Gene expression profiling of polyamine-depleted *Plasmodium falciparum*

by

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DECLARATION

I declare that the thesis/dissertation that I hereby submit for the degree in MSc (Biochemistry) at the University of Pretoria has not been previously submitted by me or anyone else for degree purposes at any other university.

Signature: _____

Date:_____

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LIST OF ABBREVIATIONS

A	Adenosine
A_{260}	Absorbance at 260nm
ABC	ATP binding cassette
ADC	Arginine decarboxylase
AdoMet	S-adenosylmethionine
AdoMetDC	S-adenosylmethionine decarboxylase
ADP	Adenosine diphosphate
ADH	Aldehyde dehydrogenase
AMA	Apical membrane protein
amp	Ampicillin
ARG	Arginine
ATP	Adenosine triphosphate
AZ	Antizyme
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
CCD	Charged coupled device
CD	Cluster of differentiation
cDNA	Complementary DNA
cg	Candidate gene
CLAG	Cytoadherence linked asexual protein
c-Myc	Nuclear transcription factor, product of *myc* proto-oncogene
CSP	Circumsporozoite protein
CTAB	Hexadecyltrimethyl-ammonium bromide
dATP	Deoxyadenosine triphosphates
dCTP	Deoxycytidine triphosphate
dcAdoMet	Decarboxylated S-adenosylmethionine
DFMO	Difluoromethylornithine
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
dGTP	Deoxyguanosinine triphosphate
DHH	Deoxyhypusine hydroxylase
DHPS	Dihydropteroate synthase
DHS	Deoxyhypusine synthase
DIG	Digoxygenin
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleiotide triphosphates
DPAP	Dipeptidyl aminopeptidase
ds	Double stranded
dT	Deoxythymidine
DTE	Dithioerythritol
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
EBA	Erythrocyte binding antigen

EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic translation initiation factor
ELAM	Endothelial cell adhesion molecule
EMP	Erythrocyte membrane protein
ER	Estrogen receptor
ERE	Estrogen receptor element
EST	Expressed sequence tags
FAD	Flavin adenine dinucleotide
FS	Forward subtracted
FSSH	Forward suppression subtractive hybridisation
FUS	Forward unsubtracted
G	Guanosine
G3PDH	Glucose-3-phosphate dehydrogenase
GPI	Glycosyl phosphatidyl inositol
GTP	Guanosine triphosphate
HEPES	(N-[2-Hydroxyethyl] piperazine-N'-[2-ethane-sulphonic acid])
Hsp	Heat shock protein
HSS	Homospermidine synthase
IC_{50}	Concentration that inhibits 50% of population
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
IPTG	Isopropyl-D-galactoside
kb	Kilo base pairs
LD-PCR	Long distance PCR
LB Broth	Luria-Bertani broth
M	Molar
mM	Millimolar
µM	Micromolar
MGBG	Methylglyoxal bis(guanylhydrazone)
MOS	Mirror orientated selection
MR	Methionine recycling pathway
mRNA	Messenger RNA
MSP	Merozoite surface protein
MTA	Methylthioadenosine
mTHF	Methylene-tetrahydrofolate
NO	Nitric Oxide
NP	Nested PCR primer
OAT	Ornithine amino transferase
OD	Optical density
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCS	PCR control subtracted cDNA
PfATPase	*P. falciparum* ATPase
pfcrt	*P. falciparum* chloroquine resistance gene
PfDHFR-TS	*P. falciparum* dihydrofolate reductase thymidylate synthase
Pfmdr	*P. falciparum* multidrug resistance gene
PlasmoDB	*P. falciparum* database
PrP^{Sc}	Scrapie protease resistant protein
Put	Putrescine
RAP	Rhoptry associated protein
RDA	Representational difference analysis
Rhop	Rhoptry protein
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per min
RS	Reverse subtracted
RSSH	Reverse suppression subtractive hybridisation
RUS	Reverse unsubtracted
RT	Reverse transcriptase
S	Svedberg constant
SAGE	Serial analysis of gene expression
SAM	S-adenosylmethionine
SAMDC	S-adenosylmethionine decarboxylase
SC	Subtracted control skeletal muscle cDNA
SD	Shine-Dalgarno
SDS	Sodium dodecyl sulphate
SERA	Serine rich antigen
Spd	Spermidine
Spm	Spermine
SpdSyn	Spermidine synthase
SpmSyn	Spermine synthase
ss	Single stranded
SSAT	Spermidine/Spermine N^1-acetyltransferase
SSC	Saline sodium citrate
SSH	Suppression subtractive hybridisation
STET	Sucrose, Triton, EDTA, Tris buffer
T	Thymidine
TAE	Tris, Acetic acid, EDTA buffer
TE	Tris, EDTA buffer
TNE	Tris, NaCl, EDTA buffer
TNF	Tumour necrosis factor
U	Uracil
UC	Unsubtracted control skeletal muscle cDNA
UTP	Uridine triphosphate
UV	Ultraviolet
V	Volts

VCAM	Vascular cell adhesion molecule
WHO	World health organisation
X-gal	5-bromo-4-chloro-indolyl-β–D-galactoside

CHAPTER 1

Literature Overview

1.1 General

Malaria is a life-threatening parasitic disease transmitted by mosquitoes. It was once thought that the disease came from fetid marshes, hence the name mal-aria, (meaning: bad air). In 1880, scientists discovered the actual cause of malaria to be a single-celled parasite called *Plasmodium* (Elford 1995). In 1897, Ronald Ross (1857-1932) discovered that the parasite was transmitted from person to person during the blood meal of a female *Anopheles* mosquito (Hagan and Chauhan, 1997). He also concluded that more than one species of parasite existed, though the mode of transmission was still unknown (Hagan and Chauhan, 1997). Of the four species that infect humans, *P. vivax* and *P. falciparum* are the most common and the latter the most deadly type of malaria infection (Richie and Saul, 2002).

Approximately 40% of the world's population, mostly those living in the world's poorest countries, is at risk of becoming infected with malaria (Malaria Foundation International [http://www.malaria.org]). The disease, once widespread, was successfully eliminated from many countries with temperate climates during the mid 20th century but is still endemic to tropical and subtropical regions of the world (Figure 1.1) (Phillips 2001 and Malaria Foundation International).

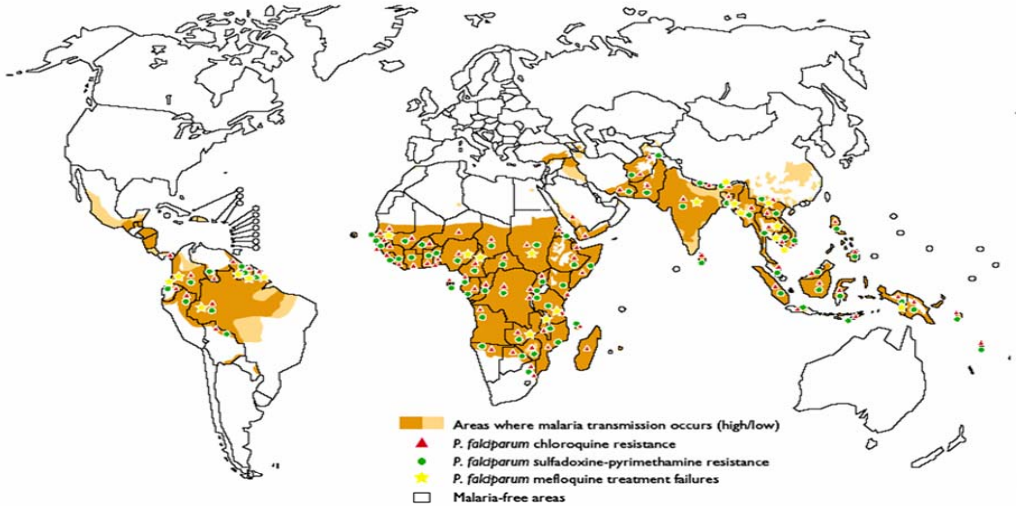


Figure 1.1: Malaria is endemic to tropical and subtropical regions and resistance to antimalarials is widespread (WHO, 2005).

Currently malaria is responsible for 300 - 500 million cases of clinical illness worldwide and accounts for between 0.7 and 2.7 million deaths annually, with Africa being the worst affected (Bozdech *et al.*, 2003a). Anaemia, low birth-weight, epilepsy, and neurological problems, all frequent consequences of malaria, compromise the health and development of millions of children throughout the tropical world (Malaria Foundation International). Malaria is Africa's leading cause of mortality in children under five years old (20%) and constitutes 10% of the continent's overall disease burden (www.rbm.who.int). It accounts for 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% of outpatient visits in areas with high malaria transmission (www.rbm.who.int).

1.2 Intraerythrocytic life cycle

P. falciparum parasites have a complicated life cycle in which they alternate between an extracellular environment in the female mosquito vector and an intracellular one in the host (Sacci and Azad, 2002). The life cycle can be divided into two distinct stages: a sexual stage which takes place within the *Anopheles* mosquito and an asexual stage within the human host (Figure 1.2) (Braun 1988). In the human host there are different phases present: the invading sporozoites, trophozoites, schizonts, merozoites that result in the disease symptoms and gametocytes (Elford 1995). The disease symptoms are associated with the asexual intra-erythrocytic stage.

Sporozoites are released from the female mosquito's salivary glands into the circulating blood of the host and subsequently enter the liver hepatocytes. The precise nature of the ligand-receptor binding interaction between the sporozoite and the hepatocyte is not known. However, it has been suggested that a major surface protein on the parasite, the circumsporozoite protein (CSP), may play a role in this interaction (Heddini 2002). Growth and division occurs within the liver, thereafter thousands of merozoites are released into the blood stream where they attach to and invade erythrocytes (Elford 1995). Recognition and attachment are via multiple receptor-ligand interactions between surface proteins on the malaria parasite (e.g. merozoite surface protein-1) and erythrocyte surface receptors (Heddini 2002). Intraerythrocytic parasites, called trophozoites, mature within a membrane bound vacuole that forms from an invagination of the host erythrocyte during the invasion process (Bannister *et al.*, 2000).

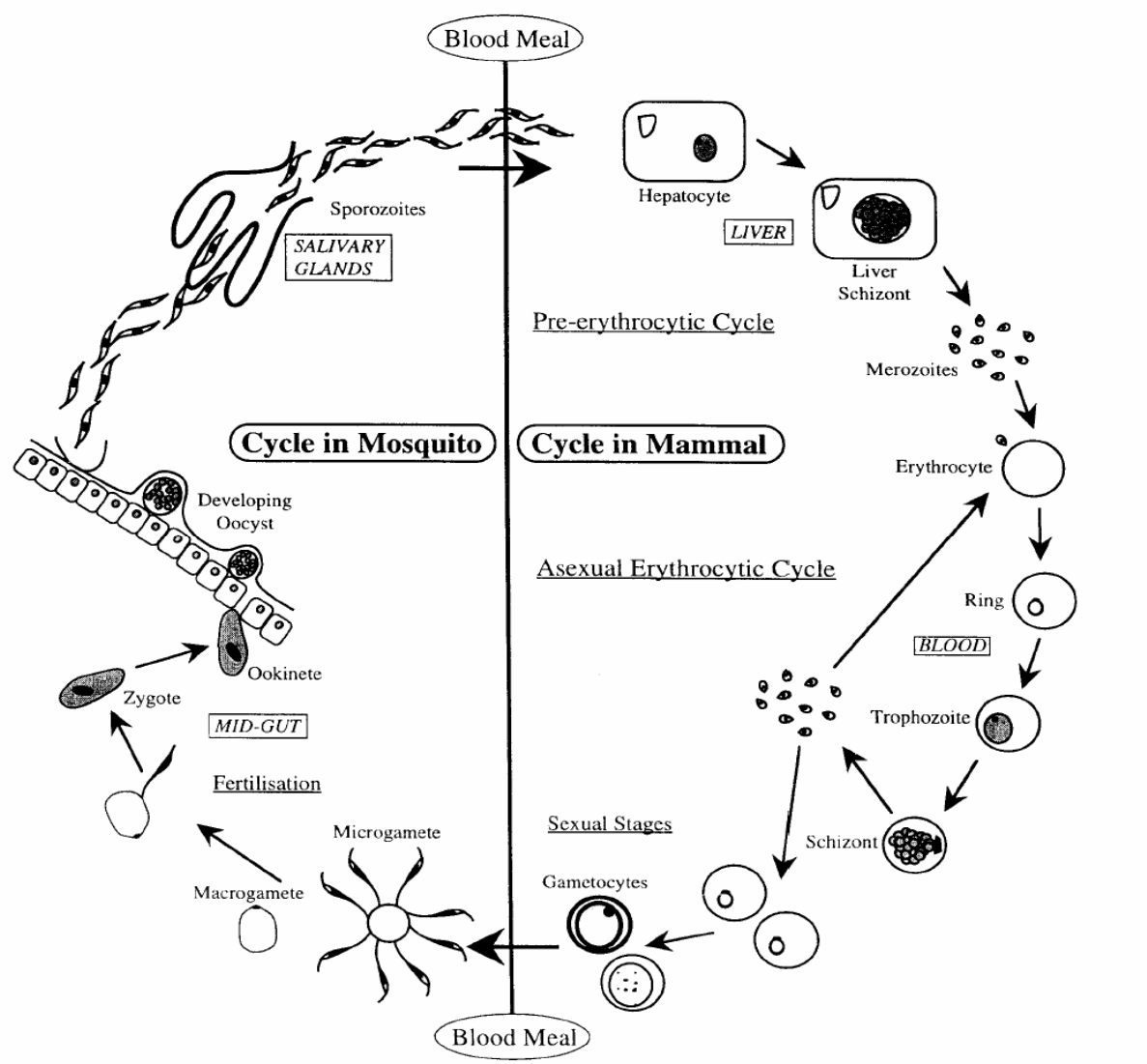


Figure 1.2: The life cycle of the malaria parasite in mammals (Phillips 2001).

After attachment and orientation on the erythrocyte surface, the merozoite releases the contents of its apical organelles (rhoptries) to generate a small endocytic vacuole. This parasitophorous vacuole buds off to form an immunologically protective environment for the ring stage parasite (or young trophozoite) (Bannister *et al.*, 2000; Chen *et al.*, 2000). Over a period of about 48 hours, the trophozoites mature to schizonts that ultimately occupy about 80 – 90% of the volume of the erythrocyte (Elford 1995). Daughter merozoites are released to invade new host erythrocytes thereby completing the asexual cycle (Phillips 2001).

Merozoites can also differentiate into male or female gametocytes that are responsible for the transmission and spread of the disease (Paton *et al.*, 1993; Dechering *et al.*, 1997). After gametocytes are ingested by mosquitoes they initiate the sexual stage of their life cycle by combining to form zygotes (Doolan and Hoffman, 1997). The zygotes

undergo sexual recombination in the mosquito midgut to produce ookinetes that penetrate into the midgut and form oocysts (Doolan and Hoffman, 1997). Thousands of new sporozoites are produced within the oocysts which then migrate to the salivary glands from where they are injected into their hosts during subsequent blood meals (Doolan and Hoffman, 1997).

1.3 Pathogenesis of Malaria

Malaria infections are complicated diseases involving many inflammatory responses. These responses may enhance cell-to-cell interaction (cytoadherence) and cell stimulation involving both malaria-derived antigens (and toxins) and host-derived factors such as cytokines (Chen *et al.*, 2000). Severe malaria is diagnosed by the presence of asexual blood stage parasites in the blood and one or more clinical features e.g. neurological impairment, pronounced anaemia, hypoglycaemia, acidosis, hyperlactaemia, circulatory collapse, multi-organ failure and coagulopathies (Planche and Krishna 2005).

Initially it was thought that a single receptor or a specific cytokine was solely responsible for the pathogenesis of the disease, however it is now believed that a number of events cause severe malaria:

1) The induction of cytokine release by parasite-derived 'toxins' (Miller *et al.*, 2002),
2) Upregulation and redistribution of endothelial receptors directly by the infected erythrocyte or by released cytokines (Ramasamy 1998),
3) Impaired or total block of local blood flow (Miller *et al.*, 1994), and
4) Severe stress of the infected organ (e.g. coma) (Heddini 2002).

P. falciparum is more pathogenic and the disease it causes is more severe than any other malaria species because it is able to invade erythrocytes of all ages resulting in very high parasitaemias and enhanced growth as well as the capacity to adhere to vascular endothelium through the process of sequestration (Bannister *et al.*, 2000; Heddini 2002). This enables the parasite to avoid detection and elimination by the immune system (Clark and Schofield, 2000; Heddini 2002).

Parasite toxins, released or formed during the trophozoite or schizont stages can either directly damage host tissue or stimulate the overproduction of host cytokines (Chen *et al.,* 2000). Moderate amounts of cytokines such as interleukin-1 (IL-1), tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are necessary for protection from invading micro-organisms but an overproduction can be harmful (Clark and Schofield, 2000; Chen *et al.,* 2000) causing fever, upsetting the immune system (by suppressing white blood cell production in the bone marrow) and resulting in the stimulation of NO (nitric oxide) production (Heddini 2002; Clark and Cowden, 1999; Ramasamy 1998). This results in a vicious cycle in which the upregulated and redistributed receptors then facilitate further cytoadherence thus promoting sequestration which does add in part to anaemia. Blood flow is blocked or at least slowed down considerably and more erythrocytes are infected and destroyed during subsequent release of the daughter merozoites (Chen *et al.,* 2000).

Nitric oxide (NO) is a short-lived, endogenously produced gas that acts as a signalling molecule in the body. NO is thought to be produced in excess by the body in order to kill the invading parasite but such an imbalance could also cause pathogenesis either through sending aberrant signals to the brain or by helping to generate toxic molecules such as peroxynitrite resulting in oxidative damage (Clark and Schofield, 2000; Heddini 2002; Clark and Cowden, 2003). An alternate view is expressed by Sobolewski and co-workers (2005) who suggest that a decrease in the bioavailability of NO might cause the pathologic activation of the immune system, the endothelium and the coagulation system factors observed in severe malaria.

Glycosyl phosphatidyl inositol (GPI) molecules are widely used as anchors of transmembrane proteins in parasitic protozoa and are considered as toxins of the malaria parasite which induce pro-inflammatory cytokines (Chen *et al.*, 2000; Clark and Schofield, 2000; Miller *et al.,* 2002). This phospholipid is found in both free form and bound to the C-terminal of the merozoite surface proteins MSP-1 and MSP-2, where it serves to anchor these proteins in the membrane (Holder and Blackman, 1994; Foley and Tilley, 1995; Chen *et al*., 2000). Antibodies to GPI anchor molecules have been associated with a lack of disease in adults but this has yet to be proved (Miller *et al.,* 2002). Another toxin produced by the parasite, hemozoin (the breakdown product of haemoglobin) is thought to induce IL-1 production (Heddini 2002; Chen *et al*., 2000).

As has been mentioned before, the overinduction of cytokines is thought to lead to upregulation and redistribution of erythrocyte receptors. The overinduction of TNF-α results in the upregulation of ICAM-1 (intracellular adhesion molecule), VCAM-1 (vascular cell adhesion molecule), ELAM-1 (endothelial cell adhesion molecule) and CD36 and thrombospondin receptors (Ramasamy 1998; Chen *et al.,* 2000). Clusters of proteins on the erythrocyte surface called PfEMP-1 (*P. falciparum* erythrocyte membrane protein) interact with the abovementioned receptors to facilitate sequestration of the infected erythrocytes (Heddini 2002; Doolan and Hoffman, 2001; Chen *et al*., 2000). During sequestration, infected erythrocytes are removed from circulation leading to decreased blood flow and impaired oxygen delivery. Reactive oxygen intermediates and NO are formed, which may result in organ failure and are also thought to be the cause of coma in cerebral malaria (Kongkasuriyachai and Kumar, 2002).

1.4 Antimalarial drugs

The emergence and spread of resistance to antimalarial drugs has highlighted the need for the discovery and development of novel antimalarials. It has also emphasised the need to understand the mechanisms by which antimalarials act. Over recent years, resistance has developed against chloroquine and sulphadoxine-pyrimethamine, two of the cheapest and most commonly used antimalarial drugs (Ridley 2003). Figure 1.3 depicts some of the compounds used as drugs in the treatment and prevention of malaria.

Quinine is the active ingredient of *Cinchona* bark, which was used for antimalarial therapy in the seventeenth century (Ridley 2002). A synthetic quinine derivative, chloroquine was developed in 1939 and has been used for decades in the treatment of malaria. Unfortunately, it has lost its effectiveness as a result of the development of worldwide resistance, the mechanism of which is still not fully understood (Winstanley 2000; Ridley 2002). Mefloquine and halofantrine, aminoalcohol quinine analogues, are structurally related drugs that are active against chloroquine resistant strains of malaria (Hastings and D'Alessandro, 2000; Phillips 2001). These drugs are more expensive and thus unaffordable to those most in need. They may also cause side effects such as fatal heart rhythms in the case of halofantrine and neurological disturbances in the case of mefloquine (Winstanley 2000).

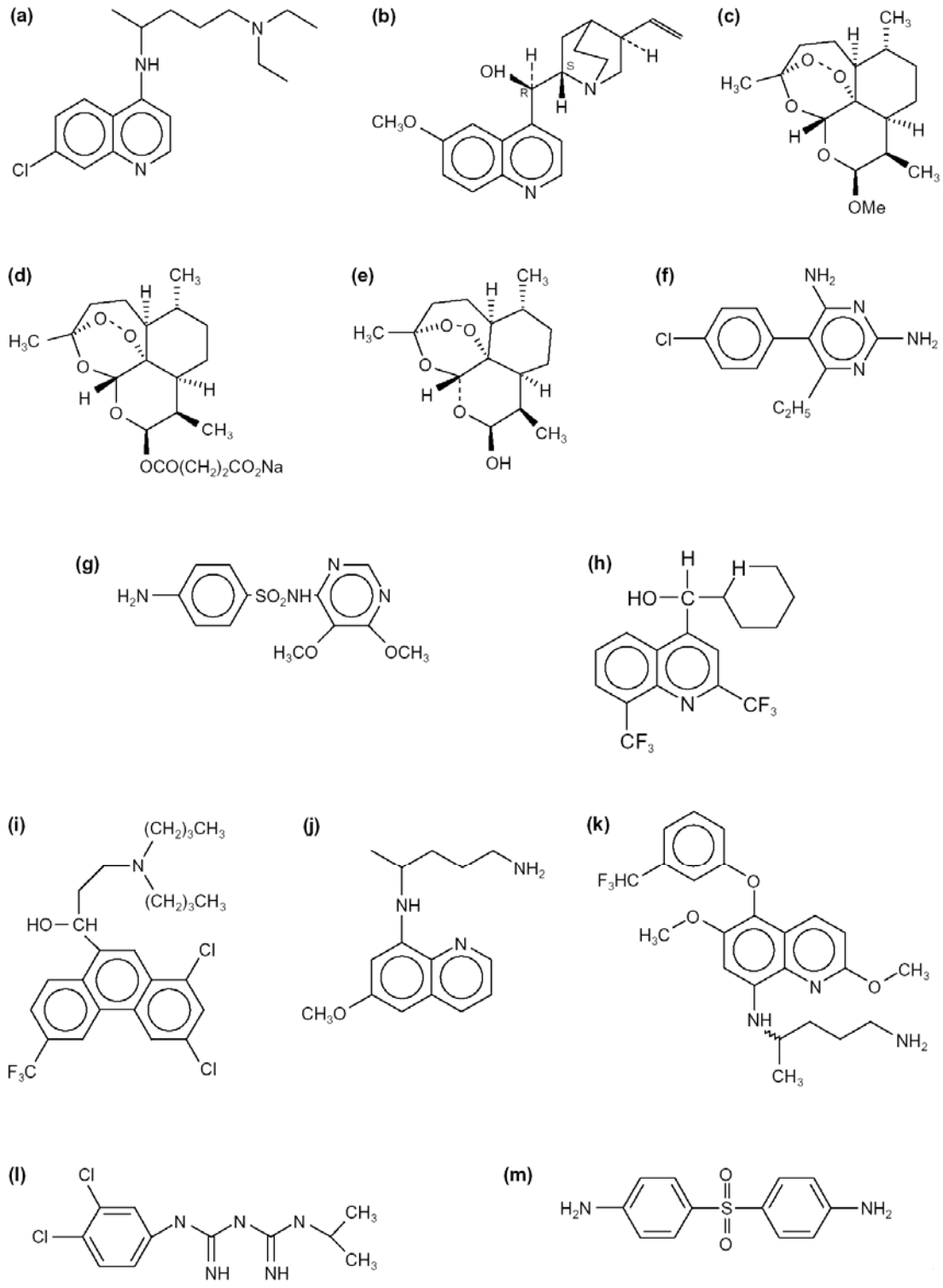


Figure 1.3: The chemical structures of drugs in widespread use for *P. falciparum* are shown here: chloroquine (a); quinine (b); artemether (c); sodium artesunate (d), dihydroartemisinin (e); pyrimethamine (f); sulfadoxine (g); mefloquine (h); halofantrine (i); primaquine (j); tafenoquine (k); chlorproguanil (l); and dapsone (m) (Winstanley 2000).

The exact mechanism and targets of the quinolines remain elusive. It has been suggested however that chloroquine works by interfering with the detoxification of heme

(ferriprotoporphyrin IX) (Olliaro 2001; Macreadie *et al.,* 2000). The binding of chloroquine to heme is thought to cause a series of toxic events in the parasite food vacuole by inhibiting heme polymerisation and preventing its degradation by glutathione-dependent and peroxidative mechanisms (Olliaro 2001).

