, enabled us to test the usefulness of the used expression system (*Pichia*) and the importance of recodoning the *Plasmodium* antigens. This recombinant protein will be used as a His-tag protein positive control and a negative control for glycosylation assays.

Chapter 3. Characterisation and screening of antigen arrays from PfEMP-1 domain expression libraries.

3.1. Aims.

The *P. falciparum* genome project has aided approaches such as the use of antigen arrays whose objective is to rapidly identify promising gene targets and thus accelerate the development of new drugs and vaccines (Young & Winzeler, 2005). Previously studies of protein or antigen activity have been conducted by studying a single protein at a time, which is time consuming and expensive. With the sequencing of entire genomes, large-scale 'proteome' analysis using protein micro-arrays has become possible (Zhu & Snyder, 2001). One of several current schools of thought with regards to the choice and subsequent production of an anti-malarial vaccine is that it should be based on the immuno-dominant parasitized erythrocyte antigens that play a role in the acquisition of natural protective immunity against the disease (Smooker *et al*, 2000, Jensen *et al*, 2004). In order to test the hypothesis that a major source of such immuno-dominant epitopes is encoded in the parasite's cell surface antigen genes, several known surface antigen specific genomic DNA (gDNA) expression libraries were produced in a form suitable for screening with human malaria immune serum.

A great deal of previous work has shown that differential screening with malaria immune sera can be used to identify parasite antigens that can be tested as vaccine candidate antigens (see, for example, Watanabe *et al*, 2001, Sowa *et al*, 2004). Genomic DNA expression libraries were chosen in preference to cDNA libraries because stage specific expression of some surface antigens may result in the production of biased libraries that exclude sequences absent, or present in very low abundance at the point of RNA extraction from cultured parasites. The use of genomic DNA thus increases the chances of expressing all surface antigens present in the genome, regardless of whether such genes are switched on in culture or not.

There are clearly technical and scientific problems with protein array technologies, sometimes caused by individual protein characteristics although some are common to high throughput methods in general. They include the problem of obtaining high

efficiencies of functional protein expression in a dense array and of selecting the best membrane for optimal signal-to-background ratios. A further consideration is that arrayed proteins should be correctly folded and functions as the naturally presented antigens as much as possible. Correlating *in vitro* with *in vivo* is difficult and requires assays that can compare the two states, which is certainly not always possible but can often be facilitated by comparing expression in different host cells which process recombinant protein in different ways. The simplest way of achieving such a comparison is by using a dual expression system in which the same plasmid vector has promoters for expression in two different host cells.

To achieve both aims, PfEMP-1 specific Duffy Binding-Like (DBL) domain expression libraries were constructed from the extracted DNA of the *P.falciparum* clone, FCR3CSA. There are estimated to be around 60 *var* genes per parasite genome. Each parasite clone is believed to have a repertoire of polymorphic but ancestrally related var genes and most (not all) *var* genes that have been sequenced have DBL-alpha domains upstream of CIDR domains. In this chapter, methods to optimise conditions for analyzing an expression array of *P.falciparum* PfEMP-1 *var* gene fragments are presented

3.2. Dual Expression Vectors.

To carry out the preliminary library-screening experiments a dual expression vector capable of expressing *P. falciparum* proteins in both prokaryotic (*E. coli*) and eukaryotic (*P. pastoris*) cells was chosen. The pPICHOLI dual expression vector (a gift of its inventor, Dr. Dolores Cahill) (Figure 3.1) combines both eukaryotic and prokaryotic promoter elements and drug selection appropriate for both hosts. A phage T7 promoter promotes bacterial expression. Protein expression in yeast is controlled by the alcohol oxidase promoter. Expression in yeast is completely repressed when grown on glucose and maximally induced when grown with methanol as the carbon source (Lueking *et al,* 2000). pPICHOLI has a multiple cloning site permitting insertion in three different reading frames within a 3.6 Kb plasmid genome. Genes cloned in pPICHOLI can be expressed in *E. coli* and *P. pastoris* as fusion proteins, with a hexa-histidine tail (the HIS tag) enabling a simple

purification step via affinity chromatography and detection by commercially available specific anti-HIS Tag antibodies. These vectors do not target the recombinant sequence into the yeast secretoary pathways. This has the disadvantage that purification is more difficult but the advantage that glycosylation during secretion is much less of a problem.

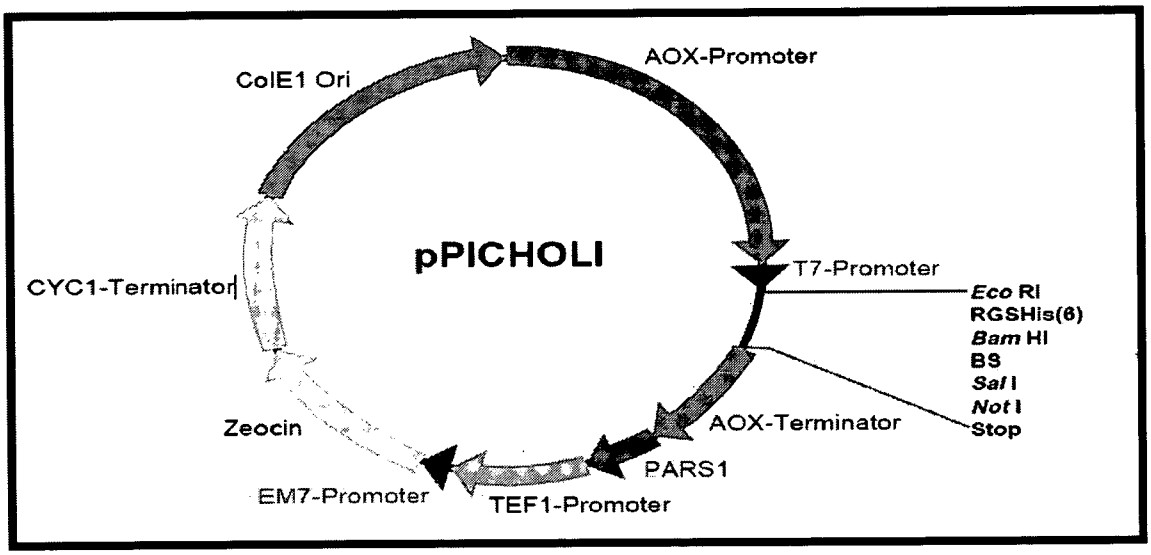


Figure 3.1. The pPICHOLI dual expression vector. It includes the 6xHis tag for detection and purification of the expressed protein. The vector contains an inducible (yeast) alcohol oxidase promoter and an *E. coli* T7 promoter as well as sequences allowing autonomous replication in both *P. pastoris* and *E. coli.*

3.3. DBL domain library construction.

The physical production of the gDNA expression libraries is a two-step process involving first amplification of PCR fragments of PfEMP1 DBL domains using FCR3CSA as the PCR template. Recombinant clones are then produced by ligating PCR products into pPICHOLI prior to transformation into BL21 codon plus cells for the *E. coli* expression library or X33 yeast cells for the *P. pastoris* library.

3.3.1 PCR amplification of PfEMP-1 specific DBL domains from FCR3CSA.

Fragments that correspond to our current estimates of where the boundaries between different DBL domains are likely to be were amplified from FCR3-CSA using degenerate primer combinations whose sequences were based on short conserved motifs within the flanking conserved blocks (see Materials and Methods for oligonucleotide sequences). All primers were designed with 5' *Sal* I and 3' *Not* I restriction sites to enable cloning into the Sal I and Not I pre-digested dual expression vector.

The PCR products (obtained using *Pfu* polymerase) were run on 1% agarose gels to confirm the presence of the amplified bands within the expected size range of 400-650bp (Figure 3.2).

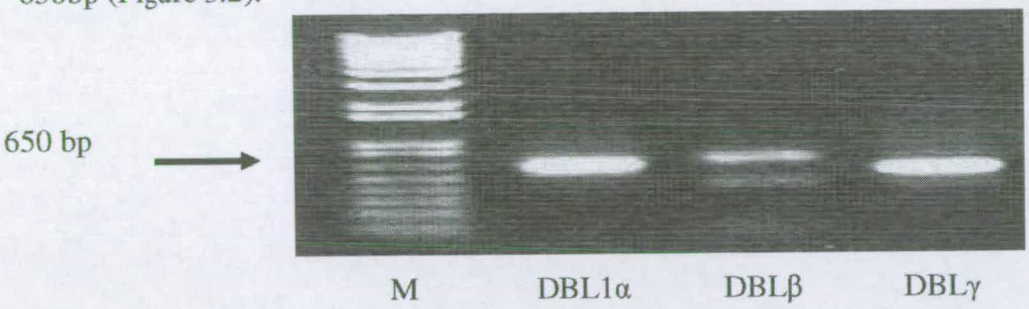


Figure 3.2: PCR products for amplified DBL domains using domain-specific degenerate primers. For DBLα domains, the PCR products are about 650bp. For DBLβ and DBLγ domains, the PCR products amplified are approximately 400-600bp. M is the 1Kb Plus DNA ladder (Invitrogen).

3.3.2 Preparation of expression plasmids.

To sub-clone into the expression vector, PCR products (flanked with *sal* 1 and *Not* 1) were first sub-cloned into the TA cloning vector. This was done to facilitate the digestion with the restriction enzymes. Double restriction digest with *Sal* 1 and *Not* 1 was used to excise the fragment from the TA cloning vector immediately prior to ligation into the same sites in the expression vector (Figure 3.3). To optimize ligation, different molar ratios of the vector and DNA were tested vector to insert ratios of 1:1, 1:3 and 1:5 were generally used. Ligation with 1:3 ratios usually generated the greatest number of clones. The ligation products were transformed into *E. coli* BL21 RIPL Codon Plus strain.

To obtain reasonable *P. pastoris* X33 strain transformation efficiency, two protocols for transformation were tested. When using freshly prepared competent yeast cells, electroporation using the Bio-Rad Gene Pulser was more efficient, with improvements in transformation efficiency of 3-10 folds when compared with the chemical method. Stored frozen cells were also used but sometimes give poor transformation results.

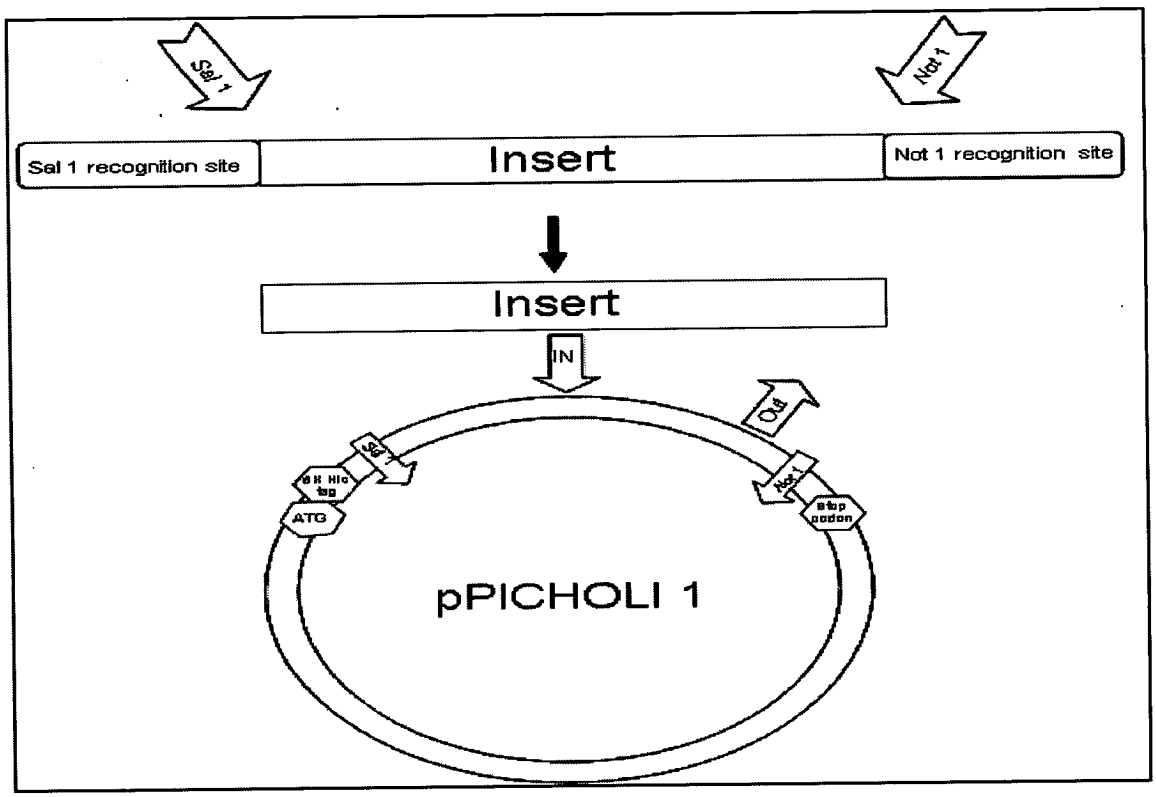


Figure 3.3. Cloning into the pPICHOLI dual expression vector.

3.3.3 *E. coli* and *P. pastoris* gDNA DBL expression libraries.

Using the method summarized in Figure 3.4, six different *P. falciparum* gDNA expression libraries were made using degenerate DBL domain primers. All libraries were stored as frozen glycerol cultures in 96-well plates at -80°C until required.

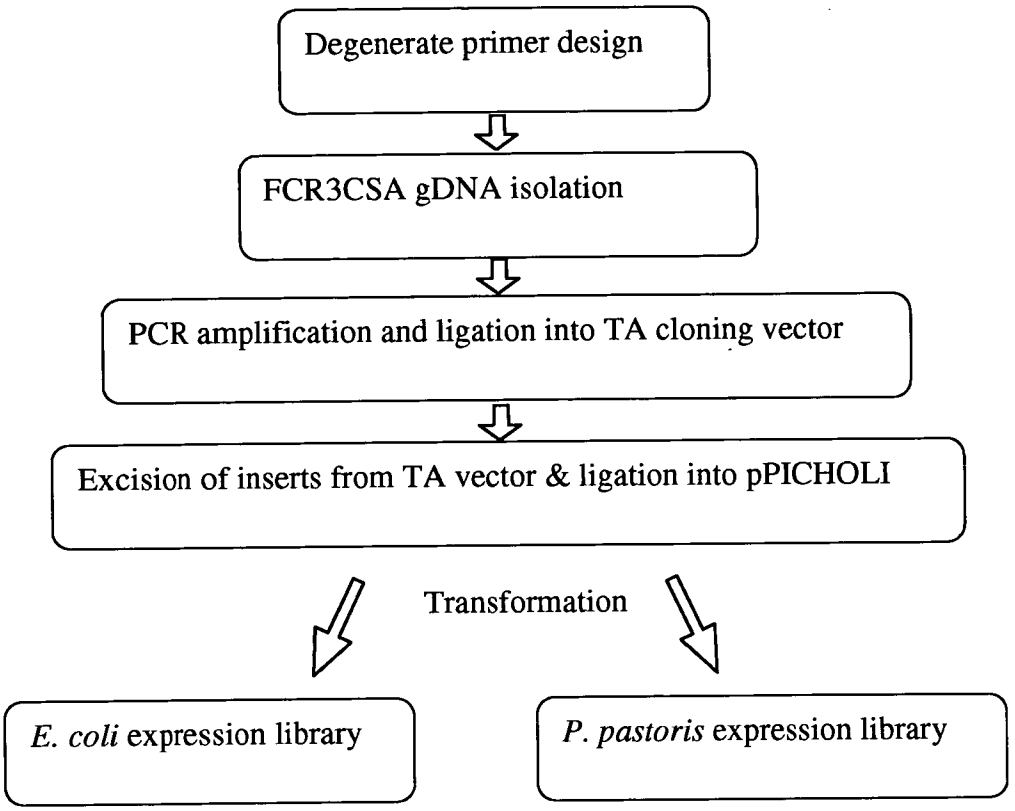


Figure 3.4. Flowchart summarizing production of protein expression libraries in either *E. coli* or *P. pastoris*.

3.3.4. Protein expression of selected clones of DBL domains in both bacteria and yeast.

After the libraries construction, a proof of concept was carried out to test whether the recombinanat plasmid is capable to express the recombinanat proteinthe and gave a strong signal with anti His tag antibodies.

The DBLα-6 clone was expressed first in bacteria and a protein band of expected size was detected by using the anti-His antibody. The *Pichia pastoris* produced recombinant also gave a strong band but of slightly higher than the expected size when probing with anti-His Tag and pooled male Ghanaian serum. A smaller band (fainter) has also been detected in these gels (Figure 3.5).This size shift could be due to glycosylation although this was not tested.

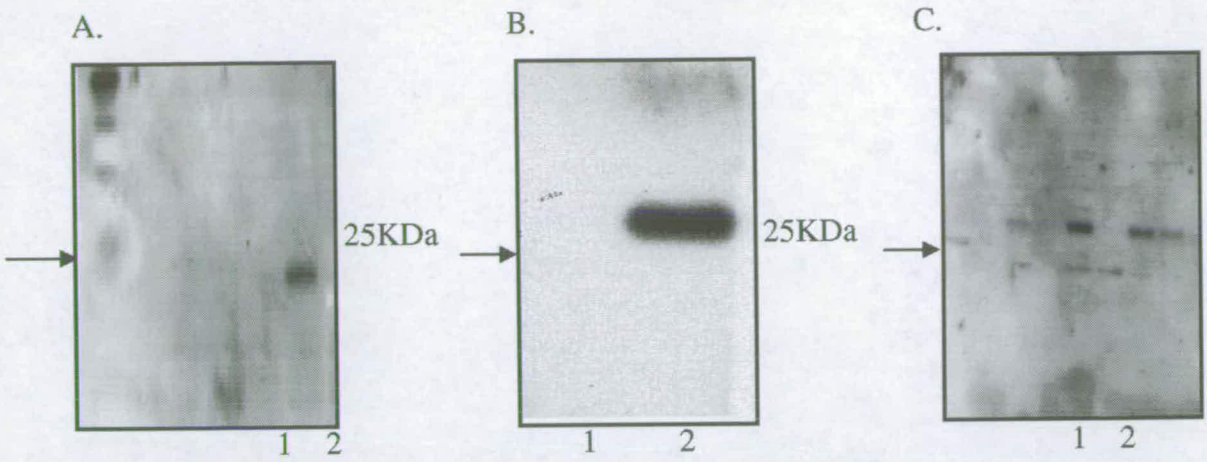


Figure 3.5: Western analysis of DBL1α-6 clone. A. The induced bacterial recombinant protein detected with anti 6XHis antibody as compared to the uninduced (1) and induced (2) clones. B. *P. pastoris* recombinant protein was detected with anti 6XHis antibody when the same plasmid was shuttled into yeast. C. Yeast recombinant proteins probed with sera from Ghana. It showed 2 bands, the stronger corresponded to the expected size.

3.4. Library characterisation.

To determine library quality before starting large-scale sequencing and protein expression studies it was necessary to characterise the libraries, based on the following criteria;

1). Percentage number of clones with inserts. 2). Size of inserts. 3). Sequence of inserts. 4). Number of domains cloned. 5). Orientation.

3.4.1 Number of recombinant clones.

Direct colony PCR was performed on 50 clones from each of the 3 *E. coli* libraries, giving results for a total of 150 clones. With the dual expression vector containing an insert in *E.coli,* only one band is expected to be amplified as *E. coli* lacks the AOX1 gene. The 3' AOX1 and 5' AOX1 primers will therefore only amplify the cloned fragment in the plasmid. Direct colony PCR was also performed on DNA isolated

from colonies from the 3 *P. pastoris* libraries. For the transformed yeast cell two PCR products were expected, a) an approximately 2.2kb fragment, equivalent to the size of the AOX1 gene amplified from the yeast genome and b) the insert in the pPICHOLI expression plasmid (Figure 3.6).

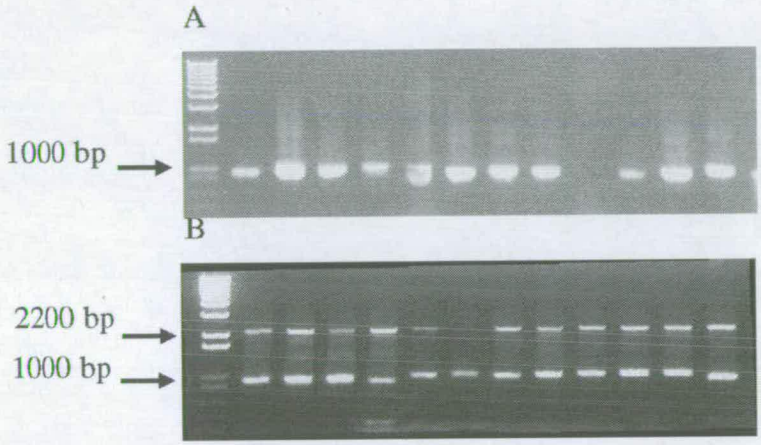


Figure 3.6: Direct colony PCR of (A) 12 bacterial clones and (B) 12 yeast clones. PCR products were separated on 1% agarose gels. M is the DNA molecular standard.

3.4.2. Insert Size.

Direct PCR screening revealed that there was size polymorphism between cloned domains and this was later confirmed by sequence analysis. In general, the current estimation of a full-length DBL domain is 900-1200bp encoding 300-400 amino acids. The degenerate primers used in this project amplified only a portion of that domain. The approximate sizes expected for DBLα1, DBLβ2 and DBLγ3 are 0.6 Kb, 0.4-0.65 Kb and 0.55 Kb respectively (Figure 3.7).

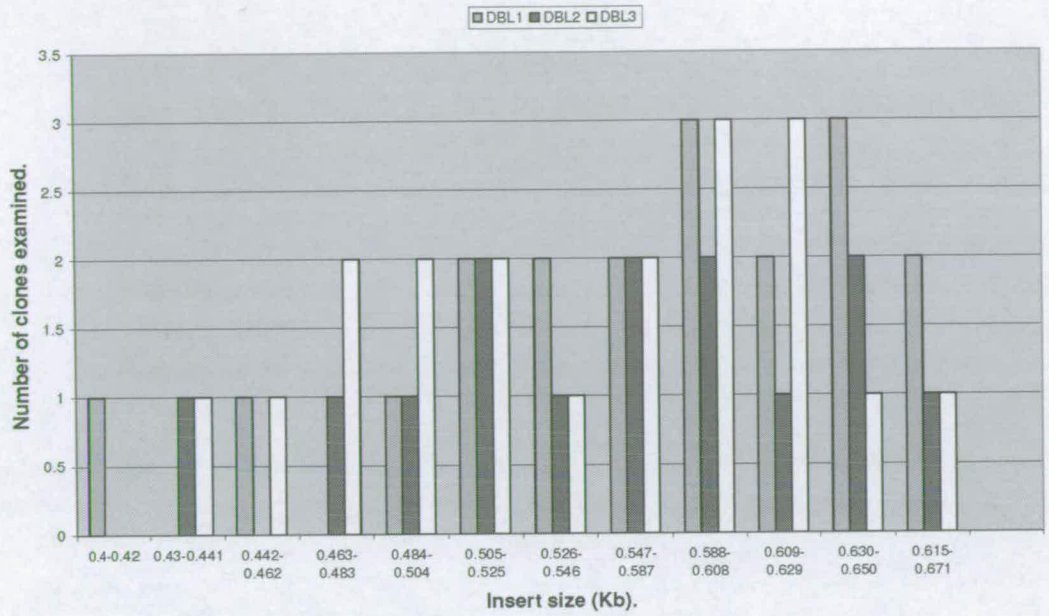


Figure 3.7. Size range of inserts for each DBL domain.

3.4.3. Sequence Representation.

A good library should have a large number of unique inserts. The nucleotide sequences were determined with a PRISM/S BigDye Terminator Cycle Sequencing Reaction Kit and the Applied BioSystems 377 DNA sequencer. Each set of primers tested amplified a number of variants whose sequences indicated that they were derived from different PfEMP-1 loci in the FCR3CSA genome. These were grouped into sub-classes according to their degree of homology to DBL domain sequences in the database. It was found that, as expected, the primers used were domain-specific in the sense that all products amplified with a particular pair of degenerate primers did amplify sequences that appeared to belong to the expected domain type.

98% of the clones with inserts gave useful sequence data. A total of 18 different DBLα, 21 different DBLβ and 23 different DBLγ sequences were isolated and sequenced from the FCR3CSA libraries. After sequencing, homology searches of the sequence databases were performed at the National Center for Biotechnology Information (http://www.ncbi.nlm.nhm.gov and www.plasmodb.net). Sequences

obtained were confirmed as *var* fragments by BLAST analysis in both the general database (NCBI) and the *P. falciparum* database (PlasmoDB). Deduced amino acid alignments of obtained sequences with the 3D7A DNA sequences and other *var* gene fragments available in the databases were made using MacVector Clustal functions (data not shown). There were three general categories of homology, identity (100%) with previously described domains, matches with between 80-100 % identity and matches with less than 80% homology (Table3.1). Specific homology of some sequenced clones are summarized in table 3.2.

Domain name	**100% homology)**	**> 80% homology.**	**< 80% homology**
DBL1	4	12	3
DBL2	3	7	5
DBL3	3	5	12

Table 3.1: The DBL domain sequence homology results compared with database DBL domain sequences.

Comparisons between sequences within each domain show that there is marked conservation of the positions of certain residues in the DBL-1 domain. Large numbers of DBL-1 sequences share short (10–20 amino acid) regions of sequence, an observation that has been reported previously (Ward *et al,* 1999). These observations support the view that the structural framework of the DBL-1 domains in most, if not all, of these molecules is highly conserved. Multiple amino acid alignments showed that DBLα and DBLβ are somewhat more similar to each other than either is to DBLγ. This may reflect ancestral relationships between domains based on ancient gene duplications. In general, full-length DBL domains are approximately 900-1200bp. The degenerate primers only amplify a portion of that domain.

Clone	Acc. No.	Reference	Isolate
DBLα /TA1	AF366354	Oguariri, *et al*, 2001.	Isolate from Gabon
DBLα /TA3	AJ319689	Duffy , *et al*, 2002.	CSA binding CS2
DBLα /TA10	AF050740	Voss , *et al*, 2000.	FCR3
DBL β /TA4	AY248887	Kraemer, *et al*, 2003.	A4
DBL β /TA12	AF193424	Smith J., *et al*, 2000b.	A4
DBL β /TA7	AY248886	Kraemer, *et al*, 2003.	A4
DBLγ /TA 3	AY248879	Kraemer, *et al*, 2003.	A4
DBLγ /TA 7	PFA133811	Buffet, *et al*, 1999.	FCR3CSA
DBLγ /TA 13	AF465509	Fried and Duffy, 2002.	Placental isolates.

