The genetics of resistance to antifolate and sulfa drugs in malaria parasites

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For the children of Africa
The genetics of resistance to antifolate and sulfa drugs in malaria parasites

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I declare that apart from the assistance mentioned above, the research described in this thesis is my own work, and that the thesis is my own composition.
Abstract

Drug resistance is one of the major obstacles facing malaria control. Resistance to the combination sulfadoxine/pyrimethamine (S/P) (Fansidar) is now widespread, although the mechanism by which this arises is still not fully understood. Therefore, the molecular basis of S/P resistance was studied in the rodent malaria parasite, *Plasmodium chabaudi*. S/P resistant mutants were selected from a clone already resistant the pyrimethamine, AS (PYR), caused by the presence of an asparagine at position 106 in its dihydrofolate reductase (DHFR). Two S/P resistant clones, AS (50S/P) and AS (75S/P), were selected and chosen for further analysis. AS (PYR) parasites were eliminated by S/P treatment, whereas AS (50S/P) and AS (75S/P) recrudesced following S/P pressure. However, each mutant possessed a different drug resistant phenotype. The AS (75S/P) clone always recrudesced before the AS (50S/P) clone following treatment with S/P, while AS (50S/P) always appeared before AS (75S/P) when treated with either sulfadoxine or pyrimethamine alone.

Mutations in the genes encoding the targets of sulfadoxine and pyrimethamine, dihydropteroate synthase (DHPS) and DHFR, have been implicated in the mechanism of S/P resistance in *P. falciparum*. The *P. chabaudi dhps* gene was cloned by homology and sequenced. The sequence analysis of the both *dhfr* and *dhps* genes of AS (75S/P) and AS(50S/P) did not reveal any polymorphisms when compared to the sequences of the AS (PYR) genes. The mechanism of resistance to S/P in these drug-selected lines is not therefore conferred by additional mutations in these genes.

To determine the genetic basis of the S/P resistance, AS (50S/P) was crossed with a drug sensitive clone, AJ. Sixteen independent recombinant progeny clones were phenotyped for their susceptibility to S/P and pyrimethamine and genotyped for the inheritance of 30 chromosome-specific markers. Linkage analysis shows that mutant *dhfr* is a major determinant of S/P resistance in *P. chabaudi*. Quantitative
trait analysis suggested that other loci, which may be involved in S/P resistance, are present on chromosomes 4, 5 and 9. In addition, the inheritance of loci on chromosomes 5 and 13 from the sensitive parent appear to contribute to the level of resistance observed. No association was found between S/P susceptibility and the inheritance of the dhps gene.
1. Introduction

Malaria is endemic throughout most of Africa, South East Asia, the Indian sub-continent and the South Pacific region. Current estimates suggest that malaria is responsible for 1.5 to 2.7 million deaths a year (WHO 1999). Almost half the world's population is exposed to infection.

Drug-resistance is well recognised as the single most important obstacle for malaria control (White et al. 1999). The genome project has lead to enormous advances in our knowledge of the basic biology of malaria parasites (Gardner 1999), but despite this, only a few genes determining resistance to the commonly used drugs have been identified. Identifying genes involved in drug-resistance is important for understanding and predicting how resistance to a given drug arises. It enables diagnostic molecular methods to be developed for identifying resistant parasites in clinical and field isolates, allows strategies to slow development of resistance to be implemented and paves the way for the pharmaceutical companies to redesign the inhibitors through rational drug design.

Resistance to the combination sulfadoxine/pyrimethamine (S/P) (Fansidar) is now widespread in the most pathogenic malaria species, *Plasmodium falciparum*, although the mechanism by which this arises is still not fully understood. The aim of this work is to identify genes conferring resistance to this drug combination, making use of the rodent model, *P. chabaudi*.

1.1 Malaria

Malaria is caused by the protozoan parasite, *Plasmodium*. There are four species which infect humans; *P. vivax*, *P. falciparum*, *P. malariae* and *P. ovale*, the most virulent of these being *P. falciparum*. There are many other malaria species which infect a variety of vertebrates including primates, rodents, birds and lizards (Garnham 1966). The rodent malaria parasites are widely used as models for human malaria. Four species, *P. berghei*, *P. chabaudi*, *P. vinckei* and *P. yoelii*, have been
isolated from thicket-rats in five countries in Africa (Killick-Kendrick and Peters 1978), all of which have been adapted to grow in laboratory rodents.

The incidence of malaria was greatly reduced in the 1960's by the use of insecticides to eliminate the mosquito from areas of human habitat, most notably dichloro-diphenyl-trichloro-ethane (DDT) (reviewed by Bruce-Chwatt 1985). However, the incidence of the disease is now increasing due to the failure of many control programmes. Attempts to control malaria include draining or treating breeding areas to reduce mosquito populations, together with the use of residual insecticides, bed nets and antimalarial drugs. However, changes in vector behaviour, mosquito resistance to insecticides, the development of drug resistance by the parasite, increased travel, lack of financial support and political unrest, especially in Africa, have resulted in the breakdown and failure of many programmes. Attempts to develop a vaccine have so far been unsuccessful and it looks increasingly unlikely that a vaccine conferring sterilising immunity will ever be feasible (Holder 1999). Until a protective vaccine or new effective chemotherapeutic drugs are developed, present malaria control measures must be relied on to control the disease.

1.2 Life-cycle of malaria parasites

Malaria parasites belong to the Apicomplexa, a phylum consisting entirely of parasitic protozoa. They are classified by the presence of an apical complex which is used by the invasive stages of these organisms to invade host cells. In all malaria species, the parasite is transmitted by the mosquito and has a complex life cycle, summarised in Figure 1 (reviewed by Garnham, 1988). Malaria parasites are ingested by the female mosquito during feeding on a vertebrate host with parasites in the circulation. In the mosquito midgut, the male gametocyte undergoes exflagellation, releasing around 8 motile gametes which fertilise the female macrogametes (Sinden 1982). Fertilisation results in the development of the motile ookinete which passes through the midgut wall (Sinden 1984). It then encysts on the outer wall of the midgut, and develops over the next 7 to 14 days as an oocyst.
Figure 1. The life-cycle of malaria parasites. Adapted from Taylor (1997).
Sporogonic development occurs within the oocyst, and sporozoite formation begins about 6 to 9 days after the infective blood meal, depending on the species (Vanderberg et al. 1967, Sinden and Strong 1978). After 8 to 15 days, the oocyst ruptures releasing 1000 to 10000 sporozoites which migrate to the salivary glands (Pringle 1965, Garnham 1966, Rosenberg and Rungsiwongse 1991). A new host becomes infected when the sporozoites are injected with saliva when the mosquito takes its next bloodmeal. The sporozoites rapidly infect hepatocytes in the liver, maturing into liver schizonts. Two to five days after infection, 5000 - 30 000 merozoites are released from the liver cells into the circulation, where they invade the red blood cells almost immediately (Shortt et al. 1951, Bray 1957a, Bray 1957b). The merozoites develop into trophozoites and then mature schizonts. One invading merozoite produces 4 to 40 daughter parasites asexually within 24 to 48 hours, depending on the parasite species (Garnham 1966). Infections are synchronous, therefore when the schizonts rupture, thousands of merozoites and their waste products are released into the bloodstream producing the fever characteristic of malaria. This asexual erythrocytic cycle is repeated many times in an infection, resulting in an increase in parasitaemia until the process is either controlled by the immune system of the host or the host dies. The intracellular environment of the red blood cell results in the parasite being relatively inaccessible to the host immune system, thus avoiding immune destruction. Furthermore, the erythrocyte provides a supply of protein in the form of haemoglobin that is utilised as a source of amino acids, nitrogen and, possibly, energy. During the course of an infection and depending on the species, a certain number of merozoites develop into the sexual stages, gametocytes, to be taken up by a mosquito for transmission to another host.

1.3 Genetics of malaria parasites

Malaria parasites are haploid for most of their life cycle. The only diploid stage is the zygote, produced by fertilisation of the gametes in the mosquito gut. Meiosis occurs within three hours of fertilisation followed by mitosis to produce the oocyst and subsequently haploid sporozoites (Sinden and Hartley 1985). All
Plasmodium spp so far examined have 14 chromosomes that range in size between 0.6 kilobases (kb) for chromosome 1 and 3400 kb for chromosome 14 and the genome is thought to encode up to 7500 genes (Kemp et al. 1987, Triglia et al. 1992, Carlton et al. 1999). Chromosomes are polymorphic both between species of malaria parasites and between isolates of the same species, while the chromosome location of different genes among the four species of human malaria is highly conserved (synteny) (Carlton et al. 1999). In addition, gene synteny is conserved between the rodent malaria parasites (Janse et al. 1994) and between P. chabaudi and P. falciparum (Carlton et al. 1998b). In addition to the nuclear genome, malaria parasites possess two extra-chromosomal DNA elements. A 35 kb genome in the apicoplast contains genes for several rRNAs, tRNA and RNA polymerase genes (Wilson et al. 1996) while a linear 6 kb element contains certain mitochondrial genes, thought to be necessary for an electron transport system. Only female gametocytes were found to have mitochondria, and thus inheritance of the 6 kb mitochondrial element is uniparental through the female gamete (Creasey et al. 1993). The presence of the apicoplast genome is unusual and it has been speculated that this is indicative of an early evolutionary origin some 800 million to 1 billion years ago at the time plants and animals diverged (Kohler et al. 1997, Denny et al. 1998).

Most P. falciparum infections consist of more than one genetically distinct genotype of the parasite. Genetic analysis using polymerase chain reaction (PCR) in Tanzania, Senegal and Papua New Guinea have shown that between 70 and 85% of infected individuals have more than one parasite genotype present (Babiker et al. 1994, Ntoumi et al. 1995, Paul et al. 1995). This has important implications for population structure, as cross-fertilisation between genetically distinct gametes can lead to the generation of parasites with novel genotypes through the processes of recombination. This has been demonstrated in P. yoelii (Walliker et al. 1971, Walliker et al. 1973), P. chabaudi (Walliker et al. 1975, Carlton et al. 1998a) and P. falciparum (Walliker et al. 1987, Wellems et al. 1990, Ranford-Cartwright et al. 1993).
Genetic recombination is important in understanding how parasites resistant to more than one drug can arise. If resistance to each drug is due to mutations in separate, unlinked genes, then a parasite must gain mutations in both genes to become double-resistant. It is unlikely that two mutations will arise simultaneously in one parasite. However, recombination can bring together genes conferring resistance to more than one drug in a single parasite, but could also dissociate such combinations during any subsequent transmissions. Therefore, predictions on how drug resistance will spread and persist in parasite populations will depend on the number of genes determining resistance, the selection pressure applied (drug use), the frequency with which recombination between the genes occurs in nature and the relative fitness of the drug resistant forms in competition with sensitive forms in the absence of drug pressure (Babiker and Walliker 1997). Mathematical modelling has shown that even when resistance is encoded by two mutant alleles at unlinked loci, as is likely for S/P resistance, the frequent breakdown of the double mutant under high transmission may not be sufficient to prevent the double mutant surviving, as selection for resistance by the presence of drug pressure may outcompete recombination to keep the mutants in the population (Mackinnon 1997).

1.4 Linkage analysis and positional cloning in malaria parasites

The identification of restriction fragment length polymorphisms (RFLPs) (Botstein et al. 1980), abundant, highly polymorphic microsatellite (short tandemly repetitive DNA) loci (Jeffreys et al. 1985) and more recently, single nucleotide polymorphisms (SNPs) (Zhao et al. 1998), has lead to chromosomal mapping of many loci through linkage analysis. The technique relies on the ability to detect a marker such as an RFLP or microsatellite, that is linked to the locus of interest. Loci are linked if they are close together on the same chromosome. Recombination is likely to separate them only rarely and they will therefore normally be inherited together. Linkage analysis involves the collection of genetic cross data or family pedigrees in which the trait of interest is co-segregating with known marker genes. The parents and progeny are phenotyped for the inheritance of the trait and genotyped
for the inheritance of multiple polymorphic markers. Markers closest to the gene will show the strongest correlation with the trait and tracking recombination events can narrow the region harbouring the gene to between 100 and several thousand kilobases (kb). Fine mapping of the gene is therefore limited by the number of informative meioses available. Final identification of the gene requires physical mapping, which has been made much easier in recent years by the development of high-throughput DNA sequencing.

As the complete sexual life-cycle of *P. falciparum* can only be studied by transmission through a primate host, classical genetics in this species can be performed only with great difficulty (Walliker *et al.* 1987). Therefore, only two genetic crosses have been made and only a few traits mapped. Detection of genetic determinants is now feasible because of the large number of genetic markers covering the genome (Su *et al.* 1999). Linkage studies of *P. falciparum* crosses have mapped genetic determinants contributing to drug resistance (Peterson *et al.* 1988, Su *et al.* 1997, Wang *et al.* 1997a) and mosquito infectivity (Vaidya *et al.* 1995). Linkage analysis in *P. chabaudi* became possible with the development of RFLPs as genetic markers and a genetic map (Carlton 1995, Carlton *et al.* 1998a).

