

NUCLEAR GENES INVOLVED IN MAINTENANCE OF THE MALARIAL PLASTID

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ABSTRACT

Plasmodium species (the causative agents of malaria), and related parasites of the phylum Apicomplexa such as *Toxoplasma gondii*, have a plastid organelle of endosymbiotic origin. This organelle is regarded as a potential new drug target. It contains its own genome encoding many of the proteins and RNAs required for protein synthesis, but its function is unclear. Nucleus-encoded proteins are likely to control the primary function and biogenesis of the plastid and I have studied two of these: FtsZ (involved in bacterial and plastid division), and Ycf16 (a protein of unknown function that may interact with Ycf24, encoded on the plastid genome).

First I set up a screen to check that any genes I identified were malarial in origin, and not from the mycoplasma that contaminate our cultures. Searches for *ftsZ* by degenerate PCR, database analysis and low-stringency hybridisation, were unsuccessful. The possibility remains that the apicomplexan plastid divides by some other mechanism, as mitochondria and some bacteria do, for example using a dynamin-related protein. A dynamin-like sequence was identified on chromosome 14 in *P. falciparum*, and its transcription at the appropriate stage of the erythrocytic cycle was confirmed. However, this protein could be involved in mitochondrial division.

A *ycf16*-like sequence was found on chromosome 14 of *P. falciparum* and expressed in *Escherichia coli* for antibody production. This sequence encodes a putative plastid-targeting amino-terminal peptide; reporter protein and immunofluorescence studies will confirm whether it is localised in the plastid. To study the function of *ycf16*, I disrupted an orthologous version in the cyanobacterium *Synechocystis* and found it is an essential gene whose partial loss was deleterious. The $\Delta ycf16$ mutant cyanobacteria were arrested in cytokinesis and resembled those found previously in our laboratory for $\Delta ycf24$ mutants, but the effects were less severe. I confirmed that *ycf24* and *ycf16* are co-transcribed in *Synechocystis*, and perhaps form part of the same operon. These findings add weight to the hypothesis that Ycf16 and Ycf24 act in the same pathway, and that the imported Ycf16 and the endogenous plastid protein Ycf24 are both essential for plastid maintenance. Based on recent additions to the literature, I propose that Ycf16 is involved in maturation of Fe-S cluster-containing proteins required for resistance to oxidative stress and the provision of reducing power for biosynthetic pathways that occur in the plastid of apicomplexans.

CONTENTS

ABSTRACT	2
CONTENTS	3
LIST OF FIGURES	7
LIST OF TABLES	8
ACKNOWLEDGEMENTS	10
ABBREVIATIONS	11

CHAPTER 1 - INTRODUCTION **13**

1.1 MALARIA	13
1.1.1 THE DISEASE	13
1.1.2 COMBATING MALARIA	14
1.1.2.1 Targeting the Parasite	14
1.1.2.2 Targeting the Vector	15
1.1.2.3 Vaccines	17
1.1.3 THE PARASITE	20
1.1.3.1 Taxonomy	20
1.1.3.2 Life Cycle	21
1.1.3.3 Genome	23
1.1.3.3.1 Nuclear Genome	23
1.1.3.3.2 Mitochondrial Genome	26
1.1.3.3.3 Plastid Genome	27
1.2 THE PLASTID	29
1.2.1 DISCOVERY	29
1.2.2 ORIGIN OF THE PLASTID	30
1.2.2.1 Endosymbiosis	30
1.2.2.2 Transfer of Organelle Genes to the Nucleus	34
1.2.2.3 Ancestry of the Apicomplexan Plastid	35
1.2.3 FUNCTION	37
1.2.3.1 Protein Synthesis	37
1.2.3.2 Porphyrin Synthesis	39
1.2.3.3 Chorismate Synthesis	40
1.2.3.4 Fatty Acid Synthesis	40
1.2.3.5 Isoprenoid Synthesis	41
1.2.3.6 Electron Transport	42
1.2.3.7 Ycf24	43
1.2.4 THE PLASTID AS A DRUG TARGET	44
1.3 PROTEIN IMPORT INTO PLASTIDS	46
1.3.1 CHLOROPLAST IMPORT	46
1.3.2 IMPORT INTO COMPLEX PLASTIDS	48
1.3.3 PROTEIN TARGETING TO THE APICOMPLEXAN PLASTID	49
1.4 AIMS OF THIS PROJECT	54

CHAPTER 2 - MATERIALS AND METHODS **55**

2.1 MATERIALS	55
2.2 MEDIA, SOLUTIONS AND BUFFERS	55
2.3 BIOINFORMATICS	55
2.4 AGAROSE GEL ELECTROPHORESIS	55
2.5 DETERMINATION OF NUCLEIC ACID CONCENTRATIONS	56
2.5.1 DNA	56
2.5.2 RNA	56
2.6 CELL CULTURE	56
2.6.1 <i>P. FALCIPARUM</i>	56
2.6.2 <i>SYNECHOCYSTIS</i> PCC6803	58
2.6.3 <i>T. GONDII</i>	58
2.7 TRANSFORMATION OF <i>SYNECHOCYSTIS</i>	58

2.8 TRANSIENT TRANSFECTION OF <i>T. GONDII</i>	58
2.9 MICROSCOPY	59
2.9.1 FLUORESCENCE MICROSCOPY	59
2.9.1.1 Autofluorescence by <i>Synechocystis</i>	59
2.9.1.2 DAPI-Staining	59
2.9.1.3 Expression of Reporter Protein in <i>T. gondii</i>	59
2.9.2 ELECTRON MICROSCOPY	59
2.10 ISOLATION OF GENOMIC DNA	59
2.10.1 <i>P. FALCIPARUM</i>	59
2.10.2 <i>SYNECHOCYSTIS</i>	60
2.11 ISOLATION OF RNA	61
2.11.1 <i>P. FALCIPARUM</i>	61
2.11.2 <i>SYNECHOCYSTIS</i>	61
2.12 PRIMER DESIGN	62
2.12.1 DEGENERATE PRIMERS	62
2.12.2 NON-DEGENERATE PRIMERS	62
2.13 AMPLIFICATION OF DNA BY PCR	62
2.14 RT-PCR	63
2.14.1 DNASE TREATMENT	63
2.14.2 cDNA SYNTHESIS	63
2.14.3 AMPLIFICATION	63
2.15 SYNTHESIS OF RADIOACTIVE DNA PROBE	63
2.15.1 PREPARATION OF TEMPLATE	63
2.15.2 LABELLING REACTION	63
2.15.3 PURIFICATION OF PROBE	64
2.16 SOUTHERN HYBRIDISATION	64
2.16.1 SOUTHERN TRANSFER	64
2.16.2 PRE-HYBRIDISATION	64
2.16.3 HYBRIDISATION OF RADIOLABELLED PROBE TO MEMBRANE	64
2.16.4 DETECTION OF HYBRIDISED PROBE	64
2.16.5 PHOSPHORIMAGING	64
2.17 CLONING INTO PLASMID VECTORS	65
2.17.1 PURIFICATION OF INSERT DNA	65
2.17.2 LIGATION REACTION	65
2.17.3 TRANSFORMATION	66
2.18 ISOLATION OF PLASMID DNA	66
2.18.1 MINIPREP	66
2.18.2 MIDIPREP	66
2.18.3 MAXIPREP	66
2.19 DNA SEQUENCING	67
2.20 EXPRESSION OF FUSION PROTEIN IN <i>E. COLI</i>	67
2.21 PREPARATION OF TOTAL PROTEIN EXTRACT FROM <i>E. COLI</i>	68
2.22 LARGE-SCALE PREPARATION OF FUSION PROTEIN	68
2.22.1 GST FUSION PROTEIN	68
2.22.1.1 Expression of Fusion Protein	68
2.22.1.2 Cell Lysis and Solubilisation of Fusion Protein	68
2.22.1.3 Purification of Fusion Protein	69
2.22.2 HEXAHIS-TAGGED FUSION PROTEIN	69
2.22.2.1 Expression of Fusion Protein	69
2.22.2.2 Cell Lysis and Purification of Inclusion Bodies	69
2.22.2.3 Purification of Fusion Protein	70
2.23 SDS-PAGE	70
2.24 WESTERN BLOTTING	72
2.24.1 TRANSFER OF PROTEINS TO NITROCELLULOSE MEMBRANE	72
2.24.2 PROBING AND DEVELOPMENT OF MEMBRANE	72
2.25 DOT BLOTTING	72
2.26 ANTIBODY PRODUCTION	73
2.27 ATP-BINDING ASSAY	73
2.28 RNASE PROTECTION ASSAY	73
2.28.1 SYNTHESIS OF RADIOACTIVE ANTISENSE RNA PROBE	73
2.28.2 RNASE PROTECTION	74
2.28.3 END-LABELLING OF MARKER OLIGONUCLEOTIDES	74

CHAPTER 3 - THE MYCOPLASMA PROBLEM	75
3.1 INTRODUCTION	75
3.1.1 MYCOPLASMA AND MALARIA CULTURES	75
3.1.2 THE ORGANISMS	75
3.1.3 THE CONSEQUENCES OF CONTAMINATION	76
3.1.4 DETECTION OF MYCOPLASMA CONTAMINATION	76
3.1.5 METHODS OF ERADICATION	77
3.1.6 AIMS	78
3.2 RESULTS	78
3.2.1 OUR CULTURES ARE CONTAMINATED WITH <i>MYCOPLASMA ORALE</i>	78
3.2.2 MYCOPLASMA DNA HAS A DISTINCTIVE MIGRATION PATTERN ON PULSED-FIELD GELS	80
3.3 DISCUSSION	80
CHAPTER 4 - DIVISION OF THE PLASTID	83
4.1 INTRODUCTION	83
4.1.1 FTSZ AND BACTERIAL CELL DIVISION	83
4.1.2 FTSZ AND PLASTID DIVISION	85
4.1.3 MITOCHONDRIAL DIVISION	87
4.1.4 DIVISION OF THE APICOMPLEXAN PLASTID	89
4.1.5 AIMS	91
4.2 RESULTS	91
4.2.1 DEGENERATE PCR RESULTS IN AMPLIFICATION OF MYCOPLASMA <i>FTSZ</i>	91
4.2.2 <i>E. COLI</i> <i>FTSZ</i> DOES NOT HYBRIDISE TO <i>P. FALCIPARUM</i> DNA	96
4.2.3 <i>P. FALCIPARUM</i> POSSESSES A DYNAMIN-LIKE PROTEIN	96
4.2.4 <i>PfDRP</i> IS ENCODED ON CHROMOSOME 10	96
4.2.5 <i>PfDRP</i> IS TRANSCRIBED BY LATE TROPHOZOITES AND SCHIZONTS	98
4.3 DISCUSSION	98
CHAPTER 5 - YCF16	103
5.1 INTRODUCTION	103
5.1.1 <i>YCF24</i>	103
5.1.2 ABC DOMAINS	107
5.1.3 AIMS	111
5.2 RESULTS	111
5.2.1 GST- <i>E.C.</i> <i>YCF16</i> WAS PURIFIED UNDER NATIVE CONDITIONS	111
5.2.2 ANTIBODIES RECOGNISE GST- <i>E.C.</i> <i>YCF16</i>	114
5.2.3 GST BINDS 8-AZIDO-ATP	114
5.2.4 <i>P. FALCIPARUM</i> <i>YCF16</i> IS ENCODED ON CHROMOSOME 14	114
5.2.5 <i>P. FALCIPARUM</i> <i>YCF16</i> CONTAINS ATP-BINDING AND ABC SIGNATURE MOTIFS	118
5.2.6 <i>P. FALCIPARUM</i> <i>YCF16</i> POSSESSES A PUTATIVE PLASTID-TARGETING SEQUENCE	118
5.2.7 <i>YCF16</i> IS TRANSCRIBED BY <i>P. FALCIPARUM</i>	118
5.2.8 <i>P. FALCIPARUM</i> <i>YCF16</i> MAY BE TARGETED TO THE PLASTID	122
5.2.9 <i>P.F.</i> <i>YCF16-6His</i> CAN BE PURIFIED UNDER DENATURING CONDITIONS	122
5.3 DISCUSSION	125
CHAPTER 6 - SYNECHOCYSTIS YCF16	132
6.1 INTRODUCTION	132
6.1.1 <i>SYNECHOCYSTIS</i> PCC6803 AS A MODEL ORGANISM	132
6.1.2 <i>SYNECHOCYSTIS</i> <i>YCF24</i>	134
6.1.3 AIMS	135
6.2 RESULTS	136
6.2.1 <i>YCF16</i> AND <i>YCF24</i> ARE CO-TRANSCRIBED IN <i>SYNECHOCYSTIS</i>	136
6.2.2 <i>SYNECHOCYSTIS</i> Δ <i>YCF16</i> MUTANTS ARE HETEROPLASMIC	136
6.2.3 Δ <i>YCF16</i> MUTANTS LOSE STREPTOMYCIN RESISTANCE IN THE ABSENCE OF SELECTION	139
6.2.4 Δ <i>YCF16</i> MUTANTS EXHIBIT A "RAGGED" COLONY MORPHOLOGY	145
6.2.5 A HIGH PROPORTION OF Δ <i>YCF16</i> MUTANTS ARE UNDERGOING DIVISION	145

6.2.6 A HIGH LEVEL OF CELL DEATH IS NOT SEEN IN $\Delta YCF16$ MUTANTS	145
6.2.7 $\Delta YCF16$ MUTANTS DO NOT CONTAIN REDUCED AMOUNTS OF DNA	145
6.2.8 $\Delta YCF16$ MUTANTS APPEAR AS WT IN ELECTRON MICROGRAPHS	145
6.2.9 $\Delta YCF16/\Delta YCF24$ MUTANTS ARE VIABLE	149
6.3 DISCUSSION	149
CHAPTER 7 - DISCUSSION	160
7.1 AN ESSENTIAL ORGANELLE IN APICOMPLEXANS	160
7.2 NUCLEUS-ENCODED PLASTID PROTEINS	160
7.3 A FUNCTION FOR YCF24 (ORF470)	161
7.4 FUTURE PERSPECTIVES	162
7.5 SUMMARY	164
REFERENCES	165
APPENDICES	202

LIST OF FIGURES

1.1	The life cycle of the malaria parasite	22
1.2	The erythrocytic cycle of <i>P. falciparum</i>	24
1.3	Complete gene map of the <i>P. falciparum</i> plastid genome	28
1.4	Subcellular location of the plastid	31
1.5	Secondary endosymbiosis	32
1.6	The evolution of plastids	38
1.7	Targeting to the apicomplexan plastid	52
3.1	<i>P. falciparum</i> cultures are contaminated with mycoplasma	79
3.2	Mycoplasma DNA remains in the wells of a pulsed-field gel	81
4.1	Mitochondrial network in <i>S. cerevisiae</i>	88
4.2	Morphology of the plastid during the erythrocytic cycle	90
4.3	The expected 600 bp product was obtained by PCR	92
4.4	<i>ftsZ</i> (600) encodes FtsZ	93
4.5	<i>ftsZ</i> (600) is similar to <i>M. pulmonis ftsZ</i>	94
4.6	<i>ftsZ</i> (600) is a mycoplasma sequence	95
4.7	The <i>P. falciparum</i> genome encodes a dynamin-like protein	97
4.8	<i>Pfdrp</i> is found on chromosome 10	99
4.9	<i>Pfdrp</i> is transcribed by late stage erythrocytic parasites	100
5.1	ABC signature motif	109
5.2	Expression of GST- <i>E.c.</i> Ycf16 in <i>E. coli</i> was detrimental	112
5.3	GST- <i>E.c.</i> Ycf16 was purified under native conditions	113
5.4	GST binds 8-Azido-ATP	115
5.5	The <i>P. falciparum</i> genome encodes Ycf16	116
5.6	<i>ycf16</i> is on chromosome 14 in <i>P. falciparum</i>	117
5.7	<i>P. falciparum</i> Ycf16 contains the expected motifs	119
5.8	<i>ycf16</i> is transcribed by <i>P. falciparum</i>	120
5.9	The sequence encoding the Ycf16 leader is transcribed by <i>P. falciparum</i>	121
5.10	Maximum <i>ycf16</i> transcription occurs in trophozoites	123
5.11	Vectors used for targeting of reporter protein in <i>T. gondii</i>	124
5.12	Expression of <i>P.f.</i> Ycf16-6His in <i>E. coli</i> was not detrimental	126
5.13	<i>P.f.</i> Ycf16-6His was purified under denaturing conditions	127
6.1	<i>ycf24</i> and <i>ycf16</i> are co-transcribed in <i>Synechocystis</i>	137
6.2	Cloning strategy for disruption of <i>ycf16</i> in <i>Synechocystis</i>	138
6.3	All transformants possess at least one copy of wt <i>ycf16</i>	140
6.4	All transformants possess at least one copy of disrupted <i>ycf16</i>	141
6.5	Recombination of disrupted <i>ycf16</i> into the genome did not disrupt <i>ycf24</i>	142
6.6	Transformants are heteroplasmic	143
6.7	Δ <i>ycf16</i> mutants exhibit a “ragged” colony morphology	146
6.8	A high proportion of Δ <i>ycf16</i> mutants are dividing	147
6.9	Electron microscopy did not reveal any differences between wt and Δ <i>ycf16</i> <i>Synechocystis</i>	148
6.10	Cloning strategy for disruption of <i>ycf16</i> in Δ <i>ycf24</i> <i>Synechocystis</i>	150
6.11	Δ <i>ycf16</i> / Δ <i>ycf24</i> mutants exhibit a “ragged” colony morphology	151

LIST OF TABLES

2.1	Lambda <i>Hind</i> III marker	57
2.2	Buffers used in purification of <i>P.f.</i> Ycf16-6His	71
5.1	<i>ycf24</i> and <i>ycf16</i> in bacterial genomes	104
5.2	<i>ycf24</i> and <i>ycf16</i> in plastid genomes	106
5.3	<i>ycf24</i> and <i>ycf16</i> in nuclear genomes	108
6.1	<i>Synechocystis</i> cannot support the loss of more than two copies of <i>ycf16</i>	144
6.2	Comparison of Δ <i>ycf16</i> <i>Synechocystis</i> with Δ <i>ycf24</i> <i>Synechocystis</i>	153

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ABBREVIATIONS

A _x	Absorbance at x nm
ABC	ATP-binding cassette
ACP	Acyl carrier protein
AIDS	Acquired immunodeficiency syndrome
ALA	5-aminolaevulinic acid
ATP	Adenosine-5'-triphosphate
ATPase	Adenosine triphosphatase
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary deoxyribose nucleic acid
CoA	Coenzyme A
cpm	Counts per minute
CTAB	Cetyltrimethylammonium bromide
CTP	Cytidine-5'-triphosphate
dA	2'-deoxyadenosine
DAPI	4,6-diamidino-2-phenylindole
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
DDT	4,4'-dichlorodiphenyltrichlorethane
dGTP	2'-deoxyguanosine-5'-triphosphate
dH ₂ O	Distilled water
DHFR	Dihydrofolate reductase
DNA	Deoxyribose nucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphate
DOXP	1-deoxy-D-xylulose-5-phosphate
dT	2'-deoxythymidine
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	Ethylenediaminetetra-acetic acid
EF	Elongation factor
EMBL	European Molecular Biology Laboratory
ER	Endoplasmic reticulum
EST	Expressed sequence tag
EtBr	Ethidium bromide
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
GTPase	Guanosine triphosphatase
HEPES	<i>N</i> -(2-hydroxyethyl)piperazine- <i>N'</i> -(2-ethanesulphonic acid)
HIV	Human immunodeficiency virus
HMG	3-hydroxy-3-methylglutaryl
IPTG	Isopropylthio-β-D-galactoside
<i>kan</i> ^R	kanamycin resistance cassette
kb	Kilobase(s)
kDa	Kilodaltons
LB	Luria-Bertani
Mb	Megabase(s)
MBq	MegaBecquerels
MES	3-(<i>N</i> -morpholino) ethane sulphonic acid
MRA	Mycoplasma Removal Agent
mRNA	Messenger ribose nucleic acid
MSP	Merozoite surface protein
N _{2(l)}	Liquid nitrogen
NADP	Nicotinamide adenine dinucleotide phosphate
NEB	New England Biolabs
NCBI	National Center for Biotechnology Information

NIMR	National Institute for Medical Research
OD _x	Optical density at x nm
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCC	Pasteur Culture Collection
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribose nucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribose nucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Svedberg units
SDS	Sodium dodecyl sulphate
<i>strep</i> ^R	Streptomycin resistance cassette
TES	<i>N</i> -Tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid
Tris	Tris(hydroxymethyl)methylamine
tRNA	Transfer ribose nucleic acid
UCL	University College, London
UK	United Kingdom
US	United States
USA	United States of America
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
v/v	Volume by volume
wt	Wild type
w/v	Weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1 - Introduction

1.1 Malaria

1.1.1 The Disease

Every 12 seconds someone dies of malaria. In terms of numbers, malaria is the most serious health problem in the world with 500 000 000 people currently ill; more than with any other disease (Cox, 1991; Wirth, 1998). A third of all humans live in areas where malaria poses a threat and every year it kills at least 2 000 000 people, mostly African children.

Malaria is an infectious disease caused by parasitic protozoa of the genus *Plasmodium* and is transmitted by the bite of the female *Anopheles* mosquito. In humans, the disease is characterised by a violent fever lasting several hours and recurring every two or three days depending on the species of *Plasmodium* causing it. Four species cause malaria in humans: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and the most dangerous *Plasmodium falciparum*, which causes the fatal “cerebral” (or “*falciparum*”) malaria, an illness which can lead to coma and death within a few days.

Malaria is predominantly a tropical disease affecting Third World countries, however, summer epidemics occurred in Mediterranean countries until as recently as the 1970s. Until the Twentieth Century, malaria caused by *P. vivax* and transmitted by *Anopheles atroparvus* occurred in the South of England (Marchant *et al.*, 1998): *P. vivax* is able to survive as hypnozoites in the liver in seasons that are too cold for mosquito transmission. Even now, up to 2 000 cases of malaria are imported into Britain every year by people infected whilst abroad.

Global warming brings the possibility of malaria once again threatening hitherto temperate regions, a rise in temperature of just a few degrees could be sufficient to allow completion of sporogony by *P. falciparum*. Although a barrier to oocyst production by *P. falciparum* has been found to exist in *A. atroparvus*, another mosquito implicated in transmission of malaria in Britain, *Anopheles plumbeus*, has been found to be susceptible to infection by *P. falciparum*, raising the frightening prospect that British mosquitoes could potentially transmit *P. falciparum* imported from the tropics (Marchant *et al.*, 1998). However, the phenomenon of global warming constitutes more variables than a simple rise in temperature and recent predictions taking these into account suggest that the distribution of *P. falciparum* is unlikely to change much if the world warms (Dye and Reiter, 2000; Rogers and Randolph, 2000; Guyatt and Robinson, 2001). For example, a reduction in summer rainfall in Britain would result in fewer breeding sites for the mosquito.

In tropical regions with rainfall throughout the year, infective *Anopheles* mosquitoes are ubiquitous. Depending on altitude, these can be regions of high transmission of malaria, where an individual may receive up to 1 000 infectious bites a year. In such “hyperendemic” areas, many people acquire a natural immunity that allows them to tolerate and pass on the parasite without developing severe symptoms. Although most adults will suffer no disease, children under the age of 18 months are at high risk of contracting malaria. After the age of 18 months they develop sufficient immunity to the parasite to be no longer at high risk of dying (Bagster Wilson *et al.*, 1950). However, such populations can lose their immunity if they experience prolonged periods of low transmission, and then risk contracting malaria if transmission rates increase again. Visitors to these endemic regions have a serious risk of contracting malaria.

1.1.2 Combating Malaria

1.1.2.1 Targeting the Parasite

The “original” antimalarial chemotherapy was the bark of the “Peruvian fever tree”, *Cinchona*. The active compound is quinine, on which many modern antimalarials are based. Whilst, the mode of action of these “quinoline” antimalarials is unclear, there is evidence to suggest that chloroquine exerts its effect by inhibiting haem-dependent protein synthesis by the parasite (Surolia and Padmanaban, 1991), or by interfering with the detoxification of haem released from the host’s haemoglobin (Slater and Cerami, 1992). If it were possible to understand the mode of action of quinoline drugs, and the mechanism of the parasite’s resistance, it might be possible to design new drugs that target the same pathway but that do not elicit the problems of resistance (Macreadie *et al.*, 2000).

Due to inappropriate or careless use of antimalarial drugs there has been a serious emergence of resistance during recent years. New drugs have been developed, but resistance to them is developing rapidly. Quinine remains effective in most areas, but approximately half the world’s production is destined for use in tonic water; continuous exposure of the parasite to low levels of quinine provides the ideal conditions for the emergence of resistance. Because of unpleasant side-effects, quinine is usually only prescribed as treatment alongside other drugs such as Fansidar or tetracycline.

Where chloroquine resistance has emerged, a combination of pyrimethamine and sulphadoxine (Fansidar) has been used as treatment for *falciparum* malaria. These antifolate drugs target dihydrofolate reductase and dihydropteroate synthase. Resistance is due either to mutations in the genes encoding these enzymes, or to the ability of the parasite to utilise a folate salvage pathway thereby bypassing the blockage of the endogenous folate biosynthetic pathway (Macreadie *et al.*, 2000). However, this drug combination is no longer routinely recommended as a prophylactic because of the risk of Stevens-Johnson

syndrome or of toxic epidermal necrolysis, although Fansidar is still used as a standby treatment (Bradley and Warhurst, 1995). Mefloquine (Lariam) is now recommended when a traveller is at a high risk of chloroquine-resistant *falciparum* malaria. Where mefloquine-resistant *falciparum* malaria exists, or where the patient cannot take chloroquine or mefloquine, doxycycline is the prophylactic of choice.

It has been suggested that to combat the increasing problem of drug resistance, a combination of inhibitors acting on pathways that converge to give a final product, such as that of the pyrimethamine and sulphadoxine antifolate combination, should be used for antimalarial chemotherapy (Macreadie *et al.*, 2000). However, where the parasite can utilise an alternative pathway (for example, the folate salvage pathway) this approach will fail. One example of a potentially successful use of drug combinations involves a herbal remedy that has been in use in China for 2 000 years: the artemisinins (Marshall, 2000a). The artemisinins, extracted from the flowering plant *Artemisia annua* (sweet wormwood), have not yet been approved for clinical use in western countries, however, it has been shown that one member of this family in particular, artesunate, can reduce the number of parasites in the blood faster than any other drug, and if given early can prevent an infection from progressing to coma. In north-western Thailand where *P. falciparum* has developed resistance to almost every drug available, trials of artesunate with mefloquine have been extremely successful (Nosten *et al.*, 2000). It has been suggested that artesunate, if used in combination with other antimalarials, could help prevent the development of drug resistance by parasites in areas of low transmission (White, 1998). However, commercial development of this drug is slow: since it is a traditional medicine no patent can be obtained on it (McFadden and Waller, 1997).

Another problem with current antimalarials is their side-effects, ranging from the minor mouth ulcers and gastrointestinal upsets caused by chloroquine and proguanil and the photosensitisation and diarrhoea caused by doxycycline, to the neuropsychiatric problems (anxiety, depression, sleep disturbances, nightmares and hallucinations) associated with mefloquine. These side-effects, coupled with the length of time these drugs need to be taken, often result in non-compliance by the user.

The problem of increasing resistance of the parasite to the antimalarials currently in use has led to an urgent need to develop new and effective drugs against established targets, and to seek out new potential drug targets.

1.1.2.2 Targeting the Vector

Ultimately, the most important way for travellers to malarious regions to protect themselves from malaria is to avoid being bitten by *Anopheles* mosquitoes. Wearing long sleeved/trouserred clothing at night, using insect repellents and sleeping under insecticide

impregnated bed nets, even when prophylactics are being taken, are all sensible recommendations (Bradley and Warhurst, 1995). To a certain extent inhabitants of malarious regions can protect themselves thus, but concerted efforts to rid such areas of the mosquito have been attempted. A campaign in the 1950s co-ordinated by the World Health Organisation resulted in the decrease, and even the elimination of malaria in several countries. During this campaign, the insect vector of malaria, the *Anopheles* mosquito, was targeted by insecticides such as 4,4'-dichlorodiphenyltrichlorethane (DDT).

Unfortunately, mosquitoes developed resistance to DDT and to other insecticides during the early years of the campaign resulting in the re-emergence of malaria where it had previously been reduced. Coupled with political instability and war in some of the countries at risk, many malaria eradication programmes failed or were abandoned. In India in the early 1950s, 75 000 000 people contracted malaria every year, with 800 000 dying annually. A decade later, after a programme of DDT treatment, there were only 100 000 new cases every year, and no deaths (Jayaraman, 1997). However, the success of the DDT programme led to complacency, the national malaria control programme was discontinued and by the end of the 1970s there were 6 500 000 new cases a year. In 1996, there was an official death toll of 3 000, although the true number of deaths was almost certainly higher. There has been increasing resistance of the parasite to chloroquine, and of the mosquito to insecticides. Moreover, *P. falciparum* is now more common than the less dangerous *P. vivax*, because it is less susceptible to chloroquine.

Because of the problems of resistance of the parasite to antimalarials, combating the mosquito has once again risen to the forefront of malaria eradication campaigns. However, increasing resistance of the mosquito to insecticides has reduced their efficacy. Furthermore, concerns about the impact of such chemicals on the environment, and the reluctance of people to have their houses repeatedly sprayed, have restricted their use.

One surprising observation is that insecticide resistant *Culex quinquefasciatus* mosquitoes are less likely to transmit filariasis. The mechanism of insecticide resistance involves high level expression of an esterase in the mosquito gut, which may interfere with the development of the parasite. McCarroll *et al.* (2000) propose that a similar situation could occur in other insect/parasite combinations. Indeed, esterase-based mechanisms of insecticide resistance have been seen in the malaria carriers *Anopheles albimanus* and *Anopheles culicifacies*. This observation could have widespread consequences for the control of human diseases transmitted by insects.

In addition to targeting the adult mosquito by house-spraying, the larvae can also be targeted. Breeding sites can be reduced by land drainage; the reduction of malaria was probably a fortuitous side-effect of the programmes of land drainage carried out in South-East England during the Nineteenth Century.

Forms of biological control have included the use of fish such as guppies which eat mosquito larvae, and the bacteria *Bacillus thuringiensis* and *Bacillus sphaericus* which produce toxins that kill mosquito larvae when ingested. Attempts have even been made to genetically engineer cyanobacteria on which mosquito larvae feed; *Synechococcus*, *Synechocystis* and *Anabaena* species have been engineered to produce the toxins from *B. thuringiensis* and *B. sphaericus* that kill mosquito larvae (Tandeau de Marsac *et al.*, 1987; Chungjatupornchai, 1990; Xudong *et al.*, 1993; Xiaoqiang *et al.*, 1997; Xu *et al.*, 2000). In the case of *Synechocystis*, although the protein produced by the cells was found to be toxic, *in vivo* levels of the protein were not sufficiently high to produce a toxic effect. However, Xu *et al.* (2000) showed that engineered *Anabaena* could keep containers of water free from *Culex* larvae for over two months, although toxicity against *Anopheles* larvae was less potent (Xudong *et al.*, 1993).

The sterilisation and release of large numbers of male *Anopheles* mosquitoes that are still capable of mating (females mate only once in their lifetime), is one strategy for reducing the population of mosquitoes. The development of insect transfection technologies has created the possibility of reducing transmission of malaria by genetically engineering the mosquito to be resistant to the parasite. Advances have been made in transforming mosquitoes using transposons with reporter genes such as that encoding green fluorescent protein (GFP) (Kidwell and Wattam, 1998; Enserink, 2000). The next target is to identify genes that prevent the mosquito from transmitting malaria. Of course, the engineered organism must have some advantage over natural mosquitoes in order for it to out-compete and result in a reduction in transmission, and there will probably be public hostility to the large-scale release of transgenic mosquitoes.

There are plans for an *Anopheles* genome sequencing project (Pennisi, 2000). Sequencing data from the *P. falciparum* genome sequencing project (see below) have already led to some fruitful research as will be described later. Information from the *Anopheles* genome, together with that from the parasite genome and the first draft of the human genome (Marshall, 2000b; International Human Genome Sequencing Consortium, 2001), will provide great scope for understanding the disease more fully, and for development of better drugs and vaccine candidates.

1.1.2.3 Vaccines

One of the major problems with developing a vaccine against malaria, is that the parasite exists in several different forms within the vertebrate host. Sporozoites and merozoites are the only forms of the parasite that are exposed to the immune system, the other stages of the life cycle being protected within host cells. Hence, research into vaccines has concentrated on these extracellular stages of the parasite.

There are two observations that lead researchers to believe that vaccination against malaria will be possible. The first is that people who live in areas of hyperendemicity develop a natural immunity to malaria over a period of a few years. If children could be protected from death by malaria, then they would have a chance to build up the natural immunity seen in adults in the same area. When blood from immune adults is transfused into children with high parasitaemia, antibodies from the transfused blood dramatically reduce the parasite load in the children (Cohen *et al.*, 1961). The second observation is that injection with irradiated sporozoites protected against malaria in birds (Mulligan *et al.*, 1941; Russell and Mohan, 1942), mice (Nussenzweig *et al.*, 1967) and monkeys (Collins and Contacos, 1972); finally some protection was seen in human volunteers vaccinated with sporozoites from irradiated mosquitoes (Clyde *et al.*, 1973). However, this approach requires exposing volunteers to several hundred mosquito bites over a period of months. Since it is not possible to culture sporozoites, it has not been possible to irradiate sporozoites directly in order to formulate a vaccine. Although this attenuated sporozoite approach is not a viable vaccine option, it has led researchers to study sporozoites to try to elucidate the mechanism triggering the immune response.

Researchers have subsequently been concentrating on the use of a single protein which could elicit an immune response similar to that caused by whole parasites (Nussenzweig and Nussenzweig, 1989). Circumsporozoite protein, the major sporozoite surface protein, was an early candidate, but did not elicit high titres of antibody. A modified version was more successful: the RTS,S vaccine consists of 189 amino acids from *P. falciparum* circumsporozoite protein fused to the surface antigen of the hepatitis B virus, and co-expressed with unfused hepatitis B surface antigen in *Saccharomyces cerevisiae* where the two polypeptides spontaneously form virus-like particles. In initial trials, six out of seven subjects were protected against malaria challenge three weeks later (Stoute *et al.*, 1997). Subsequently, however, four out of five of these developed malaria after a challenge at six months (Stoute *et al.*, 1998). In a field trial of 306 adult volunteers in The Gambia, the vaccinated group experienced 71 % fewer clinical episodes of malaria than the control group (Taubes, 2000). Researchers are now concentrating on looking for ways to boost the immune response, and hope eventually to test the current vaccine in children. Surprisingly, combining RTS,S with thrombospondin-related anonymous protein, another protein thought to play a rôle in the invasion of hepatocytes, failed to provide protection against sporozoite challenge (Anders and Saul, 2000).

The problem with targeting the sporozoite is that if just one parasite escapes the antibody response, it will reach the liver safely and develop into 30 000 merozoites, against which there will be no immune response. Therefore, most researchers agree that a multilateral attack is necessary.

Daubersies *et al.* (2000) have concentrated on proteins expressed during the liver-stage of the parasite. They used sera from humans and chimpanzees protected by immunisation with irradiated sporozoites to screen a library of *P. falciparum* polypeptides expressed during the pre-erythrocytic stage of the parasite's life cycle. They identified a clone that contained a gene that they called liver-stage antigen 3. They found that immunisation of chimpanzees (the only non-human primate susceptible to complete intrahepatic development of *P. falciparum*) with recombinant liver-stage antigen 3 provided protection against parasite challenge. Hoffman and Doolan (2000) suggest that the liver stage of the parasite's life cycle is an ideal vaccine target since this stage lasts several days, and a vaccine to this stage would prevent both clinical symptoms of malaria, and transmission of the disease. Pre-erythrocytic stage vaccines are being developed for use by non-immune travellers, and by the general population in areas of low transmission (Miller and Hoffman, 1998).

The pre-erythrocytic stage of the parasite's life cycle is asymptomatic, so vaccine research has also concentrated on targeting the merozoite, the form of the parasite that invades the erythrocyte. This approach aims to prevent disease, not infection. Mitchell *et al.* (1975) obtained promising results on immunisation of monkeys with whole, killed merozoites. One much studied protein is a merozoite surface protein of unknown function called MSP-1 with which vaccination has been found to protect rodent and primate model organisms from malaria. Certain anti-MSP-1 antibodies have been found to interfere with the asexual cycle by preventing invasion into erythrocytes (Holder and Blackman, 1994). Once inside the erythrocyte, the parasite exports proteins that are expressed externally on the erythrocyte membrane. These proteins mediate adhesion to a variety of ligands on the host endothelium, presumably to evade splenic clearance. In being unique to the surface of parasitised erythrocytes, these proteins could represent potential vaccine candidates; however, in order to avoid recognition by the immune system, the parasite has evolved a system of antigenic variation, which could be difficult to overcome (Newbold, 1999).

Transmission blocking vaccines interrupt the development of parasite gametes in the mosquito. These "altruistic" vaccines do not protect the individual, but are designed to reduce transmission of the disease. Antibodies to gamete proteins have been found to block transmission by mosquitoes to mice and rabbits, and human trials are anticipated by the end of 2001 (Taubes, 2000).

Ideally a vaccine would contain liver-stage antigens, blood-stage antigens and a transmission blocking component, for both *P. falciparum* and *P. vivax*, and would elicit both humoral and cellular immune responses (Cox, 1991; Holder, 1999). Shi *et al.* (1999) have expressed from a synthetic gene a recombinant protein containing epitopes from two sporozoite proteins, one liver stage-specific protein, five blood stage proteins and one gametocyte protein. Although rabbit antibodies induced by immunisation with this construct recognised different stages of parasites, blocked sporozoite invasion of hepatoma

cells and inhibited growth of blood-stage parasites *in vitro*, it is not known how these *in vitro* assays relate to *in vivo* functionality. DNA vaccines are thought to be another way of combining antigens from different stages of the life cycle, and Hoffman *et al.* (1998) discuss how the sequence data emerging from the *P. falciparum* genome sequencing project (see below) could be used in the development of these. DNA vaccines encoding up to 15 different malaria proteins have raised both antibody and CD8⁺ killer T cell responses in animal studies, attacking the malaria parasite in the blood stream as well as in the hepatocyte (Taubes, 2000). Prime-boosting, which involves priming the immune system with a DNA vaccine, followed by immunisation with vaccinia virus, attenuated and modified to express the same antigens as those encoded on the DNA vaccine, has been used to enhance the immune response generated by DNA vaccines (Schneider *et al.*, 1998). Phase I safety and immunogenicity trials of a multi-epitope DNA vaccine have recently been completed; testing of the DNA vaccine in combination with its vaccinia partner are underway, and a Phase I clinical trial of this vaccine has commenced in The Gambia (Ferry, 2000; Hoffman and Doolan, 2000).

1.1.3 The Parasite

1.1.3.1 Taxonomy

Malaria parasites belong to a large phylum of obligate intracellular parasites, the Apicomplexa, which includes a number of important pathogens of man and other animals. At some stage of their life cycle all apicomplexans possess an apical complex, an intracellular apparatus required for the invasion of host cells. Other important apicomplexans, besides *Plasmodium*, include the AIDS-related opportunistic human pathogens *Toxoplasma* and *Cryptosporidium*, the cattle parasites *Theileria* and *Babesia*, and *Eimeria*, the causative agent of coccidiosis in domestic fowl.

Plasmodium is further defined according to its transmission between vertebrate hosts by the *Anopheles* mosquito, the life cycle consisting of one sexual phase followed by three asexual phases, and the production of haemozoin during digestion of haemoglobin at some stage of the life cycle. There are more than 125 species of *Plasmodium* known; most infect birds, but there are also species that infect snakes, lizards, turtles, rodents and other primates as well as man. Other mammalian families appear to be free from malaria.

Studies of the density of genomic DNA and molecular analyses of small subunit ribosomal RNA (rRNA) genes have shown that the malaria parasites infecting humans are actually more closely related to the malaria parasites of other animals than they are to each other (Waters *et al.*, 1993), showing that the associations of the different human malaria parasites with humans are phylogenetically independent. *P. falciparum* in particular is more closely related to rodent and avian malaria parasites than to the other primate malaria parasites; and

in turn it appears to be more closely related to avian malaria parasites than to rodent malaria parasites (van Lin *et al.*, 2000). However, when the chimpanzee malaria parasite *Plasmodium reichenowi* was included in analysis of rRNA genes, it was found to be very closely related to *P. falciparum* (Escalante and Ayala, 1994). The time of divergence of *P. reichenowi* and *P. falciparum* is consistent with that of humans and chimpanzees, implying that they evolved separately in their different hosts.

1.1.3.2 Life Cycle

The malaria parasite is difficult to study; it has only been possible to culture it *in vitro* for the last 25 years, and then only the erythrocytic stage of *P. falciparum*. Although there are records of malaria (or “ague”) going back centuries, it was only just over 100 years ago that Sir Ronald Ross proved that it was transmitted by a particular type of mosquito. The pigment produced by parasites previously observed in the blood of malaria sufferers by Alphonse Laveran (Laveran, 1881) was eventually identified in cysts on the stomach walls of *Anopheles* mosquitoes by Ross (1897).

The life cycle of the malaria parasite can be divided into four phases: fertilisation and sporogony, which occur in the mosquito, and hepatic and erythrocytic schizogony, which take place in the vertebrate host (Figure 1.1). There are at least five phases of DNA synthesis during its life cycle (Arnot and Gull, 1998); most of the time the parasite is haploid, only becoming diploid during the zygote and ookinete stages in the mosquito.

Fertilisation is the only sexual stage of the life cycle. Shortly after uptake by the mosquito, exflagellation (the production of male gametes) occurs. The male gametes are motile, and are able to fertilise female gametes to form a zygote. The zygote develops into a mobile ookinete, an invasive form that passes through or between the epithelial cells of the mosquito stomach wall to reach the basement membrane.

During sporogony, the ookinete grows rapidly forming an oocyst that projects into the body cavity of the mosquito. After about a week, the oocyst undergoes a process of internal division during which thousands of sporozoites form. After another week, the oocyst bursts, and the sporozoites migrate to the mosquito’s salivary glands from where they can be injected into the vertebrate host the next time a blood meal is taken.

Within 10 minutes of injection into the vertebrate host, invasive sporozoites reach the liver. Hepatic schizogony lasts about a week. During this time, the hepatic trophozoite grows rapidly and divides internally to form the multinucleate hepatic schizont within which develop thousands of merozoites. In the case of *P. vivax*, some sporozoites become hypnozoites, which can lie dormant in hepatocytes causing the disease to relapse months or years later.

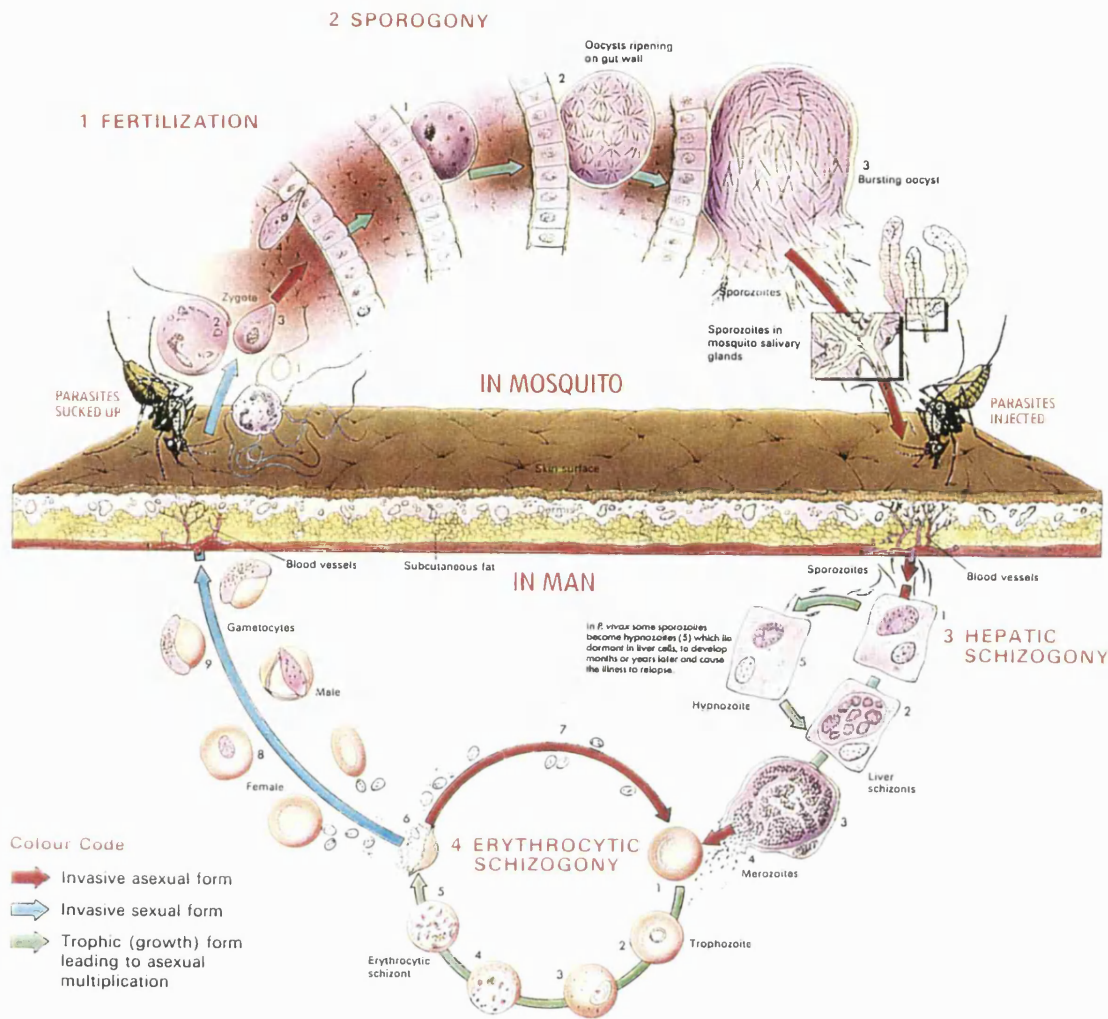


Figure 1.1: The Life Cycle of the Malaria Parasite.

1) Fertilisation. Shortly after a blood meal, motile male gametes¹ which fertilise female gametes² producing a zygote³. This develops into an ookinete⁴ which bores into the stomach wall and forms an oocyst.

2) Sporogony. The oocyst grows¹ producing thousands of sporozoites². The mature oocyst bursts³ releasing the sporozoites, which migrate to the mosquito's salivary glands.

3) Hepatic Schizogony. Sporozoites invade hepatocytes¹ and become hepatic trophozoites². These grow and divide to produce thousands of merozoites³, which are released into the blood when infected hepatocytes burst⁴. In *P. vivax*, some sporozoites become hypnozoites⁵ which can lie dormant in hepatocytes.

4) Erythrocytic Schizogony. Merozoites invade erythrocytes¹ and become erythrocytic trophozoites². These grow³ and divide⁴ into 8-32 new merozoites⁵ that are released when the erythrocyte bursts⁶, allowing the cycle to start again⁷. Some merozoites develop into gametocytes^{8,9}, which only develop further if taken up by a mosquito.

From Knell (1991).

Merozoites invade erythrocytes, becoming erythrocytic trophozoites. These trophozoites digest the host's haemoglobin as they grow. The parasite's nucleus divides three or four times to form a multinucleate schizont that fills the erythrocyte. Internal division then results in the formation of eight to 32 new merozoites, which are released and can invade new erythrocytes. It is this release of merozoites that causes the fevers characteristic of malaria. The different stages of the erythrocytic cycle (Arnot and Gull, 1998) are shown in Figure 1.2. In *falciparum* malaria, infected erythrocytes adhere to endothelial cells lining venules, a process called sequestration. Sequestration can be intense in the brain, causing a reduced blood flow and resulting in the coma and fits characteristic of *falciparum* malaria. After several cycles of erythrocytic schizogony, some merozoites will develop into gametocytes, which remain within the erythrocyte membrane, only developing further in the stomach of another mosquito.

1.1.3.3 *Genome*

1.1.3.3.1 Nuclear Genome

Malaria parasites are haploid for most of their life cycle, with a brief diploid phase prior to meiosis during the mosquito stage (Sinden and Hartley, 1985). The 30 Mb nuclear genome of *P. falciparum* consists of 14 chromosomes (Prensier and Slomianny, 1986; Kemp *et al.*, 1987) and is extremely rich in adenine and thymine nucleotides (82 % (Pollack *et al.*, 1982)). The chromosomes can be separated by pulsed-field gel electrophoresis (PFGE), although chromosomes 5 to 9 are often difficult to resolve, co-migrating as a "blob" in the middle of the gel (Gardner *et al.*, 1998a). Size polymorphism of the chromosomes caused by gene duplication or deletion can occur, and results in a characteristic karyotype for different strains, or for different clones isolated from different patients.

The nuclear genome of *P. falciparum* is currently being sequenced by the Malaria Genome Sequencing Consortium, made up of The Sanger Centre (UK), The Institute for Genomic Research/Naval Medical Research Institute (USA) and Stanford University (USA) (Fletcher, 1998; Wirth, 1998). "Shotgun" sequencing of individual chromosomes, whereby large fragments of DNA are randomly sheared into one to two kilobase fragments and the size-selected fragments are cloned into bacterial plasmids for sequencing, is the method being used for all chromosomes (Gardner *et al.*, 1998a). Chromosome 2 (947 103 bp) has been completely sequenced (Gardner *et al.*, 1998b, 1999; Jing *et al.*, 1999) and contains 209 predicted protein-encoding open reading frames (ORFs) (Wirth, 1998). Sequencing of chromosome 3 (1 060 106 bp) has been completed (Thompson and Cowman, 1997; Bowman *et al.*, 1999): it is predicted to include 215 protein-coding genes and two transfer RNAs (tRNAs). Sequencing of the remaining chromosomes is almost complete; the time-consuming task of assembling the contigs being made difficult by

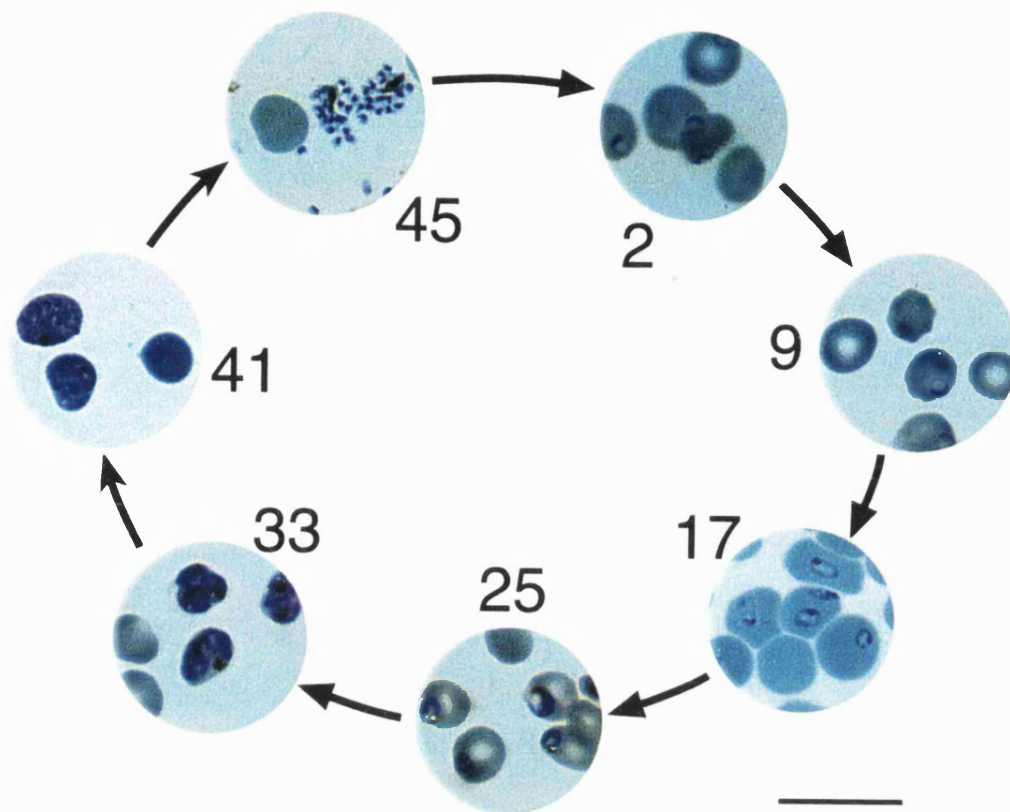


Figure 1.2: The Erythrocytic Cycle of *P. falciparum*.

P. falciparum strain C10 was synchronised by sorbitol-treatment and allowed to grow *in vitro* for 45 hours. The different time-points represent the approximate number of hours post-invasion. Scale bar = 20 μm .

the high A + T content of the genome, which reaches 97 % in places (Pennisi, 2000). Chromosomes 1 and 4 should be completed in the coming months, and it is hoped that sequencing of the entire genome will be completed by the end of 2001 (D. Lawson, personal communication).

The sequence data are being released to the scientific community *via* the internet, and annotation of all chromosomes is being carried out as the project progresses (Plasmodium Genome Database Collaborative, 2001). However, Kyrpides and Ouzounis (1999) warn against predicting functions for genes without real evidence, since errors can enter the public database and be propagated.

The sequencing of the *P. falciparum* genome, alongside improvements in bioinformatics and transfection technology, will provide researchers with a mine of information that can be exploited in many different ways (Foote *et al.*, 1998; Gardner, 1999). Sequences can be compared between species for the construction of phylogenetic trees, exons can be identified and patterns of gene expression determined, and genes can be disrupted or tagged to find out function and cellular localisation. In addition, several technologies are emerging that will facilitate the functional analysis of the *Plasmodium* genome: DNA microarrays, serial analysis of gene expression and high-throughput proteomics are already being applied to malaria research (Carucci, 2000; Hayward *et al.*, 2000; Ben Mamoun *et al.*, 2001).

Before *in vitro* culture of *P. falciparum* was possible, much of the biology and genetics of malaria parasites was studied using other species as model organisms. Other *Plasmodium* species have also been studied for the purposes of vaccine and drug development. For these reasons it has been suggested that following the completion of the *P. falciparum* genome sequencing project, the genome of *P. vivax*, or one of the rodent malaria parasites should be sequenced to provide a comparison (van Lin *et al.*, 2000). Other species of *Plasmodium* are thought to have a similar chromosome number (van Lin *et al.*, 2000).

Before sequencing of the *P. falciparum* genome started, physical maps of the genome were constructed (Triglia *et al.*, 1992; Dame *et al.*, 1996; Lai *et al.*, 1999; Thompson *et al.*, 1999). Similar studies are underway in *Plasmodium berghei* and *P. vivax* and gene sequence tag projects have commenced (Carlton and Dame, 2000); although libraries have been constructed (Camargo *et al.*, 1997), assembly and characterisation of fragments are at a preliminary stage.