Table 3.2: Clones with 100% identity to DBL domains obtained from the database.

3.4.4. Insert Orientation.

To confirm the directionality of these libraries, a total of 150 clones from the E. coli library were sequenced from both ends. None of the clones were inverted, thereby making the libraries 100% unidirectional, an important factor when considering transcription, translation and protein expression.

3.4.5. Total number of domains cloned and arrayed for screening.

The libraries made in *E. coli* comprise 475, 570 and 570 clones for DBLα1, DBLβ2 and DBLγ3 respectively, arrayed in 96-well plates. Yeast expression libraries for these domains comprise 37, 55 and 73 clones respectively, arrayed in one 96-well plate/domain (Table 3.3).

Expression system	DBLα1	DBLβ2	DBLγ3
P. pastoris	37	55	73
E.coli	475	570	570

Table 3.3: Number of colonies for each DBL domain in *E. coli* and *P. pastoris* expression libraries.

3.5. Creation of antigen arrays from the FCR3CSA gDNA library.

Membranes are currently the only practical surface for screening microbe derived protein arrays. Colonies from the bacterial and yeast libraries were picked from the master plates into 96-well growth plates (in duplicate) (Figure 3.8). The wells contained LB medium supplemented with 50μg ml^{-1} Zeocin (and 37μg ml^{-1} chloromphenicol for *E. coli* libraries). BMGY containing 100μg ml^{-1} Zeocin was used in the case of *P. pastoris* libraries. Colonies were incubated overnight at the appropriate temperature and used in inoculating membranes for subsequent growth.

Colony arrays were initially prepared by application of the liquid culture to High Bond-C Extra (Amersham) nylon filter membranes for *E. coli* expression. Protran membranes were used in the case of *P. pastoris*. Using the Replicator (a 96 prong steel pin tool) to seed the microbial cells onto the membranes and following the procedure outlined in Figure 3.9, both types of colony arrays were grown by placing the appropriate filter on either LB agar or BMGY agar. All expression arrays were grown overnight at 30°C and protein expression was induced the following day.

E. coli filters were induced for recombinant protein expression by transfer of overnight filter from 'LB agar only' plates to LB agar plates supplemented with 1mM IPTG and 50μg ml^{-1} zeocin, at 37°C for 4 hours. Yeast library induction was carried out by transferring the filter array to BMGY agar plates supplemented with 0.5% methanol and 100μg ml–1 zeocin, for 3 days at 30°C (with addition of 500μl of methanol every 24 hours). After the appropriate protein induction period, the cells were lysed in situ to liberate intra-cellular recombinant protein; the filters were blocked overnight in 3% BSA with 0.1mM of the protease inhibitor PMSF. If

necessary, after this procedure the filters may be removed from the washing buffer, wrapped in Saran Wrap and stored for up to 24 hours at 4°C.

Protein immobilized on these membranes can be remarkably stable and after incubation with one set of antibodies, these antibodies can be stripped off with strong ionic detergents and the membrane re-incubated with alternative antibodies. Some array membranes were stripped two or more times and still gave strong signals upon re-use.

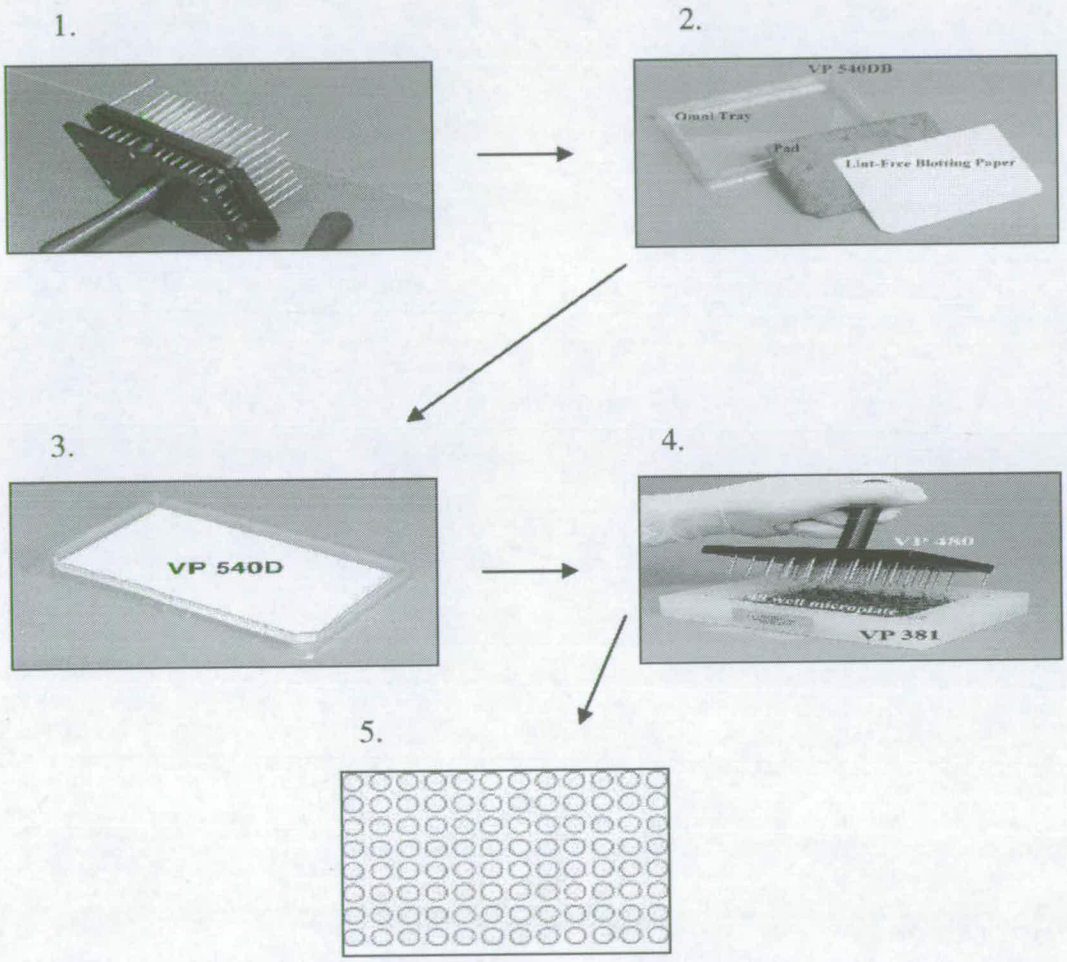


Figure 3.8. Colony printing procedure adapted from http://www.vp-scientific.com. 1. The Replicator. 2. & 3. Assembly of the membrane and the pad in a preparation tray. 4. Applying the colony array from the 96-well plate into the membrane. 5. Schematic of colonies growing on a membrane.

3.5.1. Optimisation of colony growth on different membranes.

The selection of an appropriate surface for colony seeding is the key step in recombinant protein array design. Such arrays require combining strong and durable attachment of relevant recombinant proteins with low non-specific binding of other host-derived proteins. Several membrane types were tested for this purpose. Following application colonies were 'arrayed' onto filters and grown on agar plates until clear colony growth was visible. The applicability of several different colony support filters was tested, as summarized in Table 3.4.

Colony support filter	Growth	
	E. coli	*P. pastoris*
Protran®	++	+++++
Hybond-™N+	++	+++
Hybond™- C Extra	+++++	++
Polvinylidene Difluride	+/-	-----
Cellulose Nitrate(E)	+++	----

Table 3.4. The effect of different membrane types on colony growth. Five different membranes were spotted with the same number of colonies from each library and other controls. Hybond™- C Extra was chosen for *E. coli* array and Protran®for *P. pastoris*.

After comparison of their capacity to support optimal growth of microbial colonies, a Hybond-C Extra membrane was chosen for the *E. coli* array. Protran membranes for the *P. pastoris* array proved to be superior to other membranes. Neither the Hybond-N nor the cellulose nitrate membranes resulted in clear and distinct colony-protein signals. This may be due to the lack of efficient binding of the proteins to the membranes. The PVDF based membranes could not be used for yeast or bacteria array screening as pre-soaking the membrane in 100%

methanol is required, making it impractical for use as it would kill bacteria and yeast arrays before growth and induction could commence.

3.5.2. Optimisation of signal to background ratios.

Liquid cultures were spotted onto membranes and grown on plates until colony growth was visible. High-level expression of recombinant protein was induced and the filters pre-blocked to minimize non-specific binding of detection antibodies (Table 3.5).

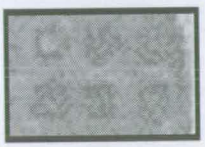
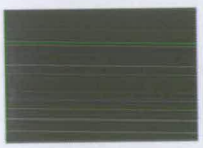
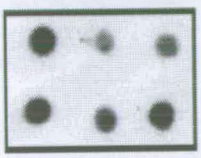
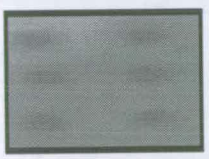
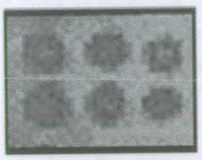
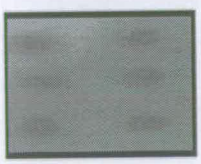
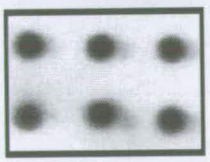
Membrane	Array	
	E. coli	*P. pastoris*
Hybond-N		
Polvinylidene Difluride		
Hybond- C Extra		
Cellulose Nitrate(E)		
Protran		

Table 3.5. Selection of membranes for antigen arrays. Membranes were spotted with *E. coli* and *P. pastoris* arrays and probed with the IgG from Ghanaian donors. Goat anti-human IgG-HRP(Dako) was used to detect antibodies.

Experiments were performed to optimize antibody concentration with respect to background, substrate color intensity and substrate color retention. For primary antibodies an appropriate dilution was chosen (generally 1:50-1:5000) and filters were incubated for 1 hour at room temperature on a shaking platform.

3.6. Preliminary screening of *Pichia* DBL libraries using anti-6XHis antibody.

When membranes containing a selection of clones from each library with controls were incubated with Anti-6X His antibody, it was found that there were strong signals for some clones and no reactivity at all for other clones. The background and signal were optimised readily (Figure 3.9). To detect intracellular expression clones of each DBL yeast expressor were grown on Protran membranes and induced on BMMY (Buffered Methanol-Complex Medium) agar plates. Cell lysis was carried out using YeastBuster™ detergent reagent for 30 minutes at room temperature. Lysed cells were removed by rinsing with PBS.

The membrane was blocked with PBS containing 0.05% Tween-20 and 5% nonfat, dry milk overnight at 4°C. Anti-6XHis-HRP was added (1:4000 dilution in PBS/5% nonfat, dry milk) for 1 hour at room temperature with moderate shaking. The blot was washed three times with PBS containing 0.05% Tween-20 (PBST) for 15 minutes each time. Positive and negative controls were clones expressing the recodoned CIDR and a clone containing the plasmid without insert. Duplicated spotting is the main means of controlling the screening results and only duplicate signals are considered as true positives.

5 DBLα clones were detected successfully, as pairs of spots for the same clone, with strong signals (42%). 5 clones were detected as one spot (42%) which considered being not true positives. The remaining clones (2 clones) could not been detected (16%). For the DBLβ clones, 5 out of 12 were detected as pairs of spots with strong signals (42%). 2 clones were detected as one spot (16 %). The remaining clones (5 clones) could not been detected (42%). In the case of DBLγ, it was found

that 7 clones were true positives (59%). One clone (7%) was detected as single spot, while the remaining clones (4 clones) could not been detected (34%).

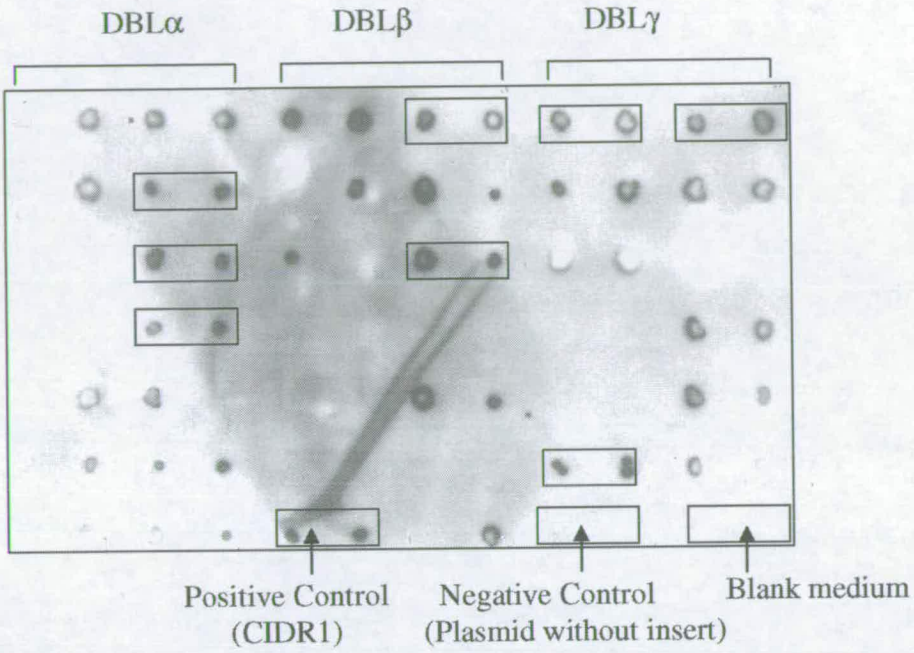


Figure 3.9: High throughput screening of a *Pichia* array of expression clones from the DBLα, β and γ libraries. Some clones gave strong signals (boxed) when probed with the anti 6XHis antibody. Controls include the positive control CIDR1 recombinant protein, while the negative control was the expression vector plasmid without insert. A blank growth medium spot was also included. Expressing colonies were detected using the ECL chemiluminescence detection kit (Amersham).

3.7. Screening the antigen arrays.

After optimising the conditions required for bacterial and yeast growth on filters and the antibody concentrations needed for Western blots, the antigen arrays were ready to be immuno-screened. In the final part of this chapter, I will discuss the steps taken to treat the sera in order to ensure that the results obtained from the screening

process were due to actual recognition of the expressed protein by the human immune sera.

3.7.1 Removal of anti-bacterial and anti-yeast antibodies from human sera for improved screening.

To decrease the possibility of obtaining 'false-positive' results due to non-specific cross-reactivity, it is imperative that the antibodies responsible for such cross reactivity be removed. Previous studies have shown that there are naturally acquired anti-bacteria and anti-yeast antibodies in some human sera. Pellets of live *E. coli* and *P. pastoris* with the expression plasmid (but without inserts) were used for the absorption of 0.1 volume of serum from a patient after dilution of the serum (1:100) in PBS-Tween 20. The mixture was incubated at 20°C for 2 h and spun at 10,000g for 15 minutes. The serum was also absorbed with lyophilized bacterial material (1 mg/ml of serum) and sonicated yeast material (Figure 3.10).

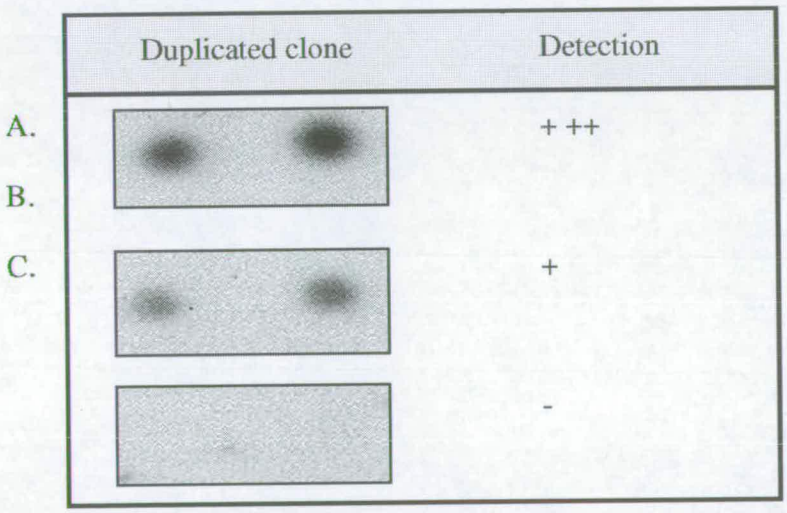


Figure 3.10. Depletion of anti E. coli antibodies from human serum. A. Clone without insert probed with undepleted human serum (before incubation with E. coli pellet and/or lysate). B. Clone without insert probed with 1X depleted serum. C. Clone without insert probed with 2X depleted serum.

Human serum antibodies from malaria endemic areas depleted of anti-bacterial and yeast antibodies were used to screen the membrane bound antigen arrays at serum dilutions from 1:50 - 1:5000 dilutions (Figure 3.11). The signals were visualized using an ECL plus detection kit with exposure times ranging from 1 to 30 minutes, the intensity of the signal was adjusted according to exposure times. Experiments were also performed to determine the best blocking agents. Different blocking agents in the blocking buffer were tested, including fetal bovine serum, non-fat dried milk and bovine serum albumin (BSA). BSA gave the lowest backgrounds with both primary and secondary antibodies and was therefore used in subsequent screening assays.

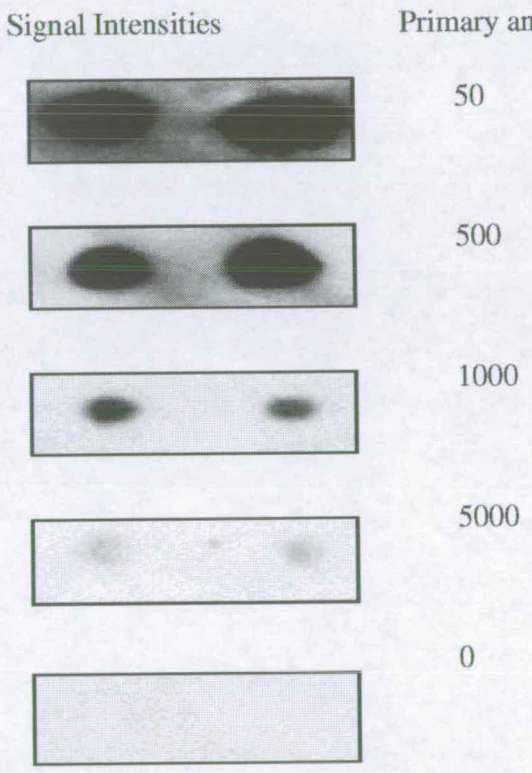


Figure 3.11. Sensitive detection of serum antibodies with goat anti-human IgG (Dako) in the antigen arrays. The membranes were incubated with varying concentrations of human serum from malaria endemic areas and then used to test the detection limit in array format.

3.7.2. Controls and extra precautions.

Both positive and negative controls were used in each assay and processed in the same manner. Pooled Scottish sera were used as negative controls. It was found that there was faint reactivity to some negative controls. Antigen arrays were also incubated with just the secondary antibodies conjugated to HRP. There was no reaction when incubated with only the secondary antibodies –HRP conjugate. The negative controls were used to confirm the specificity of the test and to assess the degree of non-specific sero-recognition present by omitting the primary antibody. Omitting the primary antibody and using antibody diluent (PBS) alone is usually sufficient for assessing the degree of nonspecific binding by the labelling (secondary) antibody.

3.8. PCR-based screening of DBLγ bacterial antigen array.

To make use of the optimised conditions an *E. coli* DBLγ expression array was tested. A clone containing the empty expression plasmid was the negative control. 189 clones were spotted in duplicate and grown by placing the filters on LB agar supplemented with glucose (1%), zeocin (25μg/ml) and chloramphenicol (34μg/ml) at 30°C overnight. Protein expression by colonies on filters was induced and expressers were detected by chemiluminescence. 19 randomly picked positive clones were cultured in 3 ml LB medium/clone overnight at 37°C. Plasmid DNA was prepared from each clone using the plasmid Qiaprep Kit (Qiagene) and sequenced using the ABI sequencer (Figure 3.12). 18 out of 19 clones that gave strong signal in the blot revealed a band of the expected size. A number of clones displayed sequence polymorphism. In fact these clones produced a diverse list of candidate antigens. The primary results indicated that the library is both representative and reliable and reliably amplifying the desired target domains. (The clones with empty vectors were removed from the master plates in preparation for immunoscreening.)

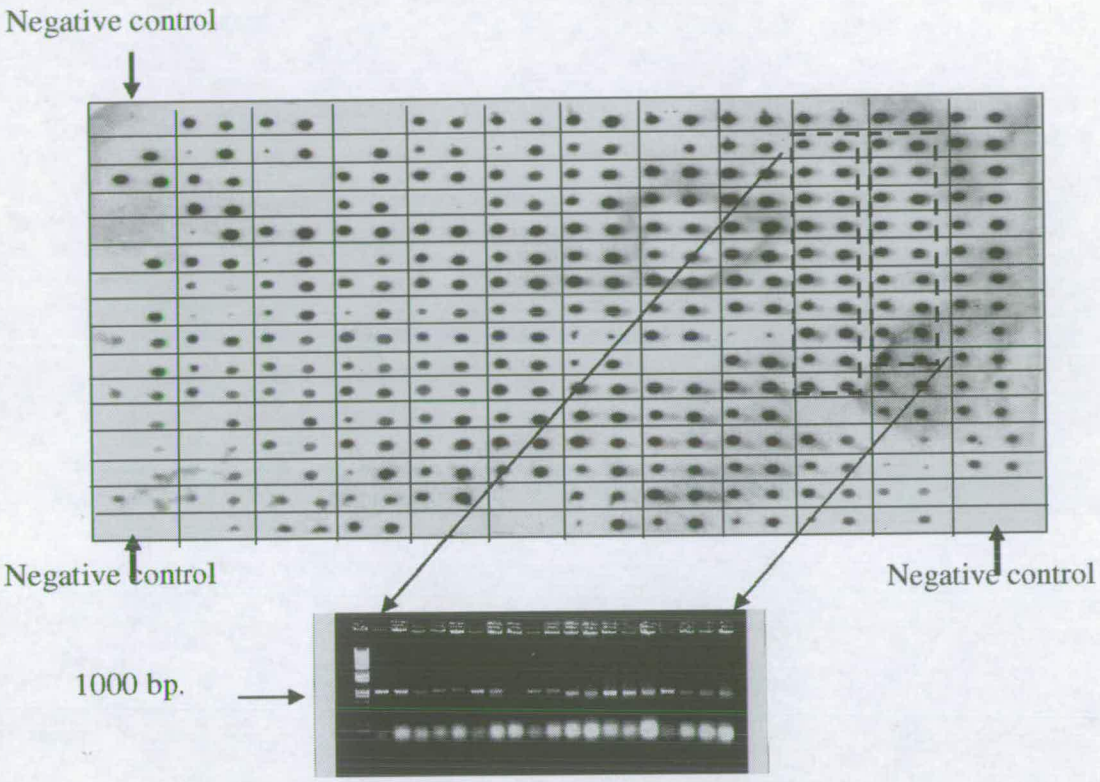


Figure 3.12: E. coli DBLγ array showing correlation between recombinant clones and detection of expressed protein. These clones were arranged in duplicate and contained inserts with size and sequence polymorphism. A negative control was used consisting of a cell containing the vector but no insert. Arrays were produced on High Bond-C Extra filters (Amersham) using an applicator with 96 steel pins. After induction and lysis, filter was incubated with pooled male serum from Ghana and was detected by goat anti-human IgG-HRP (Dako). Mark boxes clones were the randomly picked colonies from which plasmid DNA was prepared.

3.9. Conclusions.

Protein arrays allow high-throughput screening using manual or robotic methods after immobilization of antigens onto membrane. Six different *P. falciparum* gDNA expression libraries based on DBL domains from PfEMP-1 *var* genes were successfully constructed in the *E. coli* and *P. pastoris* expression systems. The shuttle library approach was exploited here in order to gain maximum flexibility and choice in optimizing the expression system. However, shuttling is not without problems and there is significantly reduced transformation efficiency in the yeast system. Transformation by electroporation became necessary for yeast work, as was the use of freshly prepared competent cells as stored frozen cells sometimes gave poor transformation results.

There was also a significant difference in the number of clones obtained from the *Pichia* libraries. In addition to reduced yeast transformation efficiencies, this may also be influenced by uncorrected codon usage differences between the two organisms (yeast and *P.falciparum*). Codon usage differences between *Plasmodium* and *E. coli* were compensated for by the use of BL21 cells which contain a plasmid that expresses a number of tRNAs that are rarely used by wild type *E.coli.* An equivalent plasmid, to compensate for the small pools of certain tRNAs in *Pichia* is not available and therefore could not be used in this project.

The primers used were able to amplify a variety of different DBL type-specific sequences from the FCR3CSA clone even although they were based on the PfEMP-1 sequence data from *P. falciparum* clone 3D7. A total of 18 DBL 1 alpha, 21 different DBL beta and 23 different DBL gamma sequences were isolated and sequenced. It is likely that more such ' *var* gene tags' could have been isolated if more clones had been sequenced but the primary objective here was to show that the libraries constructed had a good representation of different *var* genes, not to completely 'tag' label every FCR3CSA *var* gene. The indication that semi-conserved primer sequences can be used to amplify specific domain tags from a different parasite genotype provides further confirmation of earlier observations that there is extensive sequence conservation between PfEMP-1 *var* genes and some structuring of the sequences into related sub-classes. (Ward *et al*, 1999, Lavstsen *et al*, 2003).

Optimisations of antibody-based protein array format for analysis of protein expression libraries were described. To optimize the immunoscreening of antigen arrays, various membranes, antibody concentrations, and hybridization times were evaluated. The membranes were evaluated on the basis of background color reaction, retention time of the color reaction, and the durability of the membrane throughout the course of the procedure. Various problems were encountered when using antibodies to screen an expression library. Many factors should be considered if no positive clones or abundance of positives are noticed.

The antibodies used may not recognise the antigen when the latter is expressed in *E. coli.* These problems can occur when screening for eukaryotic cell protein that have posttranslational modifications such as carbohydrates, lipids and disulfide bonds. Each of these modifications is antigenic and not generally present on proteins expressed on E. coli. Monospecific polyclonal antibodies that recognise fully denatured, deglycosylated proteins should be used whenever possible to avoid these problems. This can be circumvented by utilizing a eukaryotic expression system (e.g. *P. pastoris*). The use of excess primary or secondary antibodies in the screening reactions can lead to the detection of too many positive clones. Diluting the antibody may eliminate or reduce cross-reactions without significantly affecting the binding of the antibody to its two targets. By varying the concentration of antibodies on different filters one should also be able to identify the optimal conditions and/or improved expression characteristics. Too many positives can sometimes reflect a problem with the blocking reagent; one of the other blocking reagents should be tried. Increasing the concentration of tween or the non-ionic detergent Nonidet P-40 in the rinsing and washing buffers reduced the number of seropoitives. The temperature at which the blocking, rinsing and washing steps are carried out can be increased to as high as 50°C.