It has been suggested that resistance to chloroquine could be as a result of possible genes which encode multidrug resistance proteins that actively expel chloroquine from within the parasite (Winstanley 2000). Several potential open reading frames (*cgX* for candidate gene) were analysed for possible correlation with chloroquine resistance (Hyde 2002). The *pfcrt* (*P. falciparum* chloroquine resistance transporter) gene was identified as a potential candidate, but field and *in vitro* testing indicated that either more than a single amino acid mutation was required for resistance or other genes might also be involved (Fidock *et al.,* 2000; Wongsrichanalai *et al.,* 2002; Hyde 2005; Cooper *et al.,* 2005). *Pfcrt* is thought to encode a putative transporter or channel protein that is localised to the digestive vacuole of the parasite (Fidock *et al.,* 2000). Mutations within this protein are thought to alter pH regulation and quinoline accumulation presumably within the digestive vacuole (Cooper *et al.,* 2005). Yet another gene, *pfmdr1* (*P. falciparum* multidrug resistance), is also thought to be involved in resistance to chloroquine, mefloquine, quinine and halofantrine as mutations within this gene have been found in parasites resistant to chloroquine (Duraisingh and Cowman 2005; Hyde 2005).

The antifolates are a group of synthetic compounds which were developed to inhibit DNA and RNA synthesis and amino-acid metabolism (Ridley 2002). Conversion of 2'-deoxyribosyluracil monophosphate to 2'-deoxyribosylthymine monophosphate is essential for DNA synthesis and is accomplished by donation of a methyl group by methylene-tetrahydrofolate (mTHF) (Winstanley 2000). The oxidised product, dihydrofolate, must be reduced to replenish the mTHF pool and the rate limiting step is catalysed by dihydrofolate reductase (DHFR) (Ridley 2002; Phillips 2001). Pyrimethamine competes with dihydrofolate for DHFR by binding with higher affinity to its active site and inhibiting it (Winstanley 2000; Macreadie *et al.,* 2000).

Pyrimethamine is always combined synergistically with inhibitors of dihydroopterate synthase (DHPS) such as sulfadoxine, sulfalene or dapsone which is used in combating malaria as this is cheap and only one dose is required (Ridley 2002; Hyde 2002).

However, resistance has sprouted against these combinations as well (Ridley 2002; Hyde 2002). Resistance to pyrimethamine is the result of point mutations in the *dhfr* gene of the bifunctional PfDHFR-TS enzyme (*P. falciparum* dihydrofolate reductase – thymidylate synthetase) causing reduced affinity for the drug (Zang and Rathod 2002). Amino acid mutations in the *dhps* gene have also been associated with drug resistance causing reduced binding affinity of the enzyme to sulphur-drugs (Wongsrichanalai *et al.,* 2002). Wang and co-workers (1999) demonstrated that an additional method of overcoming blockage of the endogenous folate biosynthetic pathway was employed by some *P. falciparum* lines by utilising exogenous folates.

Currently, artemisinin seems to be the last line of defence as there is no clinically relevant resistance yet (Balint 2001; Meshnick 2002). Resistance against artemisinin was delayed by using it in combination (artemisinin combination therapy) with other antimalarials (e.g. sulphadoxine) that have different targets or effects (Winstanley, 2002 and Jambou *et al.,* 2005). Jambou and colleagues found reduced *in vitro* susceptibility of West African *P. falciparum* isolates to artemisinin and certain artemisinin combinations, but state that this is not necessarily an indication of diminished therapeutic effectiveness (Jambou *et al.,* 2005). It was proposed that artemisinin is selective in targeting *plasmodia* because it is activated by heme (Meshnick 2002). It results in the formation of free radicals that cause lipid peroxidation and damage to specific intracellular targets killing the parasite (Meshnick 2002).

Krishna and colleagues have postulated that artemisinins act by irreversibly inhibiting the malarial calcium dependent ATPase (PfATP6) of *P. falciparum* (Eckstein-Ludwig 2003). Jambou *et al.* presented data on the reduced *in vitro* susceptibility of isolates from French Guiana to artemether, in which a mutation in PfATP6 (S769N) was hypothesised to decrease its affinity for artemisinins supporting the hypothesis that PfATP6 is a target for artemisinins (Jambou *et al.,* 2005; Krishna *et al.,* 2006). It was proposed that an iron-dependent mechanism generates free radicals from artemisinin which inhibit PfATP6 (Eckstein-Ludwig 2003; Ridley 2003). Robert *et al.,* (2005) suggested that heme can be one of the targets or the trigger of artemisinin but it is not the single target of its effects. Although artemisinin is thought to cause a similar effect as the quinolines it does so by a different method and appears to have different targets (Pandey *et al.,* 1999; Ridley 2003; Robert *et al.,* 2005). Artemisinin also has

gametocytocidal action and limits parasite transmission to new hosts thus slowing down the spread of resistant forms (Hyde 2002).

Antibiotics such as tetracycline, doxycycline and clindamycin are also being employed in conjunction with antimalarial treatments in order to augment their activity (Phillips 2001). These antibiotics were found to inhibit parasite growth by inhibiting 'prokaryote-like' protein biosynthesis in the apicoplast (Ridley 2002).

In order to better arm ourselves in the fight against malaria it is necessary not only to develop new drug candidates based on validated targets but also to identify new and improved targets by further studying the parasite's metabolic and biochemical processes. A possible avenue exists among the polyamines, those ubiquitous molecules required by all organisms.

1.5 Polyamines

1.5.1 General

The natural polyamines (see Figure 1.4 for structural formulas) consist of a number of diamines (putrescine and cadaverine, as they were found in putrefied and decayed carcasses and cadavers) and oligoamines (spermidine and spermine) that occur in virtually all prokaryotic and eukaryotic cells except archae (Reguera *et al.*, 2005). Spermine was discovered by Van Leeuwenhoek as early as the 17th century when he reported the existence of motile sperm (hence spermine and spermidine) in semen and a slow crystallisation of a mysterious substance later discovered to be the salt, spermine phosphate (Cohen 1998).

An important property of the polyamines is that they all are positively charged at physiological pH and thus have a high affinity toward negatively charged cellular molecules (Thomas and Thomas, 2003). Polyamines are very soluble in water and they exert strong cation-anion interactions on macromolecules, mainly DNA and RNA. This feature represents their best-known direct physiological role in cellular functions such as cell growth, division, and differentiation and also recently, cell death (Wallace *et al.*, 2003).

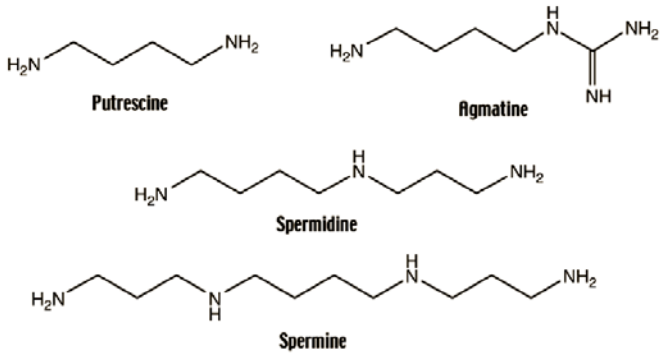


Figure 1.4: The structures of the main polyamines (Medina *et al*., 2003).

1.5.2 Synthesis and regulation of intracellular polyamine levels

In mammalian cells, putrescine is formed by decarboxylation of ornithine, a reaction catalysed by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17). Many microorganisms and higher plants are capable of using agmatine as a precursor for putrescine production as well. Arginine is decarboxylated by arginine decarboxylase (ADC, EC 4.1.1.19) and produces agmatine which is converted to putrescine by agmatinase (EC 3.5.3.11). Figure 1.5 illustrates dual pathways of putrescine synthesis, either directly from ornithine, through the activity of ODC, or from arginine and agmatine via ADC and agmatinase (EC 3.5.3.11) (Moinard *et al*., 2005). In mammalian cells, however, ornithine appears to be the exclusive precursor for *de novo* synthesis of polyamines (Reguera *et al.,* 2005).

Spermidine and spermine are synthesised by adding an aminopropyl group from decarboxylated S-adenosylmethionine to putrescine and spermidine, respectively (Figure 1.5). Therefore, synthesis of spermidine and spermine requires the action of two enzymes: first, S-adenosylmethionine decarboxylase (AdoMetDC EC 4.1.1.50) for the synthesis of the aminopropyl donor and second, a transferase enzyme, spermidine (EC 2.5.1.16) or spermine (EC 2.5.1.22) synthase which catalyses the transfer of the aminopropyl group to the primary amine groups of putrescine or spermidine, respectively (Pegg *et al*., 1995; Hoet and Nemery, 2000; Janne *et al.,* 2004).

A very specific feature of mammalian polyamine metabolism is the interconversion pathway. These interconversion pathways (metabolism-catabolism) allow the cells to provide putrescine, spermidine, and spermine when required without using external sources (Medina 2003; Urdiales *et al.,* 2001).

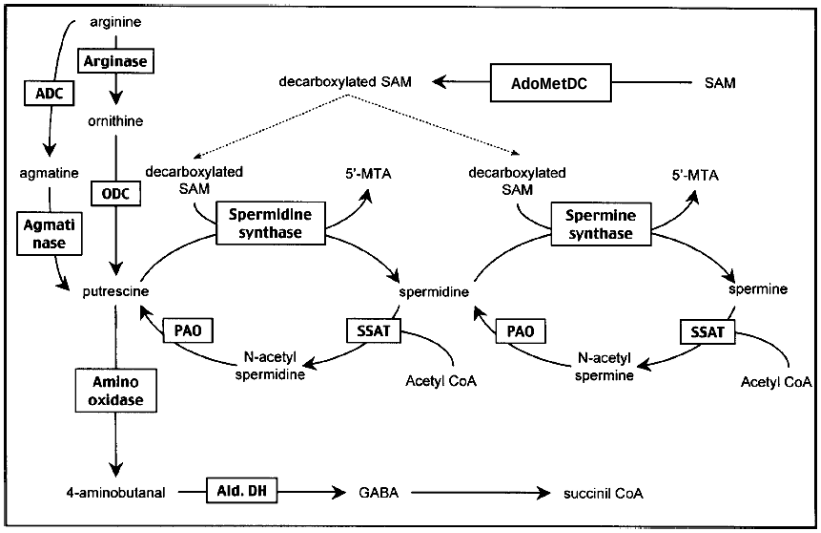


Figure 1.5: Metabolism of polyamines in mammals. Abbreviations: aldehyde dehydrogenase (Ald.DH), arginine decarboxylase (ADC), 5'-methylthioadenosine (5'-MTA), ornithine decarboxylase (ODC), polyamine oxidase (PAO), S-adenosylmethionine (SAM), S-adenosylmethionine decarboxylase (AdoMetDC), spermidine/spermine N^1-acetyltransferase (SSAT) (Adapted from Medina *et al*., 2003).

In the reverse pathway (Figure 1.5), spermidine is formed from spermine, and putrescine from spermidine. The initial reaction is an acetylation, catalysed by the enzyme spermidine/spermine acetyltransferase (SSAT, EC 2.3.1.57) yielding N1-acetylspermine or N1-acetylspermidine. These acetyl derivatives undergo an oxidative splitting by polyamine oxidase (PAO, EC 1.4.3.4) (Wallace *et al*., 2003; Janne *et al.*, 2004). The net effect of induction of the acetylase/oxidase system for interconversion is to recycle the intracellular polyamines thus preventing accumulation of toxic levels of polyamines (Wallace *et al*., 2003).

In addition to the interconversion pathway, an alternate method of regulating intracellular polyamine pools employs antizyme (AZ), a small ODC inhibiting protein that is produced when putrescine concentrations increase (Coffino 2000). AZ inhibits polyamine synthesis by both inhibiting and targeting ODC for degradation (Coffino 2001; Coffino 2000; Aubel *et al.*, 1999).

1.6 Functions of polyamines

1.6.1 General

These molecules are positively charged at the primary and secondary amino groups at physiological pH (Cohen, 1998). Thus, electrostatic interactions through the cationic

amino groups and hydrophobic interactions through the methylene bridging groups are dominant. Polyamines may act as ligands at multiple sites on DNA, RNA, proteins, phospholipids, and nucleotide triphosphates. While some of these interactions may be purely electrostatic and easily replaced by inorganic cations, others depend on the length of the aliphatic carbon chain, as all the amino groups are fully protonated at physiological pH (Wallace *et al.*, 2003; Loikkanen 2005).

Supattapone and co-workers have illustrated that branched polyamines could eliminate the protease resistant protein PrP^{Sc} from scrapie infected neuroblastoma cells *in vitro* (Supattapone *et al.,* 1999). Elevated levels of polyamines have been found in active proliferating cells and in pathological conditions other than cancer for e.g. microbial infections (Teti *et al*., 2002). Deficiencies in polyamines have been implicated in susceptibility to oxygen radicals in *E.coli* (Chattopadhyay *et al.,* 2003) and Snyder-Robinson syndrome, an X-linked mental retardation disorder (Cason *et al*., 2003).

1.6.2 Polyamine-DNA interactions

DNA is compacted (by cationic proteins known as histones for example) in order to allow its efficient packing within the small space of the cell nucleus, the bacterial cytoplasm or viral capsid (Wang *et al.*, 2001; Clark and Wall, 1996). The less compact states of DNA allow proteins access to the DNA template for a multitude of biological tasks (e.g. gene regulation, transcription and replication) (Wang *et al.*, 2001). Immunocytochemical studies of spermidine and spermine, during cell cycle progression, showed them to be associated with highly compacted mitotic chromosomes (Cohen 1998).

One of the early discoveries about polyamine-DNA interactions was that polyamines could stabilise double-stranded DNA (Saminathan *et al.,* 2002; Thomas and Thomas, 2001). This is thought to occur by neutralising the negative charges on the DNA due to electrostatic interactions with cationic polyamines (Saminathan *et al.,* 2002). It has been proposed that different polyamines have preferential binding sites (Lindemose *et al.,* 2005). Putrescine can bind to the major and minor grooves of DNA but shows preference for the major groove (Childs *et al.,* 2003). Spermidine appears to bind preferentially in the minor groove while spermine binds in the major groove of G+C rich regions and the minor groove of A+T rich regions (Childs *et al.,* 2003; Lindemose *et al.,* 2005).

It is thought that a large part of the biological function of polyamines is in the regulation of gene expression by altering either DNA structure or chemistry and that cellular polyamine metabolism may affect the degree of DNA methylation (Duranton *et al.*, 1998; Janne *et al.*, 2004). Polyamines are involved in the transition of right-handed B-DNA to left-handed Z-DNA which is thought to alter transcription as DNA sequences that form Z-DNA are frequently found near transcription start sites (Igarashi and Kashiwagi, 2000). Polyamines promote DNA bending by neutralising the negative charges on DNA phosphates thus reducing the energy required for bending (Thomas and Thomas, 2003).

1.6.3 Effects of polyamines on protein-DNA interactions

A potential target of polyamines in gene regulation is at the site of transcription factor binding to DNA (White *et al.*, 2001). Transcription factor binding to DNA and DNA bending are related processes, since many DNA binding proteins exert their action by their ability to bend DNA (Cohen 1998). This is particularly important for proteins involved in transcriptional initiation e.g. RNA polymerase II (Igarashi and Kashiwagi, 2000, White *et al.*, 2001; Chiang *et al.*, 1997).

A model system used for studies on the role of polyamines as regulatory factors that modulate DNA-protein interactions is the estrogen receptor (ER) and the estrogen response element, (ERE) (Thomas and Thomas, 2003; Childs *et al.*, 2003). In the presence of 100–500 μM of spermine, ER-ERE complex formation increased but at 1000 μM spermine there was a decrease in complex formation (Thomas and Thomas, 2003).

1.6.4 Modulation of protein synthesis

Igarashi and Kashiwagi (2000) hypothesised that the intracellular concentration of Mg^{2+} may not be optimal for protein synthesis and that it could be substituted for by polyamines since the polyamine content in cells increases during proliferation. Polyamines also have a stimulatory effect and could increase the maximal rate of protein synthesis above that seen with an optimal concentration of Mg^{2+} (Chiang *et al.*, 1997). Furthermore it was proposed that spermidine binds to a G+C-rich double-stranded region close to the Shine-Dalgarno (SD) sequence of OppA mRNA which results in a structural change of the SD sequence and the initiation codon AUG, facilitating an interaction with 30 S ribosomal subunits (Igarashi and Kashiwagi, 2000).

1.6.5 Effects of polyamines on ligand-receptor interactions

Polyamines can modulate ligand-receptor interactions and function by specific interactions with these proteins. The stability or dissociation rate of the ligand-receptor complex is important e.g. in the potency of estrogenic action (Cohen 1998). In the presence of polyamines, *in vitro*, the rate of dissociation of estradiol from the ligand-receptor complex decreased (Cohen 1998). The biological consequence was that estradiol-bound receptor was available for a longer time for transcriptional stimulation of responsive genes.

1.6.6 Ion channels and polyamines

Potassium (K^+) channels control the resting membrane potential and excitability thresholds for initiation of action potentials in many cell types (Williams 1997). A major component of the cellular machinery that controls inward rectification is intracellular polyamines (Williams 1997). An increase in the concentration of intracellular polyamines will tend to increase inward rectification of K^+ channels by binding within the channels preventing the outflow of K^+ ions and increasing cellular excitability (Loikkanen 2005).

1.6.7 Signal transduction

Protein phosphorylation plays an important role in signal transduction in eukaryotic cells. Polyamines, especially spermine, can form a ternary complex with ATP-Mg^{2+} that can affect phosphorylation by protein kinases (Bauer *et al.,* 2001). The ATPase activity of PotA, a component of a bacterial spermidine-preferential uptake system, was greatly enhanced by spermine due to formation of a ternary complex of ATP- Mg^{2+}-spermine (Igarashi *et al.,* 2001).

1.6.8 Polyamines in the cell cycle

Polyamines are known to have specific peaks of induction during cell cycle progression. The cell cycle is generally divided into four phases: G1, the first gap or growth phase; S the synthetic phase; G2 the second gap or growth phase and M the mitotic phase. During progression from the mitotic phase putrescine levels were increased during the S and G2 phases (Laitinen *et al.,* 1998; Wallace *et al.,* 2003). Spermidine increased during the entire cell cycle and spermine mainly during G1 and S phases (Laitinen *et al.,* 1998; Wallace *et al.,* 2003). Laitinen and co workers (1998) showed that Difluoromethylornithine (DFMO) prevented estradiol-stimulated degradation of cyclin

B1 in early G1 phase. They also observed stabilisation of cyclin B1 mRNA in cells treated with DFMO. Addition of putrescine or spermidine reversed the effect of DFMO on cyclin B1 and illustrated that polyamines were required for its degradation (Thomas and Thomas, 2003).

1.6.9 Apoptosis

Polyamines have dual roles in inducing and preventing apoptosis. The protective role of polyamines causes growth stimulatory effects and promotion of the cell cycle. In contrast it was found that ODC is actively involved in apoptosis induced by overexpression of c-Myc (Schipper *et al.,* 2000; Childs *et al.,* 2003). An important pathway in polyamine-induced apoptosis is the oxidation of spermidine and spermine either by serum amine oxidase or the intracellular FAD-dependent polyamine oxidase leading to the formation of aminoaldehydes and peroxide which are strong inducers of apoptosis (Song and Steller, 1999). In addition, excessive accumulation or depletion of polyamines could disrupt many cellular functions, for example DNA-protein and protein-protein interactions as well as mitochondrial stability and could also result in apoptosis (Cohen 1998; Schipper *et al.,* 2000).

By further elucidation of the roles of polyamines, their modulation of cellular functions will become less mysterious and will allow for therapeutics to be developed with regard to a variety of pathologies, most notably cancer and parasite infections.

1.7 Parasites and polyamines

Parasitic protozoa contain high levels of polyamines and recently these molecules and their associated enzymes have been investigated more closely in the search for novel and/or improved antiparasitic drug targets (Kaiser *et al.,* 2001; Muller *et al.,* 2001; Basselin *et al.,* 1997*).* The antiproliferative effects of inhibiting polyamine biosynthesis were first investigated in cancer research, but lack of success of this treatment *in vivo* led to these inhibitors being considered for antiparasitic treatment instead (Muller *et al.,* 2001; Wallace and Fraser, 2004).

DFMO is used clinically to treat sleeping sickness. DFMO is one of four active inhibitors of *T. brucei gambiense* (cause of sleeping sickness) (Burri and Brun, 2002; Docampo and Moreno, 2003) and a possible reason for this impact on the parasite

appears to be as a result of the difference in turnover rate between the ODC enzyme in the parasite compared to the human host (Muller *et al.,* 2001). Human ODC has a higher turnover rate than ODC of *T. b. gambiense*, implying that human inhibited ODC is replaced faster than the parasite inhibited ODC (Heby *et al.,* 2003). Gonzalez *et al.,* 2001 reported that inhibition using both DFMO and the spermidine synthase inhibitor, cyclohexylamine, resulted in arrest of proliferation in trypanosomatids with stable ODC enzymes as well as those with a high enzyme turnover rate (Gonzalez *et al.,* 2001).

DFMO is ineffective against *T. cruzi,* the causative agent of Chagas' disease, since ODC is absent and the parasite relies on putrescine uptake systems and the synthesis of spermidine by AdoMetDC for survival (Heby *et al.,* 2003). Experiments with the inhibitor methylglyoxal bis(guanylhydrazone) or MGBG, indicate that *T. cruzi* AdoMetDC is sufficiently dissimilar from the host enzyme so as to be a possible drug target (Heby *et al.,* 2003).

In *Leishmania* ODC, AdoMetDC and spermidine synthase are different from the human host in that they have longer half lives making them viable candidates as drug targets Roberts *et al.,* in an effort to further elucidate the polyamine biosynthetic pathways present in *Leishmania,* have cloned and characterised an *arginase* gene within this organism and report that it is necessary for parasite growth and that its sole function is to provide the parasite with vital polyamine precursors (Roberts *et al.,* 2004). They have also indicated that the parasite expresses only one ARG activity unlike its mammalian counterpart (Roberts *et al.,* 2004). Basselin *et al.,* have also shown that the *Leishmania* AdoMetDC reversible inhibitor, pentamidine, is imported into the cell via polyamine transporters and interferes with polyamine synthesis (Basselin *et al.,* 1997).

Trypanosoma and *Leishmania* synthesise a unique cofactor, trypanathione, which is a conjugate of spermidine and glutathione and is required to maintain redox balance within the cell (Tavares *et al.,* 2005; Heby *et al.,* 2003). Trypanothione is a reducing agent which has protective as well as regulatory properties for the trypanosomatids and its depletion due to inhibition of spermidine synthase by DFMO, results in parasite deterioration (Tavares *et al.,* 2005; Heby *et al.,* 2003).

In mammalian cells feedback regulation by high levels of intracellular polyamines causes the down regulation of polyamine import in an effort to prevent the accumulation

of toxic levels of polyamines (Pegg *et al.,* 1995). Polyamine transporters have also been characterised in *E. coli* and *L. major* (Igarashi *et al.,* 2001; Hasne and Ullman, 2005) and these could represent compensatory mechanisms present in the cells to overcome inhibition of polyamine biosynthesis (Bachrach 2004; Assaraf *et al.,* 1987a and b).

1.7.1 Polyamines and P. falciparum

Assaraf *et al.,* (1984) demonstrated that uninfected erythrocytes were almost devoid of polyamines but their polyamine content increased dramatically along with the activities of ODC and AdoMetDC once infected with *Plasmodium*. This was an indication that polyamine biosynthesis occurred within the parasite. Figure 1.6 illustrates the somewhat uncomplicated pathway proposed to occur in *Plasmodium* which led to the postulation that polyamine biosynthesis could be inhibited in order to control the parasite's growth (Assaraf *et al.,* 1984; Assaraf *et al.,* 1987a and b; Bitonti *et al.,* 1987).

Although putrescine production *de novo* was inhibited by DFMO the effect was cytostatic (Assaraf *et al.,* 1987a and b). The parasite could have overcome the inhibition by importing extracellular polyamines into the cell causing polyamine biosynthesis to resume (Figure 1.6 yellow putrescine and spermidine putative transporters).

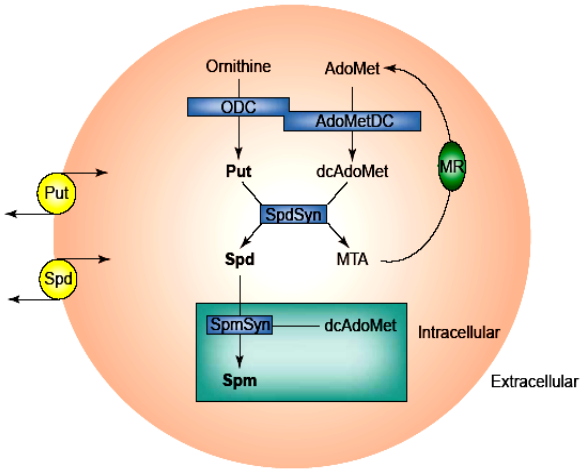


Figure 1.6: Polyamine metabolism in *P. falciparum*. Abbreviations: S-adenosylmethionine (AdoMet), S-adenosylmethionine decarboxylase (AdoMetDC), decarboxylated S-adenosylmethionine (dcAdoMet), methionine recycling pathway (MR), methylthioadenosine (MTA), ornithine decarboxylase (ODC), putrescine (Put), spermidine (Spd), spermidine synthase (SpdSyn), spermine (Spm), spermine synthase (SpmSyn), putative transporters indicated in yellow (Muller *et al.,* 2001).

