

Table of Contents

Acknowledgements

Abbreviations

List of Contents

Chapter 1 Plasmodium falciparum dihydrofolate synthase-folylpolyglutamate synthase (DHFS-FPGS): Gene synthesis and recombinant expression

by

Linda Coetzee

Submitted in partial fulfilment of the requirements for the
degree *Magister Scientiae*

in the Faculty of Natural and Agricultural Sciences

Department of Biochemistry

University of Pretoria

Pretoria

December 2003

Chapter 2

PCR-mediated synthesis of the *P. falciparum*
dhfs-fpgs gene

2.1 Introduction

2.2 Materials and methods

Table of Contents

Acknowledgements	i
Abbreviations	ii
List of tables	iv
List of figures	v

Chapter 1

Literature Review

1.1 The burden of malaria	1
1.2 Malaria pathogenesis	2
1.3 The multifaceted problem of malaria	3
1.3.1 Vector control	3
1.3.2 Host genetic factors	4
1.3.3 Pathogen control	4
1.3.3.1 Cutting-edge technologies	4
1.3.3.2 Vaccines	5
1.3.3.3 Drugs	6
1.4 Drug targets	7
1.4.1 Haemoglobin degradation in the lysosomal food vacuole	8
1.4.2 Apicoplast metabolism	9
1.4.3 Limited electron transport due to an acrystate mitochondrion	9
1.4.4 Cytosolic and membrane bound targets	9
1.5 The folate pathway	10
1.5.1 Folate metabolic organisation	10
1.5.2 Current status of the antifolates	11
1.5.3 Drug synergy	13
1.5.4 Exogenous folate utilisation	13
1.5.5 Putative drug targets within the folate pathway	14
1.5.6 Bifunctional dihydrofolate synthase-folypolyglutamate synthase (DHFS-FPGS) as a possible drug target	14
1.6 Research aims	17

Chapter 2

PCR-mediated synthesis of the *P. falciparum* *dhfs-fpgs* gene

2.1 Introduction	18
2.2 Materials and methods	21

2.2.1	Oligonucleotides and primers	21
2.2.1.1	Oligonucleotide design	22
2.2.1.2	Oligonucleotide- and primer stock solutions and nucleic acid concentration determination	22
2.2.2	PCR gene synthesis	23
2.2.3	Agarose gel electrophoresis and purification	27
2.2.4	Cloning	28
2.2.4.1	The pGEM T Easy vector system	28
2.2.4.2	The pMOS <i>Blue</i> vector system	30
2.2.5	Plasmid isolation and identification of recombinant clones	31
2.2.5.1	STET-prep plasmid isolation	31
2.2.5.2	High Pure Plasmid kit plasmid isolation	32
2.2.6	Sequencing	32
2.2.7	Construction of the full-length gene	33
2.2.8	Gene repair strategies	34
2.2.8.1	Quarter gene segments	34
2.2.8.2	Site-directed mutagenesis of point mutated PCR products	35
2.2.8.3	Restriction-ligation of correct fragments	36
2.2.8.4	Cassette mutagenesis PCR	36
2.2.8.5	Construction of the full-length gene from correct quarter fragments	38
2.3	Results	40
2.3.1	Oligonucleotide design	40
2.3.2	Optimisation of the overlap-extension assembly step	40
2.3.3	Optimisation of the number of assembly cycles	42
2.3.4	Construction of the full-length gene from half fragments	44
2.3.5	Gene repair	44
2.3.6	Construction of the full-length gene from correct quarter fragments	46
2.4	Discussion	48
2.4.1	Overlap-extension PCR as a gene synthesis method	48
2.4.2	Optimisation of the assembly PCR parameters	49
2.4.3	Optimisation of the number of assembly cycles	49
2.4.4	Construction of the full-length gene	50
2.4.5	Gene repair	51
2.4.6	Construction of the full-length gene from quarter fragments	52

Chapter 3

Expression of synthetic *P. falciparum* dihydrofolate synthase-folylpolyglutamate synthase (DHFS-FPGS)