There few non-*falciparum* sequences in the databases, so comparative genomics between different malaria parasites is at an early stage (van Lin *et al.*, 2000). The completion of the *P. falciparum* genome, along with emerging data from genomes of other malaria parasites will allow the identification of novel genes, gene classes or gene functions, a greater understanding of malaria parasite evolution and host-parasite interactions, and studies into

the differences in pathogenicity of *P. falciparum* with other species of *Plasmodium*. Although classical biology is still vital in the fight against malaria (Curtis, 2000), the information generated by parasite genome projects will increase our chances of developing new and better drugs and vaccines (Hoffman, 2000).

1.1.3.3.2 Mitochondrial Genome

The 6 kb mitochondrial genome of the malaria parasite is the smallest known genome of any mitochondrion. Its A + T content is 68 % (Feagin *et al.*, 1992) lower than the nuclear genome, and it has a limited information content, only encoding three polypeptides: cytochrome *b*, subunit I of cytochrome *c* oxidase and a poorly conserved subunit III of cytochrome *c* oxidase (Feagin, 1992). It also encodes the large and small subunit rRNAs, the genes for which are present as seemingly randomly dispersed fragments on both strands of the genome (Feagin, 1992), but no tRNAs or other common mitochondrial markers. Due to its small size, the “6 kb element” was originally thought to be just one part of a bipartite mitochondrial genome, but it is now accepted that it constitutes the entire genome (Williamson, 1998). Therefore, the remaining requirements for a functional mitochondrion must be encoded in the nucleus and imported.

Mitochondria can be seen in electron micrographs of *Plasmodium* species, but during the erythrocytic cycle these appear to have few cristae. Mammalian malaria parasites rely on glycolysis rather than aerobic respiration for energy production during the erythrocytic cycle, however, inhibitors of mitochondrial protein synthesis, and of electron transport have antimalarial activity *in vitro* (Blum *et al.*, 1984; Divo *et al.*, 1985a; Ginsburg *et al.*, 1986) so the mitochondrion appears to have some other function in these stages (Feagin, 1994). The mitochondria in insect stages of the *Plasmodium* life cycle have more cristae, indicating a more conventional role for this organelle.

There is one mitochondrion per cell in *P. falciparum* (Bannister *et al.*, 2000) and replication of the mitochondrial DNA occurs at the same time as that of nuclear DNA (Smeijsters *et al.*, 1994; Preiser *et al.*, 1996; Williamson *et al.*, 1996). There are 15 to 20 copies of the mitochondrial genome per cell in *P. falciparum*, arranged mainly in three to five, 6-30 kb, head-to-tail linear tandem arrays, with a small proportion (1-2 %) present as a covalently closed circle (Preiser *et al.*, 1996). However, Vaidya and Arasu (1987) reported that the murine malaria parasite *Plasmodium yoelii* carries approximately 150 copies of this genome.

1.1.3.3.3 Plastid Genome

Unusually for a non-photosynthetic organism, the malaria parasite possesses three genomes (Wilson *et al.*, 1991). Besides the nuclear genome are two extrachromosomal DNAs: the mitochondrial genome and a 35 kb plastid genome.

The plastid genome of *P. falciparum* (Figure 1.3) has been completely mapped and sequenced (Wilson *et al.*, 1996a). The DNA encodes many of the components required for protein synthesis including 16S and 23S rRNAs, 25 tRNAs covering all 20 amino acids, small and large subunit ribosomal proteins, three subunits of a eubacterial RNA polymerase and elongation factor (EF) Tu. It also encodes ClpC (a molecular chaperone) and a protein of unknown function, Ycf24 (ORF470), which is orthologous to a highly conserved protein encoded on the genomes of bacteria and algal plastids. There are also seven small and as yet unassigned ORFs. The genes on the 35 kb circle are frequently separated by just a few nucleotides and some even overlap.

Plastid genomes tend to be A + T-rich, and the *P. falciparum* plastid genome is even more A + T-rich than the nuclear genome (see below). The plastid DNA is passed onto the next generation through the female macrogamete in the sexual stage of the life cycle (Vaidya *et al.*, 1993; Creasey *et al.*, 1994). The plastid genome replicates at or just before onset of schizogony; timing of replication could not be distinguished from that of the mitochondrial genome, suggesting that despite their different subcellular locations, the replication of the two organellar genomes and the nuclear genome is co-ordinated in some way (Smeijsters *et al.*, 1994; Williamson *et al.*, 1996).

In the plastid genomes of the related apicomplexans *Toxoplasma gondii* and *Neospora caninum*, UGA (which normally denotes a Stop codon) is translated as a tryptophan (Denny *et al.*, 1998; Lang-Unnasch and Aiello, 1999). It is not clear whether a similar aberrant codon usage is utilised by the *P. falciparum* plastid genome: here, in all cases where UGA is predicted to denote a Stop, an in-frame UAA Stop codon is situated within 30 bp downstream of it; therefore it is possible that the UGA codon encodes a tryptophan, and the UAA is the true Stop codon (Lang-Unnasch and Aiello, 1999).

Similarities between the genome of the malarial plastid, and that of the plastid of *Epifagus virginiana*, a non-photosynthetic parasitic plant, are probably due to convergent evolution (Carter *et al.*, 1998). Either genes were retained due to the selective pressures on parasitic organisms, or they were lost through lack of need (for example, those required for photosynthesis). Selective loss of genes is an indicator of a functional genome (dePamphilis and Palmer, 1990; Wolfe *et al.*, 1992) (see below).

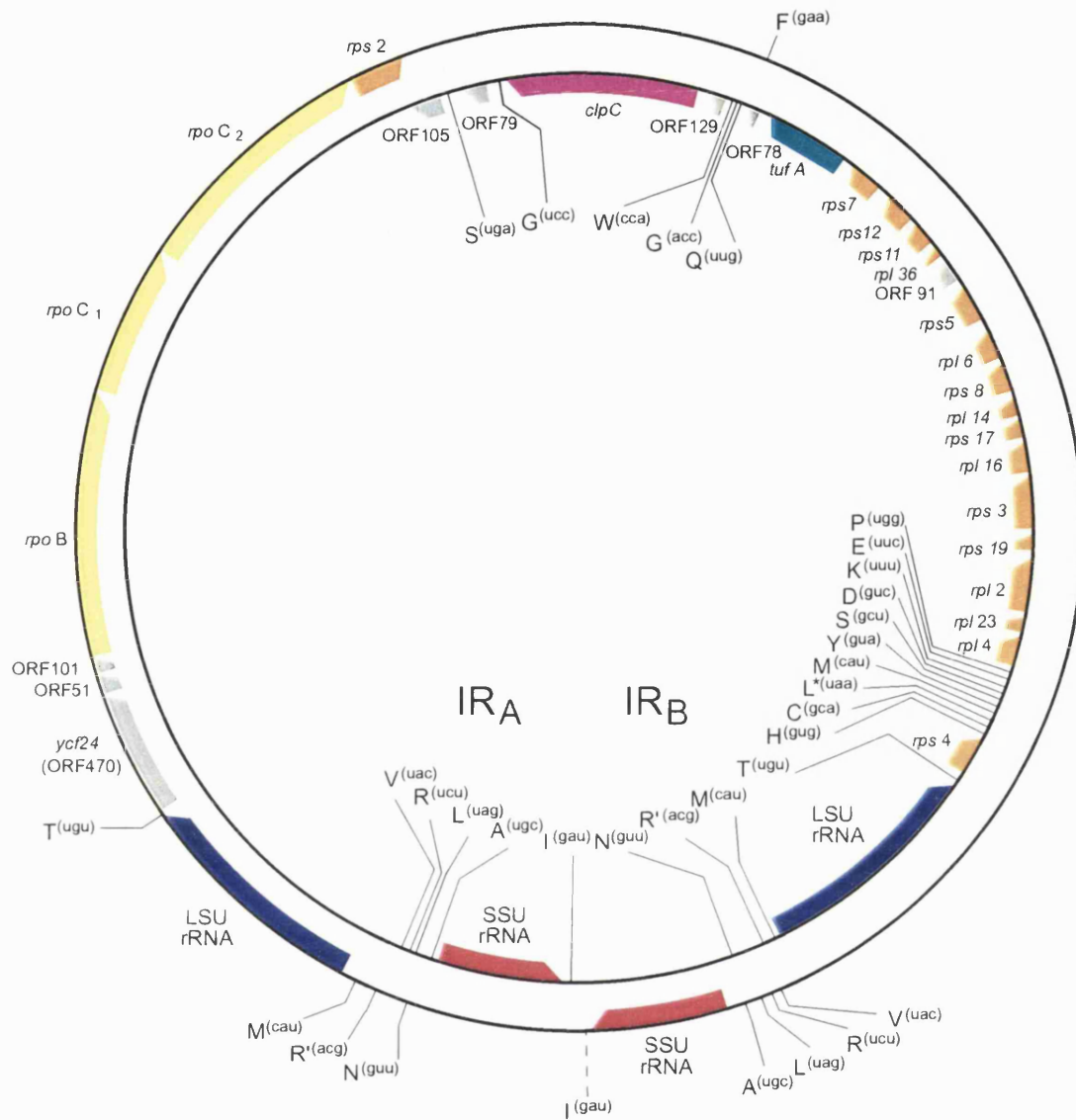


Figure 1.3: Complete Gene Map of the *P. falciparum* Plastid Genome.

The two halves (A and B) of the inverted repeat (IR) are indicated. tRNA genes are specified by the anticodon as well as the single letter amino acid code. ORFs specify the number of amino acid residues in the predicted polypeptide. Genes on the outer strand are transcribed clockwise, those on the inner strand anticlockwise. Modified from Wilson *et al.* (1996a).

1.2 The Plastid

1.2.1 Discovery

In retrospect it is evident that the plastid DNA was first observed in 1971 as a low density molecule in CsCl gradients of DNA prepared from the simian malaria parasite *Plasmodium knowlesi* (Gutteridge *et al.*, 1971). In 1972, similar DNAs were noted in two rodent malaria parasites, *Plasmodium chabaudi* and *P. berghei* (Chance *et al.*, 1972). Kilejian (1975) saw circular DNAs in electron micrographs prepared from the avian malarial parasite *Plasmodium lophurae*, with similar DNAs from *P. berghei* (Dore *et al.*, 1983)) and *T. gondii* (Borst *et al.*, 1984) being noted some years later.

Molecular studies began in 1985 when two A + T-rich DNA elements were isolated from *P. knowlesi* (Williamson *et al.*, 1985) and in 1988 the same molecules were isolated from *P. falciparum* (Gardner *et al.*, 1988). One of these was a 6 kb linear, tandemly repeated DNA (Aldritt *et al.*, 1989; Vaidya *et al.*, 1989; Feagin *et al.*, 1992); the other was a 35 kb circular DNA (Gardner *et al.*, 1988). It was understandably assumed that these extrachromosomal DNA elements both derived from the mitochondrion (Williamson *et al.*, 1985; Gardner *et al.*, 1988): bipartite mitochondrial genomes are rare, but they do exist in some plants (Small *et al.*, 1989). However, it was subsequently discovered that only the 6 kb DNA from the murine parasite *P. yoelii* co-fractionated with mitochondria (Wilson *et al.*, 1992) and encoded genes typical of mitochondria (Vaidya *et al.*, 1989). In addition, if the two extrachromosomal DNAs formed part of the same genome, they would be expected to co-operate in some way, and to have originated from a common ancestor; Feagin *et al.* (1992) found no evidence of the two genomes being evolutionarily related. The conclusion that the 6 kb linear DNA element alone was the true mitochondrial genome led researchers in the field to wonder about the function of the 35 kb circular DNA.

Evolutionary trees based on gene sequences show that apicomplexans are related to dinoflagellates (Silberman *et al.*, 1996), many of which contain plastids. This, and the genetic organisation of the 35 kb circular DNA led Wilson *et al.* (1991, 1993) to ask themselves whether it could be a plastid genome. The DNA was sequenced and characterised in this laboratory (Wilson *et al.*, 1996a) and despite the fact that the malaria parasite is non-photosynthetic, sequencing revealed many similarities between the 35 kb circular DNA and chloroplast genomes in plants and algae (Palmer, 1992). Plastid-like features of the 35 kb circular DNA include: an inverted repeat containing rRNA genes; the presence of *ycf24*; the presence of *rps2* immediately downstream of *rpoC₂*; the presence of an intron in the anticodon of the leucine tRNA gene; and ATCC (instead of GTCC) in the T-loop of the glutamic acid tRNA (Wilson *et al.*, 1996a).

It was proposed that malaria carries a vestigial plastid genome, like non-photosynthetic, parasitic plants (dePamphilis and Palmer, 1990; Wolfe *et al.*, 1992). Electron microscopic studies of apicomplexan species have shown the presence of an organelle with multiple membranes that remained undefined for over 20 years (McFadden and Waller, 1997). This organelle has previously been termed a “spherical body” (Aikawa, 1966; Kilejian, 1991), a “vacuole” (Rudzińska and Trager, 1962, 1968), a “*vacuole pleurimembranaire*” (Schrével, 1971), a “*Hohlzylinder*” (Siddall, 1992), and a “double-walled vesicle” (Hackstein *et al.*, 1995) amongst others. It seemed probable that this organelle was the location of the 35 kb circular DNA in *Plasmodium* species but it was in *T. gondii*, a closely related apicomplexan parasite of humans, that this DNA was first localised by *in situ* hybridisation of small subunit rRNA transcripts (McFadden *et al.*, 1996; Köhler *et al.*, 1997). Since it is highly unlikely that the transcripts were transported across the multiple membranes of the organelle, it was concluded that the DNA encoding the transcripts was situated within the organelle.

Other apicomplexans have been shown to harbour a homologous plastid genome and their genetic organisations appear to be similar (Wilson and Williamson, 1997). These include *T. gondii* (Borst *et al.*, 1984), *Sarcocystis* species (Hackstein *et al.*, 1995), *Eimeria tenella* (Dunn *et al.*, 1998), *N. caninum* (Gleeson and Johnson, 1999), *Theileria* species (Denny *et al.*, 1998) and *Babesia bovis* (Gozar and Bagnara, 1993, 1995). However, despite the fact that Tetley *et al.* (1998) identified putative plastid organelles in *Cryptosporidium parvum*, Zhu *et al.* (2000a), using degenerate PCR and dot-blot analysis, failed to find evidence of a plastid genome.

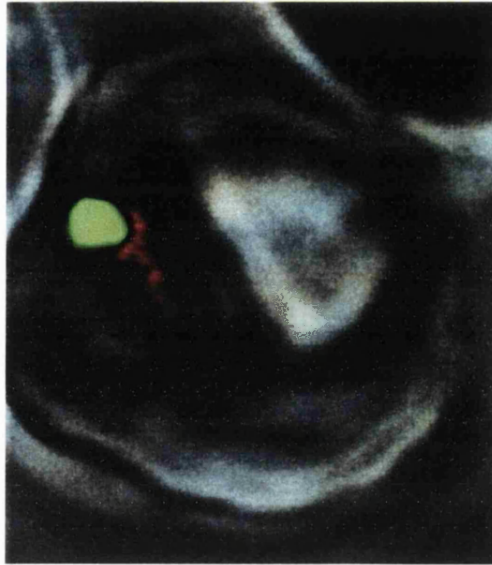
There is only one plastid per cell in apicomplexans, and there are only a few copies of the genome in *P. falciparum* (one to three per plastid) and *T. gondii* (up to eight per plastid) (Wilson, 1998). Therefore plastid DNA replication and segregation, and organelle division must be tightly controlled to ensure inheritance by daughter parasites. During erythrocytic stages of the parasite’s life cycle the plastid has been shown to be situated adjacent to the mitochondrion in avian malaria parasites (Aikawa, 1966) and in *P. falciparum*, *P. knowlesi* and *P. berghei* (Hopkins *et al.*, 1999; Bannister *et al.*, 2000; Waller *et al.*, 2000) (Figure 1.4). The significance of this association is unknown, although it may indicate metabolic interaction between the two organelles.

1.2.2 Origin of the Plastid

1.2.2.1 Endosymbiosis

Like some “algal” plastids (Gibbs, 1978, 1981), the malarial plastid is thought to have originated through secondary endosymbiosis (Wilson *et al.*, 1994) (Figure 1.5). Primary endosymbiosis resulting in the evolution of chloroplasts (and mitochondria) occurred when

a



b

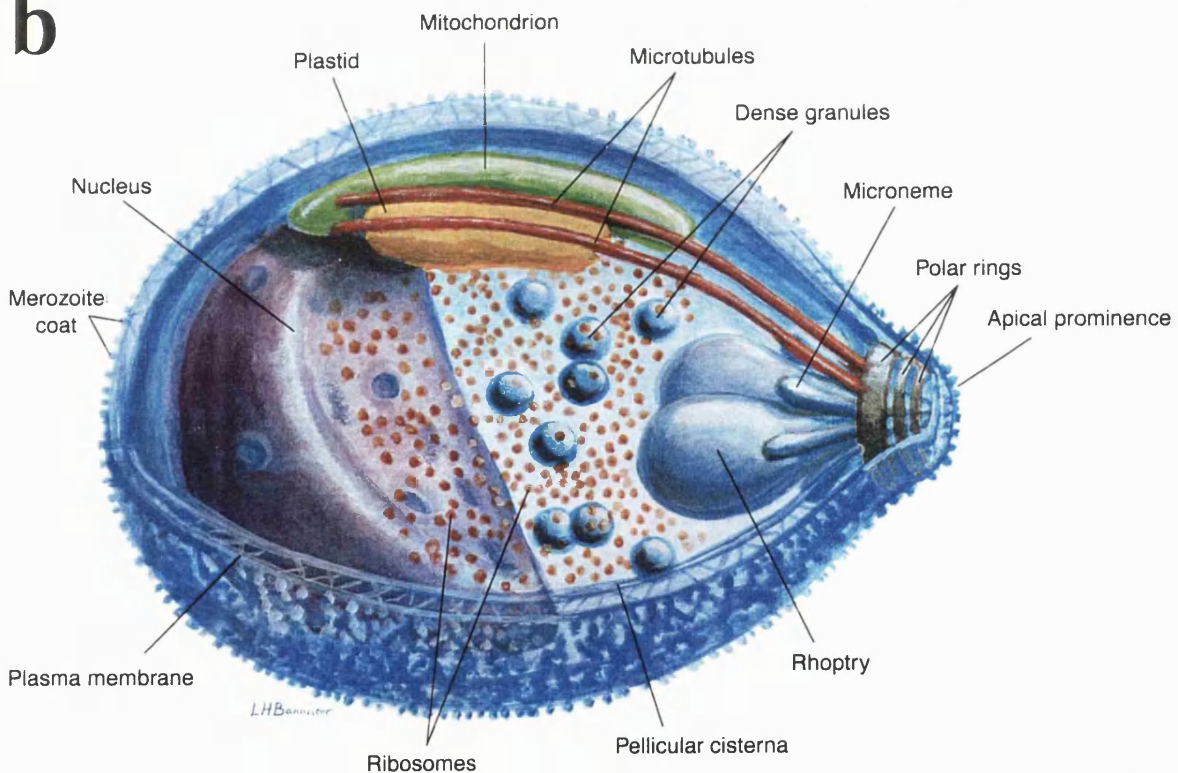


Figure 1.4: Subcellular Location of The Plastid.

Studies of electron micrographs (Hopkins *et al.*, 1999; Bannister *et al.*, 2000) and immunofluorescence (Waller *et al.*, 2000) show that the plastid is adjacent to the mitochondrion in *P. falciparum* erythrocytic stages.

a: Plastid-targeted GFP labels the plastid with green fluorescence; the mitochondrion is co-stained red. From Waller *et al.* (2000).

b: Artist's impression of the ultrastructure of a *P. falciparum* merozoite, based on electron micrographs. From Bannister *et al.* (2000).

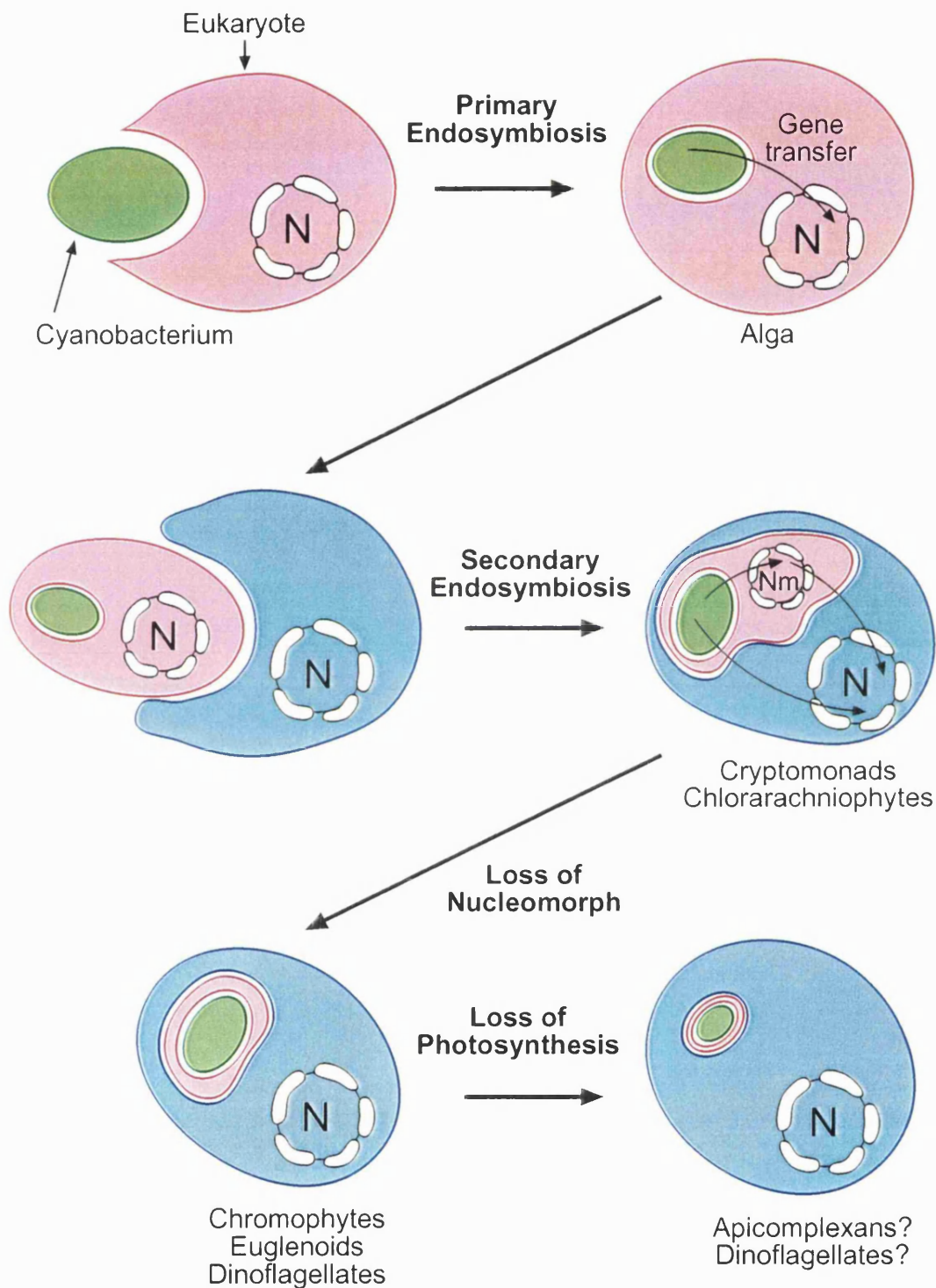


Figure 1.5: Secondary Endosymbiosis.

A heterotrophic eukaryote engulfed a photosynthetic bacterium. This process was followed by transfer of endosymbiont genes to the host nucleus, forming an alga. The alga was then taken up by another eukaryote, gene transfer again occurred and the algal nuclear genome was subsequently lost. This process resulted in a multimembraned plastid organelle. N = nucleus; Nm = nucleomorph. Modified from Wilson (1998).

an ancient eukaryotic heterotroph engulfed a cyanobacterium (probably a single event (Cavalier-Smith, 1993; Moreira *et al.*, 2000; Palmer, 2000)) forming the ancestor of modern day algae and higher plants (Gray, 1992). Genes from the genome of the endosymbiont were transferred to the host's genome in a manner that is not currently understood (Douglas, 1998; Martin *et al.*, 1998). Secondary endosymbiosis occurred when an alga, already possessing an endosymbiont (plastid), was engulfed by another eukaryotic cell. This has probably occurred many times during evolutionary history (Oliveira and Bhattacharya, 2000; Palmer, 2000). As a result of this process, the secondary endosymbiont's nuclear genome is lost, or transferred to the host cell's nuclear genome (although remnants of this eukaryotic "nucleomorph" genome are still present in chlorarachniophytes and cryptomonads (Ludwig and Gibbs, 1985; Gilson and McFadden, 1996; Palmer, 1997; Zauner *et al.*, 2000)).

An interesting example of a possible intermediate between endosymbiont and organelle is the bacterium *Buchnera* which resides within bacteriocytes in the pea aphid, *Acyrtosiphon pisum*, and is passed on to subsequent generations of aphid through the female gamete (Andersson, 2000). The endosymbiont supplies its host with essential amino acids and in return is provided with a stable, nutrient-rich environment. The genome of this organism was recently sequenced, and was found to lack "unnecessary" genes such as those required for antigenic variation or for entry into and exit from the host cell (Shigenobu *et al.*, 2000). Other genes that might be expected in a bacterial genome were missing, and it remains to be seen whether any of these have been transferred to the host's nucleus. An endosymbiont can be defined as an "organelle" once part of its genome has been transferred to the host's nucleus, thereby rendering it semi-autonomous (McFadden and Gilson, 1995), and once an organelle-specific protein import mechanism has developed (Cavalier-Smith and Lee, 1985; Cavalier-Smith, 2000).

Plastids that arose by secondary endosymbiosis are surrounded by multiple membranes (Gibbs, 1981) (for example the plastids of dinoflagellates, euglenoids, heterokonts, haptophytes, cryptomonads and chlorarachniophytes), whereas the chloroplasts of plants, green algae and red algae are surrounded by just two (Douglas, 1998). These multiple membranes arise from the two membranes (green and red; Figure 1.5) of the original plastid, the cell membrane (red; Figure 1.5) of the secondary endosymbiont (alga), and the phagocytic membrane (blue; Figure 1.5) of the host cell. The plastids of apicomplexans possess more than two membranes: three in *P. falciparum* (Hopkins *et al.*, 1999; Bannister *et al.*, 2000) and four in *T. gondii* (Köhler *et al.*, 1997). There is still some debate on the exact number and the significance of this apparent difference is not known; *P. falciparum* may have lost one of its original membranes (Hopkins *et al.*, 1999), although some groups believe that the plastid in *P. falciparum* is also surrounded by four membranes (Waller *et al.*, 2000) (see below).

1.2.2.2 *Transfer of Organelle Genes to the Nucleus*

The genes of endosymbionts have one of three fates: they may remain on the genome of the endosymbiont forming the organellar genome, they may be transferred to the host cell's nuclear genome, or if they were required for functions no longer carried out by the endosymbiont and redundant in the host cell then they may be lost from the cell altogether. Of the genes that are transferred to nuclear DNA, some of the protein products are imported back into the organelle, whereas others function in the cytosol of the host cell.

There are several theories that might explain what controls whether a gene remains in the organellar genome or is transferred to the host cell's nuclear genome. One is that consolidation of all genes onto one genome results in a more economic use of cell resources, since all genes can be expressed with one set of gene expression machinery. However, this argument would only be valid if all organellar genes had been transferred to the nucleus, since the retention of even one single protein-encoding gene on an organelle's genome requires the full complement of organellar protein synthesis components. One advantage of transferring genes to the nuclear genome is that they are subject to the same repair and recombination mechanisms as nuclear genes (Martin *et al.*, 1998), thereby avoiding the fate resulting from mutation accumulation evident in exclusively asexual populations (Muller, 1964; McFadden, 1999a, 1999b). Howe *et al.* (2000) point out that organellar genomes have a tendency to become very A + T-rich, and this causes a change in amino acid composition of the proteins (Barbrook *et al.*, 1998); they suggest these changes can become deleterious to the function of the protein, therefore transfer to the nucleus provides protection from these changes.

What dictates which genes remain on the organellar genome? It has been argued that genes remain because their products cannot be reimported back into the organelle; for example, they may be too hydrophobic to cross the organelle's membranes (Palmer, 1993, 1997) or they may contain hydrophobic regions that are confused with secretory signal peptides by the cell's export machinery (von Heijne, 1986); that aberrant codon usage prevents nuclear expression of organellar genes (Doolittle, 1998); or that the protein product may be toxic to the cell outside the organelle (Martin and Herrmann, 1998). To test these ideas, relocation studies where the products of plastid genes have been reimported into the plastid have been carried out. Kanevski and Maliga (1994) showed that the gene *rbcL* could be deleted from the chloroplast genome of tobacco, placed in the nucleus and its product, the highly hydrophilic large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, was successfully imported back into the chloroplast to form a functional enzyme. However, activity of the reimported enzyme was very low, and such mutants would probably be eliminated by natural selection in the wild (Cavalier-Smith, 2000). Allen (1993) and Allen and Raven (1996) believe that redox regulation of gene expression is the major reason for retention of genes in organellar genomes. Due to the high rate of production of oxygen free

radicals in organelles compared to the nucleus, organellar genomes mutate at a higher frequency. They propose that genes that have been transferred to the nucleus are protected from mutation, those remaining in the organellar genome may be redox regulated. Electron transport can be extremely harmful to the cell if its control breaks down, and the proteins required to mop up excess radicals must be synthesised rapidly when and where they are needed; organelle genes for structural proteins critical for redox balance, appear to be transcriptionally regulated in response to redox state (Race *et al.*, 1999).

Organellar genomes only encode a subset of the proteins required for them to carry out their function (Ellis, 1981). Throughout evolution, organellar genes are transferred to the nucleus, where the gene sequences must acquire the appropriate motifs necessary for their expression in the nuclear environment, and an in-frame upstream sequence encoding an amino-terminal organellar targeting peptide for the gene product to be targeted back to the appropriate subcellular location (dePamphilis and Palmer, 1989; Blanchard and Lynch, 2000). The genetic information to be transferred must first be present in at least duplicate copies to avoid disruption of cell metabolism during the transfer process. This is not a problem for most plastid-bearing organisms, which possess multiple copies of the plastid genome per cell, and organelle to nuclear transfer of DNA is thought to be a relatively common event (McFadden, 1999*b*). However, in order to transfer successfully the organellar DNA must integrate itself into the nuclear genome at a favourable locus. The gene must then acquire the sequence encoding an organellar targeting peptide and in some way improve the overall fitness of the cell in order that the organellar copy of the gene be lost and the nuclear version retained (Gleeson, 2000). Some groups of green algae maintain a copy of *tufA* on both the plastid and nuclear genomes, consistent with the hypothesis that this is an ongoing evolutionary process (Baldauf *et al.*, 1990).

As a result of plastid genes being transferred to the nucleus, cells have had to develop some method of co-ordinating expression from the two genomes. This is particularly important in the case of multisubunit enzymes where one subunit is nucleus-encoded and the other is plastid-encoded, for example ribulose-1,5-bisphosphate carboxylase/oxygenase. This regulation appears to be a two-way process: a large number of nuclear factors are required for expression of chloroplast genes, and the state of the plastid can influence nuclear gene expression. The molecular mechanisms of this regulation remain unknown (Somanchi and Mayfield, 1999).

1.2.2.3 *Ancestry of the Apicomplexan Plastid*

Molecular phylogeny of the nuclear small subunit rRNA genes shows that apicomplexans share a common ancestor with dinoflagellates (Gajadhar *et al.*, 1991; Kumar and Rzhetsky, 1996; Van de Peer *et al.*, 2000). The apicomplexan plastid probably arose when an alga was engulfed by a dinoflagellate-like progenitor of the Apicomplexa (Zhang *et al.*, 2000).

Dinoflagellates are a large group of aquatic protists, some of which are photosynthetic. They are thought to have acquired plastids through several independent secondary endosymbioses of chromophytic algae. It has been proposed that the progenitor of the Apicomplexa developed a parasitic lifestyle in a polychaete worm, the ancestor of the Insecta (including the mosquito) (Wilson *et al.*, 1994). Thus *Plasmodium* was well adapted to its insect vector before its vertebrate hosts evolved (Escalante and Ayala, 1995).

There is other evidence which indicates that apicomplexans share a common ancestor with photosynthetic organisms (Jeffries and Johnson, 1996). A gene encoding the glycolytic enzyme enolase has been identified in *P. falciparum*, and analysis of the encoded protein sequence showed that this version contains a pentapeptide insertion present in plant enolases, but not in those of animals or yeast (Hyde *et al.*, 1994; Read *et al.*, 1994). Similarly, a plant-like enolase and glucose-6-phosphate isomerase have been identified in *T. gondii* (Dzierszinski *et al.*, 1999); these enzymes are located in the cytosol. However, it is not known whether they were acquired *via* lateral gene transfer from the endosymbiont, or from a common ancestor of plants and apicomplexans. Hackstein *et al.* (1995) detected protochlorophyllide *a* and chlorophyll *a* bound to a photosystem reaction centre in *T. gondii*. In addition, they detected a portion of *psbA*, encoding the D1 protein of photosystem complex II in genomic DNA from *Sarcocystis muris*, *T. gondii* and *P. falciparum*. It is not known what function these proteins serve, nor their subcellular location in apicomplexans, but they are possibly the result of lateral gene transfer from the plastid to the nuclear DNA. However, no-one has been able to replicate the results of Hackstein *et al.* (1995) and the possibility remains that they are due to contamination from other organisms in their laboratory (Jeffries and Johnson, 1996).

Phylogenetic analyses of apicomplexan plastid genes have produced confusing results. The plastid has been shown to be most closely related to euglenoid plastids (Howe, 1992; Gardner *et al.*, 1994a; Egea and Lang-Unnasch, 1995), to red algal plastids (Wilson, 1993; Williamson *et al.*, 1994; Jeffries and Johnson, 1996; Cavalier-Smith, 2000; Law *et al.*, 2000; Zhang *et al.*, 2000) and to green algal plastids (Palmer and Delwiche, 1996; Köhler *et al.*, 1997). Blanchard and Hicks (1999) used both primary sequence data and genomic characters such as gene content and organisation and the presence of introns to try to determine the origins of the apicomplexan plastid. They concluded that it is derived from outside the green plastid lineage, but were unable to determine its exact origin. Results could be skewed due to the high A + T content of the apicomplexan plastid DNA. Sato *et al.* (2000) point out that adjusting phylogenetic analyses to take into account base composition can give different results, and using different genes with different methods results in different conclusions being drawn. Emerging sequence data from nuclear and organellar genomes should help to clarify the evolutionary relationships between different plastid-bearing organisms (Gray, 1999).

Lang-Unnasch *et al.* (1998) believe that although dinoflagellates probably acquired plastids several times, the apicomplexan plastid is ancient and was acquired before delineation of the Apicomplexa (Figure 1.6). This is supported by studies of gene order on plastid genomes in different apicomplexan species (Denny *et al.*, 1998). However, confirmation of the dinoflagellate ancestry of the apicomplexans will require further study of dinoflagellate chloroplast genomes, which have been found to be unusual, consisting of single-gene minicircles (Zhang *et al.*, 1999; McFadden, 1999c). Zhu *et al.* (2000a) agree with this hypothesis and propose that *C. parvum* (see above) at one time did possess a plastid organelle, but that this was subsequently lost. It would be interesting to know whether the nuclear genome of *C. parvum* contains any evidence that a plastid genome ever existed in this organism.

1.2.3 Function

1.2.3.1 Protein Synthesis

The plastid genome encodes a selection of the components required for protein synthesis (Wilson *et al.*, 1996a), but sequencing did not reveal a function for the plastid or for the genome. The original progenitor of the plastid may have been a photosynthetic alga, and conversion of the host cell from an autotrophic lifestyle to a parasitic one probably resulted in loss of the genes necessary for photosynthesis, leaving only the minimum required for protein synthesis in the plastid. As mentioned previously, the non-random loss of genes (the loss of genes required for photosynthesis, but the retention of genes required for gene expression and protein synthesis) is a strong indicator of a functional plastid genome (dePamphilis and Palmer, 1990; Wolfe *et al.*, 1992).

The plastid genome is transcriptionally active (Wilson and Williamson, 1997). Transcripts have been identified for many of the genes including 25 tRNA genes sufficient to decode all the genes present on the plastid genome (Gardner *et al.*, 1994b; Preiser *et al.*, 1995), the rRNA genes (Gardner *et al.*, 1991a, 1993), the RNA polymerase subunit genes (Gardner *et al.*, 1991b), *ycf24*, *tufA*, *clpC* and several of the ribosomal protein genes (Wilson *et al.*, 1996a). In *T. gondii*, strand specific hybridisation to rRNA transcripts also showed that the plastid genome is transcribed (Köhler *et al.*, 1997).

Electron micrographs reveal structures in the plastid of *T. gondii* comparable in size to the 70S ribosomes of plastids, mitochondria and bacteria (McFadden *et al.*, 1996, 1997), and experimental evidence has demonstrated the existence of polysomes carrying plastid-specific messenger RNAs (mRNAs) in *P. falciparum* (Roy *et al.*, 1999). It has been shown that the nucleus-encoded ribosomal proteins S9 and L28 are imported into the plastid in *T. gondii* (Waller *et al.*, 1998). In addition, the antimalarial action of plastid protein synthesis inhibitors such as thiostrepton (see below) demonstrates that protein

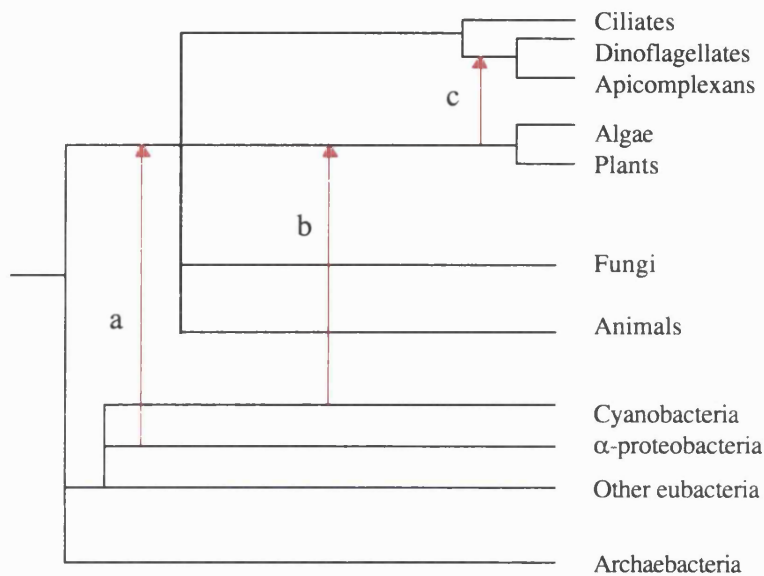


Figure 1.6: The Evolution of Plastids.

Schematic showing the processes of primary endosymbiosis (mitochondria (a) and plastids (b)), and secondary endosymbiosis (c). Distances are not phylogenetically accurate and not all branches are shown.

synthesis by the plastid is essential for the erythrocytic stage of the parasite (McConkey *et al.*, 1997). It is unlikely that the plastid would be carrying out protein synthesis purely for self-maintenance, hence it must have some other important function. Plant plastids are responsible for a range of biochemical processes as well as for photosynthesis, and even non-photosynthetic, parasitic plants retain their plastids, although their genomes are much reduced (dePamphilis and Palmer, 1990; Wolfe *et al.*, 1992).

1.2.3.2 *Porphyrin Synthesis*

Ironically, the malaria parasite is unable to salvage haem from its host's haemoglobin, and therefore relies on *de novo* haem biosynthesis (Raventos-Suarez *et al.*, 1982). It has been suggested that non-photosynthetic parasitic plants might retain their plastids for haem synthesis, since this is the only site in plants where this biosynthetic pathway is known to take place (Howe and Smith, 1991). The process of haem synthesis can be divided into two phases: 5-aminolaevulinic acid (ALA) synthesis, then conversion of ALA to protoporphyrin IX (the final common precursor of haem and chlorophyll). In plants, formation of ALA from glutamate requires activation of the glutamate by a plastid-encoded tRNA^{Glu} (Schön *et al.*, 1986; Kannangara *et al.*, 1988); the *P. falciparum* plastid genome has retained this tRNA, suggesting that this pathway could be used for porphyrin synthesis. However, an alternative pathway for ALA synthesis, using glycine as a precursor, is known to be used in the mitochondrion of the malaria parasite (Surolia and Padmanaban, 1992) and the required ALA synthase enzyme possessing a putative mitochondrial targeting sequence has been cloned (Wilson *et al.*, 1996b). Interestingly, it has been shown in *P. berghei* that ALA dehydratase is taken up from the host erythrocyte (Bonday *et al.*, 1997, 2000). It is not clear whether this enzyme is functional, but it has been proposed that at least some of the enzymes of this pathway may be provided by the erythrocyte. Conversion of ALA to haem occurs in the plastid in photosynthetic eukaryotes, with the final two steps involving the enzymes protoporphyrinogen oxidase and ferrochelatase, occurring in both the plastid and the mitochondrion (Little and Jones, 1976; Chow *et al.*, 1997). In non-photosynthetic eukaryotes, ALA is synthesised in the mitochondrion, then transported to the cytosol where it is converted to protoporphyrinogen. The final steps of haem synthesis are then carried out in the mitochondria.

A “plant-like” gene encoding ALA dehydratase has been identified in the nuclear genome of *P. falciparum* (Sato *et al.*, 2000). Although its transcription has been confirmed, the subcellular location of its product is not yet known. The predicted product of this gene possesses an amino-terminal extension that has the characteristics of a plastid-targeting peptide, and the predicted structure of the enzyme shows the active site to be conserved, so this enzyme could be functional (S. Sato, personal communication). However, ferrochelatase, the last enzyme of the haem synthesis pathway possesses a short amino-terminal sequence when compared to mitochondrial versions of this enzyme (human or

yeast). This enzyme may be targeted to the mitochondrion, or it may remain cytosolic (S. Sato, personal communication). Therefore, it is possible that in contrast to all other organisms, in *P. falciparum* ALA is synthesised in the mitochondrion (Surolia and Padmanaban, 1992), converted to protoporphyrinogen in the plastid, with the final steps of haem synthesis occurring either in the cytosol or possibly in the mitochondrion. It is interesting to note that this model depends on the transport of ALA from the mitochondrion to the plastid, and as mentioned previously these organelles are closely associated throughout the erythrocytic cycle (Figure 1.4). Future work must confirm that these enzymes are functional, and show their subcellular location by immunofluorescence or immunoelectron microscopy, or by targeting experiments.

1.2.3.3 Chorismate Synthesis

Chorismate is required for the synthesis of many aromatic compounds including 4-aminobenzoic acid and folic acid, ubiquinone, and the amino acids phenylalanine, tyrosine and tryptophan. Chorismate synthase, the final enzyme of the shikimate pathway is encoded in the nuclear genomes of both *P. falciparum* and *T. gondii*. The shikimate pathway is essential for algae, plants, bacteria and fungi, but is absent from mammals (hence “essential” amino acids). Roberts *et al.* (1998) proposed that chorismate synthesis could be a function of the plastid since the enzymes of this pathway are targeted to the chloroplast in plants (Mousdale and Coggins, 1985; Ireland, 1990; Reinbothe *et al.*, 1994). However, chorismate synthases from *T. gondii* and *P. falciparum* appear to lack a plastid-targeting signal and Keeling *et al.* (1999) suggested that this pathway occurs in the cytosol of the parasite, as is the case for fungi. Interestingly, phylogenetic analysis of chorismate synthase enzymes showed that the apicomplexan versions are more similar to fungal versions than to the plastid-targeted plant enzymes, but this fact alone is not proof that the pathway is cytosolic. It has not yet been demonstrated whether chorismate synthesis takes place in the plastid or the cytosol of the parasite. Roberts *et al.* (1999) suggest that perhaps apicomplexan chorismate synthase could function in both locations. It may have an unusual plastid-targeting sequence as is the case for some plant proteins (Hugosson *et al.*, 1995), for example its large insertions may function to target the enzyme to the plastid.

1.2.3.4 Fatty Acid Synthesis

Evidence has been obtained showing that enzymes involved in fatty acid synthesis, acyl carrier protein (ACP), FabH (3-ketoacyl-ACP synthase III) and FabZ (3-hydroxyacyl-ACP dehydrase), are imported into the plastid of *T. gondii*. Phylogenetic analysis showed that the genes encoding these enzymes are prokaryotic in nature, suggesting a plastidic origin. Antibodies to *T. gondii* ACP-glutathione S- transferase (GST) fusion proteins showed that these proteins are localised within the *T. gondii* plastid by immunofluorescence microscopy and by immunoelectron microscopy (Waller *et al.*, 1998). A gene encoding FabI (enoyl-

ACP reductase) has also been identified from the *P. falciparum* sequencing project, the predicted product of this gene possessing an amino-terminal extension that probably targets it to the plastid (Surolia and Surolia, 2001). In addition, an isozyme of acetyl-CoA carboxylase, which catalyses the first committed step of *de novo* fatty acid synthesis, has recently been shown to localise to the plastid in *T. gondii* (Jelenska *et al.*, 2001). These enzymes are of the type II fatty acid biosynthesis pathway, only found in bacteria and plastids. Waller *et al.* (1998) suggest this pathway would be a good target for combating both malaria and toxoplasmosis.

In plants and some algae, the plastid is the only site of fatty acid synthesis (Andrews and Ohlrogge, 1990). Interestingly, Zhu *et al.* (2000b) report the presence of type I fatty acid synthesis enzymes in *C. parvum*, an apicomplexan that apparently lacks a plastid (see above). *C. parvum* differs from other apicomplexans in many aspects of its biology and was found to branch off early from the apicomplexan phylogenetic tree constructed using 18S rRNA (Van de Peer and De Wachter, 1997).

Fatty acid biosynthesis by the plastid in *P. falciparum* and *T. gondii* could be a housekeeping function, but it has also been suggested that it is required for establishment of the parasitophorous vacuole during invasion of a new host cell, since the antiparasitic activity of many plastid inhibitors is only seen on invasion of a new host cell (Fichera and Roos, 1997; McFadden and Roos, 1999). This “delayed death” phenotype was also observed in a *T. gondii* transformant with defective plastid division (He *et al.*, 2001); these parasites were able to grow and divide, but stable transformants were never isolated. Parasites lacking a plastid were able to invade new host cells, although they grew very slowly and died without ever escaping from the host cell. It has been suggested therefore that the parasitophorous vacuole formed by plastid-deficient parasites is somehow dysfunctional, perhaps due to an altered lipid composition (He *et al.*, 2001).

1.2.3.5 Isoprenoid Synthesis

Different numbers of isopentenyl diphosphate molecules are condensed to form isoprenoids, which are required for the synthesis of steroids, carotenoids and chlorophyll (Stryer, 1988). In mammals and fungi, isopentenyl diphosphate is derived from the mevalonate pathway. This depends on the condensation of three molecules of acetyl coenzyme A (CoA) into 3-hydroxy-3-methylglutaryl (HMG)-CoA which is then reduced to mevalonate by HMG-CoA reductase. Mevalonate is subsequently converted to 3-isopentenyl diphosphate *via* a 5-phosphomevalonate intermediate. Only very low levels of HMG-CoA reductase activity have ever been recorded in malaria parasites (Vial *et al.*, 1984).

However, in some eubacteria, algae and plants, the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway is an alternative non-mevalonate pathway for the early steps of isoprenoid biosynthesis (Rohmer *et al.*, 1993). In the DOXP pathway, glyceraldehyde 3-phosphate and pyruvate are condensed to form DOXP, which is then converted to 2-C-methyl-D-erythritol 4-phosphate by DOXP synthase and DOXP reductoisomerase. This is then converted to isopentenyl diphosphate *via* several steps, many of which are as yet unidentified (Lange *et al.*, 2000).

Jomaa *et al.* (1999) used the sequencing data generated from chromosome 14 of *P. falciparum* to identify genes encoding two enzymes of the non-mevalonate pathway of isoprenoid biosynthesis, DOXP reductoisomerase and DOXP synthase. DOXP reductoisomerase possessed an amino-terminal extension with the features of a *P. falciparum* plastid-targeting sequence (Waller *et al.*, 1998). They subsequently showed that the amino-terminal extension of *P. falciparum* DOXP reductoisomerase could target GFP to the plastid of *T. gondii*. This suggests that this pathway takes place in the plastid of the malaria parasite.

1.2.3.6 Electron Transport

Hackstein *et al.* (1995) used fluorescence spectroscopy to show the presence of chlorophyll *a* and intermediates of chlorophyll biosynthesis bound to the photosynthetic complexes photosystems I and II in *T. gondii*. They were able to amplify *psbA*, encoding the D1 protein contained within photosystem II complexes, from genomic DNA of *S. muris*. Southern blots confirmed the presence of *psbA* in genomic DNA of *S. muris* and *T. gondii*; only a weak hybridisation was seen with *P. falciparum* DNA, but this may be because of its extreme A + T-richness. These results may be due to contamination, but if indeed they are valid it is a mystery why parasitic organisms, which spend most of their life cycle in the dark, would contain components of photosynthetic machinery. One suggestion is that it may form part of an electron transport chain, perhaps providing oxygen radicals for use in parasite invasion (Gleeson, 2000).

Recently, genes encoding ferredoxin-NADP⁺ reductase from *T. gondii* and a typical [2Fe-2S] ferredoxin from *P. falciparum* were cloned (Vollmer *et al.*, 2001). The gene encoding *P. falciparum* ferredoxin has been shown to be transcribed by the parasite. The predicted products of these genes possess amino-terminal extensions that are capable of targeting a reporter protein to the plastid. The *T. gondii* enzyme was expressed in *Escherichia coli* and shown to be functional. Ferredoxin is usually involved in electron transfer from photosystem I to NADP⁺ to form NADPH, which is needed as reducing power for the Calvin cycle for CO₂ fixation. However, this flow of electrons can also occur in reverse in non-photosynthetic plastids such as those found in roots. Reduced ferredoxin can then serve as a reducing agent for various plastid-localised enzymes. Interestingly, plants

possess different isoforms of ferredoxin and ferredoxin-NADP⁺ reductase depending on the direction of electron flow, and phylogenetic analyses of *T. gondii* ferredoxin-NADP⁺ reductase and of *P. falciparum* ferredoxin showed that these are more similar to the versions found in non-photosynthetic plastids. It has been proposed that these proteins exist in apicomplexans to provide reduced ferredoxin, perhaps for fatty acid biosynthesis or some other biochemical process, and that due to the absence of such a system in the host, this could represent a good potential drug target (Vollmer *et al.*, 2001).

1.2.3.7 *Ycf24*

Another clue to the rôle of the plastid may rest with elucidation of the function of one of the unidentified ORFs (Wilson and Williamson, 1997). For the plastid genome and protein synthesis within the plastid to be retained, they must be required for the synthesis of one of the genes encoded by the genome. Most of the ORFs on the plastid genome are small and may encode divergent ribosomal proteins (Wilson *et al.*, 1996a). However, the largest, *ycf24* (ORF470), has orthologues of unknown function on algal plastid and bacterial genomes and it is unlikely that the protein would remain so highly conserved if its function were unimportant. Gene disruption studies using the cyanobacterium *Synechocystis* (Law, 2000; Law *et al.*, 2000) have shown that Ycf24 has an essential function in this organism (see Chapter 6.1.2).

Recently, an *Arabidopsis thaliana* mutant for *ycf24*, with a reduced responsiveness towards far red light, was isolated (Møller *et al.*, 2001). It was concluded by these authors, based on the (probably erroneous) annotation of Ycf24 as a subunit of an ATP-binding cassette (ABC)-transporter in the databases, that Ycf24 is involved in intercompartmental communication for light-regulated gene expression. These findings will be discussed in more detail in Chapter 6.3.

Ycf24 in the plant pathogen *Pectobacterium (Erwinia) chrysanthemi*, has been found to form part of an operon involved in virulence (Nachin *et al.*, 2001). An orthologous operon exists in *E. coli* (Patzner and Hantke, 1999). Expression of these genes was induced under conditions of iron-deficiency, and iron levels were raised in bacteria mutant for these genes; it was therefore concluded that these genes are involved in iron metabolism, probably in Fe-S cluster assembly for resistance to oxidative stress during infection. These results will be described in more detail in Chapter 6.3, and their implications will be discussed in Chapter 7.3.

The possibility remains that *ycf24* encodes a housekeeping protein and elucidation of its function may not shed any light on the reason for the maintenance of the plastid in the malaria parasite (Wilson *et al.*, 1996a).

1.2.4 The Plastid as a Drug Target

The discovery of the plastid organelle in the malaria parasite offers the potential for development of antimalarial drugs to prokaryotic-like or plant-like targets (Soldati, 1999). As the organelle is unique to the parasite, it increases the chance of finding drugs that will not damage the host (man). Earlier work had shown that *P. falciparum* is affected by antibiotics more normally associated with antibacterial activity. Doxycycline (Bradley and Warhurst, 1995) and tetracycline (Colwell *et al.*, 1972) are actually prescribed as antimalarials and possibly target the mitochondrion.

Other antibiotics such as rifampicin (Alger *et al.*, 1970; Strath *et al.*, 1993; Pukrittayakamee *et al.*, 1994) and thiostrepton (Clough *et al.*, 1997; McConkey *et al.*, 1997) were selected for testing because they probably affect the plastid, having no known target in the mitochondrion. Rifampicin acts on the DNA-dependent RNA polymerase of prokaryotes, and was therefore assumed to target the plastid version of this enzyme in *P. falciparum* (Gardner *et al.*, 1991b). Strath *et al.* (1993) found that rifampicin was effective against ring stages of *P. falciparum in vitro* and against the murine malaria parasite *P. chabaudi in vivo*. However, in humans it was only effective in totally clearing parasitaemia when given in combination with primaquine (Pukrittayakamee *et al.*, 1994). Thiostrepton inhibits the interaction of EF-G with the ribosome by binding to a conserved region of the GTPase domain in the large subunit rRNA of bacteria and plastids, but not that found in the cytosol of eukaryotes. Only the 23S form specified on the plastid genome in *P. falciparum* has the optimal sequence for binding (Clough *et al.*, 1997). Pretreatment of mice *in vivo* with thiostrepton was found to prevent infection by *P. berghei*, and to reduce transmission to the mosquito; treatment of infected mice resulted in clearance of infected erythrocytes (Sullivan *et al.*, 2000). However, sporozoites pretreated with thiostrepton were able to develop normally.

