INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Analysis of Resistance to Inhibitors of *Plasmodium falciparum* Dihydrofolate Reductase in Yeast

by

Jason Wooden

A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

University of Washington

1996

Approved by Professor Carol Hoffman
Chairperson of Supervisory Committee

Program Authorized
to Offer Degree Genetics

Date October 9, 1996

In presenting this dissertation in partial fulfillment of the requirements for the Doctroal degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying fo this dessertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dessertation may be referred to University Microfilms, 1490 Eisenhower Place, P.O. Box 975, Ann Arbor, MI 48106, to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) prited copies of the manuscript made from microform."

Signature Jason M. Wooden

Date Oct. 9 1996

University of Washington

Abstract

Analysis of Resistance to Inhibitors of *Plasmodium falciparum* Dihydrofolate Reductase in Yeast

by Jason M. Wooden

Chairperson of the Supervisory Committee: Professor Carol Hopkins Sibley
Department of Genetics

Resistance to the malaria drugs is an increasing problem for treating the 200-300 million people infected yearly with falciparum malaria. Detailed analysis of the development of drug resistance has been complicated by difficulties in defining differences between *P. falciparum* isolates and by the difficult culture conditions required to grow parasites. The inability to distinguish *P. falciparum* isolates has complicated interpretation of drug resistance data from field isolates and hampered genetic analysis of drug resistance. A method was developed based on the polymerase chain reaction (PCR) to quickly genotype *P. falciparum* strains using polymorphisms in genes containing variable repeats (RESA, MSA-1, MSA-2, and CSP). The technique requires very small samples (100 μl of infected blood) and is suitable for field use. Additionally, the technique is sensitive enough to detect a 1% contamination of one strain with another.

Examination of populations of *P. falciparum* sensitive or resistant to the malaria drugs pyrimethamine (pyr) and cycloguanil showed that point mutations within the dihydrofolate reductase (DHFR) gene correlated with the drug resistance. To study the frequency of these changes and the effect of particular mutations on resistance, we have replaced the DHFR gene from *S. cerevisiae* with the DHFR domain of the DHFR-TS gene from a drug sensitive *P. falciparum* strain. The *P. falciparum* DHFR gene complements all normal functions of the DHFR gene from *S. cerevisiae*. Furthermore, the sensitivity of this *S. cerevisiae* to the malarial antifolates is comparable to the sensitivity seen for the original *P. falciparum* strain. A selection experiment was performed to test the ability of a plasmid-based expression system to generate DHFR point mutants. When $\approx 10^8$ drug sensitive yeast cells were plated on media containing pyrimethamine and the experimental drug WR99210 (WR), resistant colonies were recovered at frequencies of 1.3×10^{-6} and 9.5×10^{-7} for pyr and WR respectively. Subsequent analysis indicated that a large amount of the resistant colonies most likely arose from DHFR copy # increases via plasmid missegregation errors. Transferring the construct into a chromosomal location will reduce this background and facilitate the efficient isolation of DHFR point mutants.

TABLE OF CONTENTS

List of Abbreviations

bp (base pair)

C (complete media)

CSP (circumsporozoite surface protein)

cyc (cycloguanil)

cyc^R (cycloguanil resistant)

DHF (dihydrofolate; folic acid)

DHFR (dihydrofolate reductase)

DNA (deoxyribonucleic acid)

dNTP (deoxyribonucletide)

dTMP (deoxyribosylthymine monophosphate)

EDTA (ethylenediaminetetraacedit acid)

IC_{50} (50% inhibitory concentration)

M (molar)

MDR (multidrug resistance)

mg (milligram)

ml (milliliter)

mM (millimolar)

mV (millivolts)

MSA-1 (major merozoite surface antigen 1)

MSA-2 (major merozoite surface antigen 2)

MTX (methotrexate)

MTX^R (methotrexate resistant)

N (normal)

NaCl (sodium chloride)

ng (nanogram)

nl (nanoliter)

nm (nanometer)

Abbreviations Continued

PABA (para-amino benzoic acid)

PBS (phosphate buffered saline)

PCR (polymerase chain reaction)

PDR (pleiotrophic drug resistance)

PfDHFR (*Plasmodium falciparum* DHFR)

Pf-DHFR-D6 (yeast transformant containing DHFR from *P. f.* strain D6)

Pf-DHFR-Hon (yeast transformant containing DHFR from *P. f.* strain Honduras1)

Pf-DHFR-Mik (yeast transformant containing DHFR from *P. f.* strain Mikenga)

pyr (pyrimethamine)

pyr^R (pyrimethamine resistant)

RESA (ring-infected erythrocyte surface antigen)

RPM (revolutions per minute)

sec (seconds)

SDS (sodium dodecyl sulfate)

TBE (tris/boric acid/EDTA)

THF (tetrahydrofolate; folinic acid)

TE (Tris-EDTA)

Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol)

TS (thymidylate synthetase

U (unit)

μg (microgram)

μl (microliter)

WR (WR99210

WR^R (WR99210 resistant)

YEP (yeast extract/peptone)

YEPD (yeast extract/peptone/dextrose)

List of Figures

List of Figures Continued

List of Tables

Acknowledgments

The author wishes to thank Professor Carol Sibley for her enthusiasm and patience throughout the project. Thanks also to the members of the thesis committee (Professors Breck Byers, Clem Furlong, Robert Braun, and Steve Mosely) for their support during the many stages of the project. A special thanks goes to Professor Lee Hartwell for proposing and supporting the early stages of the yeast work. Finally, many thanks to the members of the research group (Kelly Hamilton, Eleanor Hankins, Tuyen Vu, Victoria Brophy, and Som Mookherjee) for many useful suggestions throughout the project. Kelly Hamilton was especially helpful during the preliminary tests of the system.

Dedication

This dissertation is dedicated to Edward and Fern Wooden for their continual encouragement and support of the author's pursuits in the scientific enterprise.

Chapter 1: Introduction

Falciparum malaria is caused by the protozoan parasite *Plasmodium falciparum* which infects 200-300 million people worldwide and is responsible for 2 million deaths a year. The life cycle of the parasite consists of two phases: a diploid sexual phase which takes place in the *Anopheles* mosquito vector and a haploid asexual phase which takes place within the human host's red blood cells (Peters, 1987). In the past, drugs targeted against the proliferation of the parasites in the human host were extremely effective for prevention and treatment. These drugs included quinine, chloroquine, and the antifolate pyrimethamine. However, in recent years, the parasite populations have been strongly selected by extensive use of these drug treatments and have evolved rapidly toward drug resistance (Wernsdorfer, 1991). Chloroquine resistant malaria was first confined to Venzuela and the Thai-Cambodia border, but by 1989 was widespread in Africa, Indonesia, and South America. Widespread resistance to pyrimethamine first appeared in Indonesia in the early 1980s and is now seen in regions of Africa and South America. Currently, most of the drugs that have been used in the past to treat or prevent malaria are no longer effective (Peters, 1987).

New antimalarials have been introduced (for example, artemesin, halofantrine, and mefloquine). However, drug resistance to mefloquine, one of newest antimalarials, evolved within two years after its introduction for clinical use (White, 1992). Detailed analysis of the development of resistance to antimalarials has been hampered for most of these drugs since the mechanism of action and resistance is unknown. For example, while the target of chloroquine is strongly thought to be the parasitic heme polymerase located in the food vacuole of the parasite, the mechanism of drug resistance is still poorly understood and widely debated (Borst, 1995). For these reasons, the class of

antimalarials most amenable to genetic analysis is the antifolates since the target enzyme, dihydrofolate reductase, is well defined.

Although the three-dimensional structure of the dihydrofolate reductase (DHFR) enzyme has been strongly conserved during evolution, subtle differences in the active sites of the bacterial, human, and parasite enzymes have allowed for the development of inhibitors specific to particular pathogens (Hyde, 1990). For example, the antibiotic trimethoprim inhibits prokaryotic, but not mammalian DHFR. Two antifolates that have been used widely against malaria are pyrimethamine and proguanil [which is metabolized in humans into the active form, cycloguanil (Peters, 1987)] (Figure 1-1). These are both competitive inhibitors of the DHFR enzyme which controls a key step in thymidylate biosynthesis (Hyde, 1990) (Figure 1-2). Inhibition of the DHFR results in depletion of the intracellular pools of thymidylate and consequently cell growth arrest (Hartman, 1993).

Drug screening studies have identified many other DHFR inhibitors that are effective *in vitro* against *P. falciparum* (Milhous, 1985). An example is the experimental drug WR99210 which is extremely effective against both pyrimethamine sensitive and resistant strains of *P. falciparum*. Table 1-1 compares the *in vitro* activities of WR99210 and pyrimethamine against a variety *of P. falciparum* strains. The remarkable potency of WR99210 is illustrated by the fact that 0.3 ng/ml of pyrimethamine is needed for inhibition of the drug sensitive strain D6-Sierra Leone while only 0.002 ng/ml of WR99210 is needed. The experimental inhibitor is 150X more potent than the traditional inhibitor. However, even more interesting is its activity against pyrimethamine resistant strains. For example, 83.3 ng/ml of pyrimethamine is required to inhibit the strain V1S Vietnam while WR99210 amounts remain low (0.002 ng/ml). In the early 1980's, the long-term potential of this remarkable inhibitor was further highlighted when experiments were done to assess

in vivo the capacity for resistance arising (Knight, 1982). Using the *P. berghei* mouse malaria model system, resistance to WR99210 was found to develop at a much slower rate than resistance to the traditional antifolates (cycloguanil and pyrimethamine) and did not lead to cross-resistance. Despite the in vitro effectiveness of this experimental drug, it was never advanced to clinical trials for use in the field. The rapid selection of resistance to pyrimethamine and cycloguanil has strongly discouraged further development of many experimental inhibitors. The assumption is that other drugs with a mode of action similar to pyrimethamine and cycloguanil will show equally strong selection for drug resistance, but it has not been possible to test this directly.

Resistance to inhibitors of DHFR develops in two major ways: over-expression of the wild type enzyme or point mutations in the DHFR gene that reduce the binding affinity of the inhibitor (Hyde, 1990). The malarial mechanism was finally revealed when the *P. falciparum* bifunctional dihydrofolate reductase-thymidylate synthetase (DHFR-TS) was cloned (Bzik, 1987). Examination of the DHFR genes from pyrimethamine resistant (pyr^R) field isolates suggested that, unlike other organisms, the major mechanism for resistance to antifolates in malarial parasites is point mutations in the DHFR gene (Cowman, 1988). Additional evidence linking pyr^R to point mutations in the gene was obtained in a genetic cross of *P. falciparum* parasites in which the resistance phenotype was found to segregate with the DHFR gene (Peterson, 1988). These DHFR mutations were presumed to result in structural changes in the enzyme giving rise to reduced drug affinity. This was eventually confirmed biochemically when the DHFR-TS was purified from pyr^S and pyr^R parasites: differences were found only in the pyrimethamine inhibition constants (Zolg, 1989). Analysis of the DHFR genes from cycloguanil resistant (cyc^R) isolates revealed that while point mutations are involved, different combinations are responsible for varying levels of resistance to cycloguanil and pyrimethamine (Peterson, 1990). Additional DHFR sequence data from 10 different field isolates further supported

this, indicating that pyrimethamine resistance and cycloguanil resistance commonly involve alternative mutations at the same site (Foote, 1990).

Currently, there is considerable agreement that resistance to pyrimethamine is associated with a mutation in the Ser^{108} to Asn and that a further increase in resistance results from changes at Ile^{51}, Arg^{59}, and Ile^{164} (Cowman, 1988; Peterson, 1988; Zolg, 1989; Sirawaraporn, 1993). The ser^{108} mutation has recently been demonstrated to be important in the acquisition of pyrimethamine resistance for the rodent malarias (*P. berghei, P. chabaudi, P. vinckei, and P. yoelii*). The resistance was found to correlate with an amino acid change of Ser to Asn at position 106 (equivalent to the 108 change in *P. falciparum*) when sensitive and isolates were compared (Cheng, 1994). Examination of the DHFR coding region of parasites from cycloguanil resistant populations have identified positions Thr^{108} and Val^{16} as possible mutations to cycloguanil resistance, but the correspondence is considerably less clear for this drug (Foote, 1990; Peterson, 1990). Nonetheless, these cyc^R and pyr^R mutations are hypothesized to surround the active site cavity of the DHFR enzyme based on structural data from *Lactobascillus casei* (Hyde, 1990). The role of structural changes as the major resistance mechanism has been further supported in laboratory experiments in which lines of *P. falciparum* parasites selected for resistance to pyrimethamine are found to contain point mutations in their DHFR genes (Tanaka, 1990; Thaithong, 1992).

Since these conclusions have been formulated from a limited set of culture-adapted reference clones of *P. falciparum*, it has become important to determine the widespread relevance of the various DHFR point mutations in field populations. Using allele-specific PCR methods, it was possible to identify DHFR mutations directly from blood samples and confirm the importance of these mutations in African populations (Plowe, 1995). A similar study focusing on pyrimethamine resistance in the Brazilian Amazon indicated that

a very high incidence of the Asn-108 mutation (90% of the collected samples) was responsible for the resistance in endemic regions (Peterson, 1991). These data, along with earlier work, highlight the importance of DHFR point mutations in *P. falicparum* and provide a strong basis for detailed analysis of the development of resistance to antifolates.

To test the assumption that resistance to promising new DHFR inhibitors will be strongly selected, I initiated studies to establish a genetic system to compare the development of drug resistance to two drugs: pyrimethamine which has been extensively used clinically and the experimental inhibitor WR99210. The approach was to select mutants resistant to each drug in parallel and then to compare the resulting mutants. Pyrimethamine would serve as an importance reference drug since it has been so well-studied and resistance to it had been studied both in the field and in laboratory drug selections with *P. falciparum*. Four specific questions formed the framework for the study (Figure 1-3):

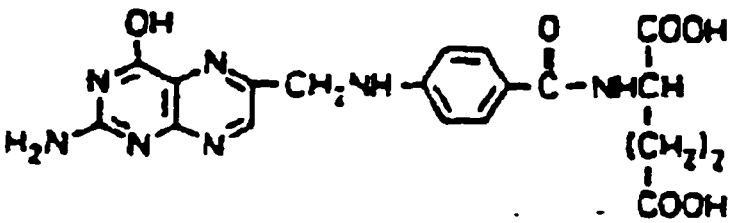
1) Is it possible to develop resistance to the new drug?
2) Would this lead to cross-resistance to other drugs of clinical importance?
3) How does the frequency of mutation to resistance to the new drug compare with that of pyrimethamine?
4) What is the mechanism of resistance to the new drug?

Two persistent obstacles have prevented the achievement of these goals. The first obstacle has been the lack of a quick and reliable method to distinguish isolates of *P. falciparum*. Confusion about the identity of commonly used strains and inadvertent contamination of one strain with another have been persistent problems in *Plasmodium* laboratory research. Additionally, the inability to assess genetic relatedness of isolates has complicated interpretation and correlation of drug resistance data. The second obstacle results from the difficulty of culturing *P. falciparum* parasites, making mutant selections

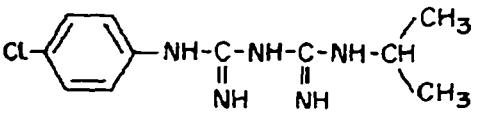
extremely time-consuming and possible only by gradually enriching a population for resistant mutants. Past efforts to select parasites resistant to pyrimethamine were successful, but resulted in a very limited number of mutant populations (Banyal, 1986; Thaithong, 1992).

These obstacles have led to three important objectives for the thesis project. The first was to create a genetic technique to quickly and reliably differentiate isolates of *P. falciparum*. The second objective was to establish a heterologous expression system for the *P. falciparum* DHFR gene that would allow quantitative study of the development of antifolate resistance at the organismal level, resulting in easier determination of resistance mechanisms and mutation frequencies. Finally, the third objective was test the ability of the heterologous expression system to select DHFR mutants conferring resistance to various drugs.

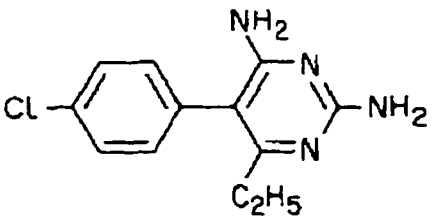
The first two objectives were met. A quick and reliable strain identification technique was developed based on the polymerase chain reaction (PCR) and polymorphisms in cloned *P. falciparum* genes. The method requires very small amounts of sample and is capable of detecting a 1% contamination of one strain with another. A *S. cerevisiae* heterologous expression system was developed for the DHFR domain of the *P. falciparum* bifunctional DHFR-TS gene. Pilot experiments using this yeast system to select DHFR mutants have revealed ways in which the system can be modified to measure efficiently the rate of mutation from drug sensitivity to drug resistance for pyrimethamine and WR99210. This system has the potential to identify drugs or combinations of drugs that do not select resistant subpopulations efficiently. Such compounds would be excellent candidates for further development of treatments that will have a longer useful life in clinical practice.



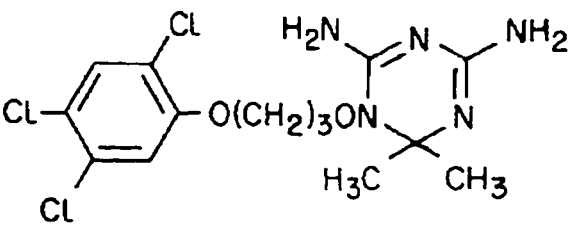
Dihydrofolate
(folic acid)



proguanil
(precursor of cycloguanil)



Pyrimethamine



WR99210
(experimental drug)

Figure 1-1. Inhibitors of the dihydrofolate reductase enzyme. Shown at top is the natural substrate of the enzyme, dihydrofolate.

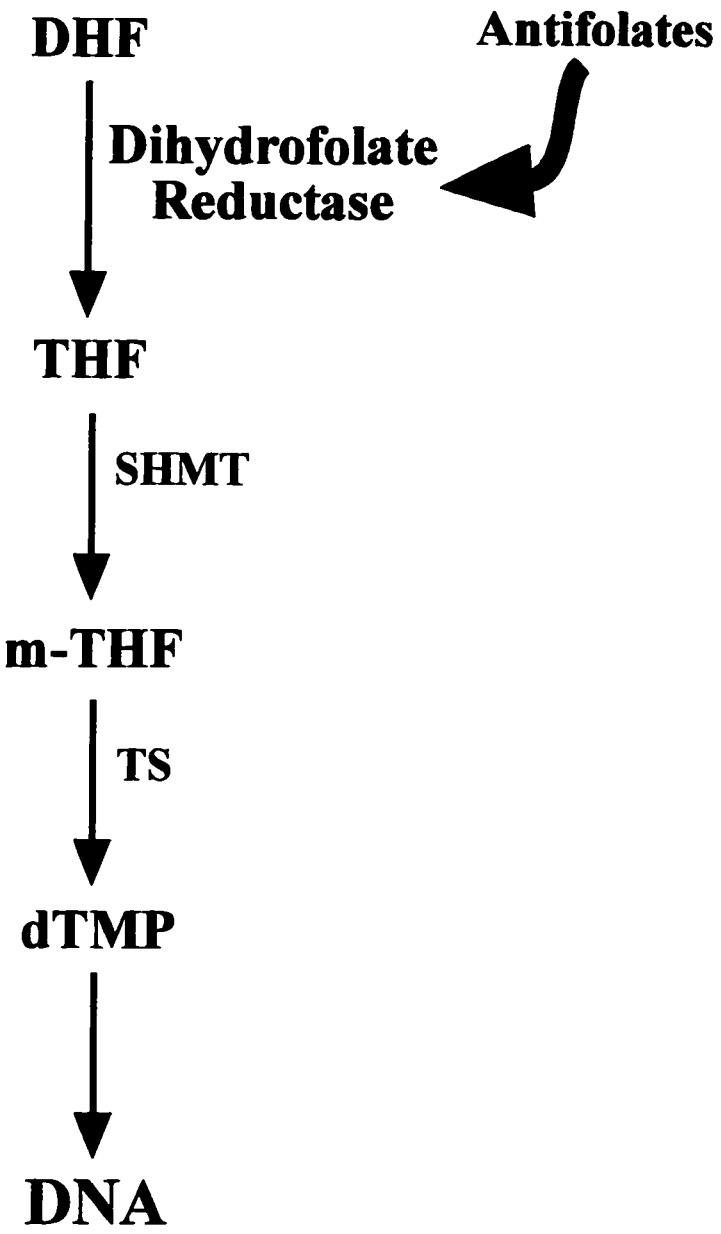


Figure 1-2. Folate pathway of metabolism and important enzymatic steps. Dihydrofolate (DHF) is converted to tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (DHFR). THF is converted to methylene tetrahydrofolate (m-THF) by the serine hydroxymethyltransferase (SHMT). Finally, m-THF is used as a cofactor in the biosynthesis of deoxyribosylthymine monophosphate (dTMP) by the enzyme thymidylate synthetase (TS).

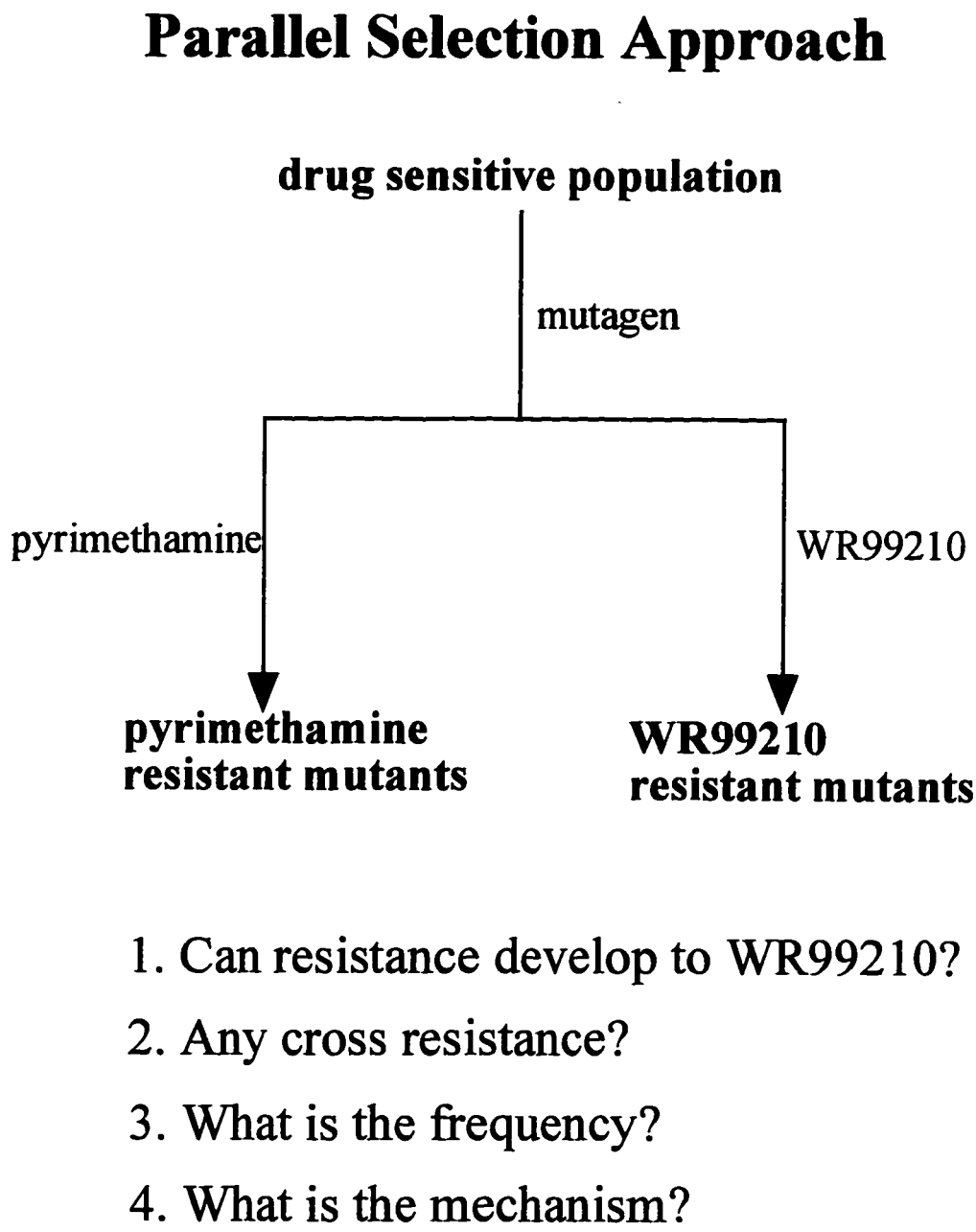


Figure 1-3. Experimental approach and questions.

Table 1-1. Comparison of *in vitro* activity for WR99210 and pyrimthamine against *P. falciparum* parasites showing various levels of resistance to pyrimethamine*.

STATUS	STRAIN	PYR (ng/ml)	WR99210 (ng/ml)
Sensitive	D6 Sierra Leone	0.3	0.002
Resistant	HB3 Honduras	21.5	0.003
	Itd12 Brazil	88.1	0.002
	W2 Indochina	33.9	0.002
Multi-Drug	V1S Vietnam	83.3	0.002
Resistant	WRAIR Kenya '91	99.4	0.002

*Data from Division of Therapeutics, Walter Reed Army Institute of Research (1991 internal report. Wilbour K. Milhous)..

CHAPTER 2: MATERIALS AND METHODS

I. Strain Identification

Strains:

Four strains were used for most of this work. FCR-8 was isolated from a West African strain by Trager and his colleagues (Trager, 1976). We obtained the strain directly from the American Type Culture Collection. 3D7 is a cloned line sent to us by Dr. Thomas Wellems; it was originally isolated from a patient in the Netherlands (Wellems, 1987). C10 and its clone, D2, were obtained from Dr. Jean Feagin (Hempelmann, 1981). In addition, DNA isolated from a number of other strains was surveyed: Sierra Leone, Tanzania 1, Honduras 1, K1 (courtesy of Dr. Randy Howard) and PLF3B11 (courtesy of Dr. Jean Feagin).

Cell growth:

Cells were grown *in vitro* with a modification of the method of Trager (Zolg, 1982). Briefly, they were grown in RPMI 1640 supplemented with 5 % v/v washed human red cells, 10% fresh human serum, 5 mg/ml hypoxanthine and 600 mg/ml reduced glutathione, and maintained in an atmosphere of 5% oxygen, 5% carbon dioxide, and 90% nitrogen.

DNA isolation:

Initial isolation of DNA was done with a modification of the standard method of Higuchi (Higuchi, 1989). In later experiments, a modification of the method of Walsh (1991) proved much easier. About 200 µl of culture was washed once in phosphate buffered saline (PBS; 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2), and the red cells

lysed by suspension in 1% saponin for 5 minutes on ice. The *P. falciparum* cells were isolated by centrifugation for 30 sec. at 12,000 x g , and a single washing in PBS. This pellet was then resuspended in a 5% w/v suspension of Chelex-100 in water, pH > 9.5 (Biorad) and incubated in boiling water for 8 minutes. The Chelex was removed by centrifugation for 30 sec at 12K RPM, and the resulting suspension precipitated once with alcohol, dried and resuspended in 50 µl water, and 2 µl used directly for PCR amplification.

PCR protocols:

The PCR protocols were all adaptations of standard procedures (Innes, 1990). The reactions contained a total volume of 100 µl, with 0.2 mM of the dNTPs, and 1.25U units of Replitherm (Epicentre). The reactions were run in a Biosycler (Bios Corporation) with the following parameters: initial denaturation 2' at 94° and 29 cycles with 20" at 94°, 20" at 55° and 20" at 72°. The primers used for each reaction are listed in Table 3-2. PCR products were analyzed on 2% agarose, run in 0.5X TBE (4.25 mM Tris, 4.45 mM borate, 1.25 mM EDTA, pH 8.2) for one hour at 100 mV. The double stranded PCR products were separated from the primers by centrifugation through a Centricon-30 membrane and the sequencing reactions performed using the Sequenase Kit (U.S. Biochemical) according to the manufacturer's instructions.

II. Yeast Expression System to Study Resistance to Malarial DHFR Inhibitors

Malaria and yeast strains:

Three strains of *Plasmodium falciparum* were used to isolate DNA. The strains SL/D6 (Oduola, 1988) and Mikenga (Kenyan isolate, personal communication) were from Dr. Wilbur Milhous (Experimental Therapeutics Division, Walter Reed Army Institute of Research, Silver Spring, MD) and were grown *in vitro* with a modification of the method of Trager (Zolg, 1982). Genomic DNA from the strain Honduras 1 (Tanaka, 1990) was provided by Dr. R.F. Howard (Seattle Biomedical Research Institute, Seattle, WA). The two *Saccharomyces cerevisiae* strains used, the wild-type parent TH1 *(MATa leu2-3,112 trp1 ura3-52 tup1*) and the *dfr1* mutant TH5 *(MATa leu2-3,112 trp1 ura3-52 dfr1::URA3 tup1*), were generously provided by Dr. Tun Huang (Huang, 1992). An inclusive list of strains used in the study is given in Table 2-1. Yeast were cultured for all experiments at 30^0 C on minimal, dropout, and rich (YEPD), and YM-1 media using standard yeast genetics techniques (Sherman, 1979). Growth of the *dfr1* mutant was supported by supplementation of the medium with 100 μg/ml dTMP (Sigma, St. Louis, MO).

Methotrexate and pyrimethamine sensitivity experiments:

Methotrexate was purchased from Sigma (St. Louis, Missouri) and dissolved at 10 mg/ml in 0.1M NaOH. Pyrimethamine was supplied by Dr. Wilbour Milhous (Therapeutics Division, Walter Reed Army Institute of Research) and made up at 1 mg/ml in absolute ethanol. Stock solutions were stored at -70°C and -20°C for methotrexate and pyrimethamine respectively. dTMP from Sigma was made up at 10 mg/ml in sterile water and stored at -20° C. All drug dilutions were made serially into YM-1 media and used immediately.

Liquid culture drug sensitivity experiments were carried out in the following manner. Overnight stock cultures were made for each test strain by inoculating a single colony into 10 ml YM-1 media and growing at 30°C to saturation ($\approx 10^8$ cells/ml). A 1:50

dilution was made for each stock into YM-1 and then aliquoted into 5 ml cultures. Methotrexate sensitivity experiments were carried out in YM-1 media supplemented with 5 mg/ml sulfanilamide (Sigma). Once the drugs and dTMP had been added at the appropriate concentrations, the cultures were incubated overnight (≈18-20 hours) on a roller (New Brunswick Scientific, New Brunswick, NJ), after which growth was determined by measuring the absorbance at 660 nm on a spectrophotometer (Gilford Instruments, Stasar II). All growth was expressed as a percentage of the growth of the control sample without drug.

Construction of plasmids:

Genomic DNA for use in polymerase chain reaction (PCR) was isolated from *Plasmodium* strains S/L D6 and Mikenga using the saponin lysis-chelex method (Wooden, 1992), and from wild-type yeast (TH1) using the glass bead method (Hoffman, 1987). Primers that incorporated useful enzyme restriction sites were designed for each DNA to be amplified based on published sequence data for the *P. falciparum* (Bzik, 1987) and *S. cerevisiae* DHFR (Lagosky, 1987) genes. PCR protocols were all adaptations of standard procedures described in Higuchi (Higuchi, 1989) and Saiki (Saiki, 1985). The reactions contained a total volume of 100 µl, with 0.2 mM dNTPs and 1.25 U of Replitherm (Epicentre, Madison, WI). The reactions were amplified in a PTC-100 thermocycler (MJ Research, Watertown, MA) with the following parameters unless noted otherwise: initial denaturation for 4 minutes at 94^{0}C and 29 cycles with denaturation at 94^{0}C for 1 minutes, annealing at 55^{0}C for 1 minute, and extension at 72^{0}C for 2 minutes The entire 1.6 kb yeast DHFR gene was amplified as a KpnI/SacI product using the upstream primer 5'-GAGGTACCCTATAGGAATCGTCACTC-3' and downstream primer 5'-TTAGAGCTCAACTGGATCCGGACAGCA-3', the yeast DHFR gene promoter as a 500 bp KpnI/BamHI product using the upstream primer 5'-

GAGGTACCCTATAGGAATCGTCACTC-3' and the downstream primer 5'-AGCGGATCCGCTCGTAGTTCGTTGCGCTCTTCA-3', the yeast DHFR gene coding region as a 600 bp BamHI/EagI product using the upstream primer 5'-CGAGGATCCATGGCTGGAGGAAAGATTCCT-3' and the downstream primer 5'-GCGCGGCCGAATAACCTTTTTCTTCCAGCGAGTA-3', and the yeast DHFR terminator/downstream flank as a 400 bp EagI/SacI product using the upstream primer 5'-TACCGGCCGGCTTCGAATTCACTCTAT-3' and the downstream primer 5'-TTAGAGCTCAACTGGATCCGGACAGCA-3'. The *Plasmodium* DHFR gene coding region was amplified (48^{0}C annealing temperature) on a Biosycler (Bios Corporation) as a 800 bp BamHI/EagI product using the upstream primer 5'-CGGGATCCTATGATGGAACAAGTCTGCG-3' and the downstream primer 5'-GCGCGGCCGTCATATGACATGTATCTT-3'.

Each PCR product was purified on a Wizard PCR Prep DNA Column (Promega, Madison, WI) and digested with the appropriate restriction enzymes. Plasmid preparations, enzyme reactions, and ligation reactions were performed according to standard methods (Sambrook, 1989), except for EagI digests which were done at 30^{0}C overnight. Restriction enzymes, phosphatase, and T4 DNA ligase were obtained from Boehringer Mannheim (Indianapolis, IN) and New England Biolabs (Beverley, MA) and used according to manufacturer's instructions. Vector and insert DNA digests were isolated on a Wizard DNA Column (Promega), ligated, and transformed into *E. coli* strain DH5α. Taking advantage of the PCR engineered restriction sites, the various components of each DHFR construct were assembled into the yeast shuttle vector pRS31 (Sikorski, 1989) in a stepwise fashion to create the constructs shown in Figure 4-8. This vector contains selectable markers for bacterial growth in ampicillin-containing medium and yeast growth in tryptophan-deficient medium. In addition, it contains yeast sequences that allow autonomous replication of the plasmid in yeast and centromere sequences that assure that

the yeast cell maintains the plasmid at about one copy/cell. To confirm that no mutations were created during the PCR, DNA fragments of interest were cloned into pKS+- BlueScript (Stratagene, La Jolla, CA) and subjected to cycle sequencing according to manufacturer's instructions (Perkin-Elmer/ABI, Foster City, CA). Plasmids used in the study, including those that were constructed, are listed in Table 2-2.

Complementation tests for DHFR enzyme function:

The constructs were transformed into the yeast *dfr1* mutant (TH5) using the lithium acetate method (Itoh, 1983). The resulting strains are listed in Table 2-1. Transformation mixes were plated on tryptophan-deficient synthetic medium supplemented with 100 μg/ml dTMP to select for plasmid-containing cells. After 3-5 days of growth, transformants that grew without tryptophan were replica plated to YEPD without dTMP to test for DHFR enzyme function. To test for auxotrophy for the other metabolites that require activity of the DHFR enzyme (adenine, histidine, and methionine), transformants for each construct were transferred to nonselective medium (YEPD + dTMP), allowed to grow for 2 days, and then replica plated to synthetic medium missing each of the metabolites and to complete synthetic medium as a control. Plates were scored for growth after 3-5 days. To insure that the observed phenotype was due to the construct used for the transformation and not a change in the biology of the yeast host, plasmid DNA was isolated from each transformant and transferred into *E. coli* (Hoffman, 1987; Sambrook, 1989). The plasmid DNA was analyzed by restriction digests and then used to transform a new yeast DHFR$^-$ mutant host.