One of the problems faced in the development of antibody-based protein arrays is the cross-reaction among different antibodies. This problem may be overcome by careful incubation of antibodies, and pre-absorption of antibodies with the expression systems of choice antigens. Automated screening of antibody or scaffold libraries against arrays of target proteins will be the most rapid way of developing the thousands of reagents required for protein expression profiling. The antigen assay

may be the most specific and easiest way to detect antibody. Other potential applications of protein array technology include identification of specific markers (antigens) for disease, and determination of differential protein expression in two different states. The antigen arrays will be screened for capacity of the target antigens to be recognized by sera collected from clinically distinct cohorts of individuals naturally exposed to malaria (Doolan *et al,* 2003). Protein microarrays are a powerful technology, which allows the protein of an organism to be screened in a single experiment. The array consists of protein released from the selected expression system after lysis and screening their immunogenecity. These assays have been used in the human malaria parasite (Sowa et al, 2004, Hayward *et al*, 2000)

The combination of DNA library construction and antibody screening methods will facilitate identification the antibody responses to immunogenic antigens of *P. falciparum* and help identify epitopes that could be exploited in malaria vaccine development (Anders, 1988). The libraries are ready for the next steps in immuno-screening.

Chapter 4. Immuno-screening DBL domain libraries with malaria immune sera.

4.1. Aims.

The experiments described in this chapter use the optimised methods for bacterial and yeast expression library construction described to identify immunogenic, 'vaccine-candidate'-type epitopes. Immuno-screening for vaccine candidate antigens in parasitology developed from antibody recognition of SDS gel fractionated parasite antigens in Western blots. This developed into a recombinant protein screening method using mono- or polyclonal anti-sera to immuno-screen cDNA or gDNA expression libraries.

A novel method to identify key PfEMP-1 genes, based on up-regulation of transcription after selection for a particular adhesion property, has recently been developed. Using gene-specific primers for all *var* genes (based on the genome sequence of 3D7) and quantitative real-time PCR, the levels of *var* gene transcription before and after selection for adhesion to CSA were measured (Salanti *et al,* 2003). The *var* gene PFL0030c (*var*2CSA) of the NF54 isolate (now thought to be identical to its daughter clone 3D7) showed a higher level of expression following CSA selection (NF54CSA). *Var*2CSA was subsequently shown to be expressed on the surface of CSA-selected parasitized cells and certain recombinant domains of this antigen were recognized by antibodies from the serum of individuals living in endemic malaria zones in a gender and parity-dependent manner (Salanti *et al,* 2004).

'Mini-libraries' of *var*2CSA domains from unselected 3D7 parasites have been constructed in *P. pastoris*. Yeast clones were induced for protein expression and preparations of induced protein were purified. ELISA studies were carried out to identify epitopes that can react with a panel of human serum samples from Eastern Sudan. The reactivity of expressed recombinant protein from *var*2CSA domains was also tested with serum antibodies from a malaria endemic region in Ghana.

4.2. *Var*2CSA yeast expression library constructs.

The *var*2CSA *P.pastoris* expression required designing specific primers, using both the *P.falciparum* 3D7 isolate genome sequence and DNA to amplify regions encode different domains of the *var*2CSA gene from that isolate. Both forward and reverse primers have similar annealing temperatures calculated using the Tm determination programme (http://alces.med.umn.edu/rawtm.html). Each primer pair has a stop codon (UAA) incorporated into the 3' sequence. 5' primers were designed to be in-frame with an ATG codon in the vector (pPICHOLI1) that initiates transcription of cloned sequences. Purification/detection tags that are thus incorporated onto the N-terminus of translated recombinant proteins. Primers usually gave a single band in 1% agarose gel separations of the amplified products. The sizes of the amplified fragments and of the expected molecular weight of *P.pastoris* produced recombinant proteins transcribed and translated from the pPICHOLI1 vector are given in Table 4.1.

Domains	PCR products	Expected Molecular Weight
DBL1X	650 bp	30.5 kDa
DBL2X	670 bp	46 kDa
DBL3X	930 bp	40.5 kDa
DBL4ε	711 bp	31.5 kDa
DBL5ε	633 bp	32.5 kDa
CIDR1	1000 bp	29.5 KDa

Table 4.1. Expected sizes of DNA fragments and molecular weights of the expressed proteins from the var2CSA gene, using the different primer pairs.

Potential N-linked glycosylation sites were avoided during the *primer* design. For asparagine to be N-glycosylated in most mammalian or yeast systems, it requires the context of the amino acid motif N-X-S/T (Marshall, 1974). X can be almost any amino acid although a motif will not be glycosylated if it contains or is followed by a proline (Gavel & von Heijne, 1990). Creating N-glycosylation sites at either end of the *var* gene fragments was therefore minimised and least one potential site was avoided in each of the *var*2CSA domain-fragments amplified (Table 4.1). Potential

internal N-glycosylation sites were not removed as the fragments were not subjected to a complete internal re-codonisation.

P. pastoris strain GS115 was used to express the *var*2CSA fragments after cloning into the pPICHOLI vector. The vector expresses heterologous sequences as fusion proteins, purified from the cytosol of the yeast cell after induction then cell lysis. The hexa-histidine 'tag' sequence is followed by a peptide sequence that can be biotinylated to facilitate purification via affinity chromatography. However this adds 34 amino acids of molecular weight (4.2kDa) to each yeast expressed recombinant protein. Rabbit anti-sera raised against bacterial recombinant material derived from *var*2CSA protein domains was also used to characterise the yeast derived recombinant proteins (a gift from Dr. Ali Salanti, Copenhagen University). Bacterial recombinant proteins, (made in Rosetta^{TM} *E. coli* strains designed to permit enhanced formation of disulphide bonds) were used to immunise the rabbits. Immunising constructs corresponded approximately to the DBL3X, DBL4ε and ID2 (CIDR-like) DBL5ε domains.

Of these rabbit antisera, the anti-DBL3X and anti-DBL5ε were surface reactive with two laboratory parasite isolates and the DBL5ε antisera reacted positively with 3 tested laboratory isolates and 1/3 field-collected placental *P. falciparum* isolates (Dr. Ali Salanti, unpublished observations). Because of the high backgrounds often observed with rabbit antisera in Western Blots, the antisera have not been used in Copenhagen in Western blots and a major effort to make monoclonal antibodies against this protein is underway in this laboratory (Dr. A. Salanti pers.com).

Figure 4.1 shows the results of Western Blots carried out here using the anti-His tag mouse monoclonal antibody (2 plates) and the anti-*var*2CSA recombinant domain polyclonal rabbit antisera (3 plates). To test for cross reactivity of rabbit serum, extracts from the yeast cells without plasmids, yeast cell with the plasmids without insert and the yeast cell with the insert that has been grown in the glycerol (i.e. before induction of recombinant protein expression) were included as controls.

The data obtained indicate that there is a problem of cross reactivity with the rabbit anti-sera. Western Blots with the anti-DBL3X antibodies indicate that there are strongly cross reactive products in all four lanes, even although only Lane 4 contains

the methanol-induced pPICHOLI plasmid containing DBL3X. The expected size of the recombinant fusion protein from this plasmid is 40.5kDa. A band of around 42-47kDa is visible in Lane 4 of plate A.1 but the rabbit antisera also cross-reacts strongly with Lanes 1-3. The Coomassie stained gels of these preparations are full of protein in this molecular weight range and this size of induced band was not easily distinguished in stained gels (data not shown).

However, when the induced material in lane 4 was purified using affinity columns and the fractions eluted off the Nickel column were run on SDS PAGE (plate B.1.1) and then Western blotted with the mouse monoclonal anti-His tag antibodies (plate B.1.2), a single ~42-45kDa band was detected. It is therefore concluded that a protein of approximately, or slightly larger than the size expected, with a hexa-His Tag sequence is being produced from the pPICHOLI 1/DBL3X plasmid vector.

The second *Pichia* expressed domain construct analysed was that containing DBL4ε. In this case the size of the induced product is expected to be 31.5 kDa. Again the rabbit antisera give background staining (somewhat less marked than in the previous experiment) but this time a distinct reaction is seen with an approximately 32-35kDa band in the methanol-induced preparation containing the pPICHOLI1/DBL4ε plasmid (Lane 4 of plate A.2). When this material was grown in larger cultures to make more protein and the Nickel column elution fractions were run on SDS PAGE gels (plate B.2.1) and Western blotted with the anti-His Tag antibodies then a distinct purified band of approximately the expected molecular weight (31.5kDa) was seen (plate B.2.2). Thus a protein of the size expected, with a hexa-His Tag sequence appears to be produced from the pPICHOLI 1/DBL4ε plasmid vector.

The third *Pichia* domain construct analysed contained DBL5ε, whose induced product is expected to be around 29.5 kDa. Again the rabbit antisera give cross-reactive binding (somewhat less marked than in the previous experiment but again showing the ~50kDa cross reacting band of unknown origin) but this time a distinct reaction is seen with an approximately 29-32kDa band in the methanol-induced preparation containing the pPICHOLI1/DBL4ε plasmid (Lane 4, plate A.3). Unfortunately, after scaled up production no His tagged product could be detected on

Western Blots (data not shown). In this case although a protein of the size expected was detected with the rabbit antibodies, no hexa-His Tag sequence appears to be attached (or free to bind detection antibody) and it can thus neither be purified on affinity columns nor detected with the antibody. A similar situation was observed with the pPICHOLI1/CIDR plasmid constructs. This is unfortunate as it is very difficult to purify and analyse these products.

These experiments demonstrated that two yeast clones with var2CSA/pPICHOLI1 plasmids, those containing the DBL3X and DBL4ε domains, yielded products that were both 'His-tagged' and recognised by the rabbit polyclonal domain-specific anti-sera. These were grown in larger quantity for further purification (1) litre cultures in 2 litre baffled culture flasks).

After following the growth protocol used for the smaller cultures, the larger samples were concentrated into pellets by centrifugation and lysed using the Yeast Buster Protein Extraction Reagent (Novagen) (with addition of benzonase nuclease to decrease viscosity of the lysate). The resulting insoluble debris fraction was pelleted by centrifugation and the lysate was then passed through a 0.2 micrometre filter. The insoluble fraction, crude and filtered lysates were fractionated by SDS PAGE. Proteins were transferred to membranes by Western blotting, which showed that recombinant proteins are present mainly in the soluble fractions.

Using the filtered lysate material, the DBL3X and DBL4ε recombinant proteins were further purified by liquid chromatography using the AKTA prime system. This exploits binding of the N-terminal histidine 'tag' to metallic nickel ions on the 'Hi-Trap' chelating column. The HiTrap column (capacity of 1ml of column material) was washed with distilled deionised water, loaded with 100mM $NiSO_4$, then washed again with water prior to loading the protein sample which was pre-dialysed into the binding buffer (20 mM sodium phosphate, 0.5M NaCl pH 7.4). The sample was filtered through a 0.2 micrometer filter before loading onto the Aktaprime column at a rate of 2ml/min. Bound protein was eluted with column elution buffer (washing buffer with 500mM imidazole added to compete for Ni binding) and collected in twenty fractions. The protein content of the fractions was measured by a Bradford assay. Fractions containing protein were run on SDS PAGE prior to Coomassie blue

staining. These gels show the presence of the expected band s for the DBL3X and DBL4ε constructs (as shown above in Figure 4.1).

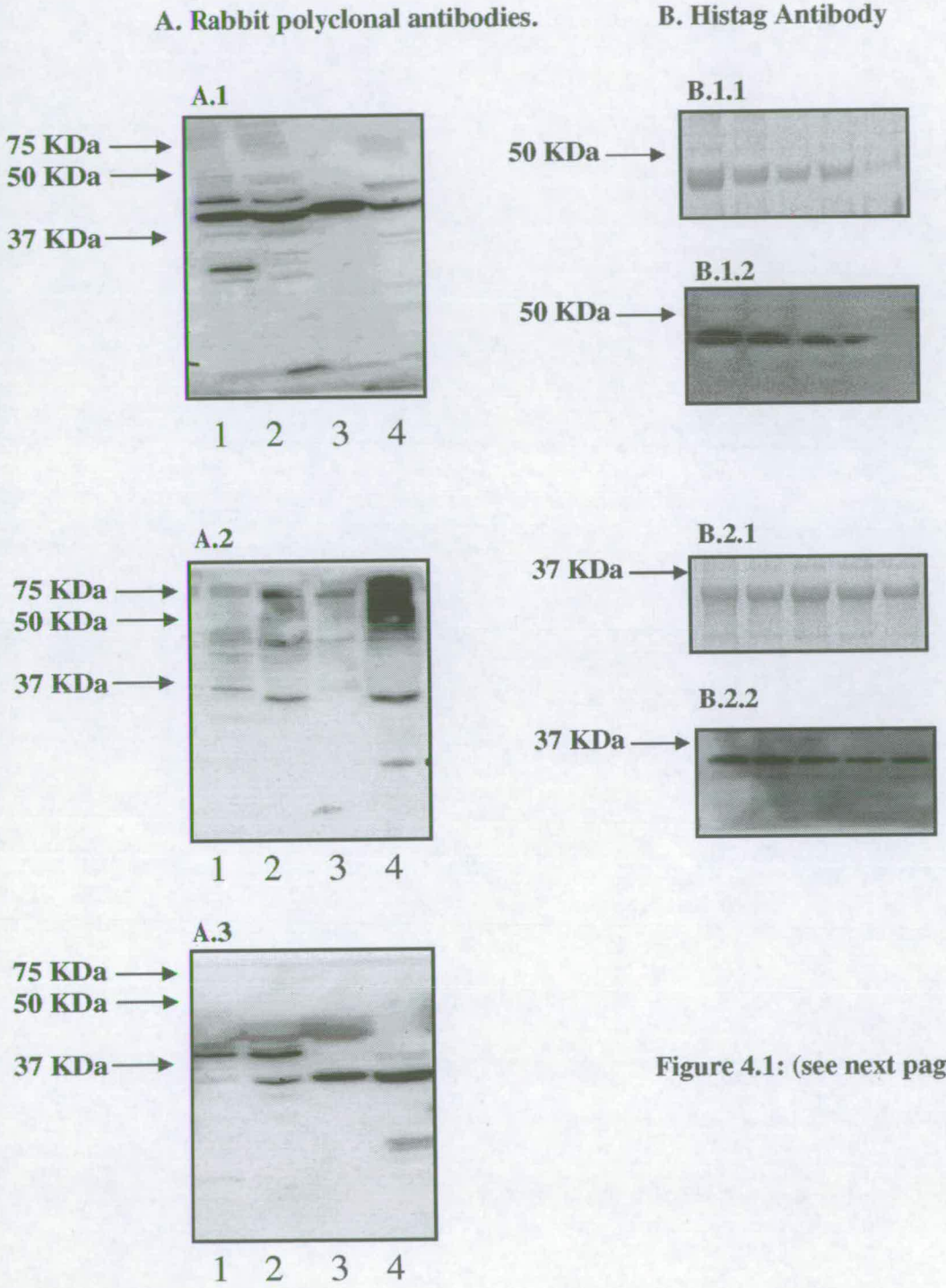


Figure 4.1: (see next page)

Figure 4.1: Western blot analysis of P. pastoris expressing pPICHOLI1 plasmids containing DBL3X, DBL4ε and DBL5ε domains. The proteins were separated by SDS-PAGE using 4-12% gels and then transferred to PVDF membranes, incubated with rabbit polyclonal anti-sera (1:250) raised against recombinant *var*2CSA DBL domains (**plate A**), followed by detection with HRP-conjugated anti-rabbit immunoglobulins (Dako) (1:1,000)(**plate B**). **Plate A**: **Plate A.1**. *Pichia* expression of pPICHOLI1 containing a DBL3X domain, probed with an anti DBL3X antiserum. **Plate A.2**. *Pichia* expression of pPICHOLI1 containing a DBL4ε domain, probed with an anti DBL4e antiserum. **Plate A.3**. *Pichia* expression of pPICHOLI1 containing a DBL5ε domain, probed with an anti DBL5ε antiserum. **Lane 1**, Untransformed *P.pastoris* extract. **Lane 2**. *P.pastoris* extract transformed with the pPICHOLI1 expression vector without cloned insert, grown on glycerol. **Lane 3.** *P.pastoris* extract transformed with the pPICHOLI1 expression vector with cloned insert, before methanol induction. **Lane 4**. Extract of transformed *P.pastoris* containing the inserts of interest, lysed after methanol induction of plasmid expression. **Plates B**: fractions eluded from scaled up protein preparations purifications, run on SDS PAGE gels and then Western blotted with mouse monoclonal anti-His Tag antibody. **Plate B.1.1**: gel analysis of the expression of pPICHOLI1 containing a DBL3X domain stained by Coomasse stain and by Western blot using 6XHis tag antibody (**B.1.2**) (Qiagene). **Plate B.2.1:** gel analysis of the expression of expression of pPICHOLI1 containing a DBL4ε domain attained by Coomasse stain and by Western blot using 6XHis tag antibody (**B.2.2**). All molecular weights are estimated from (BioRad) markers and are given in kilo Daltons.

4.3. Human antibodies to the purified DBL3X and DBL4ε recombinants.

To investigate whether humans mount an antibody response to these *var*2CSA-derived recombinant proteins following malaria infections, an ELISA assay was set up using antibodies from malaria infection samples collected in Sudan. These samples were collected by Dr. Nasreddin Abdulahi during the course of a study

investigating immune responses to *P.falciparum* antigens in 'sickling' (HbAS) and 'non-sickling' (HbAA) individuals. The serum samples were collected from adolescents and adults of both sexes as they presented with malaria symptoms at clinics in and around Gedaref, a large town in Eastern Sudan, during 1989. These samples were part of a batch that Dr. Abdulahi brought to Dr. Arnot's laboratory for analysis during a research visit in 1990. Dr. Arnot accompanied Dr. Abdulahi on a collection trip around the Gedaref health posts in 1989 and thus knows the setting and study plan. Dr. David Cavanagh identified a subset of this collection (around 20% of the samples) that had moderate-to-high levels of antibodies that recognised the C-terminal recombinant fragments of MSP-1.

The binding of antibodies to recombinant DBL3x and DBL4ε in these 'Gedaref infection sera' was assayed, in duplicate, on microtitre plates, each coated with 100 ng of purified recombinant-derived antigen prior to assay by ELISA. $MSP1_{19}$ was used as positive control and measure of that individual serum's reactivity to a defined *P.falciparum* merozoite antigen. Because a pooled malaria naïve serum was used a negative control, without standard positive serum or monoclonal antibody control it is not possible to create a standard curve for either positive or negative control sera. Nonetheless with these sera and controls it is certainly possible to detect a fairly strong and specific level of reactivity to all three recombinant antigens with the great majority of Sudanese infection serum. No such malaria antigen reactive antibodies are present in the Scottish malaria naïve serum pools.

In some ways this is a surprising result. It was not unexpected that the Sudanese serum, taken from individuals attending clinics during the malaria season, actually had quite high levels of anti-malaria major merozoite antigen antibodies. In fact these 'Gedaref clinic' sera were pre-selected because they were known to be 'high-responder' anti-MSP-1 samples, with antibody levels similar to those found in many Sudanese infection and post-infection samples from the nearby village of Daraweesh (Cavanagh *et al,* 1998). What was less expected is that so many of these individuals appear to have significant levels of antibodies to domains of a particular PfEMP-1 erythrocyte surface antigen which is known to be up-regulated by *in vitro* selection of parasites for CSA adhesion (Salanti *et al,* 2003) and probably also by *in vivo* selection for placental adhesion (Salanti *et al* 2004). Few or none of the donors of

these original blood samples were pregnant, many were males and pre-adolescent children and the low intensity transmission conditions of Eastern Sudan are not thought to lead to high levels of anti-*var*2CSA antibodies. Such antibodies have been proposed to protect adult females from the pathogenic effects of pregnancy malaria (Salanti *et al,* 2004).

2/20 samples gave low or negative responses to all three recombinant antigens (serum 11 and 20). In one third of the positive samples (6/18), responses to $MSP1_{19}$ were stronger than those to the *var*2CSA-derived antigens. However in two third of the Gedaref clinic acute infection samples (12/18) the antibody response to at least one of the var2CSA derived antigens was equal to or greater than that to the major merozoite antigen MSP1 fragment. In 7 samples, the response of both the var2CSA derived recombinant proteins was greater than that observed to $MSP1_{19}$ (Figure 4.2).

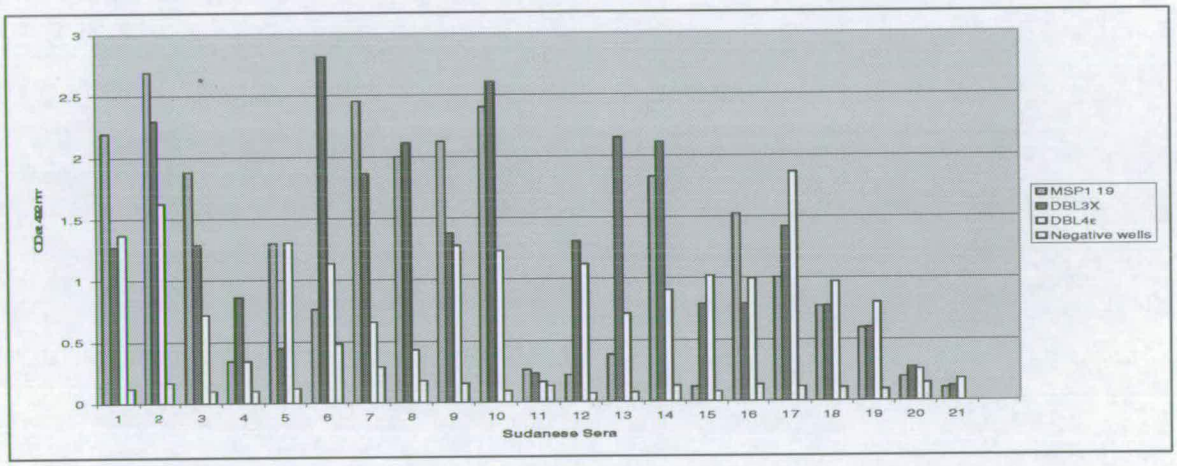


Figure 4.2: Reactivity of three recombinant produced malaria antigens (C-terminal MSP-119, PfEMP-1 var2CSA DBL3X and PfEMP1 var2CSA DBL4e) with malaria infection serum from Sudan and pooled Scottish malaria naïve serum. Samples 1-20 are individual Sudanese sera reacted with the three antigen coated wells and a blank well. Sample 21 is the Scottish serum pool reaction with these antigens. 100ng of each antigen preparation, dissolved in PBS, was used to coat the wells (overnight) of a 96 well Thermo Dynex Inc. microtitre plates. Absorbance was measured at 492nm in a Labsytems Multiscan Ascent plate reader.

4.4. Recognition of the purified yeast recombinant DBL3X and DBL4ε proteins by pooled male and pregnant female malaria immune serum from Ghana.

To further test the antigenicity of the purified yeast recombinant proteins they were then tested with a three pools of antisera to further investigate immunogenicity of the two recombinant produced *var*2CSA domains.

The experiment also assayed whether these var2CSA derived recombinant proteins are recognised in a sex and parity dependent fashion. The phenomenon of sex and parity dependent antibody recognition of placental-type *P.falciparum* parasites was first reported by Ricke *et al* (Ricke *at al,* 2000). Currently *var*2CSA is considered to be the leading candidate antigenic target of such antibodies (Salanti *et al,* 2003, 2004).

The serum pools used were the same as those previously used in studies that showed CSA binding parasites express trypsin resistant variant surface antigens (VSA) that bind the female-specific antibodies induced by pregnancy associated malaria (Sharling *et al* 2004). The pools are a 'male serum pool' of 20 serum samples from men living in a malaria endemic region of Ghana. The 'pregnant female serum pool' consists of pooled serum from 15 Ghanaian women, collected at delivery. This pool included 5 primigravidae (even not likely to have Abs against *var*2CSA binding domains), nine secundigravidae and one multigravid woman. Six Scottish malaria naïve individuals donated sera to create the 'malaria negative pool' serum. The bacterially produced $MSP1_{19}$ GST fusion recombinant protein was included as a positive control (the Ghanaian sera were the gift of Dr. Mike Ofori and Dr Trine Staalsoe, University of Copenhagen and the $MSP\text{-}1_{19}$, used above, was from Dr. David Cavanagh,).

When the PfEMP-1 *var*2CSA derived antigens were probed with both the male and the pregnant female serum pools sera, there was fairly strong reaction with both sera, although the response was stronger to the MSP-1 antigen. Thus no parity and gender specific reactivity was observed in the response to these var2CSA derived recombinant antigens (Figure 4.3). Again this finding is not exclusive as if we are to conclude a clear gender specific recognition, reasonable number of individual male

and pregnant sera should be tested , then a statistically conclusion could be reveal whither an antigen is recognised in gender specific manner.

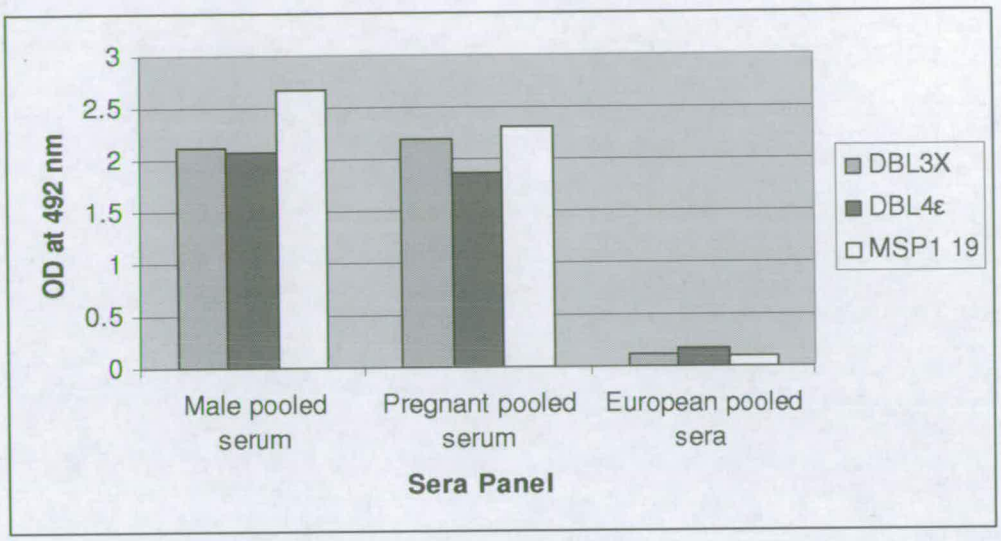


Figure 4.3: ELISA readings from pooled male, pooled pregnant female serum and Scottish sera incubated with the purified DBL3x and DBL4e domains from var2CSA protein. All sera were used at a dilution of (1:1000).