### 1.5 Quantitative trait analysis

The advent of genetic maps has permitted genetic dissection of individual quantitative traits. Mendel developed the theory of inheritance based on experiments with garden peas, studying traits that could be classified on an either-or basis, i.e. all the variation was due to a simple difference at a single locus. However, there are many traits for which this classification is impossible because the characters vary in the population along a continuum. These are quantitative characters where the individual phenotype results from the sum total of the effects of all of the contributing loci. Therefore, quantitative variation usually indicates polygenic inheritance, i.e. that there is an additive effect of two or more genes on a single phenotypic character.
Mapping quantitative trait loci (QTL) is similar to linkage analysis and involves performing a cross between two clones that differ substantially in the quantitative trait. The progeny are then scored for the trait and the inheritance of a number of genetic markers (Paterson et al. 1988). Single genetic markers are studied one at a time. The phenotypic means of the character under investigation (e.g. drug-resistance) are calculated for the two groups of progeny genotypes and compared: those inheriting the allele from parent A are compared with those inheriting the marker allele from parent B. The difference between the means provides an estimate of the phenotypic effect of inheriting either allele. A statistical test is then applied to test whether the inferred phenotypic effect is significantly different from zero; the difference in the means will be zero if inheritance of either allele has the same effect on the phenotype. If the difference is statistically significant, this implies that the marker is close to a gene or QTL that contributes to the quantitative trait of interest. Fine mapping of the region will eventually lead to the cloning of the gene underlying the trait.

The only QTL analysis study in species of *Plasmodium* reported to date, is that of Carlton et al. (1998a), on chloroquine resistance in *P. chabaudi*, although the construction of a detailed genetic map of *P. falciparum* (Su et al. 1999) now permits detailed analysis of accessible quantitative traits in the human malaria parasite (Ferdig and Su 2000).

1.6 Chemotherapy and drug-resistance

1.6.1 Drug-resistance

The failure of a drug to clear an infection can be due to either inadequate levels of drug in the patient (inappropriate dose taken, failure to complete the course, poor absorption, unusual metabolism) or to drug resistance. Resistance is selected by exposure of the organisms to the drug and can be expected to be due to a variety of mechanisms. Stable gene mutations are of most concern since they may persist in the parasite population once they have arisen.
1.6.2 Identifying genes involved in drug-resistance

The most useful starting material for identifying genes determining resistance is resistant mutants selected from sensitive clones. The sensitive and resistant forms are therefore genetically identical, except for the gene(s) determining the resistance. Direct examination of the sensitive and resistant forms can then be made to search for mutations in candidate genes which cause the resistance.

In practice, it is very difficult to select resistant \( P. falciparum \) lines which exhibit stable resistance in the absence of drug pressure. Only a few successes have been reported following many months of prolonged culture in the presence of gradually increasing doses of drug (Oduola 1988, Lim and Cowman 1996). In contrast, it is usually easy to obtain stable drug-resistant mutants of rodent parasites by exposing infected mice to various drug regimes. In \( P. chabaudi \), stable mutants have been produced in this way that are resistant to pyrimethamine (Walliker et al. 1975), chloroquine (Rosario 1976b, Padua 1981) and mefloquine (Padua and Walliker 1978). If the target(s) of the drug are known, their gene sequences are analysed from resistant and sensitive forms to search for mutations that may be associated with resistance. In the absence of a candidate gene, or to confirm the role of mutations in resistance, crosses need to be made between resistant mutants and unrelated sensitive clones and the genetic determinant mapped by linkage analysis. Transfection of candidate genes into drug-sensitive clones can then be carried out to confirm a causal relationship with resistance (van Dijk et al. 1995, Wu et al. 1996).

1.6.3 Quinine

The bark of the cinchona tree has been used to treat malaria fevers for centuries (Meshnick 1997). Extracts of this bark contain quinine, a quinolinemethanol, which is now produced synthetically. It is a low cost drug, now considered too toxic for prophylaxis or routine treatment of the disease, but it is still used under hospital supervision to treat cerebral malaria (Foley and Tilley 1997). While a few cases of quinine-resistant malaria have been reported, these have not been confirmed by \textit{in vitro} tests and quinine still remains effective (see Peters, 1987).
The reasons for this are unclear, since quinine has been used for centuries, but the short half-life is thought to minimise selection pressure on the parasite. Furthermore, as quinine is mainly administered in hospitals, patients are more likely to receive a course of quinine therapy (Meshnick 1998). Today, quinine is the last line treatment for patients infected with multi-drug resistant *P. falciparum*. Attempts to select for quinine resistance have met with little success. Therefore, it has not been possible to determine any potential mechanisms of resistance.

### 1.6.4 Chloroquine

Modern quinine derivatives, which are less toxic than quinine itself, include chloroquine, amodiaquine and more recently, mefloquine (Lariam®) and halofantrine. However, *P. falciparum* isolates resistant to almost every antimalarial drug and the various drug combinations have now been reported (see Peters, 1987). Chloroquine is one of the cheapest and most widely available antimalarials. The development of resistance to chloroquine in *P. falciparum* was thus one of the most important setbacks to malaria control. Parasites resistant to chloroquine were first reported in the early 1960's, almost simultaneously in Thailand (Harinasuta *et al.* 1962) and South America (Moore and Lanier 1961, Young and Moore 1961). Today, chloroquine is only effective in Central America and some areas in West Africa (Wernsdorfer 1991, Bloland *et al.* 1993).

The mechanism of action of chloroquine is not fully understood. The metabolism of haemoglobin by the parasite results in the production of potentially toxic, free haem. This is sequestered into the food vacuole as non-toxic crystals of haemazoin (malaria pigment). Chloroquine is proposed to inhibit the polymerisation and detoxification of haem in the vacuole, resulting in swelling of the vacuole and ultimately cell lysis (Jacobs *et al.* 1988, Goldberg *et al.* 1990, Egan *et al.* 1994).

The mechanism of resistance to chloroquine is a highly debated topic. Resistance appears to be associated with either a decreased uptake or increased expulsion of the drug. Resistant parasites have been reported to expel chloroquine 40-50 times more rapidly than sensitive parasites (Krogstad *et al.* 1987). This
resistance phenotype was found to be reversed by the calcium channel blocker verapamil, leading to the hypothesis that chloroquine resistance is similar to the multi-drug resistant (MDR) phenotype in mammalian tumour cells (Martin et al. 1987). MDR tumour cells overexpress an ATP-dependent transporter, the P-glycoprotein which can pump out a wide range of chemically dissimilar compounds (Riordan et al. 1985). Two P-glycoproteins have been identified in *P. falciparum* which are encoded by the *pfmdr1* and *pfmdr2* genes (Foote et al. 1989, Wilson et al. 1989, Cowman et al. 1991). Amplification of the *pfmdr1* gene has been observed in some chloroquine resistant isolates suggesting that increased expulsion was the mechanism of resistance (Foote et al. 1989). Further studies have cast doubts on the role of amplification as the level of chloroquine resistance in a number of strains did not correlate with the level of *pfmdr1* expression (Wilson et al. 1993, Cowman et al. 1994). Alleles of the *pfmdr1* gene identified in field isolates of *P. falciparum* have also been linked with chloroquine resistance, although there are numerous exceptions (Foote et al. 1990). Furthermore, analysis of a genetic cross between parasite clones HB3 (chloroquine-sensitive) and Dd2 (chloroquine-resistant) revealed no linkage between either of the *pfmdr* genes and chloroquine resistance (Wellems et al. 1990). Instead, a gene on chromosome 7 showed linkage with the chloroquine-resistant phenotype (Wellems et al. 1991). Further mapping identified a gene denoted cg2 on this chromosome that segregated with resistance (Su et al. 1997). More recently, transfection of resistant parasites with the 'chloroquine-sensitive' allele of cg2 was shown not to alter the chloroquine response of the recipient parasite (Fidock et al. 2000a). Instead, a gene denoted *crt*, which is closely linked to cg2, has been demonstrated to be a stronger candidate (Fidock et al. 2000b).

Other candidate genes include a chloroquine importer that has been identified in *P. falciparum* and differences in chloroquine uptake have been shown to be genetically linked to the chloroquine resistance phenotype (Sanchez et al. 1997). Recent data suggests that decreased vacuolar pH is the determinant of chloroquine resistance, which lowers the availability of free haem for chloroquine binding, resulting in decreased chloroquine uptake (Dzekunov et al. 2000, Ursos et al. 2000).
Clearly, it will be of interest to see if the locus on chromosome 7 is able to regulate vacuole pH in a verapamil reversal manner.

It is likely that there are two or more different mechanisms of chloroquine resistance, which confer different levels of resistance (Cowman and Foote 1990). Resistance to chloroquine was slow to emerge in *P. falciparum*, and may have arisen only twice, once in South East Asia and once in South America (Payne 1987). This is indicative of a multigenic trait, i.e. resulting from an additive effect of mutations in many genes. Recent transfection experiments have shown that one allele of *pfmdrl* modulates resistance to chloroquine, as well as to mefloquine and quinine (Reed *et al.* 2000), suggesting that Foote's hypothesis that the chromosome 7 locus is epistatic to *pfmdrl*, may indeed be correct (Foote *et al.* 1990).

In *P. chabaudi*, linkage analysis of progeny from crosses between chloroquine-sensitive and -resistant clones has been reported (Carlton *et al.* 1998a). Twenty independent recombinant progeny were typed for their response to chloroquine and their inheritance of 46 chromosome-specific markers. A region of chromosome 11 was linked to resistance, while no linkage was observed with the *pcmdrl* gene, the *P. chabaudi* homologue of *pfmdrl*. Quantitative trait mapping produced strong evidence for a gene determining resistance on chromosome 11, with other possible genes on chromosomes 3, 5 and 9. These genes have yet to be characterised.

**1.6.5 Mefloquine**

*Pfmdrl* has also been implicated in determining mefloquine-resistance in *P. falciparum*. Mefloquine-resistant mutants have been selected in the laboratory in which over-expression and duplication of *pfmdrl* have been observed (Cowman *et al.* 1994, Peel *et al.* 1994). Duplication and overexpression of *mdr* have also been associated with mefloquine-resistant *P. chabaudi* (Phillips 1997) and *P. berghei* (Gervais *et al.* 1999). Reed *et al.* (2000) have also shown that different allelic forms of *pfmdrl* conferred different levels of susceptibility to mefloquine.
1.6.6 Inhibitors of the folate pathway

Compounds that inhibit enzymes in the folate pathway have been used to treat many pathogens and have been widely exploited as antimalarials. Tetrahydrofolate is a cofactor essential for the transfer of one-carbon groups in various biosynthetic pathways. Folate derivatives are essential co-factors in the synthesis of DNA and certain amino acids. *Plasmodium*, like many plants and bacteria, synthesises tetrahydrofolate *de novo* from guanosine triphosphate (GTP) via the folate pathway (Figure 2). This pathway is absent from mammalian cells as dihydrofolate is derived from dietary folic acid. However, in all organisms, dihydrofolate must be reduced to tetrahydrofolate by the enzyme, dihydrofolate reductase (DHFR). Fortunately for antimalarial chemotherapy, the mammalian DHFR has a much higher substrate specificity than the *Plasmodium* enzyme and is less susceptible to inhibition by antifolates. The mode of action of the antifolates was elucidated in bacteria, where *de novo* synthesis of folate is obligatory. However, the situation is complicated in *Plasmodium* by the presence of salvage mechanisms, permitting the parasites to use exogenous folate (Chulay *et al.* 1984, Milhous *et al.* 1985, Watkins *et al.* 1985, Krungkrai *et al.* 1989, Wang *et al.* 1997c). This may even be the primary source of their folate requirements in the mammalian host. Therefore, the fact that antifolates and sulfonamides have antimalarial activity at all is somewhat surprising.

1.6.7 Antifolate resistance

The mechanism of resistance to the antifolate drugs is the only mechanism of drug-resistance in malaria parasites that is well understood. Antifolates are DHFR inhibitors and pyrimethamine is one of the most widely used inhibitors (Figure 3). Pyrimethamine was first introduced into the field in 1952, but reports of resistance in *P. falciparum* followed shortly afterwards (Peters 1987).

Pyrimethamine resistant parasites have been selected from all the rodent malaria species; from *P. berghei* (Rollo 1952, Diggens 1970, van Dijk *et al.* 1994), *P. yoelii* (Walliker *et al.* 1973), *P. chabaudi* (Walliker *et al.* 1975) and *P. vinckei*
Figure 2. The pathway for dihydrofolate and DNA synthesis from guanosine triphosphate (GTP) in Plasmodia. Adapted from Warhurst (1986). Enzymes are shown in bold, substrates in italics.
Folic Acid

\[
\text{H}_2\text{N} - \text{CH} - \text{N} - \text{CH}_2\text{NH} - \text{CH}_2\text{NH} - \text{COOH}
\]

Pyrimethamine

\[
\text{Cl} - \text{N} - \text{NH}_2 - \text{C}_2\text{H}_5
\]

Figure 3. The structures of folic acid and pyrimethamine.

(Yoeli et al. 1969). Pyrimethamine resistance became the first selectable marker available and this led to the first classical genetic studies being carried out in species of Plasmodium. Analysis of the cloned progeny of these crosses, between pyrimethamine-sensitive and resistant lines of *P. yoelii* and *P. chabaudi* respectively, demonstrated that the resistant phenotype appeared to segregate as a single locus, since progeny clones exhibited phenotypes similar to each parent with no intermediate levels (Walliker et al. 1973, Walliker et al. 1975). Subsequently, a mutation in the *dhfr* gene was identified as a probable cause of pyrimethamine resistance in *P. falciparum*. In the first genetic cross carried out in this species, all resistant progeny were shown to inherit an RFLP associated with the *dhfr* of the resistant parent (HB3), while all sensitive progeny inherited the 3D7 allele (Peterson et al. 1988). The only difference in the DHFR of each parent was residue 108, which is serine (S) in 3D7 and asparagine (N) in HB3. This result has been supported by
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numerous field surveys of *P. falciparum*, in which S108N is found in almost all confirmed pyrimethamine-resistant isolates (Siriwaraporn 1998). Convincing proof of the key role of this allele of *dhfr* has also been obtained in transfection experiments using mutant *dhfr* as a selectable marker in *P. berghei* (van Dijk et al. 1995) and *P. falciparum* (Wu et al. 1996).