Chloramphenicol, which exerts its effects by inhibiting prokaryotic 70S ribosomes, has previously been observed to have activity against *P. falciparum* (Geary and Jensen, 1983) and *T. gondii* (Beckers *et al.*, 1995). Although it has been suggested that this antibiotic acts against the mitochondrion (Geary and Jensen, 1983), Yung and Lang-Unnasch (1999) suggest that it could be inhibiting plastidic protein synthesis. They were able to target the reporter protein chloramphenicol acetyl transferase (CAT) to the plastid in *T. gondii*, and observed increased resistance to chloramphenicol in transformants compared to controls.

A range of structurally unrelated inhibitors of EF-Tu (including kirromycin, enacyloxin IIa and GE2270) was found to inhibit growth of *P. falciparum* cultures and to bind recombinant plastid EF-Tu in *in vitro* assays (Clough *et al.*, 1999). However, it remains to be proven whether these drugs act against the plastid-encoded EF-Tu, or a nucleus-encoded mitochondrial version in the parasite. Inhibitors of EF-Tu inhibited parasite growth

relatively rapidly in a similar manner to antibiotics such as tetracycline. However, others have found that some drugs specifically targeting the plastid act more slowly on the parasite than established antimalarials such as chloroquine (McFadden and Roos, 1999; Sullivan *et al.*, 2000).

Ciprofloxacin inhibits DNA gyrases in prokaryotes and chloroplasts, but does not affect mitochondrial DNA replication. Treatment of *T. gondii* tachyzoites in human fibroblasts caused a specific depletion of staining of extranuclear DNA with 4,6-diamidino-2-phenylindole (DAPI) indicating a specific action on plastid DNA replication (Fichera and Roos, 1997).

The discovery that the plastid harbours different metabolic pathways, and more importantly, metabolic pathways that do not exist in the host means that it should be a good drug target. Thiolactomycin is a selective inhibitor of type II fatty acid biosynthesis. In *E. coli* and plants it inhibits the enzymes FabB (acyl-malonyl-ACP condensing enzyme) and FabH, but has no effect on type I fatty acid biosynthesis in *S. cerevisiae*, *Candida albicans*, rat liver (Hayashi *et al.*, 1983) or the apicomplexan, *C. parvum* (Zhu *et al.*, 2000b). Thiolactomycin, has been shown to inhibit *P. falciparum* growth *in vitro* (Waller *et al.*, 1998). Triclosan, an antibacterial commonly found in deodorants, mouthwashes and toothpaste, inhibits the enzyme FabI in type II fatty acid biosynthesis. It has recently been shown to have antimalarial activity against *P. falciparum* *in vitro* and *P. berghei* *in vivo* (Beeson *et al.*, 2001; Berger, 2001; Surolia and Surolia, 2001). *P. falciparum* possesses enzymes of the DOXP, non-mevalonate pathway of isoprenoid biosynthesis and inhibitors of DOXP reductoisomerase, fosmidomycin and its derivative FR-900098, were shown to suppress the growth of *P. falciparum* *in vitro* and to clear *Plasmodium vinckei* infection in mice *in vivo* (Jomaa *et al.*, 1999).

P. falciparum has been found to be sensitive to certain herbicides, either as a result of the presence of the plastid itself, or the presence of enzymes specified by plant-like genes in the nucleus. Glyphosate (*N*-(phosphonomethyl) glycine), the herbicide “RoundUp™”, inhibits the shikimate pathway enzyme 5-enolpyruvyl shikimate 3-phosphate synthase, and has been shown to inhibit the *in vitro* growth of *T. gondii*, *P. falciparum* and *C. parvum* (Roberts *et al.*, 1998). This inhibition was reversed by addition of 4-aminobenzoic acid or of folic acid, suggesting that the major role for the shikimate pathway in apicomplexans is folate biosynthesis. Although the shikimate pathway is possibly cytosolic in apicomplexan parasites (Keeling *et al.*, 1999), this does not preclude its potential as a drug target.

All apicomplexans tested so far have been found to be sensitive to triazine herbicides such as toltrazuril. This sensitivity suggests that the chlorophyll *a*-D1 complex provides some essential function for the parasite (Hackstein *et al.*, 1995). However, triazines can also

inhibit some non-plastid-containing organisms, leading Harder and Haberkorn (1989) to suggest that these herbicides have some other target such as electron transport.

Dinitroaniline herbicides inhibit plant microtubules (Bajer and Molè-Bajer, 1986) and have also been found to have activity against a variety of protoctistans including trypanosomes (Chan *et al.*, 1993), *P. falciparum* (Kaidoh *et al.*, 1995) and *T. gondii* (Stokkermans *et al.*, 1996). Trifluralin was found to inhibit growth and differentiation of erythrocytic stage *P. falciparum*, and also to block maturation and exflagellation of gametocytes; the effect of this herbicide on malarial microtubules was highly specific and it did not bind to mammalian microtubules (Kaidoh *et al.*, 1995). Stokkermans *et al.* (1996) found that herbicide-treated *T. gondii* remained metabolically active but were unable to divide or produce infectious progeny; there was no effect on parasite invasion. Interestingly, phylogenetic analysis of tubulin protein sequences shows that apicomplexan tubulins are more similar to tubulins of plants and algae than to those of animals.

Aryloxyphenoxypropionate herbicides inhibit the single subunit acetyl-CoA carboxylase found in the plastids of monocotyledonous plants. These herbicides do not affect this enzyme in dicotyledonous plants, bacteria, man, chicken, rat or yeast. Herbicides of this type have been found to inhibit growth of *T. gondii* *in vitro* (Zuther *et al.*, 1999). Moreover, sequences encoding a single subunit acetyl-CoA carboxylase have been identified in *T. gondii*, *P. falciparum*, *P. knowlesi* and *C. parvum*; and one isozyme has been shown to localise to the plastid in *T. gondii* (Jelenska *et al.*, 2001). This provides further evidence for transfer of plant-like genes to the nuclear DNA of apicomplexan parasites.

1.3 Protein Import into Plastids

1.3.1 Chloroplast Import

In plants and algae, a transit peptide is sufficient and necessary for targeting a protein to the chloroplast (Chua and Schmidt, 1979). One possibility is that these peptides are derived from the original substrate of a bacterial exporter channel that now functions as a chloroplast importer (McFadden, 1999a; Cavalier-Smith, 2000) (see below) and that plastid genes acquired the necessary coding sequences through exon shuffling (Gantt *et al.*, 1991; Sahrawy *et al.*, 1996; Bruce, 2000). Transit peptides have been shown to comprise three distinct domains (von Heijne *et al.*, 1989; Claros *et al.*, 1997; Bruce, 2000): an uncharged amino-terminus (approximately 10 residues) beginning with a methionine followed by an alanine, and ending with glycine or proline; a central domain lacking acidic residues but rich in serine and threonine residues (von Heijne and Nishikawa, 1991), and a carboxy-terminal domain rich in arginine residues. The paucity of acidic residues results in an overall positive charge (von Heijne *et al.*, 1989). Despite predictions to the contrary (Ellis, 1981),

no consensus sequence for chloroplast transit peptides has been identified (Keegstra, 1990; Keegstra and Cline, 1999), so despite the growing availability of sequence data, identifying chloroplast-targeted proteins is not a straightforward task.

Although there does not appear to be a consensus secondary structure for plastid-targeting peptides, targeting does appear to be a function of the secondary structure of the presequence (Hugosson *et al.*, 1995). In aqueous solution, transit peptides lack discernible structure; however, in a more hydrophobic (lipid) environment, one or more regions of the transit peptide become α -helical (Bruce, 2000; Schleiff and Soll, 2000). Transit peptides have to be identified experimentally by *in vitro* import assays or *in vivo* using a reporter protein such as GFP (McFadden, 1999a). Recently a neural network-based method has become available on the internet that predicts chloroplast transit peptides (Claros *et al.*, 1997; Emanuelsson *et al.*, 1999). This type of facility, combined with emerging sequence data from a variety of organisms, should facilitate the prediction of plastid-targeted proteins.

In order for proteins to be successfully imported back into a plastid, protein import machinery is required in the membranes surrounding the organelle. This machinery (Toc (outer membrane) and Tic (inner membrane) proteins (Schnell *et al.*, 1997)) must have the ability to recognise plastid proteins (as opposed to those destined for other organelles), take up the cytosolic precursors, cleave the transit peptides, and release the processed polypeptides into the stroma (Martin and Herrmann, 1998; Caliebe and Soill, 1999). It is not known how many components make up the chloroplast import machinery; about a dozen have been identified and Keegstra and Froehlich (1999) suggest that there are probably not many more, if any, to be identified.

Transport of proteins to plastids that arose by primary endosymbiosis occurs post-translationally (Ellis, 1981). After synthesis on cytoplasmic ribosomes it is thought that interaction between the transit peptides and galactolipids in the outer chloroplast membrane induces specific secondary structures that are recognised by Toc receptor proteins (van 't Hof *et al.*, 1991; Caliebe and Soill, 1999; Chen and Schnell, 1999). Chen and Li (1998) showed that *A. thaliana* mutants deficient in digalactosyldiacylglycerol are unable to import chloroplast protein precursors. Three putative outer membrane receptors have been identified: Toc120, Toc132 and Toc159. Toc159 at least was found to be essential for chloroplast biogenesis in *A. thaliana*, leading to the conclusion that these different receptors may have different specificities for different transit peptides or different types of plastid (Bauer *et al.*, 2000). Plastid proteins are then transported in an ATP/GTP-dependent manner (Toc33/34, Toc120, Toc132 and Toc159 possess GTP-binding sites (Kessler *et al.*, 1994; Keegstra and Froehlich, 1999; Cline, 2000)) in an unfolded state through the chloroplast membranes into the stroma through junctions where the Toc and Tic

proteins associate in a supercomplex (Chen *et al.*, 2000). Hydrolysis of stromal ATP (perhaps by chaperones) is required for insertion of the translocating protein into the Tic apparatus; translocation across both membranes occurs simultaneously. Once in the stroma, a specific stromal processing metallopeptidase removes the transit peptide to form the mature protein (Smith and Ellis, 1979; Keegstra, 1990). Tobacco plants (*Nicotiana tabacum*) engineered to contain an antisense version of the gene encoding this peptidase, were found to have a severely altered phenotype including a reduced number of chloroplasts and aberrant chloroplast morphology; chloroplasts isolated from such mutants were defective in import processes (Wan *et al.*, 1998). Some proteins possess a further signal peptide downstream of the transit peptide which serves to target them to the thylakoid membrane or lumen (Cline and Henry, 1996). Deletion studies of a *Silene pratensis* chloroplast transit peptide have shown that different domains of the ferredoxin transit peptide are required for chloroplast targeting and interaction with lipids in the chloroplast outer membrane, translocation across the envelope, and processing of the protein precursor (Rensink *et al.*, 1998).

The chloroplast import machinery is thought to have evolved from bacterial protein export systems, although it does not share any structural homology with other eukaryotic import/export systems such as that of mitochondria (Caliebe and Soill, 1999; Chen and Schnell, 1999). Toc75, an ion channel found in the outer chloroplast membrane (Schnell *et al.*, 1994), is involved in protein import (Hinnah *et al.*, 1997). A homologue that has been identified in the cyanobacterium *Synechocystis* is thought to be involved in protein export (Bölter *et al.*, 1998; Reumann *et al.*, 1999). Toc75 is nucleus-encoded, possesses a cleavable bipartite transit peptide and is transported into the chloroplast, then targeted back out to the outer chloroplast membrane (Schleiff and Soll, 2000). This suggests that the channel is positioned in the membrane in the same orientation as it was in the ancestral endosymbiont, and has evolved to function in reverse for import into the organelle, rather than the channel being inserted into the membrane in the opposite orientation (Cavalier-Smith, 2000). Reumann and Keegstra (1999) argue that the chloroplast protein import apparatus is of dual origin, since they could not identify homologues of Toc159, Toc33/34 or Tic110 in the complete genome of *Synechocystis* PCC6803, although they did identify homologues of Tic22 and Tic20. Homologous components of the chloroplast import machinery (Keegstra and Cline, 1999) have been identified in *P. falciparum* (van Dooren *et al.*, 2000; Waller *et al.*, 2000).

1.3.2 Import into Complex Plastids

Protein import is further complicated in the case of plastids that arose by secondary endosymbiosis as there are more than two membranes surrounding the organelle. The outermost membrane of plastids that arose by secondary endosymbiosis, is derived from the phagosome (Figure 1.5), which means that the organelle is situated “outside” the host

within the lumen of the host's endomembrane system. Targeting of proteins to such plastids is believed to occur *via* the secretory pathway (Schwartzbach *et al.*, 1998; Bodyl, 1999; Kroth and Strotmann, 1999). In *Euglena*, which has a chloroplast surrounded by three membranes, proteins are thought to be transported to the chloroplast *via* the endoplasmic reticulum (ER) and the Golgi apparatus (Sulli and Schwartzbach, 1995; Bodyl, 1997; Schwartzbach *et al.*, 1998). The amino-terminal plastid-targeting sequence of proteins targeted to these organelles consists of two parts (Lang *et al.*, 1998; Deane *et al.*, 2000). At the extreme amino-terminus is a classic, hydrophobic signal peptide, which directs the newly synthesised protein into the ER *via* signal recognition particle-mediated translocation, this occurs co-translationally as is the case for secreted proteins (Blobel and Dobberstein, 1975). The second part of the plastid-targeting sequence resembles a plant transit peptide and directs the protein to the plastid (Schwartzbach *et al.*, 1998; Wastl and Maier, 2000).

This model accounts for transport across the outer plastid membrane (secretion) and the inner two plastid membranes (plastid import), and hence is sufficient to explain import into plastids surrounded by three membranes. However, in the case of plastids surrounded by four membranes it does not account for transport across the second plastid membrane derived from the endosymbiont's plasma membrane (Figure 1.5). Once the plastid-targeted protein has crossed the outer membrane, it is essentially outside both the host cell and the organelle. The mechanism of crossing the second membrane is unknown (McFadden, 1999a).

In some complex plastids surrounded by four membranes the outer membrane is continuous with the ER, and in some cases contains ribosomes (Gibbs, 1962, 1979; Bodyl, 1999). In these situations plastid proteins are believed to be translated on the "plastid ER" ribosomes, and co-translationally imported through the outermost plastid membrane and then transported into the plastid *via* vesicles (Douglas, 1994; Schwartzbach *et al.*, 1998; Wastl and Maier, 2000).

1.3.3 Protein Targeting to the Apicomplexan Plastid

The plastid in the malaria parasite has the most reduced plastid genome so far described. It mainly encodes proteins involved in protein synthesis, but not all the genes that would be required for a functional gene expression system are carried on the plastid genome. Structures similar in size to 70S prokaryotic and plastid ribosomes have been observed, and antibiotics that target prokaryotic protein synthesis have an antimalarial effect. This, and the fact that the plastid genome has been retained, must mean that the genome is functional. This being the case, the remaining components required for protein synthesis must be encoded in the nuclear genome and imported into the plastid.

Estimates of 1 000 to 5 000 nucleus-encoded genes required for chloroplast function have been made (Martin and Herrmann, 1998; Abdallah *et al.*, 2000). It is not known exactly how many proteins are found in the apicomplexan plastid, but by comparison to chloroplasts, and even when proteins involved in photosynthesis have been removed from the equation, there could be approximately 800 nucleus-encoded plastid proteins in apicomplexans (Waller *et al.*, 2000).

Waller *et al.* (1998) used the *P. falciparum* genome sequencing database, and the *T. gondii* expressed sequence tag (EST) database to search for nucleus-encoded proteins that are potentially targeted to the plastid. They identified the genes for ribosomal proteins S9 and L28 and the fatty acid synthesis proteins ACP, FabH and FabZ. The predicted proteins all possessed amino-terminal extensions when compared with the orthologous protein sequences from bacteria. These extensions resembled bipartite plastid-targeting sequences consisting of a signal peptide containing a hydrophobic domain followed by a “von Heijne” cleavage site (Nielsen *et al.*, 1997), followed by a transit peptide rich in serine and threonine residues in the *T. gondii* proteins, and rich in lysine and asparagine residues in *P. falciparum* proteins. The amino-terminal extension of *T. gondii* ACP was capable of targeting GFP to the plastid in *T. gondii* (Waller *et al.*, 1998). The putative amino-terminal plastid-targeting sequences of *P. falciparum* ACP and FabH were subsequently shown to target GFP to the plastid in *P. falciparum* (Waller *et al.*, 2000).

The transit peptide domains of plastid-targeted proteins in *P. falciparum* are unusual in that they do not contain a high proportion of serine and threonine residues like other transit peptides (Waller *et al.*, 1998). This is possibly a result of the high A + T-content of the *P. falciparum* genome. Moreover, Waller *et al.* (2000) were able to show that the two threonine residues present in the transit peptide of FabH (there are no serines) were not required for correct targeting of GFP. In contrast, serine and threonine residues have been shown to be essential for chloroplast targeting in plants.

Waller *et al.* (2000) created GFP fusions with different domains of the amino-terminal leader peptide of *P. falciparum* plastid-targeted ACP, and used these to study the process of protein trafficking to the malarial plastid. A fusion containing just the transit peptide domain of the leader peptide resulted in accumulation of GFP in the cytosol of the parasite, showing that the signal peptide, and thus entry into the secretory pathway, is necessary for targeting to the plastid. When only the signal peptide domain was present, GFP was exported from the parasite into the parasitophorous vacuole (and some GFP was seen within the erythrocyte). These results show that the signal peptide is necessary for entry into the secretory pathway, which in turn is necessary for plastid-targeting; if no transit peptide is present then the default secretory pathway is followed leading to export.

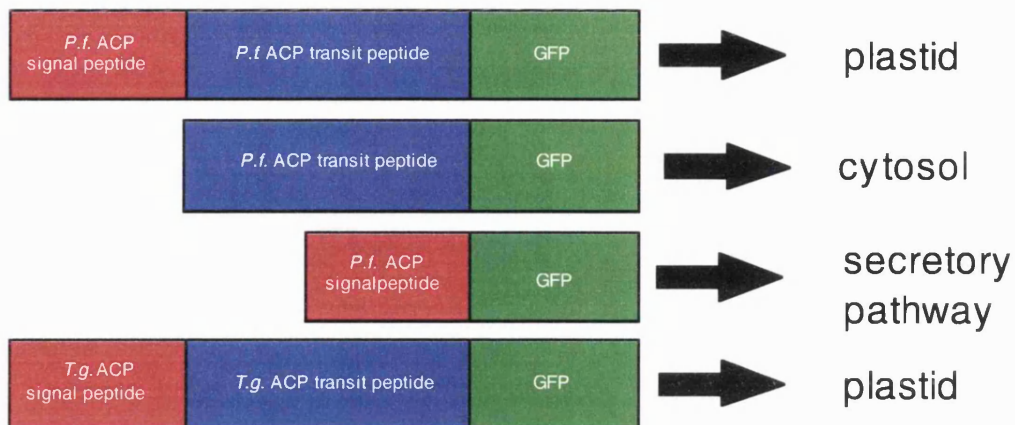
Protein targeting to the plastid in *T. gondii* has also been shown to occur *via* the secretory system, and to require a bipartite plastid-targeting sequence. The amino-terminal leader sequence of *T. gondii* ACP was used to target GFP to the plastid in *T. gondii* (Waller *et al.*, 1998). Similarly, it has been shown that the amino-terminal extensions of ribosomal proteins S9 and L28 of *T. gondii* are capable of targeting GFP to the *T. gondii* plastid (Yung and Lang-Unnasch, 1999; DeRocher *et al.*, 2000). DeRocher *et al.* (2000) carried out some deletion analysis of the S9 transit peptide and found that it has a high level of redundancy. However, if all of the putative transit peptide is absent, GFP is exported into the parasitophorous vacuole (Roos *et al.*, 1999a). Surprisingly, in fusions possessing only the transit peptide, fluorescence did not remain cytoplasmic as seen in the case of *P. falciparum* (Waller *et al.*, 2000), or as seen for GFP lacking any targeting sequence (Striepen *et al.*, 1998), but appeared to be targeted to the mitochondrion. The significance of this observation is unclear, especially since the *T. gondii* plastid is “hidden” in the secretory system and requires a signal peptide for targeting; dual targeting is rare, but there are a few examples in plants of transit peptides that direct proteins to both the mitochondria and chloroplasts (Creissen *et al.*, 1995; Chow *et al.*, 1997; Menand *et al.*, 1998; Small *et al.*, 1998; Hedtke *et al.*, 2000; Cahoon and Stern, 2001). DeRocher *et al.* (2000) were also able to demonstrate that *in vitro* translation products of the S9 transit peptide and GFP were imported into pea chloroplasts *in vitro*, confirming the functional significance of this sequence.

The process of plastid import in apicomplexans is highly conserved, both within the phylum, and between apicomplexans and other plastid-bearing organisms. *P. falciparum* and *T. gondii* plastid-targeting sequences are interchangeable. When the *P. falciparum* ACP leader sequence was replaced with the *T. gondii* ACP leader sequence, GFP was correctly targeted to the *P. falciparum* plastid (Roos *et al.*, 1999b; Waller *et al.*, 2000). *P. falciparum* leader sequences are also capable of targeting reporter proteins to the *T. gondii* plastid (Jomaa *et al.*, 1999; Roos *et al.*, 1999a, 1999b; van Dooren *et al.*, 2000).

When fused to a transit peptide, the signal sequence of P30 that directs the major surface antigen of *T. gondii* to the plasma membrane *via* the rough ER, is capable of directing GFP to the plastid (Roos *et al.*, 1999a). Moreover, when the P30 signal peptide was fused to the transit peptide of an *A. thaliana* chloroplast protein (FtsZ), GFP was targeted to the plastid in *T. gondii* (Roos *et al.*, 1999a, 1999b; van Dooren *et al.*, 2000). These targeting experiments are summarised in Figure 1.7.

Western blot analyses showed that processing of the preprotein is a two step process (Waller *et al.*, 2000). The signal peptide is probably removed during co-translational import into the ER and the transit peptide by peptidases within the plastid. Motifs denoting the cleavage site for the transit peptide have not yet been identified, however, *T. gondii* transit peptides are correctly processed by *P. falciparum*, suggesting some sort of

a



b

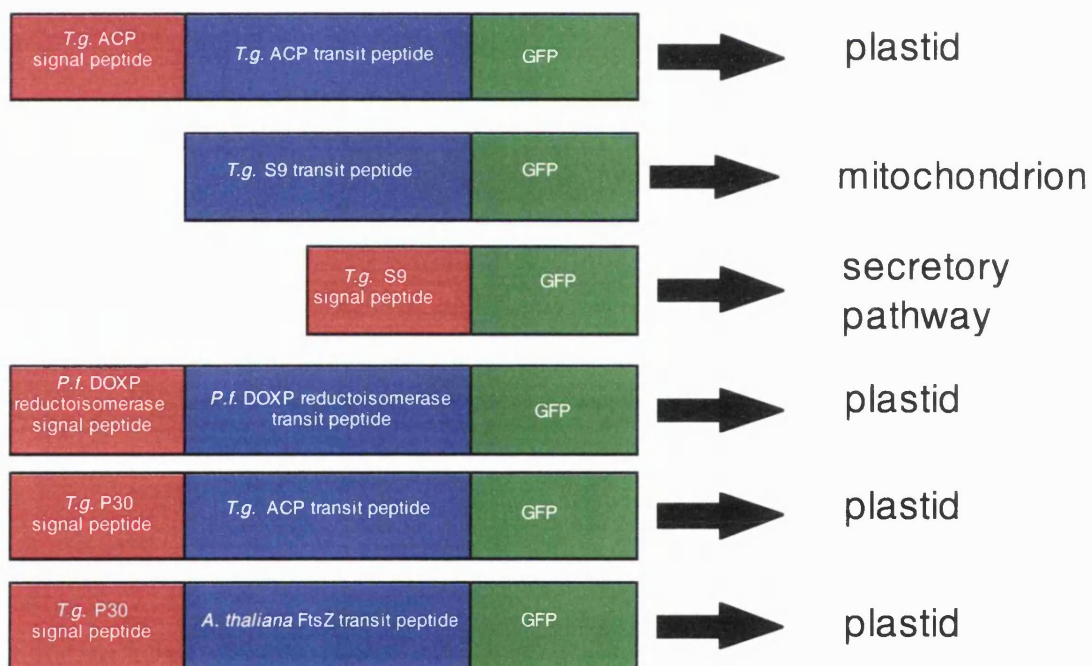


Figure 1.7: Targeting to the Apicomplexan Plastid. GFP was fused to different signal peptides and transit peptides in order to study protein trafficking to the plastid in *P. falciparum* (a) and *T. gondii* (b).

conserved motif. Hopefully this will be identified as more apicomplexan plastid-targeted proteins are discovered.

No plastid-targeted GFP was observed within the endomembrane network in *P. falciparum*, so targeting to the plastid must be a highly efficient process. This also means that the exact route to the plastid is unknown. The outer membrane of the plastid is thought to form part of the parasite's endomembrane system, and it has been suggested that the plastid lies within the secretory system and that all secreted proteins cross the outer membrane, but only those bearing a transit peptide are recognised by the plastid import apparatus (van Dooren *et al.*, 2000). Bodyl (1999) suggests that in apicomplexans, plastid proteins are transported from the ER *via* the Golgi apparatus to the plastid. No typical Golgi apparatus has been observed in *P. falciparum*, although this organelle is present in close proximity to the plastid in *T. gondii*. However, some Golgi protein homologues have been identified in *P. falciparum* suggesting that Golgi functions are retained (van Dooren *et al.*, 2000).

The model of targeting to the plastid of *P. falciparum* accounts for transport across three membranes. If there are four membranes surrounding the plastid of *P. falciparum* as there are in *T. gondii* as some suggest (Waller *et al.*, 2000), then it is not clear how imported proteins cross the second plastid membrane. Despite observations to the contrary (Hopkins *et al.*, 1999; Bannister *et al.*, 2000), the fact that the leader sequences of *P. falciparum* and *T. gondii* are interchangeable might suggest that they do possess the same number of membranes. However, the fact that a plant transit peptide is capable of targeting a protein into the apicomplexan plastid (see above), and that the extra membrane did not present a problem for this system (Roos *et al.*, 1999a) suggests it is more likely that no specific mechanism is required for a protein to cross the second membrane.

Data emerging from the *P. falciparum* genome sequencing project have allowed the identification of several plastid-targeted proteins. On analysis of the proteins predicted to be encoded by chromosome 2, 16 were found to be most similar to bacterial proteins (Gardner *et al.*, 1998b, 1999). One of these, FabH, was subsequently shown to be localised to the plastid (Waller *et al.*, 1998). The complete sequence of chromosome 3 has enabled the identification of probable organelle-localised proteins based on the presence of an amino-terminal organelle-targeting sequence (Bowman *et al.*, 1999). Three proteins were predicted to target to the mitochondrion: lipoamide acyltransferase, EF-Ts and glycerol-3-phosphate dehydrogenase, and two to the plastid: long chain fatty acid CoA ligase and ATP-dependent Clp protease (ClpP). Three other proteins that possess amino-terminal extensions, but whose subcellular localisations are unclear are: valyl-tRNA synthetase, aspartyl protease and a formate transporter. If distinctive features of apicomplexan plastid-targeting signals can be identified, then the completion of the

P. falciparum genome sequence should enable the identification of all plastid proteins, and hence the function of the plastid (Roos *et al.*, 1999a).

1.4 Aims of this Project

At the outset of this project nothing was known about nuclear genes encoding proteins imported into the malarial plastid organelle. The primary objective was to identify such genes and characterise the leader sequences directing their import. It was hoped that identification of genes encoding plastid-targeted proteins, and characterisation of their products, would provide us with more information on plastid targeting and biogenesis. The ultimate aim was to identify a function for the apicomplexan plastid and its genome. These objectives were to a large extent overtaken by events internationally as the genome sequencing project has progressed rapidly. As described above, several plastid-targeted proteins have now been identified, and shown to be targeted to the plastid in *P. falciparum* (Waller *et al.*, 1998; Waller *et al.*, 2000). In view of this, two additional areas of work were embarked upon.

Little is known about the mechanism of plastid division in apicomplexans, and this project set out to investigate the presence of genes encoding the organellar division proteins FtsZ and dynamin-related protein (Chapter 4). FtsZ was thought to be essential for prokaryotic cell division (Erickson, 1997) until very recently when it was found not to be encoded in the completed genomes of some bacteria. FtsZ is, however, involved in the division of at least some chloroplasts (Lutkenhaus, 1998). Dynamin-related protein has been found to be involved in mitochondrial division, but as discussed in Chapter 4, its rôle may be more diverse (Erickson, 2000).

It was also decided to study *ycf16*, a gene encoding a protein of unknown function, which is found adjacent to, or very close to *ycf24* (ORF470 (Wilson *et al.*, 1996a)) in most prokaryotic and algal chloroplast genomes examined (Chapters 5 and 6). The currently available genomic sequence database was searched for *ycf16* in *P. falciparum*. Following on from the work of Law (2000) and Law *et al.* (2000) the targeted disruption of *ycf16* in the surrogate organism *Synechocystis* species PCC6803, which has an amenable genetic transformation system, was to be tested.

Chapter 2 - Materials and Methods

2.1 Materials

General purpose reagents were supplied by British Drug Houses unless otherwise stated.

2.2 Media, Solutions and Buffers

Compositions of media, solutions and buffers are given in Appendix I.

2.3 Bioinformatics

Alignment and editing of nucleotide sequences were carried out using DNAsis V3.0.

Addresses of internet sites used to obtain sequences, or to carry out sequence similarity searches or sequence analysis are given in Appendix II.

Sequence data for *P. falciparum* chromosomes 5-9 were obtained from The Sanger Centre website at http://www.sanger.ac.uk/Projects/P_falciparum/. Sequencing of *P. falciparum* chromosomes 5-9 was accomplished as part of the Malaria Genome Project with support by The Wellcome Trust.

Preliminary sequence data for *P. falciparum* chromosomes 10 and 11 were obtained from The Institute for Genomic Research website (www.tigr.org). Sequencing of chromosomes 10 and 11 was part of the International Malaria Genome Sequencing Project and was supported by award from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Preliminary sequence data for *P. falciparum* chromosome 14 were obtained from The Institute for Genomic Research website (www.tigr.org). Sequencing of chromosome 14 was part of the International Malaria Genome Sequencing Project and was supported by awards from the Burroughs Wellcome Fund and the US Department of Defense.

2.4 Agarose Gel Electrophoresis (Aaij and Borst, 1972)

Fragments of DNA and RNA were separated by electrophoresis through multi-purpose agarose (Bio-Rad) (1 % (w/v) gels in 50 mL TBE buffer) containing ethidium bromide (EtBr) (Sigma) (0.5 µg.mL⁻¹). Samples were loaded in gel loading buffer III (Sambrook *et al.*, 1989) and electrophoresis was carried out at 100 V in TBE buffer.

Two molecular mass markers were used: The 123 bp ladder (GibcoBRL) (300 ng.µL⁻¹) (fragment sizes (kb)): 0.123, 0.246, 0.369, *et cetera*, and *Hind*III-digested phage λ DNA (GibcoBRL) (40 ng.µL⁻¹) (fragment sizes (kb)): 0.125, 0.564, 2.027, 2.322, 4.361, 6.557, 9.416, 23.130.

2.5 Determination of Nucleic Acid Concentrations

2.5.1 DNA

Concentration of DNA in solution was estimated by comparing the intensity of band fluorescence on an EtBr-stained agarose gel with bands of known concentration from the λ*Hind*III molecular mass marker (Table 2.1).

2.5.2 RNA

Concentration of RNA in solution was determined by measuring the absorbance at 260 nm (A_{260}) using a UV1 spectrophotometer (Unicam). An $A_{260} = 1.00$ corresponds to an RNA solution of 40 µg.mL⁻¹.

2.6 Cell Culture

2.6.1 *P. falciparum* (Trager and Jensen, 1976, 1978)

The erythrocytic stage of *P. falciparum* strain 3D7, strain C10 (derived from the Gambian BW isolate (Hempelmann *et al.*, 1981)), or strain R29 (provided by R. Pinches and A. Rowe, Institute of Molecular Medicine, Oxford) was cultured in human erythrocytes in RPMI medium containing Albumax (GibcoBRL) and supplemented with L-glutamine (Sigma) (0.2 mM). The parasites were incubated in a low oxygen atmosphere (O₂ (5 % (v/v), CO₂ (7 % (v/v)), N₂ (88 % (v/v))) at 37 °C. Parasites were visualised by light microscopy of methanol-fixed, Giemsa-stained (Giemsa's staining solution diluted 1 in 10 in dH₂O) thin blood films at 1 000 X magnification.

Synchronisation of parasites was achieved by treatment of ring-infected erythrocytes with 5-10 volumes sorbitol (5 % (w/v) in phosphate-buffered saline (PBS)) for 10 minutes at 37 °C. Sorbitol-treatment causes the selective lysis of trophozoite and schizont-infected erythrocytes (Lambros and Vanderberg, 1979).

Mycoplasma Removal Agent (MRA) (ICN) (0.5 µg.mL⁻¹) was used as directed by the manufacturer in an attempt to eradicate mycoplasma contamination in *P. falciparum*

Table 2.1: Lambda *Hind*III Marker.

Relative amounts of each band in the λ *Hind*III marker. These were used to quantify DNA in solution.

Marker Band (kb)	Per Cent of Total
0.125	0.2
0.564	1
2.027	4
2.322	5
4.361	9
6.557	13
9.416	19
23.130	48

cultures. The Mycoplasma PCR Primer Set (Stratagene) was used according to the manufacturer's instructions to detect mycoplasma contamination in culture supernatants.

2.6.2 *Synechocystis* PCC6803 (Castenholz, 1988)

Synechocystis strain PCC6803 was maintained on BG11 agar plates, supplemented with streptomycin (50 µg.mL⁻¹) or kanamycin (50 µg.mL⁻¹) where appropriate, at 27 °C in the light (illumination provided by F8T5/CW "Cool White" Preheat Fluorescent Lamps (Philips)). Where a larger quantity of cells was required for DNA or RNA extraction or for electron microscopy, cells were grown in liquid BG11 medium in conical flasks under the same conditions. Cells were counted by measuring the optical density at 750 nm (OD₇₅₀) (an OD₇₅₀ = 1.00 corresponds to 1.52x10⁸ cells.mL⁻¹).

2.6.3 *T. gondii*

T. gondii strain RH tachyzoites were cultured in Madin-Darby canine kidney epithelial cells in Modified Eagle's Medium (GibcoBRL) supplemented with foetal calf serum (1 % (v/v)) by S. Sato (NIMR) and R. Wilson (NIMR), following standard procedures (Roos *et al.*, 1994).

2.7 Transformation of *Synechocystis* (Williams, 1988)

Ten microlitres pCR®2.1 harbouring *ycf16* disrupted by a streptomycin resistance cassette (*strep^R*) (approximately 1 ng.µL⁻¹ in TE buffer), were added to 100 µL (4x10⁸ cells.mL⁻¹) wild type (wt) *Synechocystis* (provided by C. Mullineaux, UCL), or to 100 µL *Δycf24* *Synechocystis* PCC6803 (provided by A. Law, NIMR). The cells were incubated at 27 °C in the light with agitation for 4 hours, then spread onto two BG11 agar plates. Expression of the streptomycin resistance gene was allowed to continue for 3 days, after which time the agar was overlaid with 30 µL streptomycin (50 mg.mL⁻¹) in order to select for transformants.

2.8 Transient Transfection of *T. gondii*

Plasmid DNA was transfected into *T. gondii* by electroporation following standard procedures (Roos *et al.*, 1994) by S. Sato (NIMR) and R. Wilson (NIMR).

2.9 Microscopy

2.9.1 Fluorescence Microscopy

2.9.1.1 Autofluorescence by *Synechocystis*

Synechocystis cells were spread onto poly-L-lysine (Sigma) ($100 \mu\text{g}.\text{mL}^{-1}$)-coated slides and viewed by ultraviolet (UV) light microscopy (Zeiss) through a 450-490 nm filter (Zeiss). Images were captured on Ektachrome P1600x colour reversal film (Kodak).

2.9.1.2 DAPI-Staining

The DNA content of *Synechocystis* was visualised by staining with DAPI. Cells of interest were spread onto poly-L-lysine-coated slides and fixed in methanol. Thirty microlitres DAPI (Sigma) ($0.1 \mu\text{g}.\text{mL}^{-1}$) were spread onto the slides, left for 30 seconds then rinsed off with dH_2O . Coverslips were mounted using Vectashield® (Vector). Fluorescence was visualised by UV light microscopy as above.

2.9.1.3 Expression of Reporter Protein in *T. gondii*

Transiently transfected *T. gondii* were used to infect host cells (see above) growing as a monolayer on a coverslip. Reporter protein expression was either visualised directly by UV microscopy of the coverslip (GFP transformants), or by fixing of the cells in paraformaldehyde (2 % (v/v) in PBS) for 10 minutes at room temperature, followed by DAPI-staining as described above (DsRed transformants). Fluorescence images were visualised and recorded as described above by S. Sato (NIMR) and R. Wilson (NIMR).

2.9.2 Electron Microscopy (Stanier (Cohen-Bazire), 1988)

Approximately $100 \mu\text{L}$ *Synechocystis* cells were washed in BG11 medium, prefixed in glutaraldehyde (0.5 % (v/v) in sodium cacodylate (0.1 M; pH 7.2)) for 30 minutes at room temperature, then fixed in glutaraldehyde (3 % (v/v) in sodium cacodylate (0.1 M; pH 7.2)) overnight. Agarose embedding, staining and transmission electron microscopy were subsequently carried out by E. Hirst (NIMR).

2.10 Isolation of Genomic DNA

2.10.1 *P. falciparum*

Parasites were released from erythrocytes by lysis with 2 volumes ice-cold ethanoic acid (1 % (v/v)). The parasites were pelleted by centrifugation for 5 minutes at 2 500 rpm (MSE Centaur 2) and the pellet was washed with RPMI medium (GibcoBRL).

Two hundred and fifty microlitres lysis buffer (4 X) were added to approximately 750 μL pelleted parasites (from approximately 5 mL erythrocytes). After vortexing, the parasites were incubated in lysis buffer at 37 °C overnight. Following incubation, 3 mL dH_2O were added and a homogeneous solution obtained by vortexing.

DNA was extracted sequentially by adding an equal volume of Tris-equilibrated phenol (pH 8.0) (Sigma), an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated by 0.1 volumes sodium ethanoate (3 M; pH 5.2) and 2 volumes ethanol, at -20 °C overnight. Precipitated DNA was pelleted at 3 000 rpm (MSE Centaur 2) for 20 minutes, then washed with ice-cold ethanol (80 % (v/v)). DNA was resuspended in 1 mL TE buffer for every 5 mL erythrocytes used.

2.10.2 *Synechocystis*

Liquid cultures of *Synechocystis* were centrifuged in order to obtain pellets with a volume of approximately 100 μL . The pellets were subjected to 3 cycles of freezing in liquid N_2 ($\text{N}_{2(l)}$) and thawing at 37 °C, and were subsequently resuspended in 500 μL TES. The suspension was then incubated with 5 mg lysozyme at 37 °C for 30 minutes. Fifty microlitres *N*-lauroylsarcosine (Sigma) (10 % (v/v)) were added along with 700 μL phenol, and the tube was incubated at room temperature for 15 minutes, with brief vortexing once every minute during this time. The phases were separated by microfuging at 13 000 rpm for 30 minutes (IEC MicroMax®), and the aqueous layer was transferred to a fresh tube. Seven hundred microlitres phenol:chloroform (1:1) were added, and the tube was incubated at room temperature with vortexing as described above. The phases were separated by microfuging at 13 000 rpm for 1 hour. The layer of denatured proteins was scraped out of the tube with a spatula, and the phenol and phenol:chloroform extractions were repeated as above.

The resulting aqueous layer was incubated with 7.5 μg DNase-free RNase (Boehringer Mannheim) at 37 °C for 30 minutes. One hundred microlitres NaCl (5 M), 100 μL CTAB-NaCl and 600 μL chloroform were added to the resulting aqueous fraction, and incubated at room temperature for 15 minutes with brief vortexing once every minute. The phases were separated by microfuging at 13 000 rpm for 2 minutes, and the aqueous layer was transferred to a fresh tube. DNA was precipitated with 700 μL propan-2-ol for 15 minutes at room temperature, and then washed with ethanol (70 % (v/v)). After air-drying, the DNA pellets were resuspended in 100 μL TE buffer.

2.11 Isolation of RNA

2.11.1 *P. falciparum*

Standard procedures for avoiding RNase contamination were followed (Sambrook *et al.*, 1989).

RNA (enriched for mRNA) was prepared from parasites using the RNaid® PLUS Kit (Bio 101). Parasites were obtained from erythrocytes by ethanoic acid lysis as described above, and the resulting pellet resuspended in cell lysis solution provided in the kit. The manufacturer's instructions were followed for the isolation of RNA, with enrichment of mRNA. The method utilises guanidine isothiocyanate (Chirgwin *et al.*, 1979) and acid phenol extraction (Chomczynski and Sacchi, 1987) followed by binding of RNA to RNAMATRIX® (supplied in the kit). RNA was resuspended in diethyl pyrocarbonate-treated dH₂O supplied in the kit.

Alternatively total RNA was prepared using the RNeasy Midi Kit (QIAGEN). Parasites were obtained by ethanoic acid lysis, then resuspended in 3.8 mL lysis buffer provided in the kit. This method also relies on the denaturing properties of guanidine isothiocyanate. The RNA is then adsorbed to the RNeasy silica membrane in the spin columns provided in the kit. RNA was eluted in 500 µL RNase-free dH₂O supplied in the kit.

Alternatively RNA was prepared using the TRIzol method essentially as described by Kyes *et al.* (2000). Pelleted parasitised erythrocytes were resuspended in 10 volumes TRIzol (Gibco) and incubated at room temperature for 5 minutes, or stored at -70 °C until required. RNA was extracted with 2 pellet volumes chloroform at room temperature for 3 minutes, then precipitated from the aqueous phase by 0.5 pellet volumes propan-2-ol for 1 hour on ice. RNA was resuspended in 0.5 pellet volumes formamide.

2.11.2 *Synechocystis*

Standard procedures for avoiding RNase contamination were followed (Sambrook *et al.*, 1989).

Synechocystis in liquid culture was harvested by centrifugation, and the pellet resuspended in 0.6 mL or 3.8 mL Buffer RLT (QIAGEN) containing 2-mercaptoethanol (Sigma) (1 in 100 dilution). RNA was extracted twice at 65 °C with an equal volume of acid phenol (Sigma):chloroform (1:1), and once with an equal volume of chloroform.

The samples were then processed using the RNeasy midi or mini kit (QIAGEN). Ethanol was added to the aqueous phase and the RNA was purified on RNeasy midi or mini spin columns according to the manufacturer's instructions.

2.12 Primer Design

2.12.1 Degenerate Primers

Degenerate primers were designed to regions of a gene that are highly conserved in several different organisms. Codon usage was biased towards that of *P. falciparum* (Hyde *et al.*, 1989). Inosines were incorporated to minimise base mismatches and decrease degeneracy of the primer pool (Bartl, 1997).

2.12.2 Non-Degenerate Primers

Sequence-specific primers were designed using sequences from the *P. falciparum* databases.

Primer sequences are given in Appendix III.

2.13 Amplification of DNA by PCR (Sakai *et al.*, 1988)

PCR was carried out in a volume of 50 μ L PCR buffer II (Perkin Elmer) with $MgCl_2$ (Perkin Elmer) (3 mM). Each reaction contained approximately 100 ng template DNA, 50 pmoles each primer, 10 nmoles each of dATP, dCTP, dGTP and dTTP (Pharmacia), and 2.5 units AmpliTaq Gold™ polymerase (Perkin Elmer) (Moretti *et al.*, 1998). Reactions were overlaid with 50 μ L mineral oil (Sigma) to prevent evaporation where appropriate.

A Programmable Thermal Controller (MJ Research, Inc.), PCR Express (Hybaid) or T3 Thermocycler (Biometra®) thermal cycler was used. Template DNA was denatured, and AmpliTaq Gold™ polymerase activated, for an initial 12 minutes at 94 °C. This was followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at the annealing temperature appropriate to the primers and 0.5-3 minute extension time at 72 °C (allowing 1 minute per kb expected product). A final extension at 72 °C for 10 minutes was also carried out. PCR products were analysed by electrophoresis through an agarose gel.

LATaq (TaKaRa) was used to amplify sequences when high fidelity amplification was required. PCR with LATaq was carried out in 50 μ L LA PCR buffer (TaKaRa) containing $MgCl_2$ (TaKaRa) (2.5 mM). Each reaction contained approximately 100 ng template DNA, 50 pmoles each of the two primers, 10 nmoles dNTPs (TaKaRa), and 2.5 units LATaq polymerase. Reactions were overlaid with 50 μ L mineral oil where appropriate. Thermal cycling was carried out as described above, but initial denaturation at 94 °C was carried out for 2 minutes and an extension temperature of 68 °C was used.

2.14 RT-PCR

2.14.1 DNase Treatment

Approximately 50 ng total RNA from *P. falciparum* were treated with 1 unit RNase-free DNase (Promega) in 8 μ L PCR buffer II supplemented with $MgCl_2$ (3 mM). Treatment was carried out in the presence of 40 units rRNasin® (Promega), an RNase inhibitor, and 10 nmoles each of dATP, dCTP, dGTP and dTTP, at 37 °C for 30-60 minutes. DNase was subsequently inactivated by incubating at 75 °C for 5 minutes.

2.14.2 cDNA Synthesis

Three hundred and seventy-five nanogrammes random hexameric primers (GibcoBRL) and 100 units SuperScript™ II reverse transcriptase (GibcoBRL) were added to 50 ng DNase-treated RNA. Primers were allowed to anneal to RNA template for 10 minutes at 25 °C, complementary DNA (cDNA) synthesis occurred at 42 °C for 30 minutes, and reverse transcriptase was inactivated by incubation for 3 minutes at 95 °C.

2.14.3 Amplification

Amplification was carried out with AmpliTaq Gold™ as described above, using cDNA as template.

2.15 Synthesis of Radioactive DNA Probe

2.15.1 Preparation of Template

PCR product was used as template for probe synthesis. PCR was carried out as described above, and the resulting band excised from low melting point agarose (May & Baker). The gel slice was diluted by 3 volumes dH₂O, and melted at 65 °C.

2.15.2 Labelling Reaction

The Prime-It® II Random Primer Labeling Kit (Stratagene) was used to radiolabel PCR product, according to the manufacturer's instructions. Approximately 25 ng PCR product were included in the labelling reaction, and the radioisotope used was [α -³²P]-dATP (Amersham).

2.15.3 Purification of Probe

Unincorporated nucleotides were separated from labelled PCR product by purification on NICK® Spin Columns (Pharmacia), according to the manufacturer's instructions. TE was used as equilibration buffer. Probe was eluted in 150 µL TE buffer.

2.16 Southern Hybridisation (Southern, 1975)

2.16.1 Southern Transfer

After electrophoresis of digested DNA through an agarose gel, the gel was photographed with a ruler (to enable subsequent sizing of fragments). DNA was hydrolysed by incubation of the gel in $\text{HCl}_{(\text{aq})}$ (0.25 M) for 20 minutes to aid transfer of larger fragments, then denatured by incubation in denaturing solution for 90 minutes. DNA was then transferred to a Hybond™-N+ nylon membrane (Amersham) by capillary action overnight essentially as described by Sambrook *et al.* (1989) in denaturing solution. DNA was cross-linked to the membrane by exposure to UV light using the UV Stratalinker™ 1800 according to the manufacturer's instructions.

2.16.2 Pre-Hybridisation

Non-specific binding sites on the membrane were blocked by incubation for at least 1 hour in 10 mL hybridisation solution at 65 °C in a Micro-4 hybridisation oven (Hybaid™).

2.16.3 Hybridisation of Radiolabelled Probe to Membrane

After purification, probe was denatured by boiling for 10 minutes then snap-cooled on ice for 10 minutes. The probe was added to the hybridisation solution at 65 °C, and hybridisation was allowed to continue overnight.

2.16.4 Detection of Hybridised Probe

Unbound probe was removed from the membrane by washing twice for 10 minutes at room temperature in 250 mL wash solution A and once for 10 minutes at 65 °C in 250 mL wash solution B. If necessary a second wash for 10 minutes at 65 °C was carried out in 250 mL wash solution C. The membrane was exposed to XB-200 High Definition X-Ray Film (X-ograph Imaging Systems) at -70 °C overnight.

2.16.5 Phosphorimaging

To compare intensities of radioactively labelled bands a Storm 860 PhosphorImager™ (Molecular® Dynamics) was used with ImageQuant® software (Molecular® Dynamics).

The radioactive membrane was exposed to the phosphorimaging screen for 1 hour, and the resulting phosphorescence scanned into the phosphorimager for analysis. Volume integration after correction for background reading was recorded for areas of equal size around the bands of interest. From these, ratios of intensity of the different bands were calculated.

2.17 Cloning into Plasmid Vectors

2.17.1 Purification of Insert DNA

PCR products (<15 kb) were purified from an agarose gel by running onto a diethylaminoethyl-cellulose membrane (Schleicher & Schuell), followed by elution in high-salt elution buffer and phenol-chloroform extraction as described by Sambrook *et al.* (1989). This method is a modification of the methods of Girvitz *et al.* (1980) and Dretzen *et al.* (1981). PCR product was resuspended in 6 μ L dH₂O.

Alternatively PCR products were purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's protocol. Purified PCR product was eluted in 30 μ L elution buffer provided in the kit.

Where several amplification products were present, bands of interest were excised from an agarose gel and purified using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol. Purified PCR product was eluted in 30 μ L elution buffer provided in the kit.

2.17.2 Ligation Reaction

PCR products were cloned directly into pCR®2.1 or pTrcHis2-TOPO® vectors using the Original TA Cloning® Kit (Invitrogen) and the pTrcHis2-TOPO TA Cloning® Kit (Invitrogen), respectively, following the instructions provided by the manufacturer. These vectors contain single 3' dT-overhangs at their insertion sites. This enables the ligation of a PCR product without further modification, as the *Taq*-based thermostable polymerases used in PCR tend to add a single dA to the 3' ends of the PCR product in a non-template-dependant manner (Clark, 1988). Approximately 10 ng PCR product were used in ligation reactions with 50 ng TA vector.

For cloning into pGEX-3X (Pharmacia) and pACP_{Leader}-DsRed/GFP, PCR products were digested with the appropriate restriction enzymes (Boehringer Mannheim) and then purified from an agarose gel using the QIAquick Gel Extraction Kit. Ligations were carried out with one unit T4 DNA ligase (GibcoBRL) in 10 μ L T4 DNA ligase buffer (GibcoBRL) at 14 °C overnight.

2.17.3 Transformation

pCR®2.1, pGEX-3X and pACP_{Leader}-DsRed/GFP ligations were transformed into One Shot™ competent cells (*E. coli* INVαF') (Invitrogen) provided in the Original TA Cloning® Kit according to instructions supplied by the manufacturer. pTrcHis2 ligations were transformed into One Shot™ competent cells (*E. coli* TOP10) (Invitrogen) provided in the pTrcHis2-TOPO TA Cloning® Kit, according to instructions supplied by the manufacturer.

Cells transformed with pCR®2.1 were selected on 100 mm LB agar plates containing ampicillin (Sigma) (50 µg.mL⁻¹) and X-gal (Melford Laboratories Ltd.) (800 µg in 40 µL dimethylformamide spread onto LB + ampicillin plates), by resistance to ampicillin and insertional inactivation of the *lacZ* gene. Cells transformed by vector containing insert form white colonies, whereas those with no insert, and hence functional β-galactosidase, form blue colonies. Cells transformed with pGEX-3X, pTrcHis2 or pACP_{Leader}-DsRed/GFP were selected by ampicillin resistance alone.

2.18 Isolation of Plasmid DNA

2.18.1 Miniprep

Small-scale isolation of plasmid DNA from colonies of interest was carried out using the Wizard® Plus Minipreps DNA Purification System (Promega), employing a vacuum manifold according to the manufacturer's instructions. Colonies expressing the phenotype of interest were cultured overnight in 3 mL LB broth supplemented with ampicillin (50 µg.mL⁻¹) at 37 °C with agitation. For the miniprep, 1.5 mL overnight culture were used. Plasmid DNA was eluted in 50 µL dH₂O. Plasmid DNA for sequencing was subsequently concentrated by ethanol-precipitation and redissolved in 20 µL dH₂O.

2.18.2 Midiprep

Mid-scale isolation of plasmid DNA for use in further cloning experiments was carried out using the Plasmid Midi Kit (QIAGEN). DNA was purified on a QIAGEN-tip 2500 and the final pellet dissolved in 1 mL Tris (10 mM; pH 8.0).

2.18.3 Maxiprep

Large-scale isolation of plasmid DNA for transfection into *T. gondii* was carried out using the Wizard® Plus Maxipreps DNA Purification System (Promega), employing a vacuum manifold according to the manufacturer's instructions. A 100 mL culture of *E. coli* INVαF' cells was used in the maxiprep. The DNA was eluted in 1.5 mL dH₂O.

2.19 DNA Sequencing

Automated sequencing was carried out using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the manufacturer's instructions. This system is based on dideoxy chain terminator methodology (Sanger *et al.*, 1977).

Approximately 300 ng pCR®2.1 containing the insert of interest were used in each sequencing reaction with either M13 forward primer or M13 reverse primer. The TA vector contains binding sites for the M13 primers, flanking the insertion site of the ligated PCR product.

Inserts in pGEX-3X were sequenced using either the 5' pGEX Sequencing Primer or the 3' pGEX Sequencing Primer. These primers flank the multiple cloning site into which the PCR product was cloned.

pACP_{Leader}-DsRed was sequenced using primer 225 and primer 266 (provided by S. Sato, NIMR). Primer 225 binds upstream of the cloned leader sequence, primer 266 binds to the 5' of the sequence encoding DsRed. pACP_{Leader}-GFP was sequenced using primer 225 and primer 156 (provided by S. Sato, NIMR); primer 156 binds to the 5' of the sequence encoding GFP.

Inserts in pTrcHis2 were sequenced using either the pTrcHis Forward Sequencing Primer or the pTrcHis Reverse Sequencing Primer. These primers flank the insertion site of the ligated PCR product.

Excess dye terminators were removed by ethanol precipitation as instructed by the manufacturer, and the DNA pellets were resuspended in 2 µL loading buffer. Labelled DNA fragments were separated using the ABI PRISM™ 377 DNA Sequencer by M. Strath (NIMR).

2.20 Expression of Fusion Protein in E. coli

Five hundred microlitres of an overnight culture of *E. coli* INVαF' cells (Invitrogen) containing *E. coli* *ycf16* cloned into pGEX-3X, or TOP10 cells containing *P. falciparum* *ycf16* cloned into pTrcHis2 were used to seed 50 mL LB broth containing glucose (2 % (w/v)) and ampicillin (100 µg.mL⁻¹). These cultures were grown at 37 °C with vigorous agitation (250 rpm) until the OD₆₀₀ reached 0.6 (approximately 3.5 hours).