Drug sensitivity assays:

Cycloguanil and the experimental antifolate WR99210 were generously supplied by Dr. David Jacobus (Jacobus Pharmaceuticals, Princeton, NJ), and chloroquine, halofantrine, pyrimethamine and WR99210 by Dr. Wilbur Milhous. Stock solutions for pyrimethamine, cycloguanil and WR99210 were prepared by dissolving each drug at 1 mg/ml in absolute ethanol overnight. Stock solutions were aliquoted and stored at -70°C. Chloroquine was dissolved at 3.13×10^{-3} M in absolute ethanol and halofantrine at 1×10^{-3} M in 70% ethanol; both were stored at -20° C. All dilutions of drugs were made serially into medium lacking nitrogen (1.61 g yeast nitrogen base without amino acids or ammonium sulfate, 11.1 g succinic acid and 6.7 g sodium hydroxide per liter; the level of folic acid is 0.0018 mg/L and p-amino benzoic acid is 0.19 mg/l). The dilutions were stored at 4°C protected from light for up to 5 days. For drug sensitivity experiments on solid medium, drugs were spread on the surface of the plate at the appropriate concentrations and time was allowed for absorption into the medium. Mixing the drug into the medium (after it cooled to 55-60°C) before pouring the plates worked equally well. Drug sensitivity tests were made using a "double replica plating" procedure because this improved the discrimination of growth. First, yeast strains of interest were transferred to nonselective medium (YEPD), allowed to grow for 2 days, and replica plated to another YEPD plate. This plate was then immediately replica plated to a test plate containing drug and a control plate without drug. After 5 days of growth, each strain was scored for sensitivity by comparison with growth on the control plate without drug.

The quantitative drug sensitivity assays were carried out in liquid culture in the following manner. Each yeast strain was grown up to midlog phase (5×10^6 to 2×10^7 cells/ml) in YM-1 medium to insure that the test population would be actively growing at the start of the experiment. The midlog phase culture was diluted to 10^4 cells/ml in medium and aliquoted into 5 ml cultures. Drug was added to cultures at various concentrations but at constant volume to create a drug concentration series. Each

concentration point was done in duplicate. The cultures were incubated on a roller (New Brunswick Scientific, New Brunswick, NJ) for 24 hours, after which growth was determined by measuring the absorbance at 660 nm on a spectrophotometer (Gilford Instrument Stasar II). A growth period of 24 hours insured that all samples would still be actively growing in log phase at the end of the experiment. The drug sensitivity curve was generated by expressing the growth at each concentration point as a percentage of the growth of the control sample without drug.

THF and dTMP rescue experiments:

Rescue experiments were performed using the double replica plating procedure (see "Drug sensitivity assays" section). Pyrimethamine was added to complete synthetic media ("C") and synthetic media missing histidine ("C-His") at a concentration of 5×10^{-7} M. THF (Sigma) was made up at a stock concentration of 50 mg/ml in sterile water and stored at -70^{0}C. Plates were supplemented with THF and dTMP at 500 μg/ml and 100 μg/ml respectively. Yeast strains were patched out and allowed to grow up on YEPD then double replica plated to the test plate and control plate (C media). Plates were scored for growth after 5 days of incubation at 30^{0}C.

III. Selection of Drug Resistance to Malarial DHFR Inhibitors in S. cerevisae

Mutant selection:

Selections were performed as illustrated in Figure 5-1 using yeast containing a drug sensitive *P. falciparum* DHFR (pTRP-D6). 100 independent 2 ml YEPD liquid cultures were inoculated from 100 independent colonies of pTRP-D6 and allowed to grow

at 30°C to saturation ($\approx 10^8$ cells/ml). A random sampling of 10 cultures indicated that the average cell density was 1.08×10^8 cells/ml $\pm$ 0.233. 10^6 cells of each culture was plated to one YEPD plate containing 5×10^{-7} M pyrimethamine and another plate containing 5×10^{-7} M WR99210. After 5 days of incubation, the plates were scored for the number of colonies and for the size of each colony.

Characterization of drug resistance phenotype:

Drug resistant candidates were patched out onto nonselective media (YEPD) and allowed to grow for 2 days at 30°C. Strain markers were confirmed by replica plating to media lacking adenine, histidine, leucine, trptophane, and uracil. Plates were scored for growth after incubation at 30°C for 2-3 days.

To retest for the drug resistant phenotype, candidates were double replica plated from nonselective medium to medium containing the drug used for selection at the original concentration of 10X IC_{50} (5×10^{-7} M) and also at 100X IC_{50} (5×10^{-6} M). Tests were performed for cross-resistance by double replica plating to either 10X WR99210 or 10X pyrimethamine for pyr^R and WR^R candidates respectively. Candidates were also tested for growth on 10X cycloguanil (5×10^{-6} M), 100X WR99210, and 100X pyr. Tests for PDR (pleiotrophic drug resistance) were made by double replica plating to media containing 10 μg/ml cyclohexamide. Controls for cyclohexamide sensitivity included the cyclohexamide sensitive strain 5899-8a and the cyclohexamide resistant strain 5899-19b (Bryan Jensen, University of Washington). Tests for reversion at the *tup1* locus were made using media containing the drug used for the original selection at 100X with 100 μg/ml dTMP. All plates were scored for growth after 5 days of incubation.

Plasmid missegregation tests:

A single colony of each test strain that had grown up under nonselective conditions (no drug) was inoculated into a 10 ml YEPD liquid culture and grown at 30°C to saturation (≈ 10^8 cells/ml). 10 µl of each saturated culture was inoculated into 10 ml of fresh YEPD media and allowed to grow again to saturation. The final cultures were diluted down to ~ 100 cells/ml in YEP (yeast extract/peptone) medium (Sherman, 1979). Approximately 150 µl of each culture dilution (100-200 cells) was plated onto both a drug plate (YEPD + $5x10^{-7}$ M pyrimethamine or $5x10^{-7}$ M WR99210) and control plate (no drug). Plates were scored for the number of colonies after 5 days of incubation. The number of colonies of each test strain on the drug plate was expressed as a percentage of the number of colonies that were seen on the control plate. The control strain "D6-2µM" which contained the *P. falciparum* D6-S/L DHFR construct (same construct as contained in the plasmid pTRP-D6) on the multicopy vector pRS424 (Christianson, 1992) was constructed by Kelly Hamilton (University of Washington).

Plasmid Linkage:

To test for plasmid linkage of drug resistant phenotypes, plasmid DNA was isolated from each candidate using a modification of the method of Hoffman et al (Hoffman, 1987). Drug resistant candidates were patched out onto nonselective media (YEPD) and allowed to grow for 2 days at 30°C. Using a fresh sterile toothpick, a generous portion of cells from each patch were transferred into a 1.5 microfuge tube containing 0.2 ml lysis buffer (1% SDS, 2% Triton X-100, 0.1M NaCl, 10 mM Tris-HCL, pH 8.0, and 1 mM EDTA) with ≈ 0.3g acid washed glass beads (Sigma G9268, 425-600 µM). A volume of 0.2 ml of 1:1 phenol:chloroform was added followed by 2 minutes vortexing at the full speed. The samples were centrifuged at 12,000 x g for 5 minutes and

the supernatant transferred to a fresh tube. The extraction was repeated by adding 0.2 ml fresh lysis buffer to the beads and vortexing again. DNA was precipitated from the combined supernatants by adding 2.5 volumes of absolute ethanol at room temp and centrifuging 14,000 rpm in a microfuge for 5 minutes at room temperature. Pellets were washed with 1 ml 80% ethanol, allowed to dry, and resuspended into 50 µl 1X TE (Tris-EDTA). Plasmids were transformed into the *E. coli* strain DH5α (Hoffman and Winston, 1987) and isolated from bacterial transformants using a modification of the alkaline boiling lysis miniprep (Sambrook, 1989). The plasmid DNA was analyzed by restriction digestion and then used to transform a new yeast DHFR$^-$ mutant host (TH5; dfr1::URA3). The transformants were retested for the original drug resistance phenotype of each plasmid by double replica plating to plates with the appropriate drugs as described before. Plates were scored for growth after 5 days incubation.

Cycle sequencing of DHFR gene:

Plasmid DNA suitable cycle sequencing was isolated from transformants of DH5α using the Qiagen Plasmid Mini Kit (Qiagen, Inc.). The PCR sequencing reactions were carried out using the ABI Prism Dye Terminator Ready Reaction Kit (Perkin-Elmer) according to the manufacturer's instructions. The upstream primer 5'-TGAAGAGCGCAACGAACTACGAGC-3' and the downstream primer 5'-GCGCGGCCGTCATATGACATGTATCTT-3' were used to generate single stranded PCR products. The sequencing products were analyzed using an ABI automated sequencer (ABI, Foster City, CA) and the programs Phred, Phrap, and Consed from Phil Green (Department of Molecular Biotechnology, University of Washington).

Table 2-1. Yeast strains used in study.

Strain	Source	Genotype
TH1	Tun Huang	*MAT**a** ura3-52 leu2-3,112 trp1 tup1*
TH5	Tun Huang	*MAT**a** ura3-52 leu2-3,112 trp1 tup1 dfr1::URA3*
JWY1	This study	*MAT**a** ura3-52 leu2-3,112 trp1 tup1 dfr1::URA3* ***pTRP-D6***
JWY2	This study	*MAT**a** ura3-52 leu2-3,112 trp1 tup1 dfr1::URA3* ***pTRP-ScDHFR***
JWY3	This study	*MAT**a** ura3-52 leu2-3,112 trp1 tup1 dfr1::URA3* ***pTRP-Sc5D3*** (yeast DHFR "stitched")
JWY4	This study	*MAT**a** ura3-52 leu2-3,112 trp1 tup1 dfr1::URA3* ***pTRP-Hon***
JWY5	This study	*MAT**a** ura3-52 leu2-3,112 trp1 tup1 dfr1::*URA3 ***pTRP-Mik***
pTRP-2μM	This lab, Kelly Hamilton	*MAT**a** ura3-52 leu2-3,112 trp1 tup1 dfr1::URA3* ***pTRP-2μM***
5899-8a	Steve Johnson	*MAT**a** ade6 leu2 trp1 ura3* ($can^S cyh^S$)
5899-11b	Steve Johnson	*MAT**α** ade6 cyh2 his7 leu2 trp1 ura3* ($can^S cyh^R$)
5899-11d	Steve Johnson	*MAT**a** ade6 can1 his7 leu2 lys5 trp1 ura3* ($can^R cyh^S$)
5899-19b	Steve Johnson	*MAT**α** ade2 ade6 can1 cyh2 his7 leu2 lys5 trp1 ura3* ($can^R cyh^R$)

Table 2-2. Plasmids used in study.

Plasmid	Source	Description
pRS314	Sikorski and Dieter, 1987	Yeast shuttle vector with TRP maker
pJW10	This study	D6 negative control construct, inverted coding region and terminator (Pf-DHFR-D6, reverse)
pJW11	This study	pTRP-D6, complete D6 DHFR construct with 600 bp promoter (Pf-DHFR-D6)
pJW12	This study	pTRP-ScDHFR, wildtype yeast DHFR gene control construct (Sc-DHFR, unstitched)
pJW13	This study	Yeast 600 bp DHFR promoter with coding region (Sc-DHFR, no terminator)
pJW14	This study	Complete D6 DHFR construct with 500 bp yeast DHFR gene promoter
pJW15	This study	pTRP-Sc5D3, yeast DHFR promoter, coding region, and terminator (Sc-DHFR, stitched)
pJW16	This study	pTRP-Hon, Honduras1 DHFR construct (Pf-HON-DHFR)
pJW17	This study	Honduras1 negative control construct, same as pJW10 (Pf-HON-DHFR, reversed)
pJW18	This study	pTRP-D6, Mikenga DHFR construct (Pf-Mik-DHFR)
D6-2μM	Kelly Hamilton, this lab	D6-2μM, D6 DHFR construct on 2μM plasmid containing selectable Trp marker (Sikorski and Dieter, 1987)

Chapter 3: Strain Identification in *P. falciparum*

I. Introduction

Protozoan parasites have become increasingly amenable to laboratory study in recent years. As techniques have developed and more strains have become available, difficulties in defining origins and genealogies of laboratory strains and field isolates have increased. This inability to identify and monitor strains in the lab has caused several recurring problems in malarial research. First, inadvertent contamination of one strain with another has been a consistent problem for laboratory work. This was recently highlighted when mutants selected in the lab to be resistant to pyr were later revealed to be a contaminant from a pre-existing drug resistant lab strain (Tanaka, 1990). Second, these difficulties have particularly hampered genetic analysis. The inability to assess relatedness of strains has made the study of antifolate resistance in *P. falciparum* more difficult. Differences in strain backgrounds could account for either slight or major differences in sensitivity to an antifolate, for reasons not related to the genotype of the DHFR gene. An example can be seen in past studies in which pyr and cyc resistance were measured for a variety of laboratory strains. Strains having identical DHFR genotypes were found to have up to 10-fold differences in sensitivities to the two drugs (Foote, 1990; Peterson, 1990). This confusion has complicated the interpretation of drug sensitivity data in *P. falciparum*, making it difficult to derive clear genotype-phenotype relationships for resistance to DHFR inhibitors.

Before we began our genetic analysis of mutations in the *P. falciparum* DHFR gene that confer resistance to antifolates, a method was needed to distinguish field isolates and clones derived from various laboratory strains. This would facilitate comparison of data generated in the lab with data from field isolates. The established techniques for

standardizing strains use a combination of approaches and often require specific monoclonal antibodies or other reagents. For example, the *TDR News* 29, September 1989 described the characterized clones of *P. falciparum* available from the World Health Organization. These were identified by a combination of polymorphisms in electrophoresis of particular enzymes, 2D-SDS gels, antigen characterization with monoclonal antibodies, and drug sensitivity (see for example, Fenton et al). This is an extremely complex set of different assays, and a much more straightforward method was sought. The ideal method should meet several criteria: it should be rapid, easily performed on very small amounts of sample, and require simple equipment and protocols to perform.

With these criteria, it is no surprise that the chosen method was based on the polymerase chain reaction (PCR), allowing a "genetic signature" to be established for the various strains with which we routinely work. This is certainly not a new idea since PCR had been previously used to type *P. falciparum* strains for particular loci. (Foote, 1990; Wellems, 1990; Zolg, 1990). Building on this concept, the goal was simply to expand this technique into a routine screen for genotyping parasites. It was also important to adapt methods for isolation of DNA from extremely small amounts of sample, since it is not feasible to generate large amounts of parasites on a routine basis in most situations. The hope was to be able to easily purify enough DNA from 100 µl of an *in vitro* culture infected at 1% parasitemia to produce an unambiguous PCR product.

Genes whose amplified products were likely to vary in size from one strain to another were identified. Many cell surface proteins encoded by *P. falciparum* genes share a curious property: they have strongly conserved 5' and 3' ends that flank a region with blocks of repeated sequences that vary in size and DNA sequence from strain to strain (Kemp, 1987). This variation is presumably a reflection of the intense immunological selection *P. falciparum* parasites undergo in the human host, requiring them to modify

their surface proteins in order to avoid recognition by the immune system (Kemp, 1987; Anders, 1988). Published sequences were used to identify highly conserved regions so that primers could be designed which spanned the repeats in four surface antigens: the ring-infected erythrocyte surface antigen (RESA (Favalaro, 1986)), the precursor of the major merozoite surface antigen 1 (MSA-1 (Mackay, 1985)), precursor of the major merozoite surface antigen 2 (MSA-2 (Fenton, 1985)), and the circumsporozoite surface antigen (CSP (Dame, 1984)). Although not all of these genes varied among the strains that were examined, even this small set was adequate to distinguish all of our strains. It seems likely that these genes and others that are identified in studies of *P. falciparum* will allow all of the laboratories using *P. falciparum* to define the strains now in use, and perhaps to begin to determine their relationships. This standardization should be useful not only to geneticists, but to other workers as well.

II. Results

A. Isolation of the DNA

The first requirement for any technique for strain identification is that the method be rapid, easy to perform, and require a minimum of unusual equipment. The technique of isolating a small sample of DNA from blood and then analyzing it by PCR (Saiki, 1985) seemed likely to fulfill these requirements. To verify this idea, a number of procedures designed for this purpose in forensic protocols were tested (Higuchi, 1989). The PCR reactions can theoretically be realized with only one copy of the DNA sequence of interest, but we set as our target a sample of 10 ng of genomic DNA. It can be estimated that cultures are 5% by volume in red cells, that the average haploid genome of the *P. falciparum* is 3×10^7 base pairs (Weber, 1988), and that one parasite genome is 3.12×10^{-5} ng of DNA. Table 3-1 shows the volume of culture needed to isolate our target, 10

ng of DNA, when the culture is at parasitemia levels varying from 1% to 5%. The volumes required are surprisingly small, ranging from 63 µl to 13 µl. In practical terms, 200 µl was chosen as a sensible starting volume for the isolation procedure. This does, in fact, yield sufficient DNA for several PCR reactions.

Both of the procedures tested begin with 200 µl of blood from a culture parasitized with *P. falciparum* (Figure 3-1). All initial experiments were done with procedure 1, a very standard protocol for isolation of nucleic acids. A major difficulty with infected blood cultures is that heme can inhibit the DNA polymerase used in the PCR reaction (Higuchi, 1989), so it must be removed from the DNA template. Later, a much more rapid procedure based on the ion exchange resin, Chelex 100 (Walsh, 1991), was adapted (Procedure 2). This was as effective and far easier than the other protocol, and is now being used exclusively.

B. Selection of the genes and primers for PCR

In order to easily distinguish various strains of *P. falciparum*, a search was made to identify a number of genes which were likely to show size polymorphisms. The search was based on the observation that many genes which encode surface proteins in *P. falciparum* are highly homologous throughout most of their sequence, but contain regions of repeated sequence within the gene (Kemp, 1987). These repeats had been shown to be extremely variable both in sequence and in size. The variation appeared to be between strains, and not a reflection of extreme instability over short term culture.

Both the EMBL and NIH databases were searched to identify genes of this type for which several sequences were published. Primers were then designed which would be expected to recognize regions of the genes in the conserved domains flanking the repeats. None of these sequences from our standard strains were in the database, so regions were

chosen on the assumption that the conservation would extend to these genes in our strains. Four genes were selected for analysis: RESA (Favalaro, 1986), MSA-1 (Mackay, 1985), MSA-2 (Fenton, 1985), and CSP (Dame, 1984). The sequences of these primers are listed in Table 3-2. All of the primers gave consistent products in the PCR reaction for all of the strains tested. The basic conditions for the amplification reactions were optimized for each primer set, paying particular attention to the temperature of hybridization and the magnesium concentration. In fact, all of the primers can be used with one standard protocol (see Materials and Methods).

C. Specificity of the Primers

There are two elements required to determine the specificity of the primer sets. The first is the ability of the primers to amplify only the expected DNA sequence in *P. falciparum* DNA in the expected manner. One quick test is to use each primer alone in a PCR reaction, to assure that no anomalous priming is observed. Figure 3-2 shows an example of this kind of experiment using the upstream and downstream primers for RESA. At high annealing stringency (55°C), neither primer alone is sufficient to allow amplification of a product from *P. falciparum* DNA (lanes 3-4). Conversely, both primers used together yielded a single band (lane 6). As expected, the primers did not yield an amplification product in the absence of DNA (lane 2) or the presence of λ phage DNA (lane 1). At lower annealing stringency (39°C), no clear product was observed when both primers were used together.

Specificity was also assessed directly by sequencing the double-stranded products of some of the PCR reactions. This analysis focused on the products derived from the strain 3D7. In all cases the DNA sequence obtained was clearly from the gene of interest. Minor differences from the published sequences were observed in the repeat regions, and in some cases in the flanking regions as well.

Another requirement for primer specificity is the ability to discriminate between DNA templates from *Plasmodium* and unrelated organisms. The primers were tested on DNA derived from a variety of species: *Mus musculus*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, *Escherichia coli*, and λ phage. None of these DNA samples yielded a clear amplified product at either high or low stringency (data not shown). Since the *Plasmodium* DNA is derived from either a human host or an infected culture of human blood cells and serum factors, it was also important that no anomalous priming occured with human DNA sequences. Figure 3-3 shows that no product was observed when any of the primers were tested on human DNA. However, unambiguous PCR products were obtained when the primers were used with DNA from *P. falciparum* strain 3D7.

D. Different strains show size polymorphisms

All of the primer sets were tested on DNA derived from the strains listed in Table 3-3. Although there are sequence differences, RESA did not show a size polymorphism with these strains (Figure 3-4A). However, amplification of DNA from various strains using primers for MSA-1, MSA-2, and CSP yielded fragments whose sizes were easily distinguished on 2% agarose gels (Figure 3-4, B and D). The amplified fragment from the CSP gene ranged from ≈ 700-900 bp, the amplified fragment for the MSA-1 gene from ≈ 200-400 bp, and the fragment from the MSA-2 gene ranged from ≈ 400-600 bp.

While the PCR products amplified from the RESA gene were apparently identical in size for all the strains, the products from the CSP and MSA genes were extremely variable. Figure 3-5 shows the primers can be multiplexed in single PCR reaction to give distinct patterns representing a composite of the individual polymorphisms for each band. The strains are all distinguishable and the patterns provides a "genetic fingerprint" for each strain. The fact that amplification of the RESA produces fragments of the same length from all of these strains provides a convenient control to ensure that the lanes are all

running in an equivalent manner. While minor bands can be seen for some of the strains (K1, HON, and PLF), the patterns are reproducibly generated for each strain. Distinguishable patterns were also obtained for many other strains sampled (Table 3-3). Since these strains originate from very diverse geographical locations, this suggests that the PCR approach may be generally useful for identifying a wide variety of strains.

E. The size polymorphisms are stable

In order to be useful as markers of individual strains, these size polymorphisms must be stable under the standard conditions of laboratory culture. To test this, the amplified fragments generated from DNA isolated from both C10 and a subline derived from C10, called D2 (personal communication, J. Feagin), were compared. These two had been grown for at least 12 months in the laboratory before testing. Figure 3-6 summarizes the results of this experiment. As expected, all primers amplified fragments of identical size from both sources of DNA. In addition, tests were made of DNA isolated from strain 3D7 derived from two sources. One was prepared by us several months after receiving the strain from Dr. Thomas Wellems and a second from 3D7 cultured by Dr. Jean Feagin who received the strain from Dr. Barbara Sina (Biomedical Research Institute, Rockville, MD). Both strains had been separated during long period of culture, and gave identical profiles (data not shown). Thus, it seems that these size differences are sufficiently stable under laboratory conditions to make useful markers of particular strains.

F. Minor contamination between strains can be detected

Situations often arise which require a sensitive way of detecting heterogeneity in cultures of *P. falciparum*. When strains are isolated from infected humans, and then grown in vitro, it is useful to detect heterogeneity from the mixed infections commonly observed (Willet, 1991). Second, when several strains are grown simultaneously in the

laboratory, it is important to be able to distinguish the different strains so that any inadvertent contamination or substitution of one strain with another can be easily detected. To determine the sensitivity of the PCR polymorphisms, we made a series of mixtures of cultures of 3D7 and D2 in various ratios from 1:1 to $1:10^4$, prepared the DNA, and subjected each sample to amplification with the primers specific for CSP. Both the larger D2 and the smaller 3D7 product were detected (Figure 3-7, lanes 1-3). This demonstrates that a 10% contamination with either DNA is easily detected. Lane 4 shows the PCR amplified from a culture that contained only 1% D2, and even there, a clear product was observed on the original gels. This indicates that even fairly minor levels of contamination or mixed infection can be detected with this method.

III. Discussion

We have outlined a simple method for identifying size polymorphisms in the PCR products from 3 genes in *P. falciparum*. The approach is not a new one; PCR polymorphisms have been used in many studies to identify particular genes (Foote, 1990; Wellems, 1990; Zolg, 1990). However, we have used several different sets of primers, and it has allowed us to define a "genetic signature" for four different strains. Furthermore, a survey of five other strains suggests that this may provide an extremely easy way to characterize strains in the laboratory. The choice of primers was based on published sequences; we had no information about the exact sequence of those genes in our standard lab strains with which we began. Despite this uncertainty, all of the primers worked at high annealing stringency. However, amplification of the RESA gene from various strains did not show any polymorphism. This could be a result of the region against which the primers were made or reflect the lack of immunological selection for variation in this surface protein, presumably because the RESA antigen is not exposed as much to the human immune system as the surface antigens.

The same primer pairs were not equally efficient on DNA derived from all of the strains. For example, in figure 3-7 the amplification products are shown from various mixtures of DNA using the CSP primers. The smaller 3D7 product predominated even when the initial mix contained a 10-fold excess of D2 DNA. When the reactions are carried to 30 cycles, the ratio of the final products is not a measure of the starting amounts of DNA, so it is clear that no quantitative estimate of relative amounts of the starting DNA can be made with this protocol. However, this does increase the sensitivity of the method in detecting minor contaminants.

In choosing the primers, we aimed for sequences of about 20 bp, with as close to 50% G/C as possible. All of the primers are similar enough that identical conditions can be used for the reaction mix and amplification protocol, a real convenience when one has many reactions to perform! This also affords the possibility that three or more primers can be used in one reaction tube (multiplexed), greatly reducing the expense and manipulation for one analysis. This depends, of course, on the amplified products of the three primer sets being of three different sizes, but that condition is frequently met. We have tried this on a limited scale, and it seems to present no problems. The genes selected for screening were chosen only because their sequences were readily available. There may be others which would be as useful, or perhaps even more informative. These could be added to the existing collection of DNA polymorphic markers which will hopefully result in an increased ability to generate unique "genetic footprints" so that both field isolates and laboratory strains can be clearly discriminated.

The difficulty of identifying the various *P. falciparum* strains commonly used in laboratories around the world has often been an impediment to understanding their origin and interrelationships. It seems likely that with cooperation among the various laboratories using *P. falciparum* strains it would be fairly easy to define a set of primers

which could uniquely identify each of the commonly used strains. The technical ease and speed of this assay and the small amount of culture required make it ideal for monitoring of strains in the laboratory. Even clones available in very small volumes can be assayed, and the answer is available within a day. In addition, it allows careful monitoring of the integrity of populations. This is a particular concern when several strains are cultured simultaneously in the same laboratory. Even the most careful culturing techniques can sometimes prove inadequate. For example, in situations where selection for a rare phenotype like drug resistance is the goal, it is clear that contamination can be a particular problem (Tanaka, 1990). This method is sufficiently sensitive to detect even a 1% contamination, and thus is a useful check under these circumstances.

All of our work has been performed on laboratory populations. However, it seems likely that the method might be adapted fairly easily to samples collected in the field. A drop of blood dried onto filter paper is stable for long periods, and can be processed in the laboratory with a modification of the Chelex-100 preparation described here. A recent publication provides a basis for such a method (Warhust, 1991). It would be particularly useful where the temporal or spatial heterogeneity of *P. falciparum* is to be studied, or where the possibility of mixed infections in one human is important to ascertain. Using the filter paper technique, allele-specific PCR analysis of the DHFR genes of a collection of Kenyan field isolates revealed that 60% of the samples contained mixed infections (Alexis N'zlila, unpublished results). Currently, the PCR strain identification technique is being used successfully to survey field populations (Basco, 1995). Whether this approach continues to prove useful in the field or not, it clearly can solve some problems faced by workers interested in the genetics of *P. falciparum*. Being able to assess relatedness of drug resistance isolates will certainly allow a clearer understanding of the importance of various DHFR mutations to antifolate resistance.

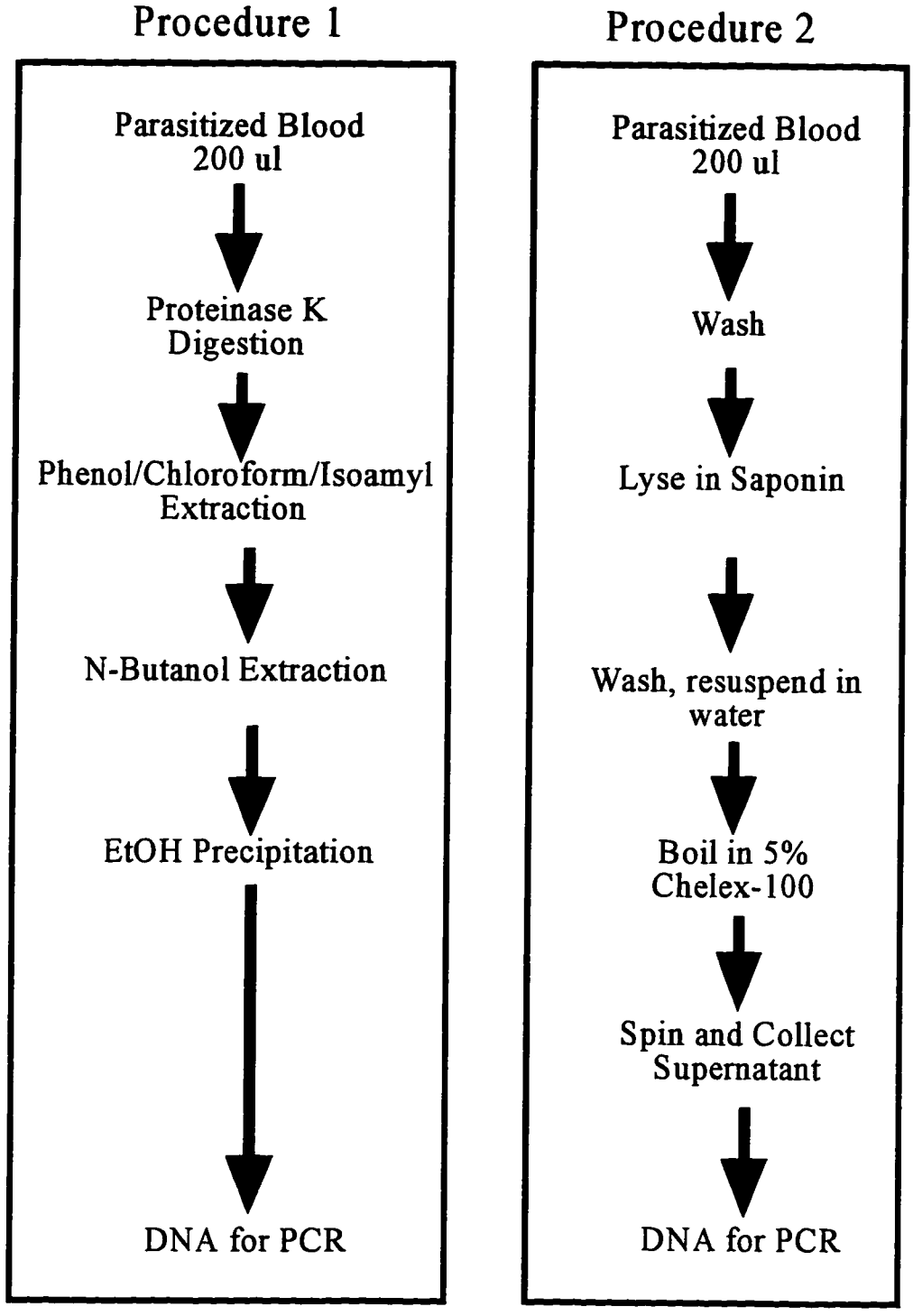


Figure 3-1. Protocols for DNA isolation. For complete description see Materials and Methods.

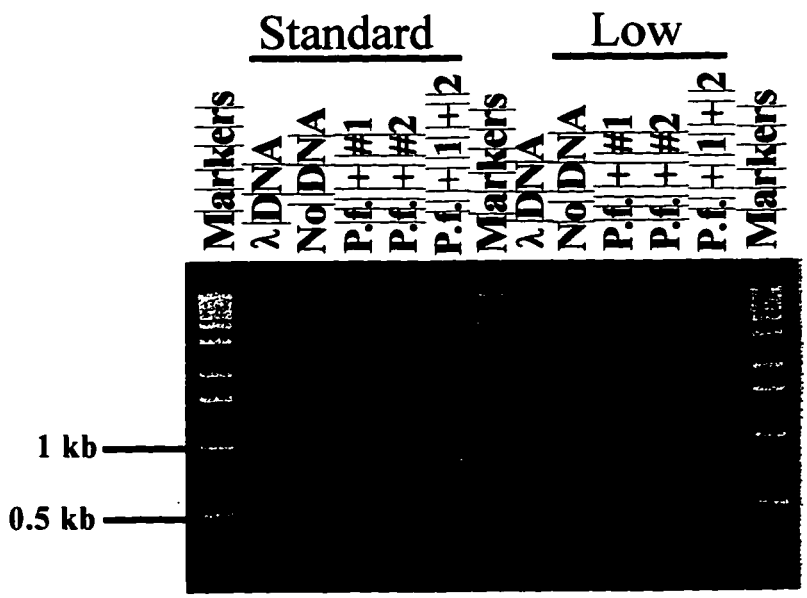


Figure 3-2. Primer dependence and specificity for PCR amplification of *P. falciparum* target DNA at standard (55°C) and low (39°C) annealing temperatures. Amplifications were performed using 5'-upstream (#1) and 3'-downstream (#2) primers for the RESA gene as described in Materials and Methods. The markers are the 1 kb ladder (BRL).

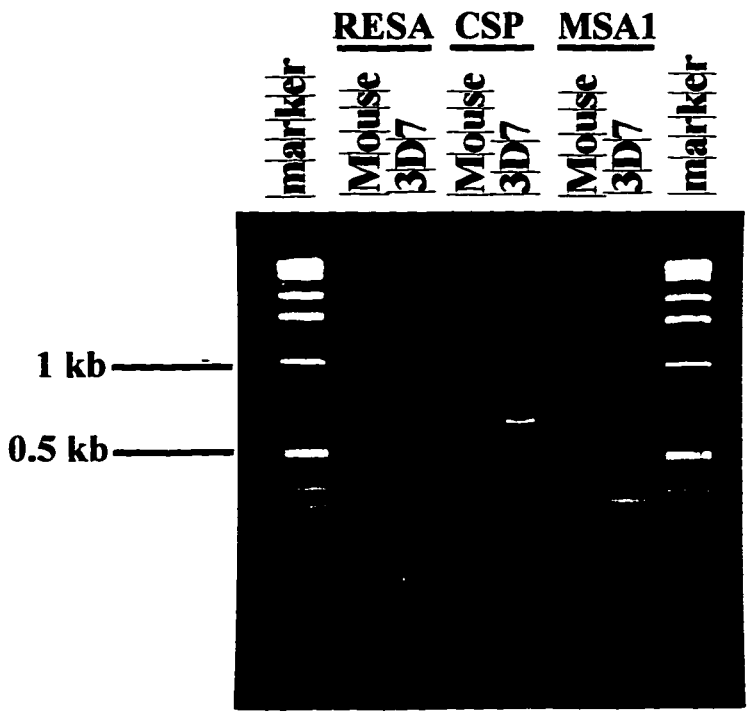


Figure 3-3. Specificity of primers for *P. falciparum* DNA. PCR amplification of mammalian (mouse) and 3D7 genomic DNA was as described in Materials and Methods. The markers are the 1- kb ladder (BRL).