The polyamine metabolic pathway of *P. falciparum* is unique when compared to its mammalian counterpart in various aspects: it appears as though the parasite lacks an interconversion pathway and ODC and AdoMetDC activities have been shown to occur in a bifunctional complex (Muller *et al.,* 2000). The parasite also seems to lack an ODC antizyme (Muller *et al.,* 2001). DFMO inhibition is reversed upon extracellular addition of putrescine and spermidine (Das Gupta *et al.,* 2005) a possible indication that polyamine uptake occurs within the parasite.

Assaraf and co-workers observed that the intracellular concentration of polyamines and the activity of enzymes involved in polyamine biosynthesis increased significantly in *P. falciparum* infected erythrocytes and that the biosynthesis of polyamines peaked during the early trophozoite stage whereas nucleic acid and protein synthesis peaked later in mature trophozoites (Assaraf *et al.,* 1987a). This finding was supported by work performed by Das Gupta *et al.,* in which the polyamine content of the uninfected host RBC, the isolated parasites and the host-parasite cell unit was measured (Das Gupta *et al.,* 2005). The infected erythrocytes contained significantly greater amounts of polyamines than the uninfected erythrocytes (Das Gupta *et al.,* 2005).

The addition of DFMO to *in vitro* synchronous *P. falciparum* cultures did not interfere with merozoite invasion or ring development, but prevented the transformation of trophozoites to schizonts (Assaraf *et al.,* 1984; Das Gupta *et al.,* 2005). This inhibited nucleic acid and subsequent protein synthesis but the effect was reversed upon addition of extracellular putrescine which is reported to penetrate *P. falciparum* infected RBCs much better than uninfected RBCs (Assaraf *et al.,* 1984; Das Gupta *et al.,* 2005). This prompted the notion that polyamine metabolism within the parasite is not as simple as initially thought.

DFMO led to a decrease in the level of spermidine in infected erythrocytes (Bitonti *et al.,* 1987). The polyamine derivative 3-aminooxy-1-aminopropane inhibited ODC and AdoMetDC activities thereby decreasing intracellular polyamine concentrations and inhibiting proliferation of *P. falciparum in vitro* (Das Gupta *et al.,* 2005). The well known inhibitor of AdoMetDC, MDL 73811 (5'-[(Z-4-amino-2-butenyl)-methylamino]-5'deoxyadenosine) (Moritz *et al.,* 2004) and MGBG have proven to be successful inhibitors of AdoMetDC to prevent the conversion of putrescine to spermidine (Das Gupta *et al.,* 2005).

Two putative enzymes involved in spermidine metabolism in *P. falciparum* have recently been identified, i.e. deoxyhypusine synthase (DHS; EC 1.1.1.249) and homospermidine synthase (HSS; EC 2.5.1.45) (Kaiser *et al.*, 2003b). In eukaryotes DHS catalyses the transfer of the aminobutyl moiety from spermidine to a specific lysine residue of the eukaryotic translation initiation factor (eIF5A) precursor forming a deoxyhypusine residue (Moritz *et al.*, 2004; Kaiser *et al.*, 2003a). This residue is hydroxylated by deoxyhypusine hydroxylase (DHH) to form the active hypusine residue on the eIF5A precursor (Kaiser *et al.*, 2003a). HSS transfers the aminobutyl group from spermidine to putrescine forming homospermidine (Kaiser *et al.*, 2001). eIF5A is vital in the translational process and preventing its synthesis by inhibiting these enzymes could also lead to inhibition of the parasite itself.

The presence of *P. falciparum* specific ODC/AdoMetDC bifunctional enzyme represents a possible parasite specific property that could be exploited in the search for novel antimalarial strategies. In order to gain better insight into the parasites' polyamine metabolic pathway, synthesis of their endogenous polyamines was inhibited by inhibiting the parasite's ODC/AdoMetDC bifunctional enzyme. The SSH technique was used to identify transcripts that were down-regulated. These transcripts represented the potential function of polyamines within the parasite which could subsequently serve as chemotherapeutic targets.

1.8 Aims

To identify the physiological effects of polyamine depletion on *P. falciparum* as well as polyamine dependent transcripts or proteins within the parasite by:

1) Inhibition of synchronised *P. falciparum* parasites with DFMO to deplete these parasites of endogenously synthesised polyamines.

2) Construction of a Suppression Subtractive Hybridisation library of up- (forward subtracted) and down-regulated (reverse subtracted) transcripts.

3) Identification and characterisation of the differentially expressed transcripts where the up-regulated transcripts represent the physiological effects of

polyamine depletion and the down- regulated transcripts represent the polyamine dependent transcripts or proteins.

In the next chapter the inhibition of *P. falciparum* parasites and the generation of up- and down-regulated transcript libraries with suppression subtractive hybridisation will be described. Chapter 3 will describe the selection, identification and characterisation of the differentially expressed transcripts.

CHAPTER 2

Suppression Subtractive Hybridisation of Polyamine-depleted *Plasmodium falciparum*

2.1 Introduction

Gene expression profiling is a rapidly emerging experimental method in which many thousands of genes can be assayed simultaneously in a single experiment (Bakay *et al.,* 2002). Expression profiling has led to dramatic advances in a variety of research areas, from understanding yeast biology (Agarwal *et al.,* 2003) to comparisons between cancerous and non cancerous cells and has even had an impact in fields such as dentistry (Bleicher *et al.,*2001).

Alterations in gene expression are associated with a large spectrum of biological and pathological processes. The identification of differentially expressed genes could result in greater insight into the molecular mechanisms which are essential for disease progression (Ji *et al.,* 2002). Furthermore, drug effects on the metabolism of targeted tissues or organisms include up- or down-regulation of the protein target(s), the up-regulation of detoxification pathways (cytotoxic response) and the up-regulation of alternative or compensatory pathways of the affected organism (Young and Winzeler, 2005). The identification and characterisation of these affected pathways using gene expression profiling could aid in the discovery of potential targets and/or drug candidates (Young and Winzeler, 2005). This is accomplished most effectively by scrutinising all facets of gene expression and intergrating the results using transciptomics, bioinformatics, proteomics and metabolomics.

To facilitate the discovery of differentially expressed genes, a variety of methods have been developed in recent years including differential display, RNA fingerprinting, serial analysis of gene expression (SAGE), representational difference analysis (RDA), subtractive suppression hybridisation (SSH) and hybridisation to gene arrays of various formats (Kozian and Kirschbaum, 1999; Ji *et al.,* 2002). Each method has advantages and drawbacks. In differential display, 3’ anchored oligo (dT) primers and short 5’ arbitrary primers are combined which allow for subsets of a transcriptome to be

amplified (Lievens *et al.,* 2001). At the time of its introduction, the advantages of this method were: it seemed fast, was highly versatile as it used already existing techniques, only a small amount of starting material was required and its sensitivity was considered to be better than other available methods (Cunningham 2001). A drawback of the technique is that it is unable to identify rare mRNAs and generates a great number of false positives (Ghosh *et al.,* 2003).

Serial analysis of gene expression (SAGE) is a sequence-based approach also used to identify differentially expressed genes. In this method, very short (10-14bp) cDNA tags are generated by restriction digestion, amplified by PCR and ligated, after which the resulting concatemers are sequenced (Byers *et al.,* 2000). Corresponding genes can be identified by sequencing the tags generated, while the incidence of occurrence of the tags can be used to determine the abundance of mRNA present in the original RNA sample. This method is considered to be the easiest as no normalisation or subtraction is needed. However, if errors occur during the sequencing procedure or not enough data has been deposited into the relevant gene banks then identification of the corresponding gene could be difficult (Chen *et al.,* 2001).

In representational difference analysis (RDA), mRNA of different origins (tester and driver) is reverse transcribed, digested with a frequently cutting restriction enzyme and then amplified via a ligated linker (Hubank and Schatz, 1999). The linkers of both cDNA pools are removed and a new linker is added to the tester cDNA. After hybridisation and amplification only those cDNAs present in the tester are amplified (Hubank and Schatz, 1999; Kozian and Kirschbaum, 1999). Although this method is similar to that employed during SSH it is more labour intensive as multiple rounds of subtraction are needed to overcome differences in mRNA abundance.

Automatic high throughput methods such as hybridisation-based gene arrays have become available as well. Since the various sequencing projects (e.g. human, mosquito and malaria genomes) are virtually completed, the generation of plates or chips consisting of many genes and expressed sequence tags (ESTs) already make array technology greatly sought after. Microarrays are capable of providing simultaneous, quantitative measurement of gene abundance and allow for a larger group of genes to be assayed for differential expression (Ji *et al.,* 2002).

Subtractive suppression hybridisation (SSH), developed by Diatchenko and co-workers (1996), is used to selectively amplify target cDNA fragments and simultaneously suppress non-target amplification. The method is based on the suppression PCR effect in which long inverted terminal repeats can selectively suppress amplification of non-target sequences by forming panhandle-like structures. This method overcomes the problem posed by the presence of high abundance mRNAs by incorporating two hybridisation steps in which differentially expressed genes can be normalised and enriched over a 1000-fold in a single round of hybridisation (Ji *et al.,* 2002; Diatchenko *et al.,* 1996). SSH is more affordable than many of the methods mentioned above. In addition, it is also capable of detecting low abundance mRNAs due to the enrichment of cDNA (Diatchenko *et al.,* 1996). SSH evaluates the expression patterns of thousands of transcripts or genes in their "natural environment".

Figure 2.1A is a representation of the SSH procedure developed by Diatchenko and co-workers (1996). The differentially expressed cDNAs (those transcripts upregulated or unique to e.g. the treated sample) are present in the ''tester'' cDNAs. These cDNAs are absent (or present at much lower levels) in the ''driver'' cDNAs (which correspond to those transcripts obtained from the control sample). The tester (treated) and driver (control) dscDNAs are digested with a fequently cutting restriction enzyme to yield blunt ended molecules. The tester cDNA fragments are subdivided into two samples and ligated with two different ds adaptors (adaptor 1 and adaptor 2), resulting in two tester populations. Sample 1 corresponds to tester cDNA fragments ligated with adaptor 1 and sample 2 to tester cDNA fragments ligated with adaptor 2. The ends of the adaptors are designed without phosphate groups, so that only the longest single strand of each adaptor can covalently attach to the 5' end of the cDNA fragments from the tester population. The reverse of the above mentioned procedure applies for the reverse subtraction.

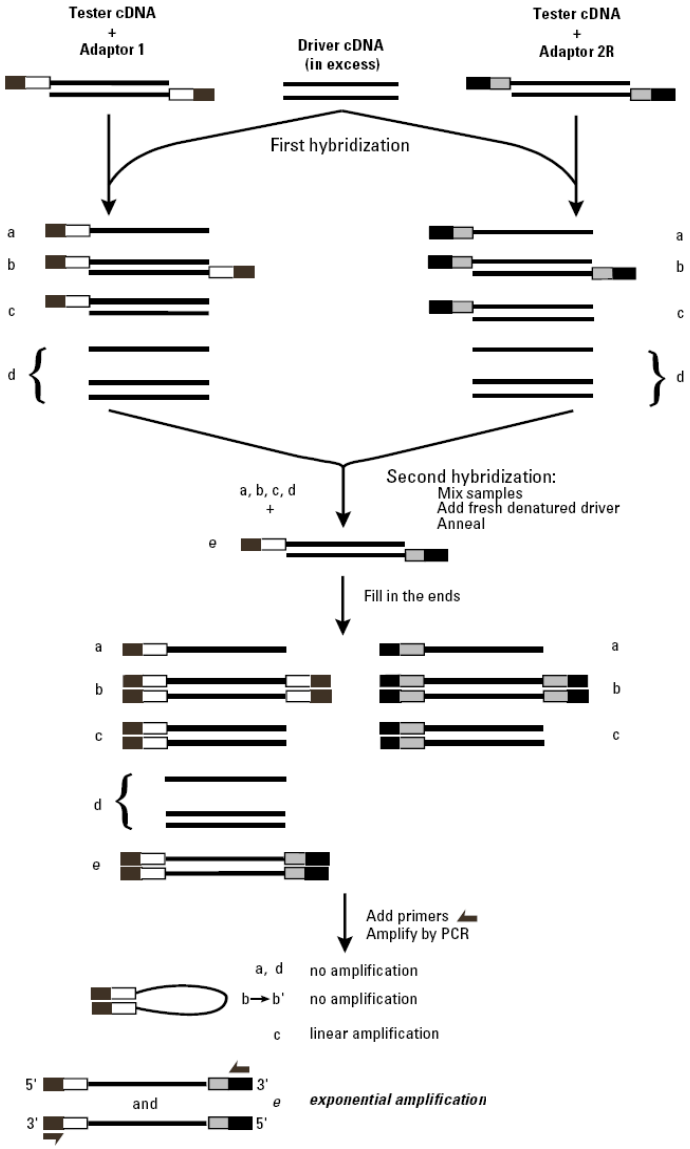


Figure 2.1A: Scheme of the SSH method. Solid lines: *Rsa*I digested tester and driver. Solid boxes: outer part of adaptor 1 and adaptor 2. Clear box: inner part of adaptors corresponding to nested primer NP1 and NP2R (light grey shaded area) (Diatchenko *et al.*, 1996, BD PCR-Select cDNA subtraction kit user manual, 2004)**. See text for further details.**

A major advantage of the SSH technique is that it combines normalisation (concentrations of high and low abundance cDNAs become roughly equal) and suppression PCR in a single step. The long inverted terminal repeats which are ligated to the ends of the tester cDNA fragments form stable panhandle-like loop structures after each denaturation and annealing cycle (Kiss *et al.*, 2003). During PCR, exponential amplification of the panhandles doesn't occur as the primers used are derived from the long terminal inverted repeats. It is kinetically more favourable/stable for intramolecular annealing between the long terminal repeats to occur than it is for intermolecular annealing between the shorter PCR primers and the long inverted repeats (Byers *et al.*, 2000).

The SSH technique (Diatchenko *et al.,* 1996) employs two hybridisation steps. During the first step, an excess of driver is added to tester sample 1 and 2 which are denatured and allowed to anneal. This results in the enrichment of differentially expressed transcripts as common transcripts between the tester and driver populations form heterohybrids (c in Figure 2.1A) leaving the uncommon fragments from the tester sample as sscDNAs (a in Figure 2.1A). This first hybridisation also leads to normalisation of the sscDNA tester fraction (a in Figure 2.1A): concentrations of high and low abundance cDNAs become roughly equal. This occurs because the reannealing process which generates the homohybrid cDNAs (those cDNAs that are the same within the tester population, b in Figure 2.1A) is faster for the more abundant molecules. In the second hybridisation, the two samples from the first hybridisation are mixed together allowing only the remaining normalised and subtracted ss tester cDNAs to reassociate and form (b), (c), and new (e) hybrids (Figure 2.1A). The subsequent addition of a second portion of denatured driver further enriches fraction (e) for differentially expressed genes by annealing to any non-target transcripts which may have been bypassed during the first hybridisation step.

The new hybrids (e) have an important feature that distinguishes them from hybrids (b) and (c) formed during the first and second hybridisations: different adaptor sequences at their 5'-ends. One is from tester sample 1 and the other from tester sample 2. The two sequences allow preferential amplification of the subtracted and normalised fraction (e) using PCR and primers that correspond to the outer part of adaptor 1 and 2, respectively. Type (b) molecules (Figure 2.1A), which contain long inverted repeats on their ends form stable panhandle-like structures (b') after each denaturation-annealing PCR step. As such they can't serve as templates for PCR (suppression effect explained above). Furthermore, type (a) and (d) molecules do not contain the required primer binding sites for subsequent amplification, and type (c) molecules are amplified at a linear rate. The result is that exponential amplification is only possible for the differentially expressed fragments (type e molecules).

The SSH procedure was used in this study to analyse the gene expression profile of polyamine-depleted *P. falciparum*. It was envisioned that the inhibition of polyamine biosynthesis, by inhibition of the ODC enzyme using DFMO, would provide some insight into the effect that the loss-of-function of the ODC enzyme has on the phenotype of the parasite. SSH was employed in order to identify unique or upregulated transcripts

present in the polyamine-depleted parasites, a procedure being defined as forward subtraction. In this instance the tester represents cDNAs originating from polyamine-depleted parasites and the driver represents those cDNAs from the control parasites. If a reverse subtraction was performed, the results would correspond to those transcripts which were downregulated due to polyamine depletion. Therefore, the cDNAs originating from polyamine-depleted parasites would represent the driver population and the cDNAs from the control parasites would represent the tester population.

2.2 Materials and methods

2.2.1 Malaria parasite cultures

P. falciparum parasites were maintained under conditions that support their intracellular growth (Trager 1994; Trager and Jensen 1976). Cryopreserved *P. falciparum* (line FAB1) infected red blood cells, were thawed quickly at 37^{o}C after which the contents were transferred to a 10 ml centrifuge tube under sterile conditions and 0.2 ml of a sterile 12% (w/v) NaCl solution was added with continuous mixing. Thereafter 1.8 ml of a sterile 1.6% (w/v) NaCl solution was added and mixed well. The solution was centrifuged at 2500xg for 5 min at room temperature to collect the parasites, thereafter the supernatant was aspirated and approximately 500 µl of fresh erythrocytes was added to the pellet and mixed well. The cells were transferred into a sterile 75cm^3 culture flask (Corning, USA) to which 10 ml of prewarmed (37^{o}C) culture medium was added (final hematocrit ~ 5%). The culture flask was gassed for approximately 30 sec with a 5% O_2, 5% CO_2 and 90% N_2 mixture and subsequently incubated at 37^{o}C. Cultures were maintained daily by examining Giemsa stained thin-blood smears using light microscopy. The parasitaemia was maintained at approximately 5% by aspirating old medium daily and replacing it with 10 ml fresh culture medium and approximately ten drops (~500 µl) of erythrocytes in suspension (final hematocrit ~ 5%).

The culture medium consisted of 1% (w/v) RPMI-1640 (with glutamine, without bicarbonate) (Sigma, South Africa), 25 mM HEPES (Sigma, Germany), 22 mM D-glucose (Sigma, South Africa), 323 µM hypoxanthine (Sigma, South Africa) and 4 mg gentamycin (Sigma, South Africa) in 900 ml sterile Milli Q water which was incubated for 30 min at room temperature. This solution was filter-sterilised through a 0.22 µm filter (Millipore, USA) and 36 ml of a 5% $NaHCO_3$ solution was added just before

filtration was complete (the final concentration of $NaHCO_3$ was 0.18%). Thereafter 50 ml of sterile human serum was added.

2.2.2 Synchronisation

Prior to inhibition of polyamine biosynthesis the culture had to be synchronised. Initial synchronisation was achieved with sorbitol treatment (http://www.malaria.mr4.org). 20 ml of culture (parasitemia > 5%) predominantly in the ring phase was transferred to a 50 ml tube and centrifuged for 5 min at 2000xg at room temperature. The supernatant was removed and 4 ml of 15% (v/v) sorbitol preheated to 37°C was added. The solution was mixed well and incubated for 5 min at 37°C. Thereafter 8 ml of 0.1% (w/v) glucose (preheated to 37°C) was added and the solution mixed by pipetting. The mixture was incubated for 5 min at 37°C and centrifuged as above. The supernatant was removed and the cells resuspended in 50 ml of culture medium, transferred to a new culture flask and approximately 50 drops of erythrocytes in suspension (hematocrit ~ 5%) added. The flask was gassed and incubated as in 2.2.1. A second synchronisation was performed 48 hours later at a parasiteamia of ~10%.

2.2.3 Inhibition of polyamine biosynthesis

The cells were centrifuged at 2000xg at room temperature after the second synchronisation and the supernatant was aspirated. The resulting pellet was resuspended in 11.5 ml of fresh culture medium 48 hours after the second synchronisation. This cell suspension was subsequently split into two culture flasks. A 10 mM (a ten fold excess of the IC_{50}, which was determined to completely inhibit ODC activity (Assaraf *et al.*, 1987a and b) solution of DFMO, prepared in culture medium at pH 7.4 (to a final volume of 50 ml), and approximately 50 drops of fresh erythrocytes in suspension (hematocrit ~ 5%) was then added to one of the culture flasks to give a final parasiteamia ~7.5%. This flask is henceforth referred to as the inhibited or polyamine-depleted culture. To the remaining flask (the control culture), 50 ml of culture medium and 50 drops of fresh erythrocytes in suspension (hematocrit ~ 5%; final parasiteamia ~ 7.5%) was added. The parasites were incubated with and without DFMO for a total of 40 hours, fresh medium including DFMO was added after 24 hours (Assaraf *et al.*, 1986). Parasites accumulated at the trophozoite stage in the DFMO treated culture.

2.2.4 Lysis of erythrocytes

The inhibited and control parasites were harvested by adding 1/100 of the volume of 10% (w/v) saponin prepared in 10 mM EDTA and incubating this solution for 5 min at room temperature to lyse the erythrocytes. The released parasites were collected by centrifugation at 1000xg for 5 min. The supernatant and erythrocyte ghosts were aspirated and the parasite pellet was resuspended in 1 ml 1x PBS (136.9 mM NaCl, 1.45 mM KH_2PO_4, 2.68 mM KCl, 10.14 mM Na_2HPO_4, pH 7) and centrifuged at 12100xg for 2 min. This 1x PBS wash was repeated 4 times in total.

2.2.5 Total RNA isolation

Total RNA was isolated from the parasites using the protocol described in the RNeasy mini kit (Qiagen, USA) for the isolation of total RNA from animal cells (Second edition, 1999). Isolation of total RNA was performed at room temperature under RNAse free conditions. Parasite membranes and organelles were disrupted completely in order to release all RNA contained within them by the addition of buffer RLT (containing guanidine isothiocyanate, which inactivates RNAses yielding intact RNA). Two homogenisation steps were performed to reduce the viscosity of the solution and shear high molecular weight genomic DNA and other cellular components. First, the sample was passed several times through a 20 gauge needle to form a lysate. The lysate was further homogenised by centrifuging it on a QIAshredder column (a unique biopolymer shredding column from Qiagen, USA) for 2 min at 16000xg.

RNA was subsequently isolated by adding 1 volume of 70% ethanol to the homogenised lysate, allowing total RNA to bind to the silica-based membrane in the RNeasy column during centrifugation at 8000xg for 15 sec. Subsequent washes removed cell debris and other contaminants leaving the RNA bound to the membrane under the high salt conditions. RNA molecules larger than 200 nucleotides bound optimally under the high salt and ethanol conditions used and allowed for their enrichment as small RNA molecules were washed away. 700 µl of buffer RW1 (Qiagen, USA) was added and the column centrifuged at 8000xg for 15 sec for the first wash cycle. The column was transferred to a new collection tube and 500 µl of buffer RPE (Qiagen, USA) was used during the second wash at 8000xg for a further 15 sec. This was repeated at 8000xg for 2 min. RNA was subsequently eluted in 30 µl of RNAse free sterile water (provided with the kit) by centrifuging the columns at 8000xg for 1 min.

The concentration of the isolated total RNA was determined using the GeneQuant *pro* UV/Vis Spectrophotometer (Biochrom, England). Absorbance values were measured at 260nm and 280nm. The absorbancy at 260 nm estimates the yield of RNA (when measured in water an A_{260} of 1 is equal to 40 µg/ml of RNA) and the ratio of absorbancies at 260 nm to 280 nm was used as an indication of the purity of the sample. Pure RNA should have an $A_{260/280}$ ratio of 1.7 – 1.9 (Sambrook *et al.*, 1989.). Standard practice requires the products to be run on an agarose gel in addition to absorbance determination, however the quantity of RNA obtained was too low to warrant this.

2.2.6 Suppression subtractive hybridisation (SSH)

Double stranded (ds) cDNA synthesis and amplification was initially performed in preparation for SSH (Figure 2.1B, sections 2.2.6.1 and 2). A modified oligo(dT) cDNA synthesis primer (CDS) (see Table 2.1 for sequence) anneals to total or poly A^+ RNA initiating first strand synthesis. SMART cDNA synthesis employs the principle that when the reverse transcriptase (RT) reaches the 5' end of the mRNA, its terminal transferase activity adds 3 - 5 template independent deoxycytidine nucleotides to the 3' end of the first synthesised strand of cDNA. A SMART II oligonucleotide (see Table 2.1 for sequence) consisting of a stretch of G residues at its 3' end is thus able to anneal to this C-rich motif and form an extended template. The reverse transcriptase then switches templates and continues replicating to the end of the oligonucleotide. The resulting sscDNA has a sequence complementary to the SMART II oligonucleotide at its 3' end and a sequence complementary to the CDS primer at its 5' end. These sequences serve as priming sites during amplification by long distance PCR resulting in dscDNA that is enriched for full length sequences.