Mutations at other sites in the *dhfr* gene have been associated with increased resistance to pyrimethamine, as well as resistance to a second antifolate drug, cycloguanil (Siriwaraporn 1998), but crossing and/or transfection experiments to confirm the role of these mutations have not been done in either *P. falciparum* or in rodent malaria species.

Molecular analysis of several pyrimethamine-resistant rodent clones has shown that the mechanisms of pyrimethamine resistance are remarkably similar to those seen in *P. falciparum*, and may provide additional insights into the means by which resistance occurs and spreads in the field. In *P. chabaudi* and *P. yoelii*, resistance is associated with a point mutation in their *dhfr* genes which causes an amino acid change at position S106N, thought to be equivalent to the S108N change in *P. falciparum* (Cowman and Lew 1990, Cheng and Saul 1994). In addition, a low level resistant line of a subspecies of *P. chabaudi*, *P. c. adami* (DS) was selected using a continuous low-pressure method and found to have an extra copy of the *dhfr* gene as a result of a partial gene duplication event (Cowman and Lew 1989) and a similar result was reported for a low-level resistant line selected from the *P. berghei* ANKA clone (van Dijk et al. 1994). Further selection to give a higher level of resistance in the *P. c. adami* mutant resulted in the loss of the duplicated gene and a point mutation at S106N in the remaining copy (Cowman and Lew 1990). The increase in resistance as a result of the point mutation was several orders of magnitude greater than the resistance conferred by the duplication, which may explain why amplification of *P. falciparum dhfr* has not been observed in the field.
1.6.8 The sulfa drugs

A variety of sulfa drugs (sulfonamides) have been developed to combat human pathogens. Sulfachryscidine and sulfanilamide were first used to treat malaria in the 1930's and many derivatives have since been used as antimalarials with varying success (for review, see Peters, 1987). These drugs had a slow and relatively short-lived action resulting in the need for high, potentially toxic doses and so their use was discontinued. Once chloroquine resistance became established and there was a need to find alternatives, the long acting sulfonamide compounds, sulfadiazine and sulfadoxine were developed. These compounds bind strongly to plasma proteins and have long half-lives. The sulfonamides act on trophozoites and schizonts, the DNA replicating stages of the asexual cycle. They are analogues of para-aminobenzoic acid (PABA) (Figure 4) and competitively inhibit the dihydropteroate synthase (DHPS) enzyme, required for folate biosynthesis. DHPS catalyses the condensation of PABA with phosphorylated pteridine to form dihydropteroate (Figure 2). This is then converted to dihydrofolate. This pathway is absent in the mammalian host where dihydrofolate is obtained from dietary folic acid, making the malaria enzymes ideal targets for rational drug design.

\[
PABA \quad \text{H}_2\text{N} - \text{COO}^- \\
\text{Sulfadoxine} \quad \text{H}_2\text{N} - \text{SO}_2\text{NH} - \text{N} \quad \text{CH}_3\text{O} \quad \text{OCH}_3
\]

Figure 4. Structures of \textit{para-aminobenzoic acid (PABA)} and the analogue, sulfadoxine.
1.6.9 Sulfonamides: mechanism of action

Sulfonamides compete with PABA for DHPS. They deplete the 7,8-dihydropteroin pool by reacting as a PABA-substrate analogue to form a 7,8-dihydropteroin-sulfonamide adduct (Roland et al. 1979). Inhibition of the next enzyme in the pathway, dihydrofolate synthase, or other folate enzymes, was found to be irrelevant at physiological concentrations and it was concluded that competition with PABA is the primary mode of action (Roland et al. 1979). The subsequent reduction in the rate of 7,8-dihydropoterate synthesis decreases the concentration of tetrahydrofolate cofactors, reducing the rate of synthesis of the products of the pathway, thus inhibiting cell growth.

The extent to which *Plasmodium* can synthesise PABA *de novo* is a much-debated topic. Milk diets, which are deficient in PABA, were shown in early work to inhibit the development of the respective malaria infections in rats, mice, chicks and monkeys (Maegraith et al. 1952, Bray and Garnham 1953, Hawking 1953, Greenberg et al. 1954). Furthermore, the addition of PABA to the diet restored growth, indicating that *Plasmodium* have a requirement for exogenous PABA. These studies have been disputed in more recent work. The majority of *P. falciparum* parasites grow *in vitro* in PABA- and folate-free medium at normal rates, suggesting that the parasites are able to synthesise the PABA they require (Milhous et al. 1985, Watkins et al. 1985, McConkey et al. 1994, Wang et al. 1997c). Dieckmann and Jung (1986) detected activities of four of the shikimate pathway enzymes, required for PABA synthesis, in *P. falciparum*. McConkey et al. (1994) were able to select for auxotrophs of *P. falciparum* that were dependent on exogenous PABA for growth, unlike the clone from which they were derived. Conclusive proof of the presence of the pathway necessary for PABA biosynthesis, the shikimate pathway, in malaria parasites was obtained when the gene encoding chorismate synthase, the final enzyme in the pathway, was identified in the genome project (Roberts et al. 1998). The precise role of the shikimate pathway in *Plasmodium* is still unclear.
1.6.10 Sulfonamides: mechanism of resistance

In bacteria, sulfonamide resistance is well characterised. Resistance in Escherichia coli is caused by a point mutation in a highly conserved region of the dhps gene (Dallas et al. 1992), while a two amino acid insertion in the DHPS of both Streptococcus pneumoniae and Neisseria meningitidis have also been shown to confer sulfonamide resistance (Lopez et al. 1987, Fermér et al. 1995). Mutations in the genes that encode DHPS have also been found to confer resistance to sulfonamides in Mycobacterium leprae (Kai et al. 1999) and Streptococcus pyogenes (Swedberg et al. 1998) and have been associated with sulfonamide failure in Pneumocystis carinii (Helweg-Larsen et al. 1999).

Therefore, it was assumed that resistance to sulfadoxine in P. falciparum would also be conferred by mutations in dhps. The dhps gene has been cloned from P. falciparum and the gene found to encode both DHPS and 7, 8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK), the enzyme prior to DHPS in the folate pathway (Figure 2) (Brooks et al. 1994, Triglia and Cowman 1994). All the protozoan DHPS enzymes characterised to date have been either bifunctional, with both PPPK and DHPS domains, as in P. falciparum and T. gondii (Pashley et al. 1997), or trifunctional as in Pn. carinii, with the domain of another enzyme from the folate pathway, dihydroneopterin aldolase (Volpe et al. 1992).

The identification of amino acid differences among alleles of dhps of P. falciparum isolates, suggested that as in other organisms, the mechanism of sulfonamide resistance involved mutations in the dhps gene. The relevance of these mutations is discussed in section 1.6.12.

1.6.11 Antifolate/sulfonamide combinations

It was observed that if pyrimethamine and sulfadoxine were administered together, a high level of synergism was displayed (Richards 1966, Chulay et al. 1984), and the rates at which resistance to these compounds developed was reduced (Peters 1974). This led to the introduction of the drug Fansidar® (S/P, Roche Pharmaceuticals, 25 mg pyrimethamine and 500 mg sulfadoxine). S/P is used both
prophylactically and therapeutically and, given the spread of chloroquine resistance, represents the only effective antimalarial affordable in Africa on a mass-scale (Foster 1991, Bloland et al. 1993, White et al. 1999).

The antimalarial activity of S/P is far greater than can be explained by simple additive effects of the two individual drugs. Wang et al (1999) explain the synergy between sulfa drugs and pyrimethamine by showing that pyrimethamine has a second site of action, in addition to inhibiting DHFR. It appears that pyrimethamine also blocks the utilisation of exogenous folate, which forces the parasite to use the endogenous folate pathway, rendering it extremely susceptible to sulfa inhibition. The mechanism by which this occurs is unknown, but it is likely that since pyrimethamine is a structural analogue of folic acid, it inhibits either the uptake or processing of exogenous folate (Wang et al. 1999).

1.6.12 Resistance to sulfadoxine/pyrimethamine

Despite evidence that resistance to antimalarial combinations is slow to arise (Peters 1974, White 1998), resistance to S/P has been quick to appear in *P. falciparum*. Reports of resistance to S/P treatment appeared in Thailand soon after it was introduced (Chongsuphajaisiddhi et al. 1979, Reacher et al. 1980). It is thought that the long half-life of SIP results in a strong selection pressure (Watkins and Mosobo 1993, Nzila et al. 2000a). By the mid-1980's, S/P was no longer effective in many areas of South America and South East Asia. Today, it is only in Africa, where extensive use of S/P is recent, that it is still effective. S/P is now the first-line antimalarial of four African countries; Botswana, Kenya, Malawi and South Africa, so the widespread development of S/P resistance in Africa would be disastrous (White et al. 1999).

While some studies suggest that S/P failure may be determined simply by high level resistance to pyrimethamine conferred by multiple mutations in *dhfr* (Watkins et al. 1997), it is also possible that it could be due to an additive effect of mutations conferring resistance to each drug separately. An association of mutations
in *dhfr* with those in *dhps* has thus been sought to explain S/P resistance (Wang *et al.* 1997b, Plowe *et al.* 1998).


More recently, transfection work has suggested that in *P. falciparum*, DHPS amino acids serine-436, glycine-437, lysine-530, alanine-581 and alanine-613 specifically confer sulfadoxine resistance, with glycine-437 being the key residue (Triglia *et al.* 1998). However, the transfection studies were complicated by the fact that an additional copy, or copies, of the *dhfr* gene were transfected into the recipient sensitive parasites, the activity of which could affect their response to sulfonamide drugs. Furthermore, the characterisation of the transfected parasites was carried out with sulfadoxine only in PABA- and folate-deficient medium, so the relevance of
these amino acid changes to S/P at physiological concentrations was unclear. Genetic crossing work has produced evidence that a dhus allele containing phenylalanine-436, glycine-437 and serine-613 is linked to sulfadoxine resistance, although another locus linked to dhfr on chromosome 4, which influences the use of exogenous folate by the parasite, has also been implicated (Wang et al. 1997a). Again, these in vitro assays were only carried out with sulfadoxine and in PABA- and folate-free medium.

The rodent malarias are more satisfactory to study resistance to these drugs because they allow levels of PABA and folate to be controlled in the animal diet. Parasites resistant to sulphonamide drugs, such as sulfadiazine, have been selected in *P. berghei* (Peters 1974) and *P. chabaudi* (MacLeod 1977). In general, sulfadiazine-resistant parasites have a reduced requirement for PABA, suggesting that they may have acquired the ability to synthesise PABA de novo (Jaswant-Singh et al. 1954, Ramakrishnan et al. 1956). Pyrimethamine-resistant parasites, however, have an increased requirement for PABA (Jacobs 1964). There are few data on the PABA requirements of S/P resistant parasites, although Merkli and Richle (1983) reported that the development of resistance to the S/P combination in *P. berghei* was accelerated by a diet containing a high level of PABA. A cross between a sensitive line of *P. chabaudi* and one resistant to both pyrimethamine and sulfadiazine suggested that resistance to these two compounds was determined by genes at two loci (MacLeod 1977). Unfortunately, there are no sequence data on the *dhfr* or *dhus* genes of any of these parasites.

1.7 Outline of present study

The object of this study was to investigate the genetic basis of S/P resistance in the rodent malaria parasite, *P. chabaudi*. Of the four rodent malaria models, *P. chabaudi* is the most similar to *P. falciparum*; both have a preference for mature erythrocytes, infections are highly synchronous and mature parasites are sequestered from the peripheral circulation (Killick-Kendrick and Peters 1978). Furthermore, the mechanisms of pyrimethamine resistance in both species have been shown to be
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identical (Peterson et al. 1988, Cheng and Saul 1994) and chromosome location of
groups of genes between the two species is highly conserved (Carlton et al. 1998b).
Therefore, once the gene(s) conferring resistance to S/P are identified in *P. chabaudi*,
examination of the two genomes will identify the corresponding *P. falciparum*
gene(s).

The first section describes selection for S/P resistant mutants. Several
mutants were obtained following single-step selection by the drug combination on a
clone already resistant to pyrimethamine, AS (PYR). Pyrimethamine resistance in
AS (PYR) is conferred by the presence of asparagine at position 106 in DHFR,
equivalent to the serine to asparagine change at position 108 conferring resistance to
pyrimethamine in *P. falciparum* (Peterson et al. 1988, Cowman and Lew 1990,
Cheng and Saul 1994). A clone already resistant to pyrimethamine was chosen as the
starting material for selection because in *P. falciparum*, pyrimethamine resistance
and hence mutations in *dhfr*, were already widespread when S/P was introduced in
the field (Peters 1987).

Mutations in the gene encoding DHPS have been implicated in the
mechanism of S/P resistance in *P. falciparum* (Triglia et al. 1998). Therefore, to
identify any possible sequence variations associated with resistance to S/P, the *P.
chabaudi pppk-dhps* homologue was isolated. Analysis of this gene and the *dhfr-ts*
of the resistant mutants did not reveal any sequence changes when compared to the
respective sequences of the AS (PYR) genes. This is presented in the second part of
the study.

In the third section, crosses were made between an S/P resistant clone, AS
(50S/P) and a sensitive clone, AJ. Sixteen independent recombinant progeny clones
were phenotyped for their susceptibility to S/P and pyrimethamine and genotyped for
the inheritance of 30 chromosome-specific markers. Linkage analysis showed that
mutant *dhfr* is a major determinant of S/P resistance in *P. chabaudi*. However at
least one other gene is involved. Quantitative trait analysis suggested that other loci
for S/P resistance could be present on chromosomes 4, 5 and 9. In addition, loci on
chromosomes 5 and 13 appear to contribute to the level of resistance observed. No
association was found between S/P susceptibility and the inheritance of the \textit{dhps} gene.
2. Materials and Methods

The composition of buffers and solutions are given in Appendix I.