4.5. Differential screening of arrayed expression libraries with male and pregnant female sera from Ghana.

As well as *var*2CSA, the DBL3 (also known as DBLγ) domain of the *var*1CSA encoded protein has been implicated in CSA binding and proposed to have a role in the pathogenic processes involved with PAM (Buffet *et al,* 1999). Therefore an experiment was designed to investigate if this class of sequences in the *P.falciparum* FCR3CSA isolate might be expressed and differentially screened with male and pregnant pooled sera from Ghana.

Colonies (90 different clones, arrayed in duplicate pairs) from a library of sequences amplified from FCR3CSA genomic DNA with 'degenerate primers' flanking a semi-conserved sequence in the DBL3 type domain were picked from the original transformation master plates into 96-well growth plates (in duplicate). The methods used in the creation of this domain sub-library were described in Chapter 3.

The pPICHOLI plasmid was used as the transformation vector. Colony arrays were prepared by seeding colonies to High Bond-C Extra (Amersham) nylon filter membranes. The *E.coli* filters were induced for recombinant protein expression and then lysed to liberate intra-cellular recombinant protein; the filters were blocked with non fat dried milk (5%) in PBS overnight. One of the membranes was then incubated with Ghanaian male sera, while the other was incubated with Ghanaian pregnant female sera. Two identical membranes containing the DBL3 type domain arrays were first tested with Scottish sera. No positive, antibody-binding colonies were observed with malaria naïve sera.

Figure 4.4 A&B shows that the Ghanaian pregnancy serum always reacted slightly more strongly with expression products than the Ghanaian male sera (a dilution of 1:5000 for the pregnant women and 1:2000 for the male sera was used to compensate for this). This stronger reactivity of infection sera from pregnant women is probably because these samples are taken from women with an active placental malaria infection who thus had significantly higher levels of anti-malaria antibodies than the relatively immune adult male who probably have not had any recent active clinical malaria infection.

Interestingly, when the duplicated expression clone arrays were screened with the different sera, there was a clear differential recognition of some clones. Certain clones are clearly reacting with the Ghanaian pregnancy serum but not with the Ghanaian male serum (Figure 4.4).

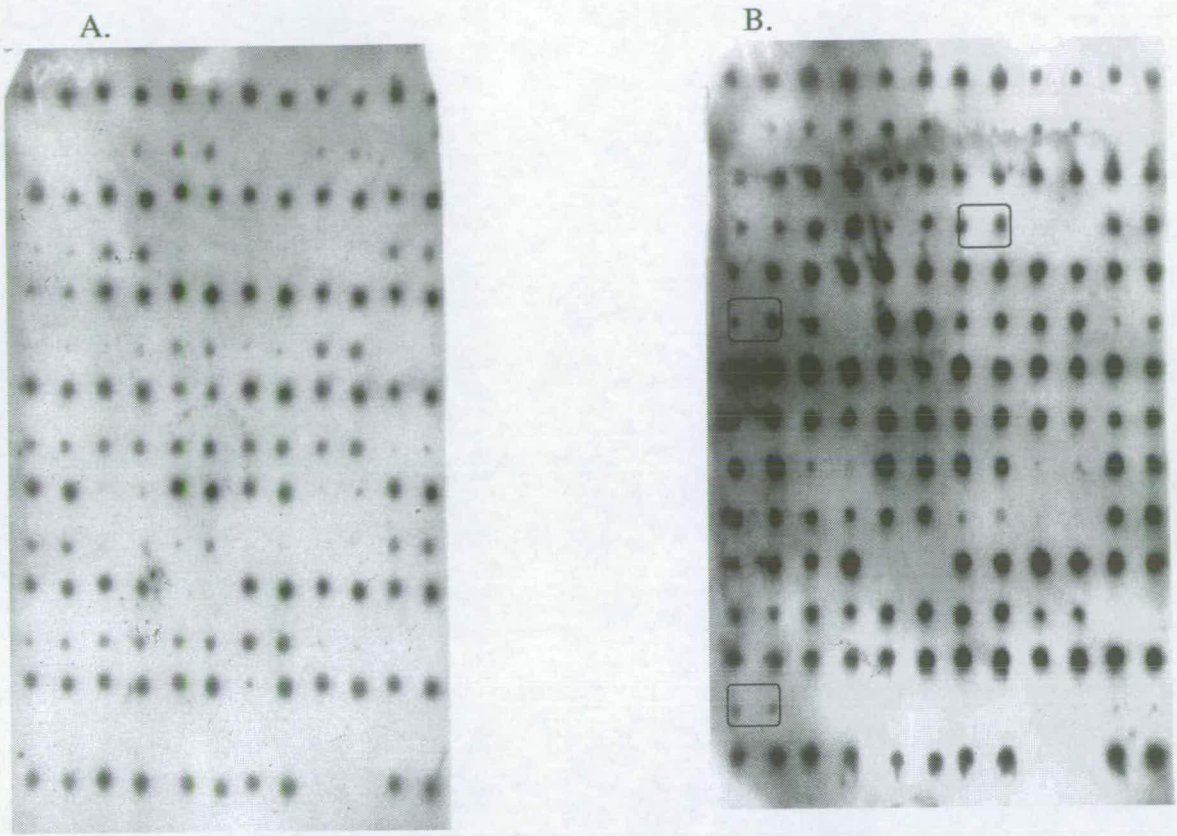


Figure 4.4: Immuno-screening of duplicated bacterial expression arrays of DBLγ type domains. A. Pooled Ghanaian male sera. B. Pooled Ghanaian pregnancy sera. After lysis, the membranes were first incubated with the primary sera at dilution of 1:5000 for the pregnancy sera and 1:2000 for the male sera. This was followed by HRP-conjugated rabbit anti-human IgG immunoglobulins (1:1,000) (Dako). Differential recognition of pairs of colonies binding female pregnancy derived antibodies but not male endemic serum antibodies is illustrated by boxes.

4.6. Characterisation of the PfEMP-1 *var*-gene fragments encoding epitopes that are differentially recognised by male and multigravid female endemic area serum antibodies.

The DBLγ expression library has been described in Chapter 3 (samples of these sequences are listed in the Appendix 1).

The three differentially recognised (i.e. pregnancy + / males -) clones were sequenced and two of the three were found to be identical (the DBLγ3/5 clone). This

serves as a good internal confirmation that the observed gender specific serum recognition is a repeatable phenomenon. The sequence of this fragment, and an identical sequence from the database, is shown in Figure 4.5. This sequence that is identical to the DBLγ3/5 over 481 nucleotides and 160 amino acids is a previously described *var* gene fragment (Accession number AY248877, from the *P.falciparum* isolate IT4/25/5 (Kraemer *et al,* 2003).

This immuno-detected 'gender-specific' clone from an FCR3CSA genomic DNA derived library is thus identical to a sequence originally identified using a novel series of type specific primers for the DBL-beta, gamma and delta types (Kraemer *et al,* 2003). The paper by Kraemer and his colleagues also states that the A4 clone used in their study as a DNA source is a derivative of the *P.falciparum* line IT4/25/5. The authors then state that, "the IT4/25/5 isolate is one of a series of isolates including the FVO, FCR3 and Palo Alto parasites that appear to have a common genetic origin due to a laboratory cross-contamination event [33]". The reference 33 is to the paper of Robson *et al* (Robson *et al,* 1990). It seems likely that part of the explanation for the 100% match with Kraemer and colleagues' sequence is that rather than working with two different isolates, we and they are actually working with the same clone. Why this clone might appear 'gender-specifically' recognised by our serum pools will be discussed later.

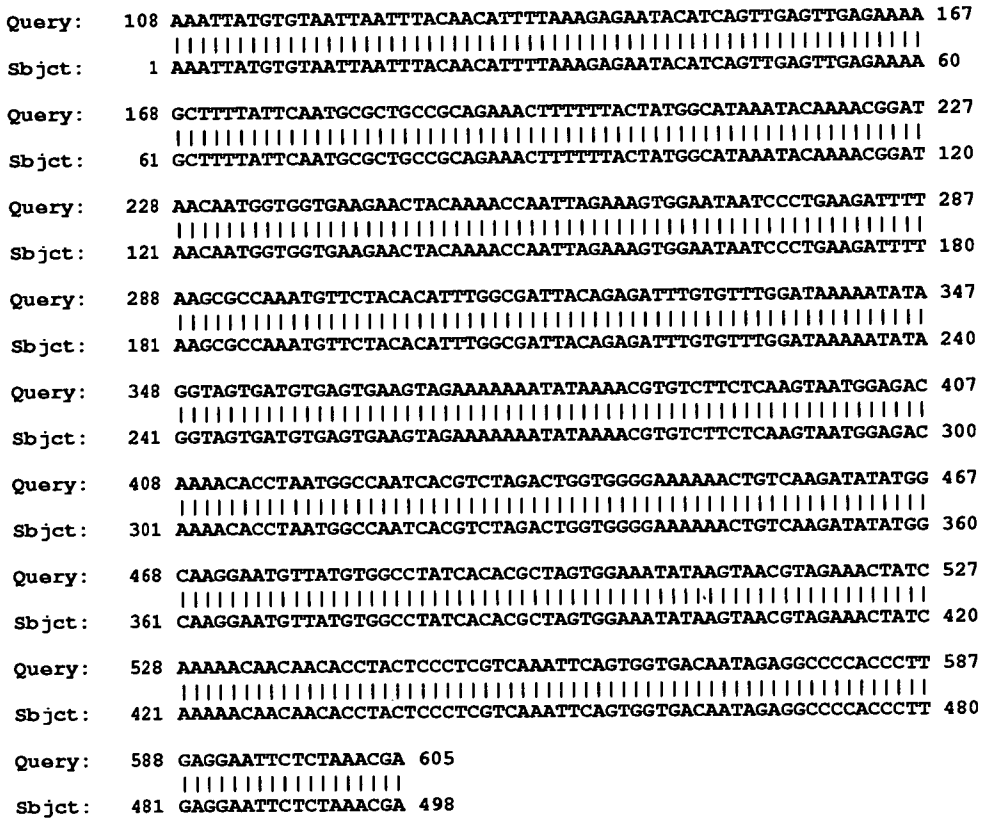


Figure 4.5. Nucleotide sequence alignment of DBLγ-5 and DBLγ type domain (Accession number AY248877) of isolate IT4/25/5 revealed that they have 100% homology.

The other sequence is a named DBL3γ-13 which, to date, has no exact homology with any sequence in the databanks, although it is has homology of (98%) of 546/553 nucleotides (Accession number AF193424) with the DBLγ domain of a PfEMP-1 *var* gene (Smith, J *et al,* 2000a), again of the IT isolate (clone A4tres), a gene encoding a protein that has been described to bind ICAM1 which has been implicated in cerebral malaria (Smith *et al,* 2000a) (Figure 4.6).

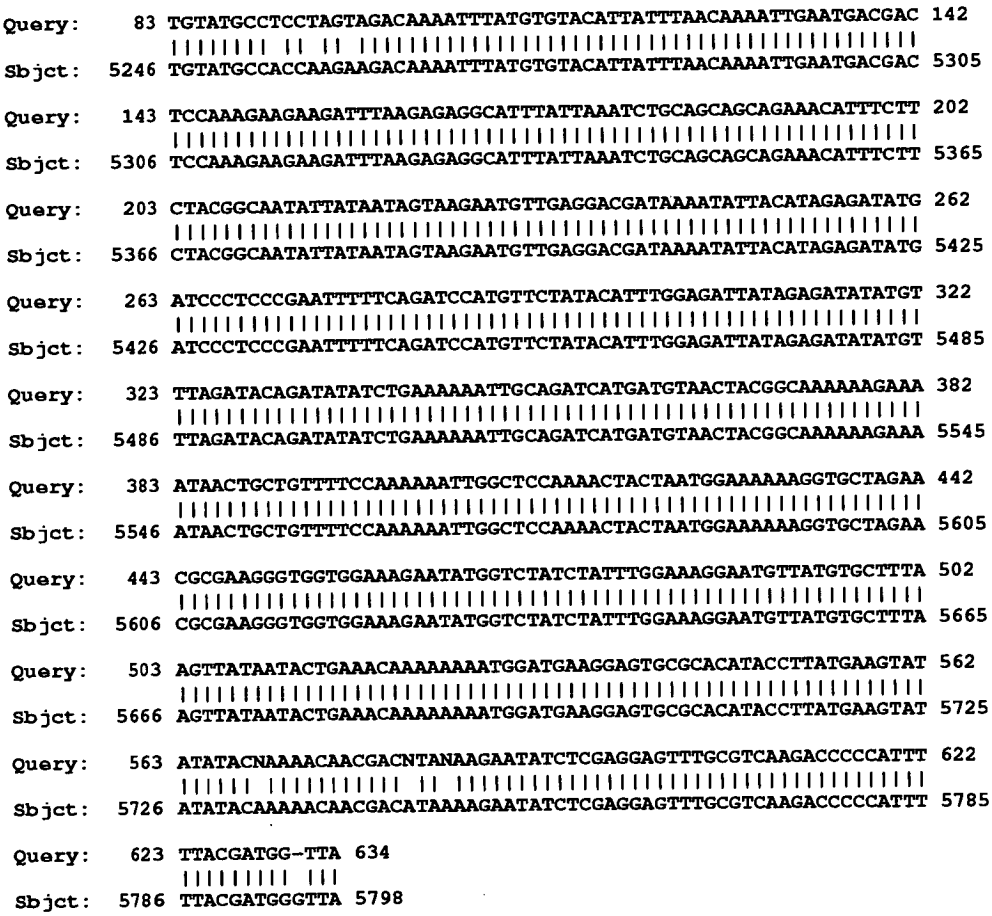


Figure 4.6: Nucleotide sequence alignment of DBLγ-13 and a fragment of *var* gene implicated in binding to ICAM1 domain (Accession number AF193424) of isolate IT revealed that they have 98% homology.

This identification of a *var* gene fragment that encodes an antigenic epitope that is recognised by multigravid female serum but not by male endemic serum is interesting. This is one, probably important, characteristic of the type of sequence that is a candidate antigen for inclusion into a malaria in pregnancy vaccine designed to give women who have never been pregnant a vaccine-induced immune response which mimics that of multigravidae who are protected against PAM. This will be discussed further in the Conclusions in Chapter 5.

4.8. Conclusions.

The sequencing of the genome of the malaria parasite *P. falciparum* has permitted much more rapid genomic and proteomic studies of this pathogen which have clearly made it easier to identify novel potential drug and vaccine targets (Hanash, 2003). The use of gene expression micro-arrays has permitted us to identify certain protein epitopes that appear to be recognised by antibodies present in pregnant women infected with *P. falciparum* but which are not recognised by compared with males. It has also been confirmed that dual expression - shuttling between two conventional expression systems can be useful in expression research. Using this vectors, and the fact it produces intracellular recombinant proteins, the glycosylation can be circumvented. Glycosylation is carried out in the endoplasmic reticulum(ER) and processed further in Golgi body to form the glycoprotein. There is, however, evidence concerning N- linked glycosylation of the extracelluar *P. falciparum* protein is contentious. Although it is suggested that it is not possible in situ due to the supposed lack of glycosylation site (Dieckmann-Schuppert *et al*, 1992), protein expressed in eukaryotic systems have in fact been shown to be glycosylated (Hodder *et al*, 1996).

When using rabbit antisera with CIDR1, DBL1X and DBL2X, rabbit antibodies appear to bind these *Pichia* expressed domains in Western blots. When using the purified recombinants for DBL3X, DBL4ε in ELISA studies, these products showed, in some individuals, strong antibody reactions to all three recombinant antigens. The purified yeast recombinant proteins were tested to further investigate whether the two yeast recombinant produced *var*2CSA domains are recognised in a sex and parity dependent fashion. When the PfEMP-1 *var*2CSA derived antigens were probed with both the male and the pregnant female serum pools sera, there was strong reaction with both sera and thus no parity and gender specific reactivity was observed.

An experiment was designed to investigate if DBL3γ class of sequences in the *P.falciparum* FCR3CSA isolate could be expressed and differentially screened with male and pregnant pooled sera from Ghana. When the two identical expression clone arrays were screened with the different sera, there was a clear differential recognition

of small group of the duplicated clones. Some clones are clearly reacting with the pooled pregnancy serum but not with the pooled male serum.

This identification of a *var* gene fragment that encodes an antigenic epitope that is recognised by multigravid female serum but not by male endemic serum is interesting. Maternal and neonatal immune responses directed to peptides corresponding to conserved regions of the DBL-γ3 domain of PfEMP1 have been analysed by other workers. It has been found that two of the four peptides were particularly immunogenic, associated with both B- and T-cell responses in pregnant women and their offspring (Brustoski *et al*, 2005).

The antigen arrays when screened for capacity of the target antigens to be recognized by sera collected from clinically distinct cohorts of individuals naturally exposed to malaria. The anticipated outcome of these studies will facilitate the identification of those proteins that correspond to immunodominant antigens and will prioritise the investigation of those antigens and epitopes on the basis of immune reactivity and according to their potential association with protection against *P. falciparum* challenge. (Doolan *et al*, 2003).

Chapter 5. Conclusions and Future Directions.

The overall aim of this study was to investigate, both singly and in a combined 'dual expression' system, the heterologous expression of *P.falciparum* PfEMP-1 antigen fragments in *E.coli* and *P. pastoris.* If this could be achieved it would be possible to first characterise and then scale–up production of candidate antigens that can be used in anti PfEMP-1 mediated cytoadhesion blocking blood stage malaria vaccines. The choice of a CIDR domain as an initial test of the *Pichia* expression system was based primarily on the fact that the CIDR1-type domains of PfEMP1 has been identified as capable of binding to CD36 (Baruch *et al,* 1995, Newbold *et al,* 1997, Beeson *et al,* 1999). It has also been shown that the same region can also mediate binding to chondroitin sulfate A (CSA) on the surface of Chinese Hamster Ovary (CHO) (Degen *et al,* 2000). It was then aimed to express this domain for parsite/host adhesion assays.

Choosing a suitable expression system for a particular protein depends primarily on the properties of the protein, the amounts required, and its intended purpose. For malaria vaccines based on PfEMP-1 the following seem to be the most important considerations;

1). Recombinant protein conformation. As near native conformation of the product as is possible is required in order for the recombinant protein to elicit antibodies that will recognise the native antigen *in vivo.*

2). The method must be high yielding and capable of being scaled up. This is important for the long-term goal of producing a GMP quality antigen for vaccination trials. Large amounts of pure material are also important for structural characterisation. Excessive modification (e.g. glycosylation) by the production cell must be avoided.

There are several protein expression systems in prokaryotes and eukaryotes. They include: bacteria - e.g. *E. coli*, Yeast (e.g. *P. pastoris* and *S. cerevisiae*), cultured insect cells and cultured mammalian cells. The advent of recombinant DNA technology makes it technically feasible to produce any protein of interest in a

number of different organisms. However technical feasibility does not guarantee production success. Factors affecting expression include initiation of transcription, RNA elongation, RNA stability, initiation of translation, translational elongation, polypeptide folding and post-translational processing including amino-terminal modification of polypeptide, disulphide bond formation, proteolytic cleavage of precursor forms, glycosylation and modification of amino acids within proteins. Production is also affected by the rate of degradation (Rai and Padh, 2001)

The expression of proteins in *E. coli* is an important and frequently used tool within malaria research although the method remains problematic. Due to lack of an appropriate redox environment within the cytoplasmic compartment that rendered it difficult to produce correctly folded molecules that require the disulfide bonds formation. Recombinant protein expression in *E.coli* can yield insoluble and misfolded or poorly expressed protein. (Yadava & Ockenhouse, 2003). Although methods have been developed to obtain correctly folded proteins from these inclusion bodies, the process of refolding cannot be successfully applied to all proteins (Pandey *et al*, 2002; Singh *et al*, 2003).

The methylotrophic eukaryotic expression host, *P. pastoris*, has been successfully developed as an alternative to the *E. coli* system (Hollenberg & Gellessien 1997, Cereghino & Cregg 2000). Yeasts are simple eukaryotes and the most frequently used alternative to bacteria. The main advantages of yeast cells are that they perform many eukaryotic, post-translational modifications. The main disadvantage of yeast cells is the presence of highly active proteases that degrade foreign proteins, so that reducing their yield. Yadava and Ockenhouse have produced two synthetic genes coding for part of the 175-kDa *P. falciparum* erythrocyte-binding antigen (EBA-175) to determine the effects of codon alteration on protein expression in homologous and heterologous host systems (Yadava & Ockenhouse 2003). Synthetic genes were designed based on *P. falciparum*, *E. coli*, and *Pichia* codon usage. Compared to the expression of the native sequence, conversion to *E. coli*- or *Pichia*-based codon usage dramatically improved the levels of recombinant protein expression in both *E. coli* and *P. pastoris*.

The majority of the protein expressed in *E. coli*, however, was produced as inclusion bodies, whilst the *P. pastoris*-expressed protein was secreted and soluble and (unlike the its *E. coli*-expressed counterpart) was functionally active (able bind to red blood cells). Consistent with these observations, antibodies generated against the *Pichia*-produced protein prevented the binding of recombinant EBA to red blood cells and recognize EBA-175 on merozoites and in sporozoites by IFA. Protein expressed in P. pastoris has been shown to be superior to that produced in *E. coli* based on its ability to bind to red blood cells (Yadava & Ockenhouse, 2003). Glycosylation in eukaryotic expression systems is carried out in the endoplasmic reticulum (ER) and processed further in the cis-Golgi to form a glycoprotein. This occurs in 0.5-1% of eukaryotic translated proteins. However, evidence concerning N- or O- linked glycosylation of extracellular *Plasmodium* proteins is contentious. Although it is suggested that it is not possible in situ due to the supposed lack of glycosylation sites (Dieckmann-Schuppert *et al,* 1992) proteins expressed in eukaryotic systems have in fact been shown to be glycosylated (Hodder et al, 1996). It is often the case that the same protein can be recognised by different glycosylation enzymes which creates a population of oligosaccharide heterogeneity (Yan *et al,* 1999). The formation of disulphide bonds and protein folding occurs in the ER and systems have been optimised with the yeast *P. pastoris* (Daly and Hearn, 2005).

The expression vector that allows protein expression in both prokaryotic and eukaryotic systems greatly reduces workload, time and costs, a good and tested examble is the dual expression vector (Leuking *et al,* 2000). It has been confirmed, to some extent, that the dual expression system is a potentially valuable reagent in malaria vaccine industry as it can be shuttled between two conventional expression systems. Due to high A/T content and frequent lysine and arginine repeats in the *P. falciparum* genome are thought to be the main reason for the frequent problem of early termination in the mRNA translation process. The majority of *P. falciparum* derived recombinant proteins are expressed only as truncated forms or as insoluble inclusion bodies within the bacterial cells and the expression of *P. falciparum* derived proteins, especially membrane-bound proteins, is still a considerable challenge (Flick *et al.*, 2004). Malaria genes to be expressed in heterologous expressition system, one has to consider the recodoning of the gene of interest to

improve the protein yield or even start the translation. Two other main methods are available to increase the expression levels of proteins which containing rare codons in host cell, which are the site-directed mutagenesis and the co-expression of the genes which encode rare TRNAs. So far, this system is perhaps the most promising one for GMP (industrial scale and guaranteed quality) productions.

The results from the first phase of this project indicate that the *Pichia* expression system can express a *P.falciparum* CIDR-domain derived cysteine-rich recombinant protein fragment. The quantity and quality of product obtained notably improved after performing a scaled-up automated fermentation run. Using this system, a time dependent increase in the amount of CIDR-like product of the expected MW (~28kDa) was consistently produced. This product was provisionally confirmed as the intended CIDR product following in Western blot using antibodies to the engineered poly-histidine tag epitope. Now that mass spectroscopy based sequencing and protein characterisation methods have become available to us in the Department of Chemistry in Edinburgh, a full sequence characterisation of this candidate product could be carried out.

Detection of the candidate CIDRI decreased with the time, suggesting that some degradation was taking place. Some evidence of aggregation was observed in the *Pichia* derived protein solutions analysed here, which quickly took on a cloudy appearance. Aggregates have been common in the production of other PfEMP-1 domains and this makes their handling and analysis problematic (Dr. Graham Bentley, Institut Pasteur, personal communication). In fact, this problem affected material that was sent to Dr. Bentley and little progress was made in the X-ray crystallographic analysis of this material, despite the fact that it was being made in sufficient quantity to carry out crystallisation experiments.

The affinity purified CIDR1 protein was also used as the target antigen in ELISA assays using $MSP1_{19}$ positive sera from malaria endemic areas. Scottish malaria-naive sera served as a negative control that was not expected to show reaction to the *P.falciparum* antigen. The positive control, expected to be recognised by most malaria infection sera, was the most conserved fragment of the major merozoite surface antigen, $MSP1_{19}$ .Results showed that most of the Sudanese sera

tested recognised both this merozoite antigen and the PfEMP-1 CIDR1 antigen fragment. The implication is that this antigen, or a similar structure which elicits antibodies which cross-react with this antigen, has been expressed during the ongoing or recent malaria infection suffered by this Sudanese malaria season acute infection cohort. Given that the CIDR domain is implicated in CD36 binding and that CD36 binding is thought to be a common adhesion phenotype, it is certainly possible that cross reactive antibodies recognising a range of different but related CIDR sequences are commonly elicited by malaria infections.

When this recombinant CIDR antigen was tested as the target antigen in another ELISA experiment using two particular pools of male and multi-gravid female sera from Ghana, each pool recognised the target antigen equally well. Given that each member of the groups used to make the serum pool must have had many different malaria infections, it is not surprising that there are antibodies in each serum pool capable of recognising this recombinant protein and the result is in accordance with what would be expected given the previous results showing widespread reaction to this antigen in the Sudanese acute infection sera. It also shows that Ag has epitopes similar to those seen by malaria exposed individuals.

An antiserum was raised in rabbits against the recombinant CIDR and the antibodies from this immunization were tested for reactivity to *P. falciparum* infected RBCs. Monoclonal antibodies to MSP-1 (a gift of Dr Jana McBride and Dr David Cavanagh, Edinburgh University) were used as positive controls. The monoclonal antibody controls were seen to strongly recognize acetone-fixed RBCs infected with late stage parasites. However none of rabbit sera from the immunization study was shown to surface label any stage of intra-erythrocytic malaria parasites (3D7) in IFA following acetone fixing of the cells. This finding was disappointing, but it has been found by many groups that it is difficult to raise antibodies that recognise PfEMP-1 on the infected erythrocyte. A few groups have however, reported success (Chen *et al,* 2004; Gamain *et al,* 2004). That the rabbit sera did not recognize 3D7 parasites infected may be due to our uncertainty that the immunising antigen is structured in its *in vivo* conformation and can thus induce 'conformation dependent' antibodies or the lack of transcription of the *var* gene that contains the nucleotide encoding this domain.