Expression of fusion protein was induced by addition of isopropylthio- β -D-galactoside (IPTG) (Calbiochem) (0.1 mM). IPTG induces expression of the fusion proteins since they are under the control of the *tac* (pGEX-3X) and *trc* (pTrcHis2) promoters. Induction was allowed to continue for 3-5 hours at 37 °C. Samples of culture were removed at intervals for preparation of total protein extract, and measurement of the OD₆₀₀.

2.21 Preparation of Total Protein Extract from E. coli

Cells from 1 mL *E. coli* culture were pelleted at 13 200 rpm for 2 minutes. The supernatant was discarded, and the pellet was resuspended in 100 μ L sodium dodecyl sulphate (SDS) loading buffer (1 X) and boiled for 10 minutes. Samples were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and western blotting.

2.22 Large-Scale Preparation of Fusion Protein

2.22.1 GST Fusion Protein

2.22.1.1 Expression of Fusion Protein

A 50 mL overnight culture of the pGEX-3X clone of interest in LB broth supplemented with glucose (2 % (w/v)) and ampicillin (100 μ g.mL⁻¹) was used to seed each of four 2 L flasks containing 500 mL LB broth supplemented with glucose and ampicillin, as described above. These cultures were incubated at 37 °C until the OD₆₀₀ reached 1-2. Expression of fusion protein was induced by addition of IPTG (0.1 mM) and was allowed to continue for 2 hours at 37 °C.

2.22.1.2 Cell Lysis and Solubilisation of Fusion Protein

Bacterial cells were pelleted by centrifugation at 5 000 g for 15 minutes at 4 °C (Beckman Model J-6B Centrifuge in a JS 5.2 rotor). The pellets were resuspended in a total of 120 mL ice-cold Solution A, on ice; to this were added 1.4 mL Protease Inhibitor Cocktail Set I (100 X) (Calbiochem). The cells were then stored at -20 °C overnight.

After thawing the cells in cold water, lysozyme (1 mg.mL⁻¹) was added before incubation on ice for 30 minutes. The cells were then subjected to four cycles (each 4 minutes with 50 % pulse) in a Vibra Cell sonicator (Sonics & Materials Inc.), freezing (N₂) and thawing (37 °C).

The sonicate was incubated with Triton X-100 (1 % (v/v)) at 4 °C on a rotator for 30 minutes, then centrifuged at 12 000 g (Sorvall® RC-5B Refrigerated Superspeed

Centrifuge (Du Pont Instruments) in an SS-34 rotor) for 10 minutes. The supernatant was transferred to a fresh tube.

2.22.1.3 Purification of Fusion Protein

The volume of supernatant was measured and 0.02 volumes of glutathione agarose (Sigma) (1:1 in Solution A) were added to the supernatant and incubated overnight at 4 °C on a rotator. The suspension was centrifuged at 1 000 rpm (MSE Centaur 2) for 5 minutes and the pellet washed three times in 10 bed volumes PBS.

Fusion protein was eluted from the glutathione agarose beads by incubation in 1 bed volume glutathione elution buffer at room temperature for 10 minutes followed by centrifugation at 1 000 rpm as above. Elution and centrifugation were carried out three times in total. The three eluates were pooled, and analysed by SDS-PAGE and western blotting.

Fusion protein was dialysed twice in Visking tubing with a molecular mass cut off of 12-14 kDa (Medicell International Ltd) against 2 000 volumes PBS at 4 °C with stirring for at least 8 hours. In order to cleave the GST tag from Ycf16, 5 µg Factor Xa (NEB) were added to approximately 0.5 mg fusion protein in 1 mL PBS, and rotated at room temperature for 8 hours. The preparation was then further dialysed against PBS and cleaved GST was removed by binding to glutathione agarose, as described above.

2.22.2 HexaHis-Tagged Fusion Protein

2.22.2.1 Expression of Fusion Protein

A 50 mL overnight culture of the pTrcHis2 clone of interest in LB broth supplemented with glucose (0.5 % (w/v)) and ampicillin (100 µg.mL⁻¹) was used to seed each of four 2 L flasks containing 500 mL LB broth supplemented with glucose and ampicillin as described above. These cultures were incubated at 37 °C until the OD₆₀₀ reached 1-2. Bacterial cells were pelleted by centrifugation at 5 000 g for 15 minutes (Beckman Model J-6B Centrifuge in a JS 5.2 rotor), then resuspended in LB broth supplemented with ampicillin and IPTG (0.1 mM). Expression of fusion protein was allowed to continue for 2 hours at 37 °C.

2.22.2.2 Cell Lysis and Purification of Inclusion Bodies

Bacterial cells were pelleted by centrifugation at 5 000 g for 15 minutes (Beckman Model J-6B Centrifuge in a JS 5.2 rotor). The pellets were resuspended in a total of 40 mL Binding Buffer (Novagen). (Imidazole concentrations of the different buffers used for this

procedure are given in Table 2.2). To this were added 50 μL Protease Inhibitor Cocktail Set III (Calbiochem). The cells were then stored at $-20\text{ }^{\circ}\text{C}$ overnight.

After thawing the cells in cold water, lysozyme ($1\text{ mg}\cdot\text{mL}^{-1}$) was added before incubation on ice for 30 minutes. The cells were then subjected to 10 cycles of sonication (10 seconds on, 10 seconds off) in order to fully resuspend the pellet and shear the DNA.

The sonicate was centrifuged at $16\,000\text{ g}$ (Sorvall® RC-5B Refrigerated Superspeed Centrifuge in an SS-34 rotor (Du Pont Instruments)) for 20 minutes to pellet inclusion bodies which were resuspended in 20 mL Binding Buffer. Sonication (to aid complete resuspension), centrifugation and resuspension in 20 mL Binding Buffer were repeated twice more in order to release more soluble proteins, and the final pellet was resuspended in 5 mL imidazole (16 mM) buffer + urea (6 M) to solubilise inclusion bodies. The sample was sonicated as above to aid complete resuspension, then incubated on ice for 1 hour to completely dissolve the protein. Insoluble material was removed by centrifugation at $16\,000\text{ g}$ (Sorvall® RC-5B Refrigerated Superspeed Centrifuge in an SS-34 rotor (Du Pont Instruments)) for 30 minutes.

2.22.2.3 Purification of Fusion Protein

One millilitre Ni-NTA His•Bind® Superflow™ resin (pre-charged with Ni^{2+}) (Novagen) (1:1 in imidazole (16 mM) buffer + urea (6 M)), was added to the supernatant and incubated on a rotator at $4\text{ }^{\circ}\text{C}$ overnight. The suspension was spun at $1\,000\text{ rpm}$ (MSE Centaur 2) for 5 minutes and the pellet washed once in 10 bed volumes imidazole (20 mM) buffer + urea (6 M) and three times in 10 bed volumes Wash Buffer (Novagen) + urea (6 M).

Fusion protein was eluted from the beads by incubation three times in 1 bed volume imidazole (81.5 mM) buffer + urea (6 M) at room temperature for 10 minutes followed by centrifugation at $13\,000\text{ rpm}$ for 2 minutes (Heraeus Biofuge *pico*). Final elutions were carried out once each in imidazole (204 mM) buffer + urea (6 M), imidazole (337 mM) buffer + urea (6 M) and Elution Buffer (Novagen) + urea (6 M). Different fractions were analysed by SDS-PAGE and western blotting.

2.23 SDS-PAGE (Laemmli, 1970)

Proteins were separated by electrophoresis through NuPAGE™ Bis-Tris ($12\text{ }\%$ (w/v)) gels (Novex) in MES Running Buffer (Novex). Samples in SDS loading buffer were boiled for 5 minutes and microfuged at $13\,000\text{ rpm}$ for 2 minutes (IEC MicroMax®) prior to loading on a gel.

Table 2.2: Buffers Used in Purification of *P.f.*Ycf16-6His.

Concentration of imidazole in the different buffers (Novagen) used for purification of *P.f.*Ycf16-6His. These buffers were combined in different proportions to make buffers of varying imidazole concentrations (see Appendix I).

Buffer	[Imidazole] (mM)
Binding Buffer	5
Wash Buffer	60
Elute Buffer	1 000

Electrophoresis was carried out at 200 V. The molecular mass marker used was the Mark 12™ Wide Range Protein Standard (Novex) (marker sizes (kDa): 2.5, 3.5, 6, 14.4, 21.5, 31, 36.5, 55.4, 66.3, 97.4, 116.3, 200). Proteins in the gel were stained by incubation in Coomassie stain for 1 hour, followed by several incubations in destain until the separated proteins could be easily visualised.

2.24 Western Blotting (Burnette, 1981)

2.24.1 Transfer of Proteins to Nitrocellulose Membrane

Proteins were electrophoretically transferred from gel to Hybond™-C Pure nitrocellulose membrane (Amersham). The Sartoblot®II-S (Sartorius) semi-dry blotting apparatus was used according to the manufacturer's instructions. Transfer was carried out at 50 mA for 3 hours in Sartoblot buffer.

2.24.2 Probing and Development of Membrane

Non-specific binding sites on the membrane were blocked by incubation with agitation for at least 1 hour in skimmed milk powder (Marvel) (5 % (w/v) in PBS/Tween 20 (Sigma) (0.1 % (v/v))). The membrane was washed three times for 10 minutes in PBS/Tween 20 (0.1 % (v/v)) and then incubated for 1 hour with agitation in primary antibody, immune serum or hybridoma culture supernatant. Unbound antibody was removed by three 10 minute washes as above. Incubation with horseradish peroxidase-conjugated secondary antibody was carried out for 1 hour with agitation. Excess antibody was removed by at least five 10 minute washes as above. Presence of peroxidase was detected using ECL™ Western Blotting Detection Reagents (Amersham) according to the manufacturer's instructions.

2.25 Dot Blotting (Hawkes et al., 1982)

Hybond™ECL™ nitrocellulose membranes (Amersham Pharmacia Biotech) were coated in antigen (4 µg.mL⁻¹ in Sartoblot buffer) by incubation with gentle agitation for 1 hour. The membrane was then washed three times for 10 minutes in PBS/Tween as described for western blotting. Non-specific binding sites on the membrane were blocked as above, followed by three 10 minute washes in PBS/Tween. The membrane was placed in the SRC 96 D Minifold I dot blotter (Schleicher & Schuell), and 50 µL culture supernatant were pipetted into each well of the apparatus. After incubation for approximately 40 minutes, vacuum was used to draw the supernatants through the membrane. Washing of the membrane, incubation with second antibody and ECL™ were carried out as described for western blotting.

2.26 *Antibody Production (Yokoyama, 1991)*

Monoclonal antibodies (Köhler and Milstein, 1975) were produced using standard procedures by M. Strath (NIMR). Approximately 125 µg fusion protein with alum adjuvant were injected intraperitoneally into BALB/c mice. Boosting was carried out at 2-3 week intervals 3-4 times and sera were tested for reaction to fusion protein by western blotting. Spleen cells from the mouse giving the best response were fused with myeloma cells (SP2/0-Ag14) and after hypoxanthine-aminopterin-thymidine selection were grown in RPMI supplemented with foetal calf serum (10 % (v/v)) and cloned by limiting dilution. Supernatants from fusions and clones were tested for recognition of Ycf16 by dot blotting and western blotting.

2.27 *ATP-Binding Assay (Czarnecki et al., 1979)*

Five microgrammes protein in 30 µL HEPES Binding Buffer were incubated in the dark for 5 minutes with 150 nmoles (0.111 MBq) 8-azido-[α -³²P]-ATP (ICN), and subsequently UV-irradiated at 366 nm for 5 minutes using a UVGL-58 Mineralight® lamp (UVP Inc.) at a distance of 10 cm. The reaction was stopped by addition of 60 nmoles ATP in 30 µL SDS-loading buffer (2 X). Radiolabelled protein was separated from unincorporated 8-azido-[α -³²P]-ATP by SDS-PAGE, and after drying, the gel was exposed to film at -70 °C overnight.

2.28 *RNase Protection Assay*

2.28.1 *Synthesis of Radioactive Antisense RNA Probe*

Probe was synthesised by *in vitro* transcription of *Ssp*I-digested PCR product in pCR®2.1, where the T7 promoter of the vector was situated at the 3' end of the insert. Approximately 200 ng template DNA were incubated with 100 nmoles dithiothreitol (Promega), 80 nmoles of each of ATP, CTP and GTP (Pharmacia), 8 nmoles UTP (Pharmacia), 1.48 MBq [α -³²P]-UTP (Amersham Pharmacia Biotech) and 30 units T7 RNA polymerase (Promega) in 20 µL T7 RNA polymerase buffer (Promega) in the presence of 40 units rRNasin at 37 °C for 1 hour. DNA template was then removed by digestion with RNase-free DNase for 15 minutes at 37 °C. Probe was purified from a denaturing (urea (8 M)) polyacrylamide (6 % (w/v) in TBE buffer) gel for use in the RNase protection assay.

2.28.2 RNase Protection

RNase protection of *P. falciparum* RNA was carried out using the RPA II™ Ribonuclease Protection Assay Kit (Ambion) essentially as directed by the manufacturer's protocol. This technique exploits the ability of a radiolabelled antisense RNA transcript to hybridise to a specific sense strand in a population of RNAs. The transcript of interest is then "protected" from digestion by RNase, which only digests single-stranded RNA.

RNase A/T1-digestion of unprotected RNA was carried out at 30 °C for 30 minutes. The protected RNA was precipitated, and the pellets were resuspended in 8 µL gel loading buffer (Solution E provided in the kit). Protected RNA was separated through a denaturing (urea (8 M)) polyacrylamide (6 % (w/v) in TBE buffer) gel at 200 V. After drying, the gel was exposed to film at -70 °C overnight.

2.28.3 End-Labeling of Marker Oligonucleotides

Marker consisted of approximately 10 000 cpm [γ -³²P]-dATP-labelled *Hinf*I-digested ϕ X174 DNA which yields bands of sizes (nucleotides): 24, 40-48 (unresolved), 66, 82, 100, 118, 140, 151, 200, 249, 311, 413-427 (unresolved), 500, 553, 713-726 (unresolved).

Fifty nanogrammes dephosphorylated *Hinf*I-digested ϕ X174 DNA marker (Promega) were end-labelled with [γ -³²P]-dATP by 10 units T4 polynucleotide kinase (NEB) in 50 µL Buffer for T4 polynucleotide kinase (NEB) at 37 °C for 30 minutes. Unincorporated [γ -³²P]-dATP was separated from labelled DNA by purification on NICK® Spin Columns as described previously.

Chapter 3 - The Mycoplasma Problem

3.1 Introduction

3.1.1 Mycoplasma and Malaria Cultures

The contamination of eukaryotic cell lines by mycoplasma represents a huge problem for researchers, and those culturing malaria parasites are no exception (Turrini *et al.*, 1997). Mycoplasma have been detected in cultures of *P. falciparum* by PCR (Rowe *et al.*, 1998), and mycoplasma DNA was detected in the supernatant of haemozoin samples, although these did not contain viable mycoplasma (Turrini *et al.*, 1997). Turrini *et al.* (1997) used MRA (see below), tylosin and enrofloxacin in an attempt to eliminate contamination, but they found that low concentrations were ineffective, high concentrations killed the parasites, and intermediate concentrations did not eliminate the mycoplasma permanently. However, Rowe *et al.* (1998) found that MRA did have the ability to permanently remove mycoplasma from their cultures and did not have any adverse effect on *P. falciparum*. Mycoplasma have no effect on parasite invasion or maturation and they are invisible in Giemsa-stained smears of malarial cultures. For these reasons mycoplasma contamination of malaria cultures is difficult to detect.

3.1.2 The Organisms

The trivial name “mycoplasma” refers to all species of the class Mollicutes, that is the genera *Mycoplasma*, *Acholeplasma*, *Spiroplasma*, *Anaeroplasma* and *Ureaplasma*. These prokaryotes are the smallest self-replicating organisms (0.3-0.8 µm diameter), and were originally thought to be viruses due to their ability to pass through 450 nm filters. Mycoplasma genomes are the smallest recorded for prokaryotes (600-1 700 kb (Razin, 1992)) and have a low G + C content (from 23-41 %). Mycoplasma lack a rigid cell wall and are therefore insensitive to many of the antibiotics that are commonly used against bacterial contamination of cell cultures.

A mycoplasma was first isolated from a contaminated cell culture almost 50 years ago (Robinson *et al.*, 1956), and it is now accepted that stable cell lines in continuous culture are frequently contaminated. Studies have shown that all cell types, from all host types examined, are subject to contamination (Rottem and Barile, 1993). Primary cell cultures are probably contaminated by the original tissue, whereas the contamination of continuous cell cultures is probably due to infection by previously contaminated cell cultures that have been maintained in the same laboratory; mycoplasma can be spread *via* contaminated laboratory media, reagents or equipment. Lacking a cell wall, mycoplasma are sensitive to chemical disinfectants and detergents, however, they are extremely resistant to environmental stresses, surviving desiccated on work surfaces for weeks (Lundin, 1994)

and frozen in liquid nitrogen for years (Turrini *et al.*, 1997). Good housekeeping practices are therefore imperative for the prevention of recontamination.

3.1.3 The Consequences of Contamination

The effect of mycoplasmal contamination on cell cultures depends on the species of mycoplasma and the type of cell infected (Rottem and Barile, 1993). Mycoplasma contamination has little visible effect on cultures, unlike bacterial or fungal contamination, and the problem can therefore go unnoticed for years. In addition, cellular morphological changes may be minimal; any effects seen are similar to those of nutrient deprivation, and can be overcome by supplementing the medium with fresh nutrients. However, this “covert” infection of cell cultures can have serious repercussions in both research and commercial environments. Although cells in culture are rarely adversely affected by mycoplasmal contamination, it does have the ability to change the properties of host cells, thereby changing the nature of experimental data. Mycoplasma can have many effects including inhibition of enzymes (Almagor *et al.*, 1983), induction of chromosomal breakage (Aula and Nichols, 1967; McGarrity *et al.*, 1984), proteolytic degradation (Chandler and Barile, 1980), changes in the metabolism of amino acids (Stanbridge *et al.*, 1971) and nucleic acids (Levine *et al.*, 1968), alteration of nucleoside transport (Plagemann, 1991), membrane aberration (Wise *et al.*, 1978), B-lymphocyte proliferation (Biberfeld and Gronowicz, 1976), cytokine induction (Kita *et al.*, 1992), superantigen activity (Cole and Atkin, 1991), retardation of growth (McGarrity *et al.*, 1980; Sasaki *et al.*, 1984), and contamination of final product (Lundin and Lincoln, 1992).

3.1.4 Detection of Mycoplasma Contamination

There have been many methods developed for the detection of mycoplasma contamination. Mycoplasma can be grown on undefined, complex media and distinguished from other bacteria by colony shape. However, the colonies are small (100-400 µm diameter (Rottem and Barile, 1993)) and the use of undefined media results in variation. Fluorophores conjugated to species-specific antibodies allow more specific identification of mycoplasma growing on agar. For some “non-cultivable” strains a cell culture method of detection is used which resembles that used to detect the presence of virus particles. Clearly, this approach is only useful where the mycoplasma species in question exerts some effect on the host cell, for example those that adsorb to the host cell surface.

DNA stains such as Hoescht 33258 (Stacey and Doyle, 1997) or DAPI (Hay *et al.*, 1989) can be used to visualise directly mycoplasma in cell cultures. DNA probes to mycoplasma rRNA (Göbel and Stanbridge, 1984; Göbel *et al.*, 1987; Hay *et al.*, 1989) have been widely used. Visualisation of mycoplasma by electron microscopy was an early, direct method of detection (Quinn *et al.*, 1977; Maul, 1978). This method, however, is time

consuming and expensive. The development of indirect methods for detecting mycoplasma meant that cultures could be routinely screened more easily.

Biochemical detection methods exploit enzyme activity that is present in infected cultures, but absent in uninfected cultures. Nucleoside phosphorylases cleave nucleosides to nucleotides, and the conversion of thymidine to thymine can be followed using a fast and simple chromatographic procedure (Gehring and Schröder, 1991; Gehring, 1994).

Antibodies have been used in the detection and identification of mycoplasma in several ways: immunofluorescence methods involving both species-specific polyclonal antisera (Del Giudice and Barile, 1974) and monoclonal antibodies conjugated to fluorescein or peroxidase; enzyme-linked immunosorbent assays (Gabridge *et al.*, 1986); immunobinding to a nitrocellulose membrane (Kotani *et al.*, 1987); and a combination of specific and non-specific staining procedures (Freiberg and Masover, 1990).

PCR is another indirect method of detecting mycoplasma contamination (Uphoff and Drexler, 1999). Blanchard *et al.* (1991) used an alignment of 16S rRNA genes to design PCR primers for the specific amplification of DNA from several *Mycoplasma* species. Their primers did not generate any product from other bacterial, or from eukaryotic DNAs. Sequencing of the amplified product allowed identification of the species of *Mycoplasma*. Spaepen *et al.* (1992) modified this method, designing universal prokaryotic primers which amplified from the 16S ribosomal DNA of prokaryotes, they then used internal primers to the variable regions, designed so that several species of mycoplasma could be detected. When the internal primers alone were used in PCR, some mycoplasma escaped detection. Spaepen *et al.* (1992) therefore recommended a nested PCR approach where the DNA of all bacterial contaminants is amplified in the first step, but just that of mycoplasma is amplified in the second. Hopert *et al.* (1993) compared nested PCR with microbiological agar culture, DAPI-staining, RNA hybridisation, immunofluorescence and enzyme-linked immunosorbent assay. They concluded that PCR was the most sensitive method; it was also highly specific, less labour-intensive, faster, less expensive, more objective and more accurate than other methods. PCR can also be used to detect mycoplasma contamination of final product produced from live tissue (Kojima *et al.*, 1997; Verkooyen *et al.*, 1997).

3.1.5 Methods of Eradication

To prevent cross-contamination from infected cell lines, all cell lines introduced into a laboratory should be quarantined and screened (Lundin and Lincoln, 1992). The accepted protocol for eradication of mycoplasma contamination is to discard infected cultures; however, this is not always desirable or practicable. There are methods available for the elimination of mycoplasma but these should only be attempted if a culture is deemed irreplaceable (Lundin and Lincoln, 1992). Antibiotic treatment may result in the emergence

of resistant mycoplasmas, and also in the selection of a subpopulation of host cells whose characteristics may differ from the original culture.

A selective method of killing mycoplasma based on the incorporation of 5-bromouracil into mycoplasma DNA, binding of the fluorochrome Hoechst 33258 to the DNA (which increases its photosensitivity) and then the induction of breaks into the DNA by visible light (Marcus *et al.*, 1980) has been successfully used. This method is enhanced by the high A + T content of mycoplasma DNA compared to that of the host cells.

MRA (4-oxoquinoline-3-carboxylic acid) (Wasserman, 1993), is a DNA gyrase inhibitor that is mycoplasmacidal rather than merely a growth inhibitor. MRA has been used with varying success by different researchers, and is more effective than alternative antibiotics such as tylosin, kanamycin or tetracyclines (Mowles *et al.*, 1989).

3.1.6 Aims

The genomes of mycoplasma are almost as A + T-rich as that of *P. falciparum*, an added complication for researchers in this field. This problem is especially pertinent when looking for prokaryote-like (plastid) genes in malaria DNA derived from blood cultures. Since the danger of identifying mycoplasma genes and assuming them to be malarial was high, I started out by setting up a screen for mycoplasma-derived PCR products.

3.2 Results

3.2.1 Our Cultures are Contaminated with *Mycoplasma orale*

The Mycoplasma PCR Primer Set was used to amplify mycoplasma-specific sequences from our malaria culture supernatants. A PCR fingerprint identical to the positive control provided in the kit (*Mycoplasma orale* DNA) was obtained (Figure 3.1). The internal control reaction (included in lanes 2 and 4) resulted in a 420 bp product, which did not out-compete the mycoplasma-specific PCR. These results indicate that the malaria cultures currently in use at NIMR are heavily contaminated with *M. orale*. I attempted to eradicate mycoplasma contamination from *P. falciparum* cultures by treatment with MRA, but was unsuccessful. This was probably because I was sharing tissue culture hoods with others whose cultures were also contaminated. I assumed, therefore, that all *P. falciparum* DNA and RNA that I prepared would be contaminated with *M. orale* DNA/RNA. Thus it was important that I should be able to confirm the origin of any PCR products that I obtained.

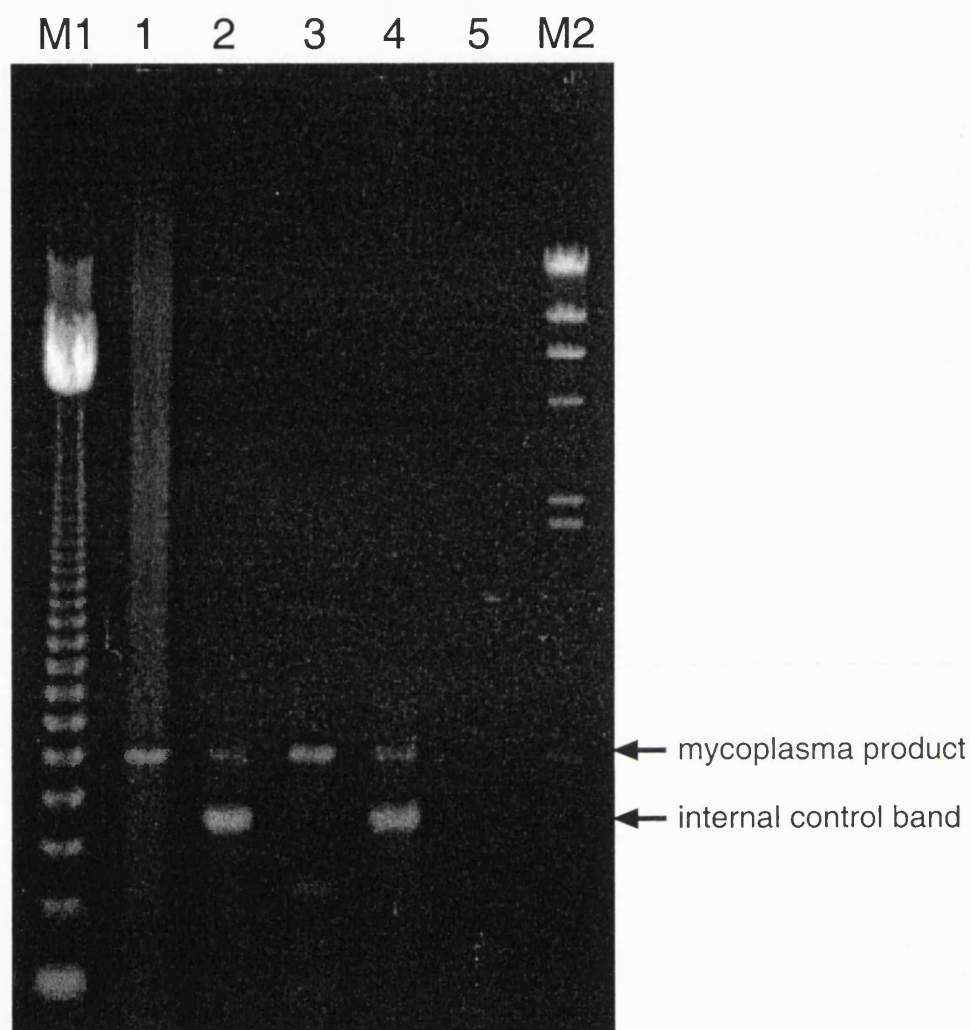


Figure 3.1: *P. falciparum* Cultures are Contaminated with Mycoplasma.

The Mycoplasma PCR Primer Set was used to test *P. falciparum* strain 3D7 culture supernatant for the presence of mycoplasma DNA. EtBr-stained gel showing the products generated. Lanes 1 and 2 are tests of culture supernatant; lanes 3 and 4 are *M. orale* positive controls; lane 5 is a no-template negative control. Lanes 2 and 4 contain an internal control; this did not completely out-compete the mycoplasma-specific PCR, which results in the 600 bp band, indicating heavy contamination of *P. falciparum* cultures. M1 = 123 bp ladder; M2 = λ HindIII marker.

3.2.2 Mycoplasma DNA has a Distinctive Migration Pattern on Pulsed-Field Gels

The 600 bp mycoplasma PCR product was radiolabelled and used to probe a nitrocellulose membrane onto which *P. falciparum* chromosomes, separated by PFGE, had been transferred (provided by P. Moore, NIMR). An autoradiograph, exposed overnight, showed that the majority of the mycoplasma DNA remained in the wells, but some migrated through the gel (Figure 3.2).

By radiolabelling any PCR product obtained in subsequent work, and using it to probe a PFGE blot of separated *P. falciparum* chromosomes it can be demonstrated that it is malarial, and not mycoplasmal, in origin.

3.3 Discussion

Because of the risks of misleading results due to mycoplasma contamination, and also the risk of contamination by mycoplasma of pharmaceutical products intended for the health market, it is imperative that researchers routinely screen their cultures for the presence of mycoplasma, and are aware of the consequences of such contamination.

Aberrant results due to mycoplasma contamination have been reported in many fields. Harper *et al.* (1988) noticed that the immunoreactivity of varicella-zoster virus glycoproteins was reduced due to contamination by glucose-fermenting mycoplasma, perhaps through the alteration of sugar metabolism. One method of detecting HIV virus particles is by assaying for reverse transcriptase activity in culture supernatants. Buckheit and Swanstrom (1991) described an HIV isolate that apparently had a low reverse transcriptase activity despite the normal production and processing of viral proteins; however, Quillent *et al.* (1994) reported that this effect was due to nucleases produced by contaminating mycoplasma. Tan *et al.* (1993) cloned what they believed to be an operon from *Chlamydia trachomatis*; they subsequently discovered that it was actually from a species of mycoplasma (Tan *et al.*, 1995). Rowe *et al.* (1998) reported that the previously described ability of *P. falciparum* strain R29 to stimulate high levels of tumour necrosis factor production from monocytes (Allan *et al.*, 1993) was greatly reduced in MRA-treated parasites. Ong and Mattes (1998) reported that mycoplasma contamination was responsible for the observed effects of “antibody dissociation” from B-cell lymphomas. They found that their cultures were contaminated with *Mycoplasma hyorhinis*, which they speculated interfered with the antibody’s ability to bind to the cell surface due to its own cytoadsorptive properties. Denecke *et al.* (1999) reported that infection with mycoplasma causes misleading results for a tetrazolium-based cytotoxicity assay, which is one of the most commonly used assays for determining the toxicity of a variety of agents.

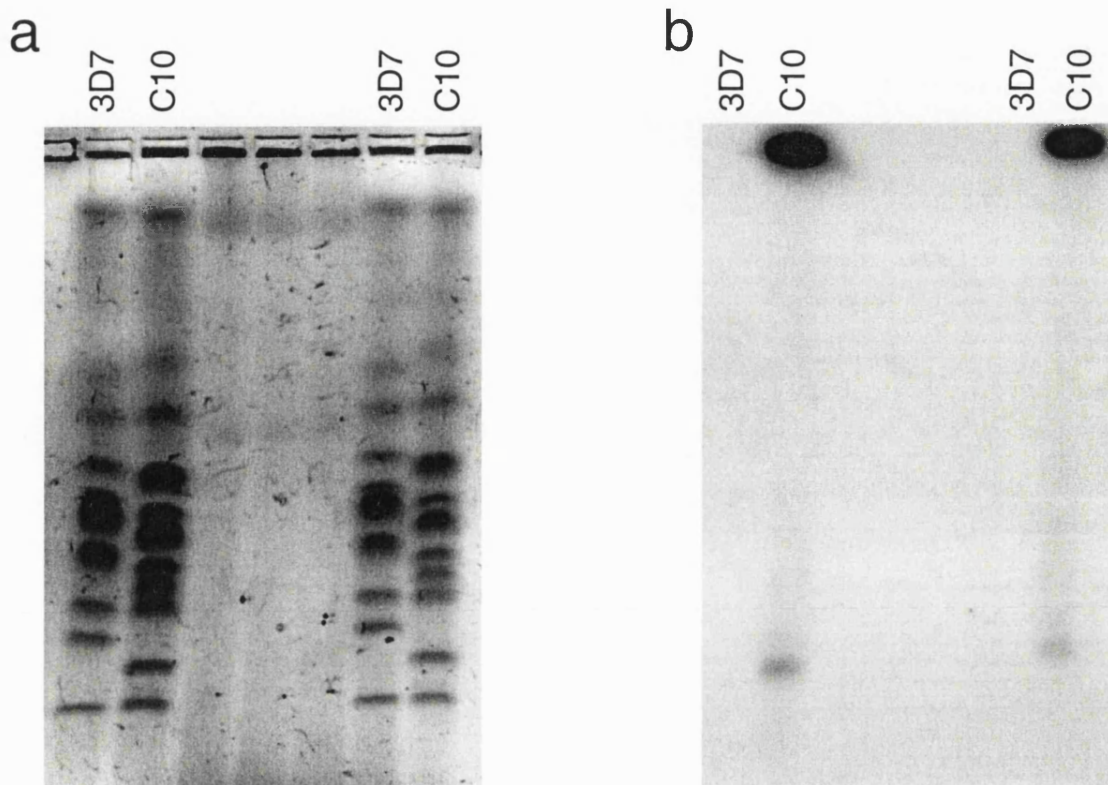


Figure 3.2: Mycoplasma DNA Remains in the Wells of a Pulsed-Field Gel. EtBr-stained pulsed-field gel showing separated *P. falciparum* chromosomes (a), and an autoradiograph showing hybridisation of a mycoplasma DNA-specific probe (b). Most of the mycoplasma DNA remains in the wells, with a small amount migrating through the pulsed-field gel.

Mycoplasma contamination appears to increase the reduction of tetrazolium, thereby simulating a higher rate of surviving tumour cells.

The contamination of commercially available products, such as vaccines or antigen preparations for serological diagnosis, by mycoplasma is also a serious concern (Stanbridge, 1971). This does not merely constitute an inconvenience, but also a potential public health problem. Kojima *et al.* (1997) described the detection of mycoplasma in avian live virus vaccines, and Verkooyen *et al.* (1997) detected mycoplasma in a commercially available *Chlamydia pneumoniae* antigen (used in serodiagnosis) after noticing high levels of non-specific background fluorescence.

The recommended solution to this problem is the discarding of all contaminated cultures. My first approach involved treating *P. falciparum* cultures with MRA; however, I was not able to clear the contamination to the point of being PCR negative, even when the MRA was used for longer than the recommended seven days. I then attempted to culture fresh, mycoplasma-free cultures (strain R29 stabilates provided by R. Pinches and A. Rowe, Institute of Molecular Medicine, Oxford), but these became contaminated within a few weeks, probably due to sharing hoods with others whose cultures were contaminated.

Figure 3.2 shows that at the time of preparation of this PFGE blot, only the strain C10 culture was contaminated with mycoplasma. This acted as an extremely useful internal control since a probe prepared from *P. falciparum* DNA would hybridise to the relevant chromosome in all malarial DNA samples. A mycoplasma probe, however, will only hybridise to contaminated samples, and may show intensities inconsistent with the loading of the malarial DNA (as shown by the photograph of the pulsed-field gel). Although the smaller mycoplasma band appears to hybridise to chromosome 2 in the strain C10 samples, if it were actually a chromosome 2 probe it would also hybridise to chromosome 2 in the strain 3D7 samples.

The examples given above, and my own experiences, highlight the need to be aware of this type of contamination, and the effects associated with it. All researchers using eukaryotic cells should exercise extreme vigilance, and screen and treat their cultures accordingly.

Chapter 4 - Division of the Plastid

4.1 Introduction

4.1.1 FtsZ and Bacterial Cell Division

Studies of cell division mutants of *E. coli* have identified many genes involved in the process of bacterial cell division. Typically the mutations resulted in long filamentous bacteria due to a continued increase in cell mass in the absence of septation, and the genes were thus designated “*fts*” (filamentous temperature sensitive). DNA replication and segregation are critical for the timing and localisation of cell division: blocking of DNA replication inhibits cell division (Burton and Holland, 1983). These filamentous mutants have regularly distributed nucleoids, indicating that the *fts* genes are specifically required for septum formation.

FtsZ is one of the essential proteins involved in prokaryotic cell division and it appears to be the target of several inhibitors (Lutkenhaus, 1993a). It is an ancestral homologue of tubulin (Erickson, 1997) and is conserved among prokaryotes. *ftsZ* has been identified in both Gram-positive and Gram-negative eubacteria, in mycoplasma (which lack a cell wall) and in archaeobacteria. In a study of the *E. coli*-like cell division genes found in other bacterial genomes, *ftsZ* was present in all of the genomes examined (Erickson, 1997).

Prokaryotic FtsZ has a short glycine-rich sequence (GGGTGTG), similar to a sequence found in tubulin (GGGTGSG), which is believed to be involved in its interaction with GTP (Hesse *et al.*, 1987; Sternlicht *et al.*, 1987; de Boer *et al.*, 1992; Mukherjee *et al.*, 1993). Although the overall sequence identity between tubulin and FtsZ is low, their three dimensional structures are almost identical (Desai and Mitchison, 1998; Löwe and Amos, 1998; Nogales *et al.*, 1998). *In vitro* studies have shown that FtsZ can polymerise into filaments similar to those formed by tubulin (Erickson *et al.*, 1996; Mukherjee and Lutkenhaus, 1997), although the mechanisms of polymerisation are different (Romberg *et al.*, 2001). In addition, the mechanism of GTP-binding and hydrolysis displayed by FtsZ and tubulin is distinct from that of other GTPases (Sage *et al.*, 1995). Polymerisation of FtsZ is GTP-dependent, but is not dependent on GTP hydrolysis (Mukherjee and Lutkenhaus, 1994); study of an *ftsZ* mutant (*ftsZ2*) in which FtsZ was defective in GTP hydrolysis showed that it was capable of polymerisation *in vitro*. However, *E. coli ftsZ2* has a complex “altered cell division activity” phenotype (Bi and Lutkenhaus, 1990).

Yu and Margolin (1998) studied the effects of tubulin inhibitors on FtsZ assembly into polymers. The most common tubulin inhibitors, colchicine, colcemid, benomyl and vinblastine, had no significant effect on FtsZ assembly. However, the hydrophobic probe 5,5'-bis-(8-anilino-1-naphthalenesulphonate) was able to inhibit polymerisation, and

promote depolymerisation of FtsZ. Sarcina and Mullineaux (2000) looked at the effects of the tubulin assembly inhibitors thiabendazole and 2-methylbenzimidazole on cell division *in vivo* in the cyanobacteria *Synechocystis* PCC6803 and *Synechococcus* PCC7942, and in *E. coli*. They found that after treatment with these inhibitors cells became elongated or enlarged and that there was an increase in the amount of DNA, with several nucleoids per cell. This morphology resembled the phenotypes of $\Delta ftsZ$ mutants suggesting that these inhibitors act on FtsZ in the same way as they do on tubulin.

The GTP-dependent formation of fibres by FtsZ has been observed *in vitro* by electron microscopy (Lutkenhaus, 1993a). Like tubulin, FtsZ can polymerise *in vitro* forming sheets and protofilaments, however, its polymerisation *in vivo* has yet to be visualised (Beech and Gilson, 2000). During bacterial cell division, FtsZ forms a ring (the “Z ring”) on the inner face of the cytoplasmic membrane at the septum between separating cells (Bi and Lutkenhaus, 1991). The Z ring is analogous to the contractile ring in eukaryotic cell division (Lutkenhaus, 1998). It is not known whether constriction of the Z ring occurs by overlapping FtsZ filaments sliding against one another, or by depolymerisation of its constituent filaments. Alternatively FtsZ may be required just as a scaffold for assembly of the division apparatus at the division site, or for signal transduction to catalyse septal invagination (Rothfield *et al.*, 1999).

FtsZ acts early on in the process of cell division. The filamentous bacterial cells formed in *ftsZ* mutants are smooth, whereas those of *ftsA*, *ftsI* or *ftsQ* mutants have indentations indicating aborted septation (Begg and Donachie, 1985). FtsZ is found at the leading edge of the septum in both rod-shaped bacteria such as *E. coli* and in cocci such as *Staphylococcus aureus* (Lutkenhaus, 1993b). Although it has not yet been shown that mycoplasma require FtsZ for cell division, Wang and Lutkenhaus (1996) propose that since they lack a peptidoglycan cell wall, the primary function of FtsZ is invagination of the cytoplasmic membrane.

FtsZ was thought to be present in all prokaryotes; however, it has recently emerged that it is not encoded on the complete genomes of the obligate intracellular parasite *C. trachomatis* (Stephens *et al.*, 1998; Kalman *et al.*, 1999), or the opportunistic human pathogen *Ureaplasma urealyticum* (*Ureaplasma parvum*) (Glass *et al.*, 2000). Moreover, *Streptomyces coelicolor* can grow and survive despite inactivation of *ftsZ* (McCormick *et al.*, 1994; Bernander, 2000). Although an FtsZ ring has been visualised and the *ftsZ* gene identified in the euryarchaeon *Haloferax mediterranei* (Poplawski *et al.*, 2000) no FtsZ homologue has been identified in an entire subdivision of the Archaea, the Crenarchaeota. Furthermore, no candidate cell division genes have been detected in the complete genome of the free-living crenarchaeon *Aeropyrum pernix* (Kawarabayasi *et al.*, 1999), or in the sequence data currently available from other crenarchaeal genomes, indicating that either the genes have diverged so much they are unrecognisable, or that division involves novel

proteins and mechanisms. Indeed, from observations of division in real time at 85 °C, the crenarchaeon *Thermoproteus tenax* was seen to undergo the “snapping division” (Horn *et al.*, 1999) often observed in coryneform bacteria (Krulwich and Pate, 1971), whereby the cell snaps in the middle after a short period of intensive vibration.

4.1.2 FtsZ and Plastid Division

Chloroplasts divide by binary fission, in a manner similar to bacteria and since it is well accepted that chloroplasts are evolutionarily derived from bacteria (see Chapter 1.2.2.1), it was thought that they might divide by a similar mechanism. A structure known as the plastid dividing ring has been identified in algae and plants (Mita *et al.*, 1986). Interestingly, the plastid dividing ring appears to be made up of two (Hashimoto, 1986) (or possibly three in red algae (Miyagishima *et al.*, 1998)) concentric rings: one on the stromal face of the inner envelope and one on the cytosolic face of the outer envelope (and possibly one found in the intermembrane space in red algae (Miyagishima *et al.*, 1998)). This contrasts with the Z ring of bacteria which is inside the cell. The plastid dividing ring of plastids derived from secondary endosymbionts appears to form between the inner and outer two membranes (Hashimoto, 1997).

Kuroiwa *et al.* (1998) believe the plastid dividing ring may comprise mainly actin-like proteins based on evidence obtained from electron microscopy, phalloidin-staining, western blotting and cytochalasin B inhibition of chloroplast division, although they concede that the evidence is by no means unequivocal (Kuroiwa, 1998). Miyagishima *et al.* (1999) have purified dividing chloroplasts and their plastid-dividing rings from the red alga *Cyanidioschyzon merolae*; this work should help to shed light on the constituents of the division apparatus of chloroplasts.

It is thought that FtsZ may be involved in the division of chloroplasts (Lutkenhaus, 1998), and an *ftsZ*-like sequence has been identified in an *A. thaliana* EST database (Osteryoung and Vierling, 1995). The protein product of this gene has a chloroplast-targeting signal, although it has not been possible to test its localisation directly *in vivo* thus far. However, *in vitro* experiments showed that the mature protein could be targeted into isolated pea chloroplasts, where the transit peptide was subsequently cleaved off. Since then, two more FtsZs have been identified in *A. thaliana* that do not possess chloroplast targeting sequences. It is thought that the original FtsZ, AtFtsZ1-1, acts at the inner plastid dividing ring, whereas AtFtsZ2-1 and AtFtsZ2-2 may act at the outer plastid dividing ring (Osteryoung *et al.*, 1998). Antisense experiments in *A. thaliana* reducing the expression of either AtFtsZ1-1 or AtFtsZ2-1 produced plants with drastically fewer chloroplasts (Osteryoung *et al.*, 1998), indicating that both forms are required.

ftsZ was isolated from the moss *Physcomitrella patens* by degenerate PCR of a cDNA library (Strepp *et al.*, 1998). *ftsZ* knockout mutants in *P. patens* had one large chloroplast

instead of the usual 50 or so, indicating disruption of chloroplast division. This FtsZ, and a second version that has been identified, have been shown to be capable of targeting GFP to the plastid *in vivo* (Kiessling *et al.*, 2000).

Gaikwad *et al.* (2000) isolated a cDNA encoding FtsZ in pea (*Pisum sativum*). The *in vitro* translated product of this cDNA was successfully translocated into intact isolated pea chloroplasts. The cDNA also complemented the thermosensitive defect of an *E. coli ftsZ* mutant. The result of a low-stringency Southern blot suggests that more than one FtsZ may be encoded in the *P. sativum* genome.

FtsZ is one of only two chloroplast genes to be found on the nucleomorph genome (the vestigial nuclear genome of the secondary endosymbiont) of *Guillardia theta* (Fraunholz *et al.*, 1998), and its presence is thought to be one of the reasons for the retention of the nucleomorph in cryptomonads (Zauner *et al.*, 2000). Recently several *ftsZ* genes from unicellular red algae have been identified (Takahara *et al.*, 1999, 2000a, 2000b; Beech *et al.*, 2000). Phylogenetic analysis shows that these cluster with other chloroplast FtsZs (Beech and Gilson, 2000). None of the predicted red algal FtsZs possesses a recognisable chloroplast-targeting peptide, but it is not yet known whether these proteins localise inside or on the surface of the chloroplast. However, in *Galdieria sulphuraria*, two FtsZs have been identified, and these may be situated inside and outside the plastid, respectively, mimicking the situation in *A. thaliana* (Takahara *et al.*, 1999).

The presence of plastid-targeted FtsZs in many plants and algae suggests a rôle for this protein in plastid division. However, immunoelectron microscopy of *G. theta*, probing with an antibody to *E. coli* FtsZ, did not identify any plastid dividing ring, FtsZ appeared to be evenly distributed throughout, although exclusively within, the chloroplast (Fraunholz *et al.*, 1998). Kuroiwa *et al.* (1999) used anti-*Bacillus subtilis* FtsZ antibodies to study the distribution of FtsZ in *C. merolae* and *Cyanidium caldarium*. They found that FtsZ was distributed throughout the chloroplast and also within the nucleus. It was concluded that FtsZ is not present in the outer plastid dividing ring, but they did not exclude the possibility that it may be present in small amounts in the inner plastid dividing ring.

A novel function of FtsZ may be as “plastoskeletal” element. Kiessling *et al.* (2000) visualised networks of FtsZ-GFP fusions in *P. patens* chloroplasts, and suggested that in addition to its rôle in plastid division, it acts as a structural element reminiscent of the eukaryotic cytoskeleton. They hypothesised that after endosymbiosis, the new organelle lost its rigid cell wall and developed an internal FtsZ plastoskeleton to maintain organelle integrity; bacteria, possessing a peptidoglycan wall, only require a transient FtsZ structure during cell division. This model would predict that the cyanelle organelles of *Cyanophora*, which still possess a peptidoglycan wall, would not contain a plastoskeleton (McFadden, 2000).

4.1.3 Mitochondrial Division

The mitochondrial dividing ring (Kuroiwa *et al.*, 1998; Kuroiwa, 2000) does not appear to require FtsZ despite the fact that mitochondria are also believed to have originated from an ancient endosymbiotic event (Erickson, 2000). Some mitochondria have been found to use dynamin-related proteins for division. Dynamins form a large protein family present in eukaryotes, but not prokaryotes (Margolin, 2000). Mutations in *OPA1*, a gene encoding a mitochondrial matrix-localised, dynamin-related protein, have been shown to be associated with the human autosomal dominant disease optic atrophy type 1 (Alexander *et al.*, 2000; Delettre *et al.*, 2000). Like FtsZ, dynamins are GTPases, and they have the ability to constrict and sever membrane tubules. There is some debate, however, as to whether dynamin is directly involved in membrane fission, or whether it acts as a regulator of membrane morphogenesis, perhaps in some way priming membranes for fission or fusion (Roos and Kelly, 1997; Sever *et al.*, 2000). Dynamins perform a variety of cellular functions, in particular those involving vesicle formation (McNiven *et al.*, 2000) during clathrin-mediated endocytosis.

Dynamin-related proteins have been found to play a rôle in mitochondrial distribution and morphology; however, these can be difficult to distinguish, particularly where the mitochondria are normally found in extended reticular networks (Yaffe, 1999) (Figure 4.1). The dynamin-related proteins Dnm1 in *S. cerevisiae* (Otsuga *et al.*, 1998; Bleazard *et al.*, 1999) and DRP-1 in *Caenorhabditis elegans* (Labrousse *et al.*, 1999), were found to be involved in mitochondrial division. Where the genes for these proteins were mutated, the mitochondria formed a network of interconnected tubules, but endocytosis was unaffected indicating that there are distinct dynamins required for different functions. Dnm1 in *S. cerevisiae* appears to act on the outside of the mitochondrion via a “squeezing” mechanism, and in *C. elegans* DRP-1 mutants, division of the inner mitochondrial compartment continued, indicating that these proteins act at the outer mitochondrial membrane. Mammalian Drp1 also appears to have a rôle exclusively in mitochondrial division, suggesting that the function of these dynamin-related proteins has been conserved through evolution (Smirnova *et al.*, 1998; van der Bliek, 2000).

Wienke *et al.* (1999) identified and disrupted dynamin A in the slime mould *Dictyostelium discoideum*. Mutants were defective in endocytosis, cytokinesis, and nuclear and endosomal morphology. They also found defects in mitochondrial form and function, however, they concluded that these were secondary consequences of a general defect in membrane transport processes, since a GFP-dynamin A fusion protein did not co-localise with mitochondria. A second dynamin from *D. discoideum*, dynamin B, which possesses an amino-terminal extension, has been identified and shown to localise to mitochondria between the inner and outer membranes. However, no obvious morphological alterations

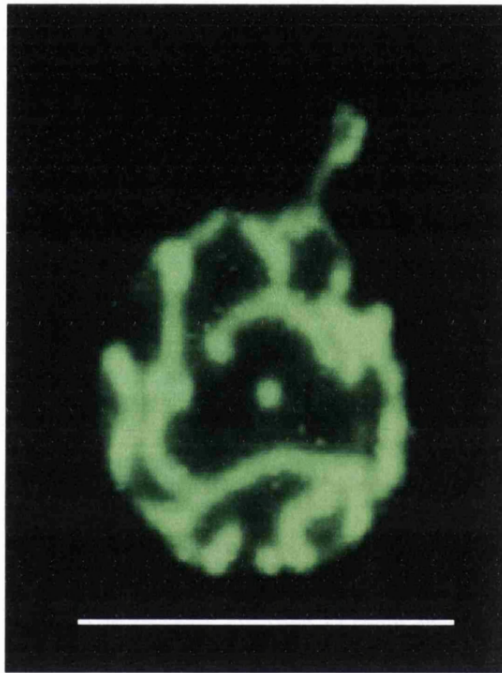


Figure 4.1: Mitochondrial Network in *S. cerevisiae*.
GFP was targeted to mitochondria in wt *S. cerevisiae* revealing an extensive mitochondrial network. Scale bar = 5 μm . From Yaffe (1999).

in cells where this protein is absent or overproduced were noted (D. Manstein, personal communication).

It was thought that mitochondria, which divide by constriction at various points along their length, did not divide using FtsZ, since no *ftsZ* sequence was found in the complete nuclear or mitochondrial genomes of *S. cerevisiae* (Erickson, 1997). However, it has recently emerged that some protists do have a mitochondrial FtsZ. Beech *et al.* (2000) identified a nucleus-encoded FtsZ in *Mallomonas splendens* (MsFtsZ-mt), which has an amino-terminal extension that resembles a mitochondrial targeting peptide. MsFtsZ-mt is most closely related to the FtsZs of α -proteobacteria, the closest known bacterial relatives of mitochondria. It was shown that a MsFtsZ-mt GFP fusion protein was imported into the mitochondria of yeast, and that MsFtsZ-mt specifically immunolocalised to *M. splendens* mitochondria *in situ*. Another candidate red algal mitochondrial FtsZ, from *C. merolae*, was also recently identified. It possesses an amino-terminal extension resembling a mitochondrial-targeting peptide, and also is more similar to FtsZs from the α -proteobacteria than to those of chloroplasts or cyanobacteria (Takahara *et al.*, 2000a). However, localisation of this protein by immunoelectron microscopy has so far been inconclusive.

The discovery of a mitochondrial FtsZ almost completes the picture. If some mitochondria, most of which divide using dynamin-related proteins, have been found to harbour FtsZ, it is possible that some plastids could utilise dynamin-like proteins to undergo division (McFadden, 1999a). Perhaps some plastids divide using both FtsZ and dynamin-related proteins. Questions remain regarding the bacteria that lack FtsZ. Since prokaryotes do not contain dynamin, there could be some other division mechanism yet to be discovered.

4.1.4 Division of the Apicomplexan Plastid

In plants, where there are many chloroplasts per cell, plastid division is autonomous: plastids divide many times during the cell cycle, in a mitotically-independent manner (Pyke, 1999). In contrast, in apicomplexans where there is only one plastid per cell, plastid division is necessarily tightly coupled to parasite division. This is particularly important in *P. falciparum* where the number of daughter cells produced by each schizont is variable.

The contractile ring involving FtsZ in prokaryotes and plastids results in a “dumbbell” shape during division and the plastid of *T. gondii* has been observed to assume a similar shape (McFadden *et al.*, 1996; Striepen *et al.*, 2000). It therefore seemed likely that the plastid of *T. gondii* divides using FtsZ.

In contrast, the dividing plastid in *P. falciparum* exhibits a very different morphology (Figure 4.2). Waller *et al.* (2000) used GFP targeted to the plastid to visualise its

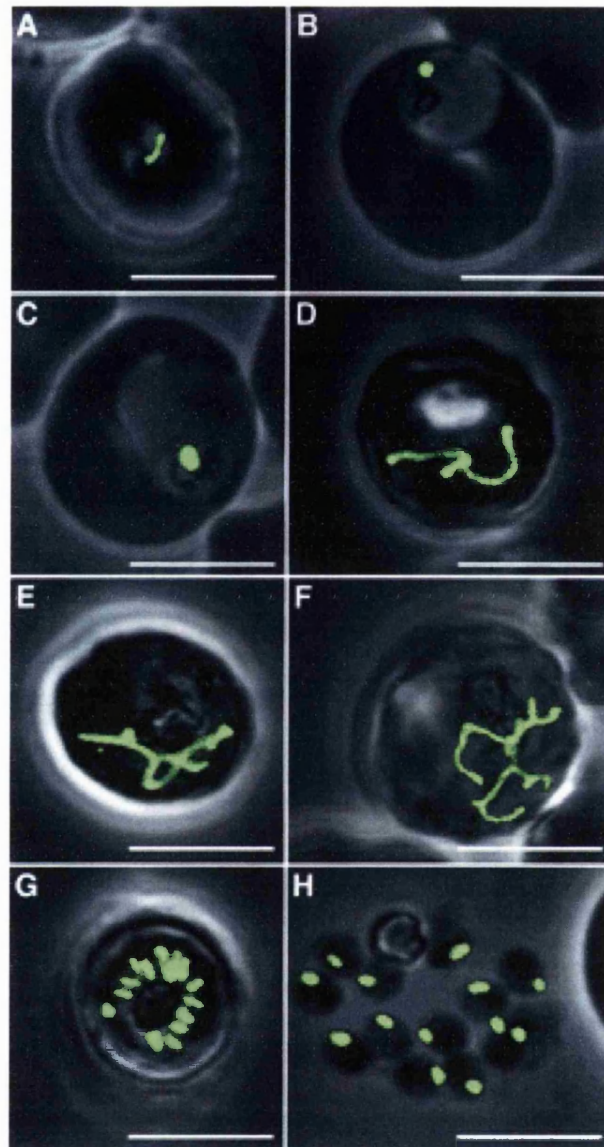


Figure 4.2: Morphology of the Plastid During the Erythrocytic Cycle.