2.1 Definition of terms

The terms describing the parasites used throughout this work are as follows (after Walliker 1983):

**Isolate** - A sample of parasites collected on a single occasion from a natural host. An isolate may contain parasites of more than one species.

**Line** - A collection of parasites that have undergone a particular laboratory passage or treatment.

**Clone** - A group of genetically identical organisms derived from a single cell by asexual multiplication.

2.2 Laboratory maintenance of *P. chabaudi*

2.2.1 Parasite clones

The *P. chabaudi* clones used in this work, AS and AJ, were cloned from isolates taken from wild thicket rats, *Thamnomys rutilans* in the Central African Republic (Carter and Walliker 1975). Both AS (SENS) and AJ are pyrimethamine and sulfadoxine/pyrimethamine (S/P) sensitive and known to be genetically distinct clones (Carter 1978, Sanderson *et al.* 1981, Carlton *et al.* 1998a). Clone AS (PYR) was derived from the AS (SENS) clone, by selection for *in vivo* resistance to pyrimethamine (Walliker *et al.* 1975). The resistance was produced by single step treatment of AS (SENS) parasites *in vivo* with 50 mg/kg pyrimethamine. AS (PYR) parasites are able to grow in the presence of 25 mg/kg pyrimethamine administered for 4 days, which normally eradicates all sensitive forms. The S/P resistant clones, AS (50S/P) and AS (75S/P), were derived from AS (PYR) for the work presented;
their derivation and characterisation will be described later in the chapter (section 2.3.3).

**Table 1. Clones of *P. chabaudi* used in this work**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Drug Sensitivity&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>PYR</td>
</tr>
<tr>
<td>AJ</td>
<td>S</td>
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<tr>
<td>AS (SENS)</td>
<td>S</td>
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<tr>
<td>AS (PYR)</td>
<td>R</td>
</tr>
<tr>
<td>AS (50S/P)</td>
<td>R</td>
</tr>
<tr>
<td>AS (75S/P)</td>
<td>R</td>
</tr>
</tbody>
</table>

<sup>a</sup>PYR, pyrimethamine; S/P, sulfadoxine/pyrimethamine; S, sensitive; LR, low-level resistance; R, resistant; HR, high-level resistance.

### 2.2.2 Rodent hosts

Laboratory mice (*Mus musculus*) were used as mammalian hosts. Clones were routinely passaged in outbred (TO) laboratory mice (Bantim and Kingman, Hull, UK). Inbred CBA/Ca male mice, 6-9 weeks old (Edinburgh University or Bantim and Kingman, Hull, UK), were used for drug susceptibility tests. Mice were routinely fed SDS Formula Number 1 (Special Diets Services Ltd, Essex, UK) supplemented with 0.05% PABA in their drinking water to promote high parasitaemia (Hawking 1953). Mice used for sulfadoxine tests were fed a folic acid-deficient diet (ICN) supplemented with water alone or with 0.005% PABA in the drinking water to assist parasite growth. Newly-weaned female Wistar rats (Bantim and Kingman, Hull, UK) were used for crossing studies. All animals were kept at 22-25°C with alternating 12 hour cycles of light and darkness, in polypropylene cages with wood shavings.
2.2.3. Mosquito hosts

Parasites were transmitted through *Anopheles stephensi* mosquitoes for crossing studies. Mosquitoes were maintained as described (Walliker *et al.* 1973) at 24-26°C and 70-90% humidity with alternating 12 hour cycles of light and darkness.

2.2.4 Syringe passage

*P. chabaudi* infections were maintained by direct blood passage from infected mice with high parasitaemias to uninfected mice. One to five drops of blood were collected from mouse tail snips into either 0.2 ml citrate saline (Appendix I) or heparinised calf serum/Ringer solution (Appendix I), and 0.1 ml injected intraperitoneally (ip). The day of inoculation was referred to as day 0. Infections were monitored by making thin blood films from tail snips. The blood films were fixed in methanol, stained with Giemsa's stain (Gibco BRL) at pH 7.2, and examined microscopically. The smears were either scored using a simple system based on that of Padua (1981) or the parasitaemia was calculated by counting the total number of red blood cells and then the number of parasitised cells. Parasitaemias were then expressed as the percent of cells infected. At least 1000 cells were examined for each count. The number of erythrocytes per ml was calculated by using a haemocytometer or a Coulter Counter (Coulter Electronics), which together with the parasitaemia, was used to calculate the number of infected cells per ml of blood.

2.2.5 Cryo-preservation of parasites

Parasites were preserved in liquid nitrogen as follows (Rowe *et al.* 1968). Mice with pre-peak parasitaemias were anaesthetised with 2-4 % volatile halothane (Rhodia Ltd, Bristol, UK), and blood was collected into citrate saline by cardiac dissection. The blood was centrifuged at 1500 x g for 10 minutes. The supernatant was removed and a volume of deep freeze solution (Appendix I) equal to that of the pelleted cells added and gently mixed. Aliquots (20-100 µl) were either pipetted into cryo-preservation ampoules (Nunc) and immediately immersed in liquid nitrogen, or
dispensed into glass capillaries which were sealed with a Bunsen flame and immersed in liquid nitrogen.

Parasites were taken from storage by thawing frozen capillaries or ampoules, diluted into 0.2 ml citrate saline and inoculated into mice (ip). Infections became patent 5-10 days after inoculation and were passaged at least once before being used in an experiment.

2.2.6 Cloning

Clonal infections were established by injecting mice (ip) with inocula containing 1.0 parasite per mouse (Walliker et al. 1975). A donor mouse with a low parasitaemia was used in order to minimise the number of cells with multiple infections. The donor was bled from the tail into fixed-volume glass micropipettes (Camlab) and serial dilutions made into citrate saline. The final dilution was made into calf serum/Ringer such that it contained 10 parasites/ml. Groups of 15-30 mice were inoculated with 0.1 ml aliquots. Blood smears were taken after 10-14 days and examined for the presence of parasites. Clonal infections were confirmed by examining a number of genetic markers (see section 2.10).

2.3 Drug-resistance studies

2.3.1 Drugs and chemicals

Pyrimethamine was originally obtained from Wellcome Research Laboratories (Kent, UK), sulfadoxine and Fansidar® were kind gifts from Roche Pharmaceuticals. Dilutions of drugs were made in dimethyl sulfoxide (DMSO) (Sigma, UK).

2.3.2 In vivo drug susceptibility tests

Parasites were tested for their susceptibility to pyrimethamine, sulfadoxine and S/P, by inoculating (ip) $10^6$ parasitised red blood cells into groups of five 6-9 week old CBA/Ca male mice. Dilutions of pyrimethamine, sulfadoxine or S/P were administered orally to three of the mice three hours after inoculation, and at the same
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time of day, for the next 3 days. Drug doses were expressed as mg per kg of mouse body weight (mg/kg). Each mouse was weighed daily before administering the drug. Blood smears were taken from day 4 onwards and parasitaemias counted microscopically. Each clone was tested for the individual drug responses in at least 2 experimental trials. Further details of drug doses and dietary regimes are described in Chapter 3.

2.3.3 Selecting for sulfadoxine/pyrimethamine resistance

TO mice were each infected (ip) with $10^6$ parasites on day 0. A high, curative dose of drug was administered orally to each mouse once daily, from day 3, for a total of 4 days. Blood smears were examined for parasites every 2 days from day 14. Any mice developing parasitaemias were sacrificed and their blood was stored in ampoules under liquid nitrogen. For subsequent work, ampoules were thawed, the contents injected (ip) into mice and the parasites' susceptibility to S/P, pyrimethamine and sulfadoxine assessed. Lines chosen for further studies were cloned as described previously.

2.3.4 Stability of resistance

To investigate the stability of resistance in the absence of drug, the resistant parasites were passaged weekly without drug pressure. Each week, the parasites were also sub-inoculated into groups of CBA/Ca mice and analysed for their responses to S/P as described in section 2.3.2. To test the stability of resistance during transmission, the drug resistant clones were transmitted through mosquitoes as described in section 2.9.1 and the resulting parasites tested for their response to S/P.

2.4 Extraction of genomic DNA

2.4.1 Phenol/chloroform extraction

Mice with high parasitaemias were anaesthetised with halothane and blood was collected into citrate saline by cardiac dissection. The blood was passed through a CF11 cellulose (Whatman) column equilibrated with 1 x PBS (Appendix I), to
remove murine leucocytes (Fulton and Grant 1956). *P. chabaudi* parasites were purified following saponin (Appendix I) lysis of erythrocytes (Kreier 1977). After washing twice with 1 x PBS, the parasite pellet was stored at -20°C. Prior to DNA extraction, the parasite pellet was incubated overnight at 42°C in an equal volume of lysis solution (Appendix I) containing 0.2 mg/ml proteinase K (Roche Diagnostics). DNA was extracted by mixing once with TE buffer (Appendix I)-equilibrated phenol (Sigma) containing 0.1% (w/v) 8-hydroxyquinoline, twice with 1:1 (v/v) phenol/chloroform (Sigma) and finally with 24:1 (v/v) chloroform/isoamylalcohol (Sigma). The DNA was precipitated in 0.3 M ammonium acetate and one volume of isopropanol by gentle mixing. The precipitate was removed with the tip of a syringe needle and resuspended in TE pH 8.0. The DNA was stored at -20°C.

2.4.2 DNA for PCR template
A few drops of infected blood were collected from tail snips into citrate saline in an Eppendorf tube and centrifuged briefly at 12,000 x g. The supernatant was removed and the red blood cell pellet stored at -20°C. To extract DNA, 6 μl of the pellet was added to 1.5 ml of sterile H2O, mixed by inversion and incubated at room temperature for 30 minutes. The tubes were spun at 12,000 x g for 2 minutes and all but 20-30 μl of supernatant removed and discarded. 200 μl of Instagene matrix (BioRad) was added to the pellet and incubated at 56°C for 30 minutes. The samples were then vortexed and placed in a heating block at 100°C for 8 minutes. After vortexing, the tubes were spun at 12,000 x g for 2-3 minutes and stored at -20°C. 1 μl of the resulting supernatant was used per PCR reaction.

2.5 Polymerase chain reaction (PCR)
2.5.1 PCR reagents and electrophoresis
Amplification reactions (Saiki *et al.* 1988) were performed on a TRIO-thermoblock PCR machine (Biometra). Reactions were carried out in 50 μl volumes containing 62.5 μM of each deoxynucleotide (Roche Diagnostics), 50 ng of each primer, 1.5 mM MgCl, 1 unit Taq polymerase (Roche Diagnostics or Promega Corp),
100-500 ng of genomic DNA in 1 x PCR buffer (Roche Diagnostics or Promega). The primers used in this work are listed in Table 2. Negative controls for each primer pair omitted the addition of DNA. Reactions were overlaid with 2 drops of light mineral oil (Sigma) and PCR performed using appropriate conditions. After cycling, 15 µl of PCR product was mixed with 5 x gel loading buffer (Appendix I) and electrophoresed on 1.5 % w/v agarose gels in 1 x TBE (Appendix I) in the presence of 0.5 µg/ml ethidium bromide (Sambrook et al. 1989). DNA size markers (Roche Diagnostics) were run alongside samples. Bands on the gel were viewed under ultra-violet light after an appropriate running time.