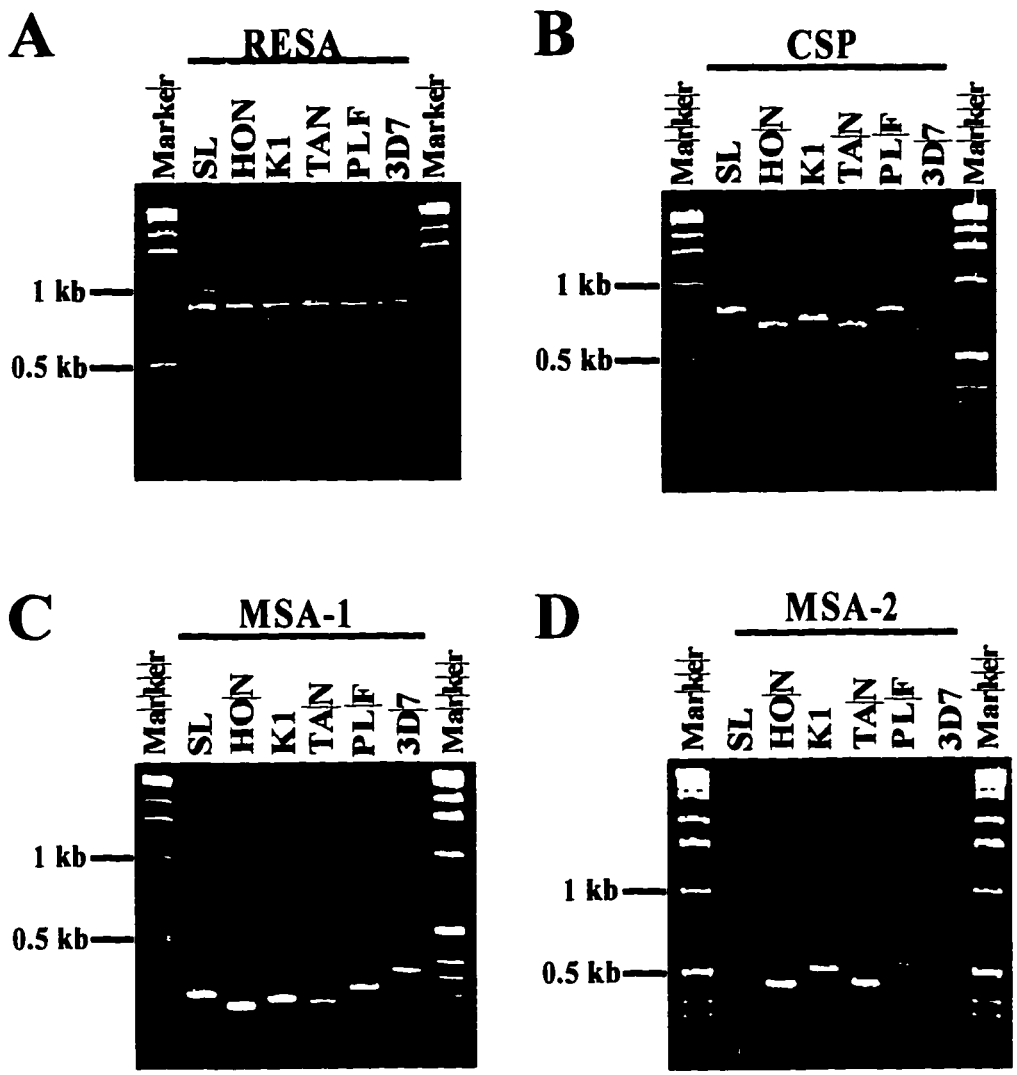


Figure 3-4. The PCR products from three genes vary in size. The PCR reactions were carried out under standard conditions using primer pairs to amplify 50 ng of DNA isolated from the Sierra Leone (SL), Honduras 1 (HON), K1, Tanzania 1 (TAN), PLF 3B11 (PLF), and 3D7 lines as template in each reaction. PCR products were analyzed on 2% agarose gels as described in Materials and Methods. The markers are the 1-kb ladder (BRL). (A) RESA; (B) CSP; (C) MSA-1; (D) MSA-2.

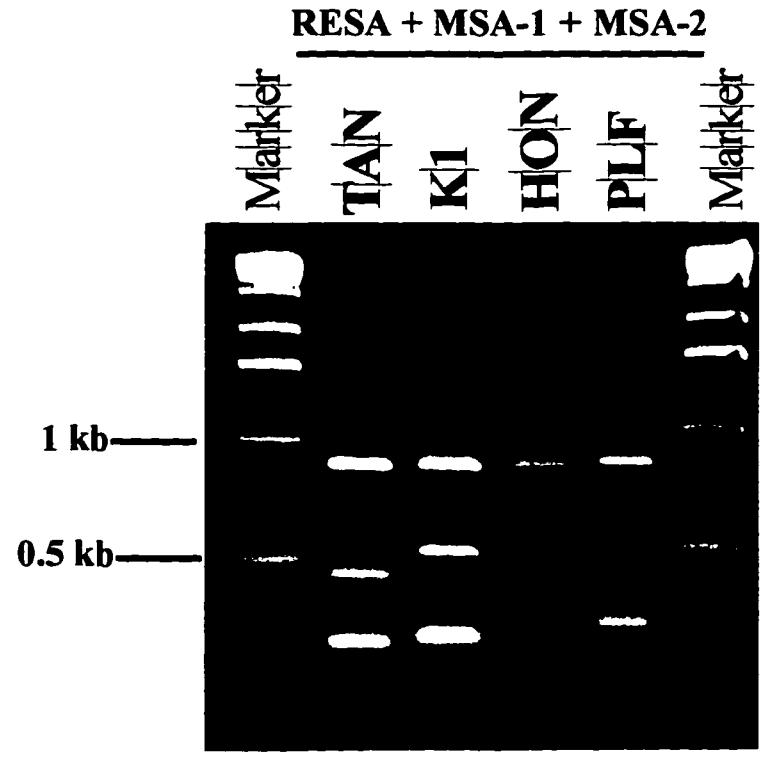


Figure 3-5 . The PCR reactions can be multiplexed. The PCR reactions were carried out under standard conditions using DNA isolated from Tanzania (TAN), K1, Honduras (HON), or PLF 3B11 (PLF) and primer pairs to amplify RESA, MSA-1, and MSA-2 in the same tube. All PCR products were anlyzed on 2% agarose gels as described in Materials and Methods. The markers are the 1-kb ladder (BRL).

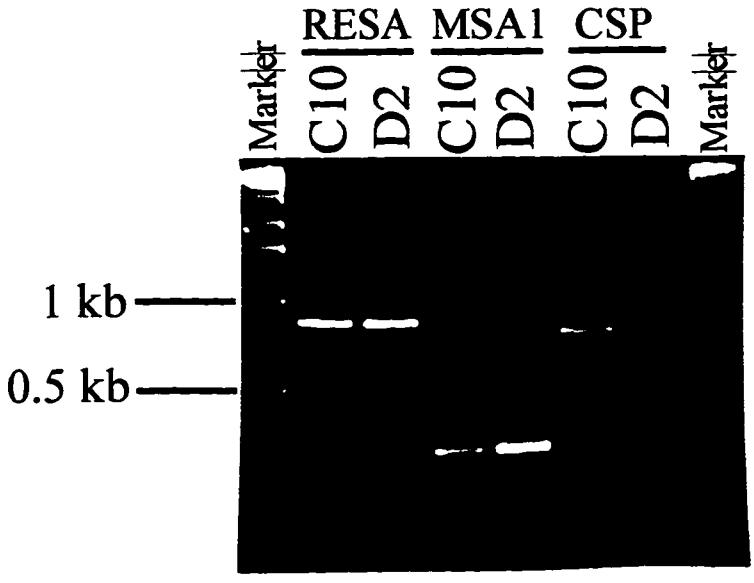


Figure 3-6. Stability of size polymorphisms between *P. falciparum* strain C10 and subline D2. The subline had been grown separately for at least 12 months. RESA, MSA-1, and CSP genes were amplified under standard conditions. The markers are the 1-kb ladder (BRL).

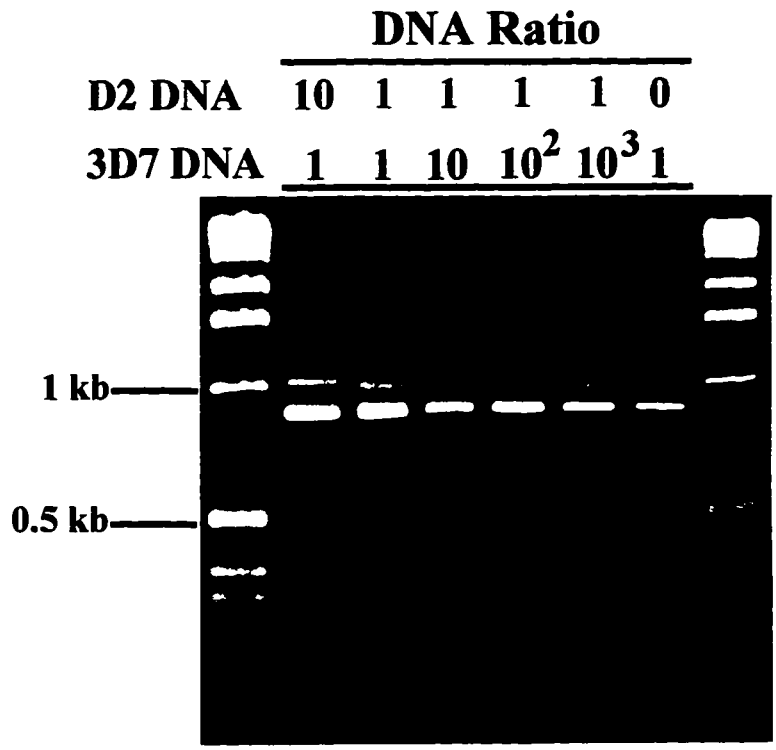


Figure 3-7. A minor contribution from a contaminating strain can be recognized. Separate cultures of the two strains, D2 and 3D7, were mixed in the ratios indicated, the DNA was prepared by the Chelex-100 method, and 50 ng of the resulting mixed prepartations of DNA was used as template for the CSP primers. All PCR products were anlyzed on 2% agarose gels as described in Materials and Methods. The markers are the 1-kb ladder (BRL).

Table 3-1. Volume of infected blood culture needed for PCR analysis.

Parasitemia	No. of haploid genomes	Vol. for 10 ng
1%	5×10^6	63 µl
2%	1×10^7	32 µl
5%	2.5×10^7	13 µl

Based on cultures containing 5% red blood cells, an average haploid genome size of 3×10^7 bp, and 1 parasite genome = 3.162×10^{-5} ng.

Table 3-2. Primers used for PCR of *P. falciparum* genes encoding surface proteins.

RESA	
5' primer	5'- GAT CAA GGA GGA GAG AAC C-3'
3' primer	5'- CAG CAT TAA CAC CAA CAC C -3'
MSA-1	
5' primer	5'- GAA GAT GCA GTA TTG ACA GG -3'
3' primer	5'- GAG TTC TTT AAT AGT GAA CAA G -3'
MSA-2	
5' primer	5'- GAG TAT AAG GAG AAG TAT GG -3'
3' primer	5'- CCT GTA CCT TTA TTC TCT GG -3'
CSP	
5' primer	5'- ATA GTA GAT CAC TTG GAG A -3'
3' primer	5'- GCA TAT TGT GAC CTT GTC CA -3'

Table 3-3. Origin of *P. falciparum* strains used in analysis.

Strain	Origin	Source
FCR-8	West Africa (?)	ATCC, Originally from Trager
C10	West Africa	J. Feagin, SBRI
D2	West Africa	J. Feagin, SBRI, clone of C10
3D7	Netherlands	T. Wellems, NIH, clone
Sierra Leone/D6	Sierra Leone	R. Howard, SBRI
Tanzania 1	Tanzania	R. Howard, SBRI
Honduras 1	Honduras	R. Howard, SBRI
K1	Thailand	R. Howard, SBRI
PLF3B11	West Africa	J. Feagin, SBRI

Chapter 4: A Yeast System to Study Resistance to Inhibitors of the *P. falciparum* Dihydrofolate Reductase

I. Introduction

Drug resistance is a complex phenomenon consisting of many components. A biological system to study the development of resistance to inhibitors of the *P. falciparum* dihydrofolate reductase should allow for the analysis of several important aspects: drug-enzyme interactions, structure-function relationships, and the genetic selection of drug resistant variants. The ideal system should permit the assessment of both mutational mechanisms and the resulting frequencies of such mutational events. A principal goal is the ability to screen new antimalarial DHFR inhibitors or combinations of inhibitors to determine the frequency with which colonies resistant to the drug arise. Since spontaneous mutation frequencies can range from 10^{-6} to 10^{-10} (Drake, 1991; Lee, 1988; Giroux, 1988; Koeberl, 1990), it is essential to be able to screen through and efficiently characterize a large population of cells in order to isolate the rare drug resistant mutant.

In the past, studies of resistance to malarial antifolates have been performed in cell culture of *P. falciparum*. Several groups have selected from drug sensitive populations for parasites resistant to the antifolate pyrimethamine (Banyal, 1986; Tanaka, 1990; Thaithong, 1992). However, this work has highlighted several important disadvantages for this approach, arising mainly from the labor intensive and lengthy process of working in cell culture. In order to recover pyr^R mutants, repeated rounds of drug selection followed by subsequent relaxation were required. It was necessary to employ lengthy limiting dilution experiments or micromanipulation to isolate clonal populations of the pyr^R mutants. Additionally, due to the technical limitations of generating large populations of *P. falciparum* parasites, it was necessary to chemically mutagenize

cultures in order to increase the chance of obtaining the rare drug resistant variant. This precludes the selection of antifolate resistant mutants under conditions which involve mutation mechanisms comparable with those seen in the field where parasites undergo spontaneous mutation processes. Because of these many disadvantages, an alternative was sought that would allow for the screening of very large populations and easier manipulation and characterization of antifolate resistant variants

Attempts made to use the bacterial *E. coli* as an heterologous expression system to study the malarial DHFR have been only partially successful. The expressed bifunctional *P. falciparum* DHFR-TS protein was found to be toxic to *E. coli* and the heterologous bifunctional DHFR-TS gene is often not stably maintained by the bacterial transformant (Hall, 1991). This problem is thought to stem principally from the differences in codon bias between *Plasmodium* and *E. coli* and the biological consequences of having excessive DHFR enzyme function (Sirawaraporn, 1993). Recently, the *P.f.* DHFR domain was successfully over-expressed in *E. coli*, but only through the use of a synthetic gene in which the codons were redesigned to more closely resemble the codon bias of *E. coli*. Even under these conditions, the enzyme produced was isolated in inclusion bodies as an insoluble product.

The budding yeast *Saccharomyces cerevisiae* offers clear advantages as a heterologous expression system for the *P. falciparum* DHFR. Yeast and *Plasmodium* have similar codon biases (both AT-rich) and many features are conserved between the two DHFRs (Table 4-1). The DHFRs share 41% homology at the DNA level and 25% at the protein level, while there is 50% protein homology between the *P. falciparum* and *P. chaubaudi* DHFRs (Figure 4-1A). Alignment of the *Plasmodium* and yeast DHFR proteins (36% similarity) suggests strong functional conservation between the two enzymes. Three out of five of the amino acids implicated as important for the development of pyrimethamine and cycloguanil resistance are conserved in the yeast

DHFR: Ala^{16}, Ser^{108}, and Ile^{164} (Figure 4-1B). These amino acids are thought to surround the active site cavity of the enzyme (Hyde, 1990). Numerous *Plasmodium* genes have been successfully expressed in yeast (Bathurst, 1993). Recently, the *P.f. mdr1* gene was used to complement the yeast *ste6* defect (D. Wirth, unpublished results). An additional advantage is that a yeast $DHFR^-$ mutant (*dfr1*::URA3) has been engineered, and this prevents complications that might arise if one had both the yeast endogenous DHFR and the P*lasmodium* DHFR expressed in the cells. Finally, yeast has been successfully used as a heterologous system to study resistance of human proteins to other therapeutic drugs of importance: topoisomerase II inhibitors (Nitiss, 1988), phosphodiesterase inhibitors (Pillai, 1993), and the antifungal/immunosuppressive agent FK520 (Raymond, 1994).

The practical limitations *P. falciparum* parasites and *E. coli* prevent the determination of the frequency with which the resistance to DHFR inhibitors develops. To circumvent these limitations, the strategy pursued was to study the *Plasmodium* DHFR in yeast by replacing the yeast DHFR gene with the gene from *P. falciparum.* There were three issues to be decided before we created this heterologous expression system. The first issue involved the design of the expression construct. The DHFR enzyme activity in *P. falciparum* is one domain of a bifunctional enzyme that includes thymidylate synthetase (TS), an enzyme that catalyzes a subsequent step in the folate pathway (Hyde, 1990, see Figure 4-2A). In most organisms, including *S. cerevisiae*, the two enzymes are encoded by unlinked genes (Lagosky, 1987). Furthermore, the promoter for *Plasmodium* DHFR gene is not known. To avoid over-expression of thymidylate synthetase in the yeast host, constructs were made in which the DHFR domain from D6/Sierra Leone, a pyrimethamine-sensitive strain of *P. falciparum* (Bzik, 1987), was linked to the 5' promoter and 3' terminator regions of the yeast DHFR gene (Lagosky, 1987) as shown in Figure 4-2A. The Pf-DHFR-D6 with the yeast DHFR promoter and 3' terminator region was amplified by PCR and assembled into a yeast shuttle plasmid that contains centromere

sequences, so that the plasmid is maintained at about one copy/cell (Sikorski and Hieter, 1989; Figure 4-2B).

The second issue for the yeast approach was whether the *Plasmodium* DHFR (Pf-DHFR) gene could supply the enzyme function for yeast. To test for this enzymatic ability, the Pf-DHFR constructs were transformed into *S. cerevisiae* that lack any endogenous DHFR activity, the TH5 (*dfr1:URA3*) mutant strain (Huang, 1992) . Finally, since yeast are impermeable to a variety of exogenous compounds (Nitiss, 1994), the third issue for this approach was whether we would observe sensitivity to the malarial DHFR inhibitors. Using the previously reported sensitivity of *S. cerevisiae* to the mammalian DHFR inhibitor methotrexate (Huang, 1992), it was possible to demonstrate that this class of drugs can cross the yeast cell wall and have an effect on growth via the folate pathway.

The results described here show that yeast are permeable to the DHFR inhibitor class of drugs and that the Pf-DHFR enzyme can substitute for the Sc-DHFR. These yeast cells are now killed by pyrimethamine and cycloguanil at concentrations in the same range as those that inhibit *P. falciparum in vitro* (Milhous, 1985; Childs, 1986; Peters, 1987; Canfield, 1993). Similar constructs were made using DHFR alleles from pyr^R strains and the drug resistance of yeast expressing these genes parallels the resistance of the *P. falciparum* strain from which the DHFR DNA was isolated. This work was the first step to establish a biological system that will allow new antimalarial drugs or combinations of drugs that target the dihydrofolate reductase enzyme to be screened quickly and quantitatively to determine the frequency with which colonies resistant to the drug arise.

II. Results

A. Yeast are permeable to the antifolate class of drugs.

If *S. cerevisiae* is to serve as a system to study resistance to malarial DHFR inhibitors, it is important that sensitivity can be seen to these drugs. This, of course, requires that the drug cross the cell wall to reach the enzyme target. Yeast have been found to be impermeable to many compounds of a diverse nature, presumably due to the presence of a tough cell wall (Nitiss, 1994). In preliminary experiments, wildtype yeast growth was found to be unaffected by the malarial antifolates pyrimethamine and WR99210 (B. Jensen, unpublished). In more controlled experiments, it was found that yeast growth was unaffected by pyrimethamine even at concentrations high enough (250 ng/ml) to kill even the most resistant of parasites (Figure 4-3). This raised the possibility that yeast were impermeable to this class of compounds. However, using the mammalian antifolate methotrexate, it was possible to confirm that yeast are permeable to the antifolates.

Methotrexate is a nonselective antifolate that has affinity for a broad spectrum of DHFRs from different organisms (Hartman, 1993). When wildtype yeast growth was examined in the presence of methotrexate, strong inhibition was seen at 25 μg/ml (Figure 4-4), a concentration in the range of sensitivity seen for mammalian cells (Pallavicini, Deteresa., 1990). The methotrexate sensitivity was dependent on the presence of sulfanilamide, an antifolate that acts further upstream in the folate pathway by inhibiting the DHPS enzyme (see Figure 4-6). This sulfanilamide dependency probably reflects either the poor affinity of methotrexate for the yeast DHFR or a high abundance of DHFR enzyme, both situations requiring that the pathway be down-regulated in order to have a measurable effect on growth. The presence of sulfanilamide did not affect the ability of pyrimethamine to inhibit yeast growth (data not shown). The yeast could be rescued from

the methotrexate-inhibition of growth by adding dTMP (Figure 4-5), the downstream product of the folate pathway. This suggests that methotrexate affected the folate pathway via the DHFR enzyme and was not by a non-DHFR related mechanism. Finally, the methotrexate sensitivity data argue that yeast are permeable to the antifolate class of drugs and that the lack of wildtype yeast sensitivity to pyrimethamine is a consequence of the differing affinities of the two drugs for DHFR enzymes. Pyrimethamine is an inhibitor selective for the *Plasmodium* DHFR enzyme. Thus, a simple prediction is that replacing the yeast DHFR with the Plasmodium DHFR should confer sensitivity to the malarial DHFR inhibitors.

B. The *P. falciparum* DHFR gene can supply the enzyme function in DHFR⁻ yeast.

To determine whether the *P. falciparum* DHFR gene could provide the enzyme function in *S. cerevisiae* that lack DHFR activity, the construct diagrammed in Figure 4-8A was made. The 500 bp region from the 5` promoter of the *S. cerevisiae* DHFR gene (Lagosky, 1987) was cloned 5' to the DHFR domain from the *P. falciparum* DHFR-TS gene from D6 (Bzik, 1987), and the translation termination and 3' termination region from the *S. cerevisiae* DHFR gene were added 3' to the Pf-DHFR-D6 coding region gene (Lagosky, 1987). This hybrid gene was inserted into the pTRP plasmid that carries *S. cerevisiae* sequences that allow autonomous replication of the plasmid in yeast, and centromere sequences to assure that the plasmid is maintained at a low copy number (Sikorski and Hieter, 1989). Several control plasmids were also made: a hybrid construct with the Pf-DHFR-D6 coding and yeast 3' regions in reverse orientation, and a hybrid construct and a yeast construct that lacked the 3' region altogether (see Figure 4-8A). In addition, a construct was made which contained the DHFR coding region from *S. cerevisiae* ligated back to the yeast 5' and 3' regions to compare with a complete Sc-DHFR gene (Sc-DHFR "unstiched"). This was designed to assure that the two restriction

sites we introduced flanking the Pf-DHFR-D6 did not adversely affect enzyme activity. To test the ability of the Pf-DHFR-D6 construct to provide the enzyme function for yeast, a yeast mutant lacking DHFR activity (*dfr1::URA3*) was utilized (Huang, 1992). The growth of the DHFR$^-$ mutant is dependent on supplementation with dTMP, the critical downstream product of the folate pathway (see Figure 4-6). If the Pf-DHFR could supply the enzyme function, transformants of this mutant containing the Pf-DHFR-D6 construct should be dTMP independent (Figure 4-7). The constructed plasmids were transformed into the DHFR$^-$ mutant and grown on plates without tryptophan but supplemented with dTMP to select for transformants. The resulting transformants were then tested for their ability to grow in the absence of dTMP.

We first determined whether complementation of the yeast DHFR defect depended on the presence of the yeast 5' and 3' control regions and a DHFR coding region. The DHFR$^-$ mutant and the transformant containing the vector alone were unable to grow on plates that lacked dTMP, but the Pf-DHFR-D6 transformant and the Sc-DHFR transformants grew equally well in the presence or absence of dTMP (Figure 4-8B). This demonstrated that the Pf-DHFR-D6 gene can supply the enzyme function. The growth of transformants that expressed the Sc-DHFR construct containing the extra restriction sites (Sc-DHFR, stitched) was indistinguishable from the growth of tranformants that contained the construct (Sc-DHFR, unstitched) that lacked the sites (data not shown), demonstrating that the introduced restriction sites did not interfere with the function of the gene. As expected, neither the hybrid gene that lacked the yeast 3' sequences nor the construct in reverse orientation showed any growth in the absence of dTMP (Figure 4-8B). While the Pf-DHFR-D6 transformant did not grow as well as the wild type parent strain, its growth was comparable to that of the transformant containing the Sc-DHFR plasmid (see Figure 4-8A). Yeast strains were made in which the Pf-DHFR-D6 gene was integrated into the DHFR locus and these showed more vigorous growth than either transformant carrying the plasmid (data not shown). These observations suggest that the reduced growth of the

Pf-DHFR-D6 transformant is probably a result of the differences between plasmid-based and chromosomal expression and are not a consequence of the heterologous nature of the *Plasmodium* enzyme. To insure that the observed phenotype was due to the construct used for the transformation and not a change in the biology of the yeast host, plasmid DNA was isolated from each transformant and transferred into *E. coli* (Hoffman and Winston, 1987; Sambrook, 1989). The plasmid DNA was analyzed by restriction digests to confirm its identity and then used to transform a new yeast DHFR$^-$ mutant host. In all cases, the original phenotype was observed in the transformant. These results together argue that the Pf-DHFR enzyme can supply the function for yeast and that these regions of the yeast DHFR gene promoter and terminator are sufficient for expression of the Pf-DHFR gene in *S. cerevisiae.*

The DHFR enzyme activity is required for the biosynthesis of other metabolites in addition to dTMP (see Figure 4-6). The yeast DHFR$^-$ mutant is auxotrophic for adenine, histidine, and methionine in addition to dTMP (Huang, 1992). Therefore, if the Pf-DHFR-D6 enzyme complements all of the functions of the yeast DHFR enzyme, the Pf-DHFR-D6 transformants should no longer be auxotrophic for these other metabolites. This prediction was tested by transferring the Pf-DHFR-D6 transformant to plates that lacked methionine, histidine or adenine (Table 4-2). Neither the DHFR mutant nor the transformant containing the vector alone was able to grow when any of these three metabolites was missing from the growth medium. However, the Pf-DHFR-D6 transformant grew as well as the Sc-DHFR transformant and wildtype yeast under all of these conditions. This suggests that all of the functions of the DHFR were restored in the Pf-DHFR-D6 transformant and that the Pf-DHFR enzyme functions in yeast in a manner that conserves the major functions of the yeast folate pathway.

C. Expression of the *Plasmodium* DHFR gene in *S. cerevisiae* confers sensitivity to inhibitors of the *Plasmodium* DHFR enzyme.

The methotrexate sensitivity data indicate that yeast are permeable to the antifolate class of drugs and argues that the lack of sensitivity to pyrimethamine was a consequence of the therapeutic specificity of this drug for the *Plasmodium* DHFR. This led to the prediction that yeast that express the *Plasmodium* DHFR should be rendered sensitive to pyrimethamine (see Figure 4-7). To test this prediction, the growth of the Pf-DHFR-D6 transformant was examined on medium containing pyrimethamine (Figure 4-9). The growth of wild type yeast and the transformant containing the yeast DHFR construct (Sc-DHFR) was unaffected by pyrimethamine concentrations as high as 4×10^{-5} M, a level high enough to kill the most resistant strains of *P. falciparum* (Peters, 1987). In contrast, the growth of the Pf-DHFR-D6 transformant was inhibited by 50%, (the IC_{50}) at 4×10^{-7} M pyrimethamine, a concentration comparable to the range of sensitivity seen in *P. falciparum* itself (Peters, 1987). This demonstrates that the sensitivity of *S. cerevisiae* to pyrimethamine does depend on the origin of the DHFR enzyme.

The drug sensitivity of the Pf-DHFR-D6 transformant was characterized more quantitatively by measuring the growth of the transformant in liquid culture. A single colony was picked, and its growth was tested in the presence of pyrimethamine at concentrations ranging from 0 to 5×10^{-5} M. The Pf-DHFR-D6 transformant was sensitive to pyrimethamine concentrations comparable to those that inhibit *P. falciparum* in the standard short-term *in vitro* assay (Figure 4-10A). The growth of the Sc-DHFR transformant was unaffected by pyrimethamine up to 1×10^{-5} M; effects at higher concentrations were identical to those observed when the solvent alone was tested. Two other DHFR inhibitors were tested on these Pf-DHFR-D6 cells: cycloguanil and WR99210, an experimental drug that is also thought to affect *P. falciparum* growth by inhibition of DHFR (Childs, 1986; Canfield, 1993). Both drugs gave comparable results; the *S. cerevisiae* cells that expressed the Pf-DHFR-D6 were sensitive to cycloguanil and WR99210 at levels comparable to the IC_{50} measured for each drug in *P. falciparum* (data

not shown). As before, the *S. cerevisiae* that expressed the Sc-DHFR were unaffected by either antimalarial drug.

To assure that the inhibition by pyrimethamine targeted specifically the Pf-DHFR-D6, the assay was used to compare the growth of the Pf-DHFR-D6 transformant in chloroquine and halofantrine, two antimalarial drugs that do not interfere with DHFR function (Peters, 1987). No inhibition of growth was observed in cultures grown in either chloroquine or halofantrine except at levels where the solvent alone caused inhibition (Figure 4-10B). Thus, the sensitivity of the PfDHFR *P. falciparum* is specific to the drugs that target DHFR.

D. The drug sensitivity of PfDHFR yeast is dependent on the DHFR allele from which the construct was derived.

If the yeast system is to be useful, then the drug sensitivity of a transformant must depend on the DHFR domain used to make the expression construct and allow discrimination of subtle differences between alleles of DHFR. To determine whether this is the case, constructs were made using the DHFR genes from the pyrimethamine-resistant strains Honduras I (Pf-DHFR-Hon) and Mikenga (Pf-DHFR-Mik). The drug sensitivity of transformants expressing these genes was then compared to that of the Pf-DHFR-D6 construct described above whose DHFR gene was isolated from the pyrimethamine sensitive strain D6/Sierra Leone. The constructs were transformed into the yeast DHFR$^-$ mutant and each DHFR construct complemented the DHFR defect as shown in Table 4-2. The sensitivity of these three Pf-DHFR transformants to pyrimethamine was compared by transferring patches of the transformed colonies to plates containing no drug or pyrimethamine ranging from 5×10^{-8} to 5×10^{-5} M (Figure 4-11). The growth of the D6-DHFR transformant was completely inhibited on plates containing 5×10^{-7} M pyrimethamine, the Hon-DHFR transformant at 5×10^{-6} M, and the Mik-DHFR

transformant at 5 x 10^{-5} M. This is the expected pattern of sensitivity based on resistance of each of these strains of *P. falciparum* (Peterson, 1988; Cowman, 1988). The growth of the transformants was also examined on media containing cycloguanil at concentrations from 5 x 10^{-8} to 3.5 x 10^{-5} M (Figure 4-12). The growth of D6-DHFR transformant was inhibited at ≈ 5 x 10^{-7} M cyc while the Hon-DHFR and Mik-DHFR transformants were inhibited at ≈ 5 x 10^{-6} M cyc, again the expected pattern of sensitivity. These data indicate that the drug sensitivity of the Pf-DHFR transformants reflects the strain of origin for each DHFR allele.

The resistance of the three transformants was compared more quantitatively by growing the cells in liquid culture. The IC_{50} of the D6-DHFR transformant was about 3 x 10^{-8} M pyrimethamine while the IC_{50} of the Hon-DHFR and Mik-DHFR transformants was around 10^{-6} M pyrimethamine for each of the strains (Figure 4-13). Growth of each culture in media containing cycloguanil revealed that D6-DHFR transformant had an IC_{50} of 5 x 10^{-8} M while the IC_{50}s for the Hon-DHFR and Mik-DHFR transformants were ≅ 8 x 10^{-7} M and 1 x 10^{-6} M cyc respectively (Figure 4-14). These IC_{50}s for both pyr and cyc are comparable with the values that have been determined in *P. falciparum*. The growth on plates containing drug gave slightly different results from the inhibition of growth in liquid; a higher concentration of drug was required to completely inhibit growth on plates. However, the data from both the replica plating and liquid culture assays demonstrates that the yeast system discriminates differences in drug resistance over the full range of sensitivity observed in populations of *P. falciparum*.

E. Inhibition of the growth by pyrimethamine can be overcome by tetrahydrofolate.

If pyrimethamine acts directly on DHFR as expected, one can make several predictions based on the folate pathway diagrammed in Figure 4-6. First, yeast with the Pf-DHFR-D6 allele should return to the original phenotype of the $DHFR^-$ mutant in the

presence of pyrimethamine and require supplementation with dTMP, adenine, methionine and histidine for growth. Second, since tetrahydrofolate (THF) is the downstream product of DHFR and is required for synthesis of all of the metabolites listed in Figure 4-6, inclusion of THF in the medium should overcome the effects of pyrimethamine. Conversely, the presence of dTMP should rescue from the effects of pyrimethamine since dTMP is downstream of THF. These predictions are easily tested in yeast since the pathway is well known and the tests of function involve simple replica plating to medium with defined nutritional requirements. The growth of transformants carrying three different plasmids was compared: pyrimethamine-sensitive Pf-DHFR-D6, pyrimethamine-resistant Pf-DHFR-Hon, and the yeast Sc-DHFR (Table 4-3). The growth of the Pf-DHFR-D6 transformant was inhibited by pyrimethamine, while the growth of the Sc-DHFR and Hon-DHFR transformants was not. The pyrimethamine-inhibition of growth was reversed by either THF or dTMP when the D6-DHFR transformant was tested on plates containing methionine, histidine and adenine. However, supplementing the medium with dTMP did not permit growth in pyrimethamine if histidine was also withheld. Under the same conditions, the transformant that expressed the Hon-DHFR did grow, although somewhat weakly. Comparable results were observed when either adenine or methionine was withheld from the medium (data not shown). Conversely, the D6-DHFR transformant was able to grow on pyr media lacking histidine if supplemented with THF. No effect on growth of the Sc-DHFR transformant was observed under any conditions. Since the only difference between these transformants is the DHFR gene they express, these results serve as strong functional evidence that the drug sensitivity of the Pf-DHFR yeast results from inhibition of the DHFR enzyme in the folate pathway

F. The Pf-DHFR yeast can be used to predict response to experimental drugs that may act as inhibitors of DHFR.

For the yeast system to be useful, it is important to see the appropriate sensitivity to experimental DHFR inhibitors. The D6, Honduras, and Mikenga strains of *P. falciparum* from which the three DHFR alleles were isolated are all efficiently killed by the experimental antimalarial drug, WR99210 (Childs, 1986; Canfield, 1993; Milhous, 1985). In contrast, the Sc-DHFR yeast is insensitive to WR99210, even at 10^{-5} M (data not shown). Examination of the growth of the three transformants on media containing WR99210 revealed that all three were completely inhibited at 5×10^{-6} M (Figure 4-15), while some growth can still be seen for the D6-DHFR transformant and weak growth for the Mik-DHFR transformant on media containing 5×10^{-7} M pyr (see Figure 4-11). When the liquid culture assay was used to compare the drug sensitivity of each transformant to WR99210, all three were efficiently inhibited at $\approx 10^{-8}$ M WR99210 (Figure 4-16). In fact, the transformants containing DHFR alleles from the pyrimethamine resistant strains, Honduras and Mikenga, were more sensitive to WR99210 than the D6-DHFR transformant, a phenomenon known as collateral sensitivity (Genther, 1977). The IC_{50} values for inhibition of growth of the three Pf-DHFR strains in liquid cultures for pyrimethamine, WR99210, and cycloguanil are compared in Table 4-4. Several conclusions can be drawn. First, the small standard deviations of the measurements demonstrates that this system provides a very precise measurement of the drug sensitivity of a DHFR allele. This degree of precision is much greater than that currently obtainable in *P. falciparum* using standard *in vitro* drug sensitivity assays (Peters, 1987). Second, the expected patterns of sensitivity were observed for all three drugs. The *P. falciparum* strains Honduras 1 and Mikenga are moderately resistant to cycloguanil but they are two to three orders of magnitude more resistant to pyrimethamine than is D6. In contrast, all three strains of *P. falciparum* are very efficiently killed by WR99210. These are exactly the patterns of sensitivity that were observed when these DHFR alleles were transferred into yeast. These results argue that the yeast system is a good predictor of the response of *P. falciparum* strains to experimental DHFR inhibitors.