The plastid-targeting sequences of ACP and FabH were used to target GFP to the plastid in different erythrocytic stages of *P. falciparum*. The plastid becomes highly branched in the schizont before dividing into distinct individual plastids, one per merozoite. Scale bars = 5 μ m. From Waller *et al.* (2000).

development in the erythrocytic cycle of the parasite. They found that in ring stages the plastid is a thin crescent shape, it then rounds up to form a sphere as the trophozoite grows. As the parasite enlarges to fill most of the erythrocyte, the plastid elongates and becomes highly branched, this form persisting until cytokinesis. In mature schizonts, just before merozoite release, each merozoite inherits one plastid. This morphology is similar to that previously observed for mitochondrial division in erythrocytic *P. falciparum* (Divo *et al.*, 1985b). Since the plastid of *P. falciparum* does not divide by binary fission it is possible that it does not divide using FtsZ but by some other mechanism, such as that of some mitochondria, involving a dynamin-related protein.

4.1.5 Aims

When this project commenced it was believed that FtsZ was an essential and ubiquitous protein involved in the division of prokaryotes. Evidence had started to accumulate that it was also involved in plastid division. Hence, we believed that FtsZ was likely to be involved in division of the apicomplexan plastid. It was decided to use degenerate PCR and genome database searching in order to identify *P. falciparum* *ftsZ*. Since then it has emerged that some prokaryotes do not contain FtsZ, and that mitochondria utilise dynamin-related proteins. The search of the *P. falciparum* database was therefore extended for a dynamin-related protein.

4.2 Results

4.2.1 Degenerate PCR Results in Amplification of Mycoplasma *ftsZ*

Primers were designed for degenerate PCR of *P. falciparum* DNA; the primers were biased towards the codon usage of *P. falciparum* (Hyde *et al.*, 1989). Primers FtsZ7 and FtsZ10 were used in PCR with total DNA from *P. falciparum* strain 3D7. Several PCR products were obtained (Figure 4.3). Amongst these was a band of approximately 600 bp (*ftsZ*(600)): the expected size for the product of these two primers.

ftsZ(600) was excised from an agarose gel and cloned into pCR®2.1. Sequencing revealed that this product did indeed potentially encode FtsZ (Figure 4.4). A BLAST search showed that it was most similar to a mycoplasma (*M. pulmonis*) version of *ftsZ* (Wang and Lutkenhaus, 1996), but it was not 100 % identical (Figure 4.5).

The PCR product was radiolabelled and used to probe a blot of *P. falciparum* chromosomes separated by PFGE (Figure 4.6). The pattern of hybridisation (most of the signal in the wells, a small amount towards the bottom of the gel) resembled the hybridisation pattern of a mycoplasma probe (see Figure 3.2). In addition, the probe did not hybridise to all of the DNA samples used to prepare the blot, indicating that the probe is

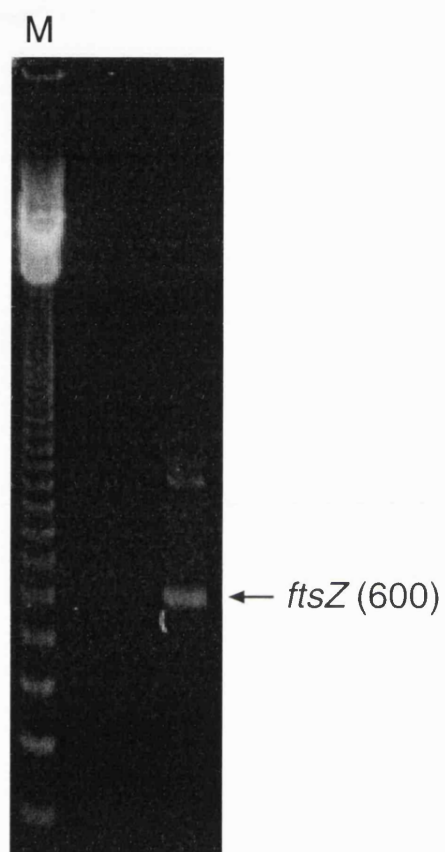


Figure 4.3: The Expected 600 bp Product was Obtained by PCR.
EtBr-stained agarose gel showing the 600 bp product obtained by PCR with the degenerate primers FtsZ7 and FtsZ10. M = 123 bp ladder.

<i>Mp</i> FtsZ FtsZ(600)	MSDLENFIPTANIKVIGVGGGGNNVETMIQAGIQGVEFIVANTDIQALQ -----XLGVGXGGNNSIKTLV????GLEFIIANTDKQVLE :*** *:*: *:***:*** *.*:
<i>Mp</i> FtsZ FtsZ(600)	RSSAPNFIHLGENKRGLGAGANPEVGKKAAEESIVEIKEKLKGADMVIIT QFDKPLRLQLGD-KKGLGAGAKPEIGRAAALASADDIKSRLKGADLVIIT : * *:***: *:*****:***: ** * *:**.:*****:***
<i>Mp</i> FtsZ FtsZ(600)	SGMGGGTGTGASPIIAKIARELGALTISIVTTPFEFEGNLRNKNAQEGIK AGMGGGTGTGASPVIAKIAKECGALVVAIVTTPFEFEGPKRTKIANDGMQ :*****:*****.* **.:***** *.* *:*::
<i>Mp</i> FtsZ FtsZ(600)	NLRAVSDSIITISNNKLEQYGDAPMKDSFLFADTILKHTVKTITDIIAI EL????DSYIIISNNKLLMQYGNVSYKDAFVCANNVLKQTNFKLM-LLLF :* ** * ***** ***:.. **:*: *:..**:* .: : :
<i>Mp</i> FtsZ FtsZ(600)	PAHINLDFADVKTVMKDKGDALIGIGRASGKDRAVKAAIHAISSPIIETS QGIINLXDVT----- **** ***
<i>Mp</i> FtsZ FtsZ(600)	IQGASHTIINITGSANLTLTEVHSAVNVIKNAVGPENNTIFGATINESIG -----
<i>Mp</i> FtsZ FtsZ(600)	DEIYVSVIATGLSSSKKFNSEQEIKDEVSSMLKTMEIDLQASETKTILIN -----
<i>Mp</i> FtsZ FtsZ(600)	DPLPKDEKMLTSLDDRDSKILEKDDSQDDTLPPFLKRN -----

Figure 4.4: *ftsZ*(600) Encodes FtsZ

Alignment of the amino acid translation of *ftsZ*(600) with *M. pulmonis* FtsZ (accession number: AAC44093). Identical (*), conserved (:) and semi-conserved (.) residues are indicated.

Mp ftsZ AGTTATCGGAGTTGGTGGTGGAGGAAATAATTCTGTTGAGACAATGATTCAAG
ftsZ(600) GTWTATGGGTGTTGGGGWGGTGGAAATAATAGTATTAAACTTTAGTTGGAA
 *** ** ***** ** ** ***** * ** * ** * ** *

Mp ftsZ CTGGTATTCAAGGTGTTGAATTTATTGTTGCAAACACTGATATTCAAGCACTT
ftsZ(600) CTAAATTAGATGGACTTGAATTTATAATAGCTAATACCGATAAAACAAGTTTAA
 ** * * ** ***** * *

Mp ftsZ CAAAGAAGCAGCGCTCCAAACTTTATTCACCTTGGTGAAAACAAAAGAGGTCT
ftsZ(600) GAACAATTTGATAAACCATTAAGATTACAATTAGGCGAT---AAAAAGGTTT
 ** * *** * ** * ** ** ***** ***** *

Mp ftsZ AGGAGCTGGGGCCAATCCTGAAGTTGGTAAAAAGCAGCAGAAGAGAGCATTG
ftsZ(600) AGGCGCAGGTGCAAAGCCTGAAATAGGTAGAGCAGCTGCACTAGCAAGTGCTG
 *** ** ** ** ** ** ** ** ** ***** * ***** * *** *

Mp ftsZ TTGAGATTAAAGAAAACTTAAAGGCGCAGATATGGTTATTATCACCTCAGGA
ftsZ(600) ATGACATTAAATCAAGATTAAAGGGTGCGGATTTAGTTATAATAACAGCAGGA
 *** ***** ** * * ** ** ** ***** * ***** ** ** *****

Mp ftsZ ATGGGTGGTGGAAACCGGAACCTGGAGCTAGTCCAATTATTGCTAAAATCGCAAG
ftsZ(600) ATGGGTGGAGGCACTGGAACCTGGTGCTTCTCCTGTTATTGCTAAAATAGCAA
 ***** ** ** ***** ***** ***** ***** ***** *****

Mp ftsZ GGAAGTAGGAGCTCTTACAATTTCAATTGTTACAACCTCCTTTTGAATTTGAAG
ftsZ(600) AGAATGTGGCGCTTTAGTAGTTGCCATAGTAACTACACCATTTGAATTTGAAG
 *** ** ** * * ** * ** ** ** ** ** ** ** ** ** ** *****

Mp ftsZ GTAATTTAAGAAATAAAAATGCCCAAGAGGGAATTAAAAACCTTAGGGCAGTT
ftsZ(600) GACCAAAACGTACTAAAATTGCAAACGATGGAATGCAAGAATTAATAAAATAT
 * * * * ***** ** * ** ***** ** * * * * *

Mp ftsZ AGCGATTCAATTATAACTATTTCTAATAATAAGTTACTTGAACAATATGGTGA
ftsZ(600) GTTGATTCATATATTATTATTTCTAATAATAAACTTCTTATGCAATATGGTAA
 ***** *** * ***** * *** ***** *

Mp ftsZ TGCTCCTATGAAAGATTCATTTCTTTTGCAGACACCATATTAAAGCACACCG
ftsZ(600) TGTTTCTTATAAAGATGCATTTGTTTGTGCAAATAATGTACTTAAACAAACAA
 ** * ** ***** ***** ***** * * ** * ** ** **

Mp ftsZ TT---AAAACCATAACAGATATAATTGCCATTCCAGCACAT-ATCAACCTTG-A
ftsZ(600) ATTAGAACTTTAAATTGATGTTATTGCTGTTCCAAGGGATTATTAATTTAGTA
 * ** ** *** * ***** ***** ** ** ** * * *

Mp ftsZ TTTTGCTGACG
ftsZ(600) CCTTGCCGATG
 ***** ** *

Figure 4.5: *ftsZ(600)* is Similar to *M. pulmonis ftsZ*.

Alignment of *ftsZ(600)*, amplified by primers FtsZ7 and FtsZ10, with nucleotides 42 to 631 of *M. pulmonis ftsZ* (accession number: U34931). Identical bases are indicated (*).

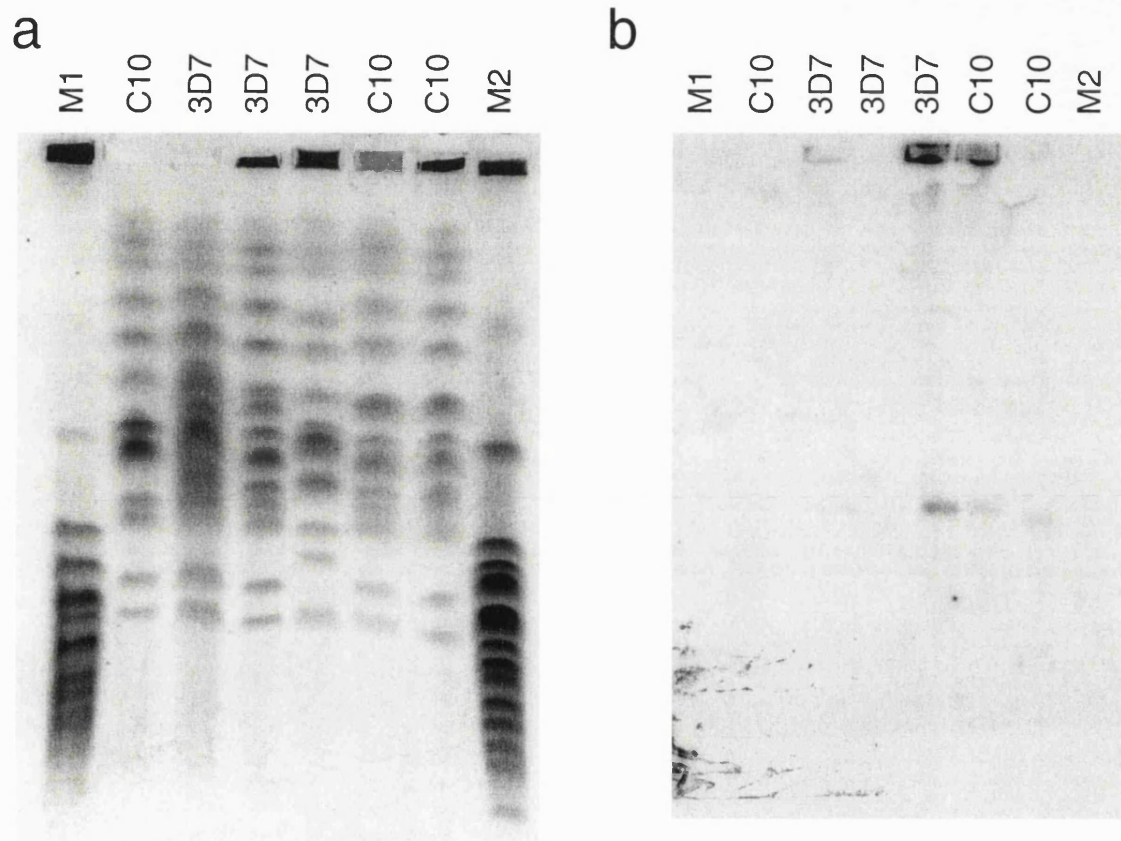


Figure 4.6: *ftsZ*(600) is a Mycoplasma Sequence.

EtBr-stained gel of *P. falciparum* chromosomes separated by PFGE (a), and autoradiograph showing hybridisation of *ftsZ*(600) to a blot of this gel (b). Most of the signal is in the wells, with a small amount migrating down the gel. This is the pattern of mycoplasma DNA on a PFGE blot. In addition, it does not hybridise to the DNA of all the cell lines used to prepare the blot, it only hybridises to the DNA of those cell lines that were contaminated at the time of preparation. M1 = *S. cerevisiae* 6H12 DNA; M2 = *S. cerevisiae* 148 DNA.

from contaminating DNA and not from a malarial gene. This confirmed the PCR product was from a mycoplasma *ftsZ*.

4.2.2 *E. coli ftsZ* Does Not Hybridise to *P. falciparum* DNA

FtsZ5 and FtsZ6 primers were used to amplify a region of *ftsZ* from *E. coli* genomic DNA (provided by A. Law, NIMR) by PCR. A product of the expected size (640 bp) was generated, radiolabelled, and used to probe a PFGE blot of *P. falciparum* genomic DNA. No signal was obtained even under low stringency (results not shown). This was either because *ftsZ* is not present in *P. falciparum*, or because the more GC-rich *E. coli ftsZ* cannot cross-hybridise with the *P. falciparum* gene.

4.2.3 *P. falciparum* Possesses a Dynamin-Like Protein

The possibility remained that *P. falciparum* does not contain FtsZ but utilises a dynamin-related protein for division. A TBLASTN search of the *P. falciparum* chromosomes 10 and 11 sequencing databases at The Institute for Genomic Research and the *P. falciparum* Sanger contigs database at The Sanger Centre was carried out with the amino acid sequence of *S. cerevisiae* Dnm1 protein (accession number: NP_013100). Contigs encoding dynamin-like sequences were identified from chromosome 10 (contig number: 21), from chromosome 11 (contig number: 3142) (sequence data were obtained through early release from The Institute for Genomic Research at www.tigr.org) and from the chromosomes 5–9 “blob” (mal_BU-114g07.p1c) (these sequence data were produced by the *P. falciparum* Sequencing Group at the Sanger Centre and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/malaria2/unfinished_contigs/BLOB). The three sequences were almost identical. The nucleotide sequences were aligned, and adjusted for frame-shifts (predominantly in mal_BU-114g07.p1c). The nucleotide sequence (henceforth referred to as *Pfdrp*) was then translated (PfDRP), and the resulting translation aligned with the dynamin-like proteins involved in mitochondrial division in *S. cerevisiae* (Dnm1) and *C. elegans* (DRP-1 (accession number: AAD49861)) (Figure 4.7). The *P. falciparum* sequence does not have an amino-terminal extension in comparison to the *S. cerevisiae* and *C. elegans* sequences (Figure 4.7).

4.2.4 PfDRP is Encoded on Chromosome 10

Primers Dyn1 and Dyn2 were used in PCR to amplify an 870 bp portion of *Pfdrp*. A product of the expected size was obtained, excised from low melting point agarose, radiolabelled and used to probe a blot of *P. falciparum* chromosomes separated by PFGE (provided by P. Moore, NIMR).