Table 2.1: Sequences of primers used during dscDNA synthesis and amplification

Primer Name	**Primer Sequence**
CDS	5' AAGCAGTGGTAACAACGCAGAGTACT$_{(30)}$N$_{-1}$N 3', where N= A, C, G or T and N$_{-1}$= A, G or C
SMART II oligonucleotide	5' AAGCAGTGGTAACAACGCAGAGTACGCGGG 3'
PCR primer	5' AAGCAGTGGTAACAACGCAGAGT 3'

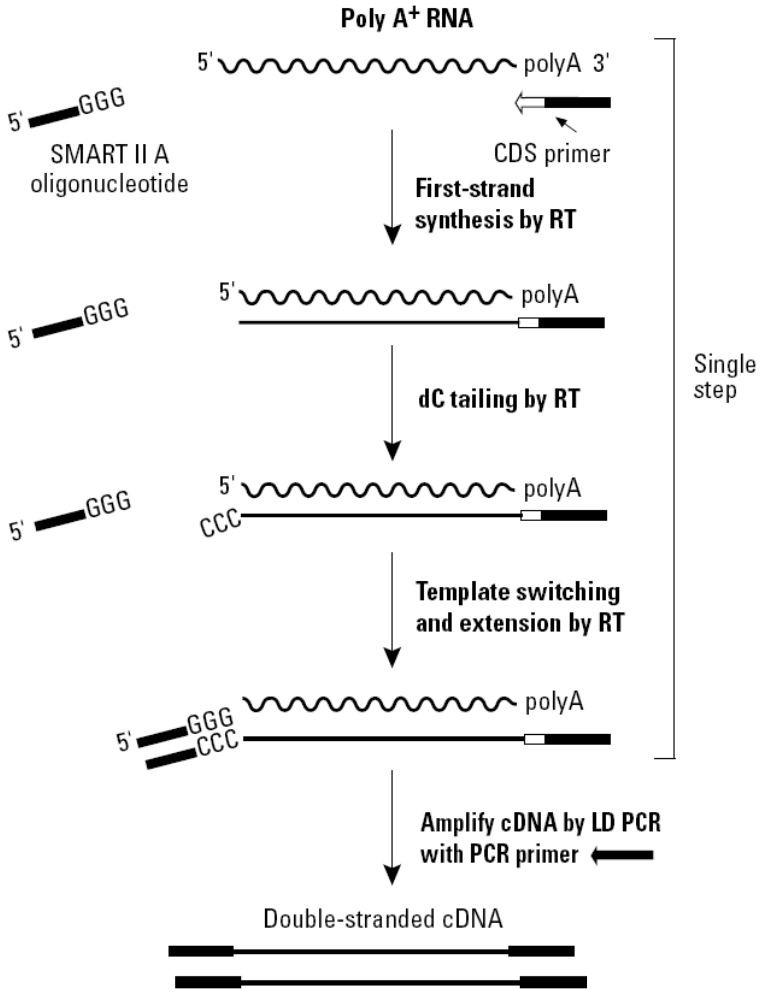


Figure 2.1B: Schematic representation of dscDNA synthesis from RNA (SMART PCR cDNA synthesis kit user manual, 2006**).**

2.2.6.1 First strand cDNA synthesis.

First strand cDNA was synthesised from approximately 528 ng of total RNA isolated as described in 2.2.5 from both the polyamine-depleted and control parasite cultures using the SMART PCR cDNA synthesis kit (Clontech, USA). 1 µM of CDS primer and 1 µM of SMART II oligonucleotide were mixed with the total RNA (total volume of 5 µl), incubated at 70^{o}C for 2 min and briefly centrifuged. To this was added 1x first strand buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 30 mM $MgCl_2$), 2 mM dithiothreitol (DTT), 5x dNTP (10 mM of each) and 1 µl of Powerscript Reverse Transcriptase (Clontech, USA) (final volume of 10 µl). The reactions were subsequently incubated at 42^{o}C for 1 hr whereafter the products were diluted five-fold with 1x TE buffer (10 mM Tris, pH 7.6; 1 mM EDTA) and heated at 72^{o}C for 7 min.

2.2.6.2 cDNA amplification by Long Distance (LD) PCR

2 µl of each of the first strand cDNA synthesis products was used for LD-PCR again according to the SMART PCR cDNA synthesis kit protocol (Clontech, USA). This reaction contained 1x Advantage 2 PCR buffer (40 mM Tricine-KOH pH 8.7, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg/ml BSA, 0.005% Tween 20, 0.005% Nonidet P40), 0.2 µM of PCR primer (see Table 2.1 for sequence), 1x dNTP (10 mM of each) and 1x Advantage 2 polymerase mix (1% glycerol, 0.3 mM Tris-HCl pH 8, 1.5 mM KCl, 1 µM EDTA) to give a final reaction volume of 100 µl. Thermal cycling was performed on a Gene Amp PCR 9400 cycler (Perkin Elmer, USA) with the following cycling parameters: 95°C for 1 min; 25 cycles of 95°C for 5 sec, 65°C for 5 sec and 68°C for 6 min with samples being taken at 15, 17, 19, 21 and 25 cycles to determine the optimum number of cycles. Once the optimum number of cycles was determined, the tubes, which had already undergone 15 cycles of PCR and were stored at 4°C, were subjected to additional cycles until the previously determined optimum had been reached. To terminate the reaction, 0.01 M EDTA was added to each tube.

These products were then purified using phenol:chloroform:isoamyl alcohol (25:24:1) and n-butanol extraction resulting in DNA being separated into the aqueous phase, proteins into the interphase and other components separating into the organic phase (Sambrook *et al.,* 1989.). n-Butanol extraction allowed the PCR products to be concentrated to a volume of 40 – 70 µl by removing water from the sample. The resulting solution was purified using a Chroma spin 1000 column (removes 50% of fragments as large as 1000bp, DNA and proteins are eluted from the column in order of decreasing molecular size, Clontech, USA) which is packed with gel filtration resin to rapidly purify double-stranded DNA from contaminants such as salts, solvents, enzymes, or proteins. The DNA was eluted in 320 µl of 1x TNE buffer (10 mM Tris-HCl, pH 8; 10 mM NaCl; 0.1 mM EDTA). This resulted in purified dscDNA of polyamine-depleted and control parasite cultures.

2.2.6.3 Tester and driver preparation

The dscDNA was subsequently used for suppression subtractive hybridisation according to the manufacturers protocol in the Clontech PCR-Select cDNA subtraction kit user manual (2000).

To prepare the dscDNA for the forward and reverse subtractions, 1 µg of each of the two dscDNA populations was digested in 1x *Rsa*I restriction buffer with 15 U of *Rsa*I (in a final volume of 50 µl). This mixture was incubated at 37°C for 3 hr and the reaction was stopped with EDTA (final concentration of 0.08 M EDTA) . This mixture was then purified using the QIAquick PCR purification kit (Qiagen, USA) in which DNA adsorbs to the silica membrane in the presence of a high concentration of chaotropic salts at a pH ≤ 7.5 while contaminants pass through the column. Impurities are washed away and DNA is eluted in Tris buffer. Briefly, 5 volumes of buffer PB (Qiagen, USA) was added to 1 volume of the *Rsa*I digested DNA and centrifuged at 10000xg for 60 sec. The flow-through was discarded and 750 µl of buffer PE added and centrifuged at 10000xg for 60 sec. The DNA was subsequently eluted in 30 µl of buffer EB (Qiagen, USA) (10 mM Tris-Cl, pH 8.5).

The digested tester cDNA (1 µl) was diluted six-fold in sterile water (provided in the subtraction kit). Subsequently, 2 µl of this was ligated to either adaptor 1 or adaptor 2 (final concentration 2 µM) (see Figure 2.1C for sequences) in 1x ligation buffer (Promega, USA) and 400U of T4 DNA ligase (Promega, USA) at 16°C overnight in separate 10 µl ligation reactions. A reaction consisting of skeletal muscle tester cDNA (provided in the kit) was also ligated to either adaptor 1 or adaptor 2 as a control (as per manufacturer's instructions). The ligation reactions were stopped by adding EDTA/glycogen (final concentration 0.02 M EDTA and 0.1 mg/ml glycogen) and heating the samples at 72°C for 5 min to inactivate the ligase. An unsubtracted tester control consisting of a mixture of the digested tester cDNA ligated to both adaptors was also included. These controls underwent subsequent primary and secondary PCR reactions and later served as the unsubtracted probes for the forward and reverse subtractions.

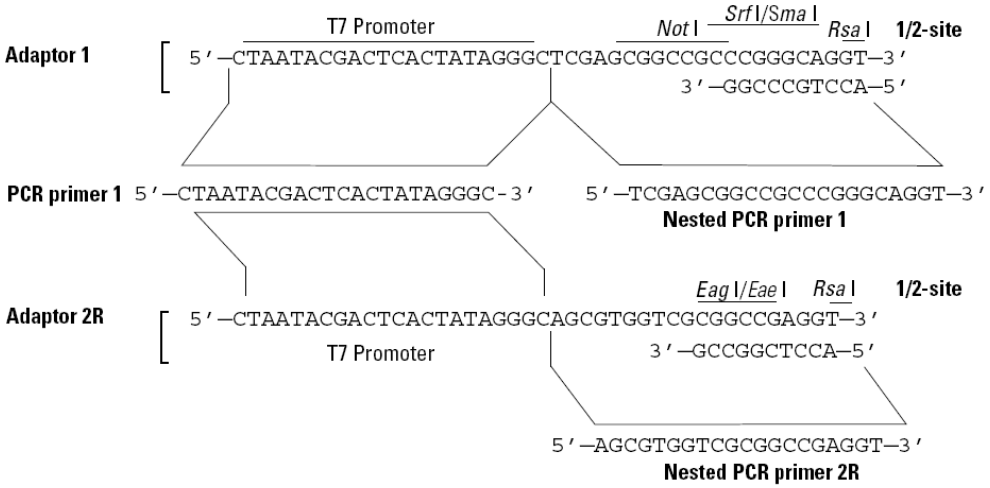


Figure 2.1C: Schematic representation of adaptor composition (Clontech PCR-Select cDNA subtraction manual, 2004).

The digested dscDNA with ligated adaptors from the inhibited parasite culture served as the tester in the forward subtraction (subtraction of control from inhibited). The digested dscDNA without ligated adaptors from the control culture served as driver in these reactions. The digested dscDNA with adaptors from the control parasite culture served as the tester in the reverse subtraction (subtraction of the inhibited from the control) now with the digested dscDNA without ligated adaptors from the inhibited parasite culture as driver. The driver dscDNAs were prepared by *Rsa*I digestion as described above and cleaned in the same manner but adaptors were not ligated to these digested cDNA populations.

2.2.6.4 Subtractive hybridisation

During the first hybridisation, 1.5 µl of digested driver dscDNA was added to each of two tubes containing either 1.5 µl of adaptor 1- (hybridisation sample 1) or adaptor 2-ligated tester cDNA (hybridisation sample 2) and 1x hybridisation buffer (4 M NaCl, 200 mM HEPES pH 8.3, 4 mM hexadecyltrimethyl- ammonium bromide (CTAB)) to a final volume of 4 µl. These solutions were denatured at 98^{o}C for 1.5 min and allowed to anneal at 64^{o}C for 8 hours (the annealing temperature was decreased from 68^{o}C suggested in the manual to 64^{o}C as suggested by Spielmann and Beck, 2000. This is due to the high A+T content (~82%) in the *P. falciparum* genome (http://www.malaria.mr4.org). The second hybridisation consisted of mixing freshly denatured driver dscDNA with hybridisation sample 1 and 2 simultaneously to ensure that the 2 hybridisation samples mixed together only in the presence of freshly denatured driver. Digested driver dscDNA (1 µl), prepared in 1x hybridisation buffer

and sterile water (provided in the subtraction kit) to a final volume of 4 µl, was denatured at 98°C for 1.5 min. 1 µl of this freshly denatured driver was combined with the two hybridisation samples from above and allowed to anneal at 64°C overnight. This second hybridisation mix was diluted 40x with dilution buffer (20 mM HEPES-HCl pH 8.3; 50 mM NaCl; 0.2 mM EDTA pH 8.0) after which the solution was heated at 68°C for 7 min to allow for suppression to proceed and to ensure the stringency of hybrids formed.

2.2.6.5 PCR amplification

For each subtraction i.e. the forward and reverse subtractions, two PCR amplifications were performed. This allowed for differentially expressed cDNAs to be amplified. The first reaction exponentially amplified only those dscDNAs with different adaptor sequences on each end. The second reaction used nested PCR to reduce background and enrich for differentially expressed sequences. Figure 2.1D illustrates a differentially expressed dscDNA with two different adaptor sequences on each end containing the annealing sites for the primary and secondary PCR primers. The sequences for the adaptors and primers are illustrated in Figure 2.1C.

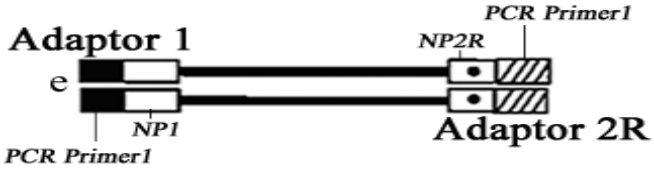


Figure 2.1D: Representation of differentially expressed dscDNA with adaptors (1 and 2R) with annealing sites for PCR primer1 (used during the primary PCR) and Nested Primers -1 (NP1) and -2R (NP2R) (used during the secondary PCR). (adapted from Diatchenko *et al.*, 1996)**.**

The primary PCR consisted of 1 µl of the 40x diluted, subtracted cDNA and 24 µl of PCR master mix (19.5 µl sterile water (provided in the subtraction kit), 1x PCR reaction buffer, 0.4 µM of PCR primer 1, 0.5 µl dNTP mix (10 mM of each) and 1x Advantage 2 cDNA polymerase mix (Clontech, USA)). The samples were incubated at 75°C for 7 min to fill in the missing strands of the adaptors. The primary PCR (final volume 25 µl) was then performed with the following cycling parameters: 94°C for 25 sec; 27 cycles at 94°C for 10 sec; 66°C for 30 sec and 72°C for 1.5 min. The amplified products were diluted 10-fold and 1 µl was used as template during the secondary PCR (25 µl) which consisted of 12 cycles of amplification mix using the same conditions as for the primary PCR except that PCR primer 1 was replaced by 0.4 µM of nested PCR primer 1 and 0.4 µM of nested PCR primer 2R.

2.2.6.6 Agarose gel electrophoresis of PCR products

All PCR products were analysed on 2% (w/v) agarose (Promega, USA)/ TAE (40 mM Tris-acetate, 1 mM EDTA) gels by electrophoresis in 1x TAE at 79 V (5.3 V/cm) in a Minicell EC370M electrophoretic system (E-C Apparatus Corporation, USA). The DNA was loaded in 6x loading dye (30% (v/v) glycerol, 0.025% (w/v) Bromophenol blue). Gels were stained in ethidium bromide (10 µg/ml) and bands visualised on a UV transilluminator at 312 nm. Images were captured with a charged-coupled device (CCD) camera linked to a computer system.

2.2.6.7 Cloning subtracted cDNA into pGEM T-Easy

3 µl of each of the products from the secondary PCR of the forward and reverse subtractions was ligated into the A/T cloning vector pGEM T-Easy (Promega, USA). Positive and negative controls were included as per manufacturer's instructions (pGEM-T and pGEM-T Easy Vector Systems technical manual, 1999). It was difficult to quantify the exact vector to insert ratio as a population of inserts was present in the secondary PCR product, thus the volume suggested in the Clontech manual of 3 µl was used during ligation. Ligation was performed using 1x rapid ligation buffer (Promega, USA), 50 ng of pGEM T-Easy vector (Promega, USA), 3 µl of secondary PCR product and 3U of T4 DNA ligase (Promega, USA). The reaction was incubated at 4^{o}C for 60 hr followed by inactivation of the ligase enzyme at 70^{o}C for 15 min.

Transformation was performed according to the standard heat shock method (Sambrook *et al.,* 1989). Ultracompetent XL1 blue (Stratagene, England) cells were thawed on ice and the entire ligation mixture (10 µl) was added to 100 µl of these cells. The cells were incubated on ice for 30 min, heat shocked at 42^{o}C for 45 sec and incubated for 2 min on ice. 900 µl of prewarmed LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) containing 20 mM glucose was added to the cells which were subsequently incubated for 1 hr at 30^{o}C while shaking at 250 rpm. LB-agar plates (1.5% (w/v) agar) containing 100 µg/ml ampicillin (Roche, Germany) were prepared on which 8 mg X-gal (5-bromo-4-chloro-indolyly-β-D-galactoside, 20 mg/ml in dimethylformamide, DMF) and 0.4 mM IPTG (isopropyl-D-galactoside) were spread. The plates were subsequently left at room temperature to absorb the DMF. 100 µl of the transformed cells were plated out and the plates were incubated at 30^{o}C for 16 hr.

2.2.6.8 cDNA insert analysis by PCR

384 white colonies were randomly selected from each cloned, subtracted library. These were inoculated into 100 µl of LB-amp (LB broth with 50 µg/ml ampicillin) in 96 well flat-bottomed Corning culture plates (Corning, USA). The bacterial cultures were grown for 16 hours at 30°C while shaking at 250 rpm. PCR analysis was performed in a 96 well PCR plate (Promega, USA). 1 µl of each bacterial culture served as template for the reaction and was transferred to each well of the PCR plate. The 20 µl PCR reactions consisted of 0.2 mM dNTP, 1x Taq DNA polymerase buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 6 µM of nested PCR primer 1 (section 2.2.6.5) and 2R (section 2.2.6.5), 1.5 mM of $MgCl_2$ and 1.25U of rTaq DNA polymerase (Takara Biomedicals, Japan). The PCR cycles were as follows: 94°C for 30 sec, then 23 cycles of 95°C for 10 sec and 68°C for 3 min. The amplified inserts were analysed on a 2% (w/v) agarose/ TAE gel as described in 2.2.6.6.

2.3 Results

Inhibition of polyamine synthesis was confirmed to halt schizogony of *P. falciparum* in the late trophozoite stage via light microscopy using Giemsa stained thin blood smears. Total RNA was isolated from *P. falciparum* cultured parasites under conditions of polyamine depletion (treated with DFMO) as well as from control, untreated cultures. The control culture yielded 7.92 µg of total RNA and the polyamine depleted culture 5.28 µg of total RNA. Pure RNA should have an $A_{260/280}$ ratio of 1.7 – 1.9 (Sambrook *et al.*, 1989.). The $A_{260/280}$ ratio of RNA from the control culture was determined as 1.7 and that of the polyamine-depleted culture as 1.5.

The RNA was subsequently used in the synthesis of dscDNA with SMART technology as described. It was necessary to determine the optimum cycling parameters for synthesis/amplification during long distance PCR of the dscDNA from both the polyamine-depleted and control cultures to obtain sufficient quantities and quality of the amplified dscDNAs.

Figure 2.2 indicates the intensities of the LD-PCR products with increasing numbers of cycles used. In general, cDNA synthesised from mammalian total RNA should appear as a moderately strong smear from 0.5-6 kb with some distinct bands. The numbers of cycles were optimised such that the dscDNA remained in the exponential phase of

amplification without reaching a plateau when the yield of PCR products stopped increasing. In Figure 2.2 it is shown that a smear ranging from 500 bp to ~2 kb was observed. The optimum number of cycles for synthesising both species of dscDNA was found to be 17. Bands were less intense when the number of cycles was either increased or decreased.

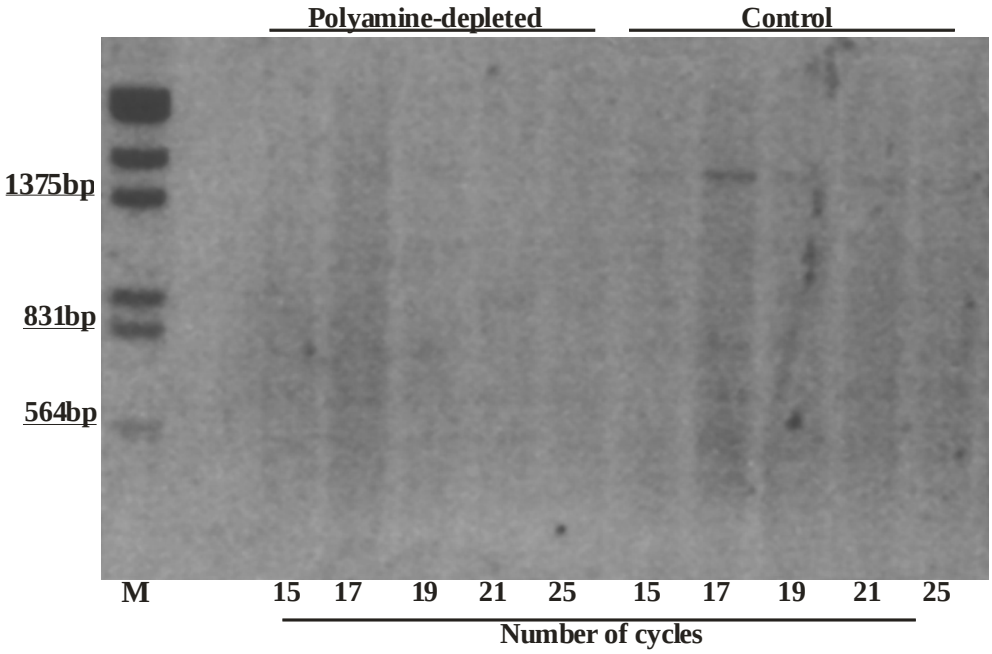


Figure 2.2: Determining the optimum number of PCR cycles for SMART dscDNA synthesis. M: *Eco*RI + *Hin*dIII digested phageλ DNA used as molecular markers. 5 µl of each LD-PCR product was analysed by 1.2% (w/v) agarose/TAE gel electrophoresis.

Figures 2.3A and B provide a brief overview of the forward (represented in green) and reverse SSH (represented in pink) procedures applied during the completion of these experiments. These figures (2.3A and B) illustrate the progression from *in vitro* parasite cultures, RNA isolation, dscDNA synthesis, to the SSH procedure itself and the subsequent array hybridisations in an attempt to clarify how the four probes utilised during the array hybridisation procedure (see section 3.2.1) were generated.