Six different sub-libraries were constructed using primers designed to hybridise to regions flanking the different PfEMP-1 domain types in the *E.coli* and *P.pastoris* expression systems. This was atchived by desingning degenerate primersfor DBLα, DBLβ and DBLγ domain of PfEMP1. These primers were able to recognize a great variety of different sequences and have also amplified DBL type-specific products. These primers could be used broadly and were found to be less biased against particular sequences. Even these primers based in different *P. falciparum* clone (3D7), thus, these degenerate primers could be used for other *falciparum* parasite genotypes (e.g. FCR3CSA). This could be even improved by including other specific or degenerate primers to make these libraries much representative ones, which in turn will help in generation of more collection of *var* gene fragments. The dual *E.coli*/yeast expression shuttle vector (pPICHOLI1) has been shown to be useful for quickly alternating between two conventional expression systems. Direct PCR screening and sequencing of clones showed that different variants of each of the domains in the FCR3CSA genomic DNA are present and growth on the filters, induction, lysis and immunoscreening of these libraries has been optimized. Various membranes, antibody concentrations, and hybridization times were evaluated. Various problems associated with high background reactivity were encountered when using antibodies to screen an expression library. These were mainly resolved by diluting the antibody and increasing the temperature at which the blocking, rinsing and washing steps are carried out (to as high as 50°C). Pre-absorption of antibodies with the the lysates from expression systems of choice also helped to remove cross-reacting specificities.

In addition to making the domain specific sub-libraries, a series of constructs were made using the sequence of a particular PfEMP-1 var gene, the pregnancy associated malaria vaccine candidate, *var*2CSA. Some of these domains were expressed in *P. pastoris* and when using rabbit anti-sera raised in Copenhagen (a gift of Dr. Ali Salanti) against the so-called CIDR1, DBL1X and DBL2X domains, these rabbit antibodies bound the *Pichia* expressed domains in Western blots, although there were clearly problems with high background and widespread cross-reactivity of yeast proteins with these rabbit antisera. When using purified recombinant antigen corresponding to the DBL3X and DBL4ε domains in ELISA studies, these products

bound some antibodies present in most Sudanese acute infection sera but did not react to Scottish malaria naïve serum pools. This could be due to the existence of serological cross-reactivity between *VAR*2CSA antibodies with other PfEMP-1 antigens. It is certainly not likely that any of these serum donors had recently suffered from pregnancy-associated malaria. There are few reports in the literature that appear to shed further light on this phenomenon but it should be followed up by testing with further groups of well characterised antisera, such as samples from the Daraweesh study.

These purified yeast recombinant proteins were tested to further investigate whether the yeast recombinant produced *var*2CSA domains are recognised by serum pools that are derived from individuals living in a much higher malaria transmission zone than Sudan, such as rural southern Ghana. When the PfEMP-1 *var*2CSA derived antigens were probed with both the male and the pregnant female serum pools, there were clearly antibodies in both serum pools that recognised the target PfEMP-1 antigens and gave quite strong reactions in the ELISA assays when compared to the Scottish malaria naïve serum pool. This experiment, using different var2CSA derived antigens than those previously used in a study by Salanti and co-workers (Salanti *et al* 2004), gives a somewhat different result than that observed in this earlier work. This earlier work showed some evidence that anti-VAR2CSA domain antibodies against DBL3X and DBL5ε were significantly more strongly recognised in ELISA assays by plasma antibodies from women, particularly term-pregnant women, than male Ghanaian plasma antibodies (Salanti *et al* 2004). The results presented here can tentatively be interpreted as indicating that the two different Var2CSA derived recombinant antigens used here (DBL3X and DBL4e) can induce either specific or cross-reactive antibodies that occur at some frequency throughout the population, regardless of sex or pregnancy and parity status. However, these results using pooled serum are more difficult to interpret than results obtained using well-characterised individual sera. These experiments with these constructs should be repeated using individual male and female serum although these were not available in quantity during this study.

To explore the parity dependent antigen recognition phenomenon from a different perspective and to use the system and optimised conditions worked out in the course

of the thesis work, an experiment was designed to investigate if DBL3γ class of sequences in the genome of the *P.falciparum* FCR3CSA isolate might be expressed and then differentially screened with the male and pregnancy serum pools from Ghana used in the ELISA experiments. Interestingly, when duplicated FCR3-Dbl3γ domain expression arrays were screened with the different sera, there was a clear differential recognition of a small subset of the clones such that on the first screening, three clones clearly bind antibodies present only in the pooled pregnancy serum.

Further sequencing showed that two of these three 'gender-specifically recognised' clones are identical, a useful internal control showing that the phenomenon is repeatable. The sequence is called DBLγ3/5 has been shown to be identical sequence with a previously described *var* gene fragment (Accession number AY248877, from the *P.falciparum* isolate IT4/25/5 (Kraemer *et al,* 2003). The other sequence is a named DBL3γ-13 which has no exact homology with any sequence in the databanks, although it is has homology of (98%) of 546/553 nucleotides (Accession number AF193424) with the DBL3γ domain of a PfEMP-1 *var* gene (Smith *et al,* 2000a), again of the IT isolate (clone A4tres), a gene encoding a protein that has been described to bind ICAM1.

The significance of these apparently 'gender specifically recognised' DBLγ derived fragment expression proteins is not clear. Time did not permit carrying out more definitive experiments in which first the induced expression clones, and then recombinant antigen purified from these expressors, was tested against a large panel of individual endemic male and endemic pregnancy malaria derived antibodies. It seems unlikely that no adult male would be able to mount an antibody response against an epitope of an ICAM-binding PfEMP-1 protein. At the risk of over-interpreting the data, these results do seem to support the view that DBLγ regions of PfEMP-1 genes can give rise to cross-reactive antibodies.

For various reasons including time constraints and difficulties encountered the possible directions that could be pursued are continue to investigate the gender specific clones. This could be done by screening the whole DBL3γ library, in automated way, screening for target proteins will be the most rapid way then isolate

the clones that are recognised in sex pattern. These antigens are now ready, together with the DBL3X, DBL4ε and CIDR fragments already expressed in *Pichia pastoris* to be used in scaled-up production runs in fermentors for purification of the large quantities of recombinant antigen required for further structural analysis and vaccinological studies. This work and the possibility of constructing larger recombinant antigens which may more closely mimic the complex epitopes that seem to be formed by these large parasite adhesion proteins could form the logical next phase of this work.

Chapter 6: Materials and Methods.

This chapter describes all of the materials and methods used during the course of this project.

6.1 Materials.

This section lists all materials used in this research. Unless stated, all reagents were purchased from Sigma. Suppliers can be found in Appendix 1.

6.1.1 *P. falciparum* parasites:

Name	Phenotype	Source
3D7	standard laboratory clone, Amsterdam Airport strain	cloned and maintained in Edinburgh
FCR3CSA	CSA binding parasite	selected in Arnot laboratory from FCR3

6.1.2 *E. coli* strains:

Different *E. coli* strains have been used in this project for different purposes.

Name	Application	Genotype
XL1 blue Stratagene	Cloning plasmids	(recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)])
TOP10 Invitrogen	Cloning plasmids	(F-*mcr*AΔ(*mrr-hsd*RMS-*mcr*BC)φ80*lacZ*ΔM15 *araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G)
BL21RIPL Stratagene	Protein expression	(*E. coli* B F– ompT hsdS(rB– mB–) dcm+ Tetr gal λ(DE3) endA Hte [argU proLCamr] [argU ileY leuW Strep/Specr)

6.1.3 *P. pastoris* strains:

Two *P. pastoris* strains have been used in this project for protein expression experiments.

Name	Application	Source
X33 (Invitrogen)	Protein expression	*wild-type*
GS115 (Invitrogen)	Protein expression	his4

6.1.4 Vectors.

Three different vectors were used during this work. Two of these vectors are commercially available, while the third was a kind gift from Dr. Dolores Cahill (Berlin).

Name	Purpose	Source
The TA Cloning® vector	Holding vector for sequencing and sub-cloning.	Invitrogen.
pPICZαA	Protein expression.	Invitrogen.
pPICHOLI	Protein expression.	Gift from Dolores Cahill.

6.1.5 Monoclonal and conjugated monoclonal antibodies:

Primary and secondary antibody reagents are listed below. These antibodies have been used in Western blots and in immuno-histochemical assays (ELISA and IFA).

Host	Specificity	Conjugate	source
Rabbit	Human IgG	HRP	DAKO
Mouse	6X His tag	HRP	Qiagen
Swine	Rabbit IgG	HRP	DAKO

6.1.6 Polyclonal sera:

Polyclonal sera were used in Western blots, in immuno-histochemical assays (ELISA and IFA) and in the immuno-screening of the expression libraries.

Host	Source
Pooled human sera	Malaria exposed adults from Sudan and Ghana.
Individual human sera	Malaria exposed adults from Sudan and Ghana.
Pregnant sera	Ghana
Rabbit polyclonal sera against the yeast recombinant CIDR1.	Rabbits immunized with the recombinant CIDR1 antigen.
Individual Scottish sera	Dr. David Cavanagh lab.

6.1.7 Reagents, Equipment and consumables.

The majority of chemicals and reagents were purchased from Sigma. Reagents supplied by others are listed in Appendix A.

6.1.8 Media, buffers and solutions.

Media, buffers and solutions for culture work were prepared with Millipore water, and sterilized by filtration through 0.22 micrometre filters. Uncompleted (Incomplete) medium was stored for up to 3 months at 4°C. A list of all prepared media, buffers and solutions is given in the following Table.

Media, buffers and solutions	Composition	Usage
SOC medium, 1 litre	5 g Bacto yeast extract 0.5 g NaCl 10 ml 250 mM KCl 20 g Bacto tryptone Make up to: 1 litre with water and adjust to pH 7.0, autoclave then add: 20 ml 1 M Glucose	Culture medium for E. coli.
LB medium	10 g Bacto tryptone 5 g Bacto yeast extract 10 g NaCl Make up to: 1 liter with water and autoclave.	Culture medium for liquid *E. coli* cultures
LB-Agar 1 litre	15 g LB medium Agar, make up to 1 litre with water, then autoclave.	Solid culture medium for *E. coli* cultures

LB / 50% Glycerol	50% Glycerol, 50% LB	Freezing medium for *E. coli* cultures
Minimal Dextrose Medium + Histidine (1 liter) (MD and MDH)	800 ml of water then add: 100 ml of 10X YNB 2 ml of 500X B 100 ml of 10X D To make MDH, add 10 ml of 100 X H stock solutions.	Determination of *Pichia* mutant phenotype.
Minimal Methanol + Histidine (1 liter) (MMH or MM)	800 ml of water then add: 100 ml of 10X YNB 2 ml of 500X B 100 ml of 10X M To make MMH, add 10 ml of 100 X H stock solutions.	Determination of *Pichia* mutant phenotype.
Buffered Minimal Medium containing methanol and/or histidine plates (BMGH and BMMH)	690 ml water 100 ml 1 M potassium phosphate buffer, pH 6.0 100 ml 10X YNB 2 ml 500X B 100 ml 10X M Add 10 ml of 100X(H) histidine stock solution. Mix and store at +4°C	Protein expression using *Pichia*

Buffered Methanol-complex Medium (1 liter) (BMMY)	Dissolve 10 g of yeast extract and 20 g peptone in 700 ml water. Autoclave, then add: 100 ml 1M potassium phosphate buffer, pH 6.0 100 ml 10X YNB 2 ml 500X B 100 ml 10X GY 100 ml 10X M	Protein expression Using Pichia
Yeast Extract Peptone Dextrose Medium (YPD) (1 liter)	Dissolve 10 g yeast extract and 20 g of peptone in 900 ml of water. Autoclave, then add: 100 ml of 10X D.	General growth and storage of *Pichia*
Yeast Extract Peptone Dextrose Sorbitol Medium with Zeocin(YPDS with Zeocin) (1 liter)	Dissolve 10 g yeast extract, 20 g of peptone, 182.2 g of sorbitol in 900 ml of water. Autoclave, then add: 100 ml of 10X D. Then add 1.0 ml of 100 mg/ml Zeocin™. Pour plates. Store YPDS plates with Zeocin™ at +4°C in the dark.	Selection of *Pichia* Zeocin resistance transformants.
10 x PBS	1.37 M NaCl 27 mM KCl	Various or General

	120 mM Na2HPO4 15 mM KH2PO4 adjust to pH 7.0	laboratory reagent
5 x TBE running buffer	5 M Tris base 4 M boric acid 10 mM EDTA, pH 8.0	Gel electrophoresis
Gel loading buffer (blue juice)	30% glycerine 10 mM Tris-HCl, pH 8.5 10 mM EDTA, pH 8.0 0.1 mg of bromophenol blue 0.1 mg of xylene cyanol10 mM Tris-HCl, pH 8.5 1 mM EDTA	Gel electrophoresis
TE (storage buffer)	10 mM Tris-HCl, pH 8.5 1 mM EDTA	Elution and storage of DNA
10X YNB	Dissolve 134 g of yeast nitrogen base (YNB) with ammonium sulphate in 1000 ml of water and filter sterilize.	Media component required for *Pichia* expression system
Lysis buffer	50 mM NaH2PO4 300 mM NaCl	Protein purification

	adjust to pH 8.0	
500X Biotin	Dissolve 20 mg biotin in 100 ml of water and filter sterilize.	Media component required for *Pichia* expression system
100X Histidine.	Dissolve 400 mg of L-histidine in 100 ml of water.	Media component required for *Pichia* expression system
10X Dextrose.	Dissolve 200 g of D-glucose in 1000 ml of water then autoclave.	Media component required for *Pichia* expression system
10X Methanol.	Mix 5 ml of methanol with 95 ml of water. Filter sterilizes.	Media component required for *Pichia* expression system
10X Glycerol.	Mix 100 ml of glycerol with 900 ml of water. Sterilize by autoclaving	Media component required for *Pichia* expression system
1 M potassium phosphate	Combine 132 ml of 1 M K2HPO4, 868 ml of 1 M KH2PO4 and confirm that the pH = 6.0.	Media component required for *Pichia* expression system

Buffer B	8 M Urea 100 mM NaH2PO4 10 mM Tris-HCl, pH 8.0 Adjust to pH 8.0.	Protein purification
2 x SDS-PAGE sample buffer	2.5 ml 0.5 M Tris-HCl, pH 6 2.0 ml glycerol 0.4 g SDS 0.31 g DTT 1 mg bromophenol blue add to: 10 ml with Millipore water.	Protein separation
5 x running buffer	15 g Tris base 72 g Glycine 5 g SDS Make up to: 1 litre with Millipore water	Protein separation
1 x transfer buffer	5 ml0.1 M CAPS, pH 11.0 5 ml MeOH 40 ml Millipore water.	Western blot
Giemsa solution	Azure II-eosin 3g	Staining of malaria

	Azure II 0.8g Glycerol 200 ml Methanol absolute 300 ml	parasites
Deep-freeze solution	28% glycerol 3% sorbitol or mannitol 0.65% NaCl	Storage of iRBCs with *P. falciparum.*
1%agarose gel	Measure 1 g Agarose powder and add it to a 500 ml flask Make up to 100 ml with TAE. Melt the agarose in a microwave or hot water bath until it has fully dissolved.	DNA electrophoresis
Ethidium bromide solution	Dissolve 10mg of ethidium bromide in 1ml of ddH2O).	DNA electrophoresis
6X DNA loading buffer	Combine 1 ml sterile H2O And 1 ml Glycerol. Add bromophenol blue powder.	DNA electrophoresis
TAE Buffer	4.84 g Tris Base 1.14 ml Glacial Acetic Acid 2 ml 0.5M EDTA (pH 8.0)	DNA electrophoresis
Complete RPMI 1640	74.75ml of RPMI 1640 and add: 50mls human serum	Culturing *P. falciparum.*

12.5ml HEPES buffer 5ml 200mM L-glutamine 5ml 20% glucose 1.25ml of 10mg/ml gentamicin 1ml of 1M NaOH (pH to approx 7.2-7.4)

6.1.9 Commercially available kits.

During this work, several kits were used. The kits are listed the table below:

Kit name	Source
Mega plasmid purification kit	Qiagen
Qiaprep Spin Plasmid	Qiagen
Kit Qiaquick Gel Extraction Kit	Qiagen
Pro-Q Fuchsia Glycoprotein Gel Stain Kit	Molecular Probes
Qiaquick PCR purification kit	Qiagen
Quick ligation kit	New England Biolabs
ECL Plus Detection System	Amersham

6.1.10 Filter membranes:

Different filter membranes have been used in this work as support matrices for growth and high-throughput immunoscreening.

Colony support filter	Supplier	Purpose
Hybond™-N+	Amersham	Growth support filter.
Cellulose Nitrate(E)	Schleicher Schuell	Growth support filter.
Polvinylidene Difluoride	Sigma	Growth support filter.
Hybond™- C Extra	Amersham	Growth support filter.
Protran™	Schleicher Schuell	Growth support filter.

6.1.11 Enzymes.

A. Restriction enzymes.

Most restriction enzymes and corresponding buffers used in this work were obtained from Promega . A list of all used enzymes used is given in Table below:

Enzyme	Recognition site	Source	Usage
Sal1	G^TCGAC	Promega	When using pPICHOLI cloning strategy requiring an upstream primer containing a Sal I site and
Not1	GC^ GGCCGC	Promega	A downstream primer containing a Not I site.
Sac1	GAGGCT^ C	Promega	Linearization of vector for efficient integration into the *Pichia* genome.

Table 2.10: List of restriction enzymes used in this project.

B. DNA polymerases and dNTPs.

To perform PCR reactions, three different DNA polymerases were used: Taq DNA polymerases (Promega), Pfu DNA polymerase (Stratagene) and KOD Hot Start DNA Polymerase (Novagen).

C. Other enzymes.

Name	Supplier
Lysozyme	Sigma
Proteinase K	Qiagen
T4 DNA Ligase	Promega
Lyticase	Sigma
RNAase	Sigma
DNAase	Sigma
Benzonase	Novagen

6.1.12 Primers.

A. Custom-made primers.

The Oligonucleotides were synthesized by MWG Biotech, at the 40nmol or 0.1nmol synthesis scale and HPLC purified. A complete list of the primers used is listed below:

Primer name	sequence 5'–3
SAL1/DBL-1X/F	AAAAGTCGACCCCTTGTGATAGAATACCACCTCCT
NOT1/DBL-1X/R	AAAAAGCGGCCGCTAATTGATTTTCCCATTCGGAT TTCCA

SAL1/CIDR1/F	AAAAGTCGACCCAAAACAAAAACGAAACTTCGCA A
NOT1/CIDR1/R	AAAAGCGGCCGCTAAGGTCCTAATATTCTTAAGTT CTTGTGT
SAL1/DBL-2X/F	AAAAGTCGACCGAGTGGATAATTGCTGCATTTCA T
NOT1/DBL-2X/R	AAAAAGCGGCCGCTAACCAAGGAGCTTTGTCCGC GCCACA
SAL1/CIDR2/F	AAAAGTCGACCGATGACAAGGATGTGACGTTC
NOT1/CIDR2/R	AAAAAGCGGCCGCTAAACAGGCACCATTTGTATC TCCATG
SAL1/DBL-3X/F	AAAAGTCGACCGCCTGTATCCCTCCAAGAACACA A
NOT1/DBL-3X/R	AAAAAGCGGCCGCTAATTGTTCGCACGAACATAT ACTGCT
SAL1/DBL-4ε/F	AAAAGTCGACCGTCCCCCCAAGACGGCAACAATT A
NOT1/DBL-4 ε /R	AAAAAGCGGCCGCTAACTCGCAAAACTCATTTGT CCATTC
SAL1/DBL-5 ε /F	AAAAGTCGACCTGGAATGATGTGGATATGCGAGA T
NOT1/DBL-5 ε /R	AAAAAGCGGCCGCTAACGATTCTTGTGCCCCTAA ATTAGT

SAL1/DBL-5 ε /F	AAAAGTCGACCTGGAATGATGTGGATATGCGAGAT
NOT1/DBL-5 ε /R	AAAAAGCGGCCGCTAACGATTCTTGTGCCCCTAAATTAGT
SAL1/DBL-6 ε /F	AAAAGTCGACCATCGGAAATGAAGCATTTAAGCAA
NOT1/DBL-6 ε /R	AAAAAGCGGCCGCTAAATCTTTGAAATATTCTGGGGATTC
DBLα/F	TGYGCNCCNTAYMGNMGN
DBLα/R	TTTNCKRCAAAARTCTTC
DBLβ/F	WSAACAHWTKTGTACMTC
DBLβ/R	TCDTTGNGGDATRTARTC
DBLγ/F	TATGCCWCCHAGDAGAVAA
DBLγ/R	CATCKHARRAAYKGDGG
DBLδ /F	CCVMGSAGRCGAMRANTATA
DBLδ /R	GCCCCATTCWTVWARSYAWCG

Abbreviations

R=A/G	Y=C/T	M=A/C	K=G/T
S=G/C	W=A/T	H=A/C/T	B=C/G/T
V=A/C/G	D=A/G/T	N=A/C/G/T	

B. Commercially available primers:

Primer name	sequence 5'–3	Purpose
M14/F	GTAAAACGACGGCCAG	Primers for sequencing from any A/T *E. coli* cloning vector
M13/R	CAGGAAACAGCTATGAC	
AOX1/F	GCAAATGGCATTCTGACATCC	Primers for sequencing from any *Pichia* expression vector
AOX1/R	GACTGGTTCCAATTGACAAGC	

6.1.13 Molecular weight markers:

All markers were used according the supplier's instructions.

Marker	usage	Source
Ready-load 1Kb plus DNA ladder	Estimation of the size of DNA.	Invitrogen
Precision plus protein standards	Estimation of the size of proteins.	BIO-RAD

6.2 Methods.

This section describes all the methods used during the course of this project. Unless stated, all centrifugation was carried out in a bench top centrifuge. All media and experimental materials were pre-warmed to 37°C before use. Liquid waste was disinfected in 1% Virkon before disposal. Contaminated consumables were disposed of by incineration or inactivated by autoclaving..

6.2.1 Preparation of the serum.

Serum was removed from coagulated bloods pack using sterile technique into sterile centrifuge tubes. It was centrifugated at 690g(using a centrifuge IEC/ Centra® MP4R from International Equipment Company, rotor 215 ans radius of 15.4) for 5 minutes to spin down any remaining red cells. Serum from at least 6 patients' blood was pooled and aliquoted and stored frozen at -70°C.

6.2.2 Preparation of Red Blood Cells.

Blood (O+) was taken into a pack containing anticoagulant. Aliquots of blood were removed from the pack using sterile technique into sterile centrifuge tubes. It was then spun at 690 g for 5 minutes to remove serum and as much of the Buffy coat as possible. An equal amount of incomplete medium was then added, mixed well and centrifuged again at 690 g for 5 minutes. this wash was repeated twice more. After the last wash, red cells were re-suspended in an equal volume of complete medium to obtain a 50% haematocrit stock.

6.2.3 Asexual parasite culturing.

Cultures may be started from patients' blood, from deep-frozen samples or from a sub-culture of an existing live culture. Normally a sub-culture is obtained from an existing culture and set up as follows: a stock of 5% haematocrit blood is prepared in complete medium i.e. from the washed 50% haematocrit blood prepared as above, take 1 ml and dilute this in 9 ml complete medium. The parasitaemia of the original culture is measured and diluted with the stock 5% haematocrit red cells to make the required sub-culture. e.g. from an original culture of 5% parasitaemia take 1ml and add 4 ml stock red cells to make a new flask of 1% parasitaemia. The flask is then gassed (10 seconds for small, and 20 seconds for large flask)with a gas mixture of 1% oxygen, 3% carbon dioxide and 96% nitrogen, then incubated at 37°C with culture medium being changed daily. Parasitaemia is checked using a Giemsa-stained thin blood smear. (Smears are fixed for 30 seconds in methanol and stained with 10% Giemsa in PBS pH 7.2 for 45 minutes). Cultures were diluted as necessary using O+ red blood cells (pre-washed 3 times in incomplete medium).

6.2.4 Deep-freezing.

For preservation, samples of PRBCs were frozen in cryoprotectant in liquid nitrogen. Mature stage parasites undergo modifications that render the PRBC membrane relatively inflexible. This means that only young, ring stages can be successfully frozen. Cultured cells were pelleted by centrifugation and the supernatant drawn off. The pellet was slowly re-suspended in an equal volume of deep-freeze solution (28% glycerol, 3% sorbitol, 0.65% sodium chloride) at room temperature and transferred in 0.5ml aliquots to freezing vials, which were then plunged in to liquid nitrogen (-196°C). Addition of the solution should be done at room temperature for efficient penetration of the glycerol into the red cells. Aliquots of the final mixture were pipetted into small screw-topped ampoules - 0.5-1ml per ampoule and rapidly frozen by plunging into liquid nitrogen. Parasites were maintained in continuous culture in human red blood cells (obtained from the Scottish Blood Transfusion Service, Edinburgh, UK), incomplete RPMI-1640 medium (cRPMI) supplemented with 10% human serum, 37.5mM HEPES, 100µlM hypoxanthine, 20mM glucose, 2mM glutamine, 2mg/ml NaHCO3, 25µlg/ml gentamycin sulphate and sodium hydroxide to pH 7.2.

6.2.5 Thawing of deep frozen *P. falciparum.*

Thawing was performed carefully using a series of salt solutions to minimise lysis and return the cells to normal osmolarity. Samples were thawed at 37°C and transferred to 50ml centrifuge tubes. 0.2 volumes for every 1ml thawed blood of 12% (w/v) sodium chloride were added drop-wise over 5 minutes, with continuous mixing. After incubation at room temperature for 3 minutes, 10 volumes of 1.6% (w/v) sodium chloride was added drop-wise over 5 minutes. Following centrifugation at 690 g for 5 minutes, supernatant was removed and 10 volumes of 0.2% dextrose/0.9% (w/v) sodium chloride was added drop-wise over 5 minutes. After a final centrifugation at 690 g for 5 minutes, the supernatant was removed and cells were re-suspended in complete RPMI-1640 to 5% haemtocrit and the culture maintained under normal conditions

6.2.6 Synchronisation of Asexual Parasite Cultures.

PRBCs older than 18-20 hours post invasion undergo changes that render them permeable to sorbitol. A method for the preferential lysis of older forms was, based on osmotic stress using sorbitol (developed by Lambros and Vanderberg, 1979) was used. To maintain parasites within a narrow age range for experiments, parasites were enriched for ring stages by treatment with sorbitol. Parasite cultures with a high proportion of young ring stages were centrifuged at 1000 g in a microfuge for 5 minutes at room temperature and the medium removed by aspiration. The culture was washed by adding 10 volumes incomplete RPMI-1640, followed by centrifugation at 690 g. The cells were then re-suspended in 5% (v/v) sterile sorbitol/PBS and incubated at 37°C for 10 minutes. The suspension was centrifuged at 690 g and washed twice in incomplete RPMI-1640. The parasites were then transferred back to culture and examined by blood smear after 24 hours by which time they should all be large parasites (trophozoites).