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Sc MASLEDLIPTVNKLQDVMYDSG--IDTLDLPILAVVGSQSSGKSSILETLVGRDFLPRGTGIVTRRPL
Ce ---MENLIPVVNKLQDVFATLGRKEDQIQLPQIVVGSQSAAGSSVLENLVGRDFLPRGTGIVTRRPL
Pf ---MDKLVPIXNKLQNLSSFIS--SETLSXPHIAVVGASXGKTSLLESVLXLXFMKGEDIXTXTX
    :.:*: * ****: : : : * :.:****:* *:*:**.* * *:*: * . *
Sc VLQLNNISPN SPLIEEDDNSVNPHEVTKISGF EAGTKPLEYRGKERNHADEWGEFLHIPGKRIFYDFD
Ce ILQLNHVALD-----DESKRRRSNGTLLT-----DDWAMFEHTGSKVFTDFD
Pf IIQX-----TNSXSDDXYCTLT-----CDYDNNRVEKHIDDFS
    :.* : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *
Sc DIKREIENETARIAGKDKGISKIPINLKVFSPHVLNLTLDLPGITKVPIGEPPDIEKQIKNLILDY
Ce AVRKEIEDETDRVTGVNKGISLLPISLKIYSHRVSLSLVDLPGITKIPVGDQPVNIEEQIREMILLY
Pf XLNEILIDVTEETGGNCKIKETPIIIIEIHKNDVXDLTLDLPGLTKVPVGNQPNVEEQIVNLVNKY
    :. . : : * :.:* : * * . * :.:. * .*:*:****:**:*:*: * :*: * : *
Sc IATPNCLILAVSPANVDLVNSESLKLAREVDPQGKRTIGVITKLDLMDSGTNALDILSGKMYPLKLG
Ce ISNPSSIILAVTPANQDFATSEPIKLAREVDAGGQRTLAVLTKLDLMDQGTAMDVLGMKVIPVKLGI
Pf IKNPNCIILAVSSANIDLANSDSLKMARNVDPKHERTIGVITKCDMVEKPEIWKMKMISGLYPLKKGF
    * .*:*:****:* * :.:* :.:* :.:* :.:* :.:* :.:* :.:* :.:* :.:* :.:* :.:*
Sc VGVNRSQQDIQLNKTVEESLDKEEDYFRKHPVYRTIST--KCGTRYLAKLLNQTLTSHIRDKLPDI
Ce IGVNRSQQNILDNKLIVDAVKDEQSFQMKK--YPTLAS--RNGTPYLAKRLNMLLMHHIRNCLPAL
Pf VAVCRSQKDVEDDITIXMSIKKEEYFSQCIDFSNQMECIMECGIKNLAKKLNNILIEHIKNTVPFL
    :.* * ****: : : : :.:* :.: : : . * * * * * *: * *: * : * :
Sc KTKLNTLISQTEQELARYGGVGATTNESRAS----LVLQLMNKFSTNFISSIDGTSSDIN--TKELCG
Ce KARVSIMNAQCQSDLVAFG---EPVEDKNR----TLLQIITRFATAYTSTIEGTARNIE--TTELCG
Pf KPKIDSLKSIEEERLLELG---EPMDNMNRSEYLAVLVNYITKFSQQYQDIIDGKVIFYKDRVDELKG
    * :.: : : : * . : . : : : : : : : : : : : : : : * : * : * : *
Sc GARIYYIYNNVFGNSLKSIDPTSNLSVLDVRTAIRNSTGPRPTLFVPELAFDLLVKPQIKLLLEPSQR
Ce GARICYIFHDTFGRSLESVNPLENLTQLDILT AIRNATGPRPALFVPEVSFELLVKRQIQRLLEPSLR
Pf GARIHYIFNDWYIKSLNDFSPLEMLTDEEIRIAIRNSSGPRGALFVPESAFXXLIKLLXNCLKEPSLR
    * * * * * : : .*:*:..* . * : : * * * : * * * : * * * : * * * : * * *
Sc CVELVYEELMKICHKCG---SAELARYPKLKSMLIEVISELLRERLQPTRSYVESLIDIHRAIYINTNH
Ce CVELVHEEMQRMVQHCGFTTQEQEMIRFPRLYDKINEVSVGLKERLKPTELVENLVAIELAYINTKH
Pf CADQVYEELLKIVDNCR---IADMERFTNLKSAINEQVKILLKDCLOPTKEMIKNMLIELSYINTSH
    *.: * :*: : : : * : : * : * . : * : : : * : * : * : * : * : * : *
Sc PNFLSATEAMDDIMKTRRKRNRQELLKSKLSQ--QENGQTNGINGTSSISSNIDQDSAKNSDYDDGDIDA
Ce PEFTEAN--LVTLKEELLDDRHRGRSRNRH--ASTGERAQLQPVPGVNG--VDLNAVLOQQQQQ---S
Pf PDFLNEHFMKNVYDKNDYIDELDTVVHPKHNHMKLQRPKREYQETCGSNMNYSTIHNNTYK---E
    * : * . * : : : : : : : : : : : : : : : : : : : : : : : : :
Sc ESKQTKDKFLNYFFGKDKGQPVFDASDKKRSIAGDGNIEDFRNLQISDFSLGDIDDLXNAEPPLTER
Ce QNQRASNGFLG-LFG-----NAAASSKTSPQEKQSANFL-PEVPETQLG-----RKLTSR
Pf EMKMWKHKEDAKSFR-----NKEGTKITNINNRENVFVLPVPEKIIP-----EYSSSSK
    : : . . * : : : : : : : * : : : : : : : : : : : : : : : :
Sc EELECELIKRLIVSYFDIIREMIEDQVPKAVMCLLVNYCKDSVQNRLVTKLYKETLFEELLVEDQTLA
Ce EQRDVAIIERLIRNYFIIVRKNIQDSVPKAIMALLVNFVRDNLQSELVRQLYKPDMDLLAETEDMA
Pf EIIEIDLKSLINNYFNIVRK-----TYSRCGT-----
    * : : * : * . * * * : * : : : . .
Sc QDRELVCVKSGLGVYKKAATLISNIL----- 757
Ce QRRRDTLETMKALQQASVIIEVRETQVW 705
Pf ----- 644

```

Figure 4.7: The *P. falciparum* Genome Encodes a Dynamin-Like Protein. Alignment of PfDRP (Pf) with Dnm1 from *S. cerevisiae* (Sc) and DRP-1 from *C. elegans* (Ce). Sequences encoding dynamin-like proteins were found in the *P. falciparum* chromosomes 10, 11 and “blob” databases. The nucleotide sequences were aligned and adjusted, then translated. Identical (*), conserved (:) and semi-conserved (.) residues are indicated.

Figure 4.8 shows that this gene is indeed malarial in origin and not the result of contamination. It also enabled me to narrow down the location of this gene to chromosome 10 and to confirm that the three sequences found in the *P. falciparum* genome sequencing database are probably the same sequence.

4.2.5 *Pfdrp* is Transcribed by Late Trophozoites and Schizonts

The PCR product generated by primers Dyn1 and Dyn2 was cloned into pCR®2.1 and sequenced. A clone containing the *Pfdrp* sequence in a 3' to 5' orientation with respect to the T7 promoter was selected. This construct was digested with *Ssp*I, a blunt cutter that cuts 321 bp from the 3' end of the amplified *Pfdrp* fragment (see Appendix III) but that does not cut the vector. A radiolabelled antisense *Pfdrp* probe was generated by *in vitro* transcription from the T7 promoter, and subsequently gel purified. RNase protection was carried out on RNA samples prepared from different stages of synchronised *P. falciparum* strain C10. Protected fragments were run on a denaturing polyacrylamide gel, which was subsequently exposed to film.

Although there is a high background of presumably undigested RNA, a band of the expected size (321 nucleotides) can be seen in Figure 4.9. This shows that transcription of this sequence occurs during the late trophozoite stage of the erythrocytic cycle (see Figure 1.2), with maximal amount of transcript in the schizont, and some remaining in free merozoites. By the time the merozoite has invaded a fresh erythrocyte and formed a ring, no transcript is present.

4.3 Discussion

Degenerate primers have been used by several researchers to isolate *ftsZ* from various organisms: *Rhizobium meliloti* (Margolin *et al.*, 1991), *Streptomyces griseus* (Dharmatilake and Kendrick, 1994), *Micrococcus luteus* (McCormick *et al.*, 1994), *Acholeplasma laidlawii*, *Haloferax volcanii*, *M. pulmonis* (Wang and Lutkenhaus, 1996), *Brevibacterium flavum* (Kobayashi *et al.*, 1997), *Brevibacterium lactofermentum* (Honrubia *et al.*, 1998), *P. patens* (Strepp *et al.*, 1998), *G. sulphuraria* (Takahara *et al.*, 1999), *P. sativum* (Gaikwad *et al.*, 2000), *C. caldarium* (Takahara *et al.*, 2000b), *C. merolae* (Takahara *et al.*, 2000a) and *Thermoplasma acidophilum* (Yaoi *et al.*, 2000). Since FtsZs from different organisms possess several highly conserved sequence motifs, and codon usage of *P. falciparum* has been characterised (Hyde *et al.*, 1989), I used this approach to try to identify a malarial *ftsZ*.

The alignment of the amino acid translation of *ftsZ*(600) with *M. pulmonis* FtsZ (Figure 4.4) shows that primers FtsZ7 and FtsZ10 do indeed amplify *ftsZ*. However, I was not able to amplify a malarial *ftsZ* despite the fact that the primers were biased towards

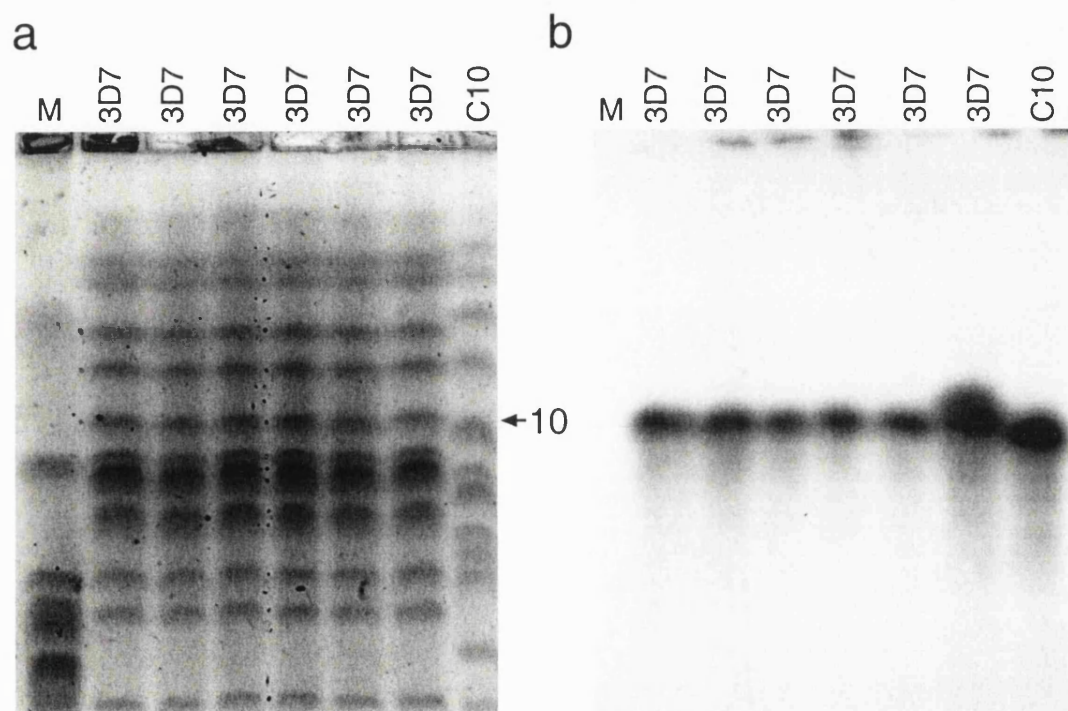


Figure 4.8: *Pfdrp* is Found on Chromosome 10.

EtBr-stained gel of *P. falciparum* chromosomes separated by PFGE (a), and autoradiograph showing hybridisation of *Pfdrp* to a blot of this gel (b). This confirms that this gene is malarial in origin, and that it is found on chromosome 10. M1 = *S. cerevisiae* 148 DNA.

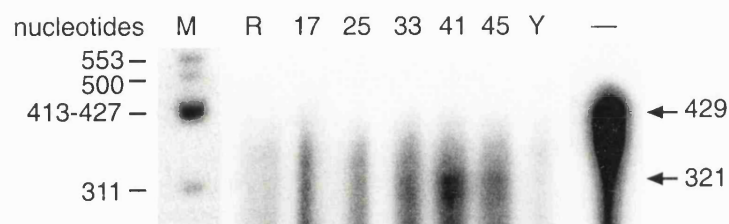


Figure 4.9: *Pfdrp* is Transcribed by Late Stage Erythrocytic Parasites.

RNase protection of RNA samples from synchronised *P. falciparum* shows that *Pfdrp* is transcribed by late trophozoites and schizonts, with some transcript persisting in free merozoites. R = ring stages less than 12 hours old; other time points refer to approximate time post-invasion; Y = yeast DNA control; - = no-RNase control; M = *Hinf*I-digested ϕ X174 DNA.

P. falciparum codon usage to take into account the A + T-richness of malarial DNA. In fact this approach probably facilitated the amplification of mycoplasma *ftsZ* since the DNA of these organisms is also extremely A + T-rich (see Chapter 3.1.2).

As previously described (Chapter 3.2.1) testing of malaria cultures at NIMR revealed heavy contamination by mycoplasma. From the PCR fingerprint obtained with the Mycoplasma PCR Primer Set (Figure 3.1), I concluded that my cultures are probably contaminated with *M. orale*, hence my malarial DNA preparations are contaminated with *M. orale* DNA. Figure 4.5 shows that *ftsZ*(600) is very similar, although not identical, to *M. pulmonis* *ftsZ* at the nucleotide level. Although at present there is no *M. orale* *ftsZ* sequence in the databases, I conclude that the *ftsZ* sequence amplified is *M. orale* *ftsZ*.

Degenerate PCR has been used with great success by many researchers, however, I have been unsuccessful in my search for a *P. falciparum* *ftsZ* gene. If the *P. falciparum* genome carries *ftsZ*, the PCR method employed here is unable to detect it, especially whilst there are contamination problems with our cultures. Striepen *et al.* (2000) have also failed to amplify any *ftsZ* sequence from *P. falciparum* or *T. gondii* by degenerate PCR, or to identify an *ftsZ* in the *P. falciparum* genome database. Until the genome of *P. falciparum* has been fully sequenced and annotated, it is not possible to state definitively that *ftsZ* is not present. However, the methods I employed have been unable to detect it, and there is not as yet any *P. falciparum* *ftsZ* sequence in the database.

Dynamins have been implicated in a variety of cellular events, in particular during endocytosis, secretion and mitochondrial division. The frequently observed mitochondrial networks (Figure 4.1) (Yaffe, 1999) are evocative of the highly branched morphology of the malarial plastid just before it divides to give one plastid per merozoite (Figure 4.2) (Waller *et al.*, 2000). Could a dynamin-related protein be involved in the “pinching off” of separate plastids? I have identified a sequence encoding a dynamin-like protein from the *P. falciparum* sequencing databases at The Institute for Genomic Research and the Sanger Centre, and determined that this sequence is present on chromosome 10 in *P. falciparum*.

RNase protection showed that this sequence is transcribed late in the erythrocytic cycle by *P. falciparum* (Figure 4.9). *Pfdrp* is transcribed by late trophozoites, with maximal transcription in schizonts, and some transcript persisting in merozoites (see Figure 1.2). When compared with the morphology of the plastid during the erythrocytic cycle (Figure 4.2) (Waller *et al.*, 2000), this result does not preclude a rôle for this protein in plastid division.

I cannot say whether this potential dynamin-like protein is necessarily involved in plastid division. It could be involved in mitochondrial division in *P. falciparum*, or it could be involved in one of the many other functions of dynamins in eukaryotic cells. The lack of an

organellar targeting sequence is no proof that it is not involved in organelle division since it could act on the outside of the relevant organelle in much the same way that some chloroplast FtsZs do (Osteryoung *et al.*, 1998). It would be interesting to generate antibodies to this protein for use in immunofluorescence or immunoelectron microscopy, in order to study its subcellular localisation in the malaria parasite. This could give some clue to its function within the cell.

Recently, evidence has been obtained that the plastid in *T. gondii* has hijacked the pre-existing cell division machinery in order to ensure its inheritance by each daughter cell. Striepen *et al.* (2000) used plastid-targeted GFP to study division of the plastid in *T. gondii*. They noted that replication of the plastid genome occurred early in organellar division, and that the genome was associated with the ends of the organelle. Plastid division occurred in close synchrony with nuclear division, with daughter plastids separating just before division of the nucleus, which in turn occurred just prior to the emergence of two daughter parasites. In contrast, plastid division in *P. falciparum* is probably delayed until after nuclear division, since the number of daughter parasites per schizont is variable. Indeed the branched form of the plastid has been observed to persist in multinucleate parasites by immunoelectron microscopy (Waller *et al.*, 2000). The *T. gondii* plastid is closely associated with the apical end of the nucleus throughout this process (Striepen *et al.*, 2000). It was also noted that there appears to be a close connection between the plastid and the centrosome throughout the parasite life cycle, and this association is probably particularly important during cell division.

Dinitroaniline herbicides were used to look at the effect of disrupting α -tubulin and hence microtubule organisation (Stokkermans *et al.*, 1996) on plastid division. Striepen *et al.* (2000) found that herbicide-treated *T. gondii* contained multiple plastids, or large reticulate plastids, which resembled the form of the plastid in *P. falciparum* schizonts (Waller *et al.*, 2000) (Figure 4.2). There were also multiple centrosomes, closely associated with the reticulate plastid. No plastid dividing ring has yet been identified in apicomplexans (Striepen *et al.*, 2000). If this structure does not exist, the mechanical force necessary to divide the plastid is postulated to come purely from the cell division machinery.

Whatever the mechanism of division of the apicomplexan plastid, specific, nucleus-encoded proteins involved in this process remain to be discovered. The mechanism of ensuring organelle inheritance by daughter parasites, particularly where there is need for tight regulation of this process, remains an intriguing problem.

Chapter 5 - Ycf16

5.1 Introduction

5.1.1 *ycf24*

ycf24 (ORF470), the largest ORF of unknown function found on the plastid genome in *P. falciparum* and other apicomplexan parasites (Wilson *et al.*, 1996a; Denny *et al.*, 1998), is a highly conserved “hypothetical chloroplast frame”. Its predicted product is orthologous to Ycf24s encoded on red algal plastid genomes and bacterial genomes suggesting that it has an essential function. Although absent from plastid genomes of the green lineage, *ycf24* has been identified on chromosome 4 of *A. thaliana*, and it has been shown that its product is targeted to plastids (Møller *et al.*, 2001).

In our laboratory, *ycf24* has been studied in two model organisms to try to establish a function for its product (Law, 2000; Law *et al.*, 2000). Firstly, *ycf24* was disrupted in the cyanobacterium *Synechocystis* PCC6803. This showed it is essential with a possible rôle in cell division; the results from these experiments are described in more detail in Chapter 6.1.2. Secondly, attempts were made to knock out *ycf24* in *E. coli*, but these were unsuccessful. In this experiment, a construct containing *ycf24* was disrupted by *strep^R* and transformed into *E. coli*, but it was found that the construct had integrated at sites other than the *ycf24* locus in streptomycin-resistant colonies. This could indicate an essential function for Ycf24. Thirdly, overexpressing Ycf24 as a maltose-binding protein fusion in *E. coli* caused the cells to be elongated and swollen and no FtsZ division ring was detected by immunofluorescence microscopy, although western blotting showed that FtsZ was present in normal amounts. These effects were also seen where maltose-binding protein was overexpressed with no fusion, indicating that these effects were due to the maltose-binding protein not to Ycf24. By contrast, DAPI-staining showed that although the nucleoids had divided, they were clustered together in the middle of the cell, rather than regularly spaced along its length. Overexpression of maltose-binding protein alone did not affect nucleoid partitioning. Immunofluorescence microscopy showed that the overexpressed Ycf24 fusion protein localised around the nucleoids in *E. coli*, leading to the conclusion that it blocked nucleoid partitioning. However, more recent immunoelectron microscopic evidence suggests that this fusion protein may be localising to inclusion bodies (R. Wilson, personal communication). The overall conclusion, taking into account the results obtained in both *Synechocystis* and *E. coli* was that Ycf24 is an essential protein with a rôle in prokaryotic cell division, and hence plastid biogenesis in eukaryotic organisms.

In all the chloroplast and bacterial genomes that possess *ycf24*, *ycf16* is found immediately adjacent or very close by (Tables 5.1 and 5.2). Neither *ycf16* nor *ycf24* have been

Table 5.1: *ycf24* and *ycf16* in Bacterial Genomes.

Genes with products similar to Ycf16 and Ycf24 were identified by TBLASTN searches of the GenBank and EMBL databases. * = one or more genes between *ycf16* and *ycf24*.

Organism	<i>ycf24</i> (gene name) (location)	<i>ycf16</i> (gene name) (location)	Source
<i>Aeropyrum pernix</i>	APE1703 88222-89652	APE1702 87392-88153	<i>A. pernix</i> genomic DNA (Accession: AP000062) (Kawarabayasi <i>et al.</i> , 1999)
<i>Anabaena</i>	Yes	Yes	<i>Anabaena</i> sequencing project, CyanoBase (contig c292)
<i>Archaeoglobus fulgidus</i>	AF2365 8551-9660	AF2364 7829-8593	<i>A. fulgidus</i> complete genome (Accession: AE001113) (Klenk <i>et al.</i> , 1997)
<i>Bacillus halodurans</i>	BH3467 complement (286957-288354)	BH3471 complement (291396-292187)*	<i>B. halodurans</i> genomic DNA (Accession: AP001518) (Takami <i>et al.</i> , 2000)
<i>Bacillus subtilis</i>	<i>yurU</i> complement (157544-159049)	<i>yurY</i> complement (162054-162839)*	<i>B. subtilis</i> complete genome (Accession: Z99120) (Kunst <i>et al.</i> , 1997)
<i>Chlamydia muridarum</i>	TC0056 5044-6495	TC0057 6498-7265	<i>C. muridarum</i> complete genome (Accession: AE002273) (Read <i>et al.</i> , 2000)
<i>Chlamydia pneumoniae</i>	CPn0692 complement (6439-7893)	abcX complement (5663-6433)	<i>C. pneumoniae</i> complete genome (Accession: AE001651) (Kalman <i>et al.</i> , 1999)
<i>Chlamydia trachomatis</i>	CT684 203-1654	CT685 1657-2424	<i>C. trachomatis</i> complete genome (Accession: AE001339) (Stephens <i>et al.</i> , 1998)
<i>Deinococcus radiodurans</i>	DR2106 complement (454-1860)	DR2107 complement (1955-2713)	<i>D. radiodurans</i> complete chromosome (Accession: AE002046) (White <i>et al.</i> , 1999)
<i>Escherichia coli</i>	<i>ynhE</i> complement (5394-6920)	<i>ynhD</i> complement (4638-5384)	<i>E. coli</i> K-12 complete genome (Accession: AE000263) (Blattner <i>et al.</i> , 1997)
<i>Halobacterium</i> species NRC-1	VNG0525C 5602-7026	<i>yurY</i> 4639-5565	<i>Halobacterium</i> NRC-1 complete genome (Accession: AE005004) (Ng <i>et al.</i> , 2000)
<i>Mesorhizobium loti</i>	Yes	Yes	<i>M. loti</i> complete genome (Accession: AP002994) (Kaneko <i>et al.</i> , 2000)
<i>Methanobacterium thermoautotrophicum</i>	MTH1150 9887-11119	MTH1149 9150-9905	<i>M. thermoautotrophicum</i> complete chromosome (Accession: AE000884) (Smith <i>et al.</i> , 1997)

Table 5.1 (continued):

<i>Methanococcus jannaschii</i>	MJ0034? complement (1961-2911)	MJ0035 complement (3053-3805)	<i>M. jannaschii</i> complete genome (Accession: U67462) (Bult <i>et al.</i> , 1996)
<i>Mycobacterium leprae</i>	MLCL536.28c complement (20425-23034)	MLCL536.26c complement (18471-19253)*	<i>M. leprae</i> cosmid L536 (Accession: Z99125) (Cole <i>et al.</i> , 2001)
<i>Mycobacterium tuberculosis</i>	Rv1461 7343-9883	Rv1463 11070-11870*	<i>M. tuberculosis</i> H37Rv complete genome (Accession: AL021184) (Cole <i>et al.</i> , 1998)
<i>Pectobacterium (Erwinia) chrysanthemi</i>	<i>sufB</i> 1378-2877	<i>sufC</i> 2966-3712	<i>P. chrysanthemi</i> ATPase operon (Accession: AJ301654) (Nachin <i>et al.</i> , 2001)
<i>Pyrococcus abyssi</i>	PAB1856 complement (117814-119154)	PAB1855 complement (119147-119890)	<i>P. abyssi</i> complete genome (Accession: AJ248285)
<i>Pyrococcus furiosus</i>	ORF4 2481-3908	ORF3 1748-2491	<i>P. furiosus</i> sequences (Accession: AF156097) (Jenney Jr. <i>et al.</i> , 1999)
<i>Pyrococcus horikoshii</i>	PH1385 83216-84556	PH1384 82480-83223	<i>P. horikoshii</i> OT3 genomic DNA (Accession: AP000006) (Kawarabayasi <i>et al.</i> , 1998)
<i>Streptomyces coelicolor</i>	SCC22.07c complement (4299-5720)	SCC22.04c complement (1968-2732)*	<i>S. coelicolor</i> cosmid C22 (Accession: AL096839) (Redenbach <i>et al.</i> , 1996)
<i>Synechocystis</i> species PCC6803	slr0074 2908-4350	slr0075 4492-5262	<i>Synechocystis</i> PCC6803 complete genome (Accession: D64004) (Kaneko <i>et al.</i> , 1996)
<i>Thermoplasma acidophilum</i>	TA0203 203117-204562	TA0202 202376-203116	<i>T. acidophilum</i> complete genome (Accession: AL445063) (Ruepp <i>et al.</i> , 2000)
<i>Thermoplasma volcanium</i>	Yes	Yes	<i>T. volcanium</i> complete genome (Accession: AP000996) (Kawashima <i>et al.</i> , 1999)
<i>Thermotoga maritima</i>	TM1369 829-2223	TM1368 92-832	<i>T. maritima</i> complete genome (Accession: AE001791) (Nelson <i>et al.</i> , 1999)
<i>Treponema pallidum</i>	TP0612 4854-6293	TP0611 3994-4773	Complete genome sequence of <i>T. pallidum</i> (Accession: AE001236) (Fraser <i>et al.</i> , 1998)
<i>Xylella fastidiosa</i>	XF1476 complement (6483-7940)	XF1475 complement (5510-6346)	Complete genome sequence of <i>X. fastidiosa</i> (Accession: AE003977) (Simpson <i>et al.</i> , 2000)

Table 5.2: *ycf24* and *ycf16* in Plastid Genomes.

Genes with products similar to Ycf16 and Ycf24 were identified by TBLASTN searches of the GenBank and EMBL databases.

Organism	<i>ycf24</i> (gene name) (location)	<i>ycf16</i> (gene name) (location)	Source
<i>Antithamnion</i> sp.	<i>orf3</i> (partial) complement (5672->6565)	<i>orf2</i> complement (4893-5648)	Chloroplast large ATP synthase operon (Accession: X63382) (Kostrzewa and Zetsche, 1992)
<i>Cyanidium caldarium</i>	<i>ycf24</i> 114386- 115837	<i>ycf16</i> 115838- 116602	<i>C. caldarium</i> RK1 chloroplast sequence (Accession: AF022186) (Glöckner <i>et al.</i> , 2000)
<i>Cyanophora paradoxa</i>	<i>ycf24</i> complement (67893-69353)	<i>ycf16</i> complement (67117-67896)	<i>C. paradoxa</i> cyanelle, complete genome (Accession: U30821) (Stirewalt <i>et al.</i> , 1995)
<i>Eimeria tenella</i>	436->1597	nucleus- encoded?	<i>E. tenella</i> plastid DNA (Accession: Y12333) (Denny <i>et al.</i> , 1998)
<i>Guillardia theta</i>	RF24 61067-62518	RF16 62544-63305	<i>G. theta</i> complete plastid genome (Accession: AF041468) (Douglas and Penny, 1999; Leitsch <i>et al.</i> , 1999)
<i>Odontella sinensis</i>	<i>ycf24</i> 69086-70546	<i>ycf16</i> 70546-71301	<i>O. sinensis</i> complete chloroplast genome (Accession: Z67753) (Kowallik <i>et al.</i> , 1995)
<i>Plasmodium berghei</i>	ORF470 complement (<1-711)	nucleus- encoded?	<i>P. berghei</i> plastid (Accession: U79731) (Yap <i>et al.</i> , 1997)
<i>Plasmodium falciparum</i>	ORF470 5142-6554 <i>P. falciparum</i> complete plastid genome (Accession: X95275) (Wilson <i>et al.</i> , 1996a)	<i>ycf16</i> complement (13934-17716) <i>P. falciparum</i> chromosome 14 preliminary sequence data (contig c14m5)	
<i>Porphyra purpurea</i>	<i>ycf24</i> 40948-42411	<i>ycf16</i> 42408-43163	<i>P. purpurea</i> chloroplast, complete genome (Accession: U38804) (Reith and Munholland, 1995)
<i>Toxoplasma gondii</i>	ORF470 complement (28289-29686) <i>T. gondii</i> plastid genome (Accession: U87145) (Denny <i>et al.</i> , 1998)	nucleus- encoded?	

identified on the chloroplast genomes of higher plants; however, I have found *ycf16*- and *ycf24*-like sequences in the nuclear genomes of *A. thaliana* (Møller *et al.*, 2001), soybean (*Glycine max*), tomato (*Lycopersicon esculentum*) and maize (*Zea mays*) (Table 5.3). In higher plants, *ycf16* and *ycf24* are not closely associated and can be found on different chromosomes.

There is some evidence that *ycf16* and *ycf24* form part of the same operon in the red alga *Porphyra purpurea* (M. Reith, personal communication). They are often situated very close together on the genome, occasionally overlapping, even where recombinational events have caused strand-switching, which indicates they could be co-transcribed (see Tables 5.1 and 5.2, and also Chapter 6.2.1). Operons tend to contain groups of genes that are associated with the same biosynthetic pathway, thus it is possible that their products interact in some way. If this is the case then *ycf16* must be encoded in the nuclear DNA of *P. falciparum*.

5.1.2 ABC Domains

ycf16 is a highly conserved gene found on many chloroplast and bacterial genomes. Studies of the sequence of its translation product suggest that it encodes an ABC protein.

ABC proteins contain at least one ATPase domain, which couples the energy from ATP hydrolysis to many, often transport-related, physiological processes. ABC proteins form one of the largest protein families, and have been identified in all organisms studied so far (Klein *et al.*, 1999). When an ABC protein associates with a protein containing a hydrophobic membrane domain for the purposes of trafficking, an ABC transporter is formed (Young and Holland, 1999). In fact, most ABC proteins are involved in membrane transport, translocating various substances to different cellular compartments.

ABC domains share 30-40 % sequence identity from one transporter to another, irrespective of their substrate specificity or species of origin (Higgins, 1995). Like all nucleotide-binding proteins they possess Walker A and B motifs (Walker *et al.*, 1982). In addition, they contain a third conserved sequence, the ABC signature motif (Figure 5.1), which is located between the Walker A and B sequences. The function of this is not completely understood, but it is possibly involved in coupling ATP-dependent conformational changes to function (Hyde *et al.*, 1990). This motif distinguishes an ABC protein from other nucleotide-binding proteins (Theodoulou, 2000). A fourth motif consisting of a highly, although not universally, conserved histidine residue preceded by four hydrophobic residues, is situated around 30 residues downstream of the Walker B motif (Decottignies and Goffeau, 1997; Schneider and Hunke, 1998; Holland and Blight, 1999).

Table 5.3: *ycf24* and *ycf16* in Nuclear Genomes.

Genes with products similar to Ycf16 and Ycf24 were identified by TBLASTN searches of the GenBank and EMBL databases.

Organism	<i>ycf24</i> (gene name) (location)	<i>ycf16</i> (gene name) (location)
<i>Arabidopsis thaliana</i>	AT4g04770 complement (join (155014-155232, 155347-156801)) Chromosome 4 contig fragment 13 (Accession: AL161501) (Mayer <i>et al.</i> , 1999; Arabidopsis Genome Initiative, 2000; Møller <i>et al.</i> , 2001)	F13M14.4 join (10053-10412, 10605-10655, 10761-10982, 11111-11233, 11681-11857, 11949-12032) <i>A. thaliana</i> chromosome 3 BAC F13M14 (Accession: AC011560) (Arabidopsis Genome Initiative, 2000; Salanoubat <i>et al.</i> , 2000)
<i>Chlamydomonas reinhardtii</i>	CL49b11_r (Accession: AV397710) chlamyEST database (Asamizu <i>et al.</i> , 1999)	?
<i>Glycine max</i>	?	EST partial, Public Soybean EST Project (Accession: AI442209)
<i>Lycopersicon esculentum</i>	EST276686 <i>L. esculentum</i> cDNA clone cLEC25J19 (Accession: AW033127)	EST253344 <i>L. esculentum</i> cDNA clone cLER2I7 (Accession: AI772244)
<i>Zea mays</i>	?	EST 605096H12.x2 (Accession: AI833904)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
L	S	S	G	D	K	K	K	L	P	L	S	F	K	L
I	A	A		E	R	R	R	I	H	I	A	Y	R	I
V		P		N	Q	N	A	V	Y	V	G	W	H	V
M		G		Q	A	Q	C	M		M	C	H	P	M
F		L		M	S	S	L	F		F	L	P		F
Y		V		W	P	T	V	Y		W	I			Y
C		F			C	A	M	P			V			W
		Y			L	V		A			P			S
		K			I	M		N						T
		Q			M									A
		H			F									
					W									

Figure 5.1: ABC Signature Motif.

The 15 residue ABC signature consensus was obtained from Prosite. Permissible amino acid residues for each position of the motif are shown in green; amino acids not found at a particular position are shown in red.

Studies of specific residues within the Walker A motif have been carried out. The conserved serine or threonine residue found towards the end of the Walker A motif was found to be essential for liganding Mg^{2+} in the transition state, and hence for ATP hydrolysis in the mammalian ABC protein P-glycoprotein (Urbatsch *et al.*, 2000). The lysine residue immediately upstream of this also has been found to have a direct function in nucleotide binding and catalysis (Urbatsch *et al.*, 1998). Both Walker sites of an ABC domain must be intact for normal function (Senior and Bhagat, 1998; Urbatsch *et al.*, 1998); however, ATPase activity in the ABC protein is not dependent on the other components of the ABC transporter (Delepelaire, 1994).

Typically, an ABC transporter is made up of two hydrophobic transmembrane domains and two ATP-binding domains (Theodoulou, 2000). These four domains are arranged in a characteristic fashion, the transmembrane domains, each usually with six membrane-spanning segments, form the pathway through which a substance can pass. Two ABC domains at the cytoplasmic face of the cell membrane couple ATP hydrolysis to the transport process (Higgins, 1992).

ABC transporters are thought to have arisen from a single ancestor, with different functions deriving from gene duplications. The ABC domain and the transmembrane domain may be expressed as separate polypeptides, or be fused in one of various configurations (Higgins, 1995). In prokaryotes the different domains are usually encoded separately, with the component genes arranged in a single operon. This situation also occurs in ABC transporters of eukaryotic organelles, reflecting the endosymbiotic origin of plastids and mitochondria.

ABC transporters are involved in the transport of a huge variety of substances, ranging from ions to large molecules such as glycoproteins. The term “allocrite” has been coined to refer to the substance that is transported by an ABC transporter (Young and Holland, 1999), to distinguish it from the “substrate”, which is ATP. Allocrite recognition is thought to be a function of the transmembrane domain of an ABC transporter or of an associated solute-binding protein. Intriguingly, many ABC transporters are relatively specific, but others are able to handle several chemically dissimilar compounds (Theodoulou, 2000). An ABC transporter can be involved in import or export, but not both (Higgins, 1992).

There are several medically important ABC proteins, and some could be targets for drug discovery (Demolombe and Escande, 1996). Mutations of many human ABC proteins cause genetic diseases, for example cystic fibrosis (Klein *et al.*, 1999). Overexpression of an ABC protein, P-glycoprotein, has been implicated in the phenomenon of multidrug resistance in tumour cells (Prasad *et al.*, 1996). It is thought that these transporters act as pumps, preventing the intracellular accumulation of drugs to an effective cytotoxic

concentration. This problem is not restricted to mammalian cells: in malaria parasites, the ABC transporter Pgh1 (pfMDR), which is located on the digestive vacuole membrane (Cowman *et al.*, 1991), is implicated in resistance to chloroquine (Foote *et al.*, 1989; Higgins, 1995).

5.1.3 Aims

In order to gain an insight into the function of Ycf16, and possibly Ycf24, it was decided to express *E. coli* Ycf16 as a GST fusion protein. The *E. coli* version was chosen firstly because a *P. falciparum* version had not been identified, and secondly because it should be relatively easy to express as a recombinant protein in *E. coli*. In the course of this work, the emerging *P. falciparum* sequencing data were searched for any nuclear gene that might encode a plastid-targeted Ycf16.

5.2 Results

5.2.1 GST-*E.c.*Ycf16 was Purified Under Native Conditions

Primers Ycf161 and Ycf162 were used to amplify the whole of *E. coli* *ycf16* (747 bp) from *E. coli* genomic DNA. These primers were designed such that the resulting PCR product could be digested with *Bam*HI and *Eco*RI and cloned in-frame into pGEX-3X for expression of a GST fusion protein. Sequencing the resulting construct showed the insert to be error-free. *E. coli* INV α F' cells into which the construct had been transformed were grown to an OD₆₀₀ of 0.6 and induced to express GST-*E.c.*Ycf16 by addition of IPTG.

Samples were taken from the induced cultures at the time of induction ($t = 0$), then every hour for three hours. The OD₆₀₀ was compared at these time points between induced cultures, uninduced cultures and host cells containing no vector. Figure 5.2 shows that expression of GST-*E.c.*Ycf16 in *E. coli* was detrimental to the host cell. Untransformed host cells grew at the same rate whether or not IPTG had been added. However, induction of GST-*E.c.*Ycf16 expression caused *E. coli* to grow more slowly; uninduced cultures grew at the same rate as host cells. Induced cultures stopped growing approximately 1 hour after addition of IPTG.

GST-*E.c.*Ycf16 was found to be soluble, and was therefore purified from induced culture by binding to glutathione agarose under native conditions (Figure 5.3). Purified protein was dialysed into PBS for Factor Xa cleavage of GST from Ycf16 but cleavage was unsuccessful. Only uncut fusion protein could be seen by SDS-PAGE and western blotting (results not shown).

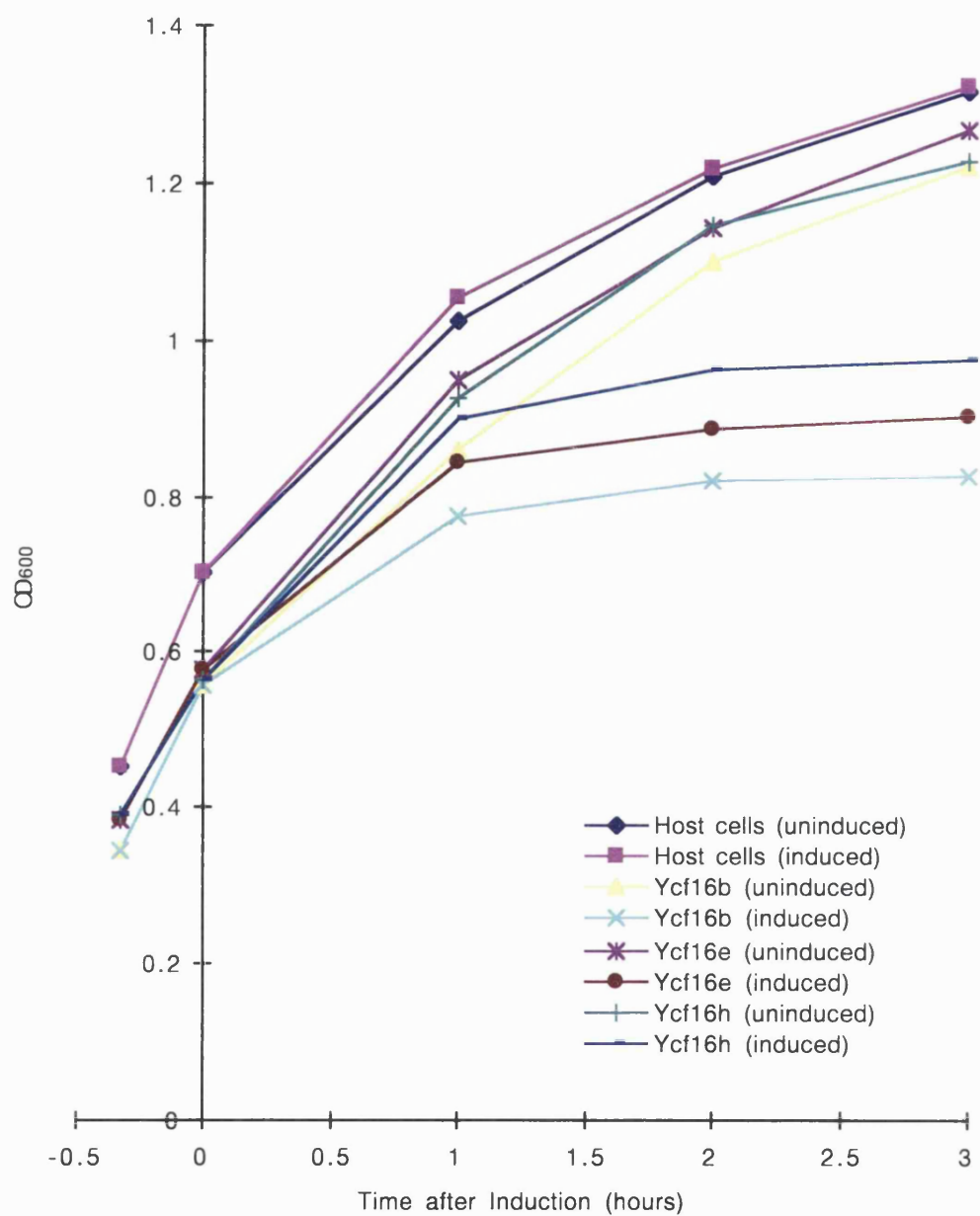


Figure 5.2: Expression of GST-*E.c.*Ycf16 in *E. coli* was Detrimental.

Host cells grew at the same rate whether or not IPTG had been added to the culture (induced *versus* uninduced). Induction of GST-*E.c.*Ycf16 expression caused *E. coli* to grow more slowly: uninduced cultures grew at the same rate as host cells, whereas induced cultures stopped growing approximately 1 hour after addition of IPTG.

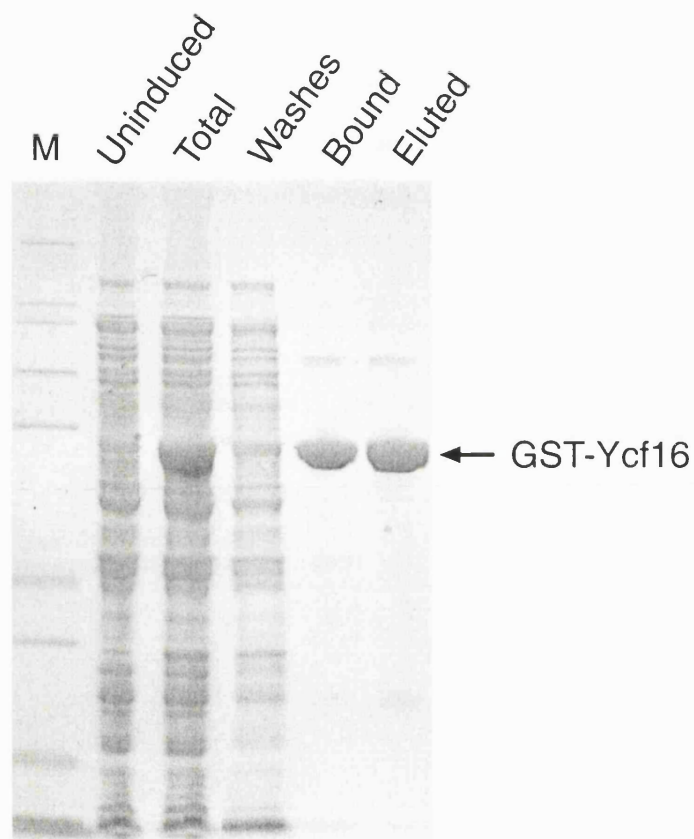


Figure 5.3: GST-*E.c.*Ycf16 was Purified Under Native Conditions.

Coomassie-stained polyacrylamide gel showing purification of GST-*E.c.*Ycf16 (~54 kDa). GST-*E.c.*Ycf16 was expressed in *E. coli* INV α F' cells. It was purified on glutathione agarose under native conditions and eluted in glutathione elution buffer. M = Mark 12™ Wide Range Protein Standard.

5.2.2 Antibodies Recognise GST-*E.c.*Ycf16

Four mice were immunised with GST-*E.c.*Ycf16 with the aim of producing Ycf16-specific monoclonal antibodies. Sera from the mice were tested for an immune response to the fusion protein by western blotting and the mouse giving the best response was selected for hybridoma production.

Hybridoma supernatants were tested by dot blotting for antibodies recognising *E.c.*Ycf16, but not GST. Promising fusions were then cloned by limiting dilution. Monoclonal antibodies were tested by dot blotting and western blotting for recognition of *E.c.*Ycf16, but not GST. This process is ongoing; once monoclonals recognising the protein of interest have been identified, they will be used for immunofluorescence studies on *E. coli*.

5.2.3 GST Binds 8-Azido-ATP

The ATP analogue 8-Azido-[α -³²P]-ATP was used to label GST-*E.c.*Ycf16 by UV crosslinking (Figure 5.4a). In order to check that the labelling was specific, the experiment was also carried out with GST and bovine serum albumin (BSA) as negative controls and actin (provided by M. Anson, NIMR) as a positive control.

Actin was labelled as expected, and the labelling seemed to be specific since BSA was not labelled (Figure 5.4b). However, GST was labelled; this rendered the result of the GST-*E.c.*Ycf16 experiment meaningless since it was impossible to say whether the labelling was due to Ycf16 or to GST.

5.2.4 *P. falciparum* Ycf16 is Encoded on Chromosome 14

The amino acid sequence of *G. theta* Ycf16 (accession number: AAC35665) was used in a TBLASTN search of the *P. falciparum* chromosome 14 database at The Institute for Genomic Research. A highly similar *P. falciparum* sequence, possessing a putative amino-terminal leader sequence, was identified. Figure 5.5 shows this sequence aligned with two prokaryotic Ycf16s and four chloroplast-encoded Ycf16s.

PCR primers Ycf1611 and Ycf1612 were used to amplify the DNA sequence from *P. falciparum* strain 3D7 genomic DNA. A product of the size predicted from the database sequence (762 bp) was obtained, cloned into pCR@2.1 and sequenced. The sequence of the PCR product was found to be identical to that given in the chromosome 14 database. The PCR product was radiolabelled and used to probe a blot of *P. falciparum* chromosomes separated by PFGE. Figure 5.6 shows that this sequence is indeed found on chromosome 14, and confirms it is not mycoplasmal in origin.



Figure 5.4: GST Binds 8-Azido-ATP.

GST-*E.c.* Ycf16 was found to be labelled by 8-Azido- $[\alpha\text{-}^{32}\text{P}]$ -ATP (a). This appeared to be a specific reaction since as expected actin was labelled whereas BSA was not (b). However, GST was also labelled (b). Labelling only occurred upon irradiation by UV light (UV) (duplicates); no labelling was seen when reactions were incubated in the dark.

Pf MKRRKWLNITWAVTIYIFFGMINVSSYNEGRKRKNKMYSFVINGYNEYKNKYSF LRKPK

S -----MSQTILSIKNLTASVDG--NQILK
Ec -----MLSIKDLHVSVED--KAILR
Os -----MNTNYPILEIKNLKACINE--NEILK
Cp -----MSTEKTILEVKNLKAQVDG--TEILK
Gt -----MKKKILEVTNLHA AVNE--IKIVK
Pp -----MSDYILEIKDLHASVND--VPILK
Pf NVVHMENNNFMDRLSLLKNLEESKIITEGEGWDKTHPLLEIKDLHAIEIEGEKEILK
: * . : : * . * :

S GVNLEIKAGEVHAIMGRNGSGKSTLSKVITGHPDYEITGGEIIYQGQDLSALEPHERAL
Ec GLSLDVHPGEVHAIMGPNGSGKSTLSATLAGREDYEVTTGGTVEFKGKDLLALSPEDRAG
Os DLNLKIHKGEIHAIMGPNGSGKSTFSKVLAGHPAYNILSGDILFKGSSILNLDPEERSH
Cp GVNLTINSGEIHAIMGPNGSGKSTFSKILAGHPAYQVTGGEILFKNKNLLELEPEERAR
Gt GLNLVVNAGEIHAIMGKNGSGKSTFAKIIAGHPDYTTITNGDITYQHTSILELTPEDRAK
Pp GVNLSIKPGEIHAIMGPNGSGKSTLSKVIAGHPAYSLLNGDILFYGRSILKMEPDERAR
Pf GINLEIYLGEKHTIMGRNGSGKSTLAKVIAGHPYYKITKGIIKYGLDLINLPVNVRSL
 . . . * : ** * : *** ***** : : : * : * : : . : : . * :

S AGIFLAFQYPLEIPGVSNLDFLR IAYNAKRKHLGLEELDTDFDFEDLIQEKL DVVKMNP
Ec EGIFMAFQYPVEIPGVSNQFFLQTALNAVRSYRGQETLDRDFDQDLMEEKIALLKMPED
Os MGIFLAFQYPIEIPGVS NEDFLRLAYNSKQKFLNKDEVDPISFFT IINEKLKLVDMSPV
Cp AGVFLAFQYPIE IAGVSNIDFLRLAYNNRRKEEGLTELDPLTFYSIVKEKLVVKMDPH
Gt RGIFLSFQYPIEIPGV TNADFLRLACNARRIYQGLPEMEPLEFFEYINSKLP LVDLKPS
Pp AGIFLAFQYPVEIPGVS NADFLRIALNARRKFRELPELSPLEFFQLITEKINLVGMKES
Pf CGIFLAFQYPVELPMVK NNEFLRAALNSHRHRNEEEISVSEFNLM MIEEIKKVGLSSE
 * : * : : * * * * : * : : * : * * : * * : : : : : * : : : : : : :

S FLERSLNEGFSGGEKKRNEILQMAILEPTLSILDEIDSGLDIDALRIVSEGVNFLKNPD
Ec LLTRSVNVGFSGGEKKRNDILQMAVLEPELCILDES DSGLDIDALKVVADGVNSLRDGR
Os FLSRVNVEGFSGGEKKRNEILQMILLDSEL SILD ETDSDGLDIDALKII SKGINTFMNQN
Cp FLNRNVNEGFSGGEKKRNEILQMALLNP SLAILDETDSGLDIDALRIVAEGVNQLSNKE
Gt FLTRDVNEGFSGGEKKRNEILQMSILT KLA VLDETDSGLDIDALRTVANGIKSLANDG
Pp FLSRVNVEGFSGGEKKRNEILQMALLES NLS ILDETDSGLDIDALKIIAQGINTLATST
Pf FLDRPVNYGFSGGEKKRNEILQMLILKPTFCILDETDSGLDVDSFKLT SNVITNF SNDN
:
* * : * ***** : ***** : * . : . : *** ***** : * : . : . : . :

S NATLVITHYQRLNLYIIPDHIHVMYDGIKIVMSGGKELALELEEKGYDFLDEQVLAPI
Ec RSFIIIVTHYQRILDIKPDYVHVLYQGRIVKSGDFTLVKQLEEQQYGWLTEQQ---
Os KAIILITHYQRLLDYVQPNYVHVMQNGKIIKTGTADLAKELESKGYEWLK-----
Cp NSIILITHYQRLLDYIVPDYIHVMQNGRILKTGGAELAKELEIKGYDWLNELEMVKK
Gt NAIILITHYQRLLDYIKPDFVHVMQEGKIIKTGSASLALDLEKYGYDWLKNELK---
Pp NSIVLITHYQRLLDYIIPNFVHIMSDGIKIVKTGNVNLAQDLEKHGYDWIKST-----
Pf NSFLIVTHYKKLLELLKPNFIHIMHQGKIIESGDFSLVDKIENKGYSQFLKE-----
 . : : : * * * : : : * : : : * : : : * * : : * * : : * : : : * * : :

Figure 5.5: The *P. falciparum* Genome Encodes Ycf16.

A sequence identified on *P. falciparum* chromosome 14 encodes a protein that is similar to Ycf16s from chloroplast and bacterial genomes. ClustalW was used to align Ycf16s from *Synechocystis* PCC6803 (*S*), *E. coli* (*Ec*), *O. sinensis* (*Os*), *C. paradoxa* (*Cp*), *G. theta* (*Gt*), *P. purpurea* (*Pp*) and *P. falciparum* (*Pf*). Identical residues (*), conserved residues (:) and semi-conserved residues (.) are indicated.

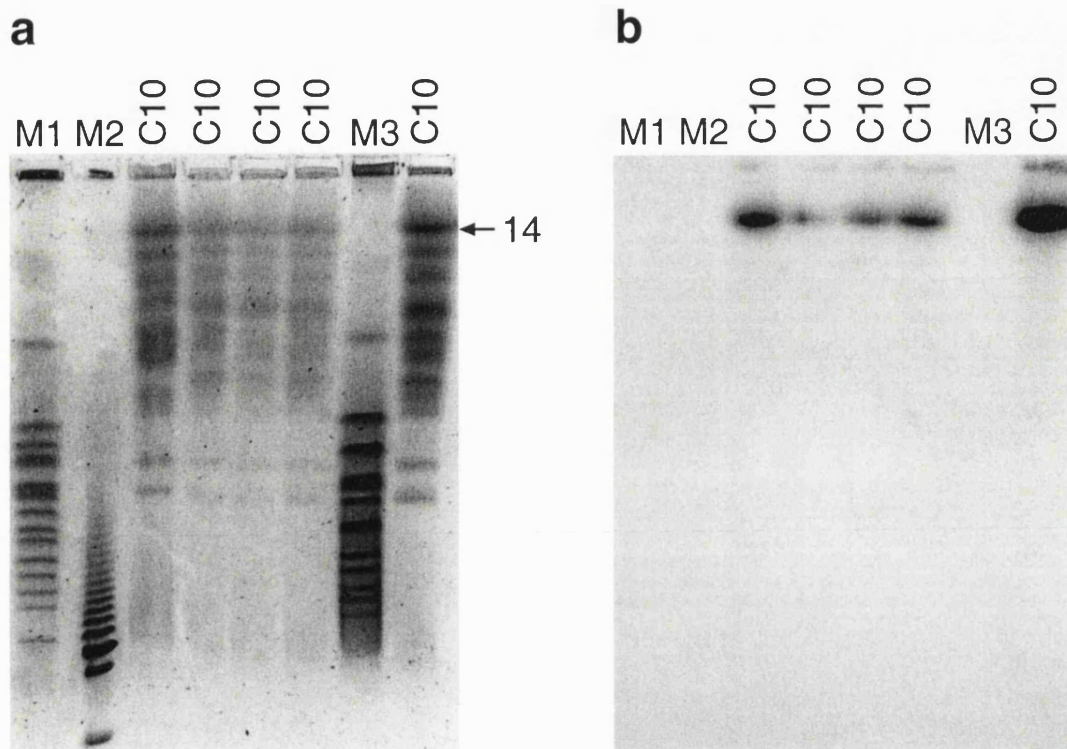


Figure 5.6: *ycf16* is on Chromosome 14 in *P. falciparum*.

Pulsed-field gel showing separated *P. falciparum* chromosomes (a) and blot of this gel showing that *P. falciparum* *ycf16* hybridises to chromosome 14 (b). M1 = *S. cerevisiae* 148 DNA; M2 = Low Range Pulsed-Field Gel Marker (NEB); M3 = *S. cerevisiae* 6H12 DNA.

5.2.5 *P. falciparum* Ycf16 Contains ATP-Binding and ABC

Signature Motifs

The deduced translation product of *ycf16* was scanned against the program Prosite (Hofmann *et al.*, 1999). Amongst the domains identified were a Walker A motif for ATP/GTP-binding, and an ABC signature motif (Figure 5.7). When patterns with a high probability of occurrence were excluded from the analysis, these were the only motifs identified. There is also a sequence that is probably a Walker B motif, which is not found in Prosite. Thirty residues downstream of the second aspartate residue of the putative Walker B motif is a conserved histidine preceded by hydrophobic residues (Figure 5.7). This motif has also been described as characteristic of ABC domains (Linton and Higgins, 1998) (see above).

5.2.6 *P. falciparum* Ycf16 Possesses a Putative Plastid-Targeting Sequence

A signal peptide cleavage site between amino acids 27 and 28 was predicted by the program SignalP (Nielsen *et al.*, 1997) (Figure 5.7). The sequence following the predicted signal peptide contains a high proportion of asparagine and lysine residues, as described for *P. falciparum* transit peptides (Waller *et al.*, 1998) (Figure 5.7). This indicates that Ycf16 in *P. falciparum* is probably a plastid protein as it is in many other plastid-bearing organisms.

5.2.7 *ycf16* is Transcribed by *P. falciparum*

Primers Ycf1611 and Ycf1612 were used in RT-PCR of *P. falciparum* strain C10 RNA. The expected 762 bp product was obtained, as predicted by the sequence in the database, indicating that it does not contain any introns (Figure 5.8). To confirm that the sequence encoding the amino-terminal extension is transcribed, RT-PCR with primers Ycf1615 and Ycf1616 was carried out on strain C10 RNA. Figure 5.9 shows that this part of the sequence is indeed transcribed, and that the transcribed sequence is the same size as the genomic sequence.

RNase protection was used to examine the transcription levels of *ycf16* by *P. falciparum* at different stages during the erythrocytic cycle. The PCR product generated by Ycf1611 and Ycf1612 was cloned into pCR®2.1 and sequenced. A clone containing *ycf16* sequence in a 3' to 5' orientation with respect to the T7 promoter was selected. This construct was digested with *SspI*, a blunt cutter that cuts 272 bp in from the 3' end of the amplified *ycf16* fragment (see Appendix III), but that does not cut the vector. A radiolabelled antisense *ycf16* probe was generated by *in vitro* transcription from the T7 promoter and subsequently gel purified. RNase protection was carried out on RNA samples prepared from different stages of synchronised *P. falciparum*, strain C10. Protected fragments were run on a

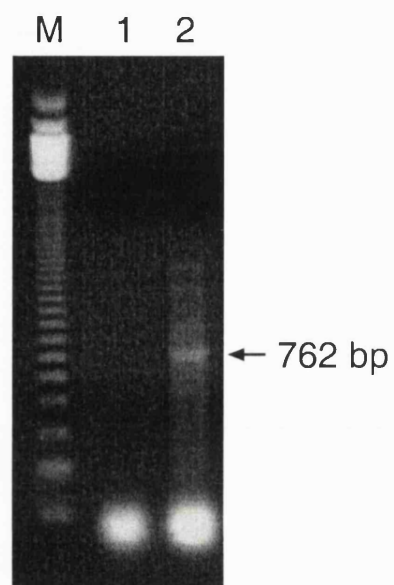


Figure 5.8: *ycf16* is transcribed by *P. falciparum*.

The expected 762 bp product was obtained by RT-PCR with primers Ycf1611 and Ycf1612. Lane 1 is a no-reverse transcriptase negative control, lane 2 shows the RT-PCR product. M = 123 bp ladder.

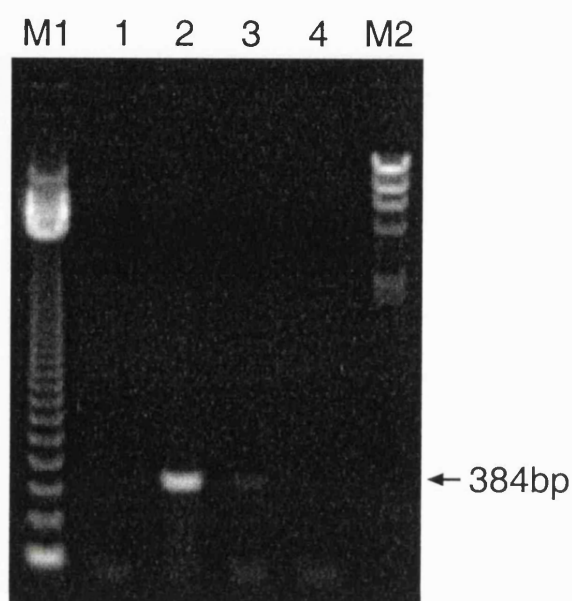


Figure 5.9: The Sequence Encoding the Ycf16 Leader is Transcribed by *P. falciparum*.

RT-PCR on *P. falciparum* strain C10 RNA was carried out with primers Ycf1615 and Ycf1616. A product of the expected size (384 bp) was obtained. Lane 1 is a no-reverse transcriptase negative control, lane 2 is the RT-PCR product, lane 3 is a strain 3D7 genomic DNA positive control, lane 4 is a no-template negative control. M1 = 123 bp ladder; M2 = λ HindIII marker.

denaturing polyacrylamide gel, which was subsequently exposed to film. Figure 5.10 shows that transcription of this sequence occurs in late ring stages through to the schizont stage of the erythrocytic cycle (see Figure 1.2) and that maximal amount of transcript is seen in early trophozoites.

5.2.8 *P. falciparum* Ycf16 May Be Targeted to the Plastid

pACP_{Leader}-GFP (Figure 5.11) was constructed by modification of the *T. gondii* transfection vector *ptubP30-GFP/sag-CAT* (Striepen *et al.*, 1998) (provided by B. Striepen, University of Pennsylvania) by S. Sato (NIMR). The sequence encoding P30 was replaced by a sequence encoding the *T. gondii* ACP leader sequence between the *Bgl*III and *Bln*I restriction sites. Subsequently, a construct consisting of a sequence encoding the *T. gondii* ACP leader, upstream of a sequence encoding DsRed (a red fluorescent protein from *Discosoma* coral (Matz *et al.*, 1999)) was prepared. This ACP_{Leader}-DsRed construct was bounded by an upstream *Bgl*III restriction site and a downstream *Pst*I site, and was used to replace the ACP_{Leader}-GFP sequence in pACP_{Leader}-GFP to form pACP_{Leader}-DsRed (Figure 5.11).

A nucleotide sequence encoding the first 119 amino acid residues of *P. falciparum* Ycf16, optimised for *T. gondii* codon usage and engineered to possess a 5' *Bgl*III restriction site and a 3' *Bln*I site was synthesised by PCR using nine overlapping oligonucleotides as template (carried out by R. Wilson). The PCR product and pACP_{Leader}-DsRed were digested with *Bgl*III and *Bln*I, purified from an agarose gel and ligated to form pYcf16_{Leader}-DsRed. Sequencing showed that there were three errors in the sequence encoding the Ycf16 leader sequence. Two of these occurred in the third base of two codons that did not alter the amino acid sequence and formed codons that are still commonly used in *T. gondii*. The third error changed a serine to a threonine residue, which should not have any functional significance. The sequence encoding the Ycf16 leader was excised from this construct by *Bgl*III and *Bln*I and also cloned into *Bgl*III/*Bln*I-digested pACP_{Leader}-GFP. pYcf16_{Leader}-DsRed and pYcf16_{Leader}-GFP were purified by maxiprep and used to transfect *T. gondii*.

Unfortunately, so far results for this targeting experiment have been inconclusive, as no reporter protein expression has been observed. Conditions for expression of GFP and DsRed need to be optimised.

5.2.9 *P.f.*Ycf16-6His can be Purified Under Denaturing Conditions

A 708 bp portion of the *P. falciparum* *ycf16* gene (excluding the sequence encoding the putative plastid-targeting sequence) was amplified by PCR using primers Ycf1617 and Ycf1618. The PCR product was ligated into pTrcHis2-TOPO®, transformed into TOP10

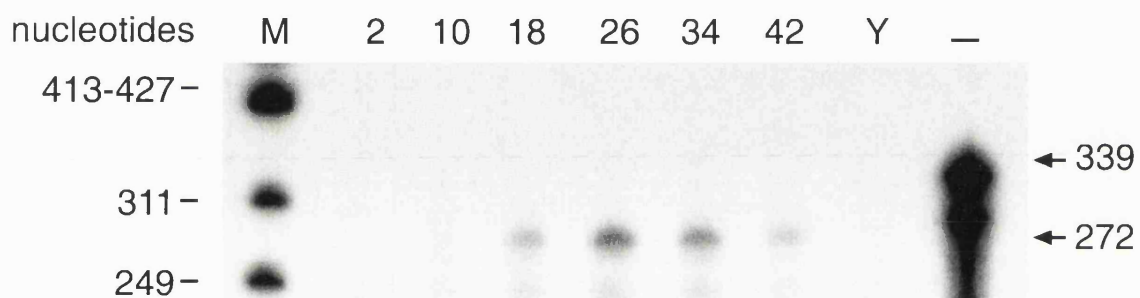
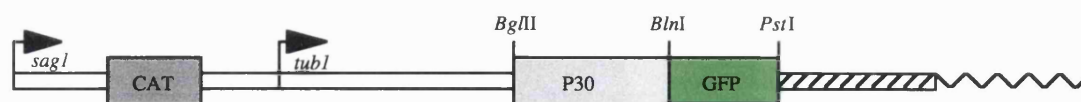
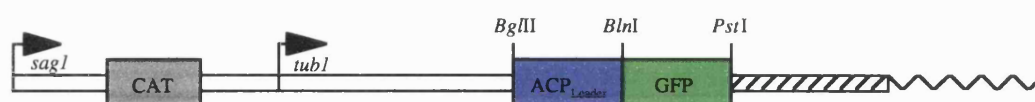


Figure 5.10: Maximum *ycf16* Transcription Occurs in Trophozoites.

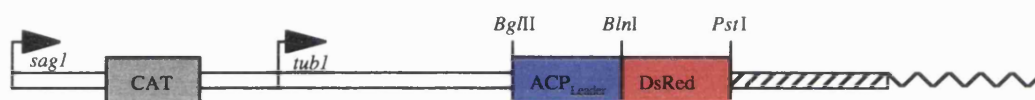
RNase protection was used to look at the transcription of *ycf16* in synchronised parasites. *ycf16* was found to be transcribed in late ring stages, trophozoites and schizonts with maximal transcription in early trophozoites. Time points refer to approximate time post-invasion; Y = yeast DNA control; - = no-RNase control; M = *Hinf*I-digested ϕ X174 DNA.



ptubP30-GFP/sag-CAT (Striepen *et al.*, 1998)



pACP_{Leader}-GFP



pACP_{Leader}-DsRed

Figure 5.11: Vectors Used for Targeting of Reporter Protein in *T. gondii*. Plasmid maps of the original *ptubP30-GFP/sag-CAT* vector (Striepen *et al.*, 1998), and the modified versions produced by S. Sato (NIMR). Coding sequences are shown as wide rectangles, untranslated regions used to drive expression as narrow rectangles and bacterial vector sequences as zig-zag lines. Promoters are shown by arrows. Untranslated regions derived from the *DHFR* locus are hatched, and CAT is flanked by the 5' and 3' regions of the *SAG1* gene.

cells, and sequenced. Transformed cells were grown to an OD₆₀₀ of 0.8 and expression of *P.f.Ycf16-6His* was induced by addition of IPTG. The OD₆₀₀ was measured every hour for 5 hours, and compared to that of untransformed host cells and *E. coli* TOP10 cells transformed by a control expression plasmid (pTrcHis2-TOPO@/*lacZ*). Figure 5.12 shows that expression of *P.f.Ycf16-6His* does not appear to be toxic to *E. coli*. However, a slowing of growth rate is often used as an indicator of expression, so this apparent lack of toxicity could indicate lack of expression.

Western blotting showed that *P.f.Ycf16-6His* was indeed expressed by *E. coli*, but the band corresponding to this protein could not be distinguished from total *E. coli* proteins by Coomassie-staining, indicating that expression levels were low. This perhaps explains the lack of toxicity indicated by the OD₆₀₀ measurements. *P.f.Ycf16-6His* was found to be insoluble and so was purified from inclusion bodies under denaturing conditions (urea (6 M)) (Figure 5.13).

P.f.Ycf16-6His was bound to Ni²⁺-charged resin in imidazole (16 mM) buffer + urea (6 M), and eluted in imidazole (81.5 mM) buffer + urea (6 M). Although western blotting with an anti-pentaHis antibody showed the eluate to be relatively clean (Figure 5.13b), Coomassie-staining revealed contaminating protein bands in the eluate (Figure 5.13a).

5.3 Discussion