**Table 2. Oligonucleotide primers used for polymerase chain reaction.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHPS1</td>
<td>TGATTG(T/G)TT(T/A)(C/T)AGATGG</td>
<td>pppk-dhps</td>
</tr>
<tr>
<td>DHPS2</td>
<td>G(A/T)GG(A/C)TT(C/A)CC(A/T)CC(A/T)ATATC</td>
<td>pppk-dhps</td>
</tr>
<tr>
<td>DHPS3</td>
<td>GAAAAAAAAATAGTGTGGGC</td>
<td>pppk-dhps</td>
</tr>
<tr>
<td>DHPS4</td>
<td>AATTCTATACCTAGGTATGCC</td>
<td>pppk-dhps</td>
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<tr>
<td>DHPS5</td>
<td>TTTCTTCGACAAATTGCATG</td>
<td>pppk-dhps</td>
</tr>
<tr>
<td>DHPS6</td>
<td>TATGAGGATGTGTATATG</td>
<td>pppk-dhps</td>
</tr>
<tr>
<td>PPPK1</td>
<td>GAAAAGCATAAAAAATAAATACTACAC</td>
<td>pppk-dhps</td>
</tr>
<tr>
<td>PPPK2</td>
<td>GAAACTTTTTAAAAACTACGGC</td>
<td>pppk-dhps</td>
</tr>
<tr>
<td>PPPK3</td>
<td>CTCAGATAGTCTATTTAAATGTAAG</td>
<td>pppk-dhps</td>
</tr>
<tr>
<td>DHFR1</td>
<td>GAAGATATCTCTGAAATATTCGATATATG</td>
<td>dhfr-ts</td>
</tr>
<tr>
<td>DHFR2</td>
<td>TTCTTATATCTTGGAATAATAACATCAC</td>
<td>dhfr-ts</td>
</tr>
<tr>
<td>DHFR4</td>
<td>CAAGTATAAGCATTATTATTTTCTTG</td>
<td>dhfr-ts</td>
</tr>
<tr>
<td>DHFR5</td>
<td>GATTTTGGTAATGGGTTAAATTTTGGAG</td>
<td>dhfr-ts</td>
</tr>
<tr>
<td>TS1</td>
<td>AGAGAAGTTAATGACTTAGGGTCC</td>
<td>dhfr-ts</td>
</tr>
<tr>
<td>TS2</td>
<td>TAAGCTGCATATCCATACTG</td>
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</tr>
<tr>
<td>ASN</td>
<td>TGTAGTAATGGGAAAGGCA</td>
<td>dhfr-ts</td>
</tr>
<tr>
<td>MSP1#3</td>
<td>GTTACAAACAAAATCGAGCT</td>
<td>msp1</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Ambiguity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP1#4 TTGAGCATAAAGTTCAGC</td>
<td>msp1</td>
</tr>
<tr>
<td>AJMDR CAATTAGCATTTATTTATGGATGCGATTTTTC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>mdr1</td>
</tr>
<tr>
<td>ASMDR CAATTAGCATTTATTTATGGATGCGATTTTTC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>mdr1</td>
</tr>
<tr>
<td>MDR1 CAGCTCTATCTTTTAATATCAACAATAG</td>
<td>mdr1</td>
</tr>
<tr>
<td>Pccg10/19 CTCTGGTCATATTTGAAAAGC</td>
<td>cg10</td>
</tr>
<tr>
<td>Pccg10/20 GCAAACCTATTGGAATGAAATG</td>
<td>cg10</td>
</tr>
<tr>
<td>vecDHPS1 GGTCTTTAATTCTCTTGGACTAAACC</td>
<td>Vectorette (outer)</td>
</tr>
<tr>
<td>vecDHPS2 GGACATAATCTTAAATAACTTGTCTTC</td>
<td>Vectorette (nested)</td>
</tr>
<tr>
<td>vecDHPS3 GGAGCAGAGGACTCACCACC</td>
<td>Vectorette (outer)</td>
</tr>
<tr>
<td>vecDHPS4 ATTCGCTGCACAGCAATATCCGG</td>
<td>Vectorette (nested)</td>
</tr>
<tr>
<td>vecDHPS5 CCTCGTCTCCTGTAGTTGGG</td>
<td>Vectorette (outer)</td>
</tr>
<tr>
<td>vecDHPS6 AAGACGCATGTCTGTTATAGGCC</td>
<td>Vectorette (nested)</td>
</tr>
<tr>
<td>vecDHPS7 ATTAGCTCATTTCTTGGCTATAATAGC</td>
<td>Vectorette (outer)</td>
</tr>
<tr>
<td>vecDHPS8 CACAGTTGGATTAGGCATTGCC</td>
<td>Vectorette (nested)</td>
</tr>
<tr>
<td>vecPPPK1 CAATTATTGCACATTCTATTTG</td>
<td>Vectorette (outer)</td>
</tr>
<tr>
<td>vecPPPK2 ACACATAATTGCATAAAAC</td>
<td>Vectorette (nested)</td>
</tr>
<tr>
<td>vecPPPK3 CATATGAGGTTCCCTCATAGG</td>
<td>Vectorette (outer)</td>
</tr>
<tr>
<td>vecPPPK4 CCAATAAGTTATGTACATATACGC</td>
<td>Vectorette (nested)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Oligonucleotides were supplied by Oswel DNA Service (Southampton, UK) or PE Applied Biosystems (Warrington, UK).

<sup>b</sup> Mutation-specific primers; specific-mismatches to either wild-type (ASN) or to mutant (SER) dhfr alleles, or AS-type and AJ-type mdr alleles are underlined. Lower-case letters indicate nucleotides mismatched to both alleles introduced to destabilise further the annealing of primer to the non-target sequence.

#### 2.5.2 Mutation-specific dhfr PCR

In AS (PYR), resistance to pyrimethamine is conferred by an asparagine at position 106 in its DHFR. The wild-type allele codes for a protein with serine at position 106. Therefore, two allele-specific amplification assays were designed that were able to detect either the wild-type *P. chabaudi* dhfr allele, or the presence of the mutation at nucleotide 319. The oligonucleotides for the serine-specific amplification (SER and DHFR1) and the asparagine-specific amplification (ASN and DHFR4) are described in Table 2. Cycling conditions; serine-specific, 94°C for 30 s,
46°C for 30 s, 65°C for 30 s for 35 cycles; asparagine-specific, 94°C for 30 s, 43°C for 30 s, 65°C for 30 s for 35 cycles.

2.5.3 Mutation-specific mdr1 PCR

Sequencing studies of the P. chabaudi mdr1 gene by P. Cravo (University of Edinburgh) revealed a polymorphism between the AS and AJ allele at a nucleotide denoted position 183. The AJ allele has a C at this position, while a T is present in the AS allele. Therefore, two allele-specific amplification assays were designed that were able to detect either the AJ or AS (50S/P) mdr1 allele. The oligonucleotides for the AJ-specific amplification (AJMDR and MDR1) and the AS-specific amplification (ASMDR and MDR1) are described in Table 2. Cycling conditions; AJ-specific, 94°C for 30 s, 50°C for 30 s, 65°C for 30 s for 35 cycles; AS-specific, 94°C for 30 s, 48°C for 30 s, 65°C for 30 s for 35 cycles.

2.5.4 Allele-specific msp1 PCR

The allele-specific msp1 PCR was adapted from Taylor (1997). The primers MSP1#3 and MSP1#4 (Table 2) were used to amplify the polymorphic region of the P. chabaudi msp1 gene using cycling parameters of 94°C for 30 s, 56°C for 30 s and 65°C for 60 s for 35 cycles. Size polymorphisms in the resulting PCR products were analysed on a 1.5% agarose gel as described.

2.6 Isolation of the P. chabaudi AS dhps gene

2.6.1 Pcdhps PCR using degenerate primers

Mutations in the gene encoding DHPS have been implicated in the mechanism of S/P resistance in P. falciparum (Triglia et al. 1998). Therefore, the P. chabaudi dhps-pppk was cloned and sequenced. Subsequently, the dhps gene was sequenced from all the S/P selected lines to identify any possible mutations associated with resistance to S/P.

Sequences of dhps genes from P. falciparum (Brooks et al. 1994, Triglia and Cowman 1994), Toxoplasma gondii (Pashley et al. 1997), Pneumocystis carinii
(Volpe et al. 1992), *Escherichia coli* (Dallas et al. 1992), *Bacillus subtilis* (Slock et al. 1990), and *Streptococcus pneumoniae* (Lopez et al. 1987) were aligned using the University of Wisconsin Genetics Computer Group DNA analysis software (UWGCG) to identify regions of sequence conservation. Two highly conserved regions were identified and degenerate oligonucleotides were designed for use in PCR. The primers DHPS1 and DHPS2 (Table 2) were used to amplify the homologous gene from genomic DNA of the AS clone of *P. chabaudi* using cycling parameters of 94°C for 30s, 45°C for 120s and 65°C for 120s for 35 cycles. The resulting PCR products were analysed on a 1.5% agarose (Roche Diagnostics) gel. A band of approximately 100 bp was observed and was sequenced as described in section 2.6.2.

### 2.6.2 ABI Prism® BigDye™ terminator cycle sequencing

PCR products were purified using a Concert kit (Gibco GBL) according the manufacturer's instructions, and sequenced directly using BigDye™ (Perkin Elmer) chemistry on an ABI 377 Prism® Sequencer. 30-90 ng of template DNA was mixed with 4 µl of BigDye™ terminator ready reaction mix (Perkin Elmer) and 1.6 pmol of appropriate primer. Reactions were overlaid with one drop of light mineral oil and subjected to 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. The annealing temperature occasionally needed to be reduced for primers with a low melting temperature. After cycle sequencing, unincorporated terminators were removed by adding 25 µl of 95% ethanol and 1 µl of 3M sodium acetate pH 4.8 to the reaction mixture. Tubes were incubated at room temperature for 15 minutes and centrifuged at 12 000 x g for 30 minutes. The supernatant was removed and discarded and the pellet carefully rinsed with 125 µl of 70% ethanol and dried by incubating at 90°C for 2 minutes. Pellets were stored at -20°C. Immediately before loading, pellets were resuspended in 3 µl of sequencing gel loading buffer (Appendix 1) and denatured by heating at 95°C for 2 minutes. 2 µl of each sample were loaded into a separate lane of the gel and the ABI 377 Prism® Sequencer was run according to the manufacturer's instructions. Sequencing was repeated at least three times on
different templates to minimise artefacts due to \textit{Taq} polymerase errors. DNA sequences were entered into UWGCG and submitted to BLAST (Altschul \textit{et al.} 1997) to search for homology to known sequences in the EMBL\textsuperscript{TM} and Genbank\textsuperscript{TM} databases.

\textbf{2.6.3 Construction of \textit{P. chabaudi} Vectorette libraries}

The \textit{P. chabaudi \textit{dhps}} sequence was extended by PCR-walking using \textit{P. chabaudi} Vectorette II (Genosys) libraries generated from genomic AS (SENS) DNA, digested with either \textit{AluI}, \textit{ClaI}, \textit{EcoRI}, \textit{HindIII}, \textit{MfeI}, or \textit{TaqI} (Roche Diagnostics) restriction enzyme (Figure 5) (Arnold and Hodgson 1991). 100 ng of AS DNA were added to 20 units of restriction enzyme in 50 \textmu l reactions containing the appropriate buffer. To ligate the Vectorette adapters, 5 \textmu l of the restriction enzyme-specific adapter were added to the digest with 1 \textmu l 100 mM ATP (Sigma), 1\textmu l 100 mM dithiothreitol (DDT) (Sigma) and 1 unit of T4 DNA ligase (Roche Diagnostics). Ligations were subjected to three cycles of 20°C for 60 minutes, followed by 37°C (60°C for \textit{TaqI}) for 30 minutes. The original restriction site is not reformed by the target-Vectorette construct. During cycling any ligated target-target molecules will therefore be cut by the restriction enzyme, increasing the overall yield of Vectorette constructs. After ligation, 200 \textmu l of sterile H\textsubscript{2}O were added to the ligation reaction and stored at -20°C in 20 \textmu l aliquots.

\textbf{2.6.4 Extending the \textit{P. chabaudi dhps} sequence}

Gene-specific primers (Table 2) were designed from sequences obtained from the initial \textit{dhps} PCR product, and these were used with the Vectorette outer and nested adapter primers (Genosys) to generate additional PCR fragments from the libraries (Figure 3). Separate outer and nested primers were designed for each round of Vectorette amplification, such that the nested primer was internal to the outer primer.
1. DNA is digested with a restriction enzyme (R).

2. Vectorette adapters are ligated to the DNA fragments, forming a Vectorette library.

3a). In the first round of amplification, extension is from the DHPS-specific primer only. Extension from the Vectorette primer is avoided initially because mismatches have been introduced into the Vectorette adapters. Therefore, it will only hybridise to the product of the first round of amplification.

3b) In the second and all subsequent rounds of PCR, priming is from both the DHPS-specific primer and Vectorette primer.

4) Step 3 is repeated in a second (nested) round of PCR to eliminate non-specific amplification products.

5). The purified amplification product is sequenced using the Vectorette sequencing primer. The novel sequence is used to synthesise new DHPS-specific primers for use in step 3.

**Figure 5. The Vectorette system.** Diagram to illustrate the construction of a Vectorette DNA library (1 & 2) and one-sided PCR amplification (3 & 4) in order to generate new sequence by chromosome walking (5).
Outer reactions were carried out in 100 µl volumes containing 50 µM of each dNTP, 250 nM of gene-specific primer, 250 nM of Vectorette outer primer (Genosys), 1.5 mM MgCl₂ and 1 µl of Vectorette library in 1 x PCR buffer. Reactions were incubated at 94°C for 3 minutes before the addition of 2.5 units of Taq polymerase and were subjected to 40 cycles of 94°C for 60s, 40-60°C (depending on the annealing temperature of the gene-specific primer) for 120s and 65°C for 240s. Nested reactions were set up with 1 µl of a 1 in 10000 dilution of the product from the outer reaction and cycling conditions were identical to the outer reaction, except they contained 1 µM of gene-specific primer and 1 µM of Vectorette nested primer (Genosys).

2.7 Manipulation of recombinant DNA

2.7.1 Cloning PCR products

Some PCR products, such as the initial dhps fragment and occasions when the sequences in the immediate proximity of the primer were desired, were cloned into the pCRTM™II vector using the TA Cloning™ System (Invitrogen) according to the manufacturer's instructions.

2.7.2 Transformations

Competent E. coli strain INVαF' (Invitrogen) were transformed with vector DNA following the manufacturer's protocol and plated onto LB (Appendix I) agar containing 50 µg/ml kanamycin (Sigma) and 50 µg/ml 5-bromo-4-chloro-3-indolyl-α-D-galactosidase (X-Gal) (Sigma). Recombinant colonies were identified by blue/white colour screening (Sambrook et al. 1989).

2.7.3 Long-term storage of transformed cells

E. coli transformed with recombinant vector DNA were preserved as glycerol stocks at -70°C (Sambrook et al. 1989). 0.15 ml of sterile glycerol were added to 0.85 ml of bacteria culture, briefly vortexed and flash-frozen in liquid nitrogen. Tubes were then transferred to -70°C for long term storage. Viable bacteria were
later recovered by scratching the surface of the frozen cells and streaking onto LB agar plates containing the appropriate antibiotic.

2.8 Sequencing the \textit{pppk-dhps} and \textit{dhfr-ts} alleles

2.8.1 Sequencing the \textit{pppk-dhps} alleles

Fragments of \textit{pppk-dhps} gene were amplified using oligonucleotides designed from the deduced sequence. The primers PPPK1, PPPK3, DHPS3, DHPS5 and vecDHPS4 were used to amplify the \textit{pppk-dhps} gene from genomic DNA from AS (PYR) and all the parasites that survived S/P selection, including AS (50S/P) and AS (75S/P). The cycling parameters were as follows; DHPS3 and DHPS5, 94°C for 30s, 40°C for 60s, 65°C for 120s for 35 cycles; DHPS4 and PPPK1, 94°C for 30s, 40°C for 60s, 65°C for 120s for 35 cycles; PPPK3 and vecDHPS4, 94°C for 30s, 45°C for 60s, 65°C for 120s for 35 cycles. Amplification products were purified and both strands sequenced as described previously.