3.1	Introduction	53
3.2	Materials and methods	56
3.2.1	Constructs, vectors and cell lines	56

3.2.2 Protein expression	57
3.2.3 Determination of the protein concentration	58
3.2.4 SDS PAGE analysis	58
3.2.5 Functional complementation	59
3.2.6 Partial protein purification	60
3.2.6.1 Unfolding/refolding protocol	60
3.2.6.2 Affinity chromatography	60
3.2.6.3 Size exclusion high performance liquid chromatography	61
3.3 Results	62
3.3.1 Expression of a variety of <i>dhfs-fpgs</i> constructs	62
3.3.2 Functional complementation	64
3.3.3 Preliminary purification studies	66
3.3.3.1 Affinity purification of refolded C-terminal His ₆ -tagged DHFS-FPGS from inclusion bodies	66
3.3.3.2 Affinity purification of soluble C-terminal His ₆ -tagged DHFS-FPGS	67
3.3.3.3 Size exclusion high performance liquid chromatography	67
3.4 Discussion	70
3.4.1 Expression of various DHFS-FPGS constructs	70
3.4.2 Functional complementation	71
3.4.3 Partial purification	72
3.4.3.1 Unfolding and refolding of inclusion bodies	72
3.4.3.2 Affinity purification of C-terminal His ₆ -tagged DHFS-FPGS.	72
3.4.4 Future prospects	73

Chapter 4

In silico analysis of dihydrofolate synthase-folylpolyglutamate synthase (DHFS-FPGS)

4.1 Introduction	74
4.2 Methods	77
4.2.1 Sequence alignments	77
4.2.2 Structure predictions	77
4.3. Results	79
4.3.1 Inter-species DHFS and FPGS alignments and phylogenetic analysis	79
4.3.2 Conservation of DHFS-FPGS within the <i>Plasmodium</i> species and comparison with human FPGS	86
4.3.3 Secondary structure prediction of <i>P. falciparum</i> DHFS-FPGS	88
4.3.4 DHFS-FPGS hydrophobicity profile	90
4.4 Discussion	91
4.4.1 Sequence conservation of DHFS-FPGS	91
4.4.2 Predicted secondary structure	92

Chapter 5

Acknowledgements

Concluding discussion

5.1 'Discover, Develop, Deliver'	93
5.2 Antifolates: New targets, old pathway	93
5.3 <i>P. falciparum</i> DHFS-FPGS: an attractive drug target	94
5.3 The aims of this study: obtaining sufficient amounts of active <i>P. falciparum</i> DHFS-FPGS	94
5.5 A look into the near and distant future	96

Summary	98
---------	----

Opsomming	100
-----------	-----

References	102
------------	-----

Appendices	114
------------	-----

Acknowledgements

To Professor Louw, my promoter and mentor, who made this project as much his own as it was mine, shared in all the ups and downs, from whom I learnt all about science and much about life in general, who believed in my abilities and gave me opportunities far beyond that and to Prof. Neitz, my co-promoter, for his sharp mind and valuable comments. To the NRF for rewarding me a scholarship that enabled these studies.

To Professor Hyde, Dr. Tanya Aspinall and the rest of the UMIST research team, for receiving and hosting me with such warmth, for all their good advice and assistance and their willingness to cooperate and work with me towards our common goal.

To all our staff, lecturers and students at UP, especially Jaco de Ridder for the design of the oligonucleotides, who gave me a new understanding and appreciation of computers, Gordon Wells who generously donated time from his own thesis to help me in the Bioinformatics department, as well as Sandra, Ben and Christine for helping me with the technical and experimental detail and Lyn-Marie for her much valued advice and insights.

To my parents, who realised the importance of a good education and who set some of their dreams aside to provide me with this, for their unconditional love and support. To my grandparents, family and friends for their continual support, interest and understanding. To Heinrich for his undaunted support and belief in my abilities, who shared with me all my failures and successes and loved me nevertheless.

Lastly, to our Father, Who loved us enough to become one of us, Who created this wonderful universe we have yet to begin to understand, Who knows all the answers to our myriad of questions, Who grants us brief glimpses of His perfection through the subjects we study and explore...