No ABC transporter can function with fewer than the four core domains required to form the basic unit for allocrite translocation (Higgins, 1992). Analysis of predicted ABC proteins encoded by *E. coli* showed most were associated with typical transmembrane domains (Linton and Higgins, 1998). However, *ycf16*'s putative partner, *ycf24*, does not encode a typical transmembrane domain (Law *et al.*, 2000). Whilst most ABC proteins constitute a subunit of a membrane transporter, a few apparently do not have a transport function. In *E. coli*, there are twelve identified ABC proteins that have no obvious association with transport-related proteins, and of these, only one has a known function: the DNA repair enzyme UvrA (Doolittle *et al.*, 1986; Linton and Higgins, 1998). The other proteins may form transporters with other components encoded elsewhere on the genome, or they may be involved in non-transport related processes, presumably functioning by coupling ATP hydrolysis to the relevant biological event.

It is the transmembrane domain of an ABC transporter that is involved in allocrite recognition, therefore it is impossible to predict what, if anything, Ycf16 is involved in transporting. Even if a transmembrane domain were identified, it would not be possible to determine the allocrite, or even the chemical class of allocrite, by primary sequence data alone. Only if the transmembrane domain is closely related to that from a different species

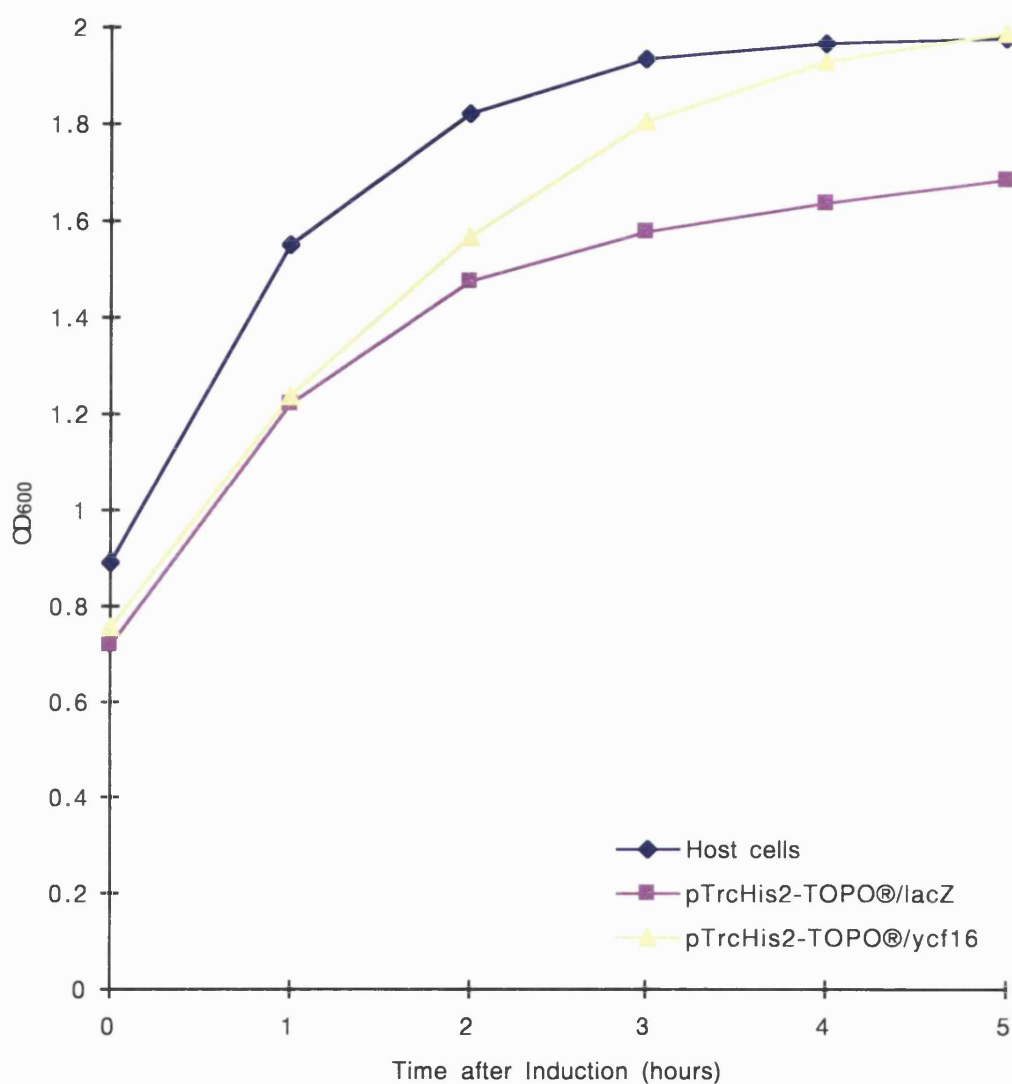


Figure 5.12: Expression of *P.f.Ycf16-6His* in *E. coli* was Not Detrimental.

E. coli TOP10 cells expressing *P.f.Ycf16-6His* grew as well as host cells, and better than control cells. This indicated that expression of this protein was not detrimental to host cells, or that there was no expression occurring (compare control).

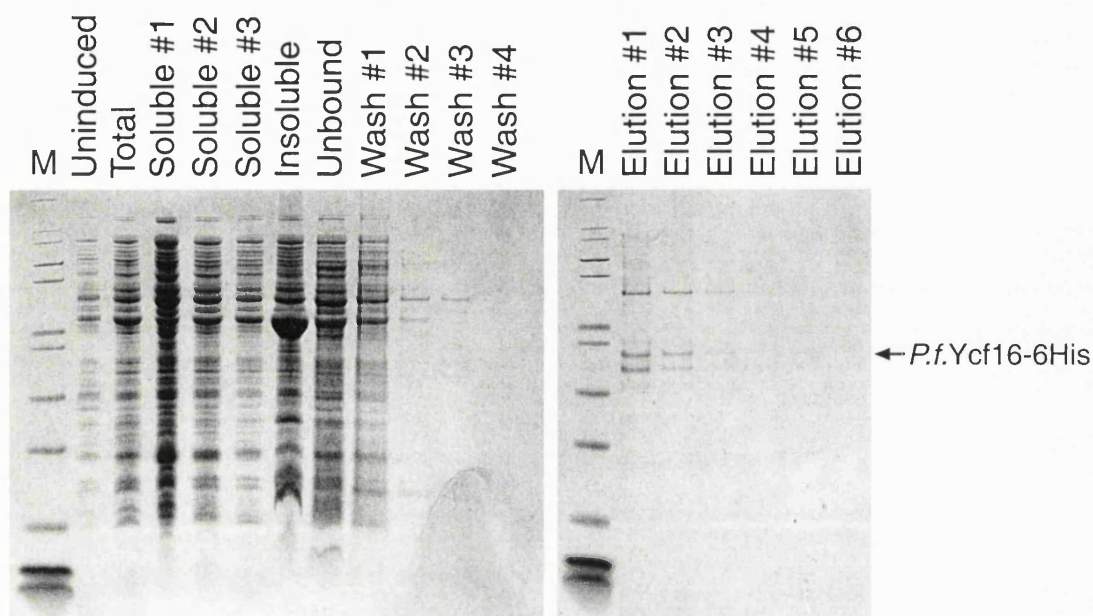
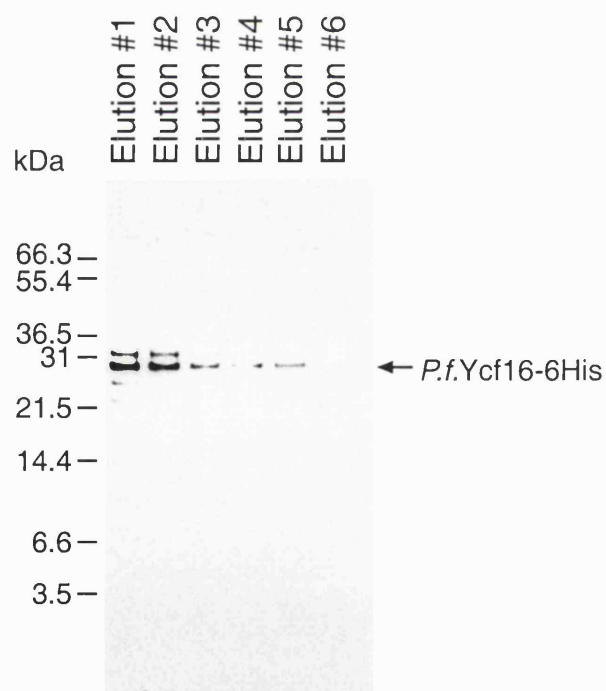
a**b**

Figure 5.13: *P.f.Ycf16-6His* was Purified Under Denaturing Conditions.

P.f.Ycf16-6His (≈ 30 kDa) was found to be insoluble and was purified on Ni-NTA resin in the presence of urea (6 M). Protein eluted in imidazole (81.5 mM) buffer + urea (6 M) can be seen on a Coomassie-stained gel (a), but contaminating bands can also be seen. The eluted protein was also detected by western blot probed with an anti-pentaHis antibody (b).

is it possible to tentatively guess that they transport similar allocrites. The function of an ABC transporter can sometimes be determined by looking at the proteins associated with it. For example, periplasmic-binding proteins are involved in solute uptake systems (Linton and Higgins, 1998).

Since no transmembrane domain has been identified in Ycf16 or its potentially associated proteins in *P. falciparum* (Law *et al.*, 2000) or in *O. sinensis* (Wittpoth *et al.*, 1996), the possibility remains that Ycf16 is an ABC domain with a non-transport related function. It was concluded that in *O. sinensis*, Ycf16 is neither exported from the plastid nor transported to the thylakoids but is located in the stromal fraction of the plastid (Wittpoth *et al.*, 1996). However, Wittpoth *et al.* (1996) remained convinced that Ycf16 is indeed a subunit of an ABC transporter; they believed it may be loosely associated with the inner chloroplast membrane.

GST-*E.c.*Ycf16 was successfully purified under native conditions from *E. coli* (Figure 5.3) and was used in an ATP-binding assay in an attempt to establish whether the putative nucleotide-binding sites are functional. 8-azido-ATP is an ATP analogue that will bind irreversibly to an appropriate active site upon exposure to UV light (Czarnecki *et al.*, 1979). Unexpectedly I found that GST alone also gave a positive result in this assay thereby nullifying the test (Figure 5.4). No nucleotide-binding sites were identified in GST from *Schistosoma japonicum* by Prosite, so this binding is probably non-specific. This is something that should be repeated, either with *E. coli* Ycf16 lacking the GST tag, or with *P. falciparum* Ycf16 once larger amounts have been produced. *P. falciparum* Ycf16 has been produced with a hexaHis tag, which should not interfere with the ATP-binding assay, although it may need to be resolubilised first. The fact that the preparation is not yet totally pure should not matter, since the products are separated by SDS-PAGE before autoradiography, it will be possible to determine whether the band corresponding to Ycf16 has indeed been radiolabelled. Alternatively, it may be possible to carry out an ATPase assay with GST-*E.c.*Ycf16. A GST fusion of the haemolysin exporter HlyB has been successfully used in an ATPase assay, and it was found that GST alone did not have ATPase activity (Koronakis *et al.*, 1993).

In order to confirm the specificity of the ATP-binding assay, competition with unlabelled ATP should be carried out. If the analogue specifically binds to the ATP-binding site, labelling of the protein will be abolished if an approximately 20-fold molar excess of ATP is included in the reaction (Delepelaire, 1994). Another experiment that would be interesting to carry out if Ycf16 were found to bind ATP, would be to look at the effect of mutating specific residues in the protein.

Attempts to cleave GST from *E. coli* Ycf16 with Factor Xa were made; however, these were unsuccessful as shown by western blotting: only GST-*E.c.*Ycf16 was recognised by

an anti-GST antibody. Therefore, it was decided to immunise mice with the fusion protein. GST is highly immunogenic and would help stimulate antibody production. By generating monoclonal antibodies, it was hoped that antibodies recognising Ycf16 but not GST could be generated. The first attempt to produce antibodies failed: only nine monoclonal antibodies were generated, and all were found to recognise GST, as well as GST-*E.c.*Ycf16.

The immunisation was repeated and this time many more fusions were successful. Fusions that appear to recognise *E. coli* Ycf16 but not GST have been obtained, and the process of testing monoclonals is ongoing. These will eventually be used in immunofluorescence on *E. coli* in order to study the subcellular localisation of Ycf16.

A sequence encoding Ycf16 on chromosome 14 in *P. falciparum* was identified (Figures 5.5 and 5.6). It contains the motifs (Walker A, Walker B and ABC signature) that identify it as an ABC protein (Figure 5.7) as well as an amino-terminal extension that resembles a plastid-targeting sequence (Waller *et al.*, 1998). The signal peptide is predicted to be 27 amino acids in length, and this is followed by a putative transit peptide. It is difficult to predict the exact length of the transit peptide since no transit peptide cleavage motif has yet been identified (Waller *et al.*, 2000). However, amongst the first 41 residues following the predicted signal peptide cleavage site, although there are only two serines and no threonines, there are 15 asparagines or lysines (36.6 %). Enrichment in asparagine and lysine residues is a feature of *P. falciparum* transit peptides. The serine and threonine residues required in all other plastid-bearing organisms studied, appear not to be necessary for translocation into the *P. falciparum* plastid (Waller *et al.*, 2000).

RNase protection was used to study transcription of *ycf16* by *P. falciparum*. The transcript was most abundant in early trophozoites, although transcription starts in late ring stages and continues until merozoite release (Figure 5.10). It is not known when *ycf24* is transcribed by *P. falciparum*. RNase protection could be used to look at transcription of both *ycf16* and *ycf24*, and this might give some clue as to whether the gene products interact: if they do one would expect their genes to be transcribed at the same time. However, we should keep an open mind about possible rôles of these genes in the mosquito stage of the parasite, since nothing is known about plastid biogenesis or function in this phase of the life cycle.

The putative plastid-targeting peptide of *P. falciparum* Ycf16 was used in a reporter protein targeting experiment in *T. gondii*. Unfortunately, due to unforeseen technical difficulties, results so far have been inconclusive. Once successful reporter protein expression has been established in this system, confirmation of the localisation of Ycf16 should be achieved.

Although I have been able to express and purify *P.f.Ycf16-6His*, it has not been produced in large amounts. Despite the fact that expression of this protein was not detrimental to the cells (Figure 5.12), a band corresponding to this protein could not be distinguished from endogenous *E. coli* proteins by Coomassie-staining (“Total” compared to “Uninduced”, Figure 5.13). Low expression levels could explain the apparent lack of toxicity of expression as shown by the OD₆₀₀ values (Figure 5.12). As might be expected, *E. coli* is able to synthesise a larger amount of an *E. coli* protein (Figure 5.3) than of a *P. falciparum* version (Figure 5.13). This is not altogether surprising considering the highly A + T-rich DNA of *P. falciparum*; expression of genes of this type must drain the relevant tRNA pools in *E. coli* resulting in decreased expression. Synthesising an artificial gene, encoding the same amino acids, but with an altered, *E. coli*-like codon usage would be one way of overcoming this problem.

Once it was found that *P.f.Ycf16-6His* was insoluble when expressed in *E. coli*, it was purified under denaturing conditions. This can be problematic for proteins that are not expressed at high levels since the extra steps required for purification can lead to loss of product. Fortunately I had enough product to be able to visualise it on a Coomassie-stained gel after elution from Ni-NTA resin. However, Coomassie-staining showed the elutions to be contaminated by several proteins other than the one of interest, in particular by a protein of approximately 50 kDa (Figure 5.13a). Some of the smaller bands, which appear to have a similar elution profile to the protein of interest, may be breakdown products of *P.f.Ycf16-6His* (those showing positive in western blots with the anti-pentaHis antibody presumably retaining their hexaHis tag). These would not be a problem for the production of antibodies. However, it is difficult to envisage what the larger bands might be. It would be difficult to clean up this preparation by altering imidazole concentrations, since the beads are already being washed at 60 mM, and the protein was eluted at 81.5 mM. Attempting purification using a column system rather than the “batch” method could lead to a cleaner product, and perhaps eliminate non-specific binding.

Other techniques that could be used to get rid of contaminating bands include gel filtration, which works on a size exclusion basis. This might get rid of the 50 kDa band, although it will be difficult to separate the bands found between 25 kDa and 31 kDa in the 81.5 mM elutions. One advantage of this technique is that it can be carried out under denaturing conditions. Ion exchange can also be carried out under denaturing conditions, but the calculated pI of *P.f.Ycf16-6His* is 6.48, resulting in only a small charge at neutral pH. However, it may be possible to carry this out under conditions resulting in a greater charge. In addition to the hexaHis tag, *P.f.Ycf16-His* also carries a *myc* epitope. An anti-*myc* antibody could be used in affinity purification; it may be that the proteins that bound non-specifically to the Ni-NTA resin would not bind to anti-*myc* antibodies. Unfortunately, this would probably need to be done under native conditions as the presence of denaturant could either denature the antibody, or disrupt the antibody-epitope interaction.

Once antibodies have been obtained, whether against *E. coli* Ycf16 or *P. falciparum* Ycf16, these will be useful first and foremost for localisation studies. Immunofluorescence microscopy may give an idea as to whereabouts in the cell Ycf16 is situated. For example confirming that it is plastid-localised in *P. falciparum* or whether it appears to be associated with membranes in either organism. For a more detailed analysis of its subcellular localisation, immunoelectron microscopy would be useful. Antibodies could also be useful in immunoprecipitation experiments to determine whether Ycf16 interacts with other proteins, for example Ycf24, or some other protein that may anchor it to the membrane. Such experiments could shed light on whether or not Ycf16 may be associated with a classical ABC transporter.

Chapter 6 - *Synechocystis* Ycf16

6.1 Introduction

6.1.1 *Synechocystis* PCC6803 as a Model Organism

Cyanobacteria are a structurally diverse group of Gram-negative, photosynthetic eubacteria found in a wide range of habitats. Many cyanobacteria were originally thought to be algae (Sauvageau, 1892) until electron microscopy and biochemical analyses showed that they are prokaryotic; however, they are still often referred to as “blue-green algae”. Over 1 500 species with various morphologies, ranging from unicellular to filamentous forms, have been described. *Synechocystis* PCC6803 is a freshwater species, which is classified by its spherical cells that divide by binary fission in successive planes at right angles to each other (Rippka *et al.*, 1979).

Due to their rapid growth rates, simple structure, ease of handling and simple genetic system with the availability of transformation procedures (see below), cyanobacteria are commonly used in the study of plant-related processes such as oxygenic photosynthesis and inorganic nitrogen assimilation (Porter, 1986). Their photosynthetic apparatus resembles that of plants; indeed chloroplasts of algae and plants are thought to have originated with the endosymbiotic relationship between a cyanobacterium and a non-photosynthetic eukaryote (see Chapter 1.2.2.1).

Synechocystis PCC6803 has a physiological, or natural, competence for DNA uptake that probably evolved for the purposes of genetic exchange (Grigorieva and Shestakov, 1982; Porter, 1986, 1988). Chromosomal transformation occurs when internalised donor chromosomal DNA undergoes homologous recombination with chromosomal DNA in the recipient cell. This approach can be used to carry out both targeted and random mutagenesis (Williams and Szalay, 1983; Golden, 1988). It is also possible to transform *Synechocystis* PCC6803 with plasmids that remain as independent replicons within the cell (Chauvat *et al.*, 1986; Mermet-Bouvier and Chauvat, 1994).

Synechocystis PCC6803 remains competent for DNA uptake throughout exponential growth, with a transformation potential directly proportional to cell number; this potential is dramatically reduced as a culture enters the stationary phase (Porter, 1986). *Synechocystis* PCC6803 has been shown to be transformable by DNA from other *Synechocystis* species in which the DNA has a similar G + C content (47.7 % (Kaneko *et al.*, 1996)).

Random mutagenesis can be carried out by treatment with mutagenic chemicals such as *N*-methyl, *N'*-nitro, *N*-nitrosoguanidine or diethyl sulphate, by exposure to ultraviolet light, by transposon tagging or by ectopic mutagenesis (where restriction fragments are

randomly ligated to antibiotic resistance cassettes and allowed to undergo integration into the genome (Labarre *et al.*, 1989)) (Golden, 1988). These techniques are useful when a particular phenotype is sought; the mutated genes are subsequently isolated, a relatively straightforward procedure in the cases of transposon tagging and ectopic mutagenesis.

Specific genes can be inactivated by replacement of a known gene in the chromosome with an interrupted allele (Golden, 1988). The gene of interest is cloned into an *E. coli* vector then linearised by an enzyme that cuts in the gene, but not in the vector. An antibiotic resistance gene, along with its regulatory elements, is then cloned into the gene of interest, thereby disrupting it. The resulting construct can then be transformed into *Synechocystis* where it will undergo homologous recombination into the *Synechocystis* genome, replacing the wt gene. Transformants can be selected by resistance to the antibiotic and presence of the disrupted allele can be confirmed by PCR or Southern blotting. Surprisingly, double crossover events between homologous regions of exogenous and genomic DNA (leading to gene replacement) occur more frequently than single crossover events (which would result in insertion of the entire plasmid into the genome). It has been suggested therefore that exogenous plasmid DNA is somehow linearised upon entry into the host cell (Porter, 1986; Thiel, 1994).

Each cell contains multiple copies of the genome (approximately 10 copies), which enables lethal gene knockouts to be supported (Labarre *et al.*, 1989). If a non-essential gene has been disrupted “homoplasmic” cells (where all the wt copies of the gene have been replaced by the disrupted version) should be detected within a few generations. However, if the gene is essential, then “heteroplasmic” cells will still be present after six months of selection.

It is possible to construct specific mutations within a gene. First it is deleted from the genome, and then replaced with versions that have been modified by site-directed mutagenesis. Since *Synechocystis* PCC6803 has the ability to grow photoautotrophically and photoheterotrophically, it is possible to isolate photosynthetic mutants if they are grown in the presence of glucose as the only source of carbon; the above approach has been used to obtain specific mutations in photosystem II proteins (Williams, 1988). A variation on this technique called “targeted random mutagenesis” results in a collection of mutants with random changes in a specific part of a protein (Ermakova-Gerdes *et al.*, 1996, 2001). This technique allows the identification of functionally important residues in a protein without undergoing the time-consuming process of carrying out site-directed mutagenesis.

Plasmid shuffling can be used to look at the effects of specific mutations on an essential gene (Poncelet *et al.*, 1998). With a wt copy of the gene expressed on a plasmid under selection of a different antibiotic to that used to disrupt the genomic copy of the gene, homoplasmy can be achieved. The plasmid is temperature controlled, so that expression of

the gene occurs when the cells are grown at a higher temperature than normal (35 °C to 39 °C). A second plasmid, carrying yet another antibiotic resistance gene, and constitutively expressing an altered version of the gene of interest is introduced into the cell. The cells are then grown under conditions that do not require retention of the first plasmid (lower temperature and in presence of the antibiotic for which the second plasmid carries a resistance gene). In cases where the altered version of the gene supports viable cells, the plasmid carrying the wt gene will be lost.

The entire 3 573 470 bp genome of *Synechocystis* PCC6803 has been sequenced (Kotani *et al.*, 1994; Kaneko *et al.*, 1995, 1996; Kaneko and Tabata, 1997), and the sequencing data can be accessed from “CyanoBase”, an internet database (Nakamura *et al.*, 1998, 2000). These data facilitate the use of this organism as a model for plastid biology.

6.1.2 *Synechocystis ycf24*

Boynton *et al.* (1988) established a biolistic chloroplast transformation method for *Chlamydomonas reinhardtii*, where transforming DNA is incorporated into the chloroplast genome by homologous recombination. Since then systems involving biolistics, polyethylene glycol-induced transformation of protoplasts, or microinjection, have been developed for several agronomically important higher plants (Daniell, 1999; Dix, 1999; Khan and Maliga, 1999; Knoblauch *et al.*, 1999; Sidorov *et al.*, 1999; Heifetz, 2000). Plant researchers have therefore been able to disrupt various genes on chloroplast genomes and to ascertain whether or not those genes are essential for the organism (Rochaix, 1997).

No plastid transformation system is available for *P. falciparum* or *T. gondii*, so it is not possible to look directly at the effects of disrupting the ORFs of unknown function on the apicomplexan plastid genome. However, many researchers have used cyanobacteria as a model for plastids, in particular for the study of photosynthesis and inorganic nitrogen assimilation. In our laboratory, *Synechocystis* PCC6803 was used to study *ycf24* (Law, 2000; Law *et al.*, 2000).

Part of *ycf24*, approximately 1 kb in length, was amplified from the genome of *Synechocystis* PCC6803, cloned into the *E. coli* vector pUC9 and disrupted by insertion of a blunt-ended kanamycin resistance cassette (*kan^R*) into a blunted, unique *Xba*I site. This construct was transformed into *Synechocystis*, and transformants were selected by resistance to kanamycin.

The transformants obtained grew more slowly than wt *Synechocystis* (colonies taking two weeks to be visible to the naked eye as opposed to three days for wt). The morphology of the colonies was “ragged” compared to “smooth” wt colonies. To ascertain whether *ycf24* is involved in one of the biosynthetic pathways common to plastids, the culture medium

was supplemented with various amino acids (L-phenylalanine, L-tryptophan, L-tyrosine, L-threonine or L-lysine) and with nitrite instead of nitrate as a source of nitrogen, but no improvement in growth rate or change in colony morphology was observed.

Transformants were found to be heteroplasmic for the disrupted gene, as shown by Southern blotting, and the proportion of disrupted copies never constituted more than half the total copies per cell. Homoplasmy was never observed for disrupted *ycf24*, an indication that disruption of this gene was lethal. It was concluded that the reduced growth rate and the “ragged” colony phenotype of $\Delta ycf24$ transformants were due to the death of homoplasmic cells. When transformants were grown in the absence of kanamycin, the disrupted gene was lost within two weeks. The fact that the growth of heteroplasmic mutants is impaired implies that the *ycf24* gene product is required in stoichiometric amounts.

Transmission electron microscopy of wt and $\Delta ycf24$ *Synechocystis* showed that transformants contained electron transparent plaques between the thylakoid membranes. These resembled glycogen deposits, which can accumulate in nitrogen-limited photosynthesising cells (Stanier (Cohen-Bazire), 1988), but the composition of these plaques was never confirmed.

Scanning electron microscopy and light microscopy showed that $\Delta ycf24$ mutant cells were much more likely than wt cells to be in the process of division indicating that disrupting *ycf24* in *Synechocystis* affects cell division. DAPI-staining showed that “ragged” mutants contained less DNA than wt cells, and missegregation of DNA was observed in approximately 3 % of dividing cells.

Chlorophyll in live cells fluoresces orange under ultraviolet light, whereas dead cells appear yellow or green. This autofluorescence was used to compare wt and $\Delta ycf24$ *Synechocystis*. It was found that approximately half the $\Delta ycf24$ *Synechocystis* cells were dead, and of these, half were undergoing division. Video-linked phase contrast microscopy showed that some cells failed to divide, and this could lead to “ragged” colony formation.

It was concluded that *ycf24* plays a rôle in cytokinesis, or in nucleoid replication or segregation (Law, 2000; Law *et al.*, 2000).

6.1.3 Aims

ycf16 is found in the cyanobacterium *Synechocystis* immediately adjacent to, and in the same orientation as *ycf24* (Kaneko *et al.*, 1995). The intergenic region between the two genes is only 141 bp in length, which suggested that the genes may be co-transcribed.

Having identified *ycf16* as a probable plastid-targeted nucleus-encoded gene in *P. falciparum* (Chapter 5.2), it was decided to disrupt *ycf16* in *Synechocystis* and compare the phenotype with that obtained for $\Delta ycf24$ mutants (Law, 2000; Law *et al.*, 2000).

6.2 Results

6.2.1 *ycf16* and *ycf24* are Co-Transcribed in *Synechocystis*

The short intergenic region between *ycf24* and *ycf16* (141 bp) in *Synechocystis* (see Appendix III) implies that they may be co-transcribed as part of the same operon. This hypothesis was tested by carrying out RT-PCR. Primers Ycf243 and Ycf169 were used to amplify the intergenic region from wt cDNA (wt *Synechocystis* provided by E. Thompson, UCL). Figure 6.1 shows that there is indeed a transcript that can be amplified by these primers, indicating that *ycf24* and *ycf16* are co-transcribed in *Synechocystis*.

6.2.2 *Synechocystis* $\Delta ycf16$ Mutants are Heteroplasmic

The cloning strategy for disrupting *Synechocystis* *ycf16* is illustrated by Figure 6.2. Primers Ycf243 and Ycf168 were used to amplify most of *ycf16* and part of *ycf24* (wt DNA provided by A. Law, NIMR). The resulting 1 368 bp PCR product was cloned into pCR®2.1, sequenced and found to be error-free. In order to disrupt *ycf16* this construct was linearised through a unique *HpaI* site situated 69 bp from the start of *ycf16* (see Appendix III). *strep^R* (1 481 bp), consisting of the *E. coli aadA* gene (encoding aminoglycoside 3"-adenyltransferase) downstream of 5' *atpA* sequence (Hollingshead and Vapnek, 1985; Goldschmidt-Clermont, 1991) was provided by M. Ashby, UCL. It was digested with *HindIII* and blunted by treatment with Klenow polymerase (by A. Law, NIMR), then ligated into the *HpaI* site resulting in elimination of this site. This construct was transformed into wt *Synechocystis* (provided by C. Mullineaux, UCL), where the disrupted *ycf24/ycf16* construct underwent homologous recombination into the *Synechocystis* genome. Transformants were selected by resistance to streptomycin.

Transformants were obtained that grew more slowly than wt *Synechocystis*. Typically when streaked out from a single colony onto BG11 agar plates and incubated at 27 °C, wt colonies could be seen by the naked eye after three days, whereas it took six days to see transformants.

In order to determine whether transformants were homoplasmic or heteroplasmic, two PCRs, carried out directly on colonies picked from BG11 agar plates were used. Firstly, two primers flanking the site of insertion of the construct, Ycf1613 and Ycf1614, were used. These primers were expected to give a 1 491 bp PCR product where *ycf16* was not

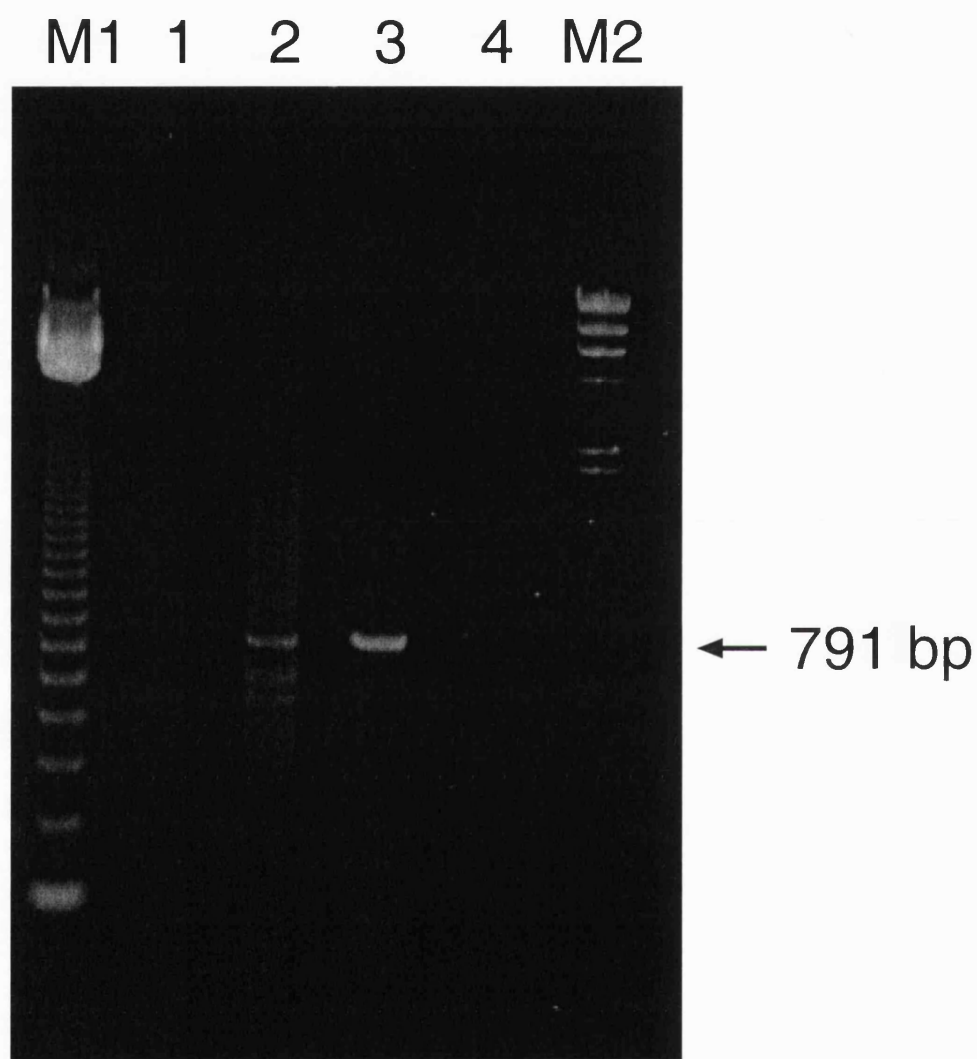
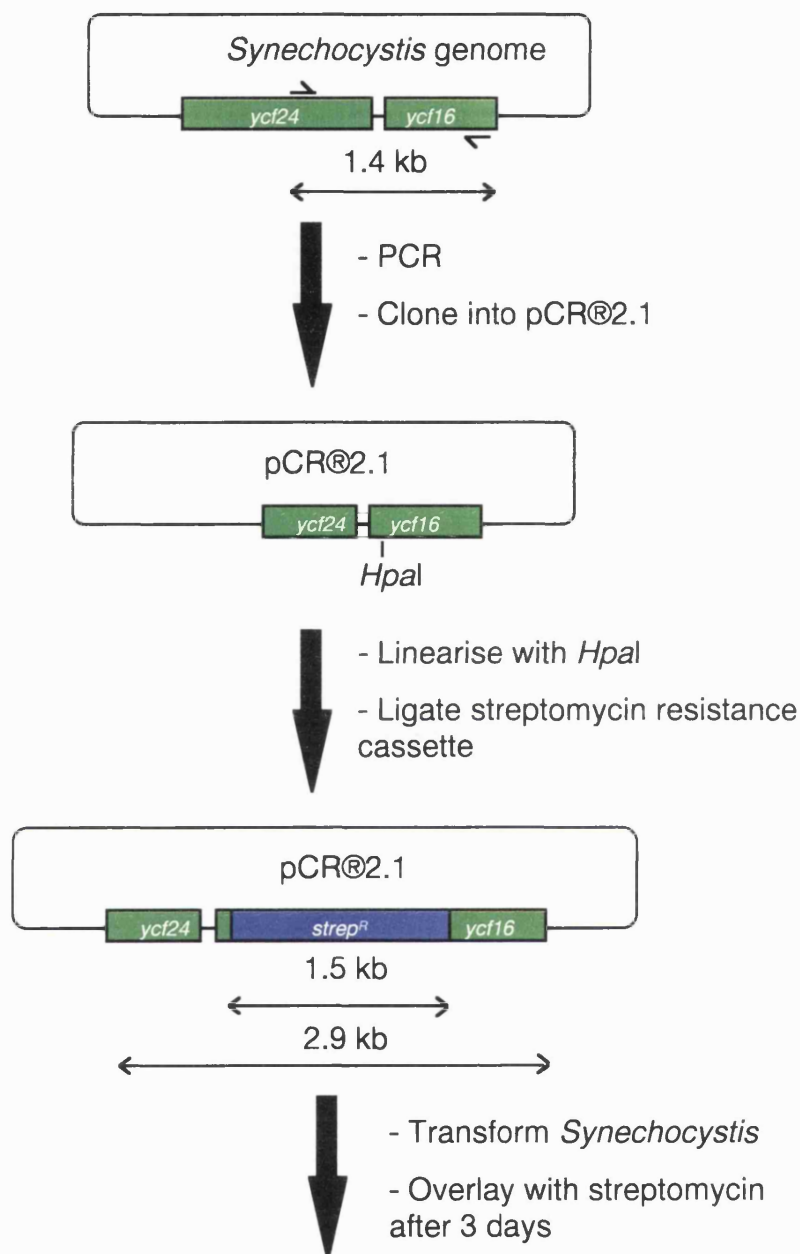


Figure 6.1: *ycf24* and *ycf16* are Co-Transcribed in *Synechocystis*.

Primers Ycf243 and Ycf169 were able to amplify the intergenic region between *ycf24* and *ycf16* from cDNA prepared from wt *Synechocystis* RNA. Lane 1 is a no-reverse transcriptase negative control, lane 2 is the RT-PCR product, lane 3 is the intergenic region amplified from wt *Synechocystis* genomic DNA and lane 4 is a no-template negative control. M1 = 123 bp ladder; M2 = λ HindIII marker.



Analyse streptomycin resistant colonies

Figure 6.2: Cloning Strategy for Disruption of *ycf16* in *Synechocystis*. Part of *ycf24* and most of *ycf16* were amplified from the *Synechocystis* genome and cloned into pCR®2.1. This construct was linearised through a unique *HpaI* site, and *strep^R* was ligated in, disrupting *ycf16*. The resulting construct was transformed into wt *Synechocystis*, and transformants were selected by resistance to streptomycin.

disrupted, and a 2 972 bp product where it was disrupted due to integration of the exogenous DNA. Figure 6.3 shows that since all transformants gave a band of the expected size for wt *ycf16*, they all contained at least one copy of the wt gene (compare with the wt samples, lanes 1 and 2). The larger PCR product expected for the disrupted *ycf16* was not seen in transformants and it was assumed that the smaller PCR product was preferentially amplified.

To confirm that the disrupted construct had recombined correctly into the *Synechocystis* genome, a second PCR with primers Sp11 and Ycf1613 was carried out. These primers gave a 792 bp product where the exogenous DNA had integrated correctly into the genome, and no product in wt DNA since wt *Synechocystis* does not contain *strep^R*. As shown in Figure 6.4, a product of the expected size was obtained in the transformants, whereas no product was obtained from either wt sample. This PCR product was cloned into pCR®2.1 and sequenced. Sequencing showed that insertion of the disrupted construct into the *Synechocystis* genome had not disrupted *ycf24* and that recombination was completely faithful (Figure 6.5).

A Southern hybridisation was carried out in order to estimate the copy number of wt *versus* disrupted *ycf16* in different heteroplasmic cell lines. Genomic DNA from wt *Synechocystis* and different mutants was digested with *HpaI*. A restriction map of the region of the genome surrounding *ycf24* and *ycf16* is shown in Figure 6.6a. The PCR product generated by primers Ycf243 and Ycf168 was used as a probe. From the resulting autoradiograph (Figure 6.6b) the pattern of bands confirms the heteroplasmic nature of the different cell lines.

The relative intensities of the bands resulting from disrupted *ycf16* compared to wt *ycf16* were analysed by phosphorimaging. The results are shown in Table 6.1 as a ratio of disrupted *ycf16* to wt *ycf16*. This shows that there are never more than two copies of the disrupted gene per cell, indicating that on average the cell cannot tolerate the loss of more than two wt copies of *ycf16*. This is further evidence that the gene product of *ycf16* has an important function.

6.2.3 Δ *ycf16* Mutants Lose Streptomycin Resistance in the Absence of Selection

Δ *ycf16* *Synechocystis* was grown on BG11 agar plates without selection. Each week it was restreaked onto a fresh BG11 plate, and also onto a BG11 plate supplemented with streptomycin. It was found that after only four weeks growth without selection, it lost its ability to grow in the presence of streptomycin.

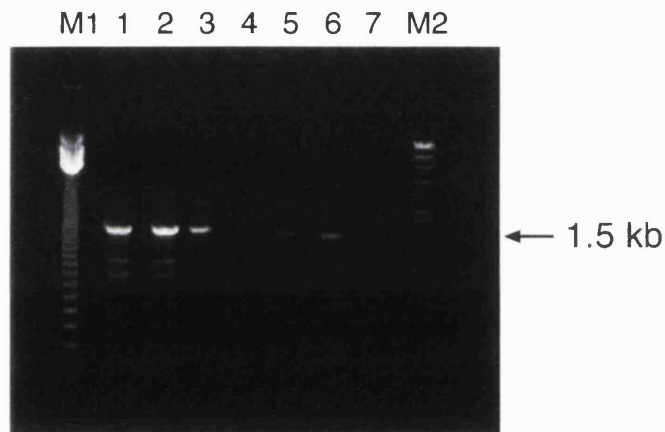
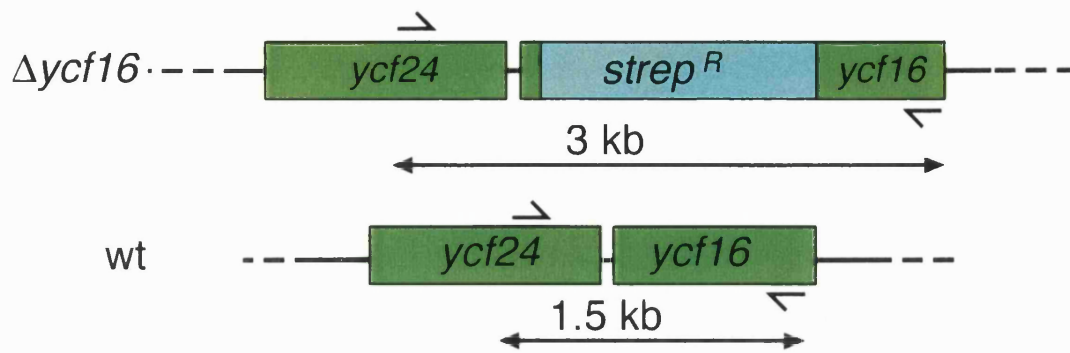


Figure 6.3: All Transformants Possess at Least One Copy of wt *ycf16*.

PCR with primers Ycf1613 and Ycf1614 was carried out directly on colonies. wt *Synechocystis* give a 1 491 bp product (lanes 1 and 2, wt genomic DNA and wt colony as template, respectively). All transformants (lanes 3 to 6, cell lines d, f, g and h) also gave a 1 491 bp product. This shows that all transformants possess at least one copy of wt *ycf16*. Lane 7 is a no-template negative control; M1 = 123 bp ladder; M2 = λ HindIII marker.

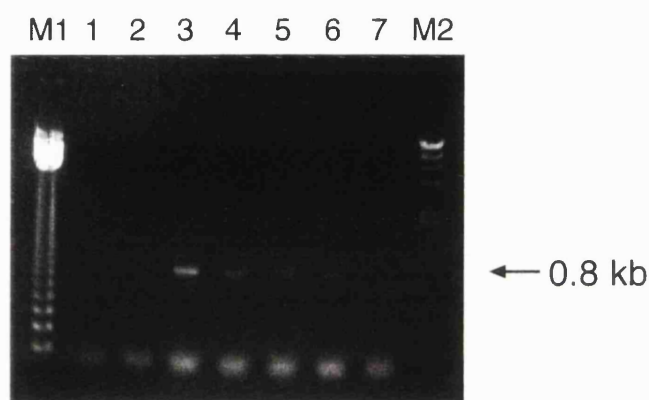
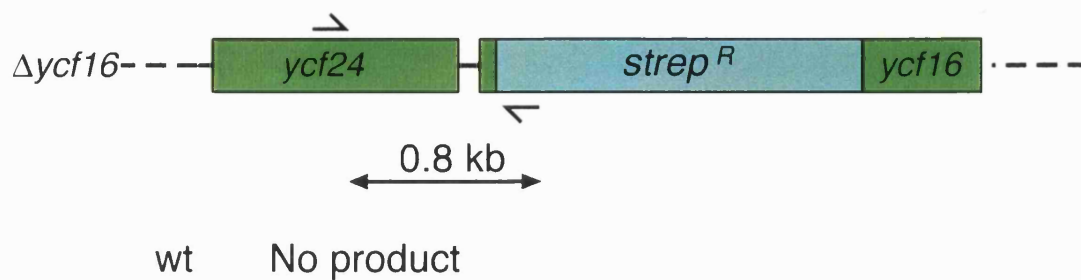
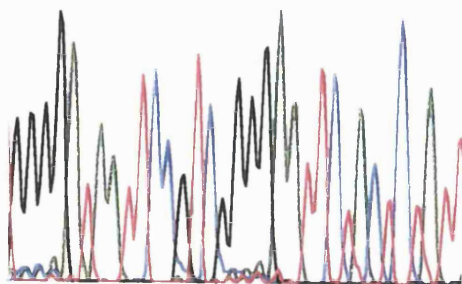


Figure 6.4: All Transformants Possess at Least One Copy of Disrupted *ycf16*.

PCR with primers Ycf1613 and Sp11 was carried out directly on colonies. Transformants (lanes 3 to 6, cell lines d, f, g and h) all give a 792 bp product indicating that they all possess at least one copy of disrupted *ycf16*. No product is obtained with wt DNA (lanes 1 and 2, wt genomic DNA and wt colony as template, respectively) since wt *Synechocystis* does not contain *strep^R*. Lane 7 is a no-template negative control; M1 = 123 bp ladder; M2 = λ HindIII marker.

a

GGGGATAATTCCGTCGGGGGAATTCTACTCTATT
160 170 180 190



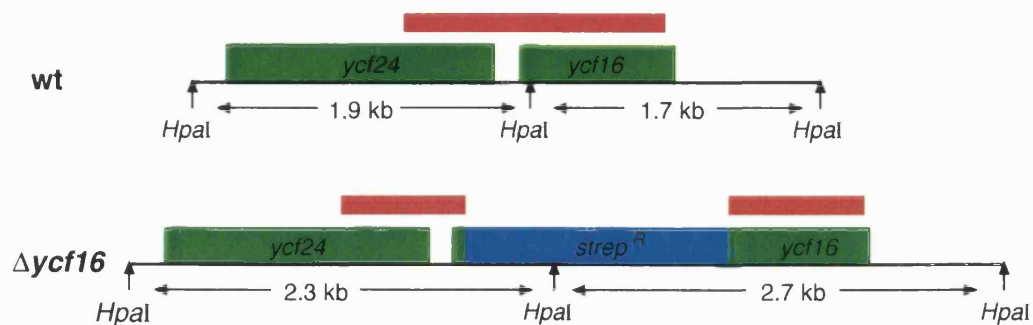
b

GGGGATAATTCCGTCGGGGGAATTCTACTCTATT

Figure 6.5: Recombination of Disrupted *ycf16* into the Genome did not Disrupt *ycf24*.

The construct used to transform *Synechocystis* possessed 473 bp *ycf24* sequence. This sequence was shown to be error-free before transformation. After transformation the Ycf1613-Sp11 PCR product generated from $\Delta ycf16$ *Synechocystis* was sequenced (a) and compared to the sequence of wt *ycf24* (b). This showed that no disruption to *ycf24* was caused by recombination of this construct into the genome. The sequence of primer Ycf243, used to create the original construct to be disrupted, is underlined in b.

a



b

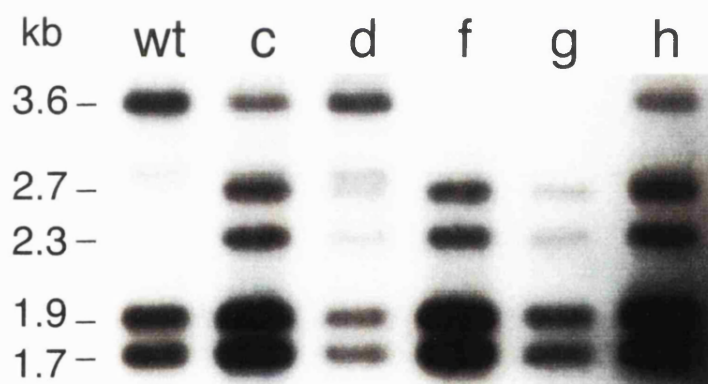


Figure 6.6: Transformants are Heteroplasmic.

Genomic DNA from wt and mutant *Synechocystis* was digested with *Hpa*I, and probed as shown by the red bars (a). The probe hybridises to fragments of 1.7 kb and 1.9 kb in both wt and transformants. In addition, in transformants the probe hybridises to bands of 2.3 kb and 2.7 kb. The 3.6 kb band corresponds to incompletely digested wt DNA. The autoradiograph (b) confirms that all transformants are heteroplasmic.

Table 6.1: *Synechocystis* Cannot Support the Loss of More than Two Copies of *ycf16*.

The relative intensities of the wt bands obtained by Southern blotting and bands resulting from the disrupted *ycf16* within each cell line were analysed. The ratios of disrupted *ycf16* to wt *ycf16* indicate that in each cell there are on average no more than two copies of the disrupted version.

Cell Line	Disrupted:wt
c	2:7
d	1:9
f	2:7/8
g	1:5
h	2:6/7

6.2.4 $\Delta ycf16$ Mutants Exhibit a “Ragged” Colony Morphology

Heteroplasmic $\Delta ycf24$ mutants were found to exhibit a “ragged” colony morphology compared to “smooth” wt type colonies (Law, 2000; Law *et al.*, 2000). Observation of $\Delta ycf16$ mutants grown on BG11 agar plates showed that they have a similar phenotype (Figure 6.7). This morphology has been attributed to death of homoplasmic cells (Law, 2000; Law *et al.*, 2000), and provides further evidence that *ycf24* and *ycf16* are essential genes in *Synechocystis*.

6.2.5 A High Proportion of $\Delta ycf16$ Mutants are Undergoing Division

It was noted that a high proportion of $\Delta ycf24$ mutants appeared to be undergoing division at any one time (Law, 2000; Law *et al.*, 2000). This was also seen in $\Delta ycf16$ *Synechocystis* by fluorescence microscopy (Figure 6.8). Both wt and $\Delta ycf16$ mutant cells were counted using a haemocytometer, and the number undergoing division recorded. Approximately 20 % wt cells were undergoing division (a typical proportion (Law *et al.*, 2000)), whereas approximately 60 % $\Delta ycf16$ mutant cells were found to be undergoing division. This suggests that some disruption to division or to the cell cycle had occurred.

6.2.6 A High Level of Cell Death is not Seen in $\Delta ycf16$ Mutants

Autofluorescence of chlorophyll under UV light in $\Delta ycf16$ *Synechocystis* did not reveal a high level of cell death. This was shown by the fact that all cells appeared orange, with no green or yellow cells (Figure 6.8).

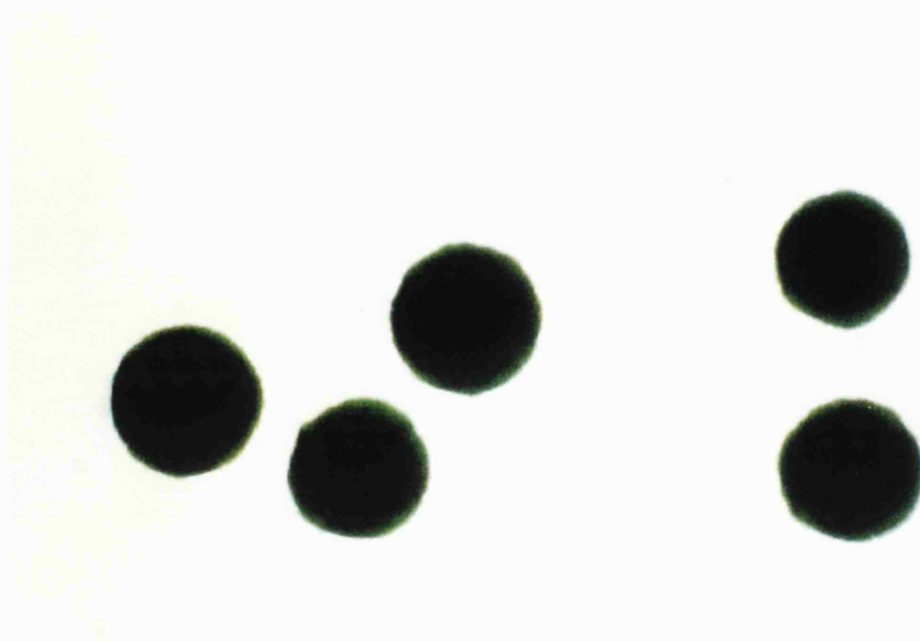
6.2.7 $\Delta ycf16$ Mutants Do Not Contain Reduced Amounts of DNA

DAPI-staining was used to compare the amount of DNA in $\Delta ycf16$ mutants with that in wt *Synechocystis*. Although $\Delta ycf24$ mutants were shown to contain less DNA than wt *Synechocystis* by a dramatic reduction in fluorescence (Law, 2000; Law *et al.*, 2000), no difference in fluorescence intensity between $\Delta ycf16$ mutant cells and wt cells was observed (results not shown).

6.2.8 $\Delta ycf16$ Mutants Appear as wt in Electron Micrographs

wt and $\Delta ycf16$ mutant *Synechocystis* were analysed by transmission electron microscopy. Although the main phenotype of $\Delta ycf24$ mutants was the presence of electron transparent plaques (Law, 2000; Law *et al.*, 2000), no such defect was observed in $\Delta ycf16$ mutants. In fact, no difference was observed at the subcellular level between wt cells and $\Delta ycf16$ *Synechocystis* (Figure 6.9).

a



b

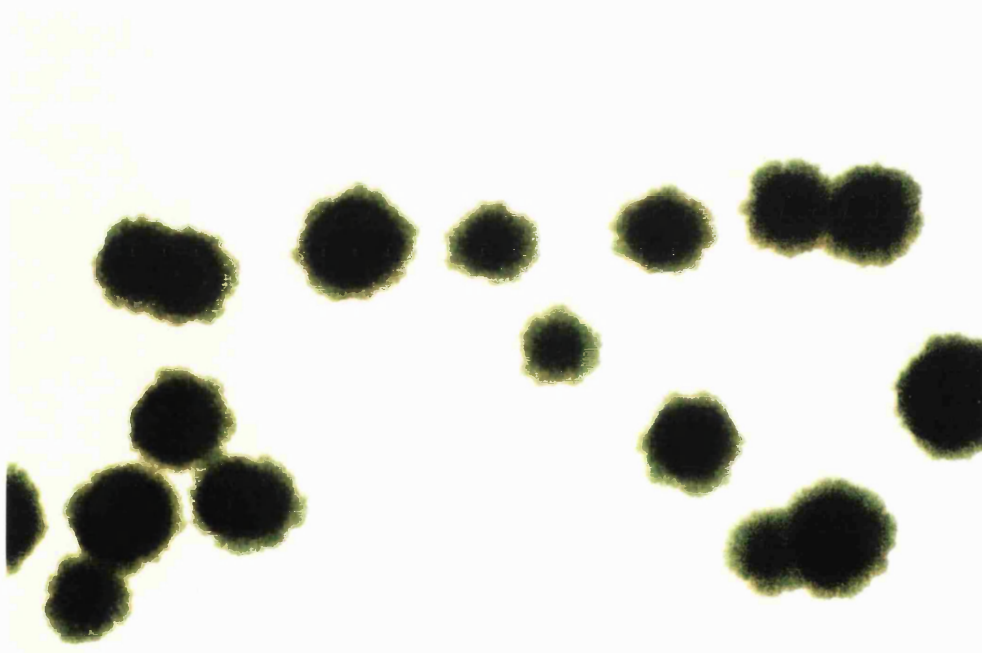


Figure 6.7: $\Delta ycf16$ Mutants Exhibit a “Ragged” Colony Morphology. wt colonies are “smooth” (a), whereas $\Delta ycf16$ mutant colonies, like those of $\Delta ycf24$ mutants, are “ragged” (b). Scale bar = 1 mm.

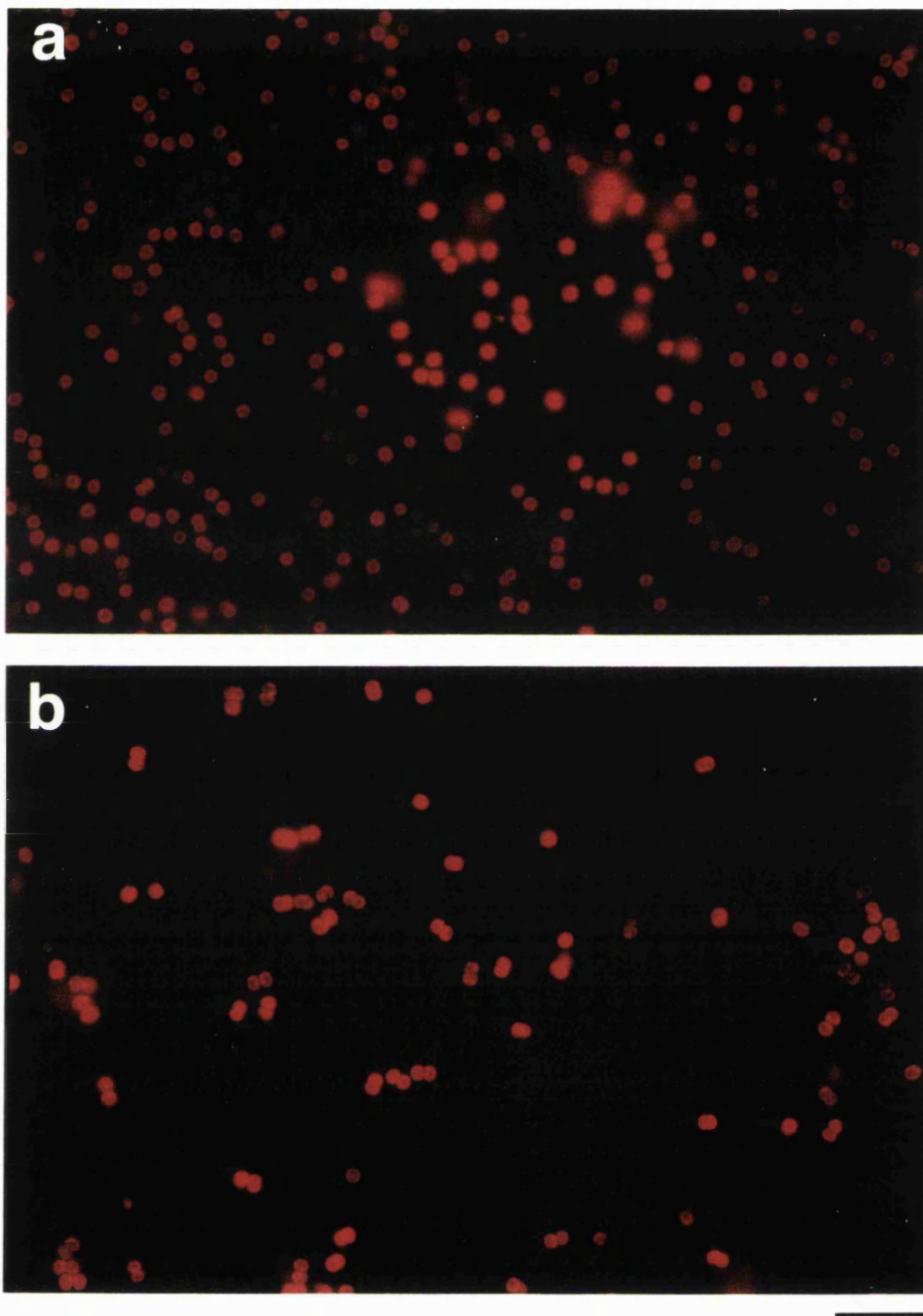


Figure 6.8: A High Proportion of $\Delta ycf16$ Mutants are Dividing. Autofluorescence of wt (a) and $\Delta ycf16$ mutants (b). Approximately 60 % mutants were undergoing division at any one time compared to 20 % wt cells. Scale bar = 20 μm .

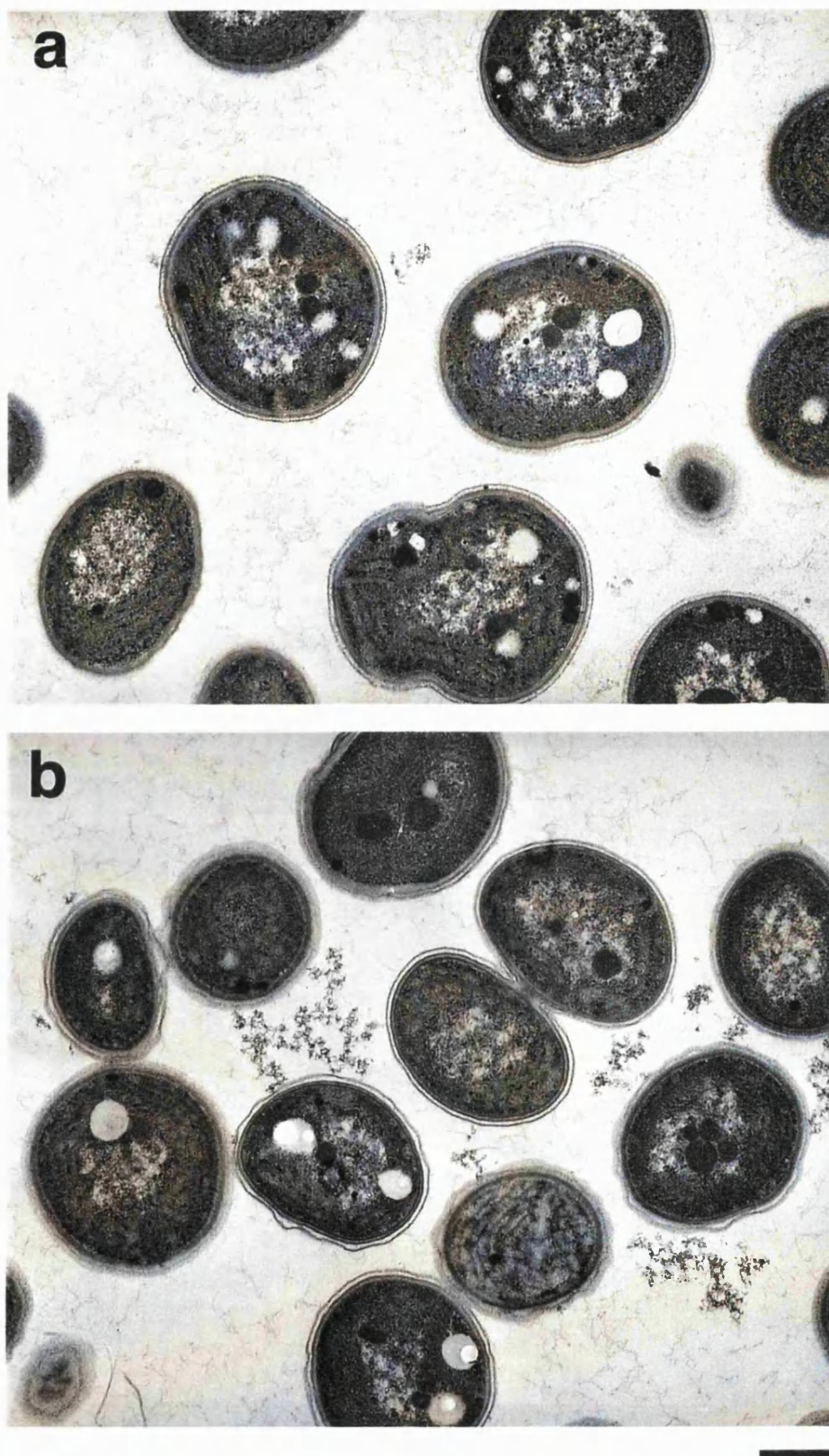


Figure 6.9: Electron Microscopy did not Reveal any Differences Between wt and $\Delta ycf16$ *Synechocystis*. Transmission electron micrographs of wt (a) and $\Delta ycf16$ *Synechocystis* (b). No differences between them were observed. Scale bar = 0.5 μm .

6.2.9 *Δycf16 /Δycf24* Mutants are Viable

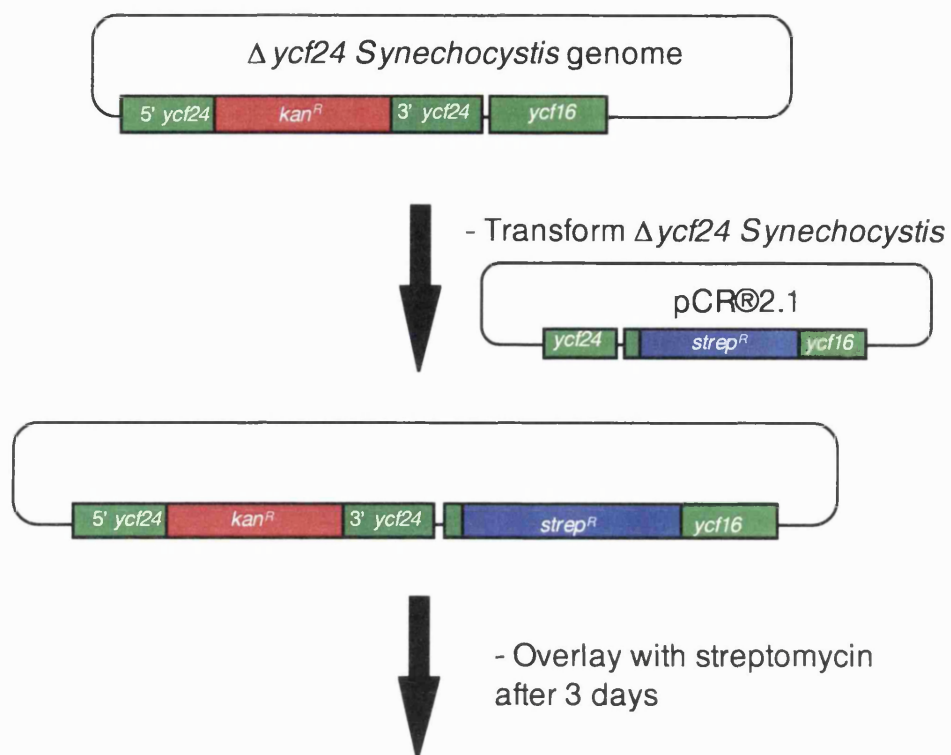
The construct used to disrupt *ycf16* in wt cells (Figure 6.2) was used to disrupt *ycf16* in *Δycf24* cells (Law, 2000; Law *et al.*, 2000) as shown by Figure 6.10. Transformants were selected by resistance to both kanamycin and streptomycin; although they grew more slowly than wt cells (taking at least four days to be visible to the naked eye on BG11 agar plates), they grew more quickly than *Δycf16* cells. In addition, although the colonies were “ragged” compared to “smooth” wt colonies, they were less “ragged” than *Δycf16* colonies (Figure 6.11).

6.3 Discussion

The components of many prokaryotic ABC transporters are encoded by separate genes, which are often arranged in operons. According to a survey of ABC transporters in the complete genomes of seven microbes, *Synechocystis* was found to lack the operon structure compared to other bacteria (Tomii and Kanehisa, 1998). This resulted in identification of a relatively large number of putative ATP-binding domains, which did not appear to be associated with other ABC components. This lack of operon structure in *Synechocystis* was also noted in a search for functionally related enzyme clusters in the genomes of ten micro-organisms (Ogata *et al.*, 2000). However, I have shown that *ycf24* and *ycf16* are co-transcribed in *Synechocystis* (Figure 6.1), as they are believed to be in some other organisms such as *P. purpurea* (M. Reith, personal communication), *E. coli* (Patzer and Hantke, 1999) and *P. chrysanthemi* (Nachin *et al.*, 2001) and therefore they could form part of an operon. In addition, other genes of this operon in *E. coli* and *P. chrysanthemi* are present in *Synechocystis*.

I disrupted *ycf16* in *Synechocystis* by insertion of *strep*^R but was unable to generate homoplasmic *Δycf16* mutants despite culturing on selective media for 12 months. This strongly suggests that *ycf16* is an essential gene; when a non-essential gene is disrupted homoplasmy can normally be achieved within one month (Williams, 1988). Moreover, when the copy numbers per cell of wt and disrupted *ycf16* were compared it was shown that the cells could not support the loss of more than one or two copies of the wt gene (Table 6.1).

I showed that when grown without selection, *Δycf16* *Synechocystis* loses the disrupted gene within four weeks. The disrupted *ycf16* is maintained purely to provide antibiotic resistance, and when this is no longer required, it is lost. This indicates there is some loss of fitness when *ycf16* is disrupted, and that when there is no selective pressure it is advantageous for the cell to revert to wt. This is further evidence that *ycf16* is an essential gene.



Analyse streptomycin and kanamycin
resistant colonies

Figure 6.10: Cloning Strategy for Disruption of *ycf16* in Δ*ycf24* *Synechocystis*.

strep^R-disrupted *ycf16* in pCR®2.1 was transformed into Δ*ycf24* *Synechocystis*. Transformants were selected by resistance to kanamycin and streptomycin. It should be noted that although this experiment was designed such that both *ycf24* and *ycf16* could be disrupted on the same copy of the genome (as shown), it may be that they are only disrupted on different copies of the genome.

a



b



Figure 6.11: $\Delta ycf16/\Delta ycf24$ Mutants Exhibit a “Ragged” Colony Morphology.

$\Delta ycf16/\Delta ycf24$ mutant colonies are “ragged” (a), but to a lesser extent than either $\Delta ycf16$ (b) or $\Delta ycf24$ mutants (Law, 2000; Law *et al.*, 2000). Scale bar = 1 mm.

Colonies of $\Delta ycf16$ *Synechocystis* exhibited a “ragged” morphology (Figure 6.7) thought to result from the death of homoplasmic cells (Law, 2000; Law *et al.*, 2000). DNA segregation in this organism is uneven, so daughter cells are not exact genetic copies of parent cells. However, presumably the “ragged” colony morphology is not due to death of cells that are homoplasmic for the wt genotype, since “ragged” colony morphology was not observed when an antibiotic resistance cassette was randomly inserted into the genome (Emlyn-Jones *et al.*, 1999). In fact, when $\Delta ycf24$ *Synechocystis* colony formation was observed by video-linked phase contrast microscopy it was shown that some cells (presumably those homoplasmic for disrupted *ycf24*) failed to divide, and this resulted in “ragged” colonies (Law, 2000; Law *et al.*, 2000).

I conclude therefore that *ycf16* is an essential gene in *Synechocystis*. However, identifying a function for its product has been less straightforward.

Disrupting *ycf16* in *Synechocystis* resulted in a similar phenotype to that found in $\Delta ycf24$ mutants, although it appeared to be less severe (Table 6.2). There are two reasons why this might be. One is due to the site of insertion of the antibiotic resistance cassette. In the $\Delta ycf24$ mutants, *kan^R* was inserted 771 bp into *ycf24* which means that there is the potential for the synthesis of a truncated version of *ycf24* 257 amino acids in length. It is possible that such a product was toxic to the cell, and it may have caused an artefactual phenotype. This situation is unlikely to have occurred in the $\Delta ycf16$ mutants since *strep^R* was inserted only 69 bp into the gene. Any product would be so small it would probably have degraded. Another possibility is that since *ycf24* and *ycf16* are co-transcribed, and *ycf16* is immediately downstream of *ycf24*, expression of *ycf16* may have also been disrupted in $\Delta ycf24$ mutants. Northern analysis of $\Delta ycf24$ *Synechocystis* compared to wt should show whether or not this is the case. Ultimately, if we had antibodies to *Synechocystis* Ycf24 and Ycf16 the levels of gene product could be compared in the different mutants by western blotting and immunofluorescence microscopy.

Since *ycf16* and *ycf24* have both been shown to be essential genes in *Synechocystis*, I decided to try to disrupt *ycf16* in $\Delta ycf24$ *Synechocystis* on the assumption that the result would be too deleterious to be supported by the cell, and that I would not be able to generate transformants. Surprisingly, however, transformants were obtained, and these grew more quickly than $\Delta ycf16$ mutants that were generated at the same time as a positive control; there was no difference in number of transformants obtained. It had previously been noted that when $\Delta ycf24$ *Synechocystis* was grown in liquid culture, “suppressor” forms were observed (Law, 2000; Law *et al.*, 2000). These, when streaked onto plates, formed “smooth” colonies. Southern blotting showed that these remained heteroplasmic, but it appeared that a mutation had occurred elsewhere in the genome that overcame the effects of losing several copies of *ycf24* (Law, 2000; Law *et al.*, 2000). It is possible that in the time between resurrecting $\Delta ycf24$ *Synechocystis* from a glycerol stock and

Table 6.2: Comparison of $\Delta ycf16$ *Synechocystis* with $\Delta ycf24$ *Synechocystis*.

A comparison of the different phenotypes observed in *Synechocystis* when *ycf24* and *ycf16* were disrupted by *kan^R* and *strep^R*, respectively.

Phenotype	$\Delta ycf24$	$\Delta ycf16$
Time for Visible Colonies to Form	14 Days	6 Days
“Ragged” Colonies	Yes	Yes
High Proportion of Dividing Cells	Yes	Yes
Cell Death as Seen by Autofluorescence	Yes	No
Loss of DNA	Yes	No
Loss of Disrupted Gene in Absence of Selection	2 Weeks	4 Weeks
Plaques Seen in Electron Micrographs	Yes	No
Homoplasmy Achieved	No	No

performing the disruption of *ycf16*, a suppressing mutation had occurred which could to some extent overcome the effects of both the *ycf24* and *ycf16* disruptions. In order to observe a true “double-disruption”, it would be preferable to perform both disruptions at the same time in wt *Synechocystis*.

In order to confirm that the phenotypes observed are due specifically to disruption of *ycf16*, it would be interesting to see whether the “ragged” colony phenotype could be rescued by complementation with wt *ycf16*. The construct used for transformation would have to possess *ycf16* with an antibiotic resistance cassette (not *strep^R*) along with flanking sequences to allow correct recombination into the *Synechocystis* genome. I have shown that recombination into the genome is completely faithful (Figure 6.5), so there should not be a problem with the construct recombining into flanking genes.

For a more precise dissection of the function of Ycf16, a technique such as plasmid shuffling (Poncelet *et al.*, 1998) to look at the effects of specific mutations in *ycf16* could be used. For example, Ycf16 resembles the ATP-binding subunit of an ABC transporter (Chapter 5.2.5), so it would be interesting to alter residues in the Walker A or B motifs, to see whether the protein remains functional. If it does not, it would indicate that the protein does indeed bind ATP, and this could give some idea of its function.

Although I have gone some way to generating antibodies to *E. coli* Ycf16 and to *P. falciparum* Ycf16 (Chapter 5.2), antibodies to *Synechocystis* Ycf16 and Ycf24 would be useful to complement the knowledge gained from the gene disruptions. They could be used in immunofluorescence and immunoelectron microscopy in order to study the localisation of the proteins both in wt *Synechocystis*, and in the mutants. Establishing the localisation of the proteins could help establish whether the effects seen are directly due to the gene disruption, or whether they are secondary effects. Antibodies may also be able to tell us whether a truncated Ycf24 is being produced that could potentially be toxic to the cell. Antibodies could also be used in immunoprecipitation experiments to try establish whether Ycf24 and Ycf16 interact with each other or with other proteins.

Law (2000) and Law *et al.* (2000) concluded that *ycf24* is an essential gene in *Synechocystis*. However, they believe that it does not specify the primary function of the apicomplexan plastid, but instead has an essential “housekeeping” function, perhaps related to the requirement for strict control of DNA segregation and plastid replication (Law *et al.*, 2000). *ycf16* and *ycf24* have been annotated as genes encoding subunits of an ABC transporter (Kaneko *et al.*, 1996). Although this annotation appears plausible in the case of Ycf16 (see Chapter 5.2.5), sequence analysis does not indicate Ycf24 is a membrane-spanning protein (Law *et al.*, 2000).

It was thought that Ycf24 might have a rôle in cell division in *Synechocystis* since a high proportion of $\Delta ycf24$ mutants appeared to be undergoing division, or appeared to be arrested in division, at any one time. Observation of single cells for several generations by video-linked phase-contrast microscopy showed that this observation was not due to a shorter cell cycle in mutants (Law, 2000; Law *et al.*, 2000). The conclusion was therefore that these mutants were defective in some division process. However, it is not known whether this is a primary effect of disrupting *ycf24*, or a symptom of sick cells. A high number of $\Delta ycf16$ mutants were also found to be undergoing division at any one time (Figure 6.8). Again, it is impossible to say whether this is a primary or secondary effect.

Although some association between Ycf24 and FtsZ was implied by the lack of an FtsZ ring in *E. coli* cells overexpressing Ycf24 as a maltose-binding protein fusion, this association is spurious since the lack of an FtsZ ring was also noted in controls (see Chapter 5.1.1). The observations in *Synechocystis* would also preclude a specific association between FtsZ and Ycf24, since $\Delta ycf24$ mutants tend to be arrested late in division, whereas FtsZ is known to function early in the division process (Chapter 4.1.1).

Interestingly, however, Ycf24 shares a motif (GX₉FX₁₀G) with the cell division protein FtsX (de Leeuw *et al.*, 1999; Law *et al.*, 2000). The function of this motif is unknown, but it is found in all the membrane proteins of group 3-1 ABC transporters of which FtsX is one. Group 3-1 transporters are unusual in that they are anchored in the membrane by only four membrane-spanning regions as opposed to six and in that their encoding operons lack a gene for a substrate-binding component (Tomii and Kanehisa, 1998). It has been proposed that all members of this group are involved in cell division.