2.8.2 Sequencing the \textit{dhfr-ts} alleles

Fragments of the \textit{dhfr-ts} gene were amplified from parasite DNA using primers designed from available sequences of the genes from \textit{P. chabaudi} AS (Cheng and Saul 1994) and \textit{P. chabaudi adami} clone DS (Cowman and Lew 1990). The primers DHFR1 and DHFR2, TS1 and TS2, and TS3 and DHFR5 (Table 2) were used to amplify \textit{dhfr-ts} from genomic DNA of AS (PYR), AS (50S/P) and AS (75S/P). The cycling conditions were as follows; DHFR1 and DHFR2, 94°C for 30s, 45°C for 60s, 65°C for 120s for 35 cycles; TS1 and TS2, 94°C for 30s, 45°C for 60s, 65°C for 120s for 35 cycles; TS3 and DHFR5, 94°C for 30s, 45°C for 60s, 65°C for 120s for 35 cycles. Amplification products were purified and both strands sequenced as described previously.
2.9 Crossing technique and analysis of the uncloned progeny

2.9.1 Conducting the crosses and recovering the progeny

In order to determine the genetic basis of resistance to S/P, the AS (50S/P) was crossed with a drug sensitive clone, AJ. Clones AJ and AS (50S/P) and AJ and AS (75S/P) were crossed by transmission through *Anopheles stephensi* mosquitoes following the basic procedure of Walliker *et al.* (1975) with modifications as described below (Figure 6). AJ and the relevant AS parasite were inoculated into separate groups of 4 CBA/Ca mice. On day 4-6, when parasitaemias were between 20-40%, mice were sacrificed and blood collected by cardiac dissection, using heparin as an anticoagulant. 0.5 ml of blood from mice infected with each clone were mixed and injected (ip) into a newly weaned, splenectomised rat; a higher number of gametocytes is produced in this host than in mice (MacLeod and Brown 1976, Cornelissen and Walliker 1985). 1.0 ml of infected blood from each clone were also injected into separate splenectomised rats as controls. When a large number of mature gametocytes were present in the blood (day 3-4), approximately 500 4-6 day old *A. stephensi* which had been starved of glucose for 24 hours, were allowed to feed on the rats, which were anaesthetised (ip) with a combination of 2.5 mg/kg Hypnovel (Roche Pharmaceuticals) and 1.25 mg/kg Hypnorm (Janssen Pharmaceuticals, Bristol, UK).

Samples of mosquito midguts were examined for mature oocysts from day 7 onwards. Sporozoite induced infections were established in CBA/Ca mice once sporozoites were present in salivary gland dissections, usually from day 16 onwards. Mice were infected either by feeding mosquitoes on mice (crosses 1 and 3), or by removing salivary glands from mosquitoes by dissection into Grace’s insect medium (Gibco GBL), releasing the sporozoites by use of a teflon pestle and injecting them into mice (ip) (crosses 2 and 4). Approximately 100 mosquitoes were used for each feed or injection.
Figure 6. Crossing methodology. Rats were infected with AJ mixed with either AS (50S/P) or AS (75SP). Mosquitoes were permitted to feed on rats on day 3 or 4 and mice were inoculated with the resulting sporozoites approximately 16 days later.
2.9.2 Testing for recombination

To test whether transmission of both parents had been successful, mutation-specific PCR of dhfr was performed on DNA from the uncloned progeny to determine whether both parent clone alleles were present. To test whether crossing had occurred, the uncloned progeny were treated with pyrimethamine in a standard drug test and then examined for the presence of parental msp1 types using allele-specific PCR of this gene (Taylor 1997). The presence of the AJ msp1 allele among drug-treated uncloned progeny would indicate recombination between the dhfr and msp1 and that crossing between AJ and AS gametes had occurred.

2.9.3 Cloning of progeny

In order to perform further genetic analysis, clones were made from the uncloned progeny of both AJ x AS (50S/P) crosses by limiting dilution, as described in section 2.2.6.

2.10 Chromosome-specific markers

2.10.1 Types of markers used in this study

Five types of probe were used as chromosome markers for the P. chabaudi genome; i) genes which had been cloned by other laboratories and developed as chromosome markers by Carlton (1995), and Carlton et al. (1998a); ii) anonymous markers from a P. chabaudi genomic DNA library (Carlton 1995, Carlton et al. 1998a), iii) mutation-specific PCR; iv) allele-specific PCR (Taylor 1997) and v) detection of polymorphisms by PCR and restriction digestion. The original source, chromosome location and probing conditions of the markers used in this study are listed in Table 3. The majority of the markers were either known genes or anonymous DNA. Polymorphisms between AS (50S/P) and AJ were detected by RFLPs on Southern blots.
### Table 3. Source of the markers used in this study, chromosome location and probing conditions

<table>
<thead>
<tr>
<th>Probe No.</th>
<th>Marker</th>
<th>P. chabaudi Chromosome Location&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference/Source</th>
<th>Type</th>
<th>RFLP Analysed</th>
<th>Washing stringency for Southern blots or PCR conditions</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ag3020</td>
<td>1</td>
<td>(Favaloro 1993, Favaloro et al. 1993)</td>
<td>RFLP</td>
<td>HindIII</td>
<td>0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>Uncharacterised P. chabaudi cDNA clone (1.3 kb in EcoRI site of pGEX-2T).</td>
</tr>
<tr>
<td>2</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; ATPase</td>
<td>2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Murakami et al. 1990)</td>
<td>RFLP</td>
<td>AsnI</td>
<td>1 x SSC, 0.1 % SDS, 60°C</td>
<td>Contains 70% of the P. yoelii Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase gene (3.3 kb in HindIII site of pUC19; clone yH4)</td>
</tr>
<tr>
<td>3</td>
<td>pBS110</td>
<td>3</td>
<td>W. Deleersnijder Institute for Molecular Biology, Brussels, Belgium</td>
<td>RFLP</td>
<td>AluI</td>
<td>0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>P. chabaudi schizont-specific gene (1.2 kb cDNA in EcoRI site of pBluescript)</td>
</tr>
<tr>
<td>No.</td>
<td>Name</td>
<td>Accession</td>
<td>Description</td>
<td>Enzyme</td>
<td>Conditions</td>
<td>Source/Details</td>
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</tr>
<tr>
<td>4</td>
<td>Ag3003</td>
<td>A</td>
<td>(Favaloro 1993, Favaloro et al. 1993) RFLP AluI 0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>AluI</td>
<td>0.5 x SSC, 0.1 %</td>
<td><em>P. chabaudi</em> cDNA (1.5 kb in EcoRI site of pGEX-2T) that expresses a protein that reacts with hyperimmune serum</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>P.1</td>
<td></td>
<td>(Carlton et al. 1998a) RFLP SspI 0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>SspI</td>
<td>0.5 x SSC, 0.1 %</td>
<td>Anonymous marker isolated from a <em>P. chabaudi</em> Sau3A genomic library</td>
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</tr>
<tr>
<td>6</td>
<td>P.9</td>
<td></td>
<td>(Carlton et al. 1998a) RFLP HindII 0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>HindII</td>
<td>0.5 x SSC, 0.1 %</td>
<td>Anonymous marker isolated from a <em>P. chabaudi</em> Sau3A genomic library</td>
<td></td>
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<tr>
<td>7</td>
<td>α-tubulin</td>
<td>4/5</td>
<td>(Holloway et al. 1989, Holloway et al. 1990) RFLP HindII 1 x SSC, 0.1 % SDS, 50°C</td>
<td>HindII</td>
<td>1 x SSC, 0.1 % SDS, 50°C</td>
<td>5' terminus of the <em>P. falciparum</em> α-tubulin gene (1.9 kb in EcoRI site of pBR322)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Ag3035</td>
<td>5</td>
<td>(Favaloro 1993, Favaloro et al. 1993) RFLP AluI 0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>AluI</td>
<td>0.5 x SSC, 0.1 %</td>
<td><em>P. chabaudi</em> cDNA (1.3 kb in EcoR1 site of pGEX-2T)</td>
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</tr>
<tr>
<td></td>
<td>DNA</td>
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<tr>
<td>9</td>
<td>polδ</td>
<td>5</td>
<td></td>
<td>RFLP</td>
<td>RsaI</td>
<td>Conserved region from <em>P. falciparum</em> DNA polymerase δ gene (1 kb in EcoRI of pUBS; clone pUBS 0/5)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>P.29</td>
<td>5</td>
<td></td>
<td>RFLP</td>
<td>AluI</td>
<td>Anonymous marker isolated from a <em>P. chabaudi</em> Sau3A genomic library</td>
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<tr>
<td>11</td>
<td>Ag3024</td>
<td>5</td>
<td></td>
<td>RFLP</td>
<td>Dral</td>
<td>Uncharacterised <em>P. chabaudi</em> cDNA clone (insert size not determined; in EcoR1 site of pGEX-2T)</td>
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<td>P.12</td>
<td>6</td>
<td></td>
<td>RFLP</td>
<td>Sau3A</td>
<td>Anonymous marker isolated from a <em>P. chabaudi</em> Sau3A genomic library</td>
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<tr>
<td>13</td>
<td>polα</td>
<td>6</td>
<td></td>
<td>RFLP</td>
<td>AluI</td>
<td>Conserved region of <em>P. falciparum</em> DNA polymerase α gene</td>
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<td></td>
<td>Gene</td>
<td>Allele</td>
<td>Reference</td>
<td>Detection Method</td>
<td>Marker Description</td>
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<tr>
<td>14</td>
<td>DHFR</td>
<td>7</td>
<td>(Cowman and Lew 1989)</td>
<td>PCR</td>
<td>94°C/30 s, 46°C/30 s, 65°C/30 s x35, ASN-specific: 94°C/30s, 43°C/30s, 65°C/30s x 35. SER-specific: P. chabaudi dhfr gene. AJ allele detected with the primer pair SER and DHFR1, AS (50S/P) allele detected with ASN and DHFR4 (Table 2).</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>P.23</td>
<td>7</td>
<td>(Carlton et al. 1998a)</td>
<td>RFLP</td>
<td>AsnI</td>
<td>0.5 x SSC, 0.1 % SDS, 65°C Anonymous marker isolated from a P. chabaudi Sau3A genomic library</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>MSP-1</td>
<td>8b</td>
<td>(Deleersnijder et al. 1990)</td>
<td>RFLP</td>
<td>N/A</td>
<td>94°C /30s, 56°C /30, 65°C /60s x 35 P. chabaudi merozoite surface protein, MSP-1 genomic DNA (5.4 kb in EcoRI site of pBluescript; clone pBS RX4)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>hsp86</td>
<td>8</td>
<td>(Su and Wellems 1994)</td>
<td>RFLP</td>
<td>HindII</td>
<td>1 x SSC, 0.1 % SDS, 50°C P. falciparum heat shock protein 86 gene (2 kb in NsiI site of pcDNA II)</td>
<td></td>
</tr>
<tr>
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<tr>
<td>18</td>
<td><strong>Ag3027</strong></td>
<td>9</td>
<td>(Favaloro 1993, Favaloro et al. 1993)</td>
<td>RFLP</td>
<td><strong>RsaI</strong></td>
<td>0.5 x SSC, 0.1 % SDS, 65°C</td>
<td><em>P. chabaudi</em> cDNA (1.3 kb in EcoR1 site of pGEX-2T)</td>
</tr>
<tr>
<td>19</td>
<td><strong>AMA-1</strong></td>
<td>9</td>
<td>(Marshall et al. 1989)</td>
<td>RFLP</td>
<td><strong>SspI</strong></td>
<td>0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>Complete <em>P. chabaudi</em> apical membrane antigen gene (2.6 kb in BamHI/EcoRI site of pBluescript)</td>
</tr>
<tr>
<td>20</td>
<td>cDNA 121</td>
<td>10(^c)</td>
<td>(Silveira et al. 1984, Sharkey et al. 1988)</td>
<td>RFLP</td>
<td><strong>AluI</strong></td>
<td>0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>Uncharacterised <em>P. chabaudi</em> cDNA clone (190 bp in PstI site pBR322)</td>
</tr>
<tr>
<td>21</td>
<td><strong>EF-1α</strong></td>
<td>11</td>
<td>D. Williamson, National Institute for Medical Research, Mill Hill, London, Genbank accession no. X60488</td>
<td>RFLP</td>
<td><strong>HindII</strong></td>
<td>1 x SSC, 0.1 % SDS, 50°C</td>
<td><em>P. falciparum</em> clone containing linked pfPK5 (yeast <em>cdc-2</em> gene homologue) and elongation factor 1-α genes (4.3 kb in <em>HindIII</em> site of EMBL8, clone pPF-1; 451 bp EF1-α gene in <em>PvuII</em> site)</td>
</tr>
<tr>
<td>22</td>
<td>PCNA</td>
<td>11</td>
<td>(Kilbey et al. 1993)</td>
<td>RFLP</td>
<td>HindII</td>
<td>1 x SSC; 0.1 % SDS, 45°C</td>
<td>P. falciparum proliferating cell nuclear antigen (PCNA) gene (800 bp in HindIII/EcoRI site of pBluescript)</td>
</tr>
<tr>
<td>23</td>
<td>P.22</td>
<td>11</td>
<td>(Carlton et al. 1998a)</td>
<td>RFLP</td>
<td>AsnI</td>
<td>0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>Anonymous marker isolated from a P. chabaudi Sau3A genomic library</td>
</tr>
<tr>
<td>24</td>
<td>mdr1</td>
<td>12</td>
<td>P. Cravo, University of Edinburgh, Edinburgh, UK.</td>
<td>MS-PCR</td>
<td>N/A</td>
<td>AS-specific: 94°c/30s, 48°C/30s, 65°C/30s x35. AJ-specific: 94°c/30s, 50°C/30s, 65°C x35.</td>
<td>P. chabaudi multi-drug resistance gene (mdr1)</td>
</tr>
<tr>
<td>25</td>
<td>aldol</td>
<td>13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(Meier et al. 1992)</td>
<td>RFLP</td>
<td>HindII</td>
<td>1 x SSC, 0.1 % SDS, 60°C</td>
<td>P. berghei aldolase-1 (aldol-1) gene (900 bp NcoI fragment)</td>
</tr>
<tr>
<td></td>
<td>cDNA 148</td>
<td>13</td>
<td>(Silveira et al. 1984, Sharkey et al. 1988)</td>
<td>RFLP</td>
<td>DraI</td>
<td>0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>P. chabaudi 37 kDa antigen gene (330 bp in PstI site pBR322)</td>
</tr>
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</tr>
<tr>
<td>27</td>
<td>G6PD</td>
<td>13b</td>
<td>(O'Brien et al. 1994)</td>
<td>RFLP</td>
<td>AluI</td>
<td>1 x SSC, 0.1 % SDS, 50°C</td>
<td>P. falciparum glucose-6-phosphate dehydrogenase (G6PD) gene (588 bp in EcoRI site of pGEM)</td>
</tr>
<tr>
<td>28</td>
<td>Pfcrk3</td>
<td>13</td>
<td>C. Doerig INSERM, Paris, France.</td>
<td>RFLP</td>
<td>AluI</td>
<td>1 x SSC, 0.1 % SDS, 50°C</td>
<td>P. falciparum mitogen-activated protein kinase-1 gene (500 bp in EcoRI site of pCR II)</td>
</tr>
<tr>
<td>29</td>
<td>Ag3040</td>
<td>14</td>
<td>(Favaloro 1993, Favaloro et al. 1993)</td>
<td>RFLP</td>
<td>RsaI</td>
<td>0.5 x SSC, 0.1 % SDS, 65°C</td>
<td>P. chabaudi cDNA (1.3 kb in EcoRI site of pGEX-2T)</td>
</tr>
<tr>
<td></td>
<td>Pccg10</td>
<td>14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>P. Hunt &amp; P. Donleavy, University of Edinburgh, Edinburgh, UK.</td>
<td>RFLP</td>
<td>EcoRV/ HindIII</td>
<td>94°C/30s, 50°C/1 min, 62°C/2 min for 35 cycles</td>
<td>P. chabaudi gene homologue of the P. falciparum chloroquine resistant transporter (CRT) (cg10)</td>
</tr>
<tr>
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</tr>
</tbody>
</table>