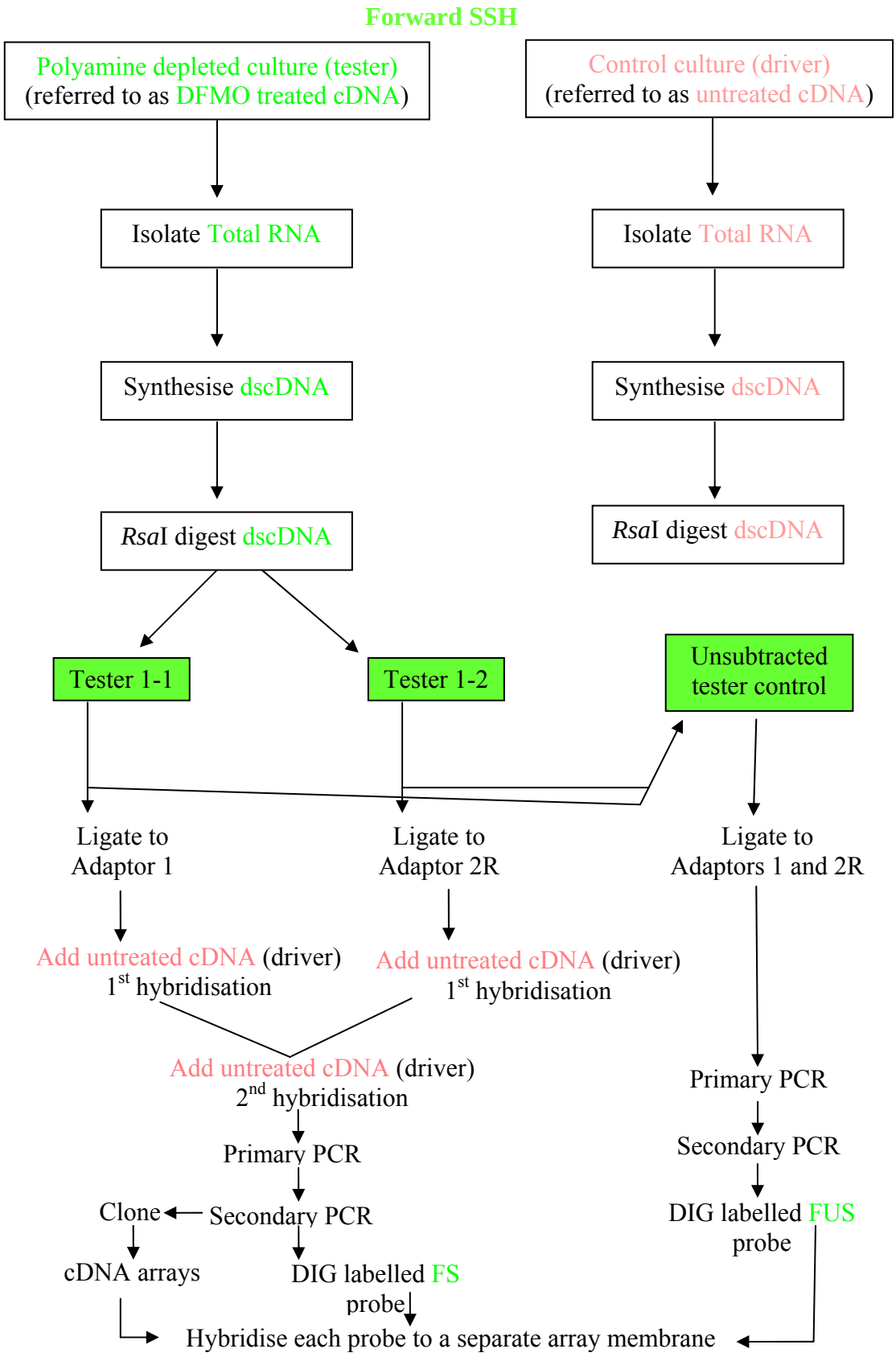


Figure 2.3A: Summary of forward SSH including production of FS and FUS probes

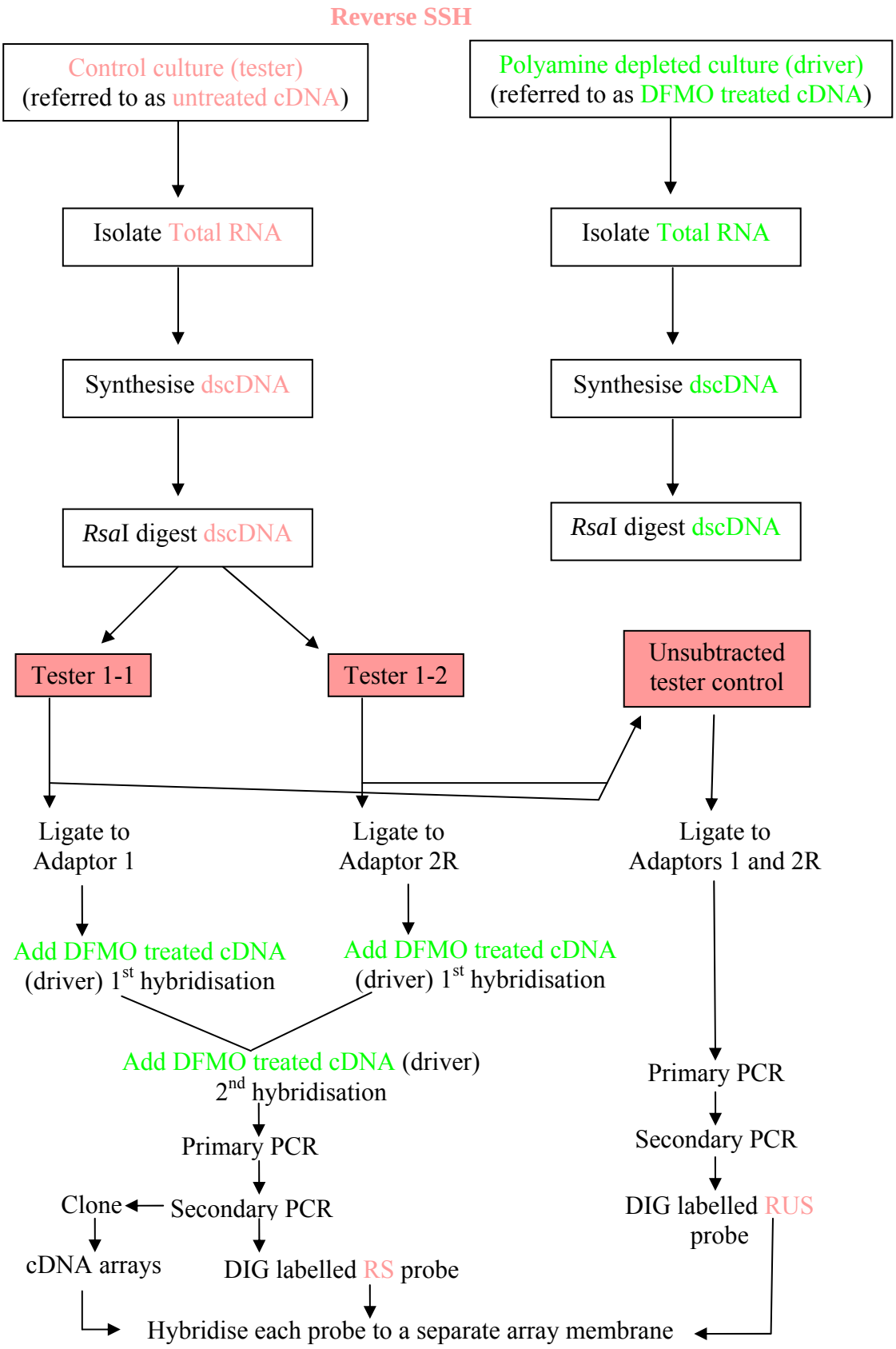


Figure 2.3B: Summary of reverse SSH including production of RS and RUS probes

Once the dscDNA was synthesised and purified it was prepared for use in the suppression subtractive hybridisation procedure by digesting with *Rsa*I to obtain shorter, blunt-ended molecules. Two tester populations were then created by ligation to different adaptors, the driver was not ligated. Hybridisation and amplification followed to produce differentially expressed products. The products of the primary and secondary PCR amplifications are represented in Figure 2.4.

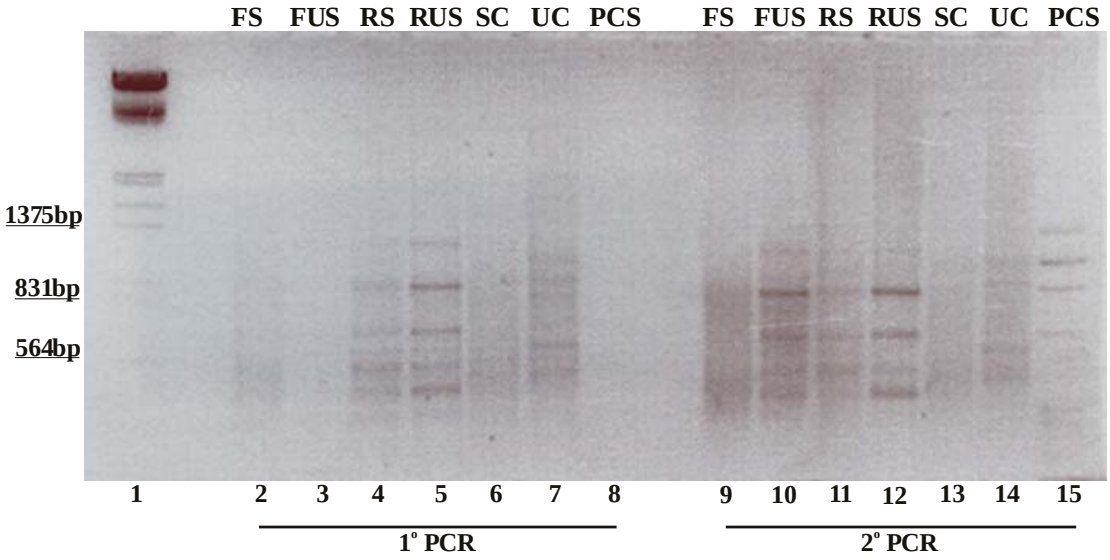


Figure 2.4: Primary and secondary PCR products after subtraction and suppression PCR. Lane 1: *Eco*RI + *Hin*dIII digested phageλ DNA used as molecular markers, lanes 2-8: 1° PCR products, lanes 9-15: 2° PCR products. Lanes 2 & 9: forward subtracted cDNA (FS), lanes 3 & 10 forward unsubtracted cDNA (FUS), lanes 4 & 11: reverse subtracted cDNA (RS), lanes 5 & 12: reverse unsubtracted cDNA (RUS), lanes 6 & 13: subtracted control skeletal muscle cDNA (SC), lanes 7 & 14: unsubtracted skeletal muscle control for the control subtraction (UC) and lanes 8 & 15: PCR control subtracted cDNA (PCS).

Bands were observed for almost all the primary PCR products. Only lanes 3 (forward unsubtracted cDNA) and 8 (PCR control subtracted cDNA) (Figure 2.4) showed no visible product. However, the Clontech PCR-select cDNA subtraction kit manual (Clontech, USA) did suggest that bands might only appear after the secondary PCR step, which was the case as is shown in lane 3 vs lane 10 and lane 8 vs lane 15. It was also suggested that the experimental PCR products obtained after subtraction should appear as a smear from approximately 0.2-2kb, with or without some distinct bands. This was indeed observed as indicated in lanes 2 and 4 for the primary PCR as well as in lanes 9 and 11 for the secondary PCR.

Control skeletal muscle tester cDNA was prepared by mixing 1 µl of control skeletal muscle cDNA with 5 µl of control *Hae*III-digested bacteriophage ϕX174 DNA (150 ng/ml). This sample was ligated to the two different adaptors (1 and 2R) and amplified during the subsequent primary and secondary PCR reactions. The driver control was prepared by synthesising dscDNA from skeletal muscle RNA (section 2.2.6.1 and 2), digesting it with *Rsa*I and purifying the product (section 2.2.6.3). The control skeletal muscle tester sample consisted of a known concentration of digested bacteriophage DNA (the profile of which is also known). After successful performance of the SSH procedure a distinct profile corresponding to that of the digested bacteriophage should appear in the secondary PCR product of the skeletal muscle control subtraction.

The manufacturers performed a successful subtraction on the control tester and driver samples and provided a subtracted PCR control sample with the kit. This subtracted control sample would display only those bands corresponding to the digested phage DNA after amplification and was a postive indication that the primary and secondary PCR reactions were optimised.The secondary PCR product of the PCR control subtracted cDNA indicated the expected ϕX174/ *Hae* IIIbands (lane 15). It was thus not necessary to optimise the PCR parameters.

The expected ϕX174/ *Hae*III bands were observed in the secondary PCR product of the PCR control subtracted DNA (lane 15), but not in the skeletal muscle control subtraction (lane 13). Instead a smear was visible which could be an indication that the subtraction efficiency was low and was probably due to inefficient ligation (Clontech PCR-select cDNA subtraction kit manual 2000).

The results from the secondary PCR suggest that the subtraction was indeed successful as the banding patterns obtained for the forward subtracted and unsubtracted fractions (Figure 2.4 lanes 9 and 10, respectively) differ from each other. The same is true for the patterns obtained for the reverse subtracted and unsubtracted fractions (lanes 11 and 12).

After the secondary PCR amplification, the products were ligated into the pGEM T-Easy vector and transformed into ultracompetent XL1 blue cells. The transformation efficiency was found to be very high as more than 10^4 colonies were obtained for each of the subtractions. This is comparable to the expected 10^4 independent colonies from

1µl secondary PCR product that was obtained by Desai *et al.*, (2000). As a result, random selection of 384 colonies from the forward and reverse subtraction was achieved with ease.

The selected colonies were incubated in LB-broth with the appropriate antibiotic and 1µl was used during screening with PCR. All 768 clones (384 each from the forward and reverse subtraction) were subjected to PCR and run on 2% (w/v) agarose/TAE gels. Figure 2.5 represents an example of the results obtained for insert analysis via PCR.

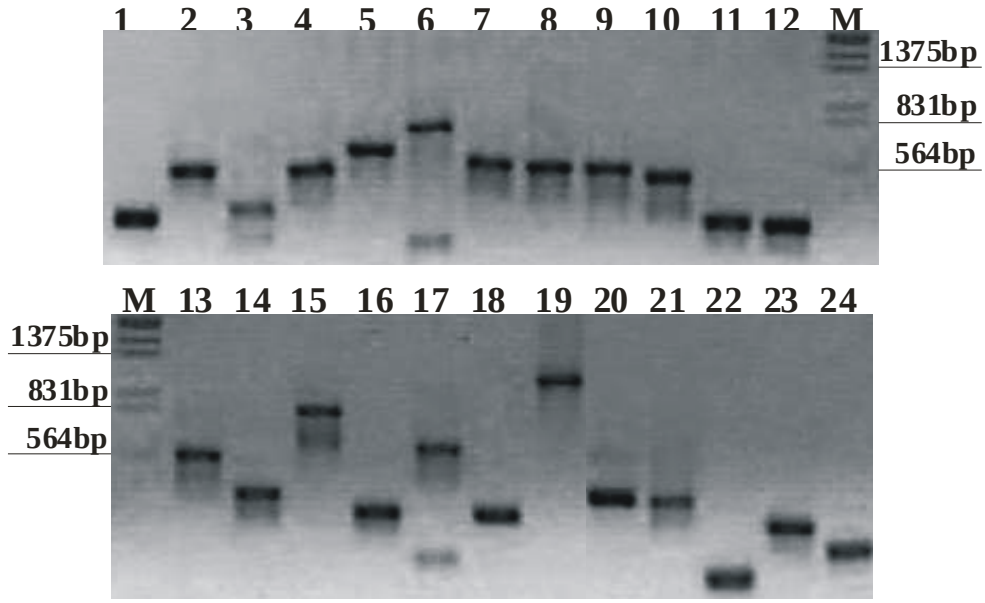


Figure 2.5: PCR screening for the presence of inserts in the cloned, subtracted libraries. The PCR products are representative of clones from the forward subtraction (lanes 1-24). Lane M: *Eco*RI + *Hin*dIII digested phageλ DNA used as molecular markers.

The lengths of bands obtained for all the inserts from both the forward and reverse subtracted libraries varied between approximately 100-1600bp which correlated with the suggested sizes obtained in the Clontech PCR-Select cDNA Subtraction manual (2000). Lanes 6, 15 and 17 (Figure 2.5) have 2 bands compared to single bands in all other lanes. Multiple bands were observed in approximately 2% of all clones amplified from both subtracted libraries. This could either have been due to selection of multiple colonies from plates or the formation of chimeric cDNAs during the suppression hybridisation and subsequent PCR procedures (Zhang *et al.*, 2000).

2.4 Discussion

Polyamines are an essential component of all living cells and inhibition of polyamine biosynthesis by DFMO, an irreversible inhibitor of ornithine decarboxylase, is capable of inhibiting cell growth and decreasing intracellular polyamine levels in *P. falciparum, in vitro* (Assaraf *et al.,* 1984; Whaun and Brown, 1985). However, this inhibition could be reversed by the addition of putrescine. Assaraf and co-workers (1984) demonstrated that polyamine synthesis reached peak values during the early trophozoite stage, whereas nucleic acid and protein synthesis occurred later in mature trophozoites. Addition of DFMO to cultures did not interfere with merozoite invasion or ring-form development, but prevented the transformation of trophozoites to schizonts. Due to polyamine depletion, the synthesis of proteins and nucleic acids was significantly inhibited (Assaraf *et al.,* 1984; Igarashi *et al.,* 1982). However, the exact molecular mechanism of action and binding targets of the polyamines in *P. falciparum* is ill-defined. Apart from the above, the precise physiological significance of polyamine depletion within the parasite is unclear. Thus SSH was employed as a method of differential gene expression profiling in order to identify differentially expressed genes under conditions of polyamine depletion, thereby indicating the compensatory mechanisms induced by the parasites in the absence of polyamines and also possibly demonstrating the function of these molecules.

The standard SSH procedure described by Diatchenko *et al.,* (1996) calls for micrograms of mRNA to be used as starting material. This was impossible to achieve as previous attempts to isolate mRNA from *P. falciparum* using a variety of methods failed to yield sufficient amounts of product (Birkholtz 2004; Clark 1998). Due to the high A + T content of *P. falciparum* oligo dT beads cannot be used to isolate mRNA by binding to its poly A tail since extended tracts of adenosine base pairs within the RNA and DNA could also bind to the beads resulting in non-specific selection (Munasinghe *et al.,* 2001). As a result, rare mRNA transcripts would be lost and abundant rRNA containing these poly-A stretches would be enriched (Birkholtz 2004; Clark 1998). It was thus decided to use total RNA during the SSH procedure.

Long DNA fragments can form complex interacting networks that could prevent the correct hybrids from forming during the SSH procedure (Lukyyanov *et al.,* 1995), necessitating the use of short cDNA fragments instead. Secondly, digesting the cDNAs

into small fragments provides better representation of individual genes as this increases the chance of similar coding sequences "bumping" into each other (Gurskaya *et al.,* 1996). These similar sequences can then cross-hybridise and be eliminated during the subtraction procedure since they could form b, c, or d type hybrids as shown in Figure 2.1A (Diatchenko *et al.,* 1996). Although some cDNAs might be lost during the SSH procedure because of non optimal driver:tester ratios during the first and second hybridisations, the generation of smaller fragments will overcome these obstacles to allow for differentially expressed genes to be identified.

The stringency of subtraction depends on the driver:tester ratio, where a high ratio will result in the preferential enrichment of those genes that are highly upregulated in tester compared to driver. However cDNAs with smaller differences in expression will be lost. If the ratio is decreased then those genes that are only slightly upregulated in the tester will be identified but the number of false positives will also increase (Desai *et al.,* 2000, Gurskaya *et al.,* 1996). Subsequently a driver:tester ratio of 30:1 was used during the SSH procedure as this ratio was also suggested to produce better results than a 50:1 ratio (Desai *et al.,* 2000).

A gene does not necessarily have to be up- or down-regulated to play a key role in certain processes, however one of the ways of elucidating such a process is to identify and isolate those genes that are differentially expressed. A problem arises when you take into consideration mRNAs that are present in abundance. The difficulty in isolating a gene responsible for a specialised function often originates from the fact that the gene is expressed at low levels whereas the majority of the cell's mRNA is made up of highly abundant transcripts (Lievens *et al.,* 2001). This snag is overcome in the current procedure by normalisation and enrichment during the hybridisations and PCR amplifications; however the results generated during this procedure are qualitative because of the relative insensitivity of the hybridisation (Diatchenko *et al.,* 1996; Lievens *et al.,* 2001).

Normalisation occurs during the first hybridisation step where tester cDNA fitted with either adaptor 1 or 2 is mixed with a large excess of driver cDNA, denatured and subjected to limited renaturation. Since the renaturation process involves the random collision of complementary strands, the likelihood of the more abundant fragments forming hybrids without the required adaptor sequences is greatly increased (Ji *et al.,*

2002). When additional driver is added during the second hybridisation, further enrichment of differential transcripts occurs because any of the similar sequences, between the tester and driver, that could have been "missed" during the previous limited renaturation process can anneal with their complementary strands (Gurskaya *et al.*, 1996).

One of the methods of confirming normalisation would be to include a control gene, for example a parasite housekeeping gene, whose levels of expression remain constant regardless of the situation. This could then be confirmed by hybridisation, to analyse the efficiency of subtraction, in which the gene should be expressed before subtraction in both the control and treated samples but should not be present in the subtracted sample. In addition, an internal SSH positive control sample of skeletal muscle cDNA, which either contains fragments of *Hae* III digested ϕX174 DNA or not, is also provided with the kit (Clontech, USA). However, no such housekeeping gene was available for use at the time of this study and the skeletal muscle controls provided with the kit had to be used instead.

A positive SSH should yield the *Hae* III digested ϕX174 bands in the subtracted control after the secondary PCR, that would not only account for SSH efficiency, but also for the efficacy of normalisation and enrichment (Diatchenko *et al.*, 1996; Ji *et al.*, 2002). However only a smear was visible (Figure 2.4 lane 13). According to the manufacturers this could have been due to poor ligation efficiency. Ligation efficiency could have been tested by performing a PCR designed to amplify fragments that span the adaptor/cDNA junction of the tester cDNAs and would have verified whether at least 25% of the cDNAs had adaptors on both ends. This test could not be performed as the G3PDH primers provided with the subtraction kit were designed to work for human, mouse and rat DNA, not for *P. falciparum* DNA.

Comparison of the secondary PCR products of the forward SSH revealed distinct differences between the subtracted (lane 9, FS) and unsubtracted (lane10, FUS) fractions. In lane 9 the forward SSH subtracted fraction (FS) appeared as a smear, whereas in lane 10 the forward SSH unsubtracted fraction (FUS) appeared as a smear and included distinct bands. Similarly, the PCR products of the reverse SSH (lane 11, RS *vs* lane 12, RUS) indicated differences in the subtracted and unsubtracted fractions. Three distinct bands in the reverse SSH unsubtracted fraction (lane 12, RUS) were more

intense than their corresponding bands in the reverse SSH subtracted fraction (lane 11, RS). If the secondary PCR products in lane 9 (FS) and lane 11 (RS) are compared, differences in their gel profiles become visible. For example the products in lane 9 appeared as a smear without any distinct bands, whereas the opposite is true for lane 11 in which a smear as well as distinct bands were visible. These results suggest that the subtraction in both the forward and reverse direction were successful.

In Figure 2.5, the analysis of different clones from the forward library is represented. However, in some instances (lanes 6 and 17), multiple bands were observed. Due to the high transformation efficiency of the ultracompetent *E. coli* cells, the density of colonies on a Petri dish of diameter ~ 8cm is also high (more than a thousand colonies were obtained per plate). This increased the possibility of overcrowding and thus of selecting more than one colony, leading to inoculation of two or more colonies into one well. In effect this could mean that all the vectors contain different inserts and upon amplification all inserts would be produced. A possible means of overcoming this is to either plate out fewer transformed cells or plate the cells onto a larger surface area.

It is interesting to note that approximately 95% of all the clones which underwent PCR contained inserts. This is comparable with the pGEM-T Easy results obtained by the manufacturer showing that 70-100% of clones they obtained in control experiments had the correct size insert (pGEM-T Easy manual 1999). It seems that even though blue/white selection was employed and no blue colonies were observed, some of the selected clones were false positives and did not contain inserts.

In the next chapter, differentially expressed transcripts were identified and characterised by dot blot analysis, restriction enzyme profiling and sequencing.

CHAPTER 3

Physiological Response to Polyamine-depletion and Putative Function of Polyamines in *P. falciparum*

3.1 Introduction

Expression data can be used to discover gene function, parasite drug response as well as general mechanisms guiding the parasite life cycle (Young and Winzeler, 2005) and this can be achieved in a number of ways including DNA microarray and real time PCR based technologies (Monis *et al.*, 2005). Little, (2005) said "Understanding a complex system requires more than assembling a parts list and wiring the parts together", which is true of all the genomic information currently available.

The relation between an organism's genome and its metabolism is of great interest and in the case of *P. falciparum* vital as metabolic pathways play an important role in resistance. However the full spectra of genes involved in conferring and overcoming resistance is unknown, exposing an interesting area for further analysis of the parasite's transcriptome. This is particularly important when trying to identify differences at the genomic level between the parasite and its host when the function of a substantial number of genes in these genomes remains to be determined. Some progress has been made in this regard given the available annotated sequence information. Genome wide expression and subsequent comparative genomics led to the identification of enoyl-ACP, a key enzyme in the type II fatty acid biosynthesis pathway of the parasite, which is absent in the human host and as such is a candidate for drug development (Ghosh *et al.*, 2003).

The latter enzyme provides a good example of reasons to analyse and elucidate the parasite's metabolome. Another reason is that cells adjust to environmental changes by altering gene expression patterns (Agarwal *et al.*, 2003) and analysis and measurement of these expression changes can be exploited to further elucidate the parasite's metabolism as well as the identification of novel drug targets. This illustrates the point that gene sequence data is useless unless it can be linked to mRNA expression profiles as well as metabolic pathways (Patil and Nielsen, 2005; Young and Winzeler, 2005).

Fairlamb, 2002 also makes the point that "in theory knowledge of the entire genome of a pathogen identifies every potential drug target", but goes on to state that this is indeed of little consequence due to the complexity of the resulting genomic information and that analysis of metabolic pathways would provide a deeper insight into the parasite's biology.

Polyamine metabolism is an ideal candidate for expression analysis as little is known about this metabolic pathway in the malaria parasite (Muller *et al.,* 2001). As discussed in Chapter 1, polyamines are also vital for survival of all organisms, which indicates that their metabolic and biosynthetic pathways are of importance and inhibition of their biosynthesis would aid in further elucidating these pathways.

This train of thought has been applied to other organisms. For example, *S. cerevisiae* was exposed to certain stress-resulting agents and its expression profile analysed for novel and differentially expressed genes (Agarwal *et al.,* 2003). An *A. gambiae* detoxification chip is also available which allows for the study of metabolic-based insecticide resistance (David *et al.,* 2005). Gunasekera *et al.* (2003) have analysed drug-induced alterations in gene expression in the asexual form of *P. falciparum*. The identification of the glucose transporter by Woodrow and colleagues, 1999, is another example which illustrates the advantage of metabolic pathway analysis as the parasite depends almost solely on glucose uptake from the host.

Nutrients often regulate signalling pathways, which in turn control transcription and translation. In *P. falciparum*, dramatic changes occur during its life cycle from its sexual stage in the mosquito vector to the asexual forms in the human host. Within the human host itself the parasite infects first liver hepatocytes and then erythrocytes and has to respond to this change in environment. Fang and co-workers, 2004, suggest that changes in glucose concentration also lead to subsequent changes in gene expression.

By inhibiting polyamine biosynthesis within *P. falciparum* and performing a subtractive suppression hybridisation on the cDNA from inhibited versus wild type/ control cultures specific transcripts were generated (Chapter 2). These transcripts were analysed in this chapter using dot blot hybridisations in which the signals produced with various probes had to conform to specific criteria before being identified as either up- or down-regulated.

This was achieved by dot blot hybridisations using a non radioactive detection method which employs the steroid hapten digoxigenin (DIG) which is usually linked to UTP (uridine triphosphates) via a linker arm (Figure 3.1). This is preferable to radioactive labelling as it is safer and more economical in terms of its longer shelf life; also the results are obtained more rapidly as shorter X-ray exposure times are required.

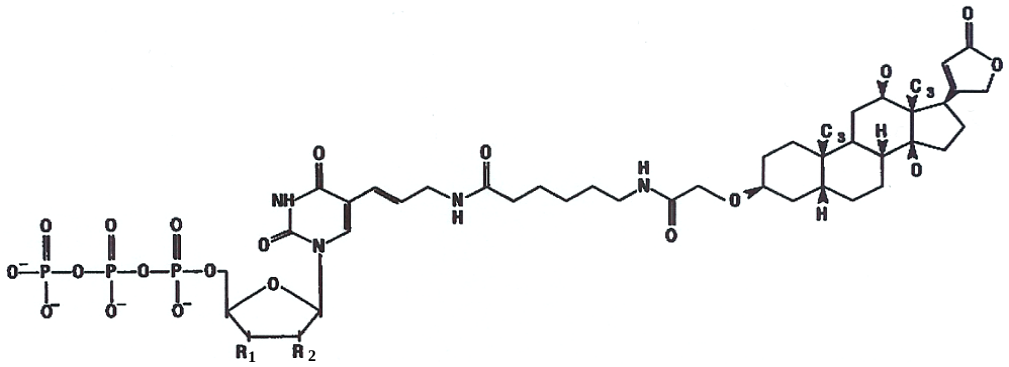


Figure 3.1: General structure of DIG-dUTP, R_1 = OH and R_2 = H
(DIG application manual for filter hybridisation, Roche, 2000).