In *E. coli*, *ftsX* is found in an operon with *ftsE* (Gill *et al.*, 1986), which encodes a typical ATP-binding domain with Walker A and B motifs for nucleotide binding (see Chapter 5.1.2). *E. coli* $\Delta ftsE$ mutants exhibit filamentation, which is suppressed by elevated NaCl levels (de Leeuw *et al.*, 1999). Overexpression of FtsE alone had no effect on cell growth, but overexpression of FtsX led to inhibition of growth, filamentation and cell death. Western blotting of different subcellular fractions of *E. coli* showed the two proteins to be membrane associated; however, consistent with computer predictions of membrane-spanning regions, FtsX is believed to be anchored in the membrane, and FtsE to be associated with FtsX. It has been proposed that these two proteins have a rôle in cell division or salt transport (de Leeuw *et al.*, 1999).

If an analogy could be drawn between FtsX/FtsE and Ycf24/Ycf16, this could perhaps provide an explanation of the division phenotypes seen in *Synechocystis* and *E. coli* (see Chapter 5.1.1). However, it remains that Ycf24 is not predicted to possess a transmembrane domain (Law *et al.*, 2000); in addition, although Ycf24 and Ycf16 have similarities to FtsX and FtsE respectively, they are not the same thing. Nevertheless, the

conservation of the GX₉FX₁₀G motif in all of the transmembrane domains of group 3-1 ABC transporters, and in Ycf24 is intriguing. If the function of this motif could be elucidated, it is possible this would shed some light onto the function of Ycf24.

The plaques resembling glycogen deposits that were observed in $\Delta ycf24$ *Synechocystis* could indicate a deficiency in nutrient uptake but supplementing the medium with a selection of different nutrients had no effect on growth of heteroplasmic $\Delta ycf24$ mutants (Law, 2000). There are several methods for visualising polysaccharides by electron microscopy, most relying on the fact that hydroxyl groups can be oxidised to aldehyde groups on treatment with periodic acid. Further treatment with thiocarbohydrazide or thiosemicarbazide, then visualisation of the thio radicals by silver proteinate treatment was found to give the best result in a comparison of four different methods (Thiéry, 1967). Carrying out such an experiment would give an indication of whether or not the plaques found in $\Delta ycf24$ *Synechocystis* are glycogen deposits. If it were found that Ycf24 did indeed form part of an ABC transporter this could explain a nutrient-deprivation phenotype. However, despite the fact that, in contrast to Ycf24, Ycf16 does look like an ABC transporter subunit, no such plaques were observed in $\Delta ycf16$ *Synechocystis*. Furthermore, in contrast to Law (2000) and Law *et al.* (2000), I did not note any plaques in wt *Synechocystis*. It is therefore possible that these were the result of transient differences in culturing conditions.

Recently, Møller *et al.* (2001) have concluded that Ycf24 is indeed part of an ABC transporter. They believe it forms part of the phytochrome A signalling pathway and is involved in the reimportation of protoporphyrin IX for chlorophyll synthesis after its oxidation at the plastid envelope by protoporphyrinogen oxidase. They conclude that this transport is critical for light regulation of co-ordinated nuclear and chloroplast gene expression, required for example, for the development of proplastids into chloroplasts. They isolated an *A. thaliana* mutant for *ycf24* (that they call *atABC1*), which was found to have a reduced responsiveness towards far red light (as shown by hypocotyl elongation), and was hence assumed to be defective in the detection of light by phytochrome A. Phytochrome A is responsible for detection of far red light, and transcription of *A. thaliana ycf24* was found to be induced upon stimulation of phytochrome A signalling by far red light. Mutant plants had a chlorophyll deficiency and increased levels of the chlorophyll (and haem) precursor protoporphyrin IX. Artificially increasing the levels of protoporphyrin IX by treatment of wt plants with flumioxazin, an inhibitor of protoporphyrinogen oxidase, resulted in plants with the same elongated hypocotyl phenotype.

A. thaliana Ycf24 was shown to be a plastid-localised protein by the ability of the transit peptide to target GFP into onion epidermal leucoplasts and by immunofluorescence microscopy of wt and mutant *A. thaliana*. Immunofluorescence microscopy showed that

Ycf24 was localised around the periphery of chloroplasts, thereby supporting the claim for association with the membrane (Møller *et al.*, 2001). This is in contrast to the evidence that Ycf16 in *O. sinensis* is not membrane-associated (Wittpoth *et al.*, 1996). Møller *et al.* (2001) suggest that since Ycf24 lacks a membrane domain, if it does form part of an ABC transporter, it is of the “small soluble” subfamily that tend to act as importers and therefore must associate with a transmembrane domain that is encoded elsewhere on the genome, although no attempt was made to identify this component (Møller *et al.*, 2001). However, it appears that these authors have assumed that *ycf24* encodes the ATPase domain of an ABC transporter, this assumption being made presumably due to its annotation as a subunit of an ABC transporter and due to the fact that it lacks transmembrane domains. If Ycf24 is eventually found to be part of an ABC transporter it will not necessarily be an importer; in addition, I propose that *ycf16* will be found to encode the ATPase domain. This, however, still leaves the precise function of Ycf24 unresolved.

It has been concluded that Ycf24, through its rôle in protoporphyrin IX import into plastids, is involved in intercompartmental communication for light-regulated gene expression (Møller *et al.*, 2001). It is possible that Ycf24 in *P. falciparum* has a rôle in transport of a precursor of haem synthesis; however, accumulating evidence suggests that protoporphyrin IX is converted to haem either in the cytosol, or the mitochondrion (see Chapter 1.2.3.2). If this is the case, Ycf24 would act as an exporter, either from the plastid to the cytosol, or from the plastid to the mitochondrion. It seems unlikely that Ycf24 would have any function in light regulation of gene expression in an intracellular parasite such as *P. falciparum*, but it is possible that it forms part of a different type of signalling pathway, transporting protoporphyrin IX for regulation of nuclear or plastid gene expression. However, it is interesting to note that light activation of *ycf24* expression has been observed in the red alga *P. purpurea* (M. Reith, personal communication).

Møller *et al.* (2001) fail to address the function of this protein in prokaryotic organisms despite acknowledging its existence in cyanobacteria. It is possible to envisage that it is involved in light regulation of gene expression in photosynthetic prokaryotes, or some other type of regulation in non-photosynthetic prokaryotes. However, the conclusion in *A. thaliana* that it is involved in the co-ordination of gene expression between two different subcellular compartments makes it difficult to see what the analogous function would be in prokaryotic organisms. Identifying whether Ycf24 in *Synechocystis* and *E. coli* is localised to external cell membranes may shed light on this mystery.

It might prove interesting to study some of the $\Delta ycf24$ *A. thaliana* phenotypes in $\Delta ycf24$ or $\Delta ycf16$ *Synechocystis*. For example, possible light regulation of *ycf24* or *ycf16* expression, whether there is any change in levels of chlorophyll, haem or their precursors when these genes are disrupted, and the effects of treating *Synechocystis* with flumioxazin.

An even more recent study has identified *ycf16* as encoding a protein called SufC involved in virulence and the SoxR-dependent response to oxidative stress, in the plant pathogen, *P. chrysanthemi* (Nachin *et al.*, 2001). It was found to form part of an operon encoding SufA, SufB (Ycf24), SufC (Ycf16), SufD, SufS (NifS), and SufE. SufA is thought to function as an iron carrier for Fe-S cluster assembly, SufD has been shown to be necessary for the stability of the [2Fe-2S]-containing FhuF protein in *E. coli*, and SufS catalyses the production of alanine and sulphur from cysteine for Fe-S cluster assembly. It was therefore concluded that the Suf proteins are involved in the formation of Fe-S clusters, and the maturation of Fe-S cluster-containing proteins.

It was found that iron accumulated within the cell when the *suf* genes were mutated, as shown by sensitivity to the iron-activated antibiotic, streptonigrin. When different *suf* genes were mutated, different sensitivities to streptonigrin were observed; the most sensitive mutant (and therefore that with the greatest accumulation of iron) was the Δ *sufC* (*ycf16*) mutant. This observation is consistent with a rôle in Fe-S cluster assembly or iron export.

Expression of the *suf* genes was found to be repressed in the presence of iron in a Fur-dependent manner; Fur is a repressor protein that is regulated by iron. During infection of plant tissue by *P. chrysanthemi*, iron is limiting, and hence, expression of the *suf* genes is induced. It has been shown that a plant-induced oxidative burst occurs in *P. chrysanthemi* during infection, and virulence of *suf* mutants was found to be impaired compared to wt bacteria.

The effects of a *sufC* (*ycf16*) mutation in *E. coli* on *soxS* expression (SoxS is involved in protecting the cell against superoxide toxicity) was tested (Nachin *et al.*, 2001). *soxS* expression depends on SoxR, a [2Fe-2S]-containing transcription factor that is activated upon oxidative stress. Induction of *soxS* expression was found to be delayed in the Δ *sufC* mutant, suggesting a defect in the assembly of the Fe-S cluster required for SoxR.

It was concluded that when there are low levels of iron, Fe-S cluster assembly is a limiting step in the formation of Fe-S cluster-containing redox proteins: the cell therefore requires additional systems to optimise Fe-S cluster formation, so the *suf* operon is induced (Nachin *et al.*, 2001). It was suggested that if Ycf16 does form part of an ABC transporter then it is likely to be involved in transporting iron, or an iron-containing molecule. They considered that Ycf16 could function as an ATPase, and along with the other Suf proteins be involved in the maturation of Fe-S cluster-containing proteins. In contrast to Møller *et al.* (2001), Nachin *et al.* (2001) discount that the Suf proteins form an ABC transporter, since they recognised that none of the other Suf proteins (Ycf24 included) contain a transmembrane domain. They do however, discuss the possibility that Ycf16 may not only function alongside the other Suf proteins, but also associate with a transmembrane protein elsewhere

to form an ABC transporter involved in iron transport. They suggest that this would explain the fact that the ΔsufC mutation was consistently more deleterious in *P. chrysanthemi* than any of the other *suf* mutations.

Several interesting experiments that could be carried out on *Synechocystis* arise from this work. These would include, for example, investigating whether expression of Ycf16 or Ycf24 is upregulated in the absence of iron, searching for a Fur-binding motif in the promoter region of Ycf24 in the *Synechocystis* genome and studying whether the Δycf16 and Δycf24 mutants are more sensitive to streptonigrin than wt. However, the fact that the *Synechocystis* mutants are heteroplasmic and probably retain small amounts of functional Ycf16 and Ycf24 must be taken into account.

Clearly *ycf24* and *ycf16* are essential genes in *Synechocystis* and this conclusion can probably be extended to the other organisms in which they are found. I remain unconvinced by the ABC transporter annotation: Ycf16 may represent a novel type of ABC-ATPase with a non-transport-related function, perhaps supplying energy for Fe-S cluster formation by the Suf proteins. More studies on localisation, the effects of specific mutations and protein-protein interactions need to be carried out before proper conclusions can be made.

Chapter 7 - Discussion

7.1 *An Essential Organelle in Apicomplexans*

The discovery of a plastid organelle in apicomplexans unites three areas of research that had been continuing independently for several years. Firstly, the identification of a mysterious membrane-bound organelle in electron micrographs in the 1960s (Aikawa, 1966); secondly, the observed antimalarial action of several antibacterial antibiotics (Geary and Jensen, 1983); and thirdly, the discovery of the 35 kb extrachromosomal DNA (Wilson *et al.*, 1991). The confirmation that the previously identified organelle housed the 35 kb DNA (Köhler *et al.*, 1997), which resembles the genomes of algal plastids (Wilson *et al.*, 1996a), provided a rationale for the action of antibiotics associated with antibacterial action.

The fact that the organelle and its genome have been retained suggests that they carry out some important, if unknown function. The plastid has been shown to be essential to the parasite since *T. gondii* parasites that do not contain it do not survive long beyond invasion of a second host cell (He *et al.*, 2001). It has been shown that enzymes of fatty acid synthesis and isoprenoid synthesis are targeted to the plastid in *P. falciparum* and *T. gondii* (Waller *et al.*, 1998; Jomaa *et al.*, 1999), indicating a rôle for this organelle in housing these biosynthetic pathways. It was proposed that fatty acid synthesis is required for the formation of the parasitophorous vacuole, and that parasites lacking a plastid form an aberrant vacuole (He *et al.*, 2001).

In addition to the plastid being essential to the parasite, it has also been shown that its genome is essential. Ciprofloxacin, which specifically inhibits prokaryotic and plastid DNA gyrases, was found to cause depletion of plastid DNA and subsequent parasite death in *T. gondii* (Fichera and Roos, 1997). Most of the genes on the plastid genome encode components of the protein synthesis machinery (Wilson *et al.*, 1996a), and antibiotics that interfere with the function of 70S ribosomes (which are found in prokaryotes and endosymbiotically-derived organelles) have antimalarial activity (Geary and Jensen, 1983). However, at least one essential protein not related to protein synthesis must be encoded on the plastid genome, otherwise there would be no need for protein synthesis to be carried out at all, and no requirement for the genome. There are two ORFs present that fill this category: ClpC, the ATP-binding subunit of Clp protease, and Ycf24 (ORF470), whose function has yet to be confirmed.

7.2 *Nucleus-Encoded Plastid Proteins*

Although most of the genes on the plastid genome in *P. falciparum* are involved in gene expression, not all of the components are present. Some of the unidentified ORFs may encode divergent ribosomal proteins, but the remainder are probably synthesised in the

cytosol and imported into the plastid. EF-G and EF-Ts function alongside EF-Tu in the elongation cycle of protein synthesis in plastids and prokaryotes (Nierhaus, 1996). Plastid EF-G in soybean is encoded in the nucleus (Hernández Torres *et al.*, 1993) and subsequently transported into the chloroplast. Antibiotic inhibitors of the elongation cycle of prokaryotic translation have been found to have antimalarial activity (Clough *et al.*, 1997; Clough *et al.*, 1999), so one would expect EF-G and EF-Ts to be encoded in the nucleus. A putative mitochondrial EF-Ts has been identified on chromosome 3 (Bowman *et al.*, 1999); a plastid version is probably encoded elsewhere.

ClpP is an ATP-dependent serine protease (Maurizi *et al.*, 1990a; Maurizi *et al.*, 1990b) and forms the proteolytic subunit of Clp protease, which is involved in the degradation of incomplete and misfolded proteins (Frees and Ingmer, 1999; Adam, 2000; Krüger *et al.*, 2000). In most plastid-bearing organisms, ClpP is encoded on the plastid genome, whereas ClpC, the regulatory subunit, is nucleus-encoded. In the red alga, *P. purpurea*, both are plastid-encoded (Reith and Munholland, 1995), whereas in *P. falciparum* only ClpC is plastid-encoded. Therefore, ClpP is probably encoded in the nucleus; indeed a *clpP*-like sequence, encoding a protein with a putative plastid-targeting sequence has been identified on chromosome 3 of *P. falciparum* (Waller *et al.*, 1998; Bowman *et al.*, 1999).

As more information about the function of the plastid is gained, further predictions about nucleus-encoded plastid proteins can be made. For example, the remaining enzymes of fatty acid synthesis and isoprenoid synthesis must also be nucleus-encoded, then post-translationally transported into the plastid. Moreover, the components of a plastid import machinery also must be encoded on the nuclear genome. Identification of several homologues of proteins from the Toc-Tic plastid import apparatus (see Chapter 1.3.1) has been reported (van Dooren *et al.*, 2000). Vollmer *et al.* (2001) recently identified a plant-like ferredoxin and ferredoxin NADP⁺ reductase that are imported into the apicomplexan plastid. In higher plants, ferredoxin is imported into plastids as an apoprotein, and the ATP-dependent addition of the Fe-S cluster occurs inside the plastid (Takahashi *et al.*, 1986, 1991). It has been suggested that this system is required for the provision of reducing power for fatty acid synthesis or for other biochemical pathways (Vollmer *et al.*, 2001).

7.3 A Function for Ycf24 (ORF470)

The identification of plastid ferredoxin in apicomplexans suggests a putative rôle for Ycf24 (Ellis *et al.*, submitted). Nachin *et al.* (2001) suggested a rôle for Ycf16 (SufC) and probably Ycf24 (SufB) in iron metabolism and resistance to oxidative stress, specifically in assembly of Fe-S clusters, and maturation of Fe-S-containing proteins. It is possible that Ycf16 and Ycf24 are involved in the assembly of Fe-S clusters for plastid proteins such as ferredoxin. It is interesting to note that formation of Fe-S cluster-containing ferredoxin

from apoferreredoxin in spinach chloroplasts was found to require ATP hydrolysis (Takahashi *et al.*, 1991). Ycf16, with its high similarity to the ATPase domains of ABC transporters, is likely to have ATPase activity although no direct evidence for this was obtained in the present study; this activity could be required for maturation of Fe-S-containing proteins.

Plastids and mitochondria have high volume redox activity resulting in a high rate of production of oxygen free radicals. Consequently, this has been used to explain the transfer of some organellar genes to the nuclear genome and the retention of other genes on the plastid genome (Allen and Raven, 1996). Despite the absence of photosynthesis in the apicomplexan plastid, the recent discovery of a plastid-imported plant-like ferredoxin of the type more commonly found in non-photosynthetic plastids, indicates that there may be redox activity within this organelle (Vollmer *et al.*, 2001). Ycf16 and Ycf24 may be required by plastids for resistance to oxidative stress (Nachin *et al.*, 2001), and such a rôle would be consistent with their continued existence in plastids, even when nucleus-encoded. However, Allen (1993) has argued that genes are retained on organellar genomes because their expression is regulated by change in redox state. Therefore, in cases where genes have been transferred to the nuclear genome there must exist some signalling mechanism (such as levels of free iron) controlling their expression.

It would be interesting to look at the effects of iron levels and oxidative stress on *T. gondii* and *P. falciparum*, and specifically on the transcription of *ycf16* and *ycf24*. I have shown that *ycf16* is transcribed by late ring stages and trophozoites during the *P. falciparum* erythrocytic cycle (Figure 5.2.7), and one major source of oxidative stress to the parasite is haemoglobin degradation, which occurs during this stage of the life cycle (Francis *et al.*, 1997). However, this degradation process occurs in the digestive vacuole, and would probably not have any effect on plastid proteins, but rather be regulated by other mechanisms. It must be recognised that the erythrocytic cycle is only one part of the parasite's complex life cycle. It may be that the plastid, and these genes, are required during the mosquito stage, but unfortunately nothing is known about the plastid during these stages of the life cycle. Another approach would be to identify other components of the putative Fe-S-cluster assembly operon, for example, the other *suf* genes (Nachin *et al.*, 2001), a gene encoding an iron sensing transcriptional repressor such as Fur (Escobar *et al.*, 1999), or a redox controlled response to oxidative stress such as the *soxRS* system (Hidalgo *et al.*, 1997).

7.4 Future Perspectives

Systems for the study of plant plastids are more developed than those for the study of the apicomplexan plastid. It is possible to isolate certain plant plastids for use in *in vitro* assays and localisation studies; indeed isolated pea chloroplasts have been used to demonstrate

plastid import of ribosomal protein S9 from *T. gondii* (DeRocher *et al.*, 2000). Transit peptides in *T. gondii* are more similar to those of plants than *P. falciparum* transit peptides are, so it is perhaps not surprising that such an assay would work for *T. gondii* proteins. It may be that *P. falciparum* transit peptides would be too divergent to be recognised by plant chloroplast import machinery, even though they are recognised by the *T. gondii* plastid import apparatus (Jomaa *et al.*, 1999). Nevertheless, such a system would certainly be worth attempting as an assay for putative *P. falciparum* plastid proteins identified from the genome sequencing project. This system has been exploited to screen for cDNA clones that encode plastid-targeted proteins in plants (Shimada *et al.*, 1998). A radish (*Raphanus sativus*) cDNA library was transcribed *in vitro*, and translated in the presence of ³⁵S methionine, and the radiolabelled proteins imported into intact chloroplasts from pea or spinach *in vitro*. Imported proteins were subsequently analysed by SDS-PAGE and autoradiography. If import of *P. falciparum* proteins into plant plastids were found to be possible, such an assay could be one way of screening for plastid imported proteins in this organism.

Transfection technology in *T. gondii* is becoming well established, and that in *P. falciparum* is developing, although transformation frequencies are extremely low in this organism. Transient transfection has been used for targeting studies and promoter mapping, and stable transfection for gene knockouts in *T. gondii*, as a model for *P. falciparum* (Roos *et al.*, 1999a). However, it is unfortunate that although there is a *T. gondii* EST project, the genome sequencing project is only in its early stages; knowledge gained from sequencing projects in combination with transfection studies will prove invaluable in years to come. In terms of studies of the apicomplexan plastid, the development of a plastid transformation system, such as exists for several plants (Heifetz, 2000), would be extremely useful. In plants it is not only possible to express foreign proteins in plastids, but also to disrupt plastid genes by homologous recombination (although homoplasmy can be difficult to achieve).

Recently, a potentially useful tool for studies of apicomplexan plastid function was inadvertently developed in *T. gondii* (He *et al.*, 2001). Parasites with a defect in plastid segregation were obtained: within each parasitophorous vacuole, only one tachyzoite retained a plastid, and this was larger than normal; vacuoles containing 128 parasites, but only a single large plastid were observed. Parasites lacking a plastid will prove useful in the testing of drugs thought to target the plastid: if a drug has antiparasitic activity on a wt parasite, but not on those lacking a plastid, then it can be concluded that the drug is indeed acting specifically on plastid processes.

7.5 **Summary**

In the course of this project, I set up a screen for mycoplasma-derived genes (Chapter 3), which has been used successfully to eliminate a degenerate PCR product from further investigation (Chapter 4). Although I failed to identify a *P. falciparum* *ftsZ* gene, I identified a sequence encoding a dynamin-related protein, which may be involved in plastid, or perhaps mitochondrial division (Chapter 4). I also investigated the gene *ycf16*, whose product is probably plastid-localised in *P. falciparum*, and confirmed that it is transcribed (Chapter 5); reporter protein targeting in *T. gondii* and antibodies to recombinant Ycf16 produced in *E. coli* will confirm its localisation. I also established that *ycf16* is co-transcribed with *ycf24* in *Synechocystis* PCC6803, and that it is an essential gene in this organism (Chapter 6). Based on recent additions to the literature, I propose that Ycf16 is involved in maturation of Fe-S cluster-containing proteins required for resistance to oxidative stress and the provision of reducing power for biosynthetic pathways that occur in the plastid in apicomplexans.

Since commencement of this project the functions of the apicomplexan plastid have started to be elucidated. It is clear that this organelle is essential to the parasite, and therefore represents a promising drug target in severe parasites of humans and other animals, including the most dangerous of all, those causing malaria.

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APPENDIX I

COMPOSITIONS OF MEDIA, SOLUTIONS AND BUFFERS

All solutions made up in dH₂O unless otherwise stated.

BG11 (100 X)

1.76 M	NaNO ₃
30 mM	MgSO ₄ ·7H ₂ O (Sigma)
25 mM	CaCl ₂ ·2H ₂ O (Sigma)
2.86 mM	citric acid
0.28 mM	EDTA (Fisons)
pH = 8.0	

BG11 MEDIUM

0.3 % (w/v)	sodium thiosulphate (Sigma)
1 % (v/v)	TES buffer
1 X	BG11
0.1 % (v/v)	trace elements
0.1 % (v/v)	iron stock
0.1 % (v/v)	phosphate stock
0.1 % (v/v)	Na ₂ CO ₃ stock

BG11 AGAR

15 g.L ⁻¹	agar (Difco)
in BG11 medium	

COOMASSIE STAIN

45 % (v/v)	methanol
10 % (v/v)	ethanoic acid
0.1 % (w/v)	Coomassie Brilliant Blue R (Sigma)

CTAB-NA₂CO₃

700 mM	NaCl
10% (v/v)	CTAB (Sigma)

DENATURING SOLUTION

1.5 M	NaCl
0.5 M	NaOH

DESTAIN

25 % (v/v)	propan-2-ol
10 % (v/v)	ethanoic acid
3 % (v/v)	glycerol

GEL LOADING BUFFER III

0.25 % (w/v)	bromophenol blue (Sigma)
0.25 % (w/v)	xylene cyanol FF (Sigma)
30 % (v/v)	glycerol

GLUTATHIONE ELUTION BUFFER

10 mM	reduced glutathione (Sigma)
50 mM	Tris
pH = 8.0	

HEPES BINDING BUFFER

50 mM	HEPES (Sigma)
80 mM	NaCl
1 mM	MgCl ₂
pH = 7.5	

HIGH-SALT ELUTION BUFFER

50 mM Tris
1 M NaCl
10 mM EDTA (Fisons)
pH = 8.0

HYBRIDISATION SOLUTION

362.5 mM NaCl
20 mM sodium phosphate buffer (pH = 7.7)
1.9875 mM EDTA (Fisons)
1 % (w/v) SDS (Bio-Rad)
0.5 % (w/v) skimmed milk powder (Marvel)
10 % (w/v) dextran sulphate (Sigma)
250 µg.mL⁻¹ denatured salmon sperm DNA (Sigma)

IMIDAZOLE (16 mM) BUFFER + UREA (6 M)

1 volume Wash Buffer (Novagen) + urea (6 M)
4 volumes Binding Buffer (Novagen) + urea (6 M)

IMIDAZOLE (20 mM) BUFFER + UREA (6 M)

4.1 volumes Wash Buffer (Novagen) + urea (6 M)
11 volumes Binding Buffer (Novagen) + urea (6 M)

IMIDAZOLE (81.5 mM) BUFFER + UREA (6 M)

1 volume Elute Buffer (Novagen) + urea (6 M)
12 volumes Binding Buffer (Novagen) + urea (6 M)

IMIDAZOLE (204 mM) BUFFER + UREA (6 M)

1 volume Elute Buffer (Novagen) + urea (6 M)
4 volumes Binding Buffer (Novagen) + urea (6 M)

IMIDAZOLE (337 mM) BUFFER + UREA (6 M)

1 volume Elute Buffer (Novagen) + urea (6 M)
2 volumes Binding Buffer (Novagen) + urea (6 M)

IRON STOCK

6 g.L⁻¹ ferric citrate (Sigma)

LB AGAR

15 g.L⁻¹ agar (Difco)
in LB broth

LB BROTH

10 g.L⁻¹ NaCl
10 g.L⁻¹ bacto-tryptone
5 g.L⁻¹ yeast extract

LOADING BUFFER

83.3 % (v/v) deionised formamide (PE Applied Biosystems)
4.2 mM EDTA (Fisons)
8.3 mg.mL⁻¹ blue dextran (PE Applied Biosystems)
pH = 8.0

LYSIS BUFFER (4 X)

40 mM Tris
80 mM EDTA (Fisons)
2 % (w/v) SDS (Bio-Rad)
2 mg.mL⁻¹ proteinase K (Sigma)
pH = 8.0

Na₂CO₃ STOCK
20 g.L⁻¹ Na₂CO₃

PBS

0.1 M	NaCl
80 mM	Na ₂ HPO ₄
20 mM	NaH ₂ PO ₄
pH = 7.5	

PHOSPHATE STOCK

30.5 g.L ⁻¹	K ₂ HPO ₄
------------------------	---------------------------------

SARTOBLLOT BUFFER

48 mM	Tris
44.8 mM	glycine
20 % (v/v)	methanol
0.037 % (w/v)	SDS (Bio-Rad)
pH = 9.2	

SDS LOADING BUFFER (1 X)

1 % (w/v)	SDS (Bio-Rad)
50 mM	Tris
2 mM	PMSF (Sigma)
1 mM	EDTA (Fisons)
10 % (v/v)	glycerol
0.5 % (w/v)	bromophenol blue (Sigma)
5 % (v/v)	2-mercaptoethanol (Sigma)
pH = 7.5	

SOLUTION A

50 mM	Tris
1 mM	EDTA (Fisons)
0.2 % (v/v)	Nonidet P40 (Sigma)
pH = 8.0	

SSPE (1 X)

120 mM	NaCl
15 mM	tri-Na citrate
13 mM	KH ₂ PO ₄
1 mM	EDTA (Fisons)

TBE BUFFER

10.8 g.L ⁻¹	Tris
22 g.L ⁻¹	boric acid
0.75 g.L ⁻¹	EDTA (Fisons)
pH = 8.0	

TE BUFFER

10 mM	Tris
1 mM	EDTA (Fisons)
pH = 8.0	

TES

5 mM	Tris
5 mM	EDTA (Fisons)
50 mM	NaCl

TES BUFFER

229 g.L ⁻¹	TES (Sigma)
pH = 8.2	

TRACE ELEMENTS

2.86 g.L ⁻¹	H ₃ BO ₃
1.81 g.L ⁻¹	MnCl ₂ .4H ₂ O (Sigma)
0.22 g.L ⁻¹	ZnSO ₄ .7H ₂ O (Sigma)
0.39 g.L ⁻¹	Na ₂ MoO ₄ .2H ₂ O (Sigma)
0.08 g.L ⁻¹	CuSO ₄ .5H ₂ O (Sigma)
0.05 g.L ⁻¹	Co(NO ₃) ₂ .6H ₂ O (Sigma)

WASH SOLUTION A

2 X	SSPE
0.1 % (w/v)	SDS (Bio-Rad)

WASH SOLUTION B

1 X	SSPE
0.1 % (w/v)	SDS (Bio-Rad)

WASH SOLUTION C

0.1 X	SSPE
0.1 % (w/v)	SDS (Bio-Rad)

APPENDIX II

INTERNET SITES USED

CLUSTAL W MULTIPLE SEQUENCE ALIGNMENT (Thompson *et al.*, 1994)

<http://www2.ebi.ac.uk/clustalw>

Alignment of amino acid sequences.

CUTTER

<http://www.medkem.gu.se/cutter/>

Generation of restriction maps.

CYANOBASE (Nakamura *et al.*, 1998, 2000)

<http://www.kazusa.or.jp/cyano/>

Genomic information on the cyanobacteria *Synechocystis* and *Anabaena*

EXPASY TRANSLATE TOOL

<http://expasy.cbr.nrc.ca/tools/dna.html>

Translation of nucleotide to amino acid sequence in six reading frames.

NCBI BASIC BLAST (Altschul *et al.*, 1990)

<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>

Search of nr or swissprot databases by inputting a nucleotide or amino acid sequence, or an accession number.

NCBI ENTREZ NUCLEOTIDE QUERY

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide>

To obtain nucleotide sequence information by description or accession number.

NCBI ENTREZ PROTEIN QUERY

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>

To obtain protein sequence information by description or accession number.

NCBI MALARIA GENETICS AND GENOMICS (Altschul *et al.*, 1997)

<http://www.ncbi.nlm.nih.gov/Malaria/plasmodiumbl.html>

Malaria genomic DNA sequences which have emerged through the malaria genome project. A nucleotide or amino acid sequence is used in a BLAST search in order to identify similar malaria sequences.

PROSITE (Hofmann *et al.*, 1999)

<http://expasy.cbr.nrc.ca/prosite/>

To scan protein sequences for biologically significant sites, patterns and profiles.

SIGNALP (Nielsen *et al.*, 1997)

<http://www.cbs.dtu.dk/services/SignalP/>

For prediction of signal peptide and cleavage site

THE ARABIDOPSIS INFORMATION RESOURCE (Huala *et al.*, 2001)

<http://www.arabidopsis.org/home.html>

Accessing *A. thaliana* sequence data.

THE SANGER CENTRE *PLASMODIUM FALCIPARUM* GENOME PROJECT

http://www.sanger.ac.uk/Projects/P_falciparum/

Accessing sequence information from chromosomes 1, 3-9 and 13, and also for general information about the *P. falciparum* genome sequencing project.

THE INSTITUTE FOR GENOMIC RESEARCH *PLASMODIUM FALCIPARUM* SEQUENCE DATABASE

<http://www.tigr.org/tdb/edb2/pfa1/htmls/>

Accessing sequence information from chromosomes 2, 10, 11 and 14.

APPENDIX III

PRIMERS AND SEQUENCES

Primer	Supplier	Sequence (5' to 3')
Dyn1	Genosys	CCCTGTTGGGAATCAGCC
Dyn2	Genosys	CCTGATCAGCACAACGAAG
FtsZ5	Genosys	GTCATCGGCGTCGGCGGCGG
FtsZ6	Genosys	GCGTACGTCTGCAAAGTC
FtsZ7	Genosys	GTWWTIGGWGTWGGIGG
FtsZ10	Genosys	IYKIACATCIGCRAARTC
M13 (-20) Forward	Genosys	GTAAAACGACGGCCAG
M13 Reverse	Genosys	CAGGAAACAGCTATGAC
5' pGEX	Pharmacia	GGGCTGGCAAGCCACGTTTGGTG
3' pGEX	Pharmacia	CCGGGAGCTGCATGTGTCAGAGG
primer 156	Genosys	CCTTCACCCTCTCCACTGACAGA
primer 225	Genosys	AAGACATCCACCAAACGGTG
primer 266	Genosys	GGTTACCTTCAGCTTTACG
pTrcHis Forward	Invitrogen	GAGGTATATATTAATGTATCG
pTrcHis Reverse	Invitrogen	GATTTAATCTGTATCAGG
Sp11	Genosys	GGTATATAAACACTGCCTC
Ycf161	Oswel	GAGCTC GGATCC TGATGTTAAGTATTAAAGATTTA
Ycf162	Oswel	GAGCTC GAATTC TACTGCTGTTCCGGTAAGCCAGCC
Ycf168	Genosys	CTGTTTCATCGAGGAAATCG
Ycf169	Genosys	GCGTCCCATATGGCGTGG
Ycf1611	Genosys	GGGATAAGACACATCCGTTG
Ycf1612	Genosys	CCTTAAGAAATTGAGAGTACC
Ycf1613	Genosys	CGGGGTCTATGTAAAGGA
Ycf1614	Genosys	TTAAATGGGAGCCAAAAC
Ycf1615	Genosys	GAGCTCAGATCTAAAATGAAAAGAAGAAAATGGCTC
Ycf1616	Genosys	GAGCTCCCTAGGTCTTTTAAATATTTCTTTCTCTCC
Ycf1617	Genosys	GGAGAGAAAGAAATATTAAAAGGA
Ycf1618	Genosys	TTCTTAAGAAATTGAGAGTACC
Ycf243	Genosys	CCGTCGGGGAATTCTACTC

A = Adenine

C = Cytosine

G = Guanine

I = Inosine

K = Guanine + Thymine

R = Adenine + Guanine

T = Thymine

W = Adenine + Thymine

Y = Cytosine + Thymine

GGATCC = *Bam*HI

GAATTC = *Eco*RI

P. falciparum Pfdrp (partial?)

atggataaattagtagtaccgattgtraacaaattacagaatgkttatcatcttttattagt
M D K L V P I X N K L Q N X L S S F I S
agtgaacaccttgagcytaccacatatagcagtggttagggctcaatcagktggaaaaacc
S E T L S X P H I A V V G A Q S X G K T
tcattactagaatcattagtaggktttaagktttatgcctaaaggagaagatatagtwact
S L L E S L V X L X F M P K G E D I X T
csgactycaatwataattcagctwacgaattctwaatcagatgattgkttattgtacctta
X T X X I I Q X T N S X S D D X Y C T L
acatatgttgattatgataataatagagtagaaaaacatatagatgatttttctwttttg
T Y C D Y D N N R V E K H I D D F S X L
aatgaaatattaattgacgttacagaagaattactgggggtaataaatgtattaaagaa
N E I L I D V T E E I T G G N K C I K E
actcctattataattgagattcataaaaaatgatgttkttagatttaactttaattgattta
T P I I I E I H K N D V X D L T L I D L
cctgggttaacaaaagtcacctgttgggaatcagccacaaaatgtagaggagcaaatagta
P G L T K V P V G N Q P Q N V E E Q I V
aattagtaaacaaatatataaagaatccgaattgtatcatttttagctgtttcatctgct
N L V N K Y I K N P N C I I L A V S S A
aatattgatttagctaattcagatagtttgaaaatggctagaaatgtagatcccaaacat
N I D L A N S D S L K M A R N V D P K H
gaaaggactataggtgttataactaaatgtgatatggttgaaaaacctgaaatatggaag
E R T I G V I T K C D M V E K P E I W K
aaaatgattagtgatcacttttatcctttgaagaaaggttttgtagctgttggttgctga
K M I S G S L Y P L K K G F V A V V C R
agtcagaaagatgtagaagatgatattactattgawatgtctattaaaaaagaagaagaa
S Q K D V E D D I T I X M S I K K E E E
tacttttagtcaatgtattgatttttcgaatcaaatggaatgtattatggaatgtggtata
Y F S Q C I D F S N Q M E C I M E C G I
aaaaatttagccaaaaaattaaataatatattaattgagcatatcaaaaaatacagtacca
K N L A K K L N N I L I E H I K N T V P
tttttaaaacctaaaattgattcattaaaaagattgaagaagaacgattattagaatta
F L K P K I D S L K S I E E E R L L E L
ggtgaacctatggataatatgaatcggttcagaaatttagcagtagtagttaattatata
G E P M D N M N R S E Y L A V L V N Y I
acaaaattttcacacagtatcaagatataatagatggaaaagttttttataaagataga
T K F S Q Q Y Q D I I D G K V F Y K D R
gttgatgaattaaagggtggcgcaagaatacattatatatttaattgattggtatataaaa
V D E L K G G A R I H Y I F N D W Y I K
tcattaaatgatttttcaccattagaaatgttaacagatgaagaaattcgaatagctata
S L N D F S P L E M L T D E E I R I A I
agaaattctagtggtccaagaggagcattattcgtaccagaaagtgccttttgammcttta
R N S S G P R G A L F V P E S A F X X L
ataaaaaaattawtaaaattgtttaaaggaaccttcacttcgttgctgatcaggatatat
I K K L X N C L K E P S L R C A D Q V Y
gaagaattattaaaaatagttgataattgtcgtatagcagatatggaaagatttactaat
E E L L K I V D N C R I A D M E R F T N
ttaagagtgctataaatgaacaagtcaaaatattattaaaagattgtttacaaccaacc
L K S A I N E Q V K I L L K D C L Q P T
aaagaaatgataaaaaatttaattgctaattgaattatcatatattaatacaagtcacct
K E M I K N L M L I E L S Y I N T S H P
gacttttttaattgaacatttttatgaaaaatgtatatgataaagataatgattatatagat
D F L N E H F M K N V Y D K D N D Y I D
gaattagatacagtagttcaccctaaacataatcataaaatgttacagcgtccaaaaaga
E L D T V V H P K H N H K M L Q R P K R
gaatatcaagaaacttggtggaagtaatatgaattattcaactatacaccataataataca
E Y Q E T C G S N M N Y S T I H H N N T
tataaagaagaatgaaaatgtggaaacataaagaagatgctaaatcttttagaaataaa
Y K E E M K M W K H K E D A K S F R N K
gaagggtaccaaattactaatataaataataatagagaaaatgtttttgttttaccagtg

Dyn1
Sspl
Sspl
Dyn2

E G T K I T N I N N N R E N V F V L P V
attccagaaaaaattataaccagaatattcatcttcatctaaagaaataatagaaattgac
I P E K I I P E Y S S S S K E I I E I D
ttaataaaatcgttaattaataattattttaatattgtagaaaaacatatagcagatgc
L I K S L I N N Y F N I V R K T Y S R C
ggtacctaagcaattagc
G T - S N -

atggtttgaaccaatggaactttaccaatgacgcggtgattaaagtcacgcgctgcggcgccg
M F E P M E L T N D A V I K V I G V G G
ggcgggcggtaatgctgttgaaacacatgggtgcgcgagcgcattgaaggtgttgaaattcttc
G G G N A V E H M V R E R I E G V E F F
gcggtaaataccgatgcacaagcgctgcgtaaaacagcggttgacagacgattcaaatac
A V N T D A Q A L R K T A V G Q T I Q I
ggtagcggtatcaccaaaggactggggcgctggcgctaatccagaagttggccgcaatgcg
G S G I T K G L G A G A N P E V G R N A
gctgatgaggatcgcgatgcattgcgtgcggcgctggaaggtgcagacatggtctttatt
A D E D R D A L R A A L E G A D M V F I
gctgcgggtatgggtggtgtaccggtacaggtgcggcaccagtcgtcgtgaagtggca
A A G M G G G T G T G A A P V V A E V A
aaagatttggtatcctgaccggttgctgtcgtcactaagcctttcaactttgaaggcaag
K D L G I L T V A V V T K P F N F E G K
aagcgtatggcattcgcggagcaggggatcactgaactgtccaagcatgtgaactctctg
K R M A F A E Q G I T E L S K H V N S L
atcactatcccgaacgacaaactgctgaaagttctgggcccgcggtatctccctgctggat
I T I P N D K L L K V L G R G I S L L D
gcgtttggcgacgcaacgatgtactgaaaggcgctgtgcaaggtatcgtgaaactgatt
A F G A A N D V L K G A V Q G I A E L I
actcgtccgggttgatgaacgtggactttgcagacgtacgcaccgtaatgtctgagatg
T R P G L M N V D F A D V R T V M S E M
ggccacgcaatgatgggttctggcggtggcgagcggtgaagaccgtgcggaagaagctgct
G H A M M G S G V A S G E D R A E E A A
gaaatggctatctcttctccgctgctggaagatatcgacctgtctggcgcgcgcgcgctg
E M A I S S P L L E D I D L S G A R G V
ctggttaacatcacggcgggcttcgacctgctgtggtgagttcgaaacggtaggtaac
L V N I T A G F D L R L D E F E T V G N
accatccgtgcatttgcttccgacaacgcgactgtggttatcggtacttctcttgaccg
T I R A F A S D N A T V V I G T S L D P
gatatgaatgacgagctgcgcgtaaccggttggtgcgacaggtatcggcattggacaaacgt
D M N D E L R V T V V A T G I G M D K R
cctgaaatcactctggtgaccaataagcaggttcagcagccagtgatggatcgctaccag
P E I T L V T N K Q V Q Q P V M D R Y Q
cagcatgggatggctccgctgacccaagagcagaagccggttgctaaagtcgtgaatgac
Q H G M A P L T Q E Q K P V A K V V N D
aatgcgccgcaaactgcgaaagagccggattatctggatatccagcattcctgcgtaag
N A P Q T A K E P D Y L D I P A F L R K
caagctgattaa
Q A D -

S MTLNNDLPLNNIGFTGSGLNLDGTEGLDDDLFSSSIVDNEPLEALVETPTFASPSPNLKRQ
A -----MENNRIQE
At MAIIPLAQLNELTISSS---SSSFLTKSISSHSLHSSCICASSRISQFRGGFSKRSDS
Bs -----MLEFETNIDG-----
Rm -----MTEYKKPIITEMR---
Ec -----MFEPMELTN---

S -----IVPSNIAKIKVIGVGGGGCNVAVNRMIASGVTGIDFWAINTDSQALTNTN FtsZ7
A -----IVPGRVANIKVIGVGGGGGNVAVNRMIESDVSGVEFWSINTDAQALTLAG
At TRSKSMRLRCSFSPMESARIKVIGVGGGGNNAVNRMISSGLQSVDFYAINTDSQALLQSS
Bs -----LASIKVIGVGGGGNNAVNRMIENEVQGVEYIAVNTDAQALNLSK
Rm -----PKITVIGVGGGGGNAINNMIAENLQGVDFAANTDAQALATSK
Ec -----DAVIKVIGVGGGGGNAVEHVMVRERIEGVEFFAVNTDAQALRKTA

S APDCIQIGQKLTRGLGAGGNPAIGQKAAEESRDEIARSLEGTDLVFIITAGMGGGTGTGAA
A APSRLQIGQKLTRGLGAGGNPAIGQKAAEESRDEIATALEGADLVFIITAGMGGGTGTGAA
At AENPLQIGELLTRGLGTGGNPLLGEQAAEESKDAIANALKGSDLVFIITAGMGGGTGSGAA
Bs AEVKMQIGAKLTRGLGAGANPEVGKKAAEESKEQIEEALKGADMVFVTAGMGGGTGTGAA
Rm AERRIQLGAAITEGLGAGSVPDIGNAAAQESIDEIMDHLGTHMCFVTAGMGGGTGTGAA
Ec VGQTIIQISGITKGLGAGANPEVGRNAADED RDALRAALEGADMVFI AAGMGGGTGTGAA
 *: *: * : * : * : * : * : * : * : *

S PIVA EVAKEMGCLTVGIVTRPFTFEGRRRAKQAE EGINALQSRVDTLV IVPNNQLLSVIP
A PIVA EVAKEMGALTGVVTRPVFEGRRRTSQA EQGIEGLKSRVDTLII IVPNNKLLLEVIP
At PVVA QISKDAGYLTGVVITYPFSFEGKRSLQALEA IEKLQKNVDTLV IVPNDRLLDIAD
Bs PVIA QIAKDLGALTGVVTRPFTFEGKRQLQAAGG ISAMKEAVDTLV IVPNDRILEIVD
Rm PVIA EAAARRAGILTVAVVTKPFSFEGQRRMQTAELGVERLRESADTV IVPNQNLFR IAD
Ec PVVA EVAKDLGILTVAVVTKPFNFEGKKRMAFAEQGITELSKHVD SLITIPNDKLLKVLG
 * : * : : : * * * : : * * * * : : * : : : : * * * : : : :

S AETPLQEA FRVADDILRQGVQG ISDIIIPGLVN VDFADVRA VMADAGSALMGIGVGS GK FtsZ10
A EQTPVQEA FRVADDVLRQGVQG ISDIIITPGLVN VDFADVRA VMADAGSALMGIGVSS GK
At EQTPLQDA FL LADDVLRQGVQG ISDIIITPGLVN VDFADV KAVMKDSGTAM LGVGVSSSK
Bs KNTPMLEA FR EADNVLRQGVQG ISDLIATPGLI NLD FADVKT IMSNKGSALMGIGIATGE
Rm AKTTFADA FM IADRVLYSGVSCITDLIVKEGL MNLD FADVKTVMKGMGRAMMGTGEATGE
Ec RGISLLDA FGAANDVLKGAVQGI AELITRPLMNVDFADV RTVMSEMGYAMMGS GVASGE

.. : ** * : * . * : : * ** : * : * : * : * * : * * : : *

S SRAKEAATAAIAISSPLL-ESSIQGAKGVVFNVTGGTDLTLHEVNVAEEIIYEVVDADANII

A SRAREAAIAAIAISSPLL-ECSIEGARGVVFNITGGSDDLTLHEVNAAAETIYEVVDPNANII

At NRAEEAAEQATLAPLI-GSSIQSATGVVYNITGGKDITLQEVNRVSQVVTSLADPSANII

Bs NRAAEAAKKAIAISSPLL-EAADGAQGVLNMITGGTNLSLYEVQEAADIVASASDQDVNMI

Rm NRAMLAAEAAIANPLLDEVS MRGAKGVLVISGGMDMTLFEVDEAATRIRREEVYDEADIV

Ec DRAEEAAEMAIAISSPLLEDIDLSGARGVLVNITAGFDRLRDEFETVGNTIRAFASDNATVV

** ** * ** : : * ** : * * : * :

S FGAVIDDRDLQGEMRITVIATGFNGE-KEKPQAKTSSKPVLSGPPAGVETVP--STTTPE
A FGAVIDDRDLQGEVRITVIATGFTGEIQAAPQQNAANARVVSAPPKRTPQT--PLTNSP
At FGAVVDTRYTG EIHVTII IATGFSQSFKTLTDPRAAKLLDKMGSSGQENKGMSLPHQK
Bs FGSVINENLKDEIVTVIATGFIE--QEKDVTKPQRPSLNQSIKTHNQSVPRDAKREEP
Rm VGAIFDRSLDGTFRVS-VATGLDS----NRSAQPTAPEAMNG-----
Ec IGTSLDPDMNDELRVTVVATGIGMD-KRPEITLVTKNQVQQPVMDRYQQHGMAPLTQE QK
* * * * *

S DPLGEIPMAPEL--DIPDFLQKRRFPRR----

A APTPEPKESGL--DIPDFLQRRRPKN----

At QSPSTISTKSSS--PRR-----LFF-----

Bs QQQNTVSRHTSQ--PADDTLDIPTFLRNRNKRG

Rm QTAAAVPSRTLQ-----

Ec PVAKVVDNAPOTAKEPDYLDIPAFLRKOAD-

strep^R

agcttatcgatgactttatttagaggcagtgtttatataaccataaacgtcaaaagtcattt HindIII/Sp1

ttataactggatctcaaaatacctataaacccattgttcttctcttttagctctaagaac

aatcaattttataaatatattttatttattatgctataataataactatataaatacattt

acctttttataaatacattttaccttttttttaatttgcattgattttaatgcttatgctat

ctttttttatttagtccataaaaaccttttaaaggaccttttcttatgggatattttatatttt

cctaacaagcaatcggcgtcataaaccttttagttgcttacgacgcctgtggacgtcccc

ccttcccccttacgggcaagtaaaccttagggattttaatgcaataaataaatttgcctct

tcgggcaaatgaatttttagtattttaaatatgacaaggggaaccattacttttgtttaaca HpaI

agtgatcttaccactcactatttttgttgaattttaaaccttattttaaaattctcgagaaa

gattttaaaaataaaccttttttaactcttttattttttttcttttttatggcaatgcgt

M A M R

actccagaagaacttagtaatcttatttaaagatttaattgaacaatacactccagaagtg

T P E E L S N L I K D L I E Q Y T P E V

aaaatgtccatggctcgtgaagcggttatcgccgaagtatcgactcaactatcagaggta

K M S M A R E A V I A E V S T Q L S E V

gttggcgatcgagcgccatctcgaaccgacgttgctggccgtacatttgtacggctcc

V G V I E R H L E P T L L A V H L Y G S

gcagtggatggcgccctgaagccacacagtgatattgatttgcgtgttacggtgaccgta

A V D G G L K P H S D I D L L V T V T V

aggcttgatgaaacaacgcggcgagctttgatcaacgaccttttgaaacttcggcttcc

R L D E T T R R A L I N D L L E T S A S

cctggagagagcgagattctccgcgctgtagaagtcaccattgttgcacgacgacatc

P G E S E I L R A V E V T I V V H D D I

attccgtggcggttatccagctaagcgcgaaactgcaatttggagaatggcagcgcaatgac

I P W R Y P A K R E L Q F G E W Q R N D

attcttgcaggatcttccgagccagccacgatcgacattgatctggctatcttgcctgaca

I L A G I F E P A T I D I D L A I L L T

aaagcaagagaacatagcgttgcttggtaggtccagcgcgagggaactctttgatccg

K A R E H S V A L V G P A A E E L F D P

gttcttgaacaggatctattttgaggcgctaaatgaaaccttaacgctatggaactcgccg

V P E Q D L F E A L N E T L T L W N S P

cccgaactgggctggcgatgagcgaaatgtagtgcttacgttgcctccgcatttggtagac

P D W A G D E R N V V L T L S R I W Y S

gcagtaaccggcaaaatcgcgccgaaggatgtcgctgccgactgggcaatggagcgctg

A V T G K I A P K D V A A D W A M E R L

ccggcccagtatcagcccgatcacttgaagctagacaggcttatcttggacaagaagaa

P A Q Y Q P V I L E A R Q A Y L G Q E E

gatcgcttggcctcgcgcgagatcagttggaagaatttgcactacgtgaaaggcgag

D R L A S R A D Q L E E F V H Y V K G E

atcactaaggtagttggcaataactgcaggcatgcaagct

HindIII

I T K V V G K -

E. coli ycf16

atgttaagtatttaaagatttacacgtcagcgtggaagataaagctatcctgcgcggatta Ycf161
M L S I K D L H V S V E D K A I L R G L
agcctcgacgttcatcccggcgaagttcacgccattatggggccaaacggttcgggcaaa
S L D V H P G E V H A I M G P N G S G K
agtaccttatcggcaacgcttgccgggcgagaagattatgaagtgcgggcggcacggtt
S T L S A T L A G R E D Y E V T G G T V
gagttcaaaggcaaagatttgcttgcgctgtcgccggaagatcgcgcgggcggaaggcatc
E F K G K D L L A L S P E D R A G E G I
tttatggccttccagttatccgggtggagattccagggtgtcagtaaccagtttttcctgcaa
F M A F Q Y P V E I P G V S N Q F F L Q
acggcacttaatgcgggtgcgcagctatcgcgccaggaaacgctcgaccgctttgatattt
T A L N A V R S Y R G Q E T L D R F D F
caggatttgatggaagagaaaaatcgctctcctgaagatgccggaagatttattaaccggt
Q D L M E E K I A L L K M P E D L L T R
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S V N V G F S G G E K K R N D I L Q M A
gtgctggaaccggagttatgcattcttgatgagtcggactccgggctggatattgacgca
V L E P E L C I L D E S D S G L D I D A
ttaaaagtggtcgccgatggcgtgaactcgctgcgtgatggcaagcgctcattcatcatt
L K V V A D G V N S L R D G K R S F I I
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V T H Y Q R I L D Y I K P D Y V H V L Y
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Q G R I V K S G D F T L V K Q L E E Q G
tatggctggcttaccgaacagcagtaa Ycf162
Y G W L T E Q Q -

Synechocystis ycf24 and ycf16

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M S S T T V K N L V N Q P Y K Y G F V T
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N I E A D A I P R G L S E D V V R L I S
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A K K N E P E F M L D F R L R A Y R H W
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L T M A E P T W P A V H Y P P I D Y Q D
attattttactactccgccccctaagcaaagtaagaaaaaactagaaagcttagatgaagtg
I I Y Y S A P K Q S K K K L E S L D E V
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D P A L L E T F E K L G I P L S E Q K R
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L S N V A V D A I F D S V S I G T T F K
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E K L A E D G V I F C S I S E A L Q E H
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P D L V Q K Y L G S V V P T A D N F F A
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A L N S A V F S D G S F V F I P K G V K
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C P M E L S T Y F R I N N G D T G Q F E
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R T L I I A E E G A S V S Y L E G C T A
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G I Y N F V T K R G L C K G V N S K I S
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W T Q V E T G S A I T W K Y P S C V L V
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D T G T K M I H I G K N T R S I I I S K
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G I S A G N S A N S Y R G L V K M G P K
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Ycf1613

Ycf243

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A S V D G N Q I L K G V N L E I K A G E
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V H A I M G R N G S G K S T L S K V I T
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HpaI

Ycf169

G H P D Y E I T G G E I I Y Q G Q D L S
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 H L G L E E L D T F D F E D L I Q E K L
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 G E K K R N E I L Q M A I L E P T L S I
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 L D E I D S G L D I D A L R I V S E G V
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 N F L K N P D N A T L V I T H Y Q R L L
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 N Y I I P D H I H V M Y D G K I V M S G
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 G K E L A L E L E E K G Y D F L D E Q V
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 L A P I -

***P. falciparum* ycf16**

atgaaaagaagaaaatggctcaacattacttgggctgttacaatatatatatattttttgga Ycf1615

M K R R K W L N I T W A V T I Y I F F G
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M I N V S S Y N E G R K R K N K M Y S F
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V I N G Y N E Y K N K Y S F L R K P K N
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V V H M E N N N F D M D R L S L L K N L
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E E E S K I I T E G E G W D K T H P L L
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N L E I Y L G E K H T I M G R N G S G K
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S T L A K V I A G H P Y Y K I T K G I I
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F L A F Q Y P V E L P M V K N N E F L R
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A A L N S H R R H R N E E E I S V S E F
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P V N Y G F S G G E K K R N E I L Q M L
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I L K P T F C I L D E T D S G L D V D S
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V T H Y K K L L E L L K P N F I H I M H
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Q G K I I E S G D F S L V D K I E N K G
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Y S Q F L K E -

Ycf1611

Ycf1616/17

SspI

Ycf1612/18