III. Discussion

The experiments described here were the first step in establishing a system for estimating rapidly and quantitatively the potential of a DHFR inhibitor for selecting populations of *P. falciparum* resistant to the drug. I have demonstrated that the DHFR enzyme from *P. falciparum* can supply the enzyme function in DHFR$^-$ *S. cerevisiae*. Furthermore, the sensitivity of the transformed yeast to antimalarial drugs that target DHFR depends only upon the allele they express. Thus, one can now treat yeast that express a drug-sensitive allele of DHFR with a drug, and select resistant yeast colonies for analysis of the frequency and mechanism of their drug resistance.

A number of choices were made in the original design of these experiments. First, the 5' and 3' regulatory elements from *S. cerevisiae* were used. The sequences of these regions were known (Lagosky, 1987), and our goal was to express the Pf-DHFR at low levels, comparable to the endogenous gene. The constructs were made with only the coding region of the DHFR domain from *P. falciparum*, even though the enzyme activity is normally part of a bifunctional DHFR-TS enzyme (Bzik, 1987). In yeast, DHFR and TS are encoded by separate genes, and the yeast host expressed normal levels of TS (Huang, 1992). Since this project focuses on mutations in the DHFR enzyme itself, and not on its interaction with TS, it was reasoned that the DHFR domain alone would be sufficient. This choice also avoided potential problems stemming from over-expression of TS and made the constructs somewhat more straightforward. For detailed studies of biochemical parameters of the DHFR enzyme, similar constructs that contain both the DHFR and TS domains from *P. falciparum* could be made.

The Pf-DHFR was purposefully kept on the pTRP plasmid for these experiments, instead of using *S. cerevisiae* in which the Pf-DHFR gene replaced that of *S. cerevisiae*. This was principally for efficiency: the plasmid system allows facile transfer of the Pf-

DHFR from one yeast cell to another and provides a quick way to localize resistance to the DHFR gene on the plasmid. The sophisticated control of plasmid copy number was another important reason for the decision to use this expression system. The ARS and centromere sequences incorporated into the plasmid design assure that the copy number of the plasmid remains near one/cell (Sikorski, 1989). The absolute amount of the enzyme produced has not been measured yet, but the Pf-DHFR transformants grow more slowly than either wild type yeast or yeast with the Pf-DHFR integrated into the DHFR locus. This suggests that expression from the plasmid is somewhat lower in the transformants. This was important, since, as one would predict, over-expression of the wild type enzyme on a high copy number plasmid does confer resistance to high levels of pyrimethamine, cycloguanil or WR99210 (K. Hamilton and S. Mookherjee, personal communication).

Another advantage is that yeast is a eukaryote. Since the ultimate goal is to study the frequency of mutations to drug resistance, the frequency of mutants observed in yeast will need to be related with the "real" frequency in *P. falciparum* itself. It seems likely that the frequency and character of mutations and their repair are more likely to be similar if both systems are eukaryotic. These parameters have been intensively studied in *S. cerevisiae* (Sherman, 1979), but there is almost no information on rates of mutation in *P. falciparum*. As more data become available on the mutation rates in *P. falciparum*, it will be possible to test the assumption that a drug to which resistance is selected rarely in Pf-DHFR-D6 yeast is also inefficient in selecting resistant populations of *P. falciparum*.

The antimalarial drugs examined in this study inhibited growth of the D6-DHFR transformants at about the same concentrations that inhibit *P. falciparum* itself, and this is an important advantage of the yeast system. It allows a fairly direct comparison of the effectiveness of particular DHFR inhibitors in the yeast transformants and *P. falciparum* itself, and will permit us to monitor the emergence of drug resistant populations at concentrations that resemble those that are likely to be operating in the actual clinical

situation. The effectiveness of low drug concentrations also demonstrated that the yeast are permeable to the antimalarial drugs. This was a concern, since *S. cerevisiae* are frequently insensitive to exogenous toxins simply because they exclude them from the cells or actively excrete them (Nitiss, 1994). In fact, yeast cells do not normally take up dTMP and the selection of DHFR$^-$ mutant cells required that the mutants be maintained by its addition to the medium. To isolate the original DHFR$^-$ mutant, Huang and his co-workers used a parental strain, TH1, that that had been first selected for the *tup1* mutation that allows thymidine uptake. It was not determined whether the *tup1* mutation was necessary for efficient uptake of the DHFR inhibitors employed, but all experiments were done in a *tup1* background.

The concentration of a DHFR inhibitor required to inhibit growth of colonies on plates did not correspond exactly to the IC_{50} measured in liquid culture. However, the assays gave comparable results with the three strains that spanned a range of three orders of magnitude in sensitivity to pyrimethamine and cycloguanil. Furthermore, Honduras 1 is somewhat less resistant to pyrimethamine than is Mikenga, and even this subtle difference was observed both on the plates and during growth in liquid. The IC_{50} was used to compare the relative effectiveness of each of the drugs in yeast and in *P. falciparum*, because it is analogous to the IC_{50} commonly used to compare the response of different malarial strains to particular drugs (Peters, 1987). It was found that the reproducibility of the IC_{50} measurements in the Pf-DHFR yeast was remarkable, far better than comparable measurements in *P. falciparum in vitro* (Peters, 1987). This is probably due to the consistency of the yeast growth medium. The presence of folate or para-aminobenzoic acid (PABA) can interfere with the measurement of the IC_{50} for a DHFR inhibitor in *P. falciparum*. The same IC_{50} for each of the drugs was obtained in rich medium or in a completely synthetic medium, so the level of these vitamins must be similar in the two media. In the defined yeast medium, the concentration of these metabolites is very low: folate is present at 0.0018 mg/L and PABA at 0.19 mg/L. This is in contrast to culture of

P. falciparum where the standard RPMI medium contains 1 mg/L of folic acid and PABA. At least some folate is required for parasite growth, so it is not surprising that the IC_{50} measured in cultures of *P. falciparum* is variable; it depends on the quality of the red cells, level of folate and PABA in the medium and in the human serum and a variety of other variables that cannot be avoided in tissue culture (Peterson, 1990). This variation in culture conditions of the *P. falciparum* means that the IC_{50} values published by different labs can differ markedly (Peters, 1987). The consistency of the IC_{50} measurement is another advantage of using the yeast system to define precisely the sensitivity of a particular DHFR allele to a DHFR inhibitor.

WR99210 is one of several promising drugs that are inhibitors of Pf-DHFR *in vitro* (Milhous, 1985). WR99210 is effective even against strains that are extremely resistant to pyrimethamine and cycloguanil, like the Honduras 1 and Mikenga strains used here. In our experiments, both of these pyrimethamine and cycloguanil resistant strains were more sensitive to WR99210 than was the pyrimethamine-sensitive D6. This suggests that the DHFR mutations responsible for the pyrimethamine and cycloguanil resistance of the Hon-DHFR and Mik-DHFR transformants has in some manner rendered them more sensitive to the experimental drug. This collateral sensitivity has been observed in other species (Genther, 1977) , and raises the hope that new DHFR inhibitors might be identified that are particularly effective against the pyrimethamine-and cycloguanil-strains that already prevail in large regions of Southeast Asia and South America and are developing rapidly in Africa (Peterson, 1991; White, 1992; Plowe, 1995). At this point, the effectiveness of WR99210 has been demonstrated *in vitro*, but it is not yet known whether all of its action is directed against DHFR (Milhous, 1985; Childs, 1986; Canfield, 1993). When the drug was tested against the yeast transformants expressing each DHFR allele, WR99210 efficiently killed all of them, but the IC_{50} was about 10^{-8}, almost an order of magnitude higher than the comparable measurement in *P. falciparum* (WRAIR internal report, Wilbur Milhous). One explanation of this discrepancy could be that WR99210

inhibits more than one function in *P. falciparum*. The yeast system affords a quick, quantitative way to determine whether a drug really does target the Pf-DHFR, and if it does, to separate that action from inhibition of other functions in the *P. falciparum*.

The basic strategy used here may be applicable to the study of resistance to a number of other antimalarial drugs. The requirements are clear. First, the drug must inhibit a particular *P. falciparum* enzyme, either as a competitive inhibitor of the enzyme or by binding to a region other than the active site. Second, the homologous enzyme activity must also operate in *S. cerevisiae*. Third, the mechanism of resistance must depend on mutations in the gene, not on increases in gene expression or changes in other genes that affect the cell biology of *P. falciparum*. Fourth, the *S. cerevisiae* must be permeable to the drug in question, and the subcellular localization of the enzyme and the drug must be the same in both systems. A number of malaria enzymes appear to conform to these requirements: dihydropteroate synthetase (the target of sulfa drugs; Brooks, 1994), topoisomerases I and II (the target of quinolones; Gamage, 1994; Tosh, 1995) and ornithine decarboxylase (the target of difluoromethylornithine and novel tetraamines; Edwards, 1991; Hibasami, 1994).

These experiments make it clear that one can study the effect of antimalarial drugs that target the DHFR enzyme of *P. falciparum* by transferring the malaria enzyme into *S. cerevisiae*. The ease of growth and manipulation of the yeast make it easy to measure the sensitivity of any particular DHFR allele to potential inhibitors of the Pf-DHFR. More importantly, it will allow the measurement of the efficiency with which resistant mutants to any particular DHFR inhibitors are selected. A drug or combination of drugs that show a very low capacity to select resistant populations in the yeast system will be a valuable lead in the development of drugs of this class that may remain clinically useful for treatment of *P. falciparum* over much longer periods than those observed for pyrimethamine and cycloguanil.

A **Sequence Homology Between the *Plasmodium falciparum* DHFR Domain and Other DHFRs of Interest**

Organism	%DNA Homology	%Protein Homology
P. chabaudi[a]	51	56
S. cerevisiae[b]	41	25
Human[c]	45	25

[a]Sequence data from Cowman and Lew, 1989, Mol. Cell. Bio. 9:5182-5188.
[b]Sequence data from Nucleic Acids Res. 15:10355-71 (1987), Gene 63:165- 174 (1988), and Gene 63:175-185 (1988).
[c]Sequence data from J. Biol. Chem. 259:3933-3943 (1984).

B **Protein Homology between the *P. falciparum* and *S. cerevisae* Dihydrofolate Reductase Proteins**

```
P.f. CMMEQVCDVFDIYAICACCKVESKNEGKKNEVFNNYTFRGLGNKGVLPWKCNSLDMKYFC50
                : .: ::   :                  :.: .: :::.    .::::
S.c.     MAGGKIPIVGIVACLQPE----------------MGIGFRGGLPWR-LPSEMKYFR39

P.f. AVTTYVNESKYEKLKYKRCKYLNKETVDNVNDMPNSKKLQNVVVMGRTSWESIPKKFKPL120
      ::.                 : :.        :: :   : ..::: .:::::: ::.::
S.c. QVTS----------------LTKD--------PNKK---NALIMGRKTWESIPPKFRPL71

P.f. SNRINVILSRTLKKE---DFDEDVYIINKVEDLIVLLG---KLNYYKCFIIGGSVVYQEF174
      :: :::.::. : .   : .  .   : .   :  :    :    . ..::::  ::
S.c. PNRMNVIISRSFKDDFVHDKERSIVQSNSLANAIMNLESNFKEHLERIYVIGGGEVYSQI131

P.f. L---EKKLIKKIYFTRINSTYECDVFFPEIN-ENEYQIISVSDVYTSNNTTLDFIIYKKT230
        .  :: ::     : :   : :      :  .       .       ..
S.c. FSITDHWLITKINPLDKNATPAMDTFLDAKKLEEVFSEQDPAQLKEFLPPKVELPETDCD191

P.f. NNKMLNEQN-CIKGEEKNNDMPLKNDDKDT                              59
     .   : :   :      :
S.c. QRYSLEEKGYCFEFTLYNRK                                        211
```

Figure 4-1. Sequence homology of *P. falciparum* dihydrofolate reductase. (A) Homology with other DHFRs of interest. (B) Alignment of lasmodium and yeast DHFR proteins. All analyses were performed on GenePro using sequence for *P. falciparum* strain 3D7. Single dots denote conserved residues and double dots identity. Residues involved in antifolate resistance shown in bold.

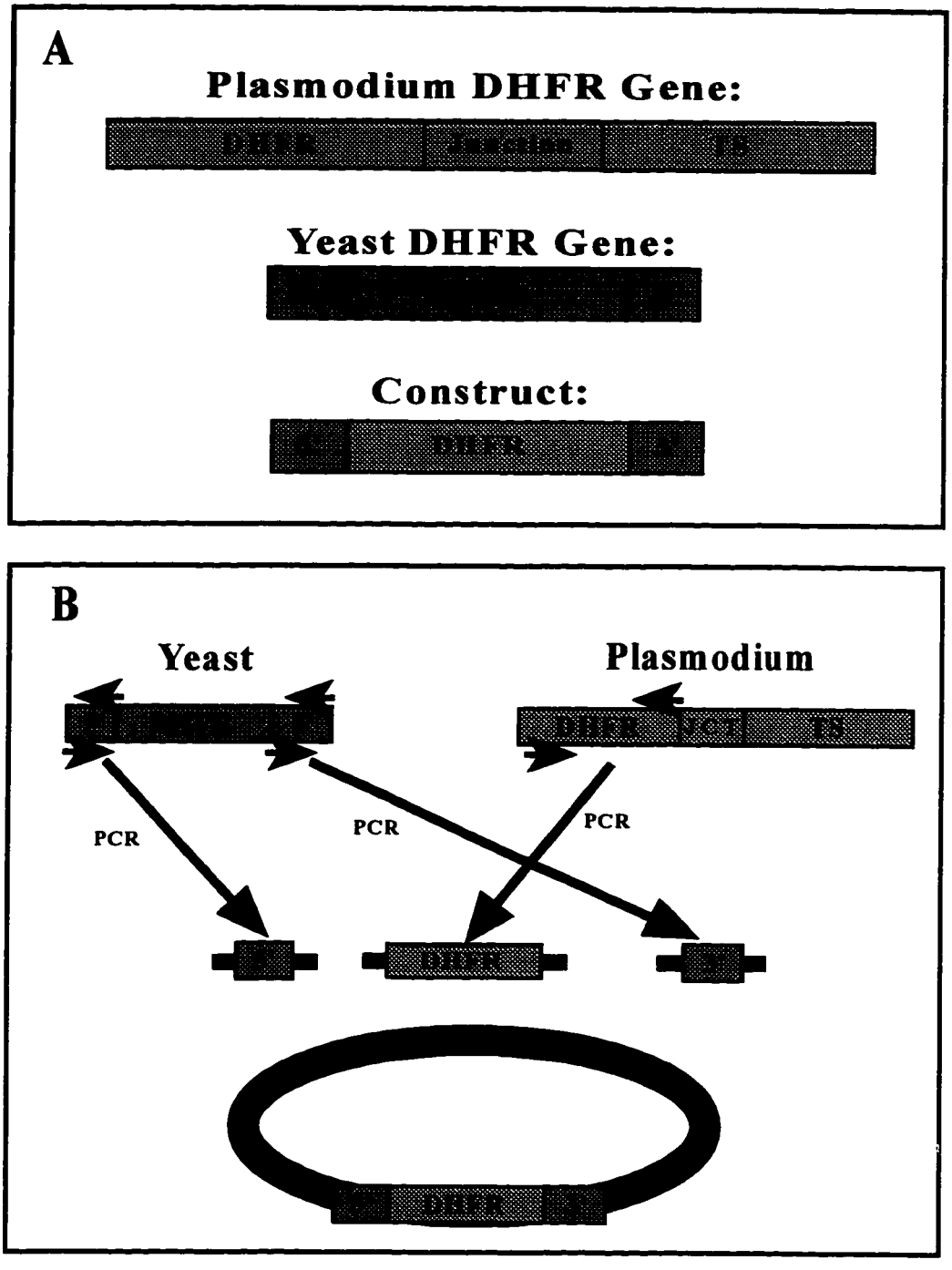
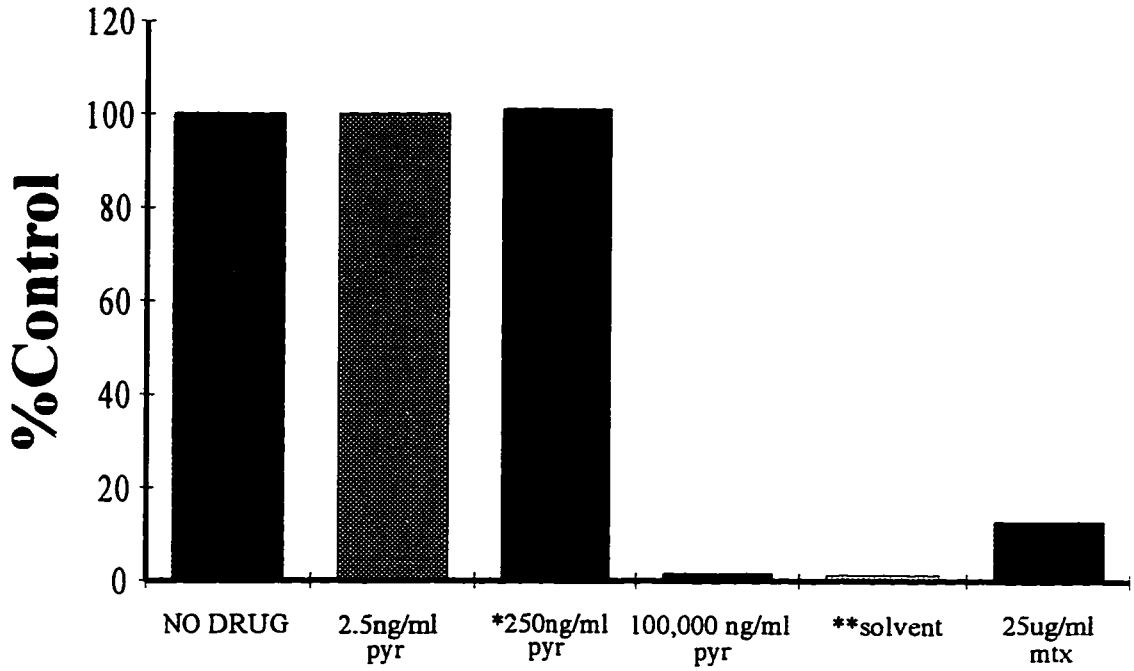


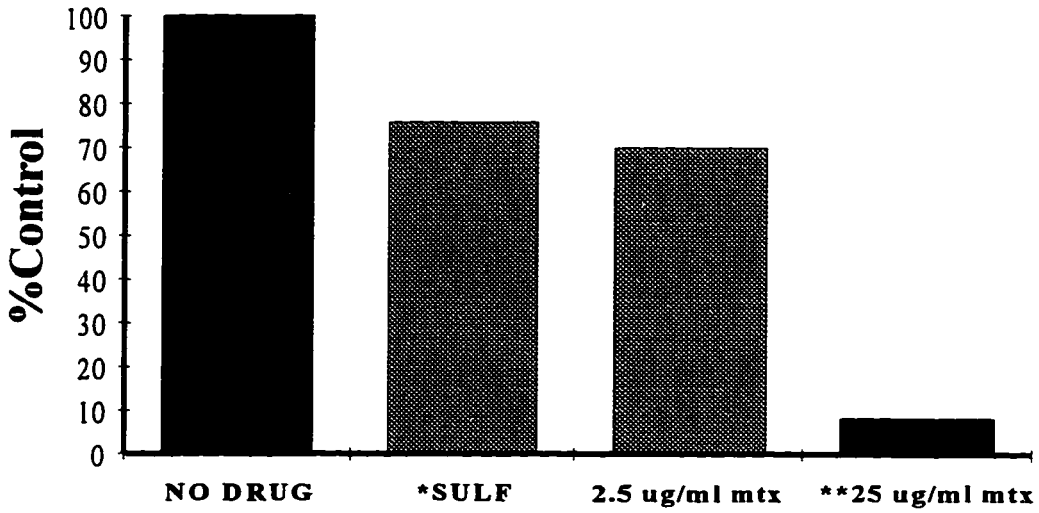
Figure 4-2. Design and construction of DHFR expression vector. (A) Components from *P.f.* and yeast DHFR genes used to make construct. (B) PCR cloning of components for construct.



*The most resistant malarial parasites have IC_{50}s of approx. 100ng/ml

**Sample with highest pyr concentration contained 9.5% EtOH

Figure 4-3. Wild-type yeast growth in pyrimethamine. Experiment were performed as described in materials and methods.



*Methotrexate-inhibition experiments done in the presence of 5ug/ul sulfanilamide

**Inhibitory amounts of methotrexate used in culture of mammalian cells are in the range of 0.1 - 45 ug/ml

Figure 4-4. Wild-type yeast growth in methotrexate. Experiments performed as described in Materials and Methods.

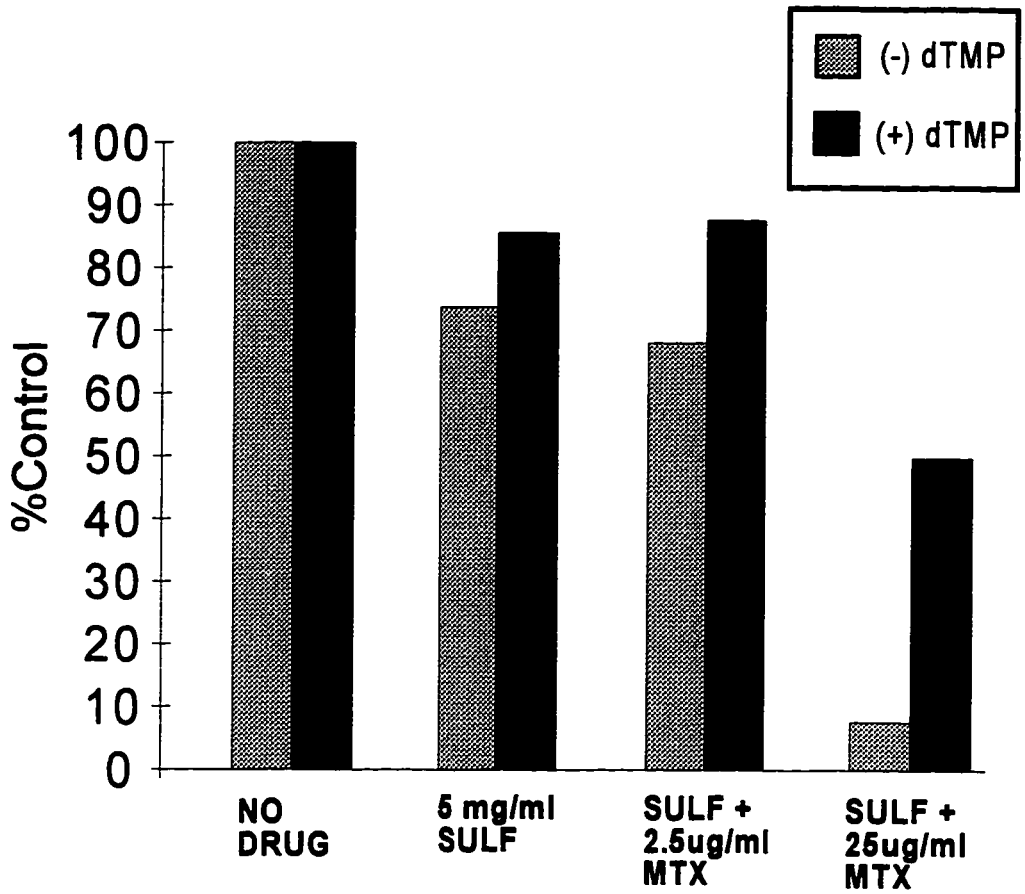


Figure 4-5. Effect of dTMP on the the ability of methotrexate to inhibit wildtype yeast growth. Experiments performed as described in materials and methods. Plates were supplemented wtih dTMP at 100 ug/ml.

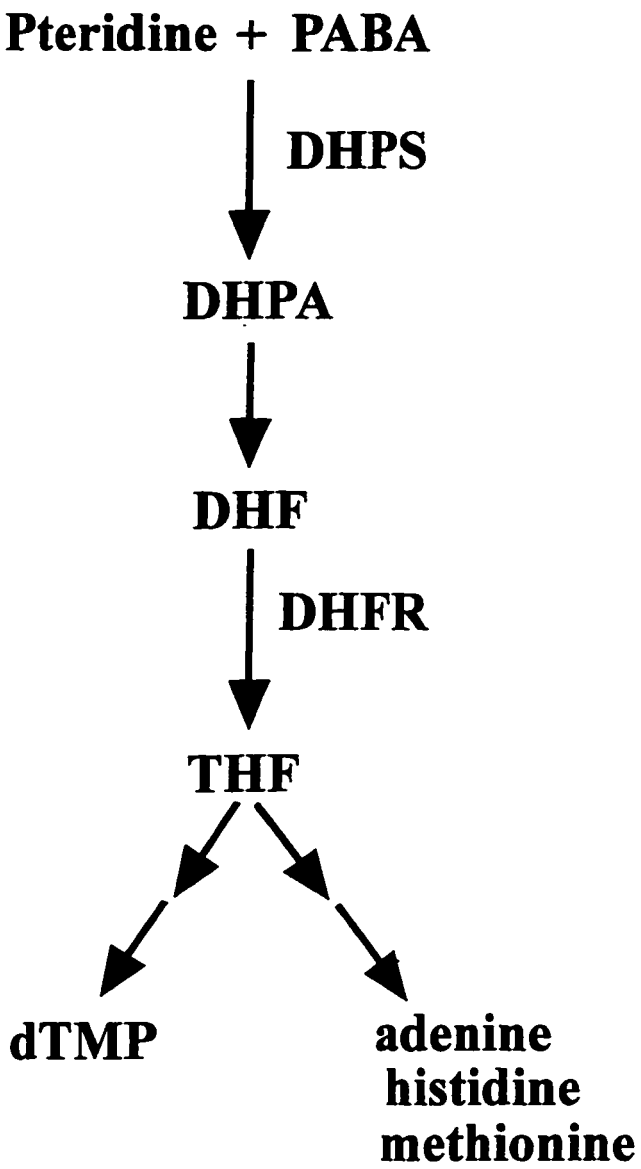
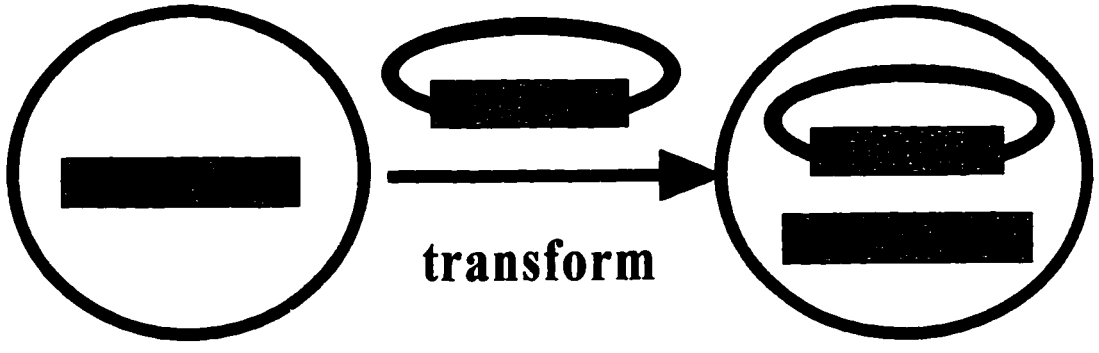


Figure 4-6. Folate pathway in yeast *S. cerivisiae* and important enzymatic steps. Pteridine and *para*-aminobenzoic acid (PABA) are combined by the enzyme diydropteroate synthetase (DHPS) to make dihydropteroic acid (DHPA), the precursor of dihydrofolic acid (DHF). The enzyme diydrofolate reductase (DHFR) converts DHF to tetrahydrofolic acid (THF), which is used as a cofactor in the biosynthesis of dTMP and other metabolites.



Properties:

- **dTMP dependent**
- **pyrimethamine-resistant**

Predicted properties:

- **dTMP independent**
- **pyrimethamine-sensitive**

Figure 4-7. Complementation of Yeast DHFR Mutant with Plasmodium DHFR.

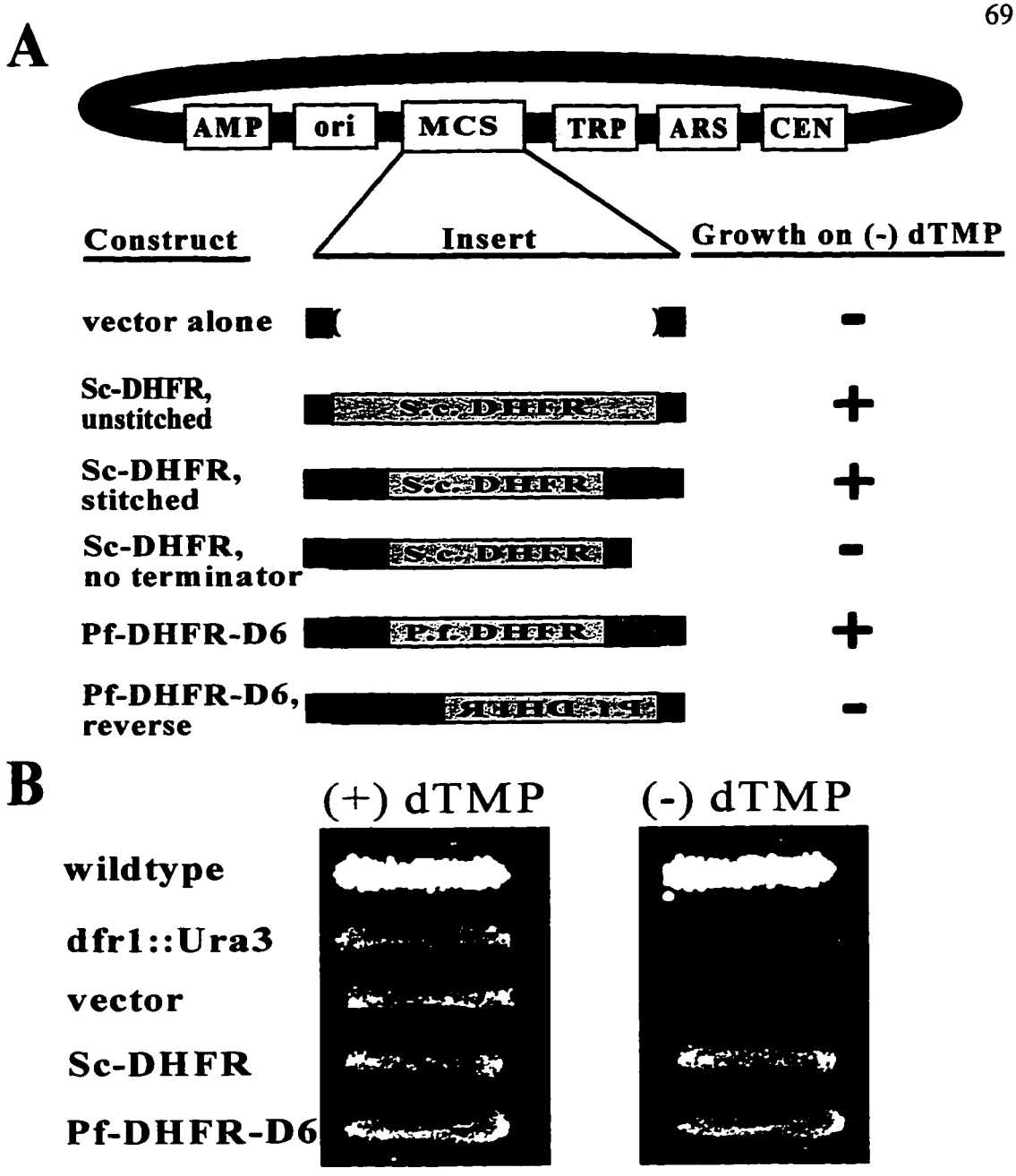


Figure 4-8. Test for DHFR enzyme function. A) Test constructs and ability to complement the yeast DHFR^{-} defect. Transformants containing test constructs were transfered onto rich medium and allowed to grow for 2 days. Yeast patches were then replica plated to a test plate without dTMP and a control plate supplemented with 100 μg/ml dTMP. The replica plates were incubated at 30°C for 3 days before scoring for growth. B) Growth with/without dTMP supplementation. The wild type, DHFR disruption mutant (dfr1::URA3), and the mutant containing either vector alone, the yeast DHFR construct (Sc-DHFR), or the *Plasmodium* DHFR construct (Pf-DHFR) were tested as described above.

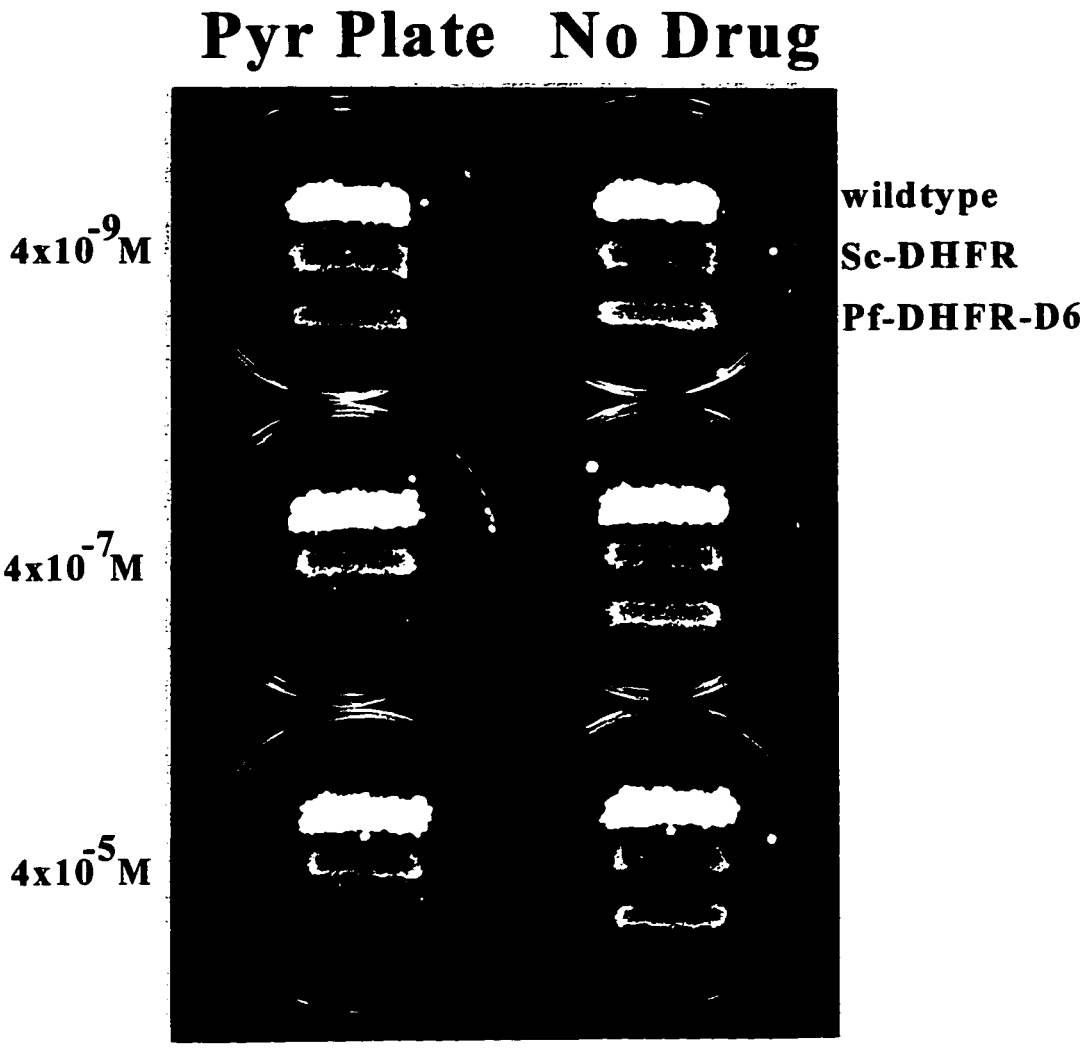


Figure 4-9. Growth of wild type yeast and two transformants on media containing pyrimethamine (pyr). Replica plating experiments were performed as described in Materials and Methods.