<sup>a</sup> Chromosomal location determined by Carlton et al. (1998a).

<sup>b</sup> As determined by Janse et al. (1994).

<sup>c</sup> As determined by Sharkey et al. (1988).

<sup>d</sup> Chromosomal location determined through inheritance of alleles in the progeny of the AJ x AS (3CQ) (Carlton et al. 1998a) and AJ x AS (50S/P) crosses.
2.10.2 Polymorphisms detected by PCR

Mutation-specific PCR and allele-specific PCR, used to determine \(dhfr\), \(mdrl\) and \(msp-1\) genotypes were performed as described in Sections 2.5.2-2.5.4. Polymorphisms between the AS (50S/P) and AJ \(P. \)chabaudi\( \) cg10 gene were detected by PCR and restriction digestion. The \(Pccg10\) primers were designed by P. Hunt and P. Donleavy (University of Edinburgh) and the cycling parameters were: 94°C for 30s, 50°C for 60s and 62°C for 120s for 35 cycles. Products were digested with \(EcoRV/HindIII\) and run on a 1.5% agarose gel to detect RFLPs as described.

2.11 Hybridisation of labelled DNA to Southern blots

2.11.1 Restriction digests of genomic DNA

\(P. \)chabaudi\( \) genomic DNA was digested following standard procedures (Sambrook \textit{et al.} 1989). Briefly, 2.5-3 \(\mu\)g of DNA was incubated overnight at the desired temperature in 5-10 units of the appropriate restriction enzyme and buffer (Roche Diagnostics).

2.11.2 Southern blotting

DNA digests were mixed with an appropriate volume of 5 x gel loading buffer and electrophoresed on 1-1.5 % agarose gels in 1 x TBE buffer. After electrophoresis, gels were depurinated in 0.25 M HCl for 15 minutes and Southern blotted (Southern 1975) onto Hybond-N+ (Amersham-Pharmacia) by alkaline transfer using a vacuum transfer system (Anachem) (Sambrook \textit{et al.} 1989).

2.11.3 DNA radiolabelling

DNA to be used for probing was labelled with \([\alpha^{32}P]\) dATP (Amersham-Pharmacia) by random hexamer priming (High Prime DNA Labelling kit, Roche Diagnostics) following the manufacturer's protocol.
2.11.4 Hybridisation

Southern blots were placed in screw-capped glass hybridisation bottles (Hybaid) and incubated in pre-hybridisation solution (Appendix I) at the desired hybridisation temperature (see Table 3) for at least one hour. Blots were incubated inside a Maxi-Oven (Hybaid) with continuous rotation.

0.1 mg sheared salmon sperm DNA (Anachem) were added to the labelled probe, the volume made up to 1 ml with sterile H₂O and the solution boiled for 5 minutes. After leaving on ice for a further 5 minutes, the probe was added to the pre-hybridising blots and hybridised overnight for at least 12 hours.

2.11.5 Washing Southern blots

Hybridisation solutions were carefully disposed of and blots were washed once at room temperature in 2 x SSC (Appendix I), 0.1% SDS for 5 minutes. Blots hybridised with homologous probes were then washed with 0.5 x SSC, 0.1% SDS at 65°C for at least one hour with one change of solution. Blots hybridised with heterologous probes were washed at a stringency appropriate to the degree of conservation between the probe and *P. chabaudi* (see Table 3).

2.11.6 Exposing and developing X-ray film

Southern blots were wrapped in cling film and exposed to autoradiography film (Kodak XAR-5) overnight at -70°C in an autoradiography cassette. The X-ray film was developed in an autoradiographer developer (Exograph). If signals were weak, the membranes were exposed to film for longer periods of time.

2.11.7 Stripping Southern blots

Southern blots were stripped of probe in 100 mM NaOH for at least 30 minutes at room temperature with agitation and at least one change of solution. Blots were washed briefly in 5 x SSC, wrapped in cling film and stored at 4°C.
2.12 Linkage analysis

Linkage was assessed by comparing the inheritance pattern of each chromosome-specific marker with the inheritance of the S/P resistance phenotype (Lander and Botstein 1989). Linkage ratios were determined as the ratio of the number of progeny showing linkage of S/P susceptibility with an individual marker to the total number of progeny analysed.

Resistance to S/P may be a complex trait, involving the additive effects of two or more genes. Therefore the data were analysed in order to map the trait to discrete quantitative trait loci (QTL). Quantitative trait data analysis was carried out by calculating the mean difference in log parasitaemias, on day 6 following treatment with either 25/1.25 mg/kg S/P, 50/2.5 mg/kg S/P or 10 mg/kg pyrimethamine, between groups of progeny clones inheriting the AS (50S/P) allele of the marker and groups inheriting the AJ allele of the marker. The mean difference in log parasitaemias could also be calculated for day 8 following treatment with 50/2.5 mg/kg S/P. The Student's t-test was used to see whether differences in the means were significant (Appendix II).
3. Results: Selecting for Resistance to Sulfonamide Drugs

3.1 Susceptibility of AS (SENS) and AS (PYR) to sulfonamide drugs

3.1.1 Susceptibility of AS (SENS) and AS (PYR) to sulfadoxine

The most useful starting material for identifying genes determining resistance is resistant mutants selected from sensitive clones. Direct examination of the sensitive and resistant forms can then be made to search for mutations in candidate genes which cause the resistance. In order to select for mutants of *P. chabaudi* resistant to sulfonamide drugs, it was first necessary to assess the level of sensitivity in the starting material, the clones AS (SENS) and AS (PYR). This allowed an appropriate drug dose for selection to be determined. However, the susceptibility of the parasites to sulfadoxine depends on levels of *para*-aminobenzoic acid (PABA) in the host diet, since the drug competes with PABA for the active site of the DHPS enzyme. A PABA- and folate-deficient mouse diet was therefore obtained (ICN) so that the levels of the antagonists could be adjusted as required. The sulfadoxine responses of parasites in mice on normal diets and mice on the PABA- and folate-deficient diet were then compared.

Groups of CBA/Ca mice were fed either on normal diet *ad libitum* supplemented with 0.05% PABA in their drinking water or on a PABA- and folate-deficient diet *ad libitum* with no PABA in their drinking water. Mice were kept on this regime for one week before being infected with either AS (SENS) or AS (PYR), and treated with varying doses of sulfadoxine in a 4 day test as described in section 2.3.2. Both AS (SENS) and AS (PYR) parasites were found to be resistant to 5 mg/kg sulfadoxine in mice fed on a normal diet (Table 4). In these mice, a dose of 25 mg/kg sulfadoxine had little effect on the growth of AS (SENS), but AS (PYR) growth was delayed relative to the undrugged control. A dose of 125 mg/kg sulfadoxine cleared AS (PYR) initially, but parasites started to recrudesce on day 7,
Table 4. The effect of PABA on the sulfadoxine sensitivities of AS (SENS) and AS (PYR).

The sulfadoxine susceptibilities of AS (SENS) and AS (PYR) were compared in mice fed on normal diet to infections in mice maintained on a folate- and PABA-deficient diet with no PABA in the drinking water.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Dose (mg/kg)</th>
<th>Normal Diet</th>
<th>PABA-and folate-deficient diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 4</td>
<td>Day 7</td>
</tr>
<tr>
<td>Sulfadoxine</td>
<td>Parasitaemia(^a)</td>
<td>Parasitaemia(^a)</td>
<td>Parasitaemia(^a)</td>
</tr>
<tr>
<td>AS (SENS)</td>
<td>0</td>
<td>++++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>++++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>+</td>
<td>+++++</td>
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<td></td>
<td></td>
<td>±</td>
<td>++++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>±</td>
<td>+++++</td>
</tr>
<tr>
<td>AS (PYR)</td>
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<td>++++</td>
<td>++++++</td>
</tr>
<tr>
<td></td>
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<td>+++</td>
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<td>++++++</td>
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<td>±</td>
<td>+++</td>
</tr>
<tr>
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<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
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<td>-</td>
</tr>
</tbody>
</table>

\(^a\)Parasitaemia scoring: - = no visible parasites; ±<0.5>0.1 parasites/500 rbcs; + > 0.5 parasites/500 rbcs; ++ > 2 parasites/500 rbcs; +++ > 10 parasites/500 rbcs; ++++ > 50 parasites/500 rbcs; +++++ > 200 parasites/500 rbcs; pp, post-peak of infection.
while AS (SENS) was resistant to 125 mg/kg sulfadoxine but peaked two days later than the undrugged control. Taken together, these results show that AS (PYR) is more sensitive to sulfadoxine than AS (SENS). In mice maintained on the folate- and PABA-deficient regime, AS (SENS) and AS (PYR) were sensitive to all doses of sulfadoxine, including the lowest dose, 5 mg/kg (Table 4). This test confirmed that susceptibility to sulfadoxine is antagonised by a high level of PABA and/or folic acid in the host diet.

In the next tests, the PABA- and folate-deficient diet was supplemented with 0.005% PABA in the rodents' drinking water. The sulfadoxine susceptibility tests were then repeated on this regime to assess an appropriate dose for selecting for resistance to sulfadoxine (Table 5). All subsequent sulfadoxine tests, unless otherwise stated, were in mice on this diet. As expected, the presence of 0.005% PABA in the drinking water resulted in both AS (SENS) and AS (PYR) having decreased sensitivity to sulfadoxine. When treated with 2 or 10 mg/kg sulfadoxine, AS (SENS) showed little reduction in its growth compared to the undrugged control; when treated with 50 mg/kg sulfadoxine, it was cleared at first, but started to recrudesce on day 9. AS (PYR) showed a reduction in its growth rate relative to the undrugged control when treated with 2 mg/kg and 10 mg/kg sulfadoxine, although the parasites recrudesced on day 6 and 9 respectively; all AS (PYR) parasites were eradicated by 50 mg/kg sulfadoxine.

The dose required to select for resistance must eradicate all parasites under normal circumstances. These results suggested that a dose higher than 50 mg/kg sulfadoxine would be needed to select for sulfadoxine resistance from clone AS (SENS). Subsequently, a dose of 75 mg/kg sulfadoxine was chosen in attempts to select for resistance. Since 50 mg/kg sulfadoxine eradicated all AS (PYR) parasites and 10 mg/kg sulfadoxine showed a marked reduction in its growth rate, 40 mg/kg sulfadoxine was chosen as an appropriate dose to select for sulfadoxine resistance in AS (PYR).
Table 5. The sulfadoxine susceptibilities of AS (SENS) and AS (PYR). Mice were maintained on a folate- and PABA-deficient diet supplemented with 0.005% PABA in the drinking water.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Sulfadoxine Dose (mg/kg)</th>
<th>Day 6 Parasitaemia&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 9 Parasitaemia&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 13 Parasitaemia&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 20 Parasitaemia&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS (SENS)</td>
<td>0</td>
<td>++++</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>+++</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+++</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-</td>
<td>±</td>
<td>+++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>±</td>
<td>+++</td>
<td>pp</td>
</tr>
<tr>
<td>AS (PYR)</td>
<td>0</td>
<td>++++</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>±</td>
<td>+++</td>
<td>pp</td>
<td>pp</td>
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<td>+</td>
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<td>pp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Parasitaemia scoring: - = no visible parasites; ± <0.5>0.1 parasites/500 rbc; + > 0.5 parasites/500 rbc; ++ > 2 parasites/500 rbc; +++ > 10 parasites/500 rbc; ++++ > 50 parasites/500 rbc; pp, post-peak of infection.