Abbreviations

ACP	acyl carrier protein
ARMS PCR	amplification refractory mutation system PCR
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	basepair
BSA	bovine serum albumin
CPG	controlled pore glass
CS	circumsporozoite
DHFR	dihydrofolate reductase
DHFS	dihydrofolate synthase
DHPS	dihydropteroate synthase
DMT	4,4'dimethoxytrityl
DNA	deoxyribonucleic acid
DOXP	deoxyxylulose phosphate
dNTP	deoxynucleoside triphosphate
dTMP	deoxythymidine monophosphate
DTT	dithiotreitol
dUMP	deoxyuridine monophosphate
E.C.	Enzyme Commission
EDTA	(ethylenedinitrilo)tetraacetic acid
FPGS	folylpolyglutamate synthase
GTP	guanosine triphosphate
GTP-CH	GTP cyclohydrolase
HLA	human leukocyte antigen
HNN	Hierarchical Neural Networks
HPLC	high performance liquid chromatography
HRP	histidine rich protein
IMAC	immobilised metal affinity chromatography
IPTG	isopropyl- β -D-thiogalactopyranoside
LB	Luria-Bertani medium
LDH	lactate dehydrogenase
MMV	Medicines for Malaria Venture
MVI	Malaria Vaccine Initiative
NMR	nuclear magnetic resonance
nt	nucleotide
OD	optical density
PABA	p-amino benzoic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PPPK	hydroxymethyl pyrophosphokinase
PYR	pyrimethamine
RBS	ribosome binding site
SDS	sodium dodecylsulfate
SDX	sulfadoxine
SERCA	sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase
SHMT	serine hydroxymethyltransferase

SNP	single nucleotide polymorphism
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris (hydroxymethyl) aminomethane
tRNA	transcription ribonucleic acid
TS	thymidylate synthetase
U	enzyme units
UTR	untranslated region
X-gal	5-bromo-4-chloro-3-indolyl-- β -D-galactopyranoside

Chapter 1

Table 1.1. Programmes used for the analysis of the data.

Table 1.2. Summary of the results of the antibiotic resistance test.

Chapter 2

Table 2.1. Summary of the results of the antibiotic resistance test.

Table 2.2. Summary of the results of the antibiotic resistance test.

Table 2.3. Summary of the results of the antibiotic resistance test.

Table 2.4. Summary of the results of the antibiotic resistance test.

Table 2.5. Summary of the results of the antibiotic resistance test.

Chapter 3

Table 3.1. Summary of the results of the antibiotic resistance test.

Table 3.2. Summary of the results of the antibiotic resistance test.

Table 3.3. Summary of the results of the antibiotic resistance test.

List of figures

List of tables

Chapter 1

Table 1.1: Problems associated with antimalarial drugs.	7
Table 1.2: Primary mutations responsible for antifolate resistance.	12

Chapter 2

Table 2.1: Forward (f) and reverse (r) oligonucleotides and forward (F) and reverse (R) primers used for the assembly of different gene fragments.	21
Table 2.2: Forward (F) and reverse (R) primers used for the generation of the quarter fragments.	34
Table 2.3: Site-directed mutagenesis primers designed for error corrections in quarter 4.	35
Table 2.4: Forward (2f/3f) and reverse (2r/3r) oligonucleotides used for the resynthesis of the quarter 2 and 3 internal fragments.	38
Table 2.5: The theoretical and actual number of cycles needed for complete template generation based on the number of oligonucleotides involved.	43

Chapter 3

Table 3.1: Vector systems used for the recombinant expression of synthetic <i>P. falciparum dhfs-fpgs</i> .	56
Table 3.2: Primers used for cloning of <i>P. falciparum dhfs-fpgs</i> into the pASK-IBA3 vector for C-terminal Strep-tagged expression.	57
Table 3.3: <i>E. coli</i> strains used as hosts for protein expression.	57

List of figures

Chapter 1

- Figure 1.1: The life cycle of the human malaria parasite *P. falciparum*. 2
- Figure 1.2: The three main links in cycle of malaria transmission. 3
- Figure 1.3: The subcellular locations of past, present and future drug targets. 8
- Figure 1.4: Folate metabolism in the human malaria parasite *P. falciparum*. 11

Chapter 2

- Figure 2.1: Schematic representation of the genetic organisation of *P. falciparum dhfs-fpgs*. 18
- Figure 2.2: Overlap PCR used during gene synthesis. 20
- Figure 2.3: Alignment of the synthetic and native (*Pf dhfs-fpgs*) *dhfs-fpgs* genes. 24
- Figure 2.4: Overlap-extension PCR. 26
- Figure 2.5: Subdivision of *dhfs-fpgs* into quarter segments with unique restriction enzyme sites in the overlaps. 34
- Figure 2.6: Cassette replacement of a mutated gene segment with a newly synthesised internal area of the quarter fragment. 37
- Figure 2.7: Graphs indicating the nucleotide composition of the synthetic *P. falciparum dhfs-fpgs* (A) gene compared to native *P. falciparum dhfs-fpgs* (B). 40
- Figure 2.8: Taguchi optimisation of the Mg^{2+} , dNTP and *Pfu* DNA polymerase concentrations for the assembly of six oligonucleotides. 41
- Figure 2.9: Refinement of the PCR parameters for the assembly PCR step. 42
- Figure 2.10: PCR products obtained for fragments assembled from 6 oligonucleotides (A) or 10 oligonucleotides (B). 43
- Figure 2.11: The full-length synthetic *dhfs-fpgs* gene obtained from overlap extension PCR of the two 750 bp half fragments. 44