DIG can be incorporated into the probe using any of the following methods: randomly primed labelling, nick translation, incorporation during PCR and oligonucleotide tailing for example. Detection is based on recognition of the DIG molecule by a specific DIG antibody, which in this case is conjugated to an enzyme, alkaline phosphatase, to allow chemiluminescent detection. The substrate used is CDP-Star, a chloro-substituted 1, 2-dioxetane which exhibits rapid light signal generation. This signal can be captured on X-ray film as enzymatic dephosphorylation of the molecule leads to a meta-stable dioxetane phenolate anion which decomposes and emits light at 466nm in the appropriate buffer (Figure 3.2).

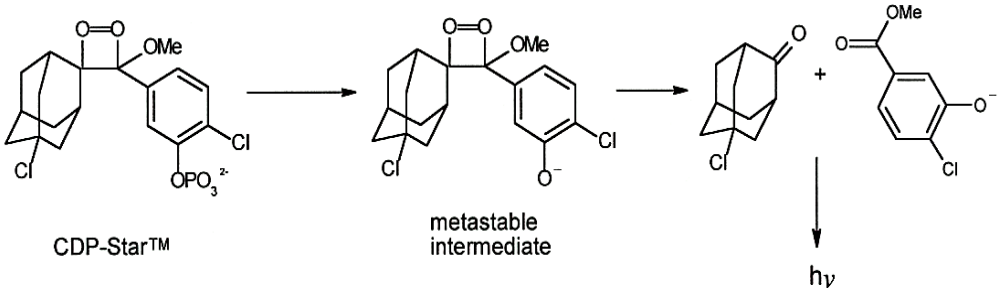


Figure 3.2: The chemical reaction of CDP-Star in the presence of alkaline phosphatase (DIG application manual for filter hybridisation, Roche, 2000).

In the following pages the differential transcriptome profiling of malaria parasites depleted of their endogenous polyamines is described. Transcripts that were up- and

down-regulated in the absence of polyamines were identified and their potential functions within the parasite and polyamine metabolism are discussed.

3.2 Materials and methods

3.2.1 Hybridisation

3.2.1.1 Synthesis of DIG-labelled DNA probes

Non-radioactive labelling was performed by incorporation of a digoxigenin (DIG)-labelled nucleotide (DIG-11-dUTP) into DNA products during PCR. DIG is detected using an enzyme-linked immunoassay with an anti-DIG antibody conjugated to alkaline phosphatase. The primary PCR products of the forward- and reverse-subtractions as well as their respective unsubtracted tester controls (sections 2.2.6.4 and 2.2.6.5) represent the forward- and reverse-subtracted as well as the forward- and reverse-unsubtracted probes.

These primary PCR products were diluted five times and used as template during a 50 µl PCR labelling reaction. The labelling reaction consisted of 6 µl of each of the four diluted DNA samples, 10 µM of nested PCR primer 1 and 2R (see 2.2.6.5), 0.8x rTaq DNA polymerase buffer (100 mM Tris-HCl pH 8.3 and 500 mM KCl), 1.5 mM $MgCl_2$ (Takara, Japan), 0.2 mM dGTP, dCTP and dATP, 0.17 mM dTTP and 0.03 mM DIG-11-dUTP (Roche, Germany) and 1.25U rTaq DNA polymerase (Takara, Japan). The PCR cycling parameters were as follows: 95^{o}C for 10 sec, 68^{o}C for 30 sec and 72^{o}C for 1.5 min for 11 cycles followed by a single cycle of 72^{o}C for 5 min. A duplicate of this reaction was repeated in tandem but without the DIG-11-dUTP nucleotide and used as a control.

The control and labelled reaction products were purified using the Sephadex G-50 column clean-up method (ABI protocol, PE/Applied Biosciences Division, USA). Briefly, the non DNA-binding column was washed by adding 800 µl of sterile Milli Q water and centrifuging at 740xg for 2 min, this was repeated twice. 650 µl of Sephadex G50 slurry (1 g of Sephadex G-50 powder was left to swell in 15 ml of sterile Milli Q water for 2-5 hours) was added to the column and centrifuged for 2 min at 740xg. The PCR products (50 µl) were pipetted onto the prepared matrix and the purified eluent collected after centrifuging at the above mentioned parameters. The control reaction was subsequently analysed on an agarose gel according to section 2.2.6.6.

3.2.1.2 Mock dot-blot of DIG labelled probes

Serial dilutions ranging from 5 ng/µl to 0.5 pg/µl of the four probes were prepared and 1µl of each was spotted onto a positively charged nylon membrane (Roche, Germany) in preparation of a dot-blot. 2.5 pmol of a DIG-labelled control oligonucleotide (Roche, Germany) was spotted as a positive control. The DNA was cross-linked to the membrane for 3 min on a UV transilluminator at 312nm. The membrane was equilibrated in Tween-20 wash buffer [0.3% (v/v) Tween-20 in maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5)] for 1 min at room temperature followed by a further 30 min in 1 X blocking solution (1% (v/v) blocking reagent in maleic acid buffer) in order to prevent non-specific binding. Subsequent incubation in antibody solution (a 1:20000 dilution of anti-DIG alkaline phosphatase Fab fragment (Roche, Germany) in 1x blocking solution) was performed at room temperature for 30 min with shaking. In order to remove any unbound blocking agent or antibody, the membrane was washed twice in Tween-20 wash buffer for 15 min each and allowed to equilibrate in detection buffer (100 mM Tris-HCl, pH 9.5; 1 mM NaCl) for 2 min.

Detection was performed using CDP-Star (Roche, Germany), an ultra-sensitive chemiluminescent for alkaline phosphatase which emits extremely rapid light signals that can be detected and recorded on X-ray film. A 1:100 dilution of CDP-Star in detection buffer (CDP-Star solution) was added to the membrane and incubated at room temperature for 5 min. The membrane was sealed in a clean plastic bag and exposed to Konica Medical X-ray film (AX; X-ray Imaging Services, RSA) for 3 min in the dark. Chemiluminescence was visualised after development and fixation of the film (3 min each). Once DIG-11-dUTP labelling of the probes was confirmed after visual inspection of the developed film, hybridisation of the subtracted products was performed.

3.2.1.3 Dot blots of the forward and reverse subtracted libraries

Once the cDNA insert analyses of the forward and reverse PCR products were completed via PCR (2.2.6.8), the 96-well PCR plates were centrifuged briefly at 1000xg. The PCR products were subsequently denatured at 95^{o}C for 10 min and immediately snap cooled on ice for approximately 10 min whereafter they were again briefly centrifuged at 1000xg. 1 µl of each denatured PCR product was spotted in a 96-well array format onto positively charged nylon membranes (Roche, Germany) and allowed to crosslink for 3 min on a UV transilluminator at 312nm.

As the inoculation and PCR analysis in 2.2.6.8 proved the most efficient to handle in a 96-well format, subsequent hybridisations remained in this format. The 384 inserts were spotted onto a total of four membranes for each subtraction and each membrane was reproduced four times. This allowed for each set of 96 spots to participate in four different hybridisations, one to each probe.

Each membrane (consisting of 96 spots) was carefully placed into a roller tube and prehybridised in 5 ml of standard hybridisation buffer (20x SSC: 3 M NaCl, 300 mM sodium citrate, pH 7; 10 % (w/v) N lauroyl sarcosine; 10 % (w/v) sodium dodecyl sulphate (SDS); 10 % (v/v) blocking buffer) at 72^{o}C for 2 hours. The probe (3.2.1.1) was denatured at 95^{o}C for 10 min and immediately snap-cooled on ice. This was added to 5 ml of prewarmed (72^{o}C) standard hybridisation buffer to a final probe concentration of 20 ng/ml. The prehybridisation buffer was discarded and the freshly prepared hybridisation buffer (containing the probe) added. Hybridisation was performed at 72^{o}C overnight in a Grant Boekel rotating hybridisation oven (Grant Instruments, Great Britain). The hybridisation solution was discarded and low and high stringency washes were performed as described below.

Low and high stringency washes were performed in roller tubes in a Grant Boekel rotating hybridisation oven (Grant Instruments, Great Britain). A low stringency (2x SSC, 0.1% (v/v) SDS) wash at 68^{o}C for 20 min was repeated four times following hybridisation. Thereafter, the membrane was washed twice for 20 min at 68^{o}C in a high stringency wash solution (0.5x SSC, 0.1% (v/v) SDS). Subsequent wash, blocking and detection steps were all performed at room temperature with shaking on a rotating shaker unless otherwise stated.

The membrane was equilibrated in Tween-20 wash buffer for 2 min followed by incubation in 1x blocking solution for an hour. The blocking solution was discarded and the membrane incubated in antibody solution for 30 min. Three washes in Tween-20 wash buffer were performed for 15 min each, followed by equilibration in detection buffer for a further 2 min. The membrane was transferred to a plastic bag and incubated in 2 ml of CDP-Star solution for 10 min after which the signal was detected as in 3.2.1.2.

3.2.2 Selection of differentially expressed transcripts

3.2.2.1 Analysis of spot intensities

Once hybridisations with all four probes were completed, spot intensities were analysed by employing the 2 D protein gel, ImageMaster 2D program (Amersham Pharmacia Biotech, South Africa). This allowed for the densitometric comparison of intensities obtained with all four probes for each spot. This program enabled the whole spot to be scanned and its total volume to be calculated as an arbitrary numerical value by defining the background level from an area of the image closest to the spots of interest and subtracting it from every pixel within the analysis area.

Criteria suggested by the Clontech PCR-Select Differential screening kit manual, 2001, was used to select spots that were potentially differentially expressed. Briefly, probes that hybridised to each spot with equal intensity were not considered as being differentially expressed. So too were spots that produced no signal when hybridised to each of the four probes.

In the forward SSH library if spots produced a signal with only the forward subtracted (FS) and unsubtracted (FUS) probes and not the reverse subtracted (RS) or unsubtracted (RUS) probes, these spots were thought to correspond to differentially expressed cDNA fragments. If a signal was only obtained for the FS probe, these spots were considered strong candidates for differential expression and were thought to correspond to low abundance transcripts that were enriched during the SSH procedure and *vice versa* for the reverse SSH library. However, when signals obtained for both the FS and RS probes differed only in intensity, the spot was considered to be upregulated if the FS probe intensity was at least 1.5-fold that of the RS probe in the forward SSH library. In the reverse SSH library the intensity of the RS probe had to be at least 3-fold that of the FS probe before it was considered to be downregulated (Daily *et al.*, 2005). This was also in accordance with the manufacturer's notes which suggested that clones could be selected for differential expression if their spot intensities differed by 1.5 to 5 fold, respectively.

3.2.2.2 Plasmid isolation using the STET method (adapted from Sambrook, 1989)

Plasmid isolation from the clones selected above had to be performed in preparation for restriction digestion screening. Thus 5 µl of each clone was inoculated into 3 ml of LB-amp (LB broth with 50 µg/ml ampicillin) and grown for 16 hours at 30^{o}C while shaking

at 250 rpm. Plasmids were subsequently isolated from 1.5 ml of culture material which was centrifuged at 3500xg for 5 min and the supernatant discarded. The remaining bacterial cells were resuspended in residual supernatant to which 300 µl of STET buffer (8% sucrose, 5 % Triton X100, 50 mM EDTA, 50 mM Tris, pH8) was added. This solution was aspirated and 25 µl of lysozyme (4 mg/ml) added, followed by boiling for 1 min and centrifuging at 16000xg for 15 min. The resulting pellet was discarded and 300 µl of isopropanol added to the supernatant which was centrifuged at 16000xg for a further 15 min. The supernatant was discarded and the pellet dried at room temperature. Resuspension of the isolated plasmid was performed in 25 µl of 1x TE (10 mM Tris-acetate, 1 mM EDTA, 0.5 mg/ml RNase) buffer.

3.2.2.3 Restriction enzyme digest screening of selected clones

A restriction enzyme digest was performed to generate a restriction profile for screening of differences without having to sequence all the selected clones. A digest was initially performed on a sample of the isolated plasmids from the forward subtraction using 10U of *Hin*dIII (Promega, USA), but produced no discernible differences in the generated restriction profiles. A double digest was subsequently performed using 10U each of *Bam*HI (Promega, USA) and *Hin*dIII restriction enzymes. This double digest screen was unsuccessful as almost all of the digested sample transcripts produced the same profile. A triple digest using the three enzymes mentioned below produced the most optimum results and subsequently 8 µl of each isolated plasmid from the forward subtraction was digested in a 20 µl reaction consisting of 10U each of *Hin*dIII and *Eco*RI and 12U of *Eco*RV in 1x Buffer C (10 mM Tri-s HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, Promega, USA). The digestion was performed for 16 hours at 37^{o}C and the products were analysed on a 1% (w/v) agarose/TAE gels as described in 2.2.6.6. An alternate method of analysing the results from the reverse subtracted library involved amplifying the inserts obtained from the STET isolation, as in 2.2.6.8 using *Taq* DNA polymerase (Promega) instead, then continuing with restriction digest and analysis as described above. Clones that produced unique digestion profiles were selected for inoculation as before and isolated using the High Pure Plasmid Isolation Kit (Roche) in preparation for sequencing.

3.2.2.4 High pure plasmid isolation (Roche)

This method entails alkaline lysis of cells followed by binding of plasmid DNA to glass fibres in the presence of chaotropic salts. The DNA is eluted using a low salt buffer or

water. 8 ml of culture was grown for 16 hours at 30°C to an approximate OD_{600} of 2 units and the cells collected by centrifuging at 3000xg for 20 min. The manufacturer's protocol was followed. Briefly, the pellet was suspended in 250 µl of suspension buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8). To this solution, 250 µl of lysis buffer (0.2 M NaOH, 1% SDS) was added and the solution incubated at room temperature for 5 min. 350 µl of chilled binding buffer (4 M guanidine hydrochloride, 0.5 M K-acetate, pH 4.2) was added and the suspension incubated for 5 min on ice whereafter it was centrifuged for 10 min at 16000xg (4°C). The supernatant was transferred to the filter-tube and centrifuged for 45 sec at 16000xg. The filter was washed with 500 µl wash buffer I (5 M guanidinium hydrochloride, 20 mM Tris-HCl, pH 6.6) and again with 700 µl of wash buffer II (20 mM NaCl, 2 mM Tris-HCl, pH 7.5). The isolated plasmid DNA was eluted in 100 µl of elution buffer (1 mM Tris-HCl, pH 8.5) and the concentration determined spectrophotometrically.

For those isolates with low DNA concentrations a sodium-acetate/ethanol precipitation was performed to concentrate the DNA. In the presence of a high concentration of salt and at a low pH the addition of ethanol allows for DNA to be concentrated by forcing it out of the aqueous solution. Sodium acetate (3 M, pH 5.2) equivalent to one-tenth the volume of isolate and three equivalent volumes of 100% ethanol were added to the solution and centrifuged at 16000xg, at 4°C for 30 min. The supernatant was discarded and 400 µl of 70% ethanol added. This was centrifuged at 16000xg, at 4°C for 10 min and the supernatant discarded. The pellet was dried *in vacuo*, dissolved in 25 µl of sterile Milli Q water and the concentration determined spectrophotometrically.

3.2.2.5 Automated nucleotide sequencing

Sequences of the clones were determined with an automated ABI Prism 3100 DNA sequencer (PE Applied Biosystems, USA). As previously mentioned in section 2.2.6.7 the cDNA fragments were cloned into the pGEM-T Easy vector system. As such the SP6 primer: (5' ATTTAGGTGACACTATAGAATAC 3'), complementary to the vector's SP6 promoter site was used for sequencing. The adaptor sequences that were ligated to the cDNA fragments during the SSH procedure had internal T7 promoter sites and the T7 primer could not be used for sequencing. An alternate primer, the SSH seq primer: (5' CGACGGCCAGTGAATTG 3'), was designed so that two-directional sequencing could be performed.

The sequencing reaction consisted of 200-700 ng of plasmid DNA, 3.2 or 5 pmol of the respective primer and 2 µl terminator ready reaction mix from the Big Dye sequencing kit (version 2.0, PE Applied Biosystems, USA) in a final volume of 20 µl. Cycle-sequencing was performed in a Perkin Elmer Gene Amp PCR system 9700 with 25 cycles of 96^{o}C for 10 sec, 50^{o}C for 5 sec and 60^{o}C for 4 min. The products were purified by the addition of 1/10 of the volume of 3 M sodium acetate (pH 5.2) and three volumes of cold absolute ethanol. The products were collected by centrifuging at 16000xg for 10 min at 4^{o}C. The supernatant was removed and 300 µl of 70% ethanol added and again centrifuged as before, this wash was repeated twice. The pellet was dried *in vacuo* and the sequencing procedure followed as per user's manual. Results were confirmed by visual inspection of the electropherograms obtained and the transcripts were analysed using the BioEdit program.

The Basic Local Alignment Search Tool, BLAST, was used to find regions of local similarity between sequences by comparing the obtained nucleotide or translated protein sequences to sequence databases and calculating the statistical significance of the matches (Altschul *et al.,* 1990). All sequences obtained were submitted to Genbank and the *Plasmodium* database, PlasmoDB, to be analysed using the non-redundant database with a low complexity filter and the BLOSSUM62 matrix for the BlastX function (for protein identification).

3.2.3 Confirmation of differential expression

Differential expression was confirmed by a reverse virtual Northern blot in which potential differentially expressed transcripts were spotted in an array fashion onto a nylon membrane and hybridised to unsubtracted probes from the forward- and reverse subtraction, respectively.

Clones containing unique transcripts were selected, inoculated, amplified and analysed by gel electrophoresis as in sections 2.2.6.8 and 2.2.6.6. The products were denatured and each was spotted onto two different membranes as in section 3.2.1.3. Serial dilutions of a DIG-11-dUTP labelled DNA control (Roche, Germany), ranging from 1 ng/µl to 0.1 ng/µl, were also spotted onto the membranes as positive controls.

The forward- and reverse-unsubtracted probes prepared in section 3.2.1.1 were subjected to restriction enzyme digestion to remove their adaptor sequences as these

sequences were also present on the target sscDNA transcripts. Although the transcripts to which the adaptors were attached were not similar to the adaptors, specific hybrids between the G+C rich probe and target adaptors were capable of forming (results not shown). Even under stringent hybridisation conditions these hybrids are difficult to melt and could give rise to false positives. Thus adaptor removal was achieved by incubating the labelled probes (44 µl) with 10U of *Rsa*I and 1x *Rsa*I buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 1 mM dithioerythritol (DTE) Roche, Germany). The products were purified using a Sephadex G50 clean-up column as described in section 3.2.1.1.

Prehybridisation was performed as in section 3.2.1.3, with the addition of 50 µl of blocking solution (10 mg/ml sheared salmon sperm DNA and 0.3 mg/ml oligonucleotides corresponding to the nested primers and their complementary sequences, Clontech, USA) to the prehybridisation solution. Although the adaptor sequences were removed from the probes by digestion, they were still present on the target and had to be blocked to prevent any non specific hybridisation in case of incomplete adaptor removal from the probes (see Figure 3.3). Probes with or without adaptors but similar in sequence to the target DNA anneal and react with the DIG chemiluminescent substrate to produce a signal. If the probes are dissimilar/non homologous to the target then annealing does not take place as even the non specific adaptor sites are blocked. This prevents a signal from being produced, thus eliminating a large number of false positives.

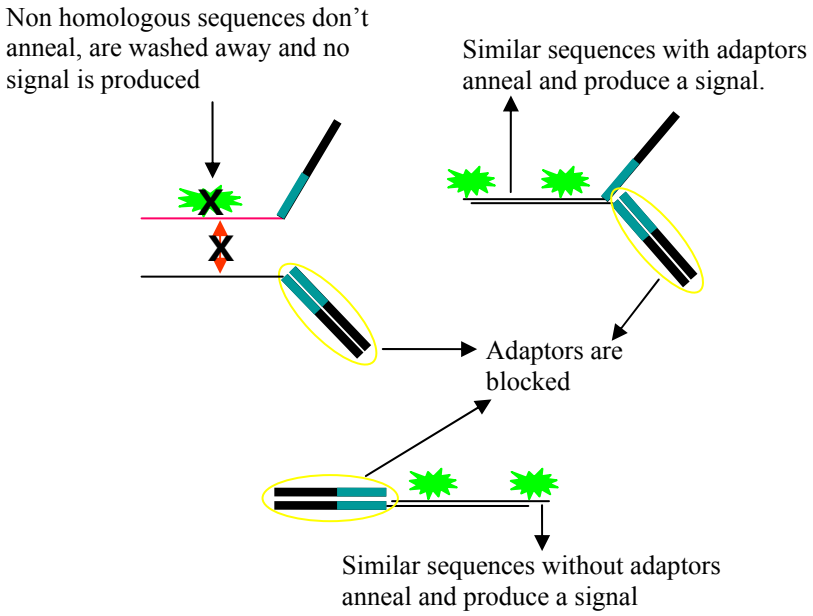


Figure 3.3: Schematic representation of hybridisation between target sscDNA and probes where adaptor sites are blocked. (represents chemiluminescent signal, (represents adaptor sequences with nested primer sites.)

Prehybridisation was performed for 2 hours at 72°C. The prehybridisation buffer was discarded and freshly prepared hybridisation buffer consisting of denatured probe (to a final probe concentration of 20 ng/ml); 50 µl of 20x SSC and 50 µl of blocking solution was prewarmed and added to the roller tube. Hybridisation and subsequent low stringency washes were performed as in section 3.2.1.3, however a more stringent high stringency wash solution (0.2x SSC, 0.5% SDS) was used.

Equilibration and detection using CDP-Star was performed as in section 3.2.1.3 with slight modifications: the membrane was incubated in CDP-Star for 2 min and exposed for 2 min to X-ray film, 2 hours post CDP-Star incubation. The film was analysed for comparison of spot intensities using the Versadoc 2000 system (Bio Rad, RSA), to confirm differential expression of the sequenced cDNA transcripts. The Versadoc 2000 system differed from the ImageMaster 2D program as it allowed a similar area for each spot to be scanned rather than the whole spot itself thus providing a more accurate indication of spot intensity. Transcripts were considered to be upregulated if the FUS probe intensity was at least 1.5-fold that of the RUS probe. For downregulated transcripts the RUS probe intensity was at least 3-fold that of the FUS probe.

3.3 Results

3.3.1 Probe synthesis and mock Dot-blot

Figure 3.4B indicates that the synthesis of DIG-labelled probes was successful. The labelled probes could not be analysed by gel electrophoresis hence a control reaction in which DIG-11-dUTP was excluded from the labelling reaction was performed in tandem. The control and labelled probes were also purified in tandem (3.2.1.1), but only the probes synthesised in the control reaction were analysed via agarose gel electrophoresis. Each probe from the control reaction was either purified or not and loaded onto an agarose gel in order to gauge the effect of the purification procedure on the resulting probes. The agarose gel in Figure 3.4A shows no differences between the respective purified (P) and unpurified probes (U) indicating that the purification procedure did not affect the probe concentrations. The purified probes having the following concentrations: forward subtracted (FS) 42 ng/µl; forward unsubtracted (FUS) 29.3 ng/µl; reverse subtracted (RS) 29 ng/µl and reverse unsubtracted (RUS) 57 ng/µl, were used during subsequent hybridisations (final probe concentration was 20 ng/ml).

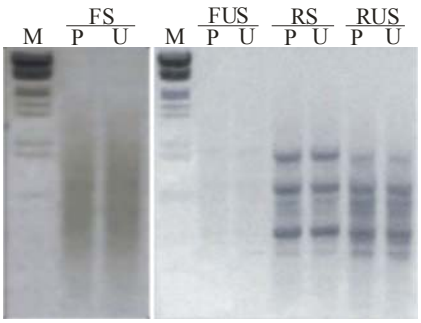


Figure 3.4A: Agarose gel of the respective control purified (P) and unpurified (U) probes, with *Eco*RI + *Hin*dIII digested phage λ DNA used as molecular marker (M). Probes: Forward subtracted (FS), forward unsubtracted (FUS), reverse subtracted (RS) and reverse unsubtracted (RUS).

The mock blot (Figure 3.4 B) indicated that probe sensitivity was good as spots were visible at a concentration of ~ 0.1 pg/µl for the FS, FUS and RUS probes and ~ 1 pg/µl for the RS probe. As a result the target transcripts needed to only be present at a minimum concentration of 1 pg/µl on the blot in order for a signal to be produced. Once the optimal probe concentrations were achieved, subsequent hybridisations were performed.

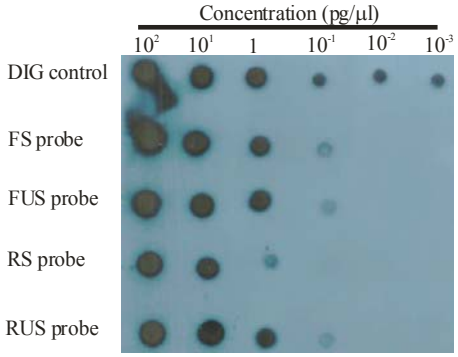


Figure 3.4B: Mock blot of DIG-11-dUTP labelled control DNA (DIG control) and probes in serial dilutions. Probes: Forward subtracted (FS), forward unsubtracted (FUS), reverse subtracted (RS) and reverse unsubtracted (RUS).