3.2 Selection for resistance to sulfadoxine

3.2.1 Results of sulfadoxine selection

One hundred mice on a PABA- and folate-deficient diet supplemented with 0.005% PABA in the drinking water, were injected (ip) with $10^6$ parasites of clone AS (SENS) and 75 mg/kg sulfadoxine was administered to the mice, from day 3, for a total of 4 days (section 2.3.3). A further 100 mice were injected with AS (PYR) and similarly drugged with 40 mg/kg sulfadoxine. The mice infected with AS (PYR) failed to develop parasites in their circulation, while 15 of the mice injected with AS
(SENS) developed patent parasitaemias by day 18. Parasites were collected and frozen from all 15 mice and are referred to here as sulfadoxine-selected lines.

### 3.2.2 Sulfadoxine responses of the sulfadoxine-selected lines

The sulfadoxine-selected lines were analysed for their responses to sulfadoxine and compared to that of AS (SENS). All 15 selected lines showed drug responses identical to AS (SENS) (data not shown). Selection for resistance therefore had not been successful.

### 3.3 Selection for resistance to sulfadoxine/pyrimethamine

#### 3.3 Susceptibility of AS (SENS) and AS (PYR) to S/P

Before selecting for resistance to the S/P combination, the sensitivities of AS (SENS) and AS (PYR) were investigated. All S/P tests were in mice fed on normal diets supplemented with the standard amount of PABA (0.05%) in the drinking water. AS (SENS) was found to be totally sensitive to 25/1.25 and 50/2.5 mg/kg S/P, and growth was reduced relative to the undrugged control when treated with 5/0.25 mg/kg S/P (Table 6). A dose of 50/2.5 mg/kg S/P was thus chosen as appropriate to select for resistance in AS (SENS).

A dose of 5/0.25 mg/kg S/P had little effect on the growth of AS (PYR) and while doses of 50/2.5 and 75/3.75 mg/kg S/P cleared parasites initially, recrudesces were observed on day 14 (Table 6). A higher dose was therefore needed to eradicate all AS (PYR) parasites; 125/6.25 mg/kg S/P was thus chosen as an appropriate dose to select for S/P resistance in this parasite.

The synergistic activity between sulfadoxine and pyrimethamine can clearly be seen in Table 6. AS (PYR) is resistant to a dose of 2.5 mg/kg pyrimethamine, while 50 mg/kg sulfadoxine results in approximately a 3 day delay to reach peak parasitaemia on normal diet. However, when these two doses are combined, AS (PYR) is eliminated from the circulation, recrudescing on day 14.
### Table 6. Susceptibility of AS (SENS) and AS (PYR) to sulfadoxine/pyrimethamine (S/P)

<table>
<thead>
<tr>
<th>Clone</th>
<th>Drug Dose</th>
<th>Day 8 Parasitaemia&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 10 Parasitaemia&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 14 Parasitaemia&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS (SENS)</td>
<td>0</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>50 mg/kg SDX</td>
<td>++++</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>2.5 mg/kg PYR</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5/0.25 mg/kg S/P</td>
<td>++</td>
<td>++++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>25/1.25 mg/kg S/P</td>
<td>±</td>
<td>++++</td>
<td>pp</td>
</tr>
<tr>
<td>AS (PYR)</td>
<td>0</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>50 mg/mg SDX</td>
<td>±</td>
<td>++++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>2.5 mg/kg PYR</td>
<td>pp</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>5/0.25 mg/kg S/P</td>
<td>++++</td>
<td>pp</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>25/1.25 mg/kg S/P</td>
<td>++++</td>
<td>++++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>50/2.5 mg/kg S/P</td>
<td>++</td>
<td>++++</td>
<td>pp</td>
</tr>
<tr>
<td></td>
<td>75/3.75 mg/kg S/P</td>
<td>-</td>
<td>±</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Parasitaemia scoring: ± < 0.5 > 0.1 parasites/500 rbcs; + > 0.5 parasites/500 rbcs; ++ > 2 parasites/500 rbcs; ++++ > 10 parasites/500 rbcs; ++++ > 50 parasites/500 rbcs; ++++ > 200 parasites/500 rbcs. SDX, sulfadoxine; PYR, pyrimethamine; pp, post-peak of infection.

### 3.3.2 Results of sulfadoxine/pyrimethamine selection

One hundred mice on normal diet, were injected with AS (PYR) and selected with 125/6.25 mg/kg S/P (section 2.3.3). Fifteen out of the 100 mice infected with AS (PYR), developed patent parasitaemias on day 18, 15 days after the last administration of S/P. Parasites were collected and frozen from all 15 mice; these are
referred to here as S/P-selected lines. A further 52 mice were injected with AS (SENS) and treated with 50/2.5 mg/kg S/P. Fourteen out of the 52 mice infected with AS (SENS) developed patent parasitaemias on day 18. These parasites were preserved, but not characterised further in the present work. The remainder of this work involves the characterisation of the S/P-selected lines that were derived from AS (PYR).

3.3.3 Sulfadoxine/pyrimethamine responses of the S/P selected lines

The growth patterns of each of the S/P-selected lines were compared under S/P pressure to that of the parent clone, AS (PYR). Eight lines showed increased resistance to S/P, while the remaining seven had drug responses comparable to that of AS (PYR). Six of the eight resistant lines showed reductions in their growth rates compared to the undrugged control following 50/2.5 mg/kg S/P treatment, but all grew more quickly than AS (PYR). These lines were classified as S/P resistant (R). The remaining two lines grew quickly under 50 mg/kg S/P and recrudesced after treatment with 75/3.75 mg/kg S/P. These parasites were denoted as having high-level S/P resistance (HR).

One R and one HR line were chosen for further analysis. These lines were cloned by limiting dilution (section 2.2.6). The S/P resistant clone was then denoted as AS (50S/P) while the clone with high-level S/P resistance was denoted as AS (75S/P). The S/P susceptibilities of AS (75S/P) and AS (50S/P) are shown in Figure 7.
Figure 7. Assessment of the sulfadoxine/pyrimethamine (S/P) sensitivities of the clones AS (PYR), AS (50S/P) and AS (75S/P). The S/P sensitivities of AS (50S/P) and AS (75S/P) were compared to AS (PYR) following treatment in a 4 day drug test with 50 mg/kg S/P. The data points are the mean ± S.E. of three mice from a representative experiment.
3.3.4 Sulfadoxine susceptibilities of the S/P resistant clones

In tests for response to 5 mg/kg sulfadoxine, AS (50S/P) grew more quickly than the clone with high-level S/P resistance, AS (75S/P), while AS (PYR) was completely eliminated (Figure 8).

Figure 8. Assessment of the sulfadoxine sensitivities of the clones AS (PYR), AS (50S/P) and AS (75S/P). The sulfadoxine sensitivities of AS (50S/P) and AS (75S/P) were compared to AS (PYR) following treatment with 5 mg/kg sulfadoxine in mice fed on PABA- and folate-deficient diet supplemented with 0.005% PABA in the drinking water. The data points shown are the mean ± S.E. of three mice from a representative experiment.
3.3.5 Pyrimethamine resistance

AS (50S/P) grew faster under pyrimethamine pressure than clone AS (75S/P), which showed a similar growth rate to AS (PYR) following 10 mg/kg pyrimethamine treatment (Figure 9).

![Figure 9. Assessment of the pyrimethamine resistance of the clones AS (PYR), AS (50S/P) and AS (75S/P). The pyrimethamine resistance of AS (50S/P) and AS (75S/P) were compared to AS (PYR) following treatment in a 4 day drug test with 10 mg/kg pyrimethamine. The data points shown are the mean ± S.E. of three mice from a representative experiment.](image)

3.4 Stability of resistance in the absence of drug

To investigate the stability of resistance in the absence of drug, the resistant parasites were passaged weekly to uninfected mice without drug pressure. Each week, the parasites were also sub-inoculated into groups of CBA/Ca mice and analysed for their responses to S/P. AS (75S/P) retained resistance after nine weekly
passages in the absence of drug, while AS (50S/P) remained resistant after 20 weekly passages without drug pressure. AS (50S/P) was successfully transmitted through mosquitoes and the subsequent sporozoite-induced infection was found to have a drug-resistance phenotype identical to AS (50S/P). Thus, S/P resistance in AS (50S/P) is stable in the absence of drug and after mosquito transmission. AS (75S/P) was eventually transmitted through mosquitoes, but the drug-resistant phenotype of the resulting parasites was not determined.

3.5 Summary of drug responses

Two S/P resistant clones, AS (50S/P) and AS (75S/P), were selected from AS (PYR) and chosen for further analysis. AS (PYR) parasites were eliminated by S/P treatment, whereas AS (50S/P) and AS (75S/P) recrudesced following S/P pressure. However, each mutant possessed a different drug resistant phenotype. The AS (75S/P) clone always recrudesced before the AS (50S/P) clone following treatment with S/P, while AS (50S/P) always appeared before AS (75S/P) when treated with either sulfadoxine or pyrimethamine alone. The drug responses of these clones are summarised in Table 7.

Table 7. Summary of drug sensitivities of the parent clone AS (PYR) and the S/P-selected clones, AS (50S/P) and AS (50S/P)

<table>
<thead>
<tr>
<th>Drug*</th>
<th>AS (PYR)b</th>
<th>AS (50S/P)b</th>
<th>AS (75S/P)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>50/2.5 mg/kg S/P</td>
<td>S</td>
<td>R</td>
<td>HR</td>
</tr>
<tr>
<td>10 mg/kg pyrimethamine</td>
<td>R</td>
<td>HR</td>
<td>R</td>
</tr>
<tr>
<td>5 mg/kg sulfadoxine</td>
<td>S</td>
<td>HR</td>
<td>R</td>
</tr>
</tbody>
</table>

*a Drug-susceptibilities as measured by the 4-day test described in section 2.3.2.

b S, sensitive; R, resistant; HR, highly resistant.
4. Results: The dihydropteroate synthase and dihydrofolate reductase genes of *P. chabaudi*.

4.1 Isolation of the *P. chabaudi pppk-dhps*

4.1.1 Cloning and sequencing the *P. chabaudi pppk-dhps* gene

Mutations in the gene coding for dihydropteroate synthase (DHPS) have been implicated in the mechanism of S/P resistance in *P. falciparum* (Triglia et al. 1998). Therefore, to identify any possible mutations associated with resistance to S/P, the *P. chabaudi* homologue was cloned and sequenced. The *dhps* gene was then sequenced from AS (PYR) and from all the S/P selected lines.

To isolate *P. chabaudi dhps*, degenerate oligonucleotide primers were used to amplify a portion of the gene from clone AS (SENS). The resulting PCR product of about 100 bp showed significant homology to the *P. falciparum dhps* sequence. The full length sequence coding for the *P. chabaudi* PPPK-DHPS was subsequently obtained through the technique of 'PCR-walking' using Vectorette genomic DNA libraries.

4.1.2 Analysis of the nucleotide and predicted amino acid sequence

A region of 2489 bp was fully sequenced and compared to the *P. falciparum pppk-dhps* sequence (Brooks et al. 1994, Triglia and Cowman 1994). Based on the structure of the *P. falciparum* gene, the *P. chabaudi* homologue is predicted to have three open reading frames from 24-130, 368-2164 and 2317-2432 bp (Figure 10). The two introns contain multiple stop codons and their positions relative to introns in the *P. falciparum pppk-dhps* gene are highly conserved. However, the comparison suggests that approximately 30 nucleotides coding for the first 10 amino acids, including the start codon, are missing from the 5' end of the *P. chabaudi pppk* sequence. Stop codons and the absence of a start codon upstream of the first codon shown in Figure 10, suggest that an additional intron may be present at the 5' end of the gene.
**Figure 10.** Nucleotide and predicted amino acid sequence of the PPPK-DHPS of *P. chabaudi*. The nucleotides are numbered from the first nucleotide in the sequence. Lower case letters indicate non-coding nucleotides and an asterisk marks the position of the predicted stop codon. The black arrows indicate the boundaries of the oligonucleotides used in either Vectorette PCR to extend the sequence, or to amplify the *pppk-dhps* for sequencing. Blue arrows indicate the junction of the PPPK and DHPS domains.
<table>
<thead>
<tr>
<th>Location</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1240</td>
<td>TTA AAA</td>
<td>DHPS3</td>
</tr>
<tr>
<td>1324</td>
<td>ATT GCT GTA GCC AGA ATA</td>
<td>vecDHPS2</td>
</tr>
<tr>
<td>1408</td>
<td>TCT CAT</td>
<td>cDHPS4</td>
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<tr>
<td>1492</td>
<td>ATT TGT</td>
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</tr>
<tr>
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<td>AGG AGG AGA TTT ATA TCG CAT ACT TTG GCA CAA AGC TGC GTT GAA ATA TGT CCT AAT ACT ATT GCC CAA AAT GAA AAT ATT CAA</td>
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<tr>
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<td>tttataaatattatatcatttttttttttcatcacagGTT GGA TTA GCC ATT GCC TCA TAT TGC TTT GAA AAA AAA GGT GAA ATG ATA</td>
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</tr>
<tr>
<td>2367</td>
<td>AGA GAT</td>
<td>vecDHPS8</td>
</tr>
<tr>
<td>2454</td>
<td>aaaaaatattatcataattagtaactatcttca</td>
<td>vecDHPS8</td>
</tr>
</tbody>
</table>
Results: The pppk-dhps and dhfr-ts genes of P. chabaudi

Chapter IV

Extensive sequencing upstream failed to