Figure 2.12: The direct relationship between the error rate and the proximity to the 5' end of each oligonucleotide.	45
Figure 2.13: Construction of the correct quarter 2 by means of cassette mutagenesis.	45
Figure 2.14: Corrected quarter fragments.	46
Figure 2.15: Isolation of the 1100bp fragment consisting of the 1 st three fragments by means of restriction digestion.	47
Figure 2.16: Full-length <i>dhfs-fpgs</i> constructs obtained after overlap-extension of the 1100bp fragment and alternative 500bp fragments.	47

Chapter 3

Figure 3.1: Expression of (A) pET15b- <i>dhfs-fpgs</i> (N-terminal His ₆ tag) and (B) pET22b- <i>dhfs-fpgs</i> (C terminal His ₆ tag) in various cell lines.	62
Figure 3.2: Solubility of C-terminal His-tagged DHFS-FPGS in A: BL21 Star (DE3), B: BL21 Gold (DE3) pLysS and C: BL21 (DE3) pLysS cell lines.	63
Figure 3.3: Solubility of tagless DHFS-FPGS in A: BL21 Star (DE3), B: BL21 Gold (DE3) pLysS and C: BL21 (DE3) pLysS cell lines.	63
Figure 3.4: Expression of C-terminal Strep-tagged <i>dhfs-fpgs</i> in A: BL21 Star (DE3), B: BL21 Gold (DE3) pLysS and C: BL21 (DE3) pLysS cell lines.	64
Figure 3.5: Complementation of DHFS-FPGS deficient <i>E. coli</i> (SF4) by different synthetic <i>P. falciparum dhfs-fpgs</i> constructs.	64
Figure 3.6: Growth of SF4 <i>E. coli</i> containing different constructs in liquid media.	65
Figure 3.7 Growth curves over 24 hours for the tagless <i>dhfs-fpgs</i> construct in Sf4 cells (tl) versus the negative control Sf4 cells without any construct (Sf4).	66
Figure 3.8: Affinity purification of resolubilised C-terminal His ₆ -tagged DHFS-FPGS obtained from BL21 Star (DE3) cells.	66
Figure 3.9: C-terminal His ₆ -tagged DHFS-FPGS affinity purified from the soluble fraction expressed by BL21 (DE3) pLysS cells.	67
Figure 3.10: Retention times of low molecular mass protein standards.	68
Figure 3.11: Size exclusion analysis of the expression of C-terminal His ₆ -tagged DHFS-FPGS from BL21 (DE3) pLysS cells.	68
Figure 3.12: Size exclusion HPLC profiles obtained for affinity purified C-terminal His ₆ -tagged DHFS-FPGS from BL21 (DE3) pLysS cells.	69

Figure 3.13: Silver stained SDS PAGE of fractions obtained after size exclusion HPLC of C-terminal His₆-tagged DHFS-FPGS. 69

Chapter 4

Figure 4.1: DHFS and FPGS enzyme reactions. 74

Figure 4.2: Schematic representation of *L. casei* FPGS. 75

Figure 4.3: Phylogenetic analysis of *P. falciparum* DHFS-FPGS based on its alignment with homologous proteins. 79

Figure 4.4: Alignment of *P. falciparum* DHFS-FPGS with homologous proteins. 84

Figure 4.5: Superimposed ribbon backbones of the preliminary homology model of *P. falciparum* DHFS-FPGS on the *L. casei* crystal structure. 85

Figure 4.6: Ramachandran plot of the *P. falciparum* DHFS-FPGS homology model. 85

Figure 4.7: *Plasmodium* DHFS-FPGS vs human FPGS alignment. 87

Figure 4.8: Secondary structure prediction of *P. falciparum* DHFS-FPGS by GOR4. 88

Figure 4.9: Alignment of independent secondary structure predictions. 89

Figure 4.10: Hydrophobicity profile for the primary amino acid sequence using Kyte and Doolittle parameters. 91