3.3.2 Suppression subtractive hybridisation and clone selection

3.3.2.1 Forward suppression subtractive hybridisation (FSSH: up-regulated transcripts)

The resulting X-rays of the FSSH cDNA dot blots (of which Figure 3.5 is an example) were analysed using the Image Master 2D program that assigned arbitrary densitometric values to each spot. This enabled the program to perform direct comparisons between the intensities generated for a particular spot with each of the probes. Results were tabulated and examples are illustrated in Figure 3.6. Based on this densitometry

analysis, potential differentially expressed transcripts were selected. As mentioned in section 3.2.2.1 certain criteria were taken into account during selection, mainly that the intensity of the FS probe was at least 1.5 fold that of the RS probe (see section 3.2.2.1). However, the fold change of certain transcripts from the FSSH library (in particular Plate 2, Figure 3.5) was less than the 1.5 fold cut off (Table 3.1A). Examination of this set of X-rays revealed that the background for the FS probe X-ray differed from that of the RS probe X-ray resulting in a lower signal value of spots on the FS probe X-ray. Thus, additional transcripts from this set of X-rays were selected by eye inspection (Table 3.1A). These transcripts were later confirmed to be differentially expressed (section 3.3.4 and Table 3.2).

Subsequently 37 clones were selected from the forward subtracted library corresponding to potentially upregulated transcripts.

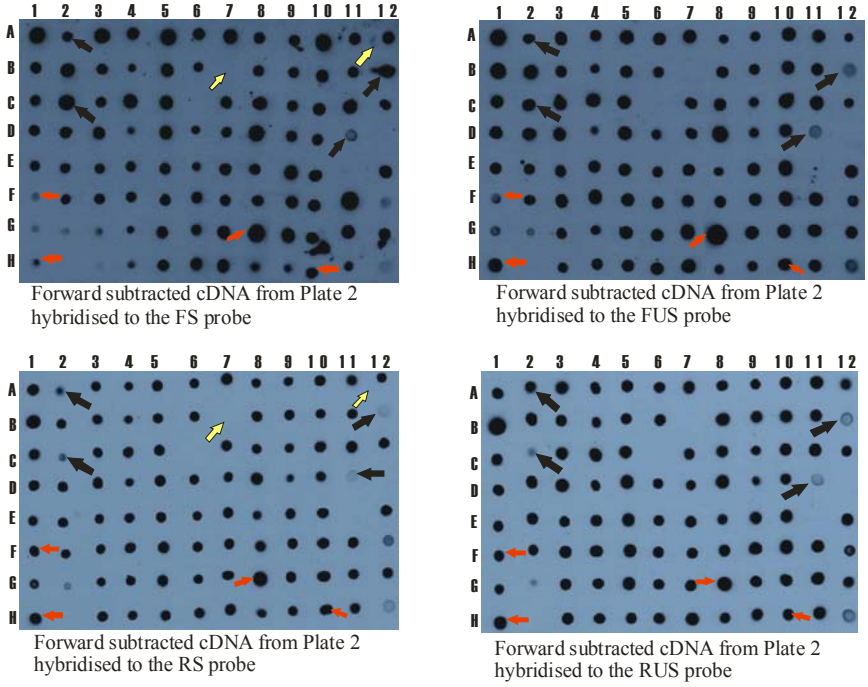


Figure 3.5: Results obtained after FSSH of a set of 96 clones from the forward subtracted library. (⬅) indicate examples of potentially upregulated cDNA transcripts and (⬅) indicate examples of non-differentially expressed cDNA transcripts. (⇦) indicates background differences between FS and RS X-rays.

Figure 3.6 illustrates that for clones A2, B12 and C2 the FS (blue) probe intensity was greater than 1.5-fold that of the RS (yellow), indicating that these transcripts were probably upregulated during inhibition of polyamine biosynthesis. The signal obtained for A2 appears to be greater with the FUS (burgundy) and RUS (light green) probes than the FS probe. This could be attributed to the transcript being present in both the

inhibited and wild type cultures prior to normalisation and equalisation. Clone C2 represents an ideal situation in which the signals with the FS and FUS probes are much greater than those obtained with the RS and RUS probes indicating a very strong candidate for differential expression. The opposite is true of clone F1, H1 and G8 where the RS probe produced the greater signal, suggesting that these are non-differentially expressed transcripts. Of the 384 spots present in the FSSH library, approximately 10% was selected as potentially differentially expressed transcripts

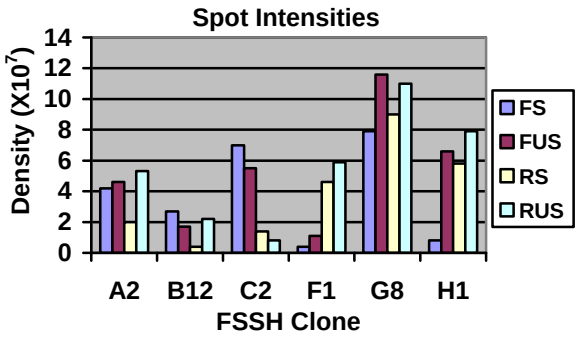


Figure 3.6: Differences in FSSH spot intensities in arbitrary density units, per clone for each of the four probes used. Three examples of upregulated transcripts (A2, B12 and C2) and three of non-differentially expressed transcripts (F1, G8, and H1) are represented.

3.3.2.2 Reverse suppression subtractive hybridisation (RSSH: down-regulated transcripts)

The X-rays of the RSSH cDNA dot blots (of which Figure 3.7 is an example) were analysed as described for the FSSH. Densitometric analysis of the intensities generated for a particular spot with each of the probes was performed and the results were tabulated as in Figure 3.8. This enabled potential differentially expressed fragments to be selected. It is important to note that the signal intensities for each spot were represented as arbitrary values only and that the spots from the FSSH were compared with each other and not with those from the RSSH.

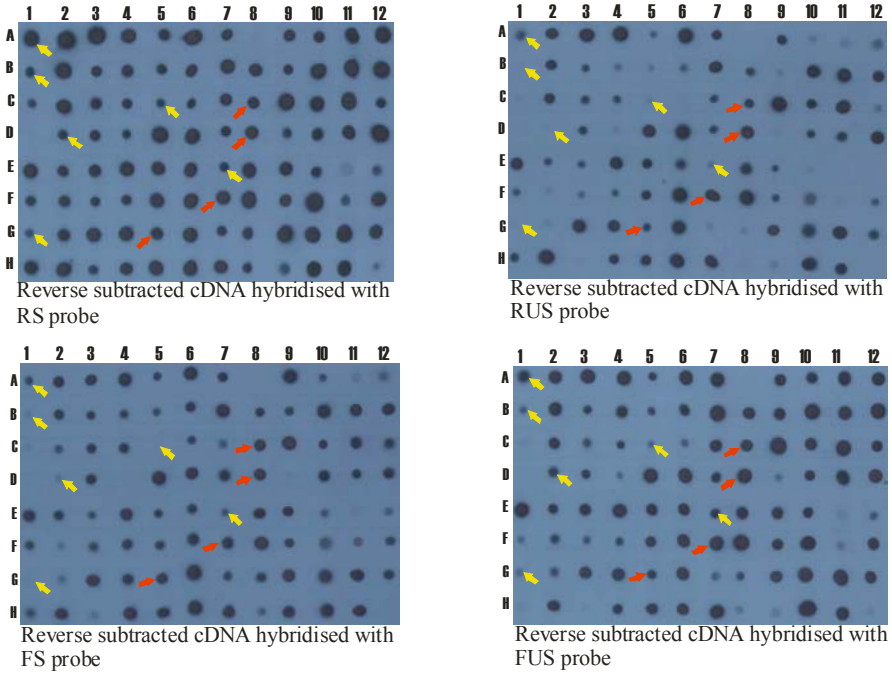


Figure 3.7: Results obtained after RSSH of a set of 96 clones from the reverse subtracted library. (←) indicate examples of potentially down regulated transcripts and (→) indicate examples of non-differentially expressed transcripts.

The results obtained with the RS probe represent those gene transcripts present in the wild type culture which are either switched off or down-regulated in the inhibited culture. During selection of potential differentially down-regulated transcripts (from the RSSH), more stringent criteria was used namely, the signal intensity of the RS probe had to be at least 3-fold that of the FS probe (blue, Figure 3.8) for a particular spot to be considered for differential expression (see section 3.2.2.1). The qualitative nature of the SSH technique required a more stringent intensity difference to increase selectivity of clones in the RSSH that were potentially down-regulated. Clones A1, C5 and G1 are examples of strong candidates for down-regulated transcripts as the signal intensity of the RS probe (yellow) is far greater than any of the other three probe signals. Clones C8, F7 and G5 are examples of non-differentially expressed transcripts as the signal intensities of the FS and RS probes differ by a smaller margin from each other, indicating that these transcripts are expressed at approximately the same levels in the wild type and inhibited cultures. Of the 384 spots present in the RSSH library, approximately 33% was selected as potentially differentially expressed transcripts.

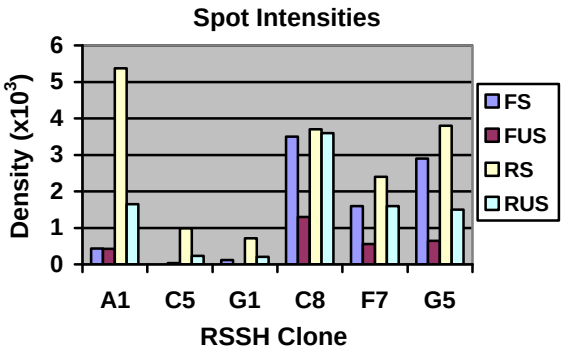


Figure 3.8: Differences in RSSH spot intensities in arbitrary density units, per clone for each of the four probes used. Three examples of down-regulated transcripts (A1, C5 and G1) and three of non-differentially expressed transcripts (C8, F7, and G5) are represented.

3.3.3 Selection and identification of transcripts

A restriction digest was performed in order to screen for possible repetitive transcripts, without having to sequence all 164 (both FSSH (37) and RSSH library (127)) transcripts, as illustrated in Figure 3.9. For example, group 1(yellow outline) consists of lanes A12, D2 and E5; group 2 (blue outline) of lanes C5, D4 and G1, group 3 (green outline) of lane A10 and group 4 (red outline) of lane B1 with each group displaying a certain restriction enzyme profile. Analysis of each clone's profile enabled the selection of a total of 59 unique transcripts for subsequent sequencing. The restriction digest indicated that approximately 36% of the transcripts that were selected from both the forward and reverse subtracted libraries were unique.

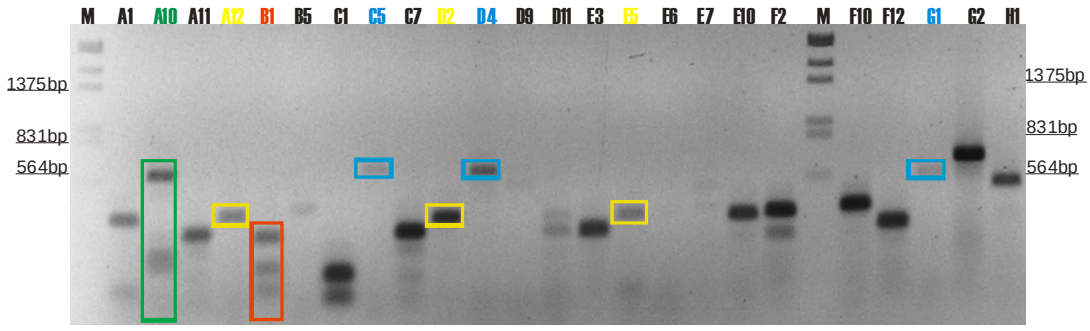


Figure 3.9: Restriction enzyme digest of clones (A1 – H1) from the first set of 96 of the reverse subtracted library, with *Eco*RI + *Hin*d III digested phage λ DNA used as molecular marker (M).

These 59 transcripts were sequenced and the genomic sequences (PlasmoDB) to which they were a good match are represented in Table 3.1. BLASTN and BLASTX searches in PlasmoDB were performed to ensure that the best matches were selected at

nucleotide and protein level. A small percentage of transcripts were found to be redundant but transcripts initially thought to be redundant were found to be a good match to different sequences (R1C5 and R1G1). Of the redundant sequences only those with the E-value closest to zero were considered further. Transcripts shorter than 190bp were excluded as well. The results yielded 21 unique transcripts from the forward SSH library (with good matches to 20 unique genes) and 24 from the reverse SSH library that (with good matches to 19 unique genes).

Table 3.1: Identification of differentially expressed transcripts (A) 20 individual transcripts that were up-regulated in the absence of polyamines and (B) 19 transcripts that was down-regulated in the absence of polyamines. Fold change by SSH was calculated by dividing spot intensities of forward subtracted libraries by reverse subtracted libraries for the up-regulated transcripts and *vice versa* for the down-regulated transcripts.

A)

Clone	Fold change (FS/RS)	CDS Name	PlasmoDB annotation	E-value	GO function
*2C2	1	PFF0435w	Ornithine amino transferase	0	Ornithine-oxo-acid aminotransferase
*2E12	1	PF07_0029	Heat shock protein 86	0	Heat shock protein activity / chaperone
*2G11	1	MAL7P1.228	Heat shock 70kDa protein	e-112	Heat shock protein activity / chaperone
*4D11	3	MAL7P1.228	Heat shock 70kDa protein	e-143	Heat shock protein activity / chaperone
2C11	5	PF08_0054	Heat shock 70kDa protein	0	Heat shock protein activity / chaperone
1B6	3	PF11_0351	Heat shock protein hsp70 homologue	0	Heat shock protein activity / chaperone
3C7	2	PF11-0111	Asparagine-rich antigen	e-162	Molecular function unknown
*2F11	1	PF13_0011	*P. falciparum* gamete antigen 27/25	0	Molecular function unknown
4F1	2	PF13_0065	Vacuolar ATP synthase catalytic subunit a	0	H^+ Transporting two-sector ATPase activity
*2H12	1	PF13_0218	ABC transporter, putative	4e-80	ATP- binding cassette (ABC) transporter activity
3A2	2	PF13_0305	Signal recognition particle receptor alpha subunit, putative	4e-60	Signal recognition particle receptor, GTP binding
4E11	3	PF14_0425	Fructose-bisphosphate aldolase	0	Fructose-bisphosphate aldolase activity
*2A2	1	PFL1125w	Phospholipid-transporting ATPase, putative	2e-88	Phospholipid translocating ATPase
2A12	2	PF11_0174	Dipeptidyl aminopeptidase 1	0	Catalytic activity
1E8	2	MAL13P1.295	Hypothetical *P. falciparum* protein	1e-88	Molecular function unknown
1F6	2	MAL6P1.139	Hypothetical *P. falciparum* protein	e-133	Molecular function unknown
1G11	2	PF10_0204	Hypothetical *P. falciparum* protein	2e-93	Molecular function unknown
2B12	7	PFA0255c	Hypothetical *P. falciparum* protein	e-178	Molecular function unknown
3C1	2	MAL13P1.249	Hypothetical *P. falciparum* protein	0	Molecular function unknown
4F10	1	MAL8P1.141	Hypothetical *P. falciparum* protein	0	Molecular function unknown
4G11	15	PF11_0347	Hypothetical *P. falciparum* protein	4e-74	Molecular function unknown

* Indicates additional clones selected from Plate 2 with eye inspection that were given a numerical fold change of 1.

B)

Clone	Fold change (RS/FS)	CDS Name	PlasmoDB annotation	E-value	GO function
4H6	7	MAL13P1.60	Erythrocyte binding antigen 140	0	Molecular function unknown
2H5	9	PF10_0203	ADP ribosylation factor	0	Purine nucleotide binding
1H8, 4E6	7, 10	PF14_0102	Rhoptry-associated protein 1	1e-52, e-161	Molecular function unknown
3A2, 2A2	23, 29	PFB0340c	Cysteine protease, putative	0, 2e-69	Cysteine-type peptidase
1B5	5	PFB0935w	Cytoadherence-linked asexual protein 2	e-154	Cell adhesion molecule
3E6	16	PFD0020c	Erythrocyte membrane protein 1	1e-33	Cell surface antigen, host-interacting/ defence, immunity protein
3D5	8	PFD0255w	*P. falciparum* blood stage membrane protein ag-1	e-179	Molecular function unknown
1B1, 2C10	27, 17	PFI0265c	Rhoptry protein, putative	e-149, 0	Molecular function unknown
1E10	8	PFI1475w	Merozoite surface protein 1 precursor	2e-19	Molecular function unknown
1A1, 2D11	12, -	MAL8P1.310	Putative senescence-associated protein	1e-92, 0.01	Molecular function unknown
2B11	32	MAL13P1.435	Conserved hypothetical *P. falciparum* protein	2e-19	Molecular function unknown
1E7	9	PFI1445w	Hypothetical *P. falciparum* protein	0	Molecular function unknown
1D2	18	PF11_0278	Hypothetical *P. falciparum* protein	e-113	Molecular function unknown
4F9	6	PFD1145c	Hypothetical *P. falciparum* protein	3e-59	Molecular function unknown
2C12, 3C5	-, 5	PFB0475c	Hypothetical *P. falciparum* protein	2e-92, e-125	Molecular function unknown
1H3	18	PF11_0513	Hypothetical *P. falciparum* protein	e-104	Molecular function unknown
3C6	10	PF14_0260	Hypothetical *P. falciparum* protein	3e-56	Molecular function unknown
2B2	7	MAL13P1.308	Hypothetical *P. falciparum* protein	0	Molecular function unknown
2D9	22	PF10_0351	Hypothetical *P. falciparum* protein	e-155	Molecular function unknown

Note: Clones with fold changes that are indicted with a dash (-) represent transcripts for which no signal was obtained with the FS probe and could correspond to genes that are switched off due to polyamine depletion.

Gene ontology (GO) is used to describe biological functionalities in all organisms by searching for gene products that share biologically meaningful attributes either from different databases or within the same database (Bada *et al.*, 2004). The results in Tables 3.1A and 3.1B indicate a high number of hypothetical proteins in both the FSSH and RSSH libraries.

The upregulation of ornithine aminotransferase is a positive indication that the changes in gene expression are drug specific. Heat shock proteins also occur with a high frequency in the FSSH. The absence of polyamines appeared to result in a metabolic response as 5% of the transcripts from the FSSH were found to be involved in polyamine metabolism and 10% were identified as possibly linked to glycolysis. These transcripts were upregulated as depicted in Figure 3.10A. There is also an increase in transcripts for transporter proteins, heat shock proteins and sexual antigens which could serve as an indication of the stress response. In Figure 3.10B, the functions of the polyamines are highlighted since the absence of polyamines enabled these novel polyamine-dependent transcripts to be identified.

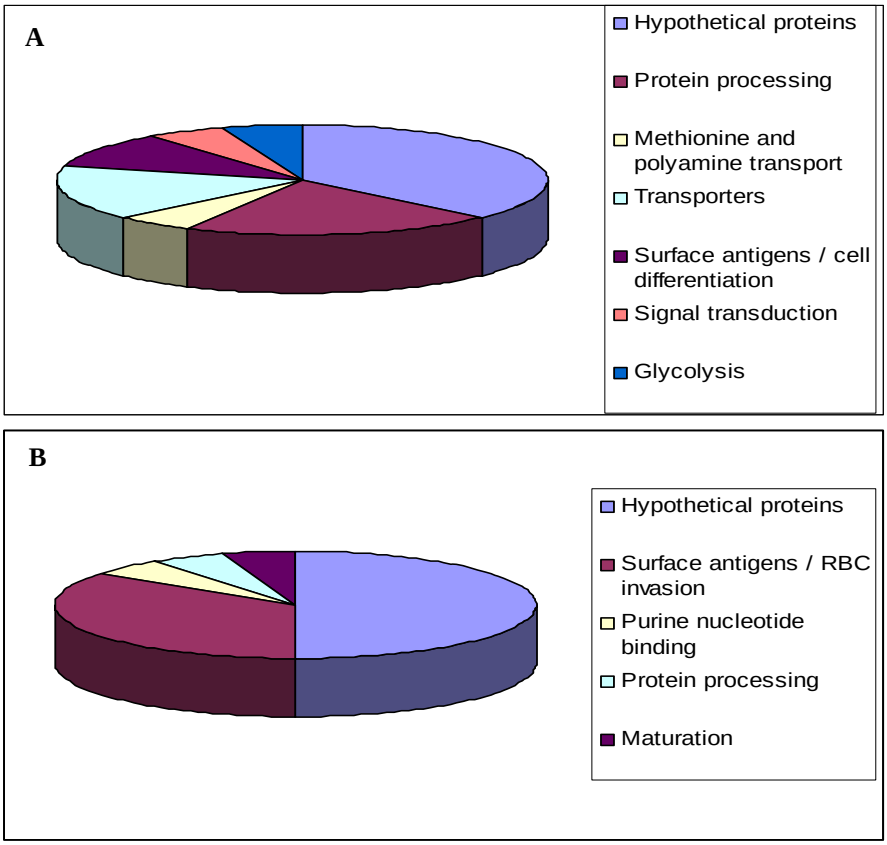
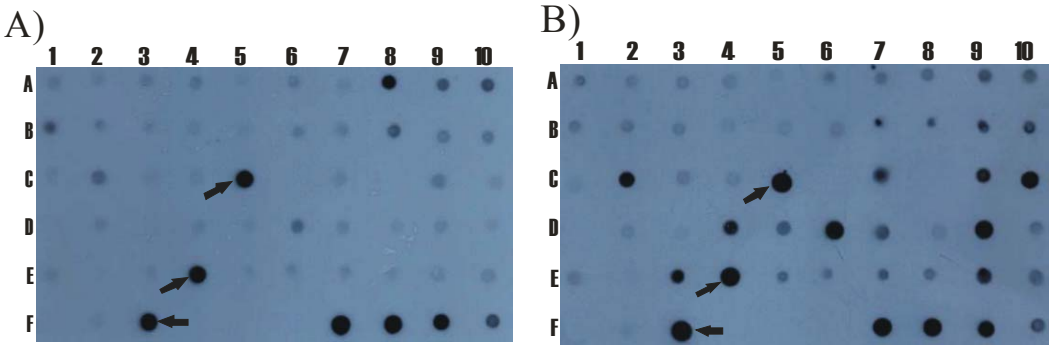


Figure 3.10: Functional annotation and clustering of the differential transcripts. (A) Transcripts upregulated in the absence of polyamines and (B) Transcripts downregulated in the absence of polyamines. Transcripts were grouped according to their GO biological function annotation as well as malaria metabolic pathway classification.

3.3.4 Confirmation of differential expression

Once sequencing was performed and individual transcripts with unique sequences identified, confirmation of differential expression was performed by reverse virtual Northern blotting to test the presence of each up/down-regulated clone (39 in total) in the comparable starting cDNA sample (wild type or inhibited cDNA).

In Figure 3.11 (A) those clones selected from the FSSH and RSSH, for confirmation of differential expression, hybridised to the cDNA from the inhibited culture with weak signals. These results indicated that although they are present they are probably down-regulated. The signals obtained with the cDNA from the wild type are much stronger and give a good indication that these transcripts are indeed down-regulated in the inhibited culture. Three distinct false positives were observed after confirmation, R1C5, R3A6 and R4H12. These transcripts are present at approximately equal expression levels (Figure 3.11, black arrows) in both the wild type and inhibited cultures and correspond to non-differentially expressed genes.



Confirmation of differential expression										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A	F1B6	F1E8	F1F6	F1G11	F2A2	F2A12	F2B12	F2C2	F2C11	F2E12
B	F2F11	F2G11	F2H12	F3A2	F3C1	F3C7	F4D11	F4E11	F4F1	F4F10
C	F4G11	R1A1	R1B1	R1B5			R1D2		R1E7	R1E10
D		R1H3	R1H8	R2A2	R2B2	R2B11	R2C10	R2C12	R2D9	R2D11
E	R2H5	R2H10	R3A2		R3C5	R3C6	R3D5	R3E6	R4E6	R4F9
F		R4H6					10X	100X	1000X	10000X

Figure 3.11: Confirmation of differential expression. A) Represents blots in which inhibited cDNA was used as probe and B) represents blots in which wild type cDNA was used as probe. Black arrows indicate false positives. The four spots in the bottom right corner of both X-rays are positive controls for hybridisation. The table above indicates the arrangement of the spots selected from the forward and reverse SSH libraries for confirmation of differential expression. Black blocks indicate position of false positives.

Spot intensities of transcripts that were confirmed to be differentially expressed were compared to determine the change in fold intensity and are represented in Table 3.2.