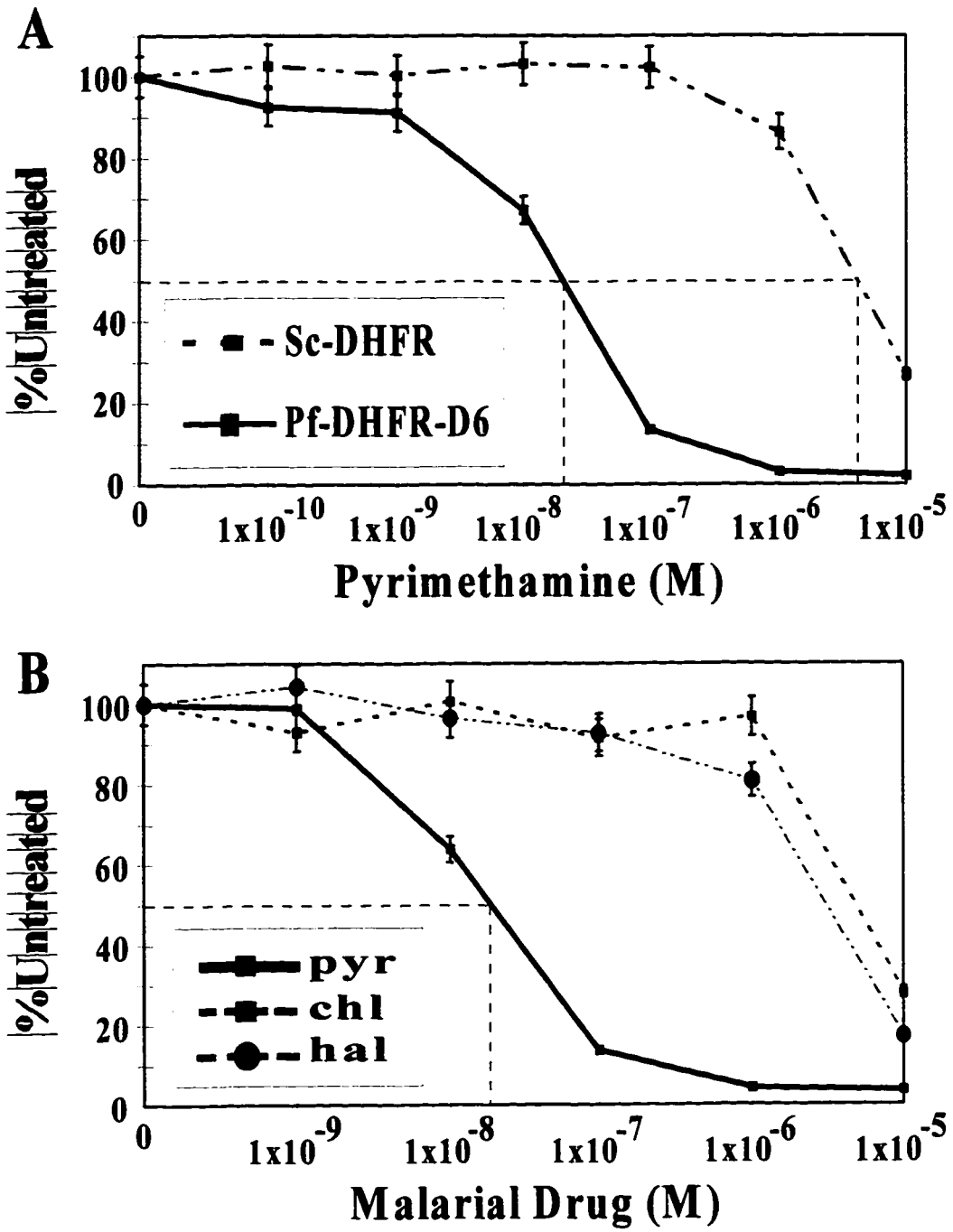


Figure 4-10. Drug sensitivity of yeast transformants. (A) Comparison of pyrimethamine sensitivities of Pf-DHFR-D6 and Sc-DHFR transformants. (B) Comparison of sensitivity of Pf-DHFR-D6 to pyrimethamine (pyr) and nonrelated malarial drugs chloroquine (chl) and halofantrine (hal). The drug sensitivity curve was generated by expressing the growth at each concentration point as a percentage of the growth of the control sample that was not treated with drug. IC_{50} indicated by dotted line intersecting x-axis.

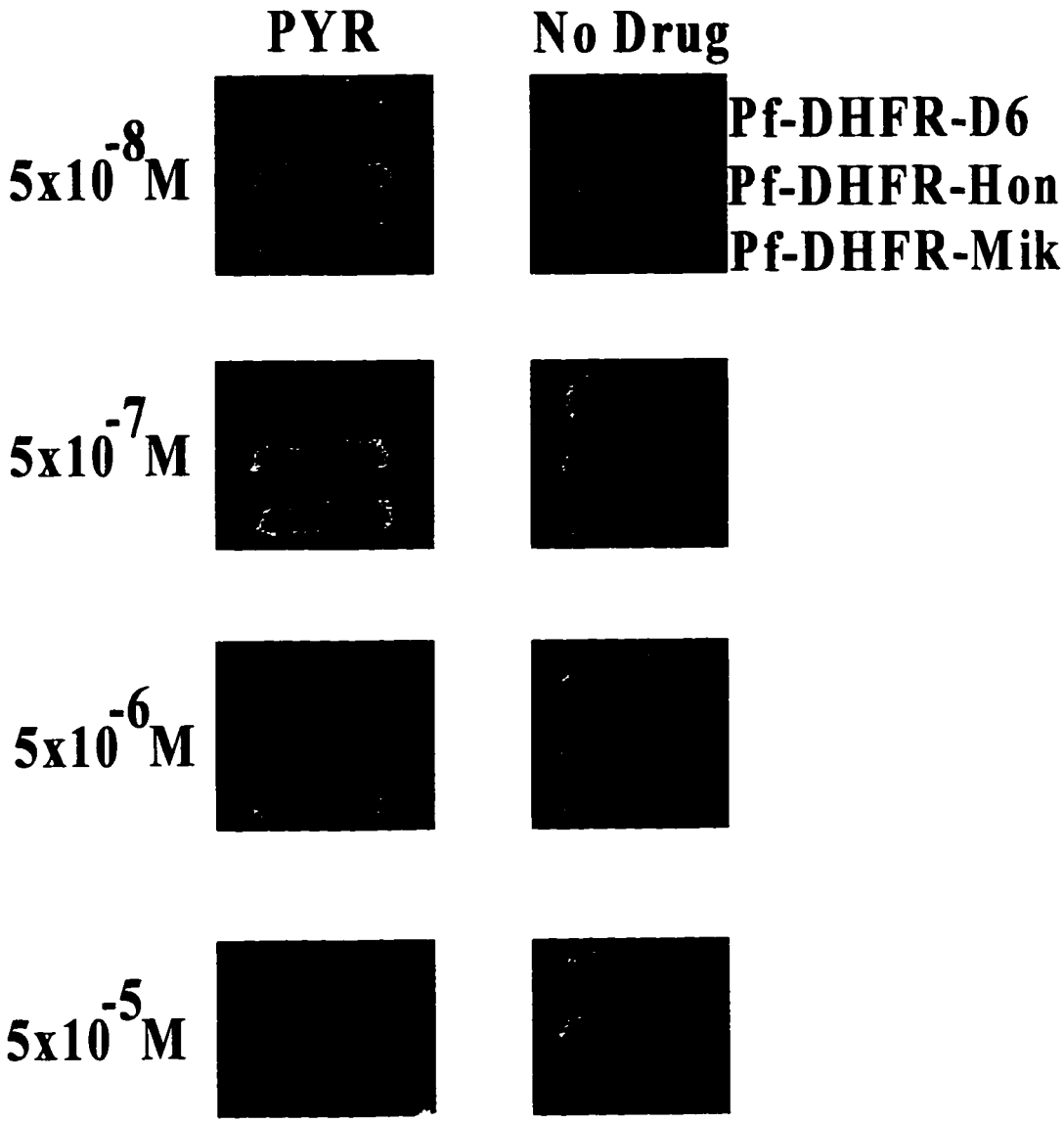


Figure 4-11. Growth of yeast containing Pf-DHFR constructs on pyrimethamine media (pyr). Relica plating tests were performed as described in materials and methods.

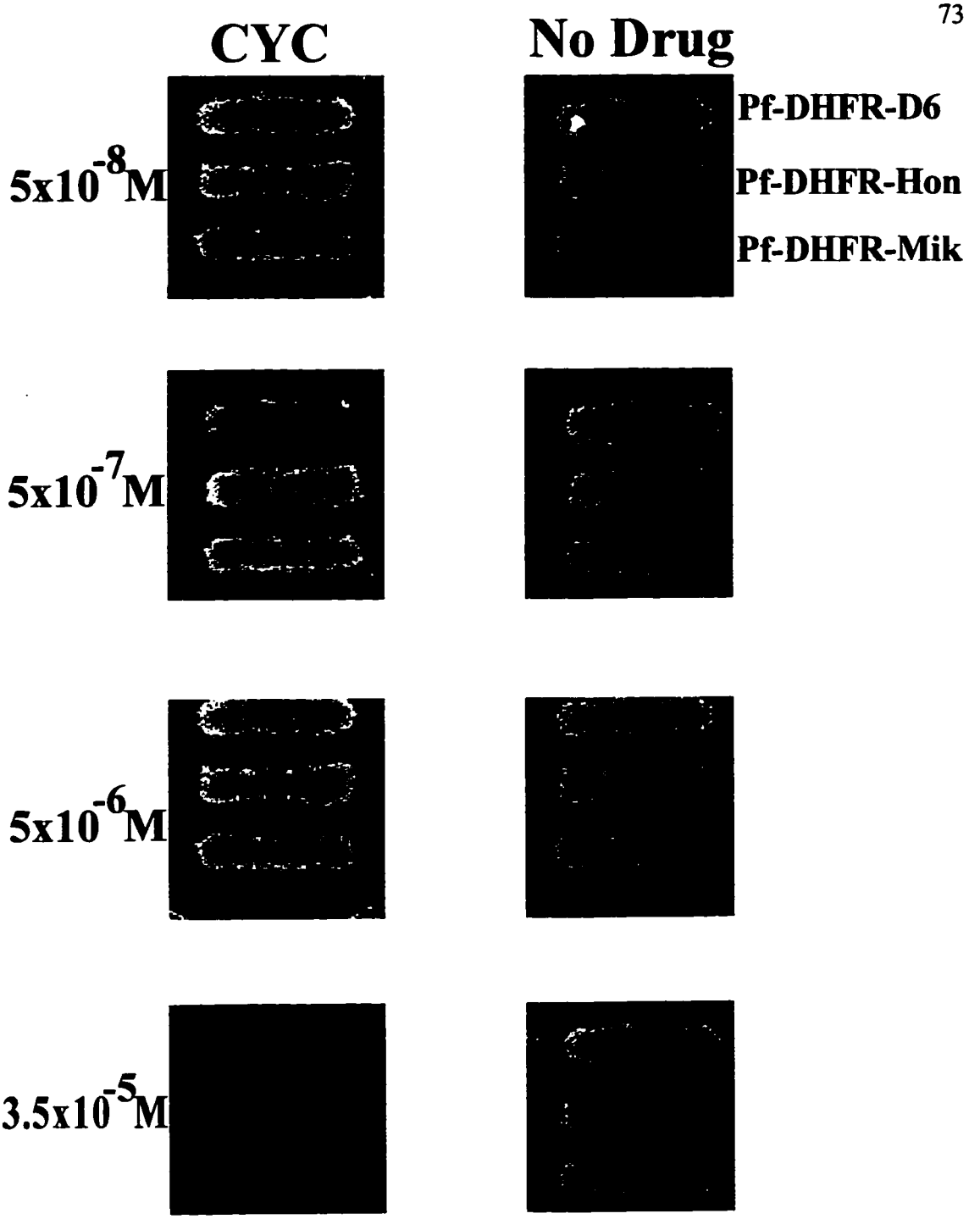


Figure 4-12. Growth of yeast containing Pf-DHFR constructs on cycloguanil media (cyc). Replica plating tests were performed as described in materials and methods.

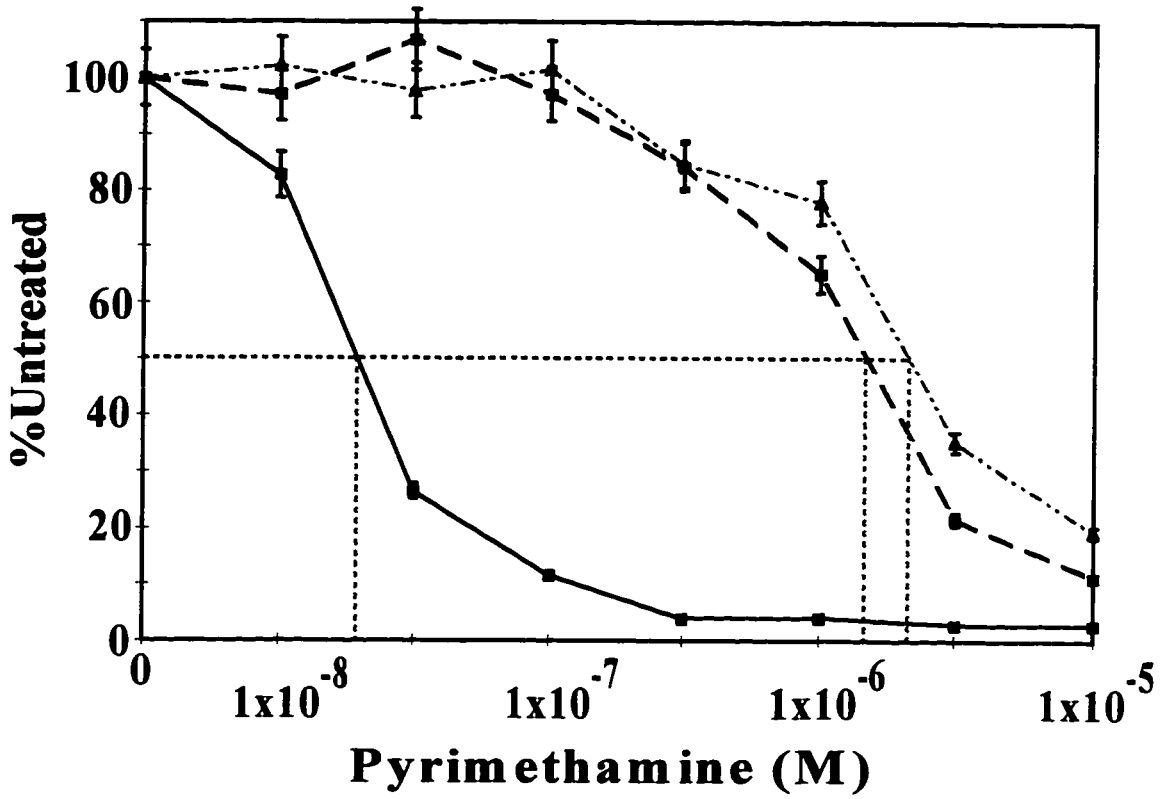


Figure 4-13. Sensitivity of P.f.-DHFR alleles in yeast to pyrimethamine. Transformants containing various DHFR constructs are noted as follows: ——■——, Pf-DHFR-D6; ——■——, Pf-DHFR-Hon; ——▲——, Pf-DHFR-Mik. Curves generated as described in figure 4 legend. IC_{50} indicated by dotted line intersecting x-axis.

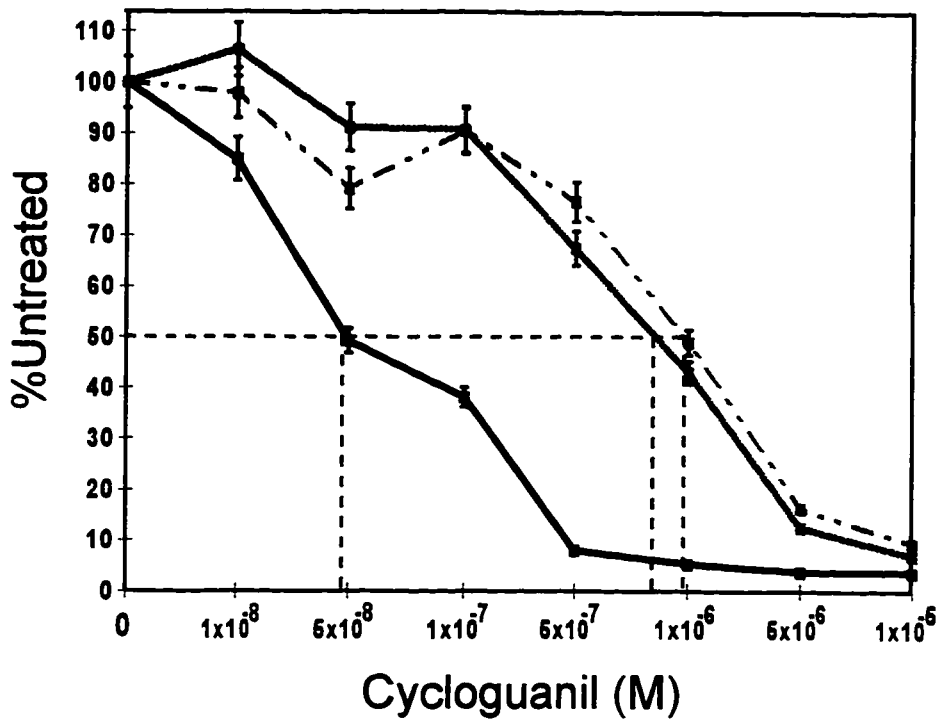


Figure 4-14. Sensitivity of P.f.-DHFR alleles in yeast to cycloguanil. Transformants containing various DHFR constructs are noted as follows: ━✚━ , Pf-DHFR-D6; ━✚━, Pf-DHFR-Hon; ━▲━, Pf-DHFR-Mik. Curves generated as described in figure 4 legend. IC_{50} indicated by dotted line intersecting x-axis.

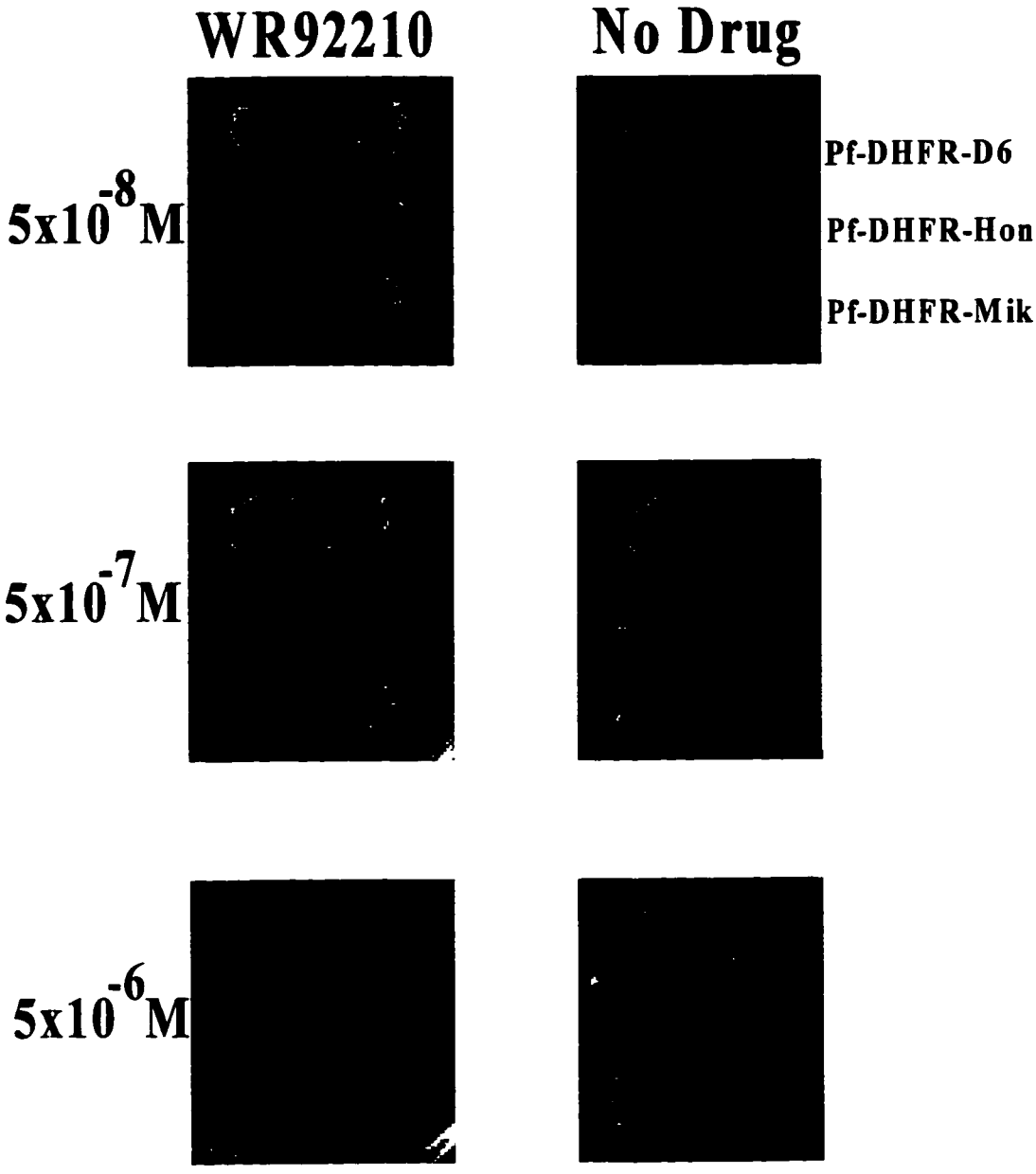


Figure 4-15. Growth of yeast containing Pf-DHFR constructs on WR99210 media. Replica plating tests performed as described in materials and methods.

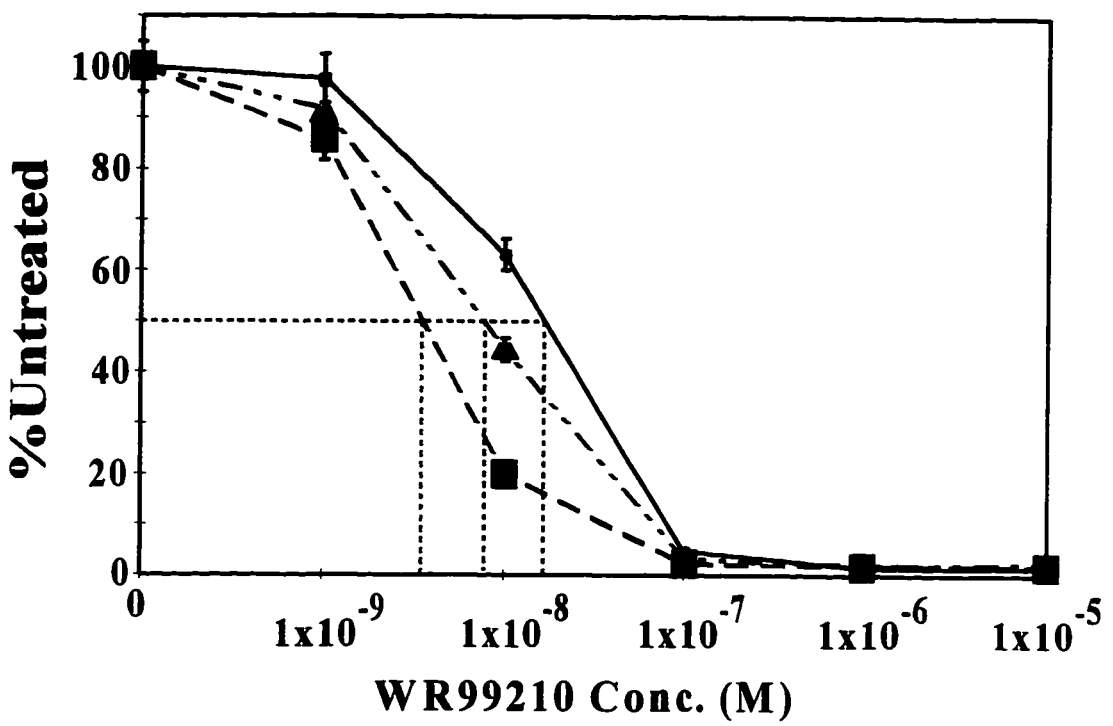


Figure 4-16. Sensitivity of P.f.-DHFR alleles in yeast to experimental malarial drug WR99210. Transformants containing various DHFR constructs are noted as follows: , Pf-DHFR-D6; , Pf-DHFR-Hon; , Pf-DHFR-Mik. Curves generated as described in figure 4 legend. IC_{50} indicated by dotted line intersecting x-axis.

Table 4-1. Comparison of the *P. falciparum* and *S. cerevisiae* DHFR Proteins[a].

Feature	P. falciparum	S. cerevisiae
amino acids	258	211
mol. wt.	30295	24260
acidic residues	14.0%	13.3%
basic residues	15.5%	13.7%
charged residues	29.5%	27.0%
net charge	1.6%	0.5%
hydrophobicity	27.1%	26.5%
potential glycosylation sites[b]	5	0
PEST sequences[c]	0	0

[a]Analaysis performed on GenePro
[b]Based on glycosylation sites for eukaryotic proteins
[c]GenePro modification of Rogers et al algorithm (Science 234:364-368 1986)

Table 4-2. Test for restoration of folate pathway-dependent metabolites.

	Yeast Strain				
Growth Medium*	wildtype	dfr1	vector	Sc-DHFR	Pf-DHFR-D6
- dTMP	+	-	-	+	+
- adenine	+	-	-	+	+
- histidine	+	-	-	+	+
- methionine	+	-	-	+	+
complete	+	+	+	+	+

* Yeast tested on various growth media as described in materials and methods.

Table 4-3. Ability of THF and dTMP to rescue from pyrimethamine inhibition.

	Transformant		
Growth Media	Sc-DHFR	Pf-DHFR-D6	Pf-DHFR-Hon
C (complete)	+	+	+
C + PYR	+	-	+
C + PYR + dTMP	+	+	+
C + PYR + THF	+	+	+
C - His + PYR	+	-	+/-
C - His + PYR + dTMP	+	-	+
C - His + PYR + THF	+	+	+

Pyrimethamine was added at $5x10^{-7}$M. Supplementation with dTMP was at 100 μg/ml and THF at 500 μg/ml. All tests were performed as described in materials and methods using defined synthetic medium.

Table 4-4. Drug sensitivities of Pf-DHFR yeast transformants.

	*IC_{50}(μM)		
Drug	Pf-DHFR-D6	Pf-DHFR-Hon	Pf-DHFR-Mik
Cycloguanil	0.15 ±0.01	1.2 ±0.21	2.9 ±0.21
Pyrimethamine	0.035 ±0.003	3.0 ±0.61	4.1 ±0.44
WR99210	0.030 ±0.0025	0.0057 ±0.0004	0.012 ±0.0038

*Each value represents an average of three independent experiments.

Chapter 5: Selection of Resistance to Malarial DHFR Inhibitors in the Budding Yeast *S. cerevisiae*

I. Introduction

The ultimate goal of the project is to develop a yeast system to screen malarial drugs that target DHFR to determine the rate at which each drug selects resistant mutants in drug sensitive population. At this point, two essential features of the PfDHFR yeast expression system have been confirmed: we have shown that the *P. falciparum* DHFR can substitute for the *S. cerevisiae* DHFR and that these yeast are sensitive to malarial antifolates. The next logical step is to test the capacity of the system to select resistant mutants arising from changes in the DHFR gene.

Several groups have selected antifolate resistant *P. falciparum* parasites in cell culture (Banyal, 1986); Thaithong, 1992), but several disadvantages prevented the careful determination of mutation frequency that is needed to measure the rate at which a given antifolate selects resistant parasites. Since it was not possible to screen large numbers of individual parasites, chemical mutagenesis was required in order to increase the mutation frequency to a level that would allow recovery of the rare DHFR mutant. The number of mutants produced was very small (3-5) and they could only be studied at the population level. Given the experimental ease of propagating and manipulating yeast, it should be possible to design a screen in a manner that would avoid the limitations of cell culture, thus making it feasible to screen large numbers of individual organisms for spontaneous DHFR mutants.

There are many ways for organisms to become resistant to drugs: changes in permeability, active efflux of the drug, sequestration of the drug, metabolic neutralization of the drug, gene amplification or transcriptional upregulation leading to increased gene

expression and increased levels of the target, and finally structural alterations in the drug target. For a yeast screening system to be useful, it must allow for efficient and effective ways to rule out mutants whose phenotypes are due to mutational mechanisms other than mutations within the coding region of the DHFR gene. The generation of mutants arising from changes in the yeast host other than the target gene may be termed "noise". One obvious source of noise is the PDR/MDR (pleiotrophic drug resistance/multidrug resistance) system. Mutations in these genes result in increases of drug efflux via changes in a membrane transporter protein, conferring resistance to a wide variety of inhibitors (Dexter, 1994). Another not so obvious source of noise involved the *tup1* mutation which is required for permeability to dTMP. Since it was not known whether this mutation was also need for permeability to the malarial antifolates, it was possible that resistance would arise if the gene reverted back to wild-type, resulting in impermeability to dTMP and the antifolates. Since in *P. falciparum* the main mechanism of resistance to antifolates is point mutations in the DHFR gene, the yeast system will only be useful if it also uses this mechanism. Therefore it was very important to incorporate tests to rule out the various possible sources of noise in order to efficiently identify mutants of interest.

There were two approaches for doing the selection experiment: (1) using a yeast in which the PfDHFR was expressed from a low copy number plasmid or (2) using one in which the gene had been integrated into the chromosome. While an integrant-based system would help ensure maintenance of the DHFR gene at 1 copy per cell, a plasmid-based system had the advantage of allowing easy mapping of the resistance phenotype to the DHFR. It was thought that the highest amount of noise would arise from non-plasmid related events such as mutations in the yeast host PDR/MDR systems. A plasmid-based system would allow for the rapid elimination of mutants whose phenotype was due to genomic mutations since any phenotype that did not follow the plasmid would not involve changes in the DHFR gene. We decided that the disadvantages of possible missegregation

events creating noise in the screen would be outweighed by the advantage of being able to rapidly identify DHFR-linked mutants.

To evaluate the usefulness of a plasmid-based approach, an experiment was done to select in parallel for spontaneous yeast mutants resistant to pyr and WR. A protocol was created to efficiently and rapidly screen through and characterize the drug resistant candidates. Interesting candidates were tested for linkage of their drug resistance phenotypes to the DHFR gene. Finally, the results of the selection were used to assess sources of noise and to determine how well the system is able to identify mutants with changes in their DHFR genes.

II. Results

A. Rationale for parameters of selection experiment.

Four parameters were considered for doing the selection experiment: plating density, drug concentration, population size, and time allowed for the appearance of drug resistant colonies. In preliminary experiments with 5×10^{-7} M pyr, plating densities greater than 5×10^6 cells / plate resulted in a partial lawn of cells growing on the drug-containing media, suggesting possible exhaustion of the drug and a poor capacity to discriminate between drug resistant and sensitive colonies under these conditions. Since the immediate effect of the antifolates is growth arrest via dTMP starvation and not cell death (Hyde, 1990), it was important to employ a plating density low enough to allow clear resolution of drug resistant colonies from the drug sensitive colonies that were simply in growth arrest. For this reason, a plating density of 1×10^6 cells / plate was utilized.

The drug concentration for doing the selection was also given careful consideration. Concentrations too high would potentially reduce the likelihood of recovering a rare drug resistant mutant since more than one point mutation in the DHFR gene would theoretically be required. On the other hand, concentrations too low would presumably make it easier for plasmid missegregation mutants to pass the screen. For several reasons, the selection was done at a concentration 10X the IC_{50} of each drug ($\approx 5x10^{-7}$ M). First, a 10-fold increase in resistance to an antimalarial is clinically significant. Second, the type of DHFR mutation required for this phenotype ought to be recoverable since a single change at position *ser108* in the DHFR protein is enough to confer resistance to pyr at this level (Banyal, 1986; Thaithong, 1992). Third, since WR had not been used clinically, there was no information either in the field or in cell culture experiments about the level of resistance conferred by point mutation. For this reason and in order to employ similar drug selection strengths for both WR and pyr, we chose a drug concentration that had been successful in selecting pyr^R parasites in the lab. Finally, a drug concentration of 10X the IC_{50} is the same as that used to select mutants in cell culture, providing for some correspondence and comparison between the selections done in *P. falciparum* and yeast.

Another important consideration was the total population size to screen for drug resistant mutants. Since spontaneous point mutation frequencies can range from 10^{-6} to 10^{-10} (Drake, 1991; Lee, 1988; Giroux, 1988; Koeberl, 1990) and it was not known how difficult it would be to generate spontaneous mutants resistant to pyr or WR, we chose a population size of 10^8 as both a reasonable range and a practical target. Also, a plating density of 10^6 cells / plate made it possible to reach this target population with only 100 plates.

The final consideration was the time to allow for the appearance of drug resistant colonies. In preliminary experiments we determined that it took 5 days for colonies to

appear when we plated yeast that express the pyr^R alleles form Honduras1 and Mikenga on media containing 5 x 10^{-7} M pyr. No experiments were performed with WR since even these strains do not grow in the presence of this drug. In addition, the drug-containing media started to lose their activity against drug sensitive yeast after 6-7 days at 30°C (Kelly Hamilton, personal communication). For these reasons, we counted colonies at 5 days for both pyr and WR.

B. Selection for yeast resistant to pyrimethamine and WR99210.

A selection for yeast resistant to pyr and WR was designed based on the parameters described above (see Figure 5-1). In brief, 100 independent cultures were inoculated from 100 independent colonies of yeast containing the drug sensitive D6 DHFR on a plasmid (pTRP-D6). The cultures were allowed to grow to saturation ($\approx 10^8$ cells/ml). A random sampling of 10 cultures indicated the average cell density to be 1.08 x 10^8 cells/ml $\pm$ 0.233. 10^6 cells of each culture were transferred to one plate containing 5 x 10^{-7} M pyr and one plate containing 5 x 10^{-7} M WR. After 5 days incubation, the plates were scored for the number of colonies and the colonies on each plate were classified for their size.