6.2.7 CSA Selection.

Sterile Chondroitin Sulphate A solution was made up at 10 microgram/ml in PBS. 2.5ml of this solution was added to Petri dishes and incubated for at least 3 hours at RT or at 4 degrees C overnight. Immediately prior to selection, the CSA was aspirated off and the plate surface blocked with 2% BSA in PBS for at least 30 mins. Parasites to be selected were synchronized and used when the parasitaemia of trophozoites was at least 5% but preferably over 10%. The 2% BSA blocking solution was aspirated off and 2-3ml. of trophozoite-enriched culture added. The plates were incubated for 60 minutes at 370C, with gentle rocking every 10 minutes by hand. After binding, the parasite suspension was gently removed and the plate washed again gently 5-7 times with PBS until the plate was visibly clear and, when viewed under the inverted microscope showed a homogeneous distribution of the expected density of parasitised cells. 3 ml of uninfected erythrocytes at 5% HCT was added, and the dish placed at 37 degrees C, under normal parasite culture conditions, i.e. placed in an incubating chamber and gassed with 1% O2, 3% CO2 and 96% N2. (If a chamber not available it is possible to culture in a 5% CO2 incubator). Culture

were left for 20-24 hours and then harvested into a normal culturing flask and grown under normal conditions.

6.2.8 Preparation of Parasites for Fixed IFA.

Cells at 5-10% parasitaemia were pelleted by centrifugation at 1000g for 5 minutes, washed twice in incomplete RPMI-1640 and re-suspended to 5% haematocrit. 25µll of this suspension was spotted onto each spot of clean, multi-spot microbiological glass slides (Hendley-Essex) and left to dry at room temperature. Slides were wrapped in Parafilm, sealed in plastic bags with dry silica gel as desiccant and stored at -20°C.

6.2.9 Parasite DNA.

5ml of asexual culture at 5-10% parasitaemia were centrifuged at 690 g for 5 minutes to pellet pRBCs then re-suspended in 1ml of 0.15% saponin in incomplete RPMI 1640 medium and mixed until lysis was observed. An equal volume of incomplete RPMI-1640 was added and the mixture spun at 1000 g for 10 minutes. DNA was extracted from the parasite pellet using QIAampR DNA Minikit, according to the manufacturer's instructions.

6.2.10 Plasmid DNA.

Plasmid DNA from 5ml overnight bacterial and yeast cultures was prepared using the QIAprep Spin Miniprep kit according to manufacturer's instructions. Final elutions were made in 50µllbuffer EB (QIAprep). For the yeast cell, acid washed glass beads were added to the P2 slusion of the kit. The QIAprep Midiprep kit (Qiagen) was used for transformation quality plasmid recovery with 25ml overnight culture and final resuspension in 200 µl water.

6.2.11 DNA quantification.

Plasmid DNA concentration was estimated by measuring its absorbance at 260nm. It was assumed that an OD260 of 1.0 is equivalent to a concentration of 50µg/ml for double stranded DNA.

6.2.12 Agarose Gel Electrophoresis.

Agarose gels were used to check the quality of genomic DNA and plasmid preparations and to analyse PCR products and restriction digests. 1% agarose was dissolved in TAE buffer by boiling. After cooling, ethidium bromide was added to a concentration of 0.5µlg/ml and the mix poured into an gel plate and left to set at room temperature before being placed in TAE buffer in an electrophoresis gel tank.. DNA was mixed with 6X DNA loading buffer and loaded into the submerged gel. Electrophoresis was carried out in TAE at 80-120 volts for an appropriate time. Fluorescence from DNA-bound ethidium bromide was visualised by short wavelength UV light and photographed.

6.2.13 Primer design.

Degenerate PCR primers were designed based on known nucleotide sequences. With the release of the genome sequence for *P. falciparum* (isolate 3D7) it has been possible to design degenerate primers to identify almost every *var* gene. To design type-specific primers to DBLα, DBLβ, DBLγ and DBLδ, *var* genes were downloaded from the databases. Primers were based on a total of 41 *var* genes from the 3D7 genome and other *var* genes from other parasite genotypes. Their predicted protein architectures were obtained. Protein motifs on each domain were determined then the nucleotide sequences rechecked to identify the codon. After codon determination, degenerate primers were designed in order to amplify the maximum numbers of each domain. As far as possible, GC content was adjusted to about 50% and Tm to above 60°C, by extending the matching regions. Sequences were analysed for hairpins, homoduplexes and Tm. Pairs of oligos were designed to be of equal (+/- 2°C) Tm. Where a restriction site was incorporated, this was at the 5' end.

6.2.14 TA cloning.

To obtain sequence for the inserts and to prepare the amplicons to be digested, PCR products were cloned into TA vectors. Using the *Pfu* polymerase, PCR products from *P. falciparum* DNA were obtained using universal degenerate primers. The *Taq*

polymerase was then used to produce amplicons with A-overhangs which are compatible with TA cloning vector.

6.2.15 Automated DNA sequencing.

Automated sequencing of plasmid DNA was carried out using dye terminators with the PRISMS/S cycle sequencing kit (Applied Biosystems Inc.) and the 377 automated sequencer (ABI). Reactions were set up using 4 µl Big Dye, 1.6pmol appropriate sequencing primer, 2µg plasmid DNA and made up to 10µl with PCR grade water. Cycle sequencing was performed with the following conditions: 25 cycles of 95°C for 30 seconds, 50°C for 20 seconds, 60°C for 4 minutes. PCR products were precipitated by centrifugation in a spin column for 2 minutes at 1500 g in a microfuge and run on an acrylamide gel. Chromatograms were viewed and data edited using the SeqEdTM program (ABI).

6.2.16 Digestion of amplicons and vector.

Restriction sites for Sal1 and Not1 in each pair of primers and in the vector were digested by Sal1 and Not1 enzymes according to the recommended protocol. To set up double digestion, 10U of each restriction enzymes were added to 16µl DNA with 2µl of the appropriate 10X buffer and the mixture made up to 20µlwith water. The digestion mixtures were incubated at 37°C for 3 hours, followed by a heat inactivation step (65°C, 15 minutes), this achieves 95% inactivation of the enzymes. BSA was added to enhance the activity of the restriction enzymes

6.2.17 De-phosphorylation of cut vector prior to cloning.

To prevent re-circularisation of cut vector during cloning, the 5' terminal phosphates were removed using SAP (shrimp alcohol phosphateas). 1µl of enzyme and 5µl 10X CIAP buffer (NEB) were added to 20µlcut vector. The mixture was made up to 50µl with water and incubated at 37°C for 2 hours, followed by inactivation of phosphatase activity at 75°C.

6.2.18 Purification of DNA fragments.

DNA fragments from restriction digests or PCR reactions were purified from agarose gels prior to cloning using a Gel Extraction kit, according to the manufacturer's instructions. PCR products were cleaned using QIAquick PCR purification kit.

6.2.19 Ligation reactions.

Typically, a 3:1 molar ratio of insert to vector results in the highest yield of transformants with a single inserts. A lower amount of insert reduces the efficiency of ligation; a higher amount increases the probability of obtaining transformants with multiple inserts. Cutting both the amplicons and the vector with the same restriction enzymes produces compatible sticky ends so that these amplicons can be inserted in the correct orientation. The ligation protocol which demonstrated the best result was Quick Ligation™ Kit (New England Biolabs). This kit uses T4 DNA ligase to catalyse the formation of phosphodiester bonds between neighbouring 3'-OH and 5'phosphate ends in double stranded DNA. Linearised vector and inserts were purified by gel-extraction and 100ng vector DNA was incubated with a 1:1, 1:3 and 1:5 molar ratio of vector:insert DNA. Controls were included using linearised vector alone. 10µlreaction mixtures were set up, with 1-3U *T4* DNA ligase (Promega) and T4 DNA ligase buffer and incubated at 16°C for 12 hours, followed by 37°C for one hour and 65°C for another one hour to denature the ligase and prevent further activity. Ligation was carried out at room temperature for 5 minutes

6.2.20 Recodoning of the CIDR1 fragment.

This part of the work was done by Toby Feagin and Peter Lawrence as part of an unpublished 4th year Honours project in the Arnot laboratory. The method for synthesizing a codon optimized version of the gene was based on the established methods (Stemmer *et al.*, 1995; Withers-Martinez *et al.*, 1999). The following parameters were taken into consideration: reduction of the A/T richness of the sequence by replacing rare by more common codons used in the host system (this was achieved with help of http:// www.kazusa.or.jp/codon), removal of potential N-

glycosylation sites (this was achieved with help of http://www.cbs.dtu.dk/services/netNGLyc) and removal of restriction sites that may affect downstream cloning techniques.

6.2.21 Creation of expression library.

To create an expression library i.e. a library that not only contains the DNA fragments of interest but one that can manufacture the protein so that it may be detected by the antibody. Essentially the process involves isolation of genomic DNA and insertion of PCR enriched fragments into an appropriate expression vector. After transformation of bacteria or yeast, growths then induction were carried out. The final step involves library screening to select particular clones from the library. For the current work, an expression vector that can be used in two expression systems, *E. coli* and *P. pastoris*, was used.

6.2.22 Transformation of Bacterial Strains.

XL-1 Blue and XL-10 Gold strains were used for cloning and propagating DNA. Based on the manufacturer's recommended protocols, cells were transformed chemically. Briefly, mercaptoethanol (1µl) was added to the cells for 10 minutes whilethe cells were kept on ice. The ligation product (2µl) was added to the cells and left for 30 minutes. The cells were subjected to heat shock for 45 seconds and transformed cells were then re-suspended in SOC medium at 37° for one hour before being spread on an agar plate containing zeocin (25µg/ml) and incubated overnight at 37°C to allow colonies to form.

6.2.23 PCR colony screening of bacterial Clones.

Successfully transformed clones were identified by direct colony PCR screening and by restriction digest of plasmid DNA. For PCR screens, a standard reaction mix was made up with PCR buffer, appropriate forward and reverse primers, 2.5mM each of dATP, dCTP, dGTP and dTTP, 1U Taq polymerase and 100µll/ml DMSO. 1µlof cell suspension was added to 9µlof reaction mix. PCR was carried out under standard conditions (94°C for 10 minutes for 1 cycle, followed by 30 cycles of 94°C for 30

seconds, 55°C for 30 seconds and 72°C for 90 seconds with a final extension cycle of 72°C for 1 minute). Amplified DNA was analysed by agarose gel electrophoresis.

When necessary, PCR conditions were optimised for different primer pairs. Clones with inserts in the correct orientation were identified using a standard PCR, with primers selected to only allow amplification of an insert in the correct orientation.

A cell suspension was first prepared using a sterile pipette tip to transfer a bacterial colony to 100µlPCR grade water. A 5-95µl of the cell suspension was used to inoculate 5ml LB with the appropriate selective antibiotic and incubated overnight at 37°C, with shaking. Plasmid minipreps were carried out on overnight cultures to recover plasmid DNA, using the QiAgen miniprep kit, according to the manufacturer's instructions. Recovered DNA was incubated with appropriate restriction enzymes to release the insert or to produce a band pattern characteristic of a particular insert when analysed by agarose gel electrophoresis.

6.2.24 Expression of in *E. coli* in liquid medium.

BL21-Codon Plus-RIPL competent cells were transformed with an appropriate concentration of DNA and transformed bacterial colonies were selected with zeocin (50µlg/ml and 70 µlg/ml chloramphenicol in the LB). Various colonies were picked and cultured separately in 50ml LB medium at 37°C, with shaking, overnight. This culture was then used to inoculate 1 litre of pre-warmed LB and incubation continued until the OD600 had reached 0.4-0.6. To induce expression, 1M IPTG was added to a final concentration of 0.1mM and the culture incubated at RT for 4 hours. Bacteria were pelleted by centrifugation at 1000 g for 20 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in 50ml BugBuster® Protein Extraction Reagent (Novagen). 50µlBenzonase® Nuclease (Novagen) (25u/ml) was added to the lysate to degrade bacterial DNA and decrease viscosity. After a spin at 1000 g for 30 minutes at 4°C, the supernatant was filtered and loaded onto the Aktaprime column at a rate of 2ml/min. Bound protein was eluted using elution buffer and collected in 2.5ml fractions. Fractions with the highest peaks of absorbance were run on an SDS gel and stained with Coomassie Blue and those fractions containing the most protein were pooled and buffer-exchanged.

6.2.25 Culture and storage of the expression library.

Individual colonies were picked from the spread plate. The picked colonies were transferred into wells of a 96 well plate containing deep-freezing LB media with the appropriate antibiotics to maintain the plasmids, and then stored at -70°C.

6.2.26 Growth and induction on filters.

Samples of DBL-α, β and γ expression libraries were cultured overnight from the frozen libraries. The colonies were grown at 30°C in a 96 well plate in LB media supplemented with glucose (1%), glycerol (8%) and zeocin (25μg/ml). Colonies were arranged in duplicate as a control measure for the Western blot to follow. A negative control was used consisting of a cell containing the vector but no insert. Following the protocol of Sowa *et al.*,(2004), High Bond-C Extra filters (Amersham) were spotted with bacterial colonies using an applicator with 96 steel pins. Colonies were grown by placing the filters on LB agar supplemented with glucose (1%), zeocin (25μg/ml) and CAM (34μg/ml) at 30°C overnight. For induction of protein expression, the arrayed filters were transferred from the glucose/ zeocin supplemented medium onto LB agar plates supplemented with IPTG (0.2%), zeocin and chloramphenicol. Induction was for 4 hours at 37.

6.2.27 Lysis of bacterial host strains.

Lysis was initiated by incubation of filters on Whatman paper pre-soaked with BugBuster™ HT (Novagen), supplemented with PMSF (0.1mM) for 20 minutes. This was to ensure that the colonies were not displaced from the filter. Following this initial lysis, each filter was incubated in 50ml of lysis buffer; Tris HCl (50 mM Tris HCl, pH 7.5), NaCl (0.15M), MgCl2 (5mM), PMSF (0.1mM), lysosyme (40μg), Benzonase (300 units/l) and BSA (3%). The filters were incubated with gentle agitation for 3 hours at room temperature. Filters were blocked overnight at 4°C in PBS-TT, PMSF (0.1mM) and BSA (3%). After overnight blocking, filters were washed 3 times in PBS-TT, 15 minutes per wash, then once in normal PBS (100ml per filter).

6.2.28 Removal of Anti-*E. Coli* and Anti-*P. pastoris* antibodies from antiserum.

For removing antibodies that react with either bacterial or yeast encoded proteins, a method adapted from De Wet *et al.*, 1984 was used. This contains two steps. Briefly, both expression cells were induced with appropriate inducer (IPTG for *E. coli* and methanol for *P. pastoris*). Live cells of both expression systems that contain just the expression vectors (i.e. ones without inserts) were pre-incubated with the panel of human sera that were to be used in the immuno-screening, to remove non-specifically binding antibodies.

6.2.29 Screening of protein arrays using Anti-His tag antibody and human sera from malaria endemic area.

Filters were probed with HRP- conjugated anti-His tag antibodies (1:2000 dilution) for one hour at room temp in the blocking buffer provided. The ECL plus Western Blotting Detection System (Amersham) was used to detect positive colonies. After lysis, blots were blocked with PBSTM (PBS, 0.5% powdered non-fat milk, 0.1% Tween-20) for one hour at RT or overnight at 4°C with gentle agitation. Antibodies were made up in PBSTM at appropriate dilutions. Membranes were incubated with antibodies for one hour at RT, with agitation, followed by three washes of 15 minutes each in PBST. Those filters to be tested with human Sera were treated as above. The primary antibody was blood sera collected from adults in malaria endemic areas, the secondary Ab was Anti-human IgG- HRP (dilution of 1:5000.) Blots were developed using an enhanced chemi-luminescence system according to the manufacturer's instructions.

6.2.30 Transformation of *P. pastoris.*

Wild-type (X33) *P. pastoris* and GS115 cells were made competent and transformed according to the manufacturer's instructions. Briefly, 100ml YPD was inoculated with 1 colony of *P. pastoris* and incubated overnight at 30°C with shaking at 250 rpm, to an OD at 600nm of 1.3-1.5. Cells were centrifuged at 1500g for 5 minutes at 4°C and re-suspended successively with 100ml, then 50ml ice-cold sterile water followed by 4ml, then 0.5ml ice-cold 1M sorbitol. Competent cells were kept

on ice and used the same day. 80µl cells per transformation were mixed with 5-10µlg of linearised DNA (in 5-10µl sterile water), transferred to an ice-cold 0.2cm electroporation cuvette and incubated on ice for 5 minutes. Electroporation was carried out using a BioRad GenePulser, at 1,500V, 25vF and 200R. After electroporation, 1ml of ice-cold 1M sorbitol was added immediately to the cuvette and the contents transferred to a sterile15ml tube. The cells were then incubated at 30°C without shaking for 1-2 hours, after which time 10, 25, 50, 100 and 100µl were plated on separate YPDS Zeocin™ (100µlg/ml) plates. Plates were incubated at 30°C for 2-3 days until colonies formed.

6.2.31 *E. coli* transformation with expression plasmids isolated from *P. pastoris.*

To prepare plasmid DNA from selected *P. pastoris* expression clone from each 2-ml cultures were grown for 2 days at 30°C. Cells were harvested by centrifugation at maximum speed for 5 mininutes. Pelleted yeast cells were incubated in 100 ml lysis buffer (1 M sorbitol, 10 mM EDTA, 5 mM DTT, and 2 mg/ml lyticase) for 30 mininutes at room temperature. Cells were lysed by the addition of 150 ml P2 buffer (Qiagen lysis buffer) and incubated on ice for 30 min. After addition of 500 ml P3 buffer (Qiagen) the suspension was further incubated on ice for 15 min and centrifuged at maximum speed for 10 minutes. The supernatant was purified according to the manufacturer's protocol (Spin Miniprep kit; Qiagen). Plasmid DNA was electroporated into XL1Blue cells and plated onto selective medium. Plasmids of each clone were isolated (QIAquick Spin Miniprep kit; Qiagen) and transformed into the bacterial expression strain.

6.2.32 Direct Screening of *Pichia* Clones.

In order to establish the presence of an insert in transformed yeast cells, a PCR screen was carried out. Yeast cells were lysed by lyticase, followed by freezing/heating treatment to release gDNA which was then used as a PCR template, according to the manufacturer's recommendations. Briefly, half of the colony was resuspended in 10µl ddH2O. 5µl of a 5U/µl lyticase solution was added, and then incubated at 30°C for 20 minutes. The PCR reaction was set up in 150µl as follow:

-H2O	85µl
-10X buffer	15µl
-MgCl2	12µl
-DMSO	15µl
-DNTPs	3µl
-F/Primer	6µl
-R/Primer	6µl
-Cell lysate	5µl
-*Taq* polymerase.	3µl
Total:	150µl

Then 30 cycles as follows;

94° C 1 minute
55° C 20 seconds 30 X
72° C 1, 20 minute

30X

72° C 7 minutes 1 X

PCR positive colonies were re-streaked on YPD plates containing 100µlg/ml, 200µlg/ml and 500µlg/ml Zeocin™. Direct PCR screening of *Pichia* was performed.

6.2.33 Selection of *Pichia* for the Methanol Utilisation (Mut+) Phenotype.

Two media were used, Minimal Dextrose with histidine (MDH) plates and Minimal Methanol with histidine (MMH). Using a sterile toothpick, one colony was

picked, and streaked on both MMH and MDH plates. The plates were incubated at 30°C for 2 days. Mut+ strains will grow normally on both plates, while Mut- strains will grow normally on MDH plate but show little or no growth on the MMH plates.

6.2.34 Screening Recombinant *Pichia* for Expression.

Three levels of induction experiments were carried out. These included small induction experiment, scale-up of expression and expression through fermentation.

A Small induction experiment.

Selected colonies were picked into 10ml (in a 50ml centrifuge tube) or 50ml (in 250ml a baffled flask) BMGY and incubated overnight at 28-30°C in a shaking incubator at 250-300rpm. When the cultures reached an OD600 of 2-6 (indicating log-phase growth), they were harvested by centrifugation at 1,500-3,000g and washed in sterile PBS to remove traces of glycerol that may adversely affect methanol induction. Cells were then re-suspended to an OD600nm of 1.0 in 100-200ml of BMMY, in a 1 litre baffled flask, to induce expression. Incubation was continued and 100% methanol was added to a final concentration of 0.5% every 24 hours for 4 days to maintain induction. 500µl samples were removed at appropriate time points, centrifuged for 2 minutes at maximum speed in a bench-top microfuge and supernatants transferred to fresh tubes. Both supernatants and cell pellets were frozen quickly in a dry ice / ethanol bath and stored at -80°C.

B Scale-up of Expression.

This involved the same set up as for the smaller inductions except that the initial inoculum (25 ml of BMGY) was added to 1 litre of BMGY in a 3 or 4 litre baffled flask and grown at 30°C with vigorous shaking until the OD = 2-6. The culture was harvested and the pellet was used to inoculate a 2-6 litres of BMMY to start induction.

C Fermentation.

The fermentation run consisted of the following stages:

- Glycerol Batch Phase – 20 hours
- Glycerol Feed Batch phase – 20 hours
- Starvation phase – 1 hour
- Methanol Feed Batch – 70 hours
- Harvest

Six litres of Basal Salts Medium were added to the fermenter. After sterilization and cooling, the temperature was set to 30°C, agitation and aeration were set to routine operating conditions (maximum rpm and 1:10 volume of gas: volume of liquid) and the pH was set to 5 with 28 % ammonium hydroxide. Maintaining sterility, 4.35ml/l of PTM was added and the inoculum seed flask was added to the fermenter culture. The culture was incubated until complete consumption of the glycerol (approximately 20 hours), after which initiation of glycerol-feed was started using 50% glycerol containing 12ml of PTM. The feed rate was adjusted to 18.15ml/hour per litre of initial fermentation volume. Glycerol feeding was terminated and 30 minutes of carbon source starvation was carried out before the production phase of the culture was started. The production phase started with 12ml/l PTM in methanol, and the feed rate was set to 3.5ml/h/l for 3 hours. After adaptation to methanol utilization, the feed rate was set at 7.3 ml/l/h for 2 hours. The methanol feed rate was changed to 11 ml/l/h to the end of the run (65 hours), at which point the culture was collected into 500 or 1000ml centrifuge bottles and centrifuged at 5000g for 30 minutes at 4° C to separate cells from supernatant.

6.2.35 Overlay Assay of *P. pastoris* colonies on the filters.

The following procedure has been optimised to screen recombinant colonies expressing the proteins of libraries of interest. After transformation, grow the cells on YPDS with 100 µg/ml Zeocin™ plates. Grow each of the growing cells on either 5 ml of MGY, BMG, or BMGY broth in 24 deep-well plates supplemented with 100µll/ml Zeocin for (approximately 16-18 hours.) at 28-30°C in a shaking incubator (250-300 rpm) until the culture reaches an OD600 = 2-6. Patch transformants onto Minimal Dextrose with histidine agar plates and YPD plates using replicating devices carrying 96 steel pins (25-50 colonies per plate) supplemented with

100µll/ml Zeocin and incubate at 30°C for 2-3 days. Replica plate from the MD (H) plates onto MM(H) Minimal Methanol with histidine. Wet the nitrocellulose filter in 0.5% methanol for 1 minute. Lay a nitrocellulose filter over the colonies with a Whatman filter on top (or paper towels, to draw liquid through the nitrocellulose filter). Invert the plates and incubate for 2 to 3 days at 30°C. Add 1 ml of 100% methanol to the lid of each plate every day.

To assay for expression, peel the nitrocellulose membrane off the plate with tweezers. To detect intracellular expression, lyse the cells by incubating the filter in Lysis Buffer. Lysis Buffer (prepare fresh, 0.1% SDS, 0.2 M NaOH, 35 mM dithiothreitol) for 30 minutes at room temperature. Remove lysed cells with rapidly flowing distilled water and rinse the filter with PBS. Block the membrane with PBS containing 0.05% Tween-20 and 5% nonfat, dry milk (PBSTM) or 2% BSA (PBS/BSA). For 100ml, 100ml PBS+50µl Tween-20+5g nonfat dry milk for 1 hour at room temperature or overnight at 4°C. Add Anti-His-HRP (1:4000 dilution in PBS+2%BSA) in blocking solution and incubate for 1 hour at room temperature with moderate shaking. Wash the blot three times with PBS containing 0.05% Tween-20 (PBST) for 15 minutes each time. Detect expressing colonies using chemiluminescence (following the manufacturer's instructions). Circle the high expressing clones and compare with the colonies that were patched onto the YPD plate. Select the appropriate patched colonies for further expression experiments. The most promising clones (those that stained the most intensively) will be picked for scale up expression in liquid culture in 50 ml plastic tubes containing 5 ml of medium.

6.2.36 SDS-PAGE.

Samples were added to 4X sample buffer and heated at 90°C for 5-10 minutes. 4-12% Bis-Tris polyacrylamide NuPAGETM Novex high-performance pre-cast gels were removed from packaging and washed three times with water, before transfer to 500ml per tank of either MOPS or MES running buffer. 6µlSeeBlue® Plus 2 Pre-stained Protein Standard (Invitrogen) and 20-30µl of each sample were loaded in each well and electrophoresis was carried out at 200V, constant voltage for

approximately 50 minutes, or until the loading dye front had reached the bottom of the gel.

6.2.37 Staining of SDS-PAGE Gels.

Two methods were used to stain the gels, Coomassie Brilliant Blue stain and Silver Salts stain. In the former the gels were fixed and stained for 1 hour in the stain solution (10% (v/v) methanol,10%(v/v) glacial acetic acid and a pitch of Coomassie Brilliant Blue). Destaining was performed in the same buffer without the dye. In the silver stain method which is more sensitive 1000-fold than the Coomassie stain. Simply this kind of stain includes fixation of the gels by the fixation solution (Ethanol: glacial acetic acid: H2O in ratio of 30:10:60). Then sensitizing was performed by sensitizing solution (30% Ethanol, sodium thiosulphate 5%, and sodium acetate). Silver nitrate is added to the gel for 30 minutes. Developing solution is then added (2.5% sodium carbonate, 0.02% formaldehyde). Stopping solution is then added to stop the reaction (1% acetic acid or EDTA-N2 .2H2O).