Table 3.2: Verification of SSH-identified differential transcripts

Gene	[A] Confirmation Fold Change	Direction of Change
Ornithine amino transferase	5.00	Up
Heat shock protein 86	1.2	Up
Heat shock 70kDa protein	1.2	Up
Heat shock 70kDa protein	1	Up
Heat shock protein hsp70 homologue	1	Up
Asparagine-rich antigen	1.1	Up
P. falciparum gamete antigen 27/25	2.2	Up
Vacuolar ATP synthase catalytic subunit a	0.3	Up
ABC transporter, putative	1	Up
Signal recognition particle receptor alpha subunit, putative	1	Up
Fructose-bisphosphate aldolase	1.5	Up
Phospholipid-transporting ATPase, putative	1	Up
Dipeptidyl aminopeptidase 1	1	Up
Hypothetical *P. falciparum* protein	1	Up
Hypothetical *P. falciparum* protein	1.4	Up
Hypothetical *P. falciparum* protein	1.9	Up
Hypothetical *P. falciparum* protein	0.4	Up
Hypothetical *P. falciparum* protein	1.2	Up
Hypothetical *P. falciparum* protein	1	Up
Hypothetical *P. falciparum* protein	1	Up
Erythrocyte binding antigen 140	1	Down
ADP ribosylation factor	1.5	Down
Rhoptry-associated protein 1	13.9	Down
Cysteine protease, putative	24.1	Down
Cytoadherence-linked asexual protein 2	1	Down
Erythrocyte membrane protein 1	2.8	Down
P. falciparum blood stage membrane protein ag-1	2.8	Down
Rhoptry protein, putative	4.4	Down
Merozoite surface protein 1 precursor	17.3	Down
Putative senescence-associated protein	3.8	Down
Conserved hypothetical *P. falciparum* protein	5.9	Down
Hypothetical *P. falciparum* protein	3	Down
Hypothetical *P. falciparum* protein	6.4	Down
Hypothetical *P. falciparum* protein	3.7	Down
Hypothetical *P. falciparum* protein	3.7	Down
Hypothetical *P. falciparum* protein	0.4	Down
Hypothetical *P. falciparum* protein	1.7	Down
Hypothetical *P. falciparum* protein	6.3	Down
Hypothetical *P. falciparum* protein	21.6	Down

A. The confirmation fold change was calculated by dividing spot intensities of forward unsubtracted libraries by reverse unsubtracted libraries for the up-regulated transcripts and *vice versa* for the down-regulated transcripts.

3.4 Discussion

It is thought that a large part of the biological function of polyamines is in the regulation of gene expression by altering either DNA structure or its chemistry (Duranton *et al.*, 1998; Janne *et al.*, 2004). The less compact states of DNA allow proteins access to the DNA template for a multitude of biological tasks (e.g. gene regulation, transcription and replication) (Wang *et al.*, 2001). Polyamines can act as transcriptional repressors by stabilising condensed chromatin and could thus effect a change in gene regulation at the site of transcription factor binding (White 2001). Polyamines can affect proteins directly (by stabilising them or affecting their degradation) or indirectly by influencing protein binding to cellular components (Childs *et al.*, 2003).

Assaraf and co-workers, 1987a, suggested that inhibition by DFMO led to a subsequent inhibition in protein, DNA and RNA synthesis. It was suggested that either spermine or spermidine was somehow responsible for inhibited protein synthesis and this does indeed seem to be the case as suggested by Kaiser and co-workers, 2001a. They showed that spermidine is an important substrate in the biosynthesis of hypusine which takes part in the activation of translation initiation factor eIF-5A. Inhibition of ODC prevents formation of spermidine which in turns inhibits hypusine formation and subsequent initiation of translation resulting in inhibited protein synthesis. eIF5A contains a unique spermidine-dependent hypusine modification on a specific lysine residue (Cohen 1998), and the hypusine modification is essential for cell growth as it plays a key role in the regulation of eIF5A function as eIF5A precursors without hypusine have little if any activity (Caraglia *et al.*, 2000; Childs *et al.*, 2003).

Bozdech and co-workers (2003a and b) suggested that 75% of the genes expressed during the intraerythrocytic life cycle of the parasite are activated only once, implying that the parasite utilises a “just in time” transcription process. They analysed the transcription of genes of known function at specific time points and found that it compared well with the respective protein’s biological function which illustrated a relationship between transcriptional regulation and developmental progression (Bozdech *et al.*, 2003a and b and Le Roch *et al.*, 2003). The “just in time” transcription process can also be utilised to elucidate the effect of antimalarials on the parasite.

3.4.1 Up-regulated transcripts

DFMO is an irreversible inhibitor of ODC and as such results in the accumulation of its substrate (ornithine) (see Figure 1.6 for polyamine metabolic pathway). In an effort to rid itself of this excess of substrate the parasite increases degradation levels thereof by increasing its complement of degradation enzymes involved in polyamine metabolism (ornithine aminotransferase, in Table 3.1A). OAT transaminates ornithine to glutamate and as such leads to a decrease in cellular content of ornithine (Gafan *et al.,* 2001).

There seems to be an overall induction of stress response genes (sexual antigens and heat shock proteins, Table 3.1A, Figure 3.10A). The asexual stages of the parasite rely on glucose and its metabolism for the production of energy. DFMO induces a cytostatic effect on the parasite, upon addition of putrescine parasite growth resumes (Assaraf *et al.,* 1987b) and could lower the demand for energy. Ohnishi and colleagues suggested a relationship between the inhibition of glycolysis and the induction of gametocytogenesis (Ohnishi *et al.,* 2001). The up-regulation of sexual antigens could indicate a move to the sexual stage of the parasite life cycle.

During this study, heat shock protein (Hsp) transcripts (Hsp 70 and Hsp 90) were found to be upregulated. *P.falciparum* Hsp90 has been shown to be essential for growth (Banumathy *et al.,* 2002) and both Hsp70 and Hsp90 expression was found to increase with high temperatures indicating its importance for parasite survival (Kumar *et al.,* 2003 and Matambo *et al.,* 2004). The results obtained in this study could broaden the possible roles for heat shock proteins in parasite development. The up-regulation of heat shock proteins could be due to a general stress response resulting from drug treatment. However, this marked up-regulation of heat shock protein transcripts was not observed during chloroquine treatment (3% of total transcripts compared to 19% obtained in this study) (Ganusekera *et al.,* 2003) a possible indication of a more important role for polyamines. The absence of polyamines could result in the destabilisation/damage of polyamine-dependent proteins and could result in the up-regulation of heat shock proteins to act as chaperones in the refolding of denatured proteins. Hsp70 and Hsp90 are inducible proteins and have been proposed to be involved in cytoprotection of the parasite by mediating the folding of newly synthesised proteins as well as the refolding of denatured proteins (Matambo *et al.,* 2004). *Hsp70* and *hsp86* share a common palindromic G-box element that acts as an upstream unique regulatory factor in Plasmodia to control the co-ordinated expression of the heat shock gene family

(Militello *et al.,* 2004). Polyamines could play a functional role in the regulation of transcription by influencing the G-box element.

Amino acids required for parasite protein synthesis are acquired from the degradation of erythrocytic haemoglobin (Rosenthal and Meshnick, 1998). Degradation occurs within the food or digestive vacuole, an acidic organelle within the parasite. The acidic pH seems to be essential for the various physiological processes of the vacuole and evidence exists for the presence of two H^+ pumping mechanisms on the vacuolar membrane, one of which is a V-type H^+- ATPase (Moriyama *et al.,* 2003; Saliba *et al.,* 2003). Table 3.1A indicates a vacuolar ATP synthase (catalytic subunit a) transcript that was upregulated in the absence of polyamines which could indicate an increased need for ATP dependent proton transport to acidify the food vacuole of parasites depleted of polyamines.

A putative ABC transporter (PF13_0218) was upregulated in the absence of polyamines. ABC transporters have been implicated in the movement of inorganic ions, sugars, amino acids, oligopeptides, proteins and complex polysaccharides as well as in multidrug resistance (Higgins 2001). PfMDR1 (multidrug transporter 1) has been localised to the food vacuole and implicated in peptide as well as chloroquine export from this organelle (Klokouzas *et al.,* 2003). BLAST analysis of the predicted amino acid sequence of this putative ABC transporter (PF13_0218) protein indicated a ~46% identity with multidrug resistance transporters and polar amino acid transport systems as well as a 48% identity with SalX, an ABC-type antimicrobial peptide transport system. This protein could be located at the food vacuole membrane and may be involved in amino acid/dipeptide transport to the cytoplasm.

Klemba *et al.,* (2004) characterised the last step in the hydrolysis of haemoglobin oligopeptides to dipeptides and illustrated that it was due to the activity of a lysosomal exopeptidase (cathepsin C) homologue: dipeptidyl aminopeptidase 1 (DPAP1). This enzyme is essential for asexual proliferation and was observed to be upregulated in the absence of polyamines.

3.4.2 Hypothetical proteins

The high number of hypothetical proteins identified in the up- and down-regulated libraries, 35% and 42% respectively, is not unusual as about 60% of the predicted open

reading frames in the *P. falciparum* genome have no functional annotation due to low homology with known proteins (Florens *et al.,* 2002; Young and Winzeler, 2005).The hypothetical proteins were analysed (Dr Birkholtz, personal communication) and 24% were found to contain signal peptides and as high as 76% contained one or more transmembrane segments indicating a relationship between the possible membrane associated proteins and the differential expression of their transcripts in the absence of polyamines. The transcript corresponding to clone 4G11 in Table 3.1A is upregulated 15 fold and PFam analysis of its predicted protein features indicated the presence of a conserved FHA domain (phosphopeptide binding domain found in many regulatory proteins) and an RNA recognition motif which could be involved in RNA stabilisation.

3.4.3 Down-regulated transcripts

Cell adhesion and erythrocyte invasion by the malaria parasites occurs at the merozoite stage. The merozoites contain specialised organelles at the apical end of the parasite and include rhoptries, micronemes and dense granules. Upon recognition and attachment to the host erythrocyte, the contents of these organelles are released and a tight junction is formed between the merozoite surface and the erythrocyte membrane (Holder 1994; Bannister *et al.,* 2000).

Transcripts of some of the proteins involved in cell-cell adhesion and invasion of the erythrocyte for example, rhoptry proteins, merozoite surface proteins, cytoadherence linked asexual proteins and erythrocyte binding proteins, as shown in Table 3.1B, were down-regulated in the absence of polyamines. Inhibition of polyamine biosynthesis does not interfere with merozoite invasion or ring development, but prevents the transformation of trophozoites into schizonts and thus the *in vitro* proliferation and development of the parasites (Assaraf *et al.,* 1984; Bitonti *et al.,* 1987).

PfEBA-140 (erythrocyte binding antigen 140) and an ag-1 blood stage membrane protein homologue (good match to apical membrane antigen 1 AMA1) are found in the microneme and were found to be down-regulated (Table 3.1B) (Sherman *et al.,* 2003). AMA1 and EBA140 are respectively involved in the initial contact at the moving junction and in the binding of host glycoproteins during the invasion process (Dowse and Soldati, 2004). Rhoptry-associated protein 1 (RAP1), rhoptry protein (RhopH3) and cytoadherence linked asexual protein 2 (clag 2) are present in the rhoptry and were also

found to be downregulated (Table 3.1B). RAP1 and RhopH3 were shown through knock-out studies to be essential for the invasion process (Cowman *et al.,* 2000).

PfEMP1 is a protein that is exported to the surface of the infected erythrocyte where it serves as an important virulence and adherence mediator during sequestration (Flick and Chen, 2004). The PfEMP1 transcript was also found to be down-regulated due to the absence of polyamines. Merozoite surface protein 1 (MSP1) is found on the surface of merozoites and thought to be involved in the initial interaction of the merozoite with the erythrocyte (Cowman *et al.,* 2000; Chen *et al.,* 2000) was observed to be downregulated.

Table 3.1B indicates that SERA 4 (serine rich antigen 4) was downregulated. This putative protein is a member of the cysteine proteases (including the falcipains) and is predicted to be involved in haemoglobin degradation and erythrocyte invasion (Rosenthal 2004). Proteolytic hydrolysis of proteins involved in invasion is essential to this process (Blackman 2004). It appears as though the SERA-4 transcript is co-regulated with other transcripts involved in invasion and therefore that its transcription is regulated by polyamines.

Polyamines are essential for cell growth and differentiation and inhibition of ODC blocks G_1 and G_2-M possibly via induction of p21 proteins in eukaryotes (Ackermann *et al.,* 2003; Wallace *et al.,* 2003). Assaraf and co-workers (1984; 1986; 1987b) illustrated that inhibition of polyamine biosynthesis prevents the parasite from differentiating further than the late trophozoite stage and thus prevents its maturation to schizonts and merozoites. Table 3.1B indicates the down-regulation of a putative senescence-associated protein that may play a role in cellular differentiation and parasite development providing a possible link between polyamine inhibition and the lack of further parasite development.

The ADP ribosylation transcript (Table 3.1B) was observed to be down-regulated. This transcript is one of several small GTPases that are required in the control and initiation of specific vesicular trafficking events (Lee *et al.,* 1997) and its down-regulation could affect multi-subunit assembly on membranes as well as bud formation.

3.4.4 Conclusion

In conclusion, it appears that polyamines are essential molecules for protein synthesis and stabilisation within the parasite. In the absence of polyamines up-regulation of transcripts for a V-type ATPase, DPAP1 (an ABC transporter) as well as heat shock proteins occurred. This could indicate increased food vacuole acidification, haemoglobin degradation and transport of amino acids to the cytosol to compensate for the decrease in protein synthesis and stabilisation brought about by the absence of polyamines.

The high number of transcripts down-regulated in the absence of polyamines indicates their importance to the parasite since they seem to be essential components in cell-cell adhesion as well as invasion and are essential for parasite survival.

These results are representative of the level of mRNA expression. They may not be an indication of the relevant corresponding protein levels. Transcriptomic and proteomic analysis undertaken by Bozdech *et al.,* (2003a and b), Nirmalan *et al.,* (2004a and 2004b) and Le Roch *et al.,* (2004) have illustrated differences regarding the pattern of protein accumulation relative to expression of their cognate mRNAs. These researchers suggested that this may be due to differences in the type, efficiency and timing of translation of different mRNAs. Nirmalan *et al.,* (2004a) illustrated that inhibiting *P. falciparum* using antifolate drugs caused no significant difference in abundance of dihydrofolate reductase thymidylate synthase (DHFR-TS) mRNA between the treated versus untreated parasites. They did confirm at the proteomic level however, that the activity of this enzyme did indeed increase upon drug exposure (Nirmalan *et al.,* 2004b).

Proteomic analysis of the differentially expressed transcripts will have to be undertaken to establish the relationship between their expression and the inhibition of polyamine biosynthesis.

CHAPTER 4

Concluding Discussion

The development and spread of resistance against current antimalarials is on the increase and as a result the risk of losing the battle against this disease is high. Lack of understanding the mechanisms of action of drugs and resistance traits of the parasite have proved deleterious in the control of malaria. Chloroquine is an example of initial antimalarial success until resistance developed and efforts to overcome it were unsuccessful as its mechanism of action was and is still not completely understood (Olliaro 2001; Hyde 2005). Further research did uncover a gene *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) that coded for a protein thought to aid the parasite in overcoming chloroquine treatment (Fidock *et al.,* 2000).

It has become ineffective to concentrate on uncovering a single gene or protein in the *P. falciparum* parasite as a potential antiparasitic target. A more reasonable approach would be to embark on a global search involving the parasite's entire genome. In this way the effects of drug pressure on multiple genes and proteins could lead to an improved understanding of various biosynthetic pathways within the parasite and ultimately parasite biology.

The elucidation of metabolic pathways within the malaria parasite can prove to be beneficial since it aids in expanding insight into resistance against antimalarials as well as in the search for novel or improved chemotherapeutics. Chapter 1 provided a brief introduction to polyamines, ubiquitous molecules that are necessary for parasite survival. Polyamines have been implicated in various processes in almost all organisms including the functioning of ion channels, nucleic acid packaging and stabilisation, DNA replication, apoptosis, transcription and translation, protein stabilisation and signal transduction (Ackermann *et al.,* 2003; Childs *et al.,* 2003; Cohen 1998; Janne *et al.,* 2004; Wallace *et al.,* 2003).

Polyamine biosynthesis is regulated by two key enzymes: Ornithine decarboxylase (ODC) and S-Adenosylmethionine decarboxylase (AdoMetDC). Inhibition of polyamine biosynthesis, using the ODC inhibitor DFMO, provided a new avenue for

consideration in the search for antimalarials- polyamine metabolism. Information regarding the exact role of the polyamines in parasitic protozoa is not as extensive as for other organisms. Molecular and biochemical characterisation of the polyamine biosynthetic enzymes confirmed that these enzymes and their by-products are indeed essential for parasite survival (Klenke *et al.,* 2003; Heby *et al.,* 2003; Kaiser *et al.,* 2001 and Muller *et al.,* 2001). Characterisation of the polyamine biosynthesis pathway has yielded results, by identifying and characterising the bifunctional ODC/AdoMetDC enzyme present in the parasite but not in the human host (Muller *et al.,* 2000; Muller *et al.,* 2001). Kaiser *et al.,* (2001) also illustrated the possible link between polyamine biosynthesis and protein synthesis in the parasite by illustrating the importance of spermidine in subsequent hypusine activation.

The suppression subtractive hybridisation (SSH) technique represents a large-scale method for detecting known and unknown transcriptionally regulated genes. It is unbiased and has the ability to bring to the fore even low-abundance transcripts by employing suppression and normalisation techniques. It can thus serve as a starting point for the cloning and analysis of existing and novel genes. This method also represents an "open system" of investigation as the entire transcriptome of the parasite is affected and examined unlike a conventional microarray which is representative of a "closed system" with a limited number of genes that can be investigated. It was difficult to isolate sufficient parasite RNA to pursue the microarray route of investigation thus SSH, which requires very little starting material (less than 1 µg was used in these experiments), was performed instead. This technique was employed as a method of transcriptome profiling to evaluate the effect of polyamine depletion on the intraerythrocytic cycle of *P. falciparum* and to further clarify the polyamine metabolic pathway within the malaria parasite.

The SSH technique is a purely qualitative method (as the results in Chapter 3 indicate) and requires alternative methods of validation, usually northern blotting and real-time PCR. In addition, the technique itself and subsequent sequencing of the subtracted genes/ transcripts are labour intensive. As SSH is a qualitative method a suitable internal parasite specific control (household gene unaffected by treatment) would additionally allow the effectiveness of the SSH (i.e. efficiency of normalisation and subtraction) technique to be gauged. This is especially important in view of the fact that *P. falciparum* has a high A+T content that has to be compensated for during subsequent

normalisation and subtractive hybridisation. Sequencing of the parasite's genome has been completed thus enabling a suitable control for future experiments to be selected with ease.

Once the SSH technique was completed clones representing subtracted transcripts were selected by densitometric analysis and comparison of X-ray film from each blot. X-ray film was used as it has a fast exposure time, however it has a poor dynamic range and must be scanned in for digital analysis. Two systems were used to accomplish this: the ImageMaster 2D program and the Versadoc 2000 system. These programs calculated the integrated density which is defined as the sum of pixel values minus a background value for each pixel within a given area. For a given area and increase in brightness or size of a spot resulted in a higher integrated density measurement. Although integrated density measurements are useful for comparing spot intensities on images where standards are not included it depends on variables such as pixel saturation, background and region of interest selected. This last point is of particular importance since increased size of a spot can result in a relative density measurement that does not correspond to its brightness. The ImageMaster 2D program allowed for background subtraction but the region of interest encompassing each spot varied and thus required eye inspection to confirm results. The Versadoc 2000 system however, not only subtracted background values but also allowed for a defined constant region of interest to be analysed for each spot providing more reliable data regarding the confirmation of differential expression.

An added disadvantage with SSH is that since it represents an "open system" the genes/ transcripts that are selected are of unknown sequence and as such redundant transcripts are indistinguishable from non-redundant ones. In an effort to exclude redundant transcripts a triple restriction digest was performed to select transcripts based on unique restriction profiles. A high number of background clones, i.e. those clones that were not differentially expressed were observed, particularly in the FSSH. This could also have been due to discrepancies in the ImageMaster 2D program analysis package regarding the region of interest. An alternative could be to rescan the X-ray films of the forward and reverse SSH libraries with the Versadoc 2000 system to compensate for the variations in spot sizes and thus regions of interest.

Rebrikov and co-workers, 2000, developed a method known as mirror orientated selection (MOS) (Figure 4.1) to decrease the number of background clones as well as

false-positives. It is based on the premise that each background transcript is ligated to the adaptors in a specific orientation (for example adaptor 1 is ligated to the 5'-end of all background transcripts). Consider the SSH procedure (see Figure 2.1A) in which type b molecules formed after the first hybridisation. It is possible that not all type b molecules were suppressed by forming pan handles and were able to anneal to their complementary sequences (also ligated to the same type of adaptor) resulting in the linear amplification of these background transcripts. The target transcripts are ligated to adaptors in both orientations (i.e. adaptor 1 can be ligated to either the 5'- or the 3'-end; the same applies to adaptor 2R). If a single adaptor is removed by restriction endonuclease (see Figure 2.1C for restriction enzyme recognition sites) and the sample is denatured and allowed to reanneal, some of the newly-formed hybrids will bear one of the adaptors (adaptor 1 or 2R) at either end. These hybrids can only be formed from complementary transcripts that are ligated to both adaptors i.e. the target sequences. The 3'-ends are filled and the molecule is amplified with a primer corresponding to the nested primer sequence on the remaining adaptor allowing only those molecules with this adaptor to be amplified exponentially. However both methods are labour intensive and expensive, making them unsuitable.

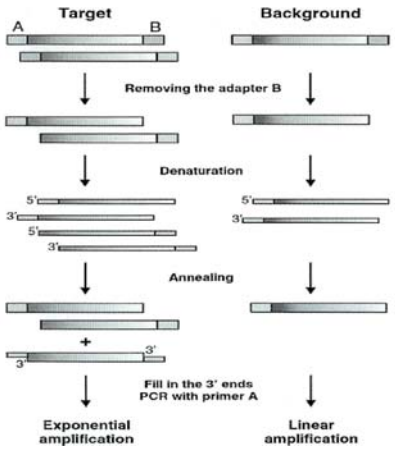


Figure 4.1 Representation of the MOS method. Broad rectangles represent dsDNA; narrow rectangles, ssDNA. The gradient of filling shows the orientation of the cDNA molecules (Rebrikov *et al.*, 2000**).**

Confirmation of differential expression posed a difficult problem as well. In order to perform a northern blot, at least 10µg of RNA was required. As can be seen from the RNA yield in Chapter 2 this was difficult to achieve. A reverse virtual northern blot was performed instead with the probes that were *Rsa*I digested to remove adaptor sequences. This was performed to reduce false positives as a result of non-specific binding by the

adaptors because of their high G+C content. The hybridisation solution was of a higher stringency as it contained sequences similar to the adaptors as well as salmon sperm DNA which improved the blocking efficiency. In future the conditions mentioned above should be introduced in order to prevent non-specific binding. These experiments were all performed in duplicate but it is important to note that the comparisons are made between spots from the same library (for example spot FA1 *versus* spot FA1 with all four probes from the FSSH only).

Further investigations (e.g. DNA microarrays) into various exposures to and doses of DFMO will allow for a larger number of drug-induced alterations to be investigated. This will aid not only in uncovering genes likely to be involved in immediately overcoming the effects of the drug but also those expected to mediate toxic consequences of exposure. These DFMO-induced alterations may also elucidate the roles of transcripts of unknown function, subsequently opening up new avenues for investigation. They may also aid in unravelling the function of known transcripts. For example, further investigation of the expression of heat shock proteins during the entire life cycle of *P. falciparum* (i.e. normal versus drug-treated parasites) could indicate that they function as more than just chaperones. Or, certain transcripts may be down regulated not as a result of polyamine depletion but rather because of the inhibition of the cell cycle. These effects can be investigated by analysing the differential expression of transcripts from all stages of the parasite's life cycle before and after addition of DFMO.

In general, the results obtained during the completion of these experiments were promising as they indicated not only that the SSH technique was successful but also that inhibiting polyamine biosynthesis did affect the parasite physiologically. However, mRNA transcript levels do not necessarily correspond to those of their subsequent proteins (Bozdech *et al.*, 2003a and b; Nirmalan *et al.*, 2004a). To confirm the effects observed at the transcriptome level, proteomic analysis under the same conditions must be performed. This will result not only in an improved understanding of polyamine function and metabolism within *P. falciparum* but may also lead to the discovery of putative/ novel antimalarial targets and thus aid in overcoming the severe effects of malaria.

SUMMARY

Polyamines play an important role in DNA, RNA and protein synthesis as well as a variety of other biological processes (cell division, differentiation and death) as outlined in Chapter 1. Assaraf and co-workers (1984) demonstrated that treatment with DFMO resulted in the inhibition of polyamine biosynthesis as well as schizogony arrest in *P. falciparum*. However, they did not elaborate on any other consequences that polyamine depletion could exert on the parasite. This dissertation aims to elucidate the significance of the inhibition of polyamine biosynthesis within *P. falciparum* by using differential transcriptome profiling.

Suppression subtractive hybridisation generated transcripts which were potentially up- and down-regulated due to endogenous polyamine depletion within the human malaria parasite *P. falciparum*. The resulting transcripts were subjected to a restriction enzyme analysis and those with unique digestion profiles were selected and sequenced. The sequences were analysed using PlasmoDB to identify the genomic sequences to which they were best matched. To confirm that the selected transcripts were indeed differentially expressed a reverse virtual Northern dot blot was performed.

Transcripts for proteins involved in protein processing, methionine and polyamine metabolism, various transporters, proteins involved in cellular differentiation and signal transduction were found to be upregulated in the absences of polyamines. This could be suggestive of a metabolic response induced by the parasite in order to overcome this deficiency. Polyamines seem to influence protein synthesis and haemoglobin degradation as well since depletion of endogenous polyamines within the parasite seems to result in increased food vacuole acidification, haemoglobin degradation, transport of proteins to the cytoplasm and protein synthesis and stabilisation.

The majority of downregulated transcripts were found to be involved in cell-cell adhesion and erythrocyte invasion, protein processing and transport indicating that these processes are dependent on polyamines. Further validation of these findings by microarray as well as proteomic analysis will need to be undertaken.

These results validate that polyamines do play an essential role in the cellular biology of the parasite. They also confirm that the inhibition of polyamine biosynthesis is a viable route to undertake in the search for new and improved antimalarial targets. This would be especially useful if it was combined with other antimalarials and their synergistic effects were investigated by transcriptomic, proteomic and bioinformatic analysis

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