Table 5-1 shows the total number of resistant colonies observed for each plate. On average, more colonies were seen on the pyr plates, although some cultures did give rise to equivalent numbers of colonies on the pyr and WR plates (for example, see tubes 23, 51, and 96). The pyr plates were classified into four groups according to the number of colonies that grew up on each: no colonies, 1-10, 11-20, and >21 colonies (see Figure 5-2). Likewise, the WR plates were classified into four groups that reflected their somewhat lower numbers of colonies: no colonies, 1-5, 6-10, and >10 colonies (see Figure 5-3). Fewer zero plates (2%) were seen for pyr while 10% of the WR plates contained no

colonies. In all, the majority of the WR plates (83%) had 10 or fewer colonies while only 46% of pyr plates contained amounts this low.

In total, 887 colonies were isolated from the pyr plates and 424 from the WR plates, indicating formal mutation frequencies of 8.96×10^{-6} and 4.24×10^{-6} for pyr and WR respectively (Table 5-2A). Since the pyr^R mutants were twice as frequent as the WR^R mutants, it immediately suggested that mutation to pyr^R was more frequent than mutation to WR^R. However, this is in the absence of specific information about the mutation mechanisms that these mutants represent. The frequencies of drug resistant colonies for each size class are shown in table 5-2B. It can be seen that the most prevalent size class for each drug was the small colonies. The selection with WR resulted in fewer medium size colonies than pyr (frequency of 1.2×10^{-6} vs. 7.7×10^{-7}), while the two drugs resulted in approximately the same frequency of large colonies. The most dramatic difference between the pyr and WR mutant collections was in the frequency of small size colonies: the pyr selection yielded almost 2-fold more small colonies.

Finally, after 9-10 days of incubation, an average of 62.5 additional colonies grew up on each pyr plate and an average of 55 additional colonies on each WR plate. These were presumed either to be plasmid missegregation mutants with enough additional DHFR copies to confer weak drug resistance or to represent the slow recovery and growth of drug sensitive yeast due to a loss in drug activity. The frequencies of these mutants were approximately 5 to 6×10^{-5}, much higher than one would expect for spontaneous point mutations. For these reasons, these colonies were not analyzed further.

C. Retest of drug resistance phenotype.

The day 5 colonies were subjected to a three level analysis to identify members of each mutant collection that arose from changes in the PfDHFR gene (Figure 5-4). Level 1

tests were performed to eliminate contaminants and retest for the original drug resistance phenotype. In level 2, the drug resistance phenotype was characterized more sensitively using a double replica plating assay along with a test for cross-resistance to other malarial antifolates. Also, tests were done to eliminate mutants whose phenotype could be due common yeast drug resistance mechanism not related to the DHFR gene. Finally, in level 3 mutants were tested to see if the resistance phenotype was linked to the DHFR gene and whether point mutations in the gene were involved.

The drug resistant isolates of each collection were patched and grown under nonselective conditions (Figure 5-4). They were then tested for the presence of the correct nutritional markers and for growth on either pyr or WR (Table 5-3). 7 candidates from both the pyr and WR collections were found to have incorrect strain markers, suggesting they were contaminants. 137/887 of pyr^R mutants did not retest as drug resistant while 29 had questionable drug resistance. Likewise, 84/424 WR^R mutants did not retest for drug resistance and 13 had questionable resistance. Altogether, the level 1 tests eliminated 15% of the pyr^R mutants and 20% of the WR^R mutants, indicating pass rates of 85% and 80% respectively.

A correlation was found between the original colony size of a mutant and the failure to retest as drug resistance. All of the colonies that failed to retest from the pyr collection were small while 89% of the WR failures were small (see Figure 5-5). 18% of the pyr small colonies and 24% of WR small colonies failed to retest as resistance. These were not contaminants as they had the correct strain markers. This suggested that the small colonies represented a borderline drug resistance class possibly resulting from slight changes in DHFR copy number via plasmid missegregation errors.

D. Characterization of drug resistance profiles for the medium/large size mutants.

In level 2 analysis, the candidate mutants were characterized in more sensitive ways for drug resistance and cross-resistance to other malarial antifolates of clinical importance. Also, the mutants were tested for common yeast drug resistance mechanism unrelated to the DHFR gene. For the following reasons, this analysis was only performed on the medium and large size mutants. First, their appearance and size on drug plates during the selection were more similar to that of yeast containing the Honduras1 and Mikenga drug resistant DHFRs. Second, their lower mutation frequencies seemed to be closer to the range of spontaneous point mutations while the frequencies for the small colonies seemed unreasonably high. Finally, the correlation between colony size and drug resistance failure suggested that the small colonies were borderline: some were past a threshold where small increases in copy number via plasmid missegregation errors resulted in marginal drug resistance for some colonies and transient resistance for others.

Removal of the small colonies from consideration resulted in a different colony distribution profile for the pyr^R and WR^R mutant collections (Table 5-4). First, more plates of the zero class can be seen: 50% instead of 2% and 59% instead of 10% for pyr and WR respectively. In classical genetic analysis of selection for rare drug resistant variants, it is expected that at least 1/3 of the plates will contain no colonies (Luria, 1943). This is necessary to be 95% confident that a colony really originated from one cell and not two or more, using the Poisson distribution. For both mutant collections, the majority of the plates are found to have 5 or fewer colonies. Finally, the mutation frequencies are reduced to 1.33×10^{-6} for pyr and 9.5×10^{-7} for WR, closer to the range of spontaneous mutations.

Table 5-5 summarizes the results of subjecting the pyr^R and WR^R medium/large size mutants to level 2 analysis. The mutants were tested for their growth on pyr, WR, and cyc. All of the candidates retested as drug resistant using this more sensitive double replica plating technique. Tests were made for high level resistance (i.e., growth at drug

concentrations 10-fold higher than used during the selection). The only candidate (pyr^R) to show resistance at a drug concentration 100X the IC_{50} was later revealed to be a contaminant. 92% of the WR^R candidates showed cross-resistance to the other malarial antifolates while only 71% of the pyr^R candidates did. Fewer showed cross-resistance to cyc: 23% of the pyr^R candidates and 51% of the WR^R candidates.

Since it was not known whether the *tup1* mutation was required for sensitivity to the antifolates, the mutants were tested for possible changes in drug permeability via reversion at the this locus. The status of the *tup1* gene is confirmed by testing for growth at an antifolate concentration (100X pyr or WR) that should result in inhibition of the drug resistant strains and then testing for growth in the presence of drug with dTMP. Rescue of growth from the drug-inhibition by dTMP indicates that the yeast are still permeable to this agent and by inference permeable to the antifolates. All candidates were found to be rescued from drug-inhibition by the addition of dTMP, indicating the status of the *tup1* gene was unchanged. Finally, it was determined whether the mutant phenotype could be ascribed to the yeast PDR/MDR drug resistance systems, using resistance to a cyclohexamide as a test (Dexter, 1994). The mutant collections were tested for their ability to grow in the presence of cyclohexamide. The growth of all candidates was inhibited by cyclohexamide suggesting that the drug resistance was specific to the antifolate class of drugs and did not involve a PDR/MDR type mechanism. Taken together, the cyclohexamide sensitivity and *tup1* reversion data suggests a very low amount of "noise" in the system due to these potential drug resistance mechanisms. In total, 102/103 pyr^R and 73/73 WR^R candidates were able to pass the level 2 tests, indicating pass rates of 99% and 100% respectively. This suggests that candidates that pass the level 1 tests are likely to pass level 2.

Based on the level 2 tests, the pyr^R and WR^R mutant collections could be separated into 3 classes according to their resistance to pyr, WR, and cyc (Table 5-6). The first

class for each collection consisted of mutants resistant solely to the drug used during the selection (class I). The second class showed cross-resistance to either WR or pyr (class II). A third class showed resistance to all 3 antifolates (class III). The most frequent class in the pyr^R mutant collection was class II ($pyr^R wr^R cyc^S$) comprising 47% of the mutants while the most frequent class for the WR^R mutant collection was class III ($wr^R pyr^R cyc^R$) comprising 50% of the collection. The least frequent class (23%) for pyr was class III ($pyr^R wr^R cyc^R$) and for WR (8.2%) class I ($wr^R pyr^S cyc^S$). Since resistance to WR was found most frequently (92%) accompanied by cross-resistance to either pyr or cyc, it suggested that changes in copy number were involved since DHFR gene amplification is predicted to confer broad spectrum resistance to all three antifolates. This was supported by the high number of WR^R mutants that showed cross-resistance.

The distribution of phenotypes for the pyr^R mutants is shown in table 5-7. While some plates gave rise to mutants with identical phenotypes (for example, see plates 1, 5, and 3), many contained mixed phenotypes. Likewise, examination of the phenotypic distributions for the WR^R mutants revealed that some plates had mixed phenotypes while others had colonies of identical drug resistance patterns (Table 5-6). Interestingly, 5 WR plates gave rise to colonies that were originally $wr^R pyr^R$ then later retested as $wr^S pyr^R$ (see plates 30 and 56 for example). This suggested that the original WR^R phenotype was not the result of a stable mutation mechanism.

E. Test for plasmid missegregation.

Representative candidates from each mutant collection were subjected to level 3 analysis in order to determine whether the drug resistance was mediated by mutations in the DHFR gene. As a first step, tests were made using a plasmid missegregation assay in order to determine whether the resistance was due to amplification of the DHFR gene via changes in plasmid copy number. It was important to eliminate mutants that utilized a

gene amplification/over-expression mechanism since this phenomenon has not been observed in resistant isolates of *P. falciparum*. The simplest yeast mechanism for changes in plasmid copy number is missegregation errors occurring during the mitotic growth of centromere-containing plasmids. Plasmid missegregation errors (plasmid lost and gain) have been observed to occur in *S. cerevisiae* at a frequency of 2.0% / division (Koshland, 1985). The underlying principle of the plasmid missegregation assay is that the extra plasmid copies should be lost along with the resistance phenotype in the absence of drug selection after many generations of outgrowth if the original increase was due to missegregation errors.

A single colony of each test candidate that had grown up under nonselective conditions (no drug) was inoculated into a liquid culture, grown to high density (i.e., saturation), a small amount transferred into a fresh culture, and then grown to high density again (see Figure 5-6). This ensured that the candidate would have undergone approximately 40 generations of outgrowth under nonselective conditions. Equivalent amounts of the final culture were plated on media containing no drug (control) and media with pyr or WR. After 5 days the control and drug plates are expected to contain equivalent amounts of colonies if the original drug resistance phenotype was due to a stable mutation mechanism. However, it the phenotype was due to a plasmid missegregation error, the extra copies will be lost and the drug plate should contain fewer colonies than the control plate.

Figure 5-7 shows potential mutation mechanisms that could give rise to a drug resistance phenotype and how the drug resistance would be maintained or lost under conditions of nonselective outgrowth. The models are based on the amount and condition of the DHFR protein present. In scenario A, a strain contains a single copy of the wild-type sensitive DHFR gene (D6) giving rise to a hypothetical 2 molecules of DHFR protein. This strain is predicted to be drug sensitive before and after outgrowth. In

scenario B, a strain contains a single copy of a DHFR gene with point mutations (Hon) giving rise to 2 structurally altered DHFR molecules which confer stable drug resistance before and after outgrowth. Scenario C involves a yeast strain which is drug resistant due to a stable increase in plasmid copy number via changes in the plasmid maintenance elements (the D6 DHFR gene on a 2μM plasmid). These changes could be mediated by point mutations in the plasmid CEN sequences which normally would insure maintenance of the plasmid at low copy number. Such mutations would lead to a stable increase in plasmid copy number and consequently stable amplification of the DHFR gene resulting in increased DHFR protein levels. The hypothetical 8 molecules of DHFR protein would be maintained under conditions of nonselective outgrowth. In scenario D, the drug resistance is mediated by increases in gene expression via stable mutations in the DHFR gene promoter. The over-expression is predicted to be maintained during outgrowth resulting in a strain that is still drug resistant at the end of the assay. It is important to note the scenarios B, C, and D are indistinguishable in the assay since all three are predicted to give a similar phenotype. Finally, scenario E denotes a situation in which an increase in DHFR protein level is mediated by an increase in plasmid copy number via plasmid missegregation mutations. The extra plasmid copies are lost during nonselective outgrowth resulting in a decrease in DHFR expression. The subsequent reduction in DHFR protein levels is predicted to result in a drug sensitive phenotype at the end of the assay.

The results of the plasmid missegregation analysis of the representative pyr^R mutants are shown in table 5-9. For all samples, the number of colonies observed on the drug plate is expressed as a percentage of the colonies found on the control plate. In the case of the controls, the results from two independent experiments are shown. The starting drug sensitive strain for the selection experiment, pTRP-D6, yielded a number of colonies on pyr that was in the range of 2-25% of the control plate. A control strain containing point mutations in the DHFR gene, pTRP-Hon, yielded 82-102% of the

colonies of the control plate. Expressing the drug sensitive D6 DHFR gene on a high copy plasmid (D6-2μM) resulted in comparable amounts of colonies (79-105% of the control plate). When the mutants were examined, the only medium/large size mutant that clearly tested as containing a stable mutation was P4-B1 ($pyr^Rwr^Rcyc^R$) which yielded equivalent amounts of colonies on the control and pyr plate (105%). The rest of the candidates were either marginal or not statistically distinguishable from the pTRP-D6 control. No class I ($pyr^Rwr^Scyc^S$) or class II ($pyr^Rwr^Rcyc^S$) behaved as containing a stable mutation. To test the validity of excluding the small size class from level 2 and 3 analysis, 3 small size candidates were examined in the assay. Two yielded numbers indicating stable mutations (see P15s-B1 and P6s-E4). These consisted of the phenotype $pyr^Rwr^Rcyc^S$. In total, 3 out of 11 candidates were suggested by the assay to have their drug resistance mediated by stable mutation mechanisms.

The results of the plasmid missegregation analysis of the representative WR^R mutants are listed in table 5-10. As before, the results from two independent experiments are shown for the controls. Both the pTRP-D6 drug sensitive control and the pTRP-Hon pyr resistant control produced no colonies on the WR plate. This was expected since WR is an efficient killer of both pyr sensitive and resistant strains. The strain expressing the drug sensitive D6 DHFR gene on a high copy plasmid (D6-2μM) yielded 56-76% of colonies on the control plate. When the medium/large size mutants were tested, the only mutant that performed as expected for a stable mutation had the phenotype $wr^Rpyr^Rcyc^R$ (W3-F1) and produced equivalent numbers of colonies on the control and drug plate. Interestingly, the mutant W1-I2 whose phenotype was $wr^Spyr^Rcyc^S$ yielded a number of colonies on the WR plate that was comparable to the D6-2μM control (75%). It is difficult to explain why this WR^S mutant would yield colonies on the WR plate. Several small size WR^R candidates were tested. A mutant of the phenotype $wr^Rpyr^Rcyc^R$ behaved as a stable mutation (W9s-B1). Also, a noncross-resistant mutant (W1s-J3) tested as

arising from a stable mutation. This is in contrast to the medium/large WR^R collection or the pyr^R collection in which none of the noncross-resistant candidates passed the plasmid missegregation test. In total, 4 out of 11 WR^S candidates tested were suggested to arise from stable drug resistance mutations. The fact that stable mutants were obtained from both the pyr and WR small size mutant collections suggests that the original assumptions of this class consisting predominantly of missegregation mutants may not be correct.

F. Tests for linkage of drug resistance to DHFR gene.

To test for linkage of drug resistance phenotypes to the plasmid and possible linkage to the DHFR gene, plasmid DNA was isolated from each candidate mutant and transferred into a new yeast host. The transformants were tested for their drug resistance phenotypes. If the original phenotype was seen, this would indicate that the mutations responsible for the resistance were contained on the plasmid. Conversely, if the original phenotype was not seen, then this would suggest that the mutations responsible for the resistance were most likely contained in the genome of the original yeast host. In table 5-11 the plasmid linkage results of representative pyr^R mutants are listed along with their respective size classes and performance in the plasmid missegregation assay. No clear relationship was seen between colony size, performance in the plasmid missegregation assay, and linkage to the plasmid. Out of 22 pyr^R candidates tested, 8 showed plasmid-linkage to some degree, but in cases of plasmid-linkage the complete resistance profile did not always follow the plasmid. For example, transformants containing a plasmid isolated from a pyr^R and WR^R mutant (P3-I1) was found to exhibit resistance to only pyr. Interestingly, a candidate (P4-E4) that was questionably mediated by a stable mutation mechanism showed plasmid-linkage for the resistance phenotype.

Plasmid-linkage analysis was also performed for representative WR^R mutants with results similar to the pyr^R candidates (Table 5-12): incomplete transfer of the resistance

phenotype and no clear relationships between size, plasmid missegregation results, and linkage to the plasmid. Only 2 out of 17 WR^R candidates tested showed linkage to the plasmid. In both mutant collections, medium/large size candidates were identified that were resistant to all three antifolates and behaved as arising from a stable mutation mechanism in the plasmid missegregation assay (see P4-B1 on Table 5-11 and W3-F1 on Table 5-12). However, both of these stable mutants were found to show no plasmid-linkage, indicating that the original resistance phenotype was mediated chromosomally by some mechanism that the screen did not account for.

Finally, cycle sequencing was performed to examine the DHFR genes of interesting mutants that showed plasmid linkage (Table 5-13). For the 12 candidates sequenced, the DHFR genes were found to contain sequences identical to that of the starting plasmid pTRP-D6 (data not shown). This suggests that the resistance phenotype could be due to other plasmid-related mechanisms such as mutations in either the promoter or centromere sequences.

III. Discussion

A screen for yeast resistant to pyr and WR was done using yeast containing a drug sensitive *P. falciparum* DHFR gene on a plasmid. A large number of mutants (887 pyr^R and 424 WR^R) was generated in a relatively short period. Simple replica plating experiments allowed for quick characterization of resistance profiles and tested against some known yeast drug resistance mechanisms. No cyclohexamide resistance was seen for any of the candidates, suggesting a low amount of noise from the PDR/MDR systems. Also, reversion of the *tup1* gene was not seen for any candidates suggesting a low amount of noise from this potential impermeability mechanism or alternatively that the *tup1*

mutation is not involved in permeability to antifolates. Since there were no estimates available about the frequency of spontaneous reversion at the *tup1* gene, it was not possible to predict how many revertants should be seen in the 10^8 cells screened.

Plasmid missegregation analysis of representative candidates indicated that the resistance phenotype of 3/11 pyr^R and 4/11 WR^R mutants was mediated by stable mutation mechanisms. The remainder tested as plasmid missegregation mutants. If this representative sampling is indicative of the complete mutant collections, it suggests a high amount of "noise" in the screen from plasmid missegregation errors. Additional sources of noise are suggested by the fact that only 1/2 of the pyr^R candidates and 1/3 of the WR^R candidates which tested as stable in the missegregation assay were found to have their phenotypes linked to the plasmid. These mechanisms presumably represent chromosomal mutations in the yeast host that the screen did not account for.

Overall, 8 out of 22 pyr^R candidates and 2 out of 17 WR^R candidates were found to have their phenotypes linked to the gene. No clear relationship was seen between size, performance in the plasmid missegregation assay, and linkage to plasmid. This suggests that the rationale for focusing solely on the large/medium size colonies may have been fallacious.

No DHFR mutations were identified in the candidates whose phenotype was linked to the plasmid. This suggests additional sources of noise such as mutations in the promoter or centromere sequences. These mutational mechanisms are indistinguishable from DHFR mutations in both the plasmid missegregation assay and the plasmid-linkage test since all three types of mutation would occur on the plasmid. Centromere mutations leading to aberrant plasmid segregations have been observed in yeast (McGrew, 1986) and could potentially result in plasmid copy number mutants. However, it is difficult to predict how stable these mutants would be in the absence of drug selection. Mutations in the

yeast DHFR promoter have not been studied, but formally represent a source of noise that would be present in both a plasmid and chromosomal based expression system for the PfDHFR construct. Finally, these results raise the possibility that the majority of the candidates for both mutant collections can be ascribed to either plasmid missegregation errors, mutations in the promoter, or mutations in the plasmid centromere sequences.

The biggest elimination of candidates (15-20%) was at level 1 in which mutants did not retest for the original drug resistance phenotype. Level 2 tests (drug resistance profile characterization, PDR/MDR tests, and *tup1* reversion tests) resulted in the elimination of only an additional 1% of the candidates. The results of the level 3 tests (plasmid missegregation and DHFR linkage analysis) indicates that potentially another 64-88% of pyr^R and WR^R candidates could be discounted as either plasmid missegregation mutants or as having mutations not contained on the plasmid. If true, these results altogether suggest that potentially up to 88% of the drug resistant candidates could be eliminated.

Plasmid copy number mutations are predicted to result in resistance to all three of the antifolates used in the study. However, for both the pyr^R and WR^R mutant collections, a class was seen with resistance only to pyr and WR (see Table 5-6, class II mutants). Figure 5-8 shows a simple model for how cumulative plasmid missegregation errors could lead to some of the observed drug resistance phenotypes. Tests for cross-resistance involved examining the growth of candidates on media containing each antifolate at a concentration 10X the IC_{50}. The cyc concentration used was later revealed to actually be 15X the IC_{50}. Therefore, different amounts of over-expression would be need to see resistance to pyr, WR, and cyc. Based on this, the starting strain was drug sensitive ($cyc^S pyr^S wr^S$) due to presence of only 1 copy (1N) of a wildtype DHFR present. Occurrence of a plasmid missegregation error led to a hypothetical 2 copies of the plasmid (2n) and enough extra DHFR enzyme present to confer resistance to pyr and WR.

Additional missegregation errors led to the accumulation of more plasmid copies (4N) which resulted in the production of enough additional enzyme to confer resistance to cyc. While the model accounts for the WR mutant collection in which there were two major phenotypes seen ($wr^R pyr^R cyc^S$ and $wr^R pyr^R cyc^R$), it fails to account for the frequency of noncross-resistant mutants (29% were $pyr^R wr^S cyc^S$) seen in the pyr mutant collection.

Since colony growth would be proportional to the amount of DHFR enzyme present, another possibility raised by the model is that the small, medium, and large size colonies represent intermediate steps in the evolution from drug sensitivity to resistance to all three drugs. The different sizes could reflect different degrees of cumulative missegregation errors. If true, the frequencies observed for each size class could reflect the probability of having enough missegregation errors to accumulate the number of plasmid copies needed to grow to a specific size. For example, the frequency of accumulating enough missegregation errors to grow to a large sized colony would be approximately 1.3×10^{-7} (recall Table 5-2).

The results of the selection experiment strongly indicate that a plasmid-based expression system for the PfDHFR gene is not the most efficient approach to obtain antifolate resistant mutants with mutations in the DHFR gene. A high amount of noise due to plasmid missegregation mutations was suggested. Also, none of the promising mutants sequenced so far were found to have mutations in their DHFR genes.

One issue remaining to be resolved is whether the *tup1* mutation is really required for permeability to the malarial antifolates. The mutation is necessary for the uptake of dTMP by yeast cells and may have fortuitously led to permeability to other molecules. To test whether this is true, isogenic strains containing the PfDHFR gene who only differ at the *tup1* locus could easily be constructed. If, for example, the strain with the wildtype

gene (*TUP1*$^+$) is found to be insensitive to pyrimethamine, this would indicate that the mutation is required and that it is important to include tests for *tup1* reversion in future yeast screens. The *tup1* mutation has been found to have many pleiotrophic effects on diverse yeast functions (Keleher, 1992). The mechanism by which this is accomplished is poorly understood. Although the classical test for reversion was used in this study, there could be other possible perturbations that could give rise to resistance given the complexity of the *tup1* transcription factor. Since this would represent a source of noise present in both a plasmid-based and integrant-based system, it will be important to determine if the classical test is able to account for these additional potential perturbations.

As described earlier, four parameters were considered for doing the selection experiment: plating density, drug concentration, population size, and the time allowed for the appearance of drug resistant colonies. With few exceptions, the plating density used (10^6 cells / plate) proved to allow easy identification and isolation of resistant yeast colonies. It is difficult to evaluate at this point whether a selection for yeast 10-fold more resistant was feasible, but the results of this study do not present any reason why this drug concentration would be impractical. Screening through a total population of 10^8 cells was clearly found to be feasible. Finally, isolating colonies that arise from the drug plates on day 5 still remains a reasonable target since after 7 days a loss of drug activity has been observed.

One interesting issue raised by the selection experiment is based on the drug sensitivities of yeast transformants containing the DHFR genes from the malarial strains D6-S/L, Honduras1, and Mikenga. The pTRP-D6 strain is completely drug sensitive while pTRP-Hon and pTRP-Mik are resistant to both pyr and cyc. These drug resistant strains have been observed to be actually more sensitive to the experimental drug WR than the drug sensitive strain pTRP-D6. A prediction that follows from this collateral

hypersensitivity is that mutants selected for resistance to pyr should be of increased sensitivity to WR. Therefore, the frequency of pyr^R mutants observed that are cross-resistant to WR should be very low. However, in the selection experiment 48% of the pyr^R mutants were found to be cross-resistant to WR. One explanation of this result is that this class of mutants represent DHFR mutations different from the ones found in the Honduras1 and Mikenga DHFRs that for some reason do permit cross-resistance WR. Another explanation for the frequency of these mutants is that they are not due to changes in the DHFR, but instead are the result of copy number changes. This would involve a mutation mechanism in which collateral hypersensitivity forces would not be in operation. Given the plasmid missegregation and linkage data, this second alternative seems more likely.

In summary, this study has demonstrated the potential of a yeast heterologous PfDHFR expression for screening and characterizing a large number of drug resistant candidates in an efficient manner. It was possible to generate mutants in a single step without mutagenesis. The results of the selection experiments have provided numerous suggestions for optimizing the yeast screening system in order to isolate the rare DHFR mutants of interest more efficiently. Possible ways to improve the system will be discussed in the concluding chapter.

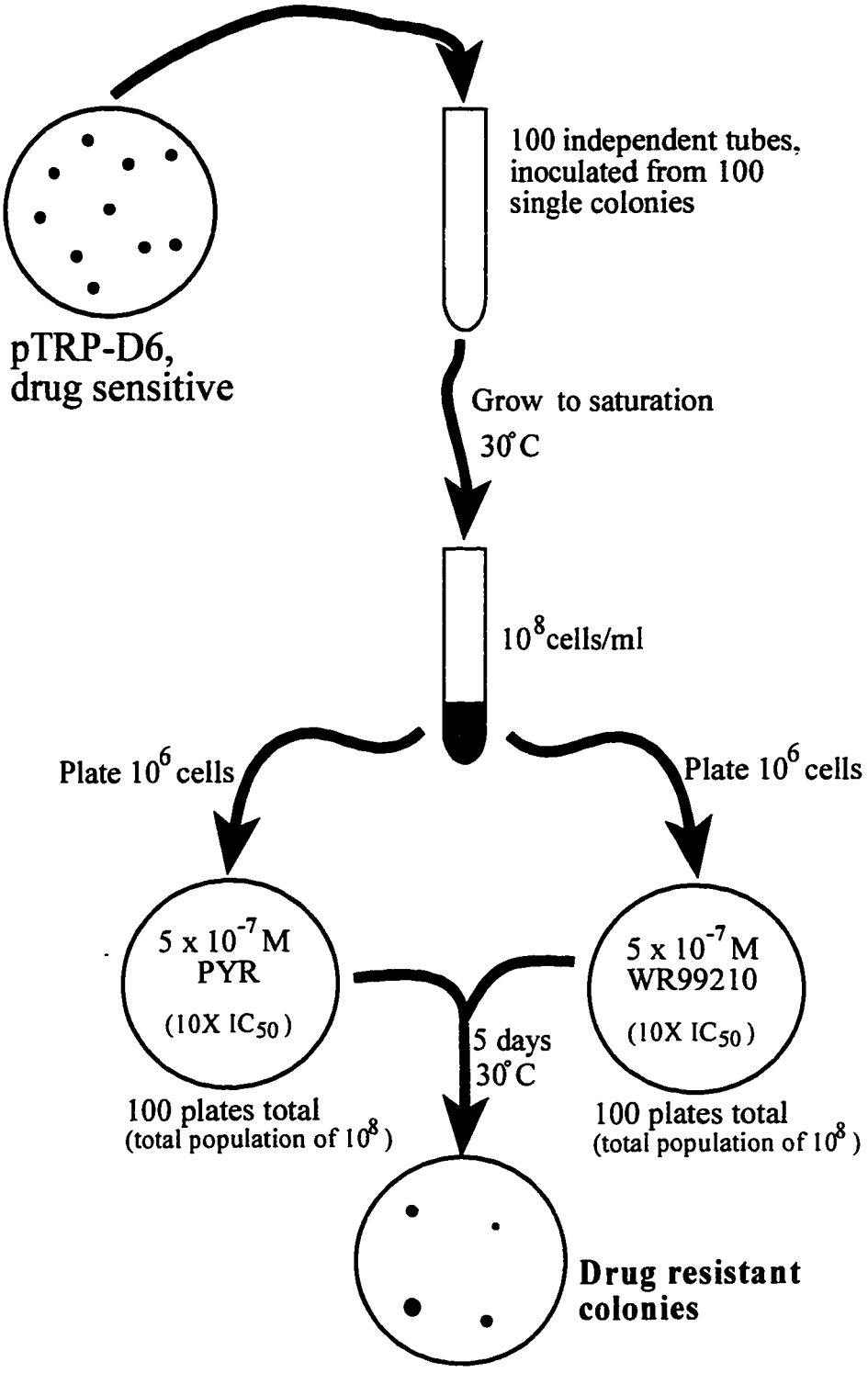


Figure 5-1. Selection for drug resistant mutants using yeast containing the plasmid-borne drug sensitive DHFR gene.

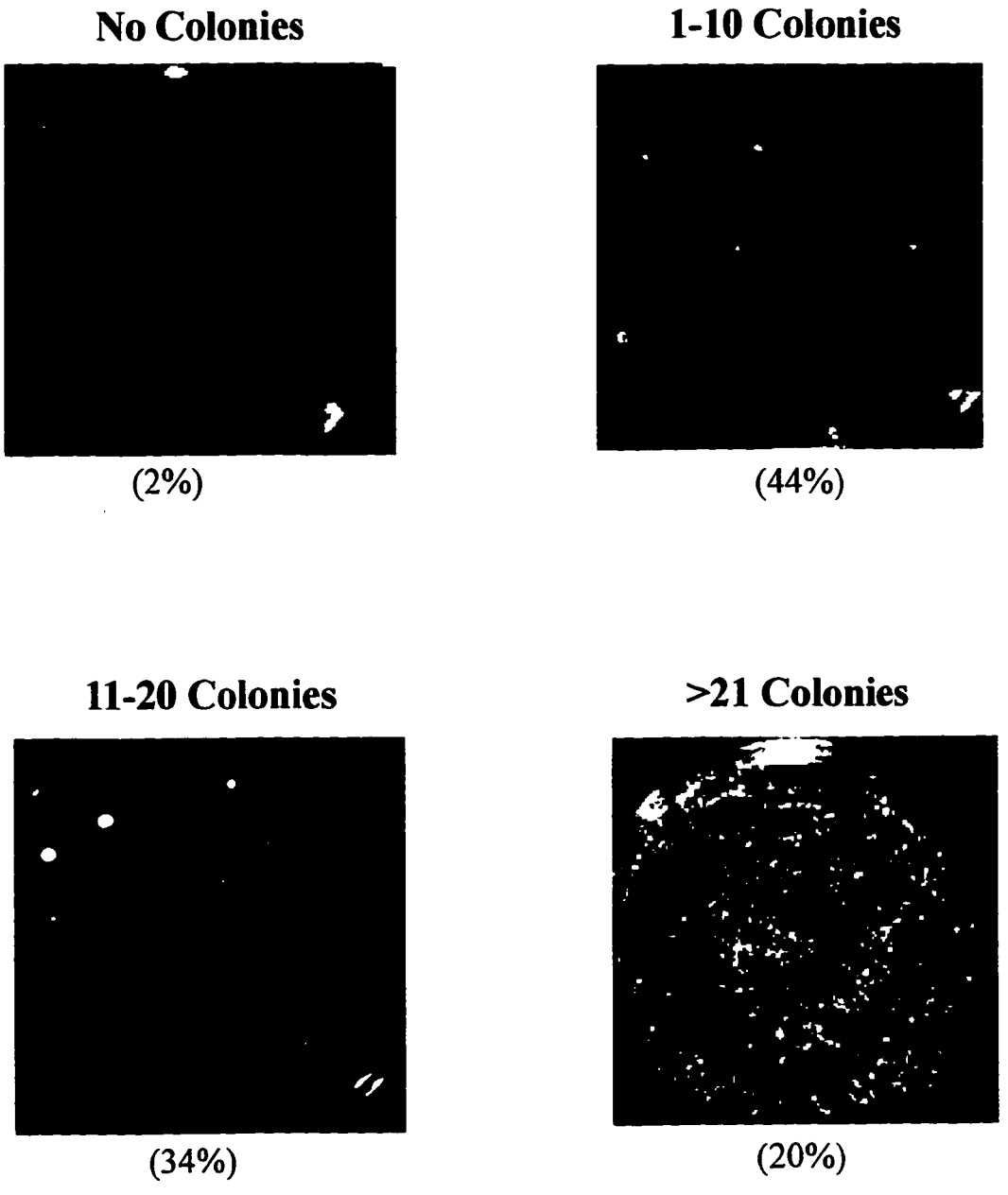


Figure 5-2. Pyrimethamine plates containing varying numbers of drug resistant colonies. Listed below are the percentages of the total number of plates that each category represents.