6.2.38 Protein Concentration Determination.

Two assays were used: Bradford Assay and Bio-Rad/DC Protein Assay (based on the Lowry assay). In the Bradford assay (maximum 100µl of sample and 1 ml of Bradford Working Buffer) were added to the sample. Readings were taken after 2 minutes at 595nm. We prepared a series of BSA standard solution between 0.2mg/ml and 2mg/ml. In Bio-Rad DC Protein Assay we added 5 of 3-5 dilutions of protein standard containing 0/2 mg/ml to 1.5mg/ml of BSA, and an appropriate dilution of the protein samples. 25 of Reagent A is added to each well, 200 of reagent B is added and mixed well. After 15 mins, readings were taken at the absorbance of 750nm.

6.2.39 Concentration of the protein sample.

Vivaspin concentrators, ammonium sulphate precipitation, and a Speed-vac concentrator were used in this project. Vivaspin concentration was used directly with molecular weight cut-off of 5000Kda. Speed-Vac concentration was also used directly for the appropriate times. 300 ml supernatant was mixed with 1200µl of saturated ammonium sulphate and left on ice for 20 minutes, followed by

centrifugation for 15 minutes at maximum speed in a microcentrifuge. Supernatant was discarded and the pellet was re-suspended in PBS.

6.2.40 Western blot.

Wet transfer of proteins to PVDF was carried out in a BioRad Mini Trans-Blot cell, according to the manufacturer's instructions. Briefly, SDS-PAGE gels were placed in a sandwich of blotting paper sheets and filter pads saturated in transfer buffer and run at 30V for 1 hour. After transfer, blots were blocked with PBSTM (PBS, 0.5% powdered non-fat milk, 0.1% Tween-20) for one hour at RT or overnight at 4°C with gentle agitation. Antibodies were made up in PBSTM at appropriate dilutions. Membranes were incubated with antibodies for one hour at RT, with agitation, followed by three washes of 15 minutes each in PBST. Blots were developed using an enhanced chemiluminescence system (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions and placed between clear acetate sheets for exposure to film.

6.2.41 Desalting and buffer exchange the purified His Tag Protein.

Dialysis and D-Salt Excellulose Plastic Desalting columns (from Pierce) were used in this project. In both methods, we have chosen the appropriate dialysis tubing for the former, and the appropriate matrix. In dialysis, the protein samples were poured into the dialysis tubing and were dialysed against PBS buffer overnight with 2 to 3 buffer exchanges. The desalting column was used to apply the sample into the gel. An aliquot of PBS buffer then was used to elute the sample. This column was used for buffer exchange as well.

6.2.42 Purification of His Tag Protein.

For small scale purification, Ni-NTA His Bind ® Risins, Hi Trap Chelating column, and gel filtration were used for purification. We incubated the clarified supernatant with Ni-NTA His-Bind Resins, which are divalent Ni++ cations. The His Tag will bind, while the other proteins which should not bind are discarded. Finally an elution buffer can be added to the beads to elute the His Tagged protein in a purified concentrated form. In the large scale purification, Hi Trap chelating columns

(1ml) was used as follows; the column was washed with H2O, loaded with NiSO4 (100mM NiSO4), then washed again with H2O, meanwhile the protein sample was dialysed against the binding buffer (20 mM Sodium phosphate, 0.5M NaCl pH 7.4). The supernatant was filtered and loaded onto the Aktaprime column at a rate of 2ml/min. Bound protein was eluted using elusion buffer and collected in 2.5ml fractions. Fractions with highest peaks of absorbance were run on an SDS gel and stained with Coomassie Blue and those fractions containing the most protein were pooled and buffer-exchanged.

6.2.43 Glycosylation assay.

To test for glycosylation, the Pro-Q Fuchsia Glycoprotein Gel Stain Kit (from Molecular Probes) was used. After fixing the gel with fixation solution (250 ml of methanol and 250 ddH2O), it was washed with ddH2O and incubated in oxidizing solution (Periodic acid). In the dark, the gel was washed in Pro-Q Fuchsia reagent, before being incubated in the reducing agent (Metabisulfate). After staining with glycoprotein staining kit, the recombinant expressed in *Pichia* then to be compared with the four positive controls in the marker provided with kit. The glycosylated recombinant should stained fushia.

6.2.43 Enzyme –Linked Immunosorbent Assay (ELISA).

Wells of 96-well plates (Immulon 4, HBX Thermo Dynex) were coated with 0.5, 1 and 5 µlg/ml of antigen to be assayed in 100ml coating buffer (15mM Na2 CO3, 35 mM NaCO3 pH 9.3). The wells were then washed 3 times in washing buffer (0.05%Tween 20 in PBS). The adherent antigens were blocked with blocking buffer (1% skimmed milk powder in washing buffer). Human plasma diluted 1:500 in blocking buffer was added in duplicates and incubated overnight at 4°C. After 3 washes, the wells were incubated for 3 hours at room temperature with horseradish peroxidase-conjugated rabbit anti-human IgG (diluted 1:5000 in washing buffer). The wells were then washed again 3 times and incubated for 15 min at room temperature with the substrate (0.1 mg -1 O-Phenylendiamine +0.012 H2O2) in developing buffer (24.5 mM citric acid monohydrate and 52 mM Na2HPO4 pH 5).

The reaction was stopped by addition of 25µl 2M H2SO4 and OD measured at 492nm.

6.2.44 Rabbit immunization.

The antigen concentrations required for each immunization was 50 - 200ug which was administered with complete Freund's adjuvant to ensure a high quality/quantity response Immunisations were carried out by the Harlan Bio-Sera Company according to their rabbit immunisation Protocol:

Day	Action
0	Pre Bleed + Immunisation
14	Boost 1
28	Boost 2
35	Test Bleed 1
42	Boost 3
49	Test Bleed 2
56	Boost 4
63	Test Bleed 3
70	Boost 5
77	Terminal Bleed approx. 50-70 ml serum

6.2.45 Live Immunofluorescence Assay on iRBCs.

Infected red blood cells at 5-8% parasitaemia (4% trophs) were washed and 10µl cell pellet per sample was re-suspended in 100µlPBS/1% BSA containing primary antibody at the appropriate dilution. Samples were incubated on ice for 30 minutes with flicking every 10 minutes to re-suspend cells. Between incubations, cells were washed three times in 1ml PBS/1%BSA and re-suspended in 100µl FITC-conjugated

secondary antibody, at the appropriate dilution in PBS/ 1% BSA. Incubation with secondary antibody was as previously described but in the dark to prevent bleaching of fluorescence. Cells were washed three times as previously and re-suspended at 20-40% haematocrit with 50:50 glycerol: PBS containing 0.1% Evans Blue, 1: 100 000 DAPI and 1.25 mgml-1 DABCO. A drop of each sample was spotted onto a clean glass slide, mounted for observation using a 9:1 solution of glycerol/PBS conatining the anti-fade reagent (Citifluor) and sealed with nail varnish to prevent drying out.

6.2.46 Fixed Immunofluorescence Assay on iRBCs.

Microspot slides were transferred from storage at –20°C and allowed to equilibrate to RT, for 15 minutes. For dry IFA they were used thus. For fixed IFA they were covered in 2% paraformaldehyde/PBS for 5 minutes, followed by 90% methanol/10% acetone for 1-2 minutes. Slides were then air-dried and re-equilibrated in PBST/0.1% Tween-20. Antibodies were made up in 1% BSA/PBS at appropriate dilutions and 30-50µl per spot was used for each incubation. To stain parasite nuclei, DAPI was added to secondary antibody solutions at a dilution of 1:1000. Incubations were for 30 minutes at 37°C followed by 3 washes in PBS, 10 minutes each, with gentle agitation. After the final wash, slides were mounted using 2-3 drops of Vectashield® and sealed with nail varnish.

Appendix 1.

A.the native and recodoned CIDR1 in the nucleotides and amino acids levels.

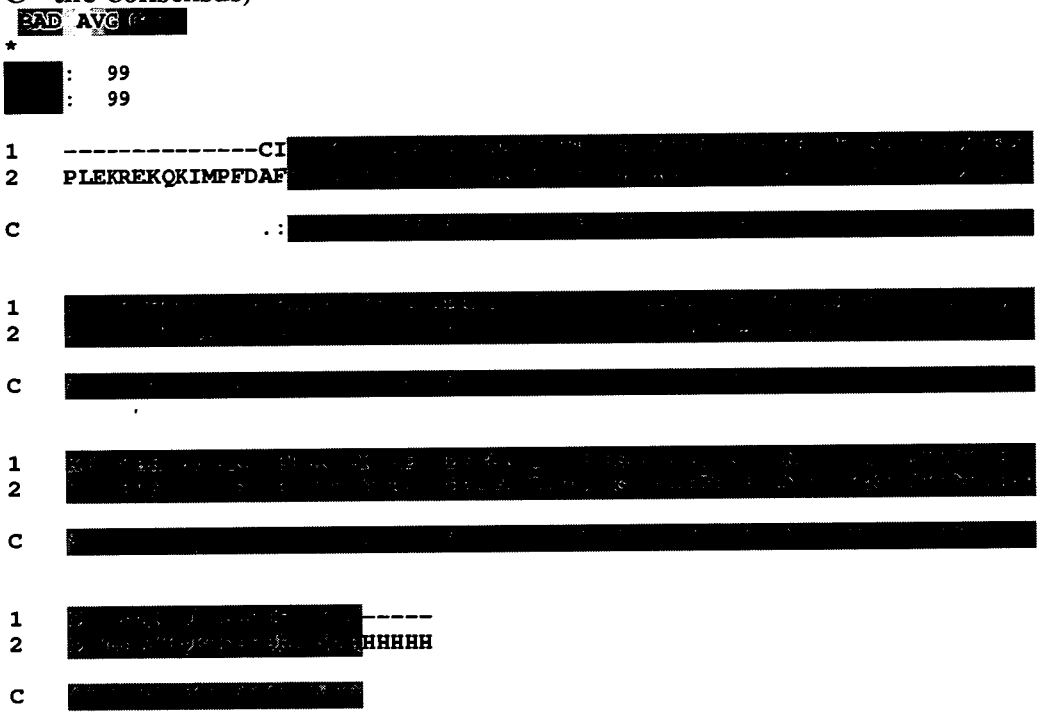
A.1. The native sequence:

```
     C  I  F  F  L  W  L  T  Q  M  L  D  D  S  I  E  W  R  K  K
3    tgcatttttttcctttggctaacacaaatgttagatgattctatagaatggagaaaaaaa  62
     L  K  T  C  I  N  N  E  K  P  T  N  X  I  R  G  C  K  K  P
63   cttaaaacctgtataaataatgaaaaaccaactaattftatacggggttgtaaaaagccc  122
     C  E  C  F  E  R  W  V  E  Q  K  E  E  E  W  I  S  I  E  K
123  tgcgaatgttttgaaagatgggttgaacaaaaagaagaagaatggatttcaattgaaaaa  182
     H  F  D  K  Q  R  D  I  S  E  E  E  R  Y  I  T  L  E  Y  I
183  cattttgacaaacaaagagatatatcagaagaagaacgttatataacacttgaatatatt  242
     L  N  E  F  F  M  D  K  I  E  K  A  Y  G  I  E  K  S  K  E
243  ttgaatgaatttttttatggataaaattgaaaaagcttatggaatagaaaaatcaaaagaa  302
     L  K  E  K  L  K  S  N  K  G  H  G  I  I  R  D  T  E  H  S
303  ttaaaggagaaattaaaatcaaataaaggtcacggaattataagagatacagaacattca  362
     Q  D  A  I  K  I  L  L  E  H  E  L  E  D  A  K  K  C  T  E
363  caggatgcaataaaaatattgctagaacatgaattagaagatgcaaaaaaatgcacagaa  422
     T  H  N  D  E  K  C  K  E  Q  E  E  S  G  G  R  S  L  N  P
423  acccataatgatgaaaaatgtaaagaacaagaagagagcggaggtcgctctttaaacccg  482
     D  P  E  S  D  D  E  E  E  T  D  N  V  K  E  N  P  C  A  V
483  gatccagaatccgacgacgaagaagaaacggataatgtaaaagagaacccgtgtgctgta  542
     G  K  K  L  T  K  T  V  K  Q  I  A  R  Q  M  H  Q  A  A  K
543  gggaaaaaactcactaaaactgtgaaacaaatcgctagacagatgcatcaagcggcaaag  602
     K  Q  L  G  S  S  S  S  R  A  L  K  A  H
603  aaacaattgggtagtagtagtagggcattgaaggctcat                      644
```

A.2. the recodoned CIDR1 sequence:

```
     P  L  E  K  R  E  K  Q  K  I  M  P  F  D  A  F  F  F  L  W
1    ccgctcgagaaaagagagaagcagaagatcatgccattcgacgccttcttcttcctgtgg  60
     L  T  Q  M  L  D  D  S  I  E  W  R  K  K  L  K  T  C  I  N
61   ctgactcagatgctggacgactccatcgagtggcgtaagaagcttaagacctgcatcaac  120
     N  E  K  P  T  N  C  I  R  G  C  K  K  P  C  E  C  F  E  R
121  aacgagaagccaaccaactgtatccgtggttgtaagaagccttgcgagtgtttcgagaga  180
     W  V  E  Q  K  E  Q  E  W  I  S  I  E  K  H  F  D  K  Q  R
181  tgggttgaacagaaggagcaggagtggatctccattgagaagcactttgacaagcagaga  240
     D  I  S  E  E  E  R  Y  I  T  L  E  Y  I  L  N  E  F  F  I
241  gacatctccgaggaagagcgttacatcaccctggagtacatcctgaacgagttcttcatc  300
     D  K  I  E  K  A  Y  G  I  E  K  S  K  E  L  K  E  K  L  K
301  gacaagatcgagaaggcttacggaattgagaagtccaaggagttgaaggagaagctgaag  360
     S  N  K  G  H  G  I  I  R  D  T  E  H  S  Q  D  A  I  K  I
361  tccaacaagggtcacggaatcatccgtgacactgaacactcccaagacgccatcaagatc  420
     L  W  S  T  N  W  R  T  L  R  S  A  P  K  P  T  T  T  R  S
421  ttgtggagcacgaattggaggacgctaagaagtgcaccgaaacccacaacgacgagaagt  480
     V  R  S  R  R  E  S  G  G  R  S  L  N  P  D  P  E  S  D  D
481  gtaaggagcaggagggagtccggtggtagatccctgaaccctgatccagaatccgacgac  540
     E  E  E  T  D  N  V  K  E  N  P  C  A  V  G  K  K  L  T  K
541  gaagaggagactgacaacgtcaaggaaaacccatgtgctgtcggaaagaagctgaccaag  600
     T  V  K  Q  I  A  R  Q  M  H  Q  A  A  K  K  Q  L  G  S  S
601  accgtcaagcaaatcgctagacaaatgcaccaagctgccaagaagcagttgggttcctct  660
     S  S  R  A  L  K  A  H  H  H  H  H  H  *
661  tcctctagagccttgaaggctcaccaccatcaccatcattaa  702
```

A.3. Alignment of the native and the recodoned version of CIDR1 using T-COFFEE, Version,1.41 (1=The native sequence, 2= the recodoned version and C= the Consensus)



B. Samples of sequences obtained from DBLα, β and γ expression libraries

B.1. DBLα sequences.

B.1.1.

```
      L  T  T  L  E  R  S  K  T  T  N  Y  S  K  D  P  T  Y  S  R
2     cttacgacacttgagagatcaaaaacaactaattattcgaaggatcctacgtattcaaga  61
      I  R  L  T  I  F  A  L  R  A  D  N  N  Y  N  R  F  N  C  E
62    atacgactcactatatttgctttgcgagcggataacaattataatagattcaattgtgag  121
      R  I  T  I  S  H  R  I  H  Y  R  G  E  I  N  Y  E  R  I  A
122   cggataacaatttcacacagaattcattatagaggagaaattaactatgagaggatcgca  181
      S  P  S  P  S  R  I  P  E  R  H  L  R  S  S  E  N  R  M  E
182   tcaccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatggaa  241
      K  V  D  N  E  A  D  L  L  A  E  V  C  M  A  A  K  Y  E  G
242   aaagtcgacaacgaagcagatttgttggcagaagtatgtatggcagccaaatacgaaggg  301
      G  S  I  K  T  H  Y  T  P  H  Q  H  K  Y  G  D  S  D  S  Q
302   ggctcaataaaaacacattatacaccacatcaacacaaatatggtgattctgattctcaa  361
      I  C  T  V  L  A  R  S  F  A  D  I  G  D  I  I  R  G  K  D
362   atatgtactgtattagcacgaagttttgcagatattggagacattatacgaggaaaagat  421
```

```
     L  Y  L  G  D  K  K  K  A  K  S  N  R  K  R  K  L  E  Q  K
422  ctgtatctcggtgataaaaaaaaagcaaaatcaaaccgaaagagaaaattagaacagaaa  481
     L  K  E  I  F  K  K  I  H  S  E  V  T  T  N  G  E  L  Q  K
482  ttgaaagaaattttcaagaaaatacatagtgaagtgacgacaaatggggagctacaaaaa  541
     R  Y  G  Q  D  G  Q  N  F  Y  Q  L  R  E  D  W  W  Y  A  N
542  cgctacggacaagacggtcaaaatttttatcaattacgagaagattggtggtatgctaat  601
     R  E  T  V  W  K  A  M  T  C  E  A  P  E  S  A  S  Y  F  R
602  cgagaaacagtatggaaagccatgacatgtgaagcaccagaaagtgcttcatattttcga  661
     K  T  C  D  G  G  E  N  P  T  A  T  Q  G  N  C  K  C  I  N
662  aaaacatgtgatggtggagaaaatccaactgcgactcagggtaactgcaaatgcattaat  721
     G  D  V  P  H  I  S  T  T  S  A  V  S  S  V  V  R  G  M  G
722  ggagatgtcccccatatttcgactacgtccgcagtatcttcggtggtccgaggaatgggc  781
     P  K  T  F  V
782  ccgaagacttttgta  796
```

B.1.2.

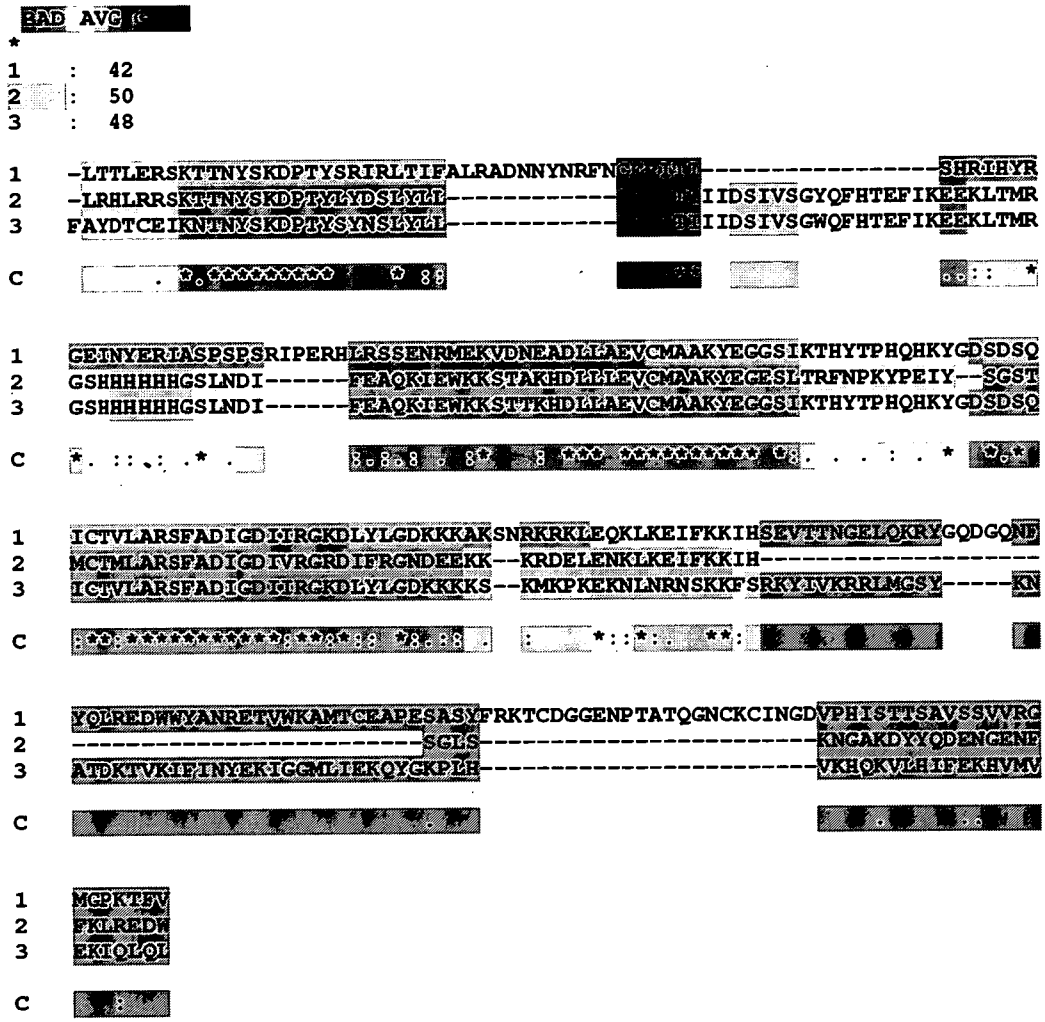
```
     L  R  H  L  R  R  S  K  T  T  N  Y  S  K  D  P  T  Y  L  Y
2    ttacgacacttgaggagatcaaaaacaactaattattcgaaggatcctacgtatttatac  61
     D  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V  S  G
62   gactcactatatttgctttgtgagcggataacaattataatagattcaattgtgagcgga  121
     Y  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S  H  H
122  taccaatttcacacagaattcattaaagaggagaaattaactatgagaggatcgcatcac  181
     H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W  K  K
182  catcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatggaaaaag  241
     S  T  A  K  H  D  L  L  L  E  V  C  M  A  A  K  Y  E  G  E
242  tcgacggctaagcatgatttgttgttagaggtgtgtatggcagccaaatatgaaggagaa  301
     S  L  T  R  F  N  P  K  Y  P  E  I  Y  S  G  S  T  M  C  T
302  tctttaacacgttttaatccaaaatatccagaaatatattctggttctacaatgtgtact  361
     M  L  A  R  S  F  A  D  I  G  D  I  V  R  G  R  D  I  F  R
362  atgttggcacgcagttttgcagatataggagatattgtaagaggaagagatatatttcgt  421
     G  N  D  E  E  K  K  K  R  D  E  L  E  N  K  L  K  E  I  F
422  ggtaatgatgaagaaaaaaaaaaaaagagatgaattagaaaataagttgaaagaaattttc  481
     K  K  I  H  S  G  L  S  K  N  G  A  K  D  Y  Y  Q  D  E  N
482  aagaaaatacatagtggattgtcgaagaacggcgcaaaagactactaccaagatgaaaat  541
     G  E  N  F  F  K  L  R  E  D  W
542  ggtgaaaatttttttaaattacgagaagattgg  574
```

B.1.3.

```
     F  A  Y  D  T  C  E  I  K  N  T  N  Y  S  K  D  P  T  Y  S
2    tttgcttacgacacttgtgagatcaaaaacactaattattcgaaggatcctacgtattca  61
     Y  N  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V  S
62   tacaactcactatatttgctttgtgagcggataacaattataatagattcaattgtgagc  121
     G  W  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S  H
122  ggatggcaatttcacacagaattcattaaagaggagaaattaactatgagaggatcgcat  181
     H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W  K
182  caccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatggaaa  241
     K  S  T  T  K  H  D  L  L  A  E  V  C  M  A  A  K  Y  E  G
242  aagtcgacaacgaagcatgatttgttggcagaagtatgtatggcagccaaatacgaaggg  301
     G  S  I  K  T  H  Y  T  P  H  Q  H  K  Y  G  D  S  D  S  Q
302  ggctcaataaaaacacattatacaccacatcaacacaaatatggtgattctgattctcaa  361
     I  C  T  V  L  A  R  S  F  A  D  I  G  D  I  I  R  G  K  D
362  atatgtactgtattagcacgaagttttgcagatattggagacattatacgaggaaaagat  421
     L  Y  L  G  D  K  K  K  K  S  K  M  K  P  K  E  K  N  L  N
422  ctgtatctcggtgataaaaaaaaaaaaaagcaaaatgaaaccgaaagagaaaaatttgaac  481
     R  N  S  K  K  F  S  R  K  Y  I  V  K  R  R  L  M  G  S  Y
482  agaaattcaaagaaattttcaagaaaatacatagtgaagagacgactaatggggagctac  541
     K  N  A  T  D  K  T  V  K  I  F  I  N  Y  E  K  I  G  G  M
542  aaaaacgctacggacaagacggtcaaaatttttatcaattacgagaagattggtggtatg  601
     L  I  E  K  Q  Y  G  K  P  L  H  V  K  H  Q  K  V  L  H  I
```

```
602  ctaatcgagaaacagtatggaaagccattacatgtgaagcaccagaaagtgcttcatatt  661
     F  E  K  H  V  M  V  E  K  I  Q  L  Q  L
662  ttcgaaaaacatgtgatggtggagaaaatccaactgcaactc  703
```

B.1.4. DBLαs amino acids Sequences alignment.



B.2. DBLβ sequences.

B.2.1.

```
     R  F  T  T  L  G  E  D  Q  K  T  T  N  Y  S  K  D  P  T  Y
2    cgctttacgacacttggagaagatcaaaaaacaactaattattcgaaggatcctacgtat  61
     Y  Y  D  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V
62   tattacgactcactatatttgctttgtgagcggataacaattataatagattcaattgtg  121
     S  G  S  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S
122  agcggatcacaatttcacacagaattcattaaagaggagaaattaactatgagaggatcg  181
```

```
     H  H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W
182  catcaccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatgg  241
     K  K  S  T  R  E  H  V  C  T  S  N  L  E  Y  L  L  S  G  R
242  aaaaagtcgacccgagaacacgtgtgtacctcaaatttagaatatttactttctggtaga  301
     G  G  Q  F  E  Q  V  P  D  D  K  A  S  D  S  F  L  G  D  V
302  ggcggacaatttgaacaagttccagatgataaagctagcgactcattcttgggcgatgtt  361
     L  V  A  A  R  Y  R  S  R  K  H  K  E  T  G  W  N  S  C  P
362  cttgttgcagcacgatatagaagcagaaaacataaagaaactggatggaactcatgcccg  421
     M  K  P  N  S  C  K  R  R  S  Y  Y  L  P  C  Y  T  L  Q  X
422  atgaagccgaattcatgtaaaagacgaagctactatttgccgtgctatacgttacagntt  481
     C  R  Y  W  R  Y  Y  S  R  N  R  F  V  G  Y  K  W  L  C  N
482  tgcagatattggcgatattattcgaggaacagatttgtgggatataaatggttatgtaac  541
     R  C  A  K  S  F  T  N  G  Y  L  E  T  S  K  S
542  aggtgtgcaaagtcatttacaaacgggtatttggaaacttcaaaaagc  589
```

B.2.2.

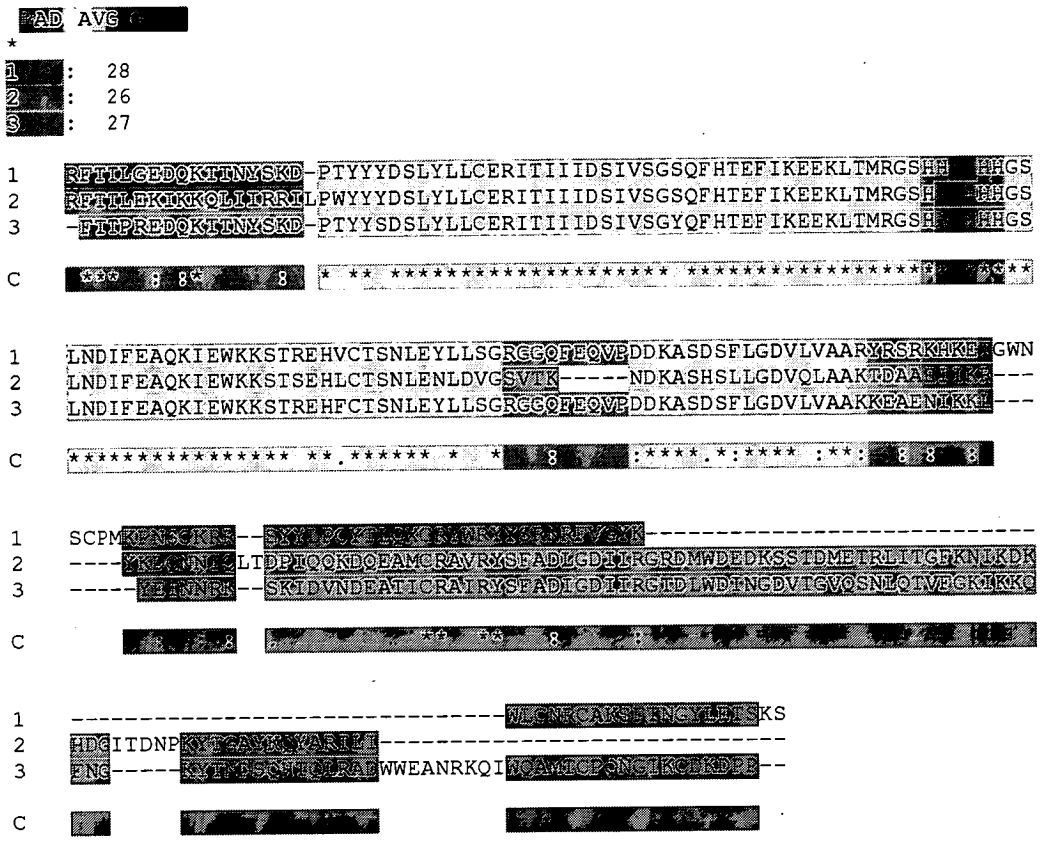
```
     R  F  T  T  L  E  K  I  K  K  Q  L  I  I  R  R  I  L  P  W
1    cgctttacgacacttgagaagatcaaaaaacaactaattattcgaaggatcctaccatgg  60
     Y  Y  Y  D  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I
61   tattattacgactcactatatttgctttgtgagcggataacaattataatagattcaatt  120
     V  S  G  S  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G
121  gtgagcggatcacaatttcacacagaattcattaaagaggagaaattaactatgagagga  180
     S  H  H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E
181  tcgcatcaccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaa  240
     W  K  K  S  T  S  E  H  L  C  T  S  N  L  E  N  L  D  V  G
241  tggaaaaagtcgaccagtgaacacttgtgtacatccaatttagaaaatttagatgttggt  300
     S  V  T  K  N  D  K  A  S  H  S  L  L  G  D  V  Q  L  A  A
301  agtgtcactaaaaatgataaggctagccactcattattgggagatgttcagctcgcagca  360
     K  T  D  A  A  E  I  I  K  R  Y  K  D  Q  N  N  I  Q  L  T
361  aaaactgatgcagctgagataataaaacgctataaagatcaaaataatatacaactaact  420
     D  P  I  Q  Q  K  D  Q  E  A  M  C  R  A  V  R  Y  S  F  A
421  gatccaatacaacaaaaagaccaggaggctatgtgtcgagctgtacgttatagttttgcc  480
     D  L  G  D  I  I  R  G  R  D  M  W  D  E  D  K  S  S  T  D
481  gatttaggagacattattcgaggaagagatatgtgggatgaggataagagctcaacagac  540
     M  E  T  R  L  I  T  G  F  K  N  I  K  D  K  H  D  G  I  T
541  atggaaacacgtttgataaccggatttaaaaacattaaagataaacatgatggaatcaca  600
     D  N  P  K  Y  T  G  A  Y  K  Q  Y  A  R  I  L  I
601  gacaaccctaaatataccggtgcatataagcaatacgcccgcatattaata  651
```

B.2.3.

```
     F  T  T  P  R  E  D  Q  K  T  T  N  Y  S  K  D  P  T  Y  Y
3    tttacgacacctagagaagatcaaaaaacaactaattattcgaaggatcctacgtattat  62
     S  D  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V  S
63   tccgactcactatatttgctttgtgagcggataacaattataatagattcaattgtgagc  122
     G  Y  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S  H
123  ggatatcaatttcacacagaattcattaaagaggagaaattaactatgagaggatcgcat  182
     H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W  K
183  caccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatggaaa  242
     K  S  T  R  E  H  F  C  T  S  N  L  E  Y  L  L  S  G  R  G
243  aagtcgacccgtgaacacttttgtacctcaaatttagaatatttactttctggtagaggc  302
     G  Q  F  E  Q  V  P  D  D  K  A  S  D  S  F  L  G  D  V  L
303  ggacaatttgaacaagttccagatgataaagctagcgactcattcttgggcgatgttctt  362
     V  A  A  K  K  E  A  E  N  I  K  K  L  Y  E  T  N  N  R  K
363  gttgcagcaaaaaaagaagcagaaaacataaagaaactgtatgaaacaaataaccgaaaa  422
     S  K  I  D  V  N  D  E  A  T  I  C  R  A  I  R  Y  S  F  A
423  agcaaaattgatgtaaatgacgaagcaactatttgccgtgctatacgttacagttttgca  482
     D  I  G  D  I  I  R  G  T  D  L  W  D  I  N  G  D  V  T  G
483  gatattggggatattattcgaggaacagatttgtgggatataaatggtgatgtaacaggt  542
     V  Q  S  N  L  Q  T  V  F  G  K  I  K  K  Q  F  N  G  K  Y
```

```
543  gtgcaaagtaatttacaaacggtttttggtaaaatcaaaaagcaatttaatggcaaatat  602
     T  N  D  S  Q  H  T  Q  L  R  A  D  W  W  E  A  N  R  K  Q
603  accaatgactcccaacatacacaattacgagcagattggtgggaggctaatagaaaacaa  662
     I  W  Q  A  M  T  C  P  Q  N  G  I  K  C  D  K  D  P  P
663  atatggcaagccatgacatgtccacaaaatggtatcaagtgtgataaagatccaccac  720
```

B.2.4. DBLβ amino acids Sequences alignment



B.3. DBLγ sequences.

B.3.1.

```
     R  L  Y  D  T  T  E  D  Q  K  T  T  N  Y  S  K  D  P  T  Y
1    cgcctttacgacactacagaagatcaaaaaacaactaattattcgaaggatcctacgtat  61
     Q  Y  H  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V
62   caataccactcactatatttgctttgtgagcggataacaattataatagattcaattgtg  121
     S  G  L  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S
122  agcggacttcaatttcacacagaattcattaaagaggagaaattaactatgagaggatcg  181
     H  H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W
182  catcaccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatgg  241
     K  K  S  T  C  M  P  P  R  R  Q  K  L  C  I  Y  K  L  T  R
242  aaaaagtcgacctgcatgcctcctagaagacaaaaattatgtatatataaattaacaaga  301
     D  N  D  T  K  D  L  K  E  L  K  M  S  F  I  K  S  A  A  L
302  gataatgacacaaaagaccttaaagaattaaaaatgtcatttattaaatctgcagcacta  361
     E  T  Y  L  A  W  E  I  Y  K  G  N  N  I  D  A  I  T  E  L
```

```
362  gaaacatatcttgcatgggaaatatataaaggaaataatattgatgccattacggaatta  421
     Q  N  G  Q  I  P  E  G  F  K  R  I  M  F  Y  T  F  G  D  F
422  caaaatggacaaattccagaaggttttaaaagaataatgttttacacatttggagatttt  481
     R  D  M  C  L  G  T  D  I  S  S  K  A  N  K  S  T  G  V  G
482  cgagatatgtgtttgggtactgatatatcatcaaaagcaaataaaagtacaggtgtaggt  541
     K  V  E  S  S  I  N  K  L  F  P  N  V  K  N  S  K  S  Q  D
542  aaagtagaatctagtataaataaacttttcccaaatgtcaaaaattctaaatcacaagac  601
     R  K  T  W  W  N  S  I  E  K  E  V  W  E  G  M  L  C  G  L
602  cgtaaaacgtggtggaattcaattgaaaaagaggtatgggaagggatgttatgtgggtta  661
     S  H  A  G  G  N  D  A  I  K  S  N  Q  D  Y  Q  Y  S  R  G
662  tcacatgctggtggcaatgacgctatcaaaagcaaccaggactaccaatactcccgagga  721
     Y  N  I  L  S  K  L  W  K  W  Y  P  H  Y  P  N  L  P  N  D
722  tataatattttatccaagttatggaagtggtatccccattatcccaatttgccgaacgac  781
     P  R  F  W  D  G  S  R  P  R  I  N  S  P  L  H  M  T
782  ccccgattctgggatggttcgcggccgcgaattaattcccccttacacatgacgg  836
 836
```

B.3.2.

```
     F  Y  D  T  E  E  D  Q  K  T  T  N  Y  S  K  D  P  T  Y  P
2    ttttacgacagaggagaagatcaaaaaacaactaattattcgaaggatcctacgtatcct  61
     Y  N  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V  S
62   tacaactcactatatttgctttgtgagcggataacaattataatagattcaattgtgagc  121
     G  L  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S  H
122  ggacttcaatttcacacagaattcattaaagaggagaaattaactatgagaggatcgcat  181
     H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W  K
182  caccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatggaaa  241
     K  S  T  C  K  I  C  G  R  E  L  I  R  L  R  H  D  C  S  S
242  aagtcgacctgcaagatctgcggccgcgaattaattcgccttagacatgactgttcctca  301
     V  Q  V  G  H  L  R  E  D  R  S  C  L  I  L  I  K  R  M  S
302  gttcaagttgggcacttacgagaagaccggtcttgcttgattctaatcaagaggatgtca  361
     E  C  H  L  P  E  R  C  R  L  H  F  R  Y  F  F  I  C  N  L
362  gaatgccatttgcctgagagatgcaggcttcattttcgatacttttttatttgtaaccta  421
     Y  S  I  G  F  F  L  S  F  C  F  F  S  Y  E  L  A  P  D  Q
422  tatagtataggatttttttgtcattttgtttcttctcgtacgagcttgctcctgatcag  481
     P  I  S  Q  L  M  N  I  L  W  S  G  F  G  K  I  I  R  V  R
482  cctatctcgcagctgatgaatatcttgtggtcggggtttgggaaaatcattcgagttcga  541
     C  F  S  W  Y  F  P  L  L  F  R  V  Q  K  I  K  L  D  L  R
542  tgtttttcttggtatttcccactcctcttcagagtacagaagattaagttagaccttcgt  601
     L  C  G  S  N  Y  Y  L  L  I  L  V  N  P  K  L  V  D  F  I
602  ttgtgcggatccaattattatttacttattttggtcaaccccaaattagttgatttcata  661
     L  G  F  I  Q  K  Y  V  V  F  L  D  L  S  I  L  S  C  I  Y
662  cttgggttcattcaaaaatacgtagtctttttagatctttcaatattatcatgtatatac  721
     Y  N  S  P  T  C  F  I  F  A  N  V  P  P  A  L  S  D  P
722  tataacagcccgacttgtttcattttcgcgaatgttcccccagctttatcggatccccc  780
```

B.3.3.

```
     L  Y  D  T  L  A  D  Q  K  T  T  N  Y  S  K  D  P  T  Y  I
3    ctttacgacactcttgcagatcaaaaaacaactaattattcgaaggatcctacgtatatt  62
     Y  D  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V  S
63   tacgactcactatatttgctttgtgagcggataacaattataatagattcaattgtgagc  122
     G  T  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S  H
123  ggaactcaatttcacacagaattcattaaagaggagaaattaactatgagaggatcgcat  182
     H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W  K
183  caccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatggaaa  242
     K  S  T  C  M  P  P  R  R  Q  S  L  C  I  H  D  L  K  V  L
243  aagtcgacctgtatgccacctaggagacaatcattatgcatacatgatttaaaagtatta  302
     T  N  T  S  S  E  K  Q  L  R  E  A  F  I  N  C  A  A  I  E
303  acgaacacttcatcggaaaaacaattaagagaagcttttattaactgtgcagcaatagaa  362
     T  H  F  L  W  I  Y  Y  K  N  K  N  S  S  I  V  D  T  Q  L
```

```
363  acacatttttatggatttattataagaataaaaatagtagtattgtagacacacagtta  422
     Q  N  G  N  I  P  D  E  F  K  S  I  M  Y  Y  T  F  G  D  Y
423  caaaatggaaatattccagacgaatttaaaagtataatgtattatacgtttggcgattat  482
     R  D  I  C  L  G  T  D  I  S  S  D  S  N  I  K  G  I  S  Q
483  agagatatatgtttgggaacagatatttctagtgattccaatattaagggaatatcacag  542
     K  V  I  D  I  L  N  S  Q  Y  G  K  T  H  E  Q  Y  I  T  P
543  aaggttattgatattttaaatagtcaatatggcaaaactcatgaacaatatattacacct  602
     N  T  W  W  E  K
603  aacacttggtgggaaaaa  620
```

B.3.4.

```
        L  R  H  L  E  K  I  K  K  Q  P  N  Y  S  K  D  P  T  S  D
3    ttacgacacttggagaagatcaaaaaacaacctaattattcgaaggatcctacgtcggat  62
     H  Y  H  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V
63   CACtaccactcactatatttgctttgtgagcggataacaattataatagattcaattgtg  122
     S  G  K  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S
123  agcggaaaacaatttcacacagaattcattaaagaggagaaattaactatgagaggatcg  182
     H  H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W
183  catcaccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatgg  242
     K  K  S  T  C  M  P  P  S  R  Q  K  L  C  V  I  N  L  K  T
243  aaaaagtcgacctgcatgcctccaagtagacaaaagttatgtgtaatttaatttaaaaact  302
     F  T  G  K  T  T  V  D  L  R  E  A  F  I  K  C  A  A  I  E
303  tttacagggaaaacaacagtggacttacgagaagcttttattaaatgtgctgcaatagaa  362
     T  H  F  L  W  K  Y  Y  K  T  K  N  S  E  A  H  D  D  L  K
363  acacatttttatggaaatattataaaactaaaaactcagaagcacatgatgatttgaag  422
     K  G  T  I  P  E  K  F  K  R  Q  M  F  Y  T  F  G  D  Y  X
423  aaaggaacaattcctgagaaatttaaacgtcaaatgttctatacatttggagattatngg  482
     D  L  C  L  D  K  N  I  G  N  D  V  S  D  A  K  N  N  I  K
483  gatttatgtttggataaaaatataggtaatgatgtgagtgatgcaaaaaataatataaag  542
     G  V  F  L  Q  M  L  T  L  P  A  N
543  ggtgtttttctccagatgctaaccctccctgctaacct  580
```

B.3.5.

```
        L  Y  D  T  D  E  D  Q  K  T  T  N  Y  S  K  D  P  T  Y  S
1    ctttacgacactgatgaagatcaaaaaacaactaattattcgaaggatcctacgtattcg  60
     Y  N  S  L  Y  L  L  C  E  R  I  T  I  I  I  D  S  I  V  S
61   tacaactcactatatttgctttgtgagcggataacaattataatagattcaattgtgagc  120
     G  E  Q  F  H  T  E  F  I  K  E  E  K  L  T  M  R  G  S  H
121  ggagaacaatttcacacagaattcattaaagaggagaaattaactatgagaggatcgcat  180
     H  H  H  H  H  G  S  L  N  D  I  F  E  A  Q  K  I  E  W  K
181  caccatcaccatcacggatccctgaacgacatcttcgaagctcagaaaatcgaatggaaa  240
     K  S  T  C  M  P  P  R  R  Q  K  L  C  V  I  N  L  Q  H  F
241  aagtcgacctgtatgccaccaaggagacaaaaattatgtgtaatttaatttacaacatttt  300
     K  E  N  T  S  V  E  L  R  K  A  F  I  Q  C  A  A  A  E  T
301  aaagagaatacatcagttgagttgagaaaagcttttattcaatgcgctgccgcagaaact  360
     F  L  L  W  H  K  Y  K  T  D  N  N  G  G  E  E  L  Q  N  Q
361  tttttactatggcataaatacaaaacggataacaatggtggtgaagaactacaaaaccaa  420
     L  E  S  G  I  I  P  E  D  F  K  R  Q  M  F  Y  T  F  G  D
421  ttagaaaagtggaataatccctgaagattttaagcgccaaatgttctacacatttggcgat  480
     Y  R  D  L  C  L  D  K  N  I  G  R  D  V  S  E  V  E  K  N
481  tacagagatttgtgtttggataaaaatataggtagagatgtgagtgaagtagaaaaaaat  540
     I  K  R  V  F  S  S  N  G  D  K  T  P  N  G  Q  S  R  L  D
541  ataaaacgtgtcttctcaagtaatggagacaaaacacctaatggccaatcacgtctagac  600
     W  W  G  K  N  C  Q  D  I  W  Q  G  M  L  C  G  L  S  H  A
601  tggtggggaaaaaactgtcaagatatatggcaaggaatgttatgtggcctatcacacgct  660
     S  G  N  I  S  N  V  E  T  I  K  N  N  N  T  Y  S  L  C  Q
661  agtggaaatataagtaacgtagaaactatcaaaaacaacaacacctactccctctgtcaa  720
     I  Q  W  L  Q  W  R  P  R  P  L  R  N  S  L  N  D  P  R  S
721  atccagtggttacaatggaggccccgcccccttgaggaattctctaaacgacccccgttcc  780
```

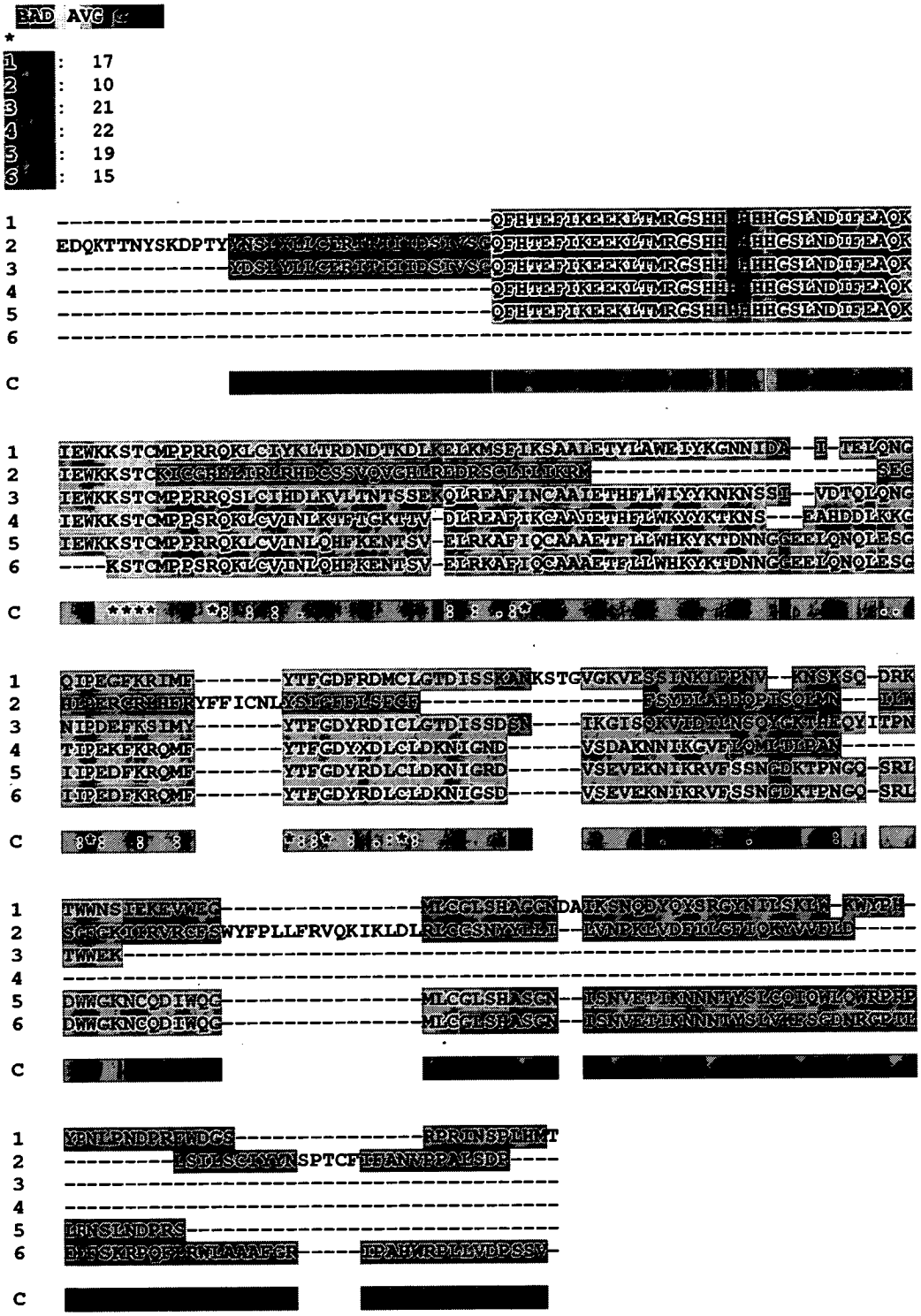
B.3.6.

```
     C  R  I  R  P  P  K  S  T  C  M  P  P  S  R  Q  K  L  C  V
1    tgcagaattcgccctcctaagtcgacctgcatgcctcctagtagacaaaaattatgtgta  60
     I  N  L  Q  H  F  K  E  N  T  S  V  E  L  R  K  A  F  I  Q
61   attaatttacaacattttaaagagaatacatcagttgagttgagaaaagcttttattcaa  120
     C  A  A  A  E  T  F  L  L  W  H  K  Y  K  T  D  N  N  G  G
121  tgcgctgccgcagaaacttttttactatggcataaatacaaaacggataacaatggtggt  180
     E  E  L  Q  N  Q  L  E  S  G  I  I  P  E  D  F  K  R  Q  M
181  gaagaactacaaaaccaattagaaagtggaataatccctgaagattttaagcgccaaatg  240
     F  Y  T  F  G  D  Y  R  D  L  C  L  D  K  N  I  G  S  D  V
241  ttctacacatttggcgattacagagatttgtgtttggataaaaatataggtagtgatgtg  300
     S  E  V  E  K  N  I  K  R  V  F  S  S  N  G  D  K  T  P  N
301  agtgaagtagaaaaaaatataaaacgtgtcttctcaagtaatggagacaaaacacctaat  360
     G  Q  S  R  L  D  W  W  G  K  N  C  Q  D  I  W  Q  G  M  L
361  ggccaatcacgtctagactggtggggaaaaaactgtcaagatatatggcaaggaatgtta  420
     C  G  L  S  H  A  S  G  N  I  S  N  V  E  T  I  K  N  N  N
421  tgtggcctatcacacgctagtggaaatataagtaacgtagaaactatcaaaaacaacaac  480
     T  Y  S  L  V  K  F  S  G  D  N  R  G  P  T  L  E  E  F  S
481  acctactccctcgtcaaattcagtggtgacaatagaggccccacccttgaggaattctct  540
     K  R  P  Q  F  L  R  W  L  A  A  A  F  W  G  R  I  P  A  H
541  aaacgaccccaattcctaagatggttagcggccgctttttgggggcgaattccagcacac  600
     W  R  P  L  L  V  D  P  S  S  V
601  tggcggccgttactagtggatccgagctcggtacc  635
```

B.3.7.

```
     G  R  W  A  L  S  C  M  L  E  R  P  P  V  K  W  I  S  I  C
3    gggcgatgggccctctcctgcatgctcgagcggccgccagtgccctggatatctatatgc  62
     R  I  R  P  s  K  S  T  C  M  P  P  S  R  Q  N  L  C  V  H
63   agaattcgccctagtaagtcgacctgtatgcctcctagtagacaaaatttatgtgtacat  122
     Y  L  T  K  L  N  D  D  S  K  E  E  D  L  R  E  A  F  I  K
123  tatttaacaaaattgaatgacgactccaaagaagaagatttaagagaggcatttattaaa  182
     S  A  A  A  E  T  F  L  L  R  Q  Y  Y  N  S  K  N  V  E  D
183  tctgcagcagcagaaacatttcttctacggcaatattataatagtaagaatgttgaggac  242
     D  K  I  L  H  R  D  M  I  P  P  E  F  F  R  S  M  F  Y  T
243  gataaaatattacatagagatatgatccctcccgaatttttcagatccatgttctataca  302
     F  G  D  Y  R  D  I  C  L  D  T  D  I  S  E  K  I  A  D  H
303  tttggagattatagagatatatgtttagatacagatatatctgaaaaaattgcagatcat  362
     D  V  T  T  A  K  K  K  I  T  A  V  F  Q  K  I  G  S  K  T
363  gatgtaactacggcaaaaaagaaaataactgctgttttccaaaaaattggctccaaaact  422
     T  N  G  K  K  V  L  E  R  E  G  W  W  K  E  Y  G  L  S  I
423  actaatggaaaaaaggtgctagaacgcgaagggtggtggaaagaatatggtctatctatt  482
     W  K  G  M  L  C  A  L  S  Y  N  T  E  T  K  K  M  D  E  G
483  tggaaaggaatgttatgtgctttaagttataatactgaaacaaaaaaaatggatgaagga  542
     V  R  T  Y  L  M  K  Y
543  gtgcgcacataccttatgaagtat  566
```

B.3.8. DBLγ amino acids Sequences alignment.



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