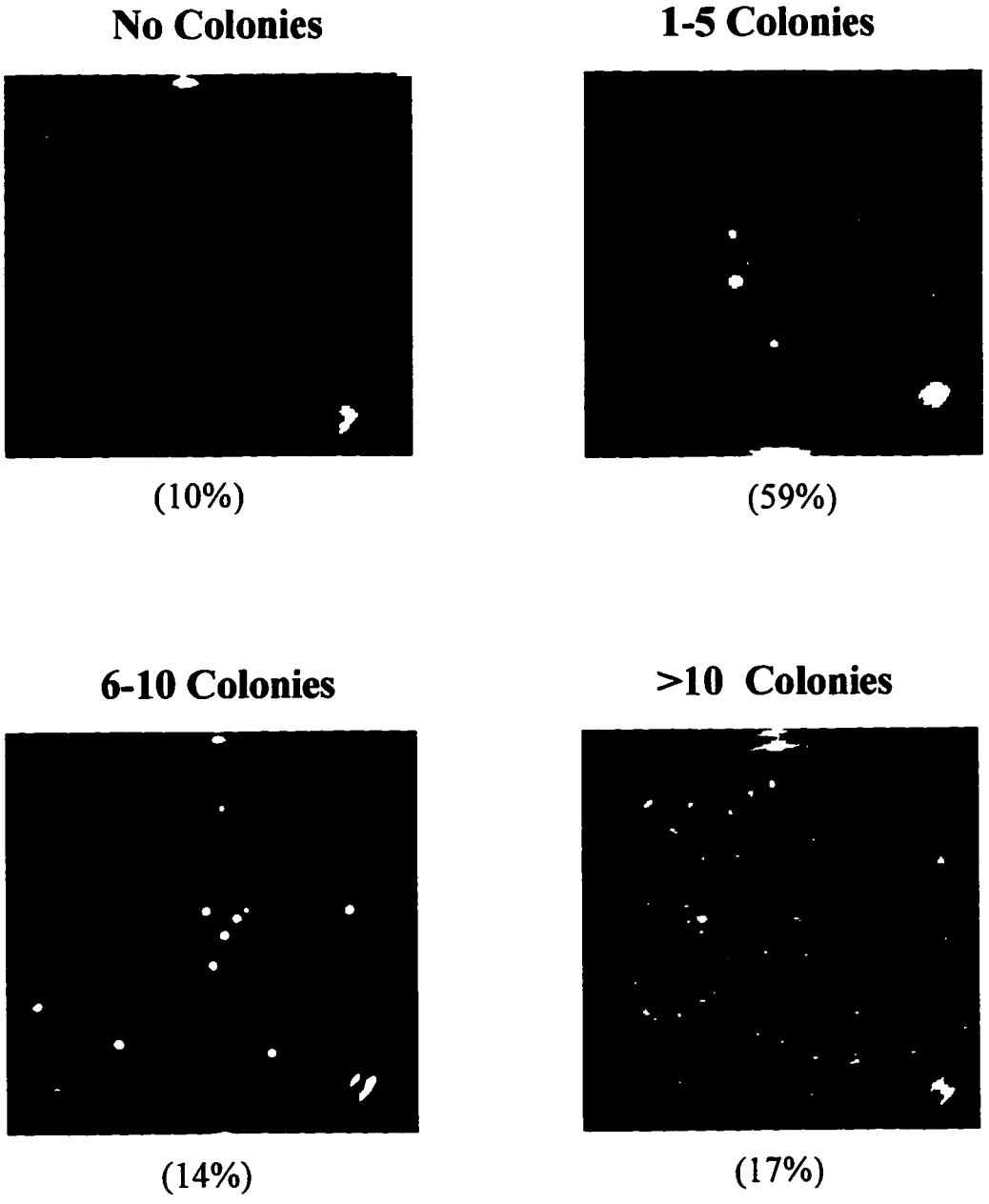
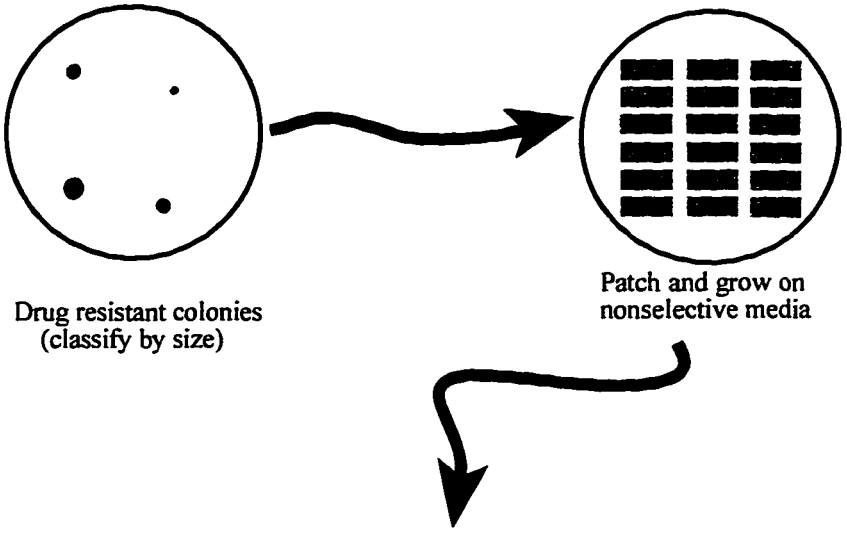


Figure 5-3. WR99210 plates containing varying numbers of drug resistant colonies. Listed below are the percentages of the total number of plates that each category represents.



Level 1 Tests:

1. Test for correct markers
2. Retest for drug resistant phenotype

Level 2 Tests: Double replica plating analysis

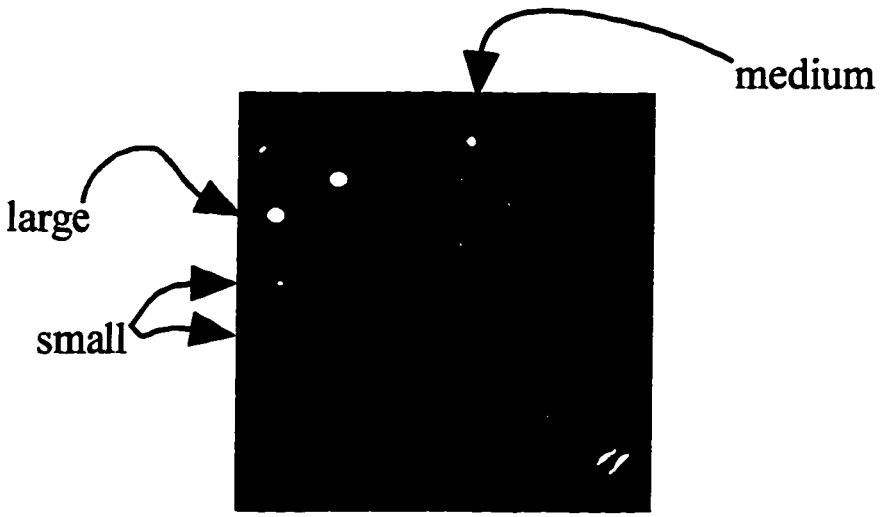
1. Growth on 10X PYR, CYC, or WR (original phenotype and cross-resistance)
2. Growth on 100X PYR or WR (high level resistance)
3. Growth on dTMP + 100X PYR or WR (reversion at tup1 locus)
4. Growth on cyclohexamide (nonspecific resistance, pleiotrophic drug resistance)

Level 3 Tests:

1. Plasmid missegregation assay (plasmid copy number changes)
2. Plasmid transfer to new yeast host (phenotype linkage to plasmid)
3. cycle sequencing (identify DHFR mutatations)

Figure 5-4. Strategy for characterization of drug resistant yeast.

A



B

	Pyrimethamine Colonies			WR99210 Colonies		
Size	Failures	Total #	%	Failures	Total	%
small	137	757	18.1	79	329	24.0
medium	0	117	0.0	5	77	0.0
large	0	13	0.0	0	18	0.0
Total	137	887	100.0	84	424	100.0

Figure 5-5. Relationship between colony size and drug resistance failure. A) Size range of resistant colonies obtained on drug plates after 5 days at 30 C. B) Number of pyr^R and wr^R colonies that failed to retest as drug resistant in each size class. Failures are expressed as a percentage of the total for each size class.

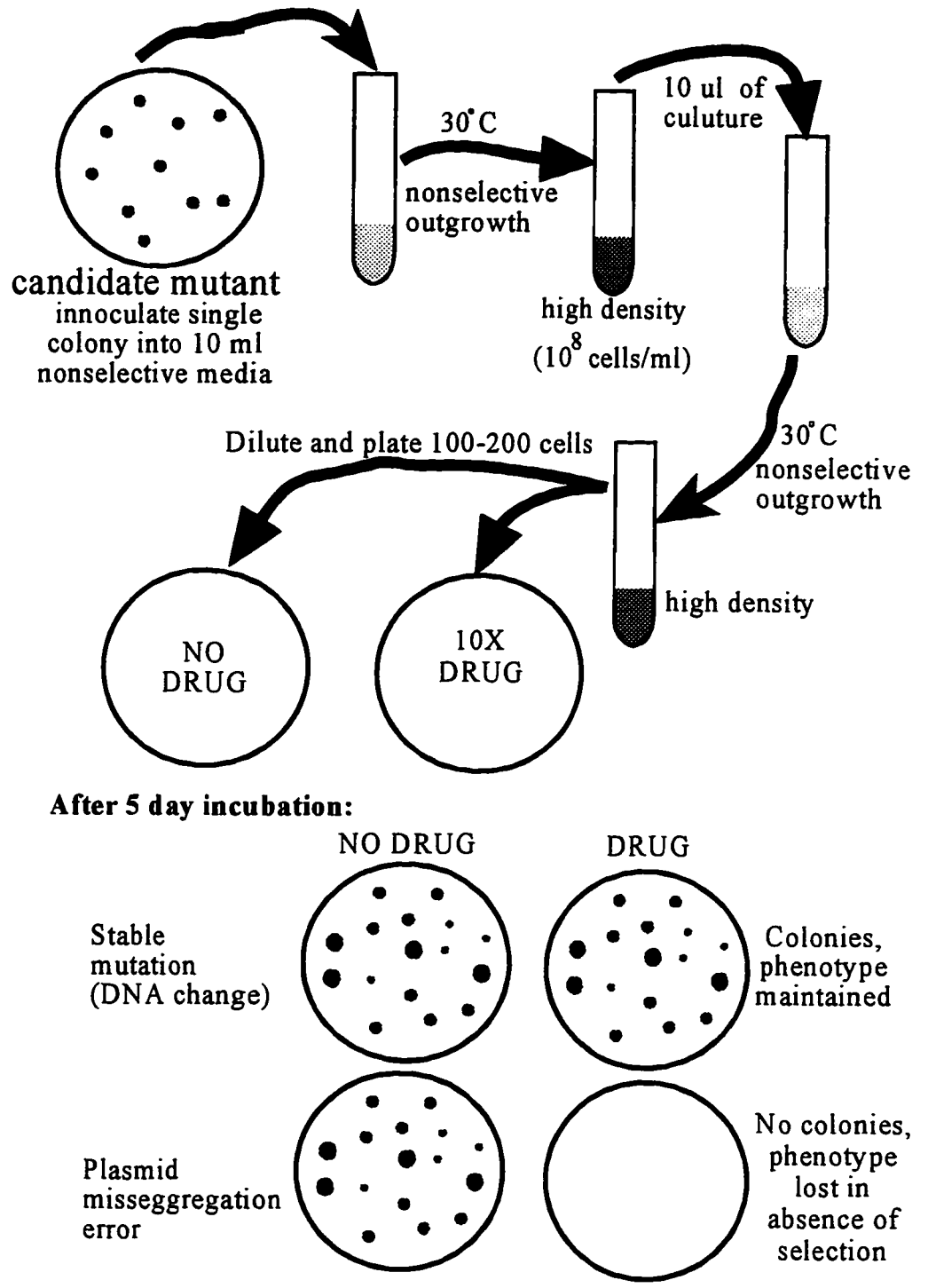


Figure 5-6. Plasmid missegregation assay and predicted outcomes. Assay was performed as described in materials and methods.

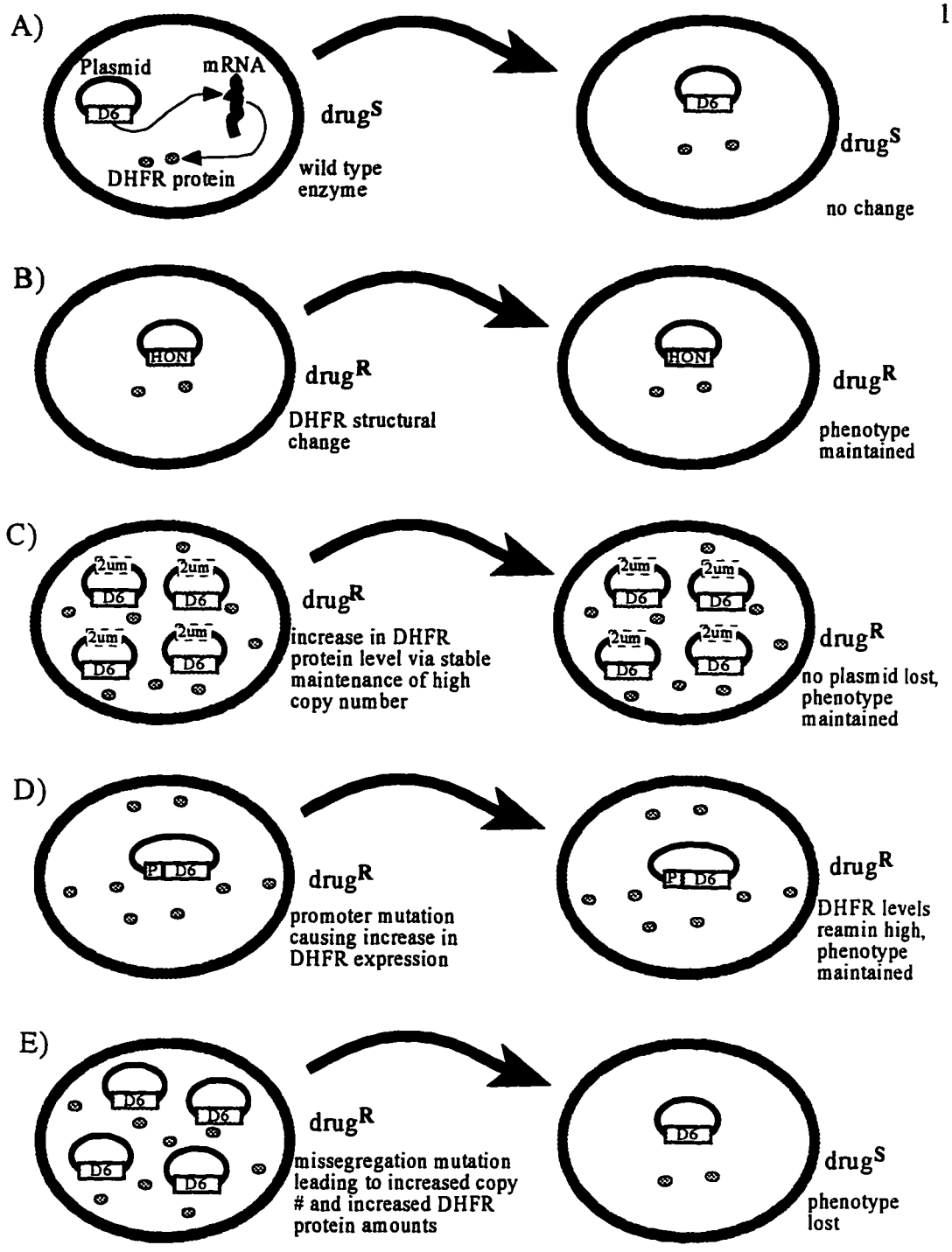


Figure 5-7. Models for maintenance in the plasmid missegregation assay of drug resistance phenotypes resulting from various mechanisms. A) Yeast containing a drug sensitive DHFR, B) drug resistant DHFR, C) drug sensitive DHFR stably maintained at high copy # (2um), D) drug sensitive DHFR expressed at high levels due to promoter mutation, and E) increased amounts of drug sensitive DHFR due missegregation error.

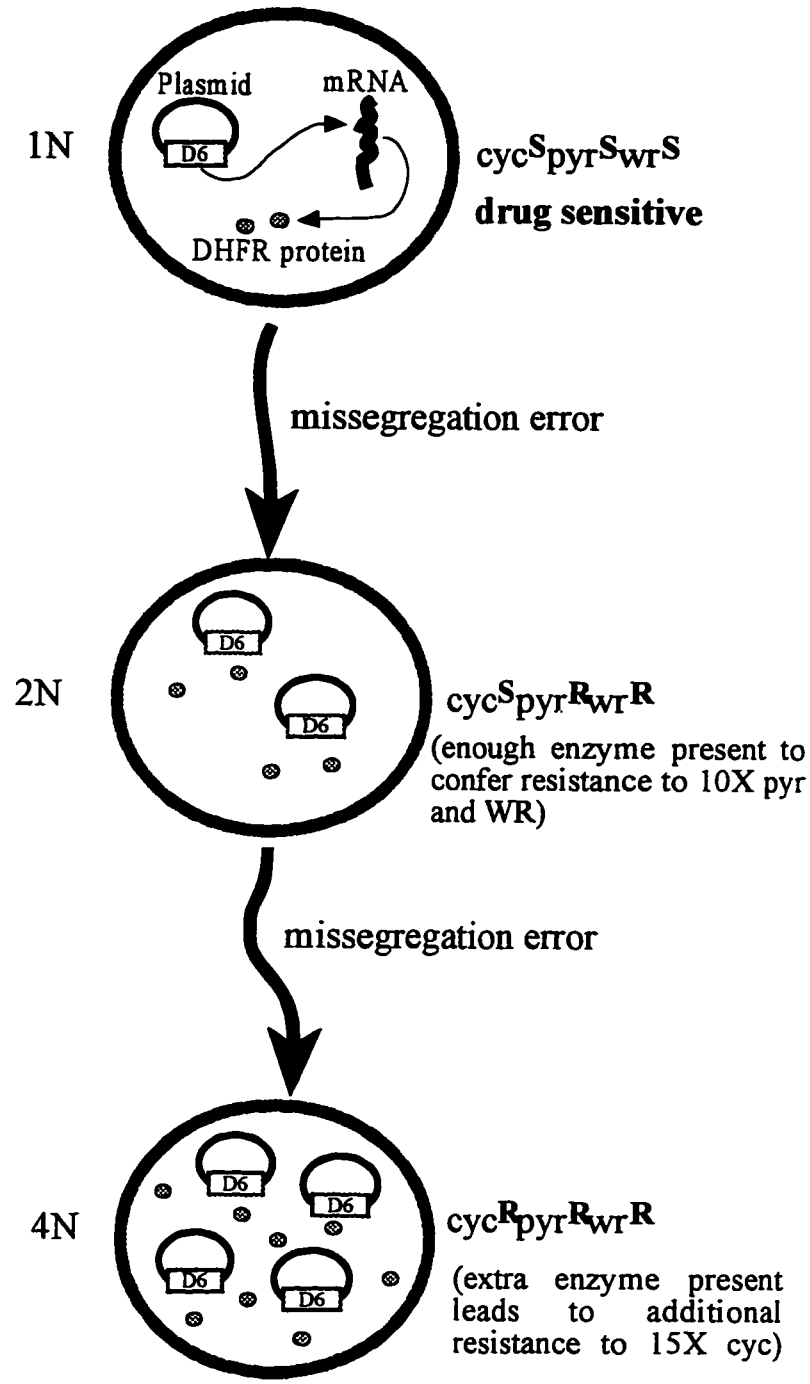


Figure 5-8. Simple model to show how culmulative missegregation errors could lead to different mutant phenotypes. 1N, 2N, and 4N represent hypothetical plasmid copy numbers based on drug concentrations used in screening for cross-resistance.

Table 5-1. Total Number of PYR and WR Resistant Colonies for Each Plate

Tube	PYR	WR	Tube	PYR	WR	Tube	PYR	WR
3	16	7	46	7	0	98	7	3
4	22	10	47	15	1	99	2	1
5	14	6	48	>20	16	100	5	10
8	0	1	50.1	11	2	101	>10	2
9	~25	9	50.2	11	1	102	12	105
10	7	0	51	11	11	103	6	0
11.1	9	4	52	52	5	104	9	3
11.2	20	3	53	>11	15	105	3	1
13	~30	4	54	5	1	106	3	1
14	11	2	55	4	2	107	7	5
15	~21	8	56	>16	~28	108	2	0
16	8	1	57	>19	1	109	12	2
17	TNC	7	60	2	1	110	11	8
18	12	4	63	4	4	112	10	2
19	4	0	65	5	2	113	16	8
20	~28	9	68	>20	~30	114	13	0
21	~19	14	69	3	3	116	10	1
23	~20	22	70	12	4	117	9	3
24	~24	4	72	>20	~20	118	8	10
25	5	4	73	>27	8	119	12	3
26	~20	4	74	14	2	120	22	6
27	>20	21	75	6	2	121	16	15
28	7	2	76	0	0	122	4	3
30	>20	14	79	8	2	123	6	1
31	18	1	84	31	15	124	7	0
32	6	3	86	21	5	125	3	0
33	4	1	87	5	5			
34	3	2	88	>20	1			
35	16	3	89	TNC	13			
36	11	2	92	5	1			
38	6	1	93	17	7			
39	11	5	94	8	1			
40	6	0	95	6	1			
42	13	2	96	6	6			
44	7	4	97	3	5			

TNC=too numerous to count

Table 5-2. Mutation Frequencies of Drug Resistant Colonies.

A. Mutation frequencies for pyrimethamine and WR99210 resistant colonies.*

Drug	# Colonies isolated	# Cells screened	Frequency
PYR	887	9.9×10^{7}	8.96×10^{-6}
WR	424	1.0×10^{8}	4.24×10^{-6}

*Values inclusive of all size classes.

B. Mutation frequencies for drug resistant colonies comprising each size class.

Colony size	PYR	WR
large	1.3×10^{-7}	1.8×10^{-7}
medium	1.2×10^{-6}	7.7×10^{-7}
small	7.7×10^{-6}	3.3×10^{-6}

Table 5-3. Summary of Results for Level 1 Tests

	Pyrimethamine	WR99210
Starting Number of Colonies	887	424
Marker failures	7	7
Drug resistance		
negative	137	84
questionable	29	13
Passed	750	340
Pass rate	85%	80%

Table 5-4. Distribution of Medium and Large Size Resistant Colonies for Pyrimethamine and WR99210

Tube	PYR	WR	Tube	PYR	WR	Tube	PYR	WR
3	6	3	46	2	0	98	4	0
4	7	2	47	3	0	99	1	1
5	2	0	48	0	0	100	0	8
8	0	1	50.1	0	0	101	0	0
9	3	0	50.2	1	0	102	3	2
10	5	0	51	0	3	103	0	0
11A	5	3	52	0	1	104	1	1
11B	4	1	53	2	0	105	0	0
13	1	2	54	2	0	106	0	0
14	1	0	55	0	0	107	0	0
15	3	1	56	0	3	108	0	0
16	3	0	57	0	0	109	0	0
17	3	3	60	0	0	110	0	4
18	2	0	63	2	0	112	0	0
19	2	0	65	1	0	113	3	6
20	2	2	68	0	0	114	0	0
21	3	5	69	0	0	116	0	0
23	1	0	70	1	0	117	0	0
24	1	0	72	0	0	118	0	3
25	0	3	73	2	0	119	0	1
26	2	0	74	0	0	120	0	1
27	0	4	75	0	0	121	0	1
28	0	1	76	0	0	122	3	1
30	0	1	79	0	0	123	1	1
31	6	0	84	0	4	124	0	0
32	0	2	86	4	0	125	0	0
33	2	0	87	2	2			
34	3	0	88	1	1			
35	2	1	89	1	6			
36	0	0	92	1	1			
38	3	0	93	0	2			
39	6	0	94	1	0			
40	0	0	95	0	0			
42	0	0	96	0	1			
44	3	0	97	0	2			

Table 5-5. Results of Double Replica Plating Tests

	Pyrimethamine	WR99210
# Tested	103	73
Resistance:		
10X	103	73
100X	1	0
100X/dTMP	0	0
Cross-resistance:		
10X WR or PYR	73	67
10X CYC	24	37
100X PYR or WR	1	0
Marker failures:	1	2
Cyclohexamide Resistant:	0	0
Passed:	102	73
Pass Rate:	99%	100%

Table 5-6. Drug Resistance Classes for Medium/Large Sized Colonies Selected on Pyrimethamine and WR99210.

Drug Selection	Resistance Class	Number Mutants Isolated	% Total
Pyrimethamine	I. $pyr^{R}wr^{S}cyc^{S}$	30	29.1
	II. $pyr^{R}wr^{R}cyc^{S}$	49	47.6
	III. $pyr^{R}wr^{R}cyc^{R}$	24	23.3
	Total # tested	103	100.0
WR99210	I. $wr^{R}pyr^{S}cyc^{S}$	6	8.2
	II. $wr^{R}pyr^{R}cyc^{S}$	30	41.5
	III. $pyr^{R}pyr^{R}cyc^{R}$	37	50.7
	Total # tested	73	100.0

Table 5-7. Distribution of Phenotypes for Pyrimethamine Resistant Mutants

Plate	Mutant	PYR	WR	CYC	Plate	Mutant	PYR	WR	CYC	Plate	Mutant	PYR	WR	CYC
1	P1-b1	+	+	-	15	P2-b2	-	-	-	70	P3-g1	+	+	+
	P1-b2	+	+	-		P2-b3	+	+	-					
	P1-b3	+	+	-		P2-b4	+	+	+	86	P3-h1	+	+	-
											P3-h4	+	-	-
3	P1-b4	+	+	+	16	P2-c1	+	+	-					
	P1-c1	+	+	-		P2-c2	+	+	-	87	P3-i1	+	+	-
	P1-c2	+	+	-							P3-i2	+	+	-
	P1-c3	+	-	-	17	P2-d1	+	-	-					
	P1-c4	+	-	-						88	P3-i3	+	+	-
	P1-d1	+	-	-	18	P2-d4	+	+	+					
										92	P3-j1	+	+	-
4	P1-d2	+	+	-	20	P2-e1	+	+	-		P3-j2	+	+	-
	P1-d3	+	+	-							P3-j3	+	+	-
	P1-d4	+	-	-	21	P2-f1	+	+	-		P3-j4	+	+	-
	P1-e1	+	+	+										
	P1-e2	+	-	-	31	P2-g1	+	+	-	94	P4-b1	+	+	+
	P1-e3	+	-	-		P2-h1	+	+	-					
	P1-e4	+	-	-						98	P4-b2	+	-	-
					34	P2-i1	+	+	-		P4-b3	+	+	-
5	P1-f1	+	-	-		P2-i2	+	+	-		P4-b4	+	+	+
	P1-f2	+	-	-		P2-i3	+	+	-		P4-c1	+	-	-
9	P1-f3	+	-	-	35	P2-j1	+	+	-	99	P4-c2	+	-	-
	P1-f4	+	+	+										
	P1-g1	+	+	-	38	P2-j2	+	+	-	102	P4-c3	+	-	-
						P2-j3	+	+	-		P4-c4	+	+	+
10	P1-g2	+	-	-		P2-j4	+	+	-		P4-d1	+	-	-
	P1-g3	+	-	-										
	P1-g4	+	+	-	39	P3-b2	+	+	+	104	P4-d2	+	+	+
	P1-h1	+	+	-		P3-b3	+	+	-					
	P1-h2	+	+	-		P3-b4	+	+	+	106	P4-d3	+	-	-
						P3-c1	+	+	-					
11A	P1-h3	+	-	-						107	P4-d4	+	+	+
	P1-h4	+	+	-	44	P3-c3	+	-	-		P4-e1	+	+	-
	P1-i1	+	+	-		P3-c4	+	+	-		P4-e2	+	+	-
	P1-i2	+	+	-		P3-d1	+	-	-					
	P1-i3	+	+	-						115	P4-e3	+	-	-
					47	P3-d4	+	-	-		P4-e4	+	+	+
11B	P1-i4	+	+	+		P3-e1	+	+	-		P4-f1	+	+	+
	P1-j1	+	+	+							P4-f2	+	+	-
	P1-j2	+	+	+	50B	P3-e3	+	-	-					
	P1-j3	+	+	+						117	P4-f3	+	-	-
					53	P3-e4	+	+	-		P4-f4	+	+	+
13	P1-j4	+	+	-		P3-f1	+	+	-					
										122	P4-g1	+	+	+
14	P2-B1	+	+	+	65	P3-f4	+	+	-		P4-g2	+	+	+
											P4-g3	+	-	-

Table 5-8. Distribution of Phenotypes for WR99210 Resistant Mutants.

Plate	Mutant	WR	PYR	CYC	Plate	Mutant	WR	PYR	CYC	Plate	Mutant	WR	PYR	CYC
3	w1-b2	+	+	+	84	w2-d3	+	+	+	104	w3-c1	+	+	+
						w2-d4	+	+	+					
4	w1-b4	+	+	-		w2-e1	+	+	-	110	w3-c2	+	+	-
	w1-c4	+	+	-		w2-e2	+	+	+		w3-c3	+	+	-
	w1-d1	+	+	-							w3-c4	+	+	+
					87	w2-e3	+	+	+		w3-d1	+	+	-
13	w1-d4	+	+	-		w2-e4	+	+	+					
										113	w3-d2	+	+	+
15	w1-e1	+	+	+	88	w2-f1	+	+	+		w3-d3	+	+	+
											w3-d4	+	+	+
17	w1-e4	+	+	-	89	w2-f2	-	+	-		w3-e1	+	+	+
						w2-f3	+	+	+		w3-e2	+	+	-
21	w1-f4	+	+	-		w2-f4	+	+	+		w3-e3	+	+	+
	w1-g1	+	+	-		w2-g1	+	+	+					
	w1-g4	+	+	-		w2-g2	+	+	+	118	w3-e4	+	+	-
						w2-g3	+	+	+		w3-f1	+	+	+
26	w1-h4	+	+	-							w3-f2	+	+	+
	w1-i2	+/-	+	-	92	w2-g4	+	+	-					
										119	w3-f3	+	+	+
27	w1-j1	+	+	+	93	w2-h1	+	+	+					
	w1-j2	+	+	-		w2-h2	+	+	+	120	w3-f4	+	+	+
28	w1-j3	+	+	-	96	w2-h3	+	+	+	121	w3-g1	+	+	+
32	w1-j4	+	+	+	97	w2-h4	+	+	-	122	w3-g2	+	+	+
						w2-i1	+	+	-					
35	w2-b2	+	+	+						126	w3-g3	+	+	+
					100	w2-i3	+	+	+					
30	w2-b3	-	+	-		w2-i4	+	+	-					
						w2-j1	+	+	-					
51	w2-b4	+	+	-		w2-j2	+	+	-					
	w2-c1	+	+	+		w2-j3	+	+	-					
	w2-c2	-	+	-		w2-j4	+	+	-					
						w3-b1	+	+	-					
52	w2-c3	-	+	-		w3-b2	+	+	-					
56	w2-c4	+	+	+	102	w3-b3	+	+	-					
	w2-d1	+	+	+		w3-b4	+	+	-					
	w2-d2	-	+	-										

Table 5-9. Plasmid Missegregation Annalysis for Pyrimethamine Resistant Mutants.

Strain*	Phenotype**	Colonies on 10X PYR (% Control)	S.D.	Possible Explanation
pTRP-D6	pyr^S	25.0	25.9	wildtype DHFR maintained at low copy #
		2.4	3.3	
pTRP-Hon	pyr^R	82.2	31.0	Mutant DHFR maintained at low copy #
		102.4	17.2	
D6-2uM	pyr^R	105.2	19.9	Over-expressed wildtype DHFR by stable high copy # maintenance
		79.1	11.5	
P3-D1	$pyr^{SR}wr^Scyc^S$	9.1	12.9	misseggregation
P4-D1	$pyr^Rwr^Scyc^S$	6.3	3.0	misseggregation
P4-E2	$pyr^{SR}wr^{SR}cyc^S$	12.7	14.4	misseggregation
P1-J4	$pyr^Rwr^{SR}cyc^S$	44.9	4.2	misseggregation?
P1-C2	$pyr^Rwr^Rcyc^S$	21.4	6.8	misseggregation
P4-E4	$pyr^Rwr^{SR}cyc^{SR}$	55.9	4.2	misseggregation?
P1-E1	$pyr^Rwr^Rcyc^{SR}$	48.7	26.0	misseggregation?
P4-B1	$pyr^Rwr^Rcyc^R$	105.2	7.9	stable mutation
P15s-B1	$pyr^Rwr^Rcyc^S$	93.0	15.4	stable mutation
P6s-E4	$pyr^Rwr^Rcyc^S$	70.0	1.8	stable mutation
P8s-J2	$pyr^Rwr^Rcyc^{SR}$	22.7	9.4	misseggregation

* Results from two independent experiments shown for control strains

** "SR" indicates slightly resistant

Table 5-10. Plasmid Missegregation Analysis for WR99210 Resistant Mutants.

Strain*	Phenotype**	Colonies on 10X WR (% Control)	S.D.	Possible Explanation
pTRP-D6	wr^S	0.0	0.0	wildtype DHFR maintained at low copy #
		0.0	0.0	
pTRP-Hon	wr^S	0.0	0.0	wr^S Hon DHFR maintained at low copy #
		0.0	0.0	
D6-2uM	wr^R	76.3	24.1	wildtype DHFR over-expressed by stable maintenance at high copy #
		56.4	6.5	
W1-I2	$wr^S pyr^R cyc^S$	75.4	2.2	stable mutation
W2-E1	$wr^{SR} pyr^{SR} cyc^S$	1.2	0.0	missegregation
W1-D4	$wr^{SR} pyr^R cyc^S$	4.3	0.7	missegregation
W1-F4	$wr^R pyr^R cyc^S$	15.9	9.0	missegregation
W2-E4	$wr^{SR} pyr^R cyc^{SR}$	12	1.3	missegregation
W2-F3	$wr^R pyr^R cyc^{SR}$	6.6	4.3	missegregation
W3-F1	$wr^R pyr^R cyc^R$	103.1	2.5	stable mutation
W9s-B1	$wr^R pyr^R cyc^R$	75.4	2.2	stable mutation
W5s-F4	$wr^R pyr^R cyc^S$	10.9	4.2	missegregation
W9s-C3	$wr^R pyr^R cyc^S$	12.0	0.0	missegregation
W1s-J3	$wr^R pyr^S cyc^S$	61.8	20.8	stable mutation

* Results from two independent experiments shown for control strains

** "SR" indicates slightly resistant

Table 5-11. Phenotypes of Pyrimethamine Resistant Mutants Subjected to Level 3 Analysis.

Class	Sub-Class *	Mutant	Culture	Size	Missegregation Assay **	Plasmid Linkage
I	**No cross-resistance:**					
	a) $pyr^{SR}wr^{S}cyc^{S}$	P3-D1	44	M	missegregation	no
	b) $pyr^{R}wr^{S}cyc^{S}$	P4-D1	102	M	missegregation	no
II	**wr cross-resistant:**					
	a) $pyr^{SR}wr^{SR}cyc^{S}$	P4-E2	107	M	missegregation	$pyr^{SR}wr^{S}cyc^{S}$
	b) $pyr^{R}wr^{SR}cyc^{S}$	P1-D3	4	L	N.D.	no
		P1-J4	13	M	maybe	no
		P2-F1	21	M	maybe	no
		P3-J1	92	M	N.D.	$pyr^{R}wr^{S}cyc^{S}$
	c) $pyr^{R}wr^{R}cyc^{S}$	P1-C2	3	L	missegregation	no
		P1-I1	11A	M	N.D.	no
		P3-I1	87	M	N.D.	$pyr^{R}wr^{S}cyc^{S}$
		P3-J3	92	XS	N.D.	$pyr^{R}wr^{S}cyc^{S}$
III	**wr^{R} and cyc^{R}:**					
	a) $pyr^{R}wr^{SR}cyc^{SR}$	P4-E4	115	M	maybe	$pyr^{R}wr^{S}cyc^{S}$
	b) $pyr^{R}wr^{SR}cyc^{R}$	N.D.	N.D.	N.D.	N.D.	N.D.
	c) $pyr^{R}wr^{R}cyc^{SR}$	P1-E1	4	L	maybe	no
		P3-G1	70	M	N.D.	no
	d) $pyr^{R}wr^{R}cyc^{R}$	P4-B1	94	L	stable mutation	no
IV	**Small-sized colonies:**					
	a) $pyr^{R}wr^{SR}cyc^{S}$	P10s-J1	52	XS	N.D.	$pyr^{SR}wr^{S}cyc^{S}$
	b) $pyr^{R}wr^{R}cyc^{S}$	P6s-B1	27	XS	N.D.	$pyr^{SR}wr^{S}cyc^{S}$
		P6s-E4	30	XS	stable mutation	$pyr^{SR}wr^{S}cyc^{S}$
	c) $pyr^{R}wr^{R}cyc^{SR}$	P8s-J2	47	S	missegregation	N.D.
	d) $pyr^{R}wr^{R}cyc^{R}$	P12s-I4	70	S	N.D.	no
		P15s-B1	88	S	stable mutation	N.D.

* "SR" indicates slightly resistant; ** "N.D." indicates not done

Table 5-12. Phenotypes of WR99210 Resistant Mutants Selected for Level 3 Analysis.

Class	Sub-Class *	Mutant	Culture	Size	Missegregation Assay **	Plasmid Linkage
I	**No cross-resistance:**					
	$wr^{S}pyr^{R}cyc^{S}$	W1-I2	26	**M**	stable mutation	no
II	**pyr^{R}:**					
	a) $wr^{SR}pyr^{SR}cyc^{S}$	W2-E1	84	**M**	missegregation	no
	b) $wr^{SR}pyr^{R}cyc^{S}$	W1-D1	11A	**M**	N.D.	no
		W1-D4	13		missegregation	N.D.
		W2-I4	100	**L**	N.D.	no
		W3-B3	102	**M**	N.D.	no
	c) $wr^{R}pyr^{R}cyc^{S}$	W3-D1	110	**M**	N.D.	$wr^{S}pyr^{R}cyc^{S}$
		W1-C4	11A	**M**	N.D.	no
		W1-F4	21	**L**	missegregation	no
		W1-J3	28	**M**	N.D.	no
III	**pyr^{R} and cyc^{R}:**					
	a) $wr^{SR}pyr^{R}cyc^{SR}$	W2-E4	87	**M**	missegregation	no
		W3-F3	119	**M**	missegregation	no
	b) $wr^{R}pyr^{R}cyc^{SR}$	W2-F3	89	**M**	missegregation	no
	c) $wr^{R}pyr^{R}cyc^{R}$	W3-F1	88	**M**	stable mutation	no
IV	**Small-sized**					
	colonies:	W1s-J3	14	**S**	stable mutation	$wr^{SR}pyr^{R}cyc^{S}$
	a) $wr^{R}pyr^{S}cyc^{S}$	W5s-F4	53	**XS**	missegregation	no
	b) $wr^{R}pyr^{R}cyc^{S}$	W9s-C3	114	**XS**	missegregation	no
		W9s-B1	110	**S**	stable mutation	N.D.
	c) $wr^{R}pyr^{R}cyc^{R}$	W9s-B4	112	**S**	N.D.	no

* "**SR**" indicates slightly resistant

** "**N.D.**" indicates not done

Table 5-13. DNA Sequencing Results for DHFR Genes of Mutants.

Drug Selection	Resistance Class	Mutant	Size Class	DHFR Gene
Pyrimethamine	$pyr^{R}wr^{R}cyc^{S}$	P3-J1	M	no mutations
		P3-I1	M	no mutations
		P3-J3	XS	no mutations
	$pyr^{R}wr^{R}cyc^{R}$	P4-E4	M	no mutations
		P4-B2	L	no mutations
	Small colonies:			
	$pyr^{R}wr^{R}cyc^{S}$	P10s-J1	XS	no mutations
	$pyr^{R}wr^{R}cyc^{S}$	P6s-E4	XS	no mutations
	$pyr^{R}wr^{R}cyc^{R}$	P15s-B1	XS	no mutations
WR99210	$wr^{S}pyr^{R}cyc^{S}$	W1-I2	M	no mutations
	$wr^{R}pyr^{R}cyc^{S}$	W3-D1	M	no mutations
	$pyr^{R}pyr^{R}cyc^{R}$	W3-F1	M	no mutations
	Small colonies:			
	$wr^{R}pyr^{S}cyc^{S}$	W1s-J3	S	no mutations

Chapter 6: Conclusions

I. Improving the yeast heterologous expression system.

To facilitate the study of antifolate resistance, this thesis project had three aims: to create a genetic technique to quickly and reliably differentiate isolates of *P. falciparum*, to establish a heterologous expression system for the *P. falciparum* DHFR gene, and to test the ability of the system for selecting point mutations that confer resistance to DHFR inhibitors. A quick and reliable strain identification technique was developed based on the polymerase chain reaction (PCR) and polymorphisms in cloned *P. falciparum* genes. A *S. cerevisiae* heterologous expression system was created by replacing the yeast DHFR gene with the DHFR domain of the *P. falciparum* bifunctional DHFR-TS. The *P. falciparum* DHFR gene was found to complement all normal functions of the yeast DHFR gene. Furthermore, the sensitivity of this yeast to the malarial antifolates was comparable to the sensitivity seen for *P. falciparum* strains.

Using yeast that carried the PfDHFR gene on a low copy number plasmid, I performed a pilot experiment to select for spontaneous mutants resistant to pyr and WR. Analysis of representative candidates suggested that most of the resistant mutants were a result of changes in plasmid copy number and not mutations in the DHFR gene. This, in turn, suggested that a plasmid-based system would not be the most efficient approach for selecting DHFR point mutations because the background of colonies drug resistant from missegregation errors would be too high. Since the largest source of noise appeared to be changes in copy number, a plasmid-based system might be optimized by using the ADE3 plasmid marker system (Koshland, 1985). Changes in the adenine biosynthetic genes affect the pigmentation of yeast *S. cerevisiae* (Roman, 1956). Strains with mutations in the *ADE3* gene are white while mutations in the *ADE2* gene result in a red pigmentation.

Since *ADE3* mutations are epistatic to *ADE2* mutations, an *ade2ade3* strain is white. Accumulation of copies of a partially defective wildtype *ADE3* gene (Koshland, 1985) results in a gradual return to a red pigmentation color. In this way, *ade2ade3* cells containing a plasmid with the *ADE3* gene range from white to red depending on the number of plasmid copies. Therefore, adding an ADE3 marker to the pTRP-D6 plasmid would presumably allow the immediate visual exclusion of mutants whose resistance phenotype is mediated by changes in plasmid copy number.

Transferring the PfDHFR construct to a chromosomal location is an alternative approach for eliminating the background due to changes in DHFR copy number. This would ensure maintenance of the PfDHFR gene at one copy per cell due to the stricter regulation of chromosomal copy number. However, this approach would not be without disadvantages. Table 6-1 compares the advantages and disadvantages for the plasmid-based and chromosomal based approaches. A plasmid-based system allows for easy isolation and transfer of the DHFR construct to a new yeast host, thus providing a simple test for linkage of a resistance phenotype to the plasmid and exclusion of mutational changes in the yeast genome. Additionally, the expression of the PfDHFR gene is low enough to see drug sensitivity at levels comparable to those seen in *P. falciparum*, presumably due to the absence of enhancer elements that would be present on the chromosome. While a chromosomal-based system would reduce the noise of copy number changes, it would not be as straightforward to screen out mutations in the yeast host since this would involve either genetic crosses for mapping or removal of the DHFR construct from the chromosomal location and reinsertion into a new host. Furthermore, a DHFR gene in the chromosome might increase its expression if it came under the influence of linked upstream activating sequences. This over-expression would decrease drug sensitivity, resulting in a resistant yeast strain. One way to prevent this effect would be to insert the construct in a chromosomal location with low transcriptional activity or to insert the construct in a manner that would minimize the influence of linked sequences. For

example, one could insert the PfDHFR construct in a chromosomal locus in reverse orientation to that of a target gene thus reducing the activity of upstream enhancer elements.

The results of the pilot selection experiment suggested that reversion at the *tup1* locus and mutations in the PDR/MDR multi-drug resistance systems were not a significant problem. The lack of *tup1* revertants in the mutant collections suggests that either *tup1* is a very stable mutation or that selection for resistance to antifolates does not select for *tup1* reversion because *tup1* is not involved in permeability. Until experiments are done to determine the role of *tup1* in drug permeability, both this mutation and the PDR/MDR drug resistance systems formally represent sources of noise that could be present in a chromosomal-based system. However, the simple tests used to discriminate these were adequate in the pilot experiment and could be incorporated into a screen based on an integrated DHFR gene.

There are many possible mechanisms for resistance to antifolates that would involve the DHFR gene. For example, drug resistance could arise from either structural changes in the enzyme via point mutations in the gene or over-expression of the enzyme such as occurs when copy number is increased. Over-expression is predicted always to confer broad spectrum resistance to the malarial antifolates. The results of the pilot experiment suggested that copy number mutants comprised a significant fraction of the drug resistant candidates. Therefore, when the selection experiment is repeated using a chromosomal-based system in which DHFR copy number is strictly maintained, one prediction is that the mutant collections will have fewer broad spectrum antifolate resistant mutants and a higher proportion of DHFR point mutants with specific resistance to the drug used for the selection.

The *Plasmodium* DHFR is part of a bifunctional enzyme complex which contains the thymidylate synthetase (TS). As a starting point, a construct was created which contains solely the DHFR domain. It has been debated whether it is important to have the complete *P. falciparum* DHFR-TS bifunctional enzyme complex present in the yeast system since there could be subunit interactions which may affect the outcome of the development of drug resistance (Hyde, 1990). Often, the presence of a multifunctional enzyme complex suggests that there are significant biological reasons for the constituent enzymes not to exist separately. For example, interactions between the subunits could be important for efficient coupling of metabolic substrates. The DHPS-PPPK enzyme complex (dihydropteroate synthetase - hydroxymethyldihydropterin pyrophosphokinase) has been conserved over long evolutionary periods, suggesting strong functional reasons for keeping these two enzymes joined. In *Pneumocystis* (Volpe, 1993), *Plasmodium* (Hyde, 1990), and *S. cerevisiae* (Sibley and Hunt, unpublished data) the two enzymes are present in a complex. However, in yeast, the DHFR and TS functions are encoded by separate genes as separate proteins. The fact that the DHPS-PKKG enzyme complex is conserved while the DHFR-TS is not suggests that subunit interactions between the DHFR and TS may not be as important as previously thought. Therefore, a yeast system which contains solely the DHFR domain may be meaningful and useful. Nonetheless, a simple experiment would be to make a construct that contains both the DHFR and TS domains for use in selection experiments. Comparison of the mutation frequencies and range of mutation observed with the PfDHFR-TS system and the information from a system based only on the DHFR domain will allow assessment of the validity of each approach.

II. Applications for the system.

There are many potential applications for the heterologous yeast PfDHFR expression system. The most obvious application is to use the system to measure the

capacity of single drugs and drug combinations to select resistant mutants. If resistance is observed, cross-resistance to other clinically important inhibitors could also be measured. This will allow assessment of the long-term efficacies of candidate DHFR inhibitors. Since yeast are inexpensive to culture and very easy to work with, the system offers a less labor-intensive alternative to traditional screening of drugs in *Plasmodium*. In a very controlled fashion, this system can be used as a routine screen for the potency of candidate inhibitors against a wide variety of sensitive and resistant DHFR alleles. The ability of one candidate DHFR inhibitor to potentiate the potency of another inhibitor against specific DHFR alleles could also be measured. Finally, it will be easy to determine whether other inhibitors show collateral hypersensitivity against drug resistant DHFR alleles as seen for WR99210.

There are potential applications beyond the original scope of the project. In the past, the number of field isolates for which the antifolate resistance could be measured *in vivo* has been limited by the need to establish primary cultures and cell lines for each isolate. This has resulted in a very limited data set from which to formulate an understanding of antifolate resistance. In fact, our current understanding of the DHFR mutations involved in resistance is based mainly on a small collection of drug sensitive and resistant standard laboratory cell lines. Furthermore, the differences in strain background between these cell lines have complicated comparison and interpretation of the drug resistance data. Both of these problems have prevented examination of a large number of isolates over a large geographic area, thus limiting the assessment of drug resistance at the population level. The yeast expression system will allow measurement of the DHFR-mediated drug resistance of isolates without the complicating differences in strain background. Blood spots of isolates can be collected in the field and used for PCR cloning of the DHFR genes (see Chapter 3 for description of technique). Each DHFR gene can be transferred into the yeast expression system so that its sensitivity to antifolates can be determined in a genetic background identical to that for all other alleles. Another

benefit of this type of analysis arises from the fact many field samples contain a mixture of parasites. PCR cloning and transfer into yeast will to help to sort out the mixed DHFR alleles contained in the original sample since each yeast transformant should contain only one type of allele. Nonetheless, the DHFR mutations can be easily identified by cycle sequencing of the cloned DHFR genes. This information can complement ongoing field studies using allele specific PCR. Furthermore, the PCR strain identification assay will help assess genetic relatedness of the isolated blood samples. Consequently, antifolate resistance could be examined at the population level over wide geographic areas.

Finally, having the PfDHFR gene contained within yeast presents opportunities for classical genetic analysis not possible in *Plasmodium*. The biology of DHFR enzyme function and interaction with drugs could be studied using structure-function analysis. Such a study would involve mutating the DHFR gene in a systematic fashion and assessing the effects of each amino acid change on enzyme function and drug interactions. This would identify residues important for normal enzyme function and residues critical for drug binding. This type of analysis would complement ongoing drug structure-activity studies which are aimed at determining the portions of a drug molecule that are important for binding with the DHFR enzyme. Table 6-2 summarizes the potential applications for the yeast system.

III. Future directions for the system.

The yeast system easily offers itself to other types of selection experiments besides single step selections for spontaneous drug resistant mutants. The possible range of DHFR mutations could be measured by mutagenesis of either the PfDHFR construct or yeast containing the PfDHFR gene, allowing the generation of a wider spectrum DHFR mutations than possible using spontaneous mutant selections. Another interesting experiment would be to measure the effect of various types of drug selection on mutation

spectrum and frequency. Selection for drug resistant parasites out in the field does not involve single step selections under idealized laboratory conditions. Therefore, it will be of interest to see how incrementally increasing the drug concentration from suboptimal to very high levels over a long period of time affects the outcome of resistant parasites. Single step selection experiments can be done to assess whether the strength of the drug selection biases mutation spectra and frequencies. It will also be of clinical importance to perform double drug selection experiments to measure how the presence of two drugs affects the outcome for drug resistance. Such experiments would have obvious implications for potential combination drug therapies. Finally, it will be useful to determine what happens to drug resistant populations in the absence of selection. Since DHFR mutations may compromise enzyme function, one experiment would be to measure the persistence of drug resistant DHFR alleles in a population when the drug selection is removed. If these alleles are selected against and lost over time, it may be possible to restore the activity of antifolates in geographic regions of the world by restricting their use.

PfDHFR yeast transformants resistant to cyc and pyr (Pf-DHFR-Hon and Pf-DHFR-Mik) were found to be more sensitive to WR99210 than the completely drug sensitive transformant (Pf-DHFR-D6). This phenomenon of collateral hypersensitivity offers tremendous potential for the development of combination drugs. Many aspects of the phenomenon are testable in the yeast system. The basic prediction is that DHFR mutants resistant to pyr or cyc should still be sensitive to WR99210. Selecting in parallel for mutants resistant to pyr and cyc in the yeast system should result in few, if any, mutants cross-resistant to WR99210. Another interesting experiment will be to select for mutants resistant to WR99210 starting with a yeast strain that is pyr^R. This will address two questions: 1) is it possible for a pyr^R strain to develop resistance to WR99210 and 2) will the wr^R mutants now be sensitive to pyr? Finally, it will be of interest to determine

how the various combinations of cyc^R and pyr^R mutations observed in the field affect the collateral hypersensitivity of the DHFR enzymes to WR99210.

Another interesting aspect of collateral hypersensitivity can be studied using the yeast system. One can assess the consequences of drug resistance DHFR mutations for enzyme function by comparing the growth rates and viabilities of the Pf-DHFR-D6 and Pf-DHFR-Hon transformants. These parameters can also be measured in the presence of pyr and WR99210 to examine enzyme function during drug selection. It is conceivable that DHFR mutations that confer drug resistance could compromise normal DHFR enzyme function in the absence of drug, resulting in reduced cellular growth. Yet under drug selection, these mutations could become favorable. Conversely, it is possible that these mutations would not have any adverse consequences on cellular growth. These experiments should reveal which of these alternatives is more likely and provide valuable insights into the mechanism of antifolate resistance and collateral hypersensitivity. This information would complement biochemical characterization of the enzyme performance in the presence and absence of drugs.

DHPS (dihydropteroate synthetase) is an enzyme that acts upstream of DHFR in the folate pathway and numerous inhibitors have been designed which mimic the structure of its natural substrate, para-amino benzoic acid (PABA). Commonly, pyr is used in combination with a PABA analog, such as a sulfonamide. Resistance to this combination has been observed and point mutations have been identified in the DHPS gene which appear to be involved in the sulfonamide resistance (Brooks, 1994). Since both the *Plasmodium* and yeast DHPS genes have been identified, a logical next step would be to create a yeast strain that contains both the *P. falciparum* DHFR and DHPS enzymes. Such a double target system would allow for screening of antifolate combination drugs with a low capacity for selecting for resistance. Additionally, this system could be used to measure the ability of candidate DHPS inhibitors to enhance the effect of specific DHFR

inhibitors when used in combination. As an example, methotrexate alone does not affect wildtype yeast growth while in the presence of sulfanilamide there is substantial inhibition of growth (recall Chapter 4, methotrexate sensitivity experiments).

Finally, it will be of importance to compare the spectrum of DHFR mutations selected in the yeast system with the mutation spectrum that has already been identified from field isolates. This will allow assessment of the relevance of the information selected in yeast to the problem of antifolate resistance in *P. falciparum*. If there are DHFR mutations selected in yeast that have not been observed in drug resistant field isolates, it will be of interest to understand why these mutations have not contributed to the acquisition of drug resistance in wild parasite populations. Another test of the validity of the DHFR mutations selected in yeast will be to transfer interesting mutant DHFR genes back into *Plasmodium*. This is now possible since successful stable transfection of *P. falciparum* parasites was recently reported (Wu, 1995). In this way, it could be demonstrated that these mutant DHFRs have biological consequences for drug resistance in the parasite.

IV. Closing:

Drug resistance is one of the major medical challenges of our time. The yeast PfDHFR expression system has the potential to make contributions to the battle against drug resistance on both a theoretical and an applied level. At the theoretical level, it will help to better define the relationship between drug selection and the frequency and spectrum of mutants selected. At the applied level, it will help to identify drugs with a low capacity for selecting DHFR mutations that confer resistance and thus serve as a model system for assessing drug performance prior to clinical use. This type of experimental approach will hopefully lead to improved drug management and the availability of drugs

that will have longer efficacy against infectious diseases like malaria. This approach will be useful for therapeutics of any infectious organism in which point mutations within the target gene are known to be the resistance mechanism and the counterpart of that target is present in yeast.

Table 6-1. Comparison of plasmid-based and chromosomal-based expression systems for the *P. falciparum* DHFR gene.

Expression System	**Advantage**	**Disadvantage**
Plasmid-based	Low DHFR gene expression resulting in drug sensitivity at levels comparable to parasite Very easy to screen out mutations in yeast host through plasmid-linkage of phenotype Easy isolation of DHFR gene	High frequency of DHFR copy number mutants via increases in plasmid copy number
Chromosomal-based	DHFR gene maintenance at 1 copy per cell	Possibility for increased DHFR gene expression resulting in decreased drug sensitivity More difficult to screen out mutations in yeast host

Table 6-2. Applications of yeast system.

I. Screening of DHFR inhibitors:

A. Effectiveness against sensitive and resistant DHFR alleles

B. Potentiation (ability to increase potency of another antifolate when present in drug combinations)

C. Potential for collateral hypersensitivity

II. Measure capacity of single and combination drugs for selecting resistance:

A. Posssible mutation mechanisms

B. Frequency of mutation to resistance

C. Potential for cross-resistance to other drugs of clinical importance

III. Survey resistance of field isolates

IV. Biology of DHFR enzyme function and drug/enzyme interaction:

A. Structure-function analysis

B. Characterization of drug inhibition mechanism

List of References

Anders, R. F. et al. (1988). "Antigens with repeated amino acid sequences from the asexual blood stages of *Plasmodium falciparum*." **Progress in Allergy 41: 148-172.**

Banyal, H. S. and Inselburg, J. (1986). "*Plasmodium falciparum*: induction, selection, and characterization of pyrimethamine-resistant mutants." **Experimental Parasitology 62: 61-70.**

Basco, L. K. et al. (1995). "Point mutations in the dihydrofolate reductase-thymidylate synthetase gene and pyrimethamine and cycloguanil resistance in Plasmodium falciparum." **Mol Biochem Parasitol 69(1): 135-8.**

Bathurst, I. C. et al. (1993). "An experimental vaccine cocktail for Plasmodium falciparum malaria." **Vaccine 11(4): 449-56.**

Borst, P. and Ouellette, M. (1995). "New mechanisms of drug resistance in parasitic protozoa." **Annu. Rev. Microbiol. 49:427-60.**

Brooks, D. R. et al. (1994). "Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthetase gene in lines of the human malaria parasite, Plasmodium falciparum, with differing resistance to sulfadoxine." **Eur J Biochem 224(2): 397-405.**

Bzik, D. J. et al. (1987). "Molecular cloning and sequence analysis of the Plasmodium falciparum dihydrofolate reductase-thymidylate synthetase gene." **Proc Natl Acad Sci U S A 84(23): 8360-4.**

Canfield, C. J. et al. (1993). "PS-15,; a potent, orally active antimalarial from a new class of folic acid antagonists." **Am. J. Trop. Med. Hyg. 49: 121-126.**

Cheng, Q. and Saul, A. (1994). "The dihydrofolate reductase domain of rodent malarias: point mutations and pyrimethamine resistance." **Mol. Biochem. Parisitol. 65:361-363.**

Childs, G. E. and C. Lambros (1986). "Analogues of N-benzyloxydihydrotriazines: in vitro antimalarial activity against Plasmodium falciparum." **Ann Trop Med Parasitol 80(2): 177-81.**

Christianson, T. W. et al. (1992). "Multifunctional yeast high-copy-number shuttle vectors." **Gene 110(1): 119-122.**

Cowman, A. F. et al. (1988). "Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthetase gene of Plasmodium falciparum." **Proc Natl Acad Sci U S A 85(23): 9109-13.**

Dame, J. B. et al. (1984). "Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*." **Science 225: 593-599.**

Dexter, D. et al. (1994). "Mutations in the yeast *PDR3*, *PDR4*, *PDR7* and *PDR9* pleiotrophic (multiple) drug resistance loci affect the transcript level of an ATP binding cassette transporter encoding gene, *PDR5*." **Genetics 136: 505-515.**

Drake, J. W. (1991). "A constant rate of spontaneous mutation in DNA-based microbes." **Proc. Nat. Acad. Sci. USA 88: 7160-7164.**

Edwards, M. L. et al. (1991). "Antimalarial polyamine analogues." **J Med Chem 34(2): 569-74.**

Favalaro, J. M. et al. (1986). "Structure of the RESA gene of *Plasmodium falciparum*." **Nucleic Acids Research 14: 8265-8277.**

Fenton, B. et al. (1985). "Protein variation in clones of *Plasmodium falciparum* detected by two dimensional electrophoresis." **Molecular Biochemical Parasitology 16: 173-183.**

Foote, S. J. et al. (1990). "Amino acids in the dihydrofolate reductase-thymidylate synthetase gene of Plasmodium falciparum involved in cycloguanil resistance differ from those involved in pyrimethamine resistance." **Proc Natl Acad Sci U S A 87(8): 3014-7.**

Foote, S. J. et al. (1990). "Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in Plasmodium falciparum [see comments]." **Nature 345(6272): 255-8.**

Gamage, S. A. et al. (1994). "Synthesis and in vitro evaluation of 9-anilino-3,6-diaminoacridines active against a multidrug-resistant strain of the malaria parasite Plasmodium falciparum." **J Med Chem 37(10): 1486-94.**

Genther, C. S. et al. (1977). "Antifolate studies: activities of 40 potential antimalarial compounds against sensitive and chloroguanide triazine resistant strains of folate-requiring bacteria and *Escherichia coli*." **J. Medicinal Chem. 20(2): 237-243.**

Giroux, C. N. et al. (1988). "DNA sequence analysis of spontaneous mutations in the *SUP4* gene of *Saccharomyces cerevisiae*." **Mol. Cell. Biol. 8: 978-981.**

Hall, S. J. et al. (1991). "Functional expression of the dihydrofolate reductase and thymidylate synthetase activities of the human malaria parasite Plasmodium falciparum in Escherichia coli." **Mol Biochem Parasitol 45(2): 317-30.**

Hartman, P. G. (1993). "Molecular aspects and mechanism of action of dihydrofolate reductase inhibitors." **J Chemother 5(6): 369-76.**

Hempelmann, E. et al. (1981). **Transaction of the Royal Society of Tropical Medicine and Hygiene 75: 855-858.**

Hibasami, H. et al. (1994). "Remarkable activation of polyamine biosynthesis in hematopoiesis and hyperplasia of spleen in mice with hemolytic anemia caused by infection with Plasmodium berghei." **Int J Parasitol 24(2): 213-7.**

Higuchi, R. (1989). Simple and rapid preparation of samples for PCR. PCR Technology: Principles and Applications for DNA Amplification. H. Erlich. New York, Stockton Press: 31-38.

Hoffman, C. S. and F. Winston (1987). "A ten-minute preparation for yeast efficiently releases autonomous plasmids for transformation of *E. coli*." **Gene 57: 267-272.**

Huang, T. et al. (1992). "The phenotype of a dihydrofolate reductase mutant in *Saccharomyces cerevisiae*." **Gene 121: 167-171.**

Hyde, J. E. (1990). "The dihydrofolate reductase-thymidylate synthetase gene in the drug resistance of malaria parasites." **Pharmac. Ther. 48: 45-59.**

Innes, M. A. et al. (1990). **PCR Protocols: A Guide to Methods and Procedures**. Academic Press, New York.

Itoh, H. et al. (1983). **J. Bacteriol. 153: 163-168**.

Keleher, C. A. et al. (1982). "Ssn6-Tup1 is a general repressor of transcription in yeast." **Cell 68(4):709-719**.

Kemp, D. J. et al. (1987). "Repetitive proteins and genes of malaria." **Annual Review in Microbiology 41: 181-208**.

Knight, D.J. and Williamson, P. (1982). "The development of resistance to BRL 6231 (4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2,4,5-trichloropropyloxy)-1,3,5 triazine hydrochloride) by Plasmodium berghei." **Annals of Trop. Med. Parisitol. 76(1):9-14**.

Koeberl, D. D. et al. (1990). "Mutations causing hemophilia B: direct estimate of the underlying rates of spontaneous germ-line transitions, transversions, and deletions in a human gene." **American Journal of Human Genetics 47: 202-217**.

Koshland, D. et al. (1985). "Genetic analysis of the mitotic transmission of minichromosomes." **Cell 40: 393-403**.

Lagosky, P. A. et al. (1987). "Molecular characterization of the *saccharomyces cerevisiae* dihydrofolate reductase gene (DFR1)." **Nuc. Acids Res. 15**: **10355-10371**.

Lee, G. S.-F. et al. (1988). "The base-alteration spectrum of spontaneous and ultraviolet radiation-induced forward mutations in the *URA3* locus of *Saccharomyces cerevisiae*." **Mol. Gen. Genet. 214: 396-404.**

Luria, S. E. and M. Delbruck (1943). "Mutations of bacteria from virus sensitivity to virus resistance." **Genetics 28: 491-511.**

Mackay, M. et al. (1985). "Polymorphism of the precursor for the major surface antigens of *Plasmodium falciparum* merozoites." **EMBO 4: 3823-3829.**

McGrew, J. et al. (1986). "Single base-pair mutations in centromere element III cause aberrant chromosome segregation in *Saccharomyces cerevisiae*." **Mol. Cell. Bio. 6(2): 530-538.**

Milhous, W. K. et al. (1985). "In vitro activities of and mechanisms of resistance to antifol antimalarial drugs." **Antimicrob Agents Chemother 27(4): 525-30.**

Nitiss, J. and J. C. Wang (1988). "DNA topoisomerase-targeting antitumor drugs can be studied in yeast." **Proc Natl Acad Sci U S A 85(20): 7501-5.**

Nitiss, J. L. (1994). "Using yeast to study resistance to topoisomerase II-targeting drugs." **Cancer Chemother. Pharmacol. 34(Suppl): S6-S13.**

Oduola, A. M. et al. (1988). "*Plasmodium falciparum*: induction of resistance to mefloquine in cloned strains by continuous drug exposure *in vitro*." . **Exptl. Parasitol. 67(2): 354-360.**

Pallavicini, M. G. et al. (1990). "Effects of methotrexate on transfected DNA stability in mammalian cells." **Molecular and Cellular Biology 10(1): 401-404.**

Peters, W. (1987). Chemotherapy and drug resistance in malaria. London, Academic Press.

Peterson, D. S. et al. (1988). "Evidence that a point mutation in dihydrofolate reductase-thymidylate synthetase confers resistance to pyrimethamine in falciparum malaria." **Proc Natl Acad Sci U S A 85(23): 9114-8.**

Peterson, D. S. et al. (1990). "Molecular basis of differential resistance to cycloguanil and pyrimethamine in Plasmodium falciparum malaria." **Proc Natl Acad Sci U S A 87(8): 3018-22.**

Peterson, D. S. et al. (1991). "Prevalence of the dihydrofolate reductase Asn-108 mutation as the basis for pyrimethamine-resistant falciparum malaria in the Brazilian Amazon." **Am J Trop Med Hyg 45(4): 492-7.**

Pillai, R. et al. (1993). "Use of a yeast expression system for the isolation and analysis of drug-resistant mutants of a mammalian phosphodiesterase." **Proc. Nat. Acad. Sci. USA 90: 11970-11974.**

Plowe, C. V. et al. (1995). "Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa." **Am J Trop Med Hyg 52(6): 565-8.**

Raymond, M., S. Ruetz, et al. (1994). "Functional expression of P-glycoprotein in Saccharomyces cerevisiae confers cellular resistance to the immunosuppressive and antifungal agent FK520." **Mol Cell Biol 14(1): 277-86.**

Roman, H. (1956). "Studies of gene mutation in *Saccharomyces*." **Cold Spring Harbor Quantitative Biology 21: 175-185.**

Saiki, R. et al. (1985). "Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia." **Science 230: 1350-1354.**

Sambrook, J. et al. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Press.

Sherman, F. et al. (1979). Methods in Yeast Genetics. Cold Spring Harbor, N.Y., Cold Spring harbor Press.

Sikorski, R. S. and Hieter, P. (1989). "A system or shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*." **Genetics 122: 19-27.**

Sirawaraporn, W. et al. (1993). "The dihydrofolate reductase domain of Plasmodium falciparum thymidylate synthetase-dihydrofolate reductase. Gene synthesis, expression, and anti-folate-resistant mutants." **J Biol Chem 268(29): 21637-44.**

Tanaka, M. et al. (1990). "Mutant dihydrofolate reductase-thymidylate synthetase genes in pyrimethamine-resistant Plasmodium falciparum with polymorphic chromosome duplications." **Mol Biochem Parasitol 42(1): 83-91.**

Thaithong, S. et al. (1992). "Pyrimethamine resistant mutations in Plasmodium falciparum." **Mol Biochem Parasitol 52(2): 149-57.**

Tosh, K. and B. Kilbey (1995). "The gene encoding topoisomerase I from the human malaria parasite Plasmodium falciparum." **Gene 163(1): 151-4.**

Trager, W. and J. B. Jensen (1976). "Human malaria parasites in continuous culture." **Science 193: 673-675.**

Walsh, P. S. et al. (1991). "Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material." **Biotechniques 10: 506-512.**

Warhust, D. C. et al. (1991). "Simplified preparation of malarial blood samples for polymerase chain reaction." **The Lancet 337: 303-304.**

Weber, J. L. (1988). "Molecular biology of malaria parasites." **Exptl. Parasitol. 66: 143-170.**

Wellems, T. E. et al. (1990). "Chloroquine resistance not linked to mdr-like genes in a Plasmodium falciparum cross [see comments]." **Nature 345(6272): 253-5.**

Wellems, T. E. et al. (1987). "A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of *Plasmodium falciparum*." **Cell 49: 635-642.**

Wernsdorfer, W. H. and Payne, D. (1991). "The dynamics of drug resistance *in Plasmodium falciparum*." **Pharmac. Ther. 50:95-121.**

White, N. J. (1992). "Antimalarial drug resistance: the pace quickens." **J Antimicrob Chemother 30(5): 571-85.**

Willet, G. P. et al. (1991). "Mixed population dynamics in human malarial parasites." **Transactions of the Royal Society of Tropical Medicine and Hygiene 85: 33-34.**

Wooden, J. M. et al. (1992). "*Plasmodium falciparum:* a simple polymerase chain reaction method for differentiating strains." **Exptl. Parasitol. 74: 207-212.**

Wu, Y. et al. (1995). "Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine." **Proc. Nat. Acad. Sci., USA.**

Zolg, J. W. et al. (1982). "*Plasmodium falciparum*: modifications of the *in vitro* culture conditions improving parasitic yields." **Journal of Parasitology 68: 1072-1080.**

Zolg, J. W. et al. (1990). "Detection of pyrimethamine resistance in Plasmodium falciparum by mutation-specific polymerase chain reaction." **Mol Biochem Parasitol 39(2): 257-65.**

Zolg, J. W. et al. (1989). "Point mutations in the dihydrofolate reductase-thymidylate synthetase gene as the molecular basis for pyrimethamine resistance in *Plasmodium falciparum*." **Mol. Biochem. Parasitol. 36: 253-262.**

Curriculum Vitae

Jason M. Wooden

Birthdate: 03/20/67

Education:

B.S. in Biology, Seton Hall University, 1989
(Minors in Chemistry and Mandarin Chinese)
Ph.D. in Genetics, University of Washington, 1996

Dissertation Research:

Genetic Analysis of the Development of Drug Resistance to Antifolates by the Malarial Parasite *Plasmodium falciparum.*

Awards and Honors:

Martin Luther King Jr. Scholarship, Seton Hall University, 1985-89
Amer. Heart Assoc. Summer Research Fellowship, Cardiology Div., Univ. of Conn., 1987
Biology Honors Program, Seton Hall Univ., 1987-89
RA Scholar of the Year, Seton Hall Univ., 1989
Student Leadership Certificate, Seton Hall Univ., 1989
Departmental Honors Citation in Biology, Seton Hall Univ., 1989
Martin Luther King Jr. Scholar University Medal, Seton Hall Univ., 1989
Graduated Magna Cum Laude, Seton Hall Univ., 1989
Ford Foundation Predoctoral Fellow, 1989-92
Amer. Soc. for Cell Biology Minority Fellowship, Marine Biological Labs, 1991
NIH Predoctoral Fellow, 1992-96
Amer. Soc. of Tropical Medicine & Hygiene Travel Awardee to present research at annual meeting, 1994

Positions Held:

Director, Environmental Education Program, Channel 3 Country Camp, Andover, CT, 1986, summer
Treasurer, Archaeology Society, Seton Hall Univ., 1987-89
Resident Assistant, Seton Hall Univ., 1988-89
President, Biological Sciences Society, Seton Hall Univ., 1988-89
Research Assistant, Biology Dept, Seton Hall Univ., 5/88 - 6/88
Teaching Assistant for General Genetics course, Univ. of Washington, 1/92 - 3/92
Volunteer for "Young Scientist in the Classroom" Program, Seattle Public Schools, 1992-93
Teaching Assistant for Human Genetics, Univ. of Washington, 3/93 - 6/93

Publications:

Wooden, J., Gould, E.E., Paul, A.T., and Sibley, C.H. 1992. ***Plasmodium falciparum*: A Simple Polymerase Chain Reaction Method for Differentiating Strains.** Experimental Parasitology 74:207-212.

Wooden, J., Kyes, S., and Sibley, C.H. 1993. **PCR and Strain Identification in *Plasmodium falciparum*.** Parasitology Today 9(8):303-305.

Wooden, J. M., Hartwell, L.H., Vasquez, B. L., and Sibley, C.H. 1996. **Analysis in Yeast of Antimalaria Drugs That Target the Dihydrofolate Reductase of *Plasmodium falciparum*.** Molecular and Biochemical Parasitology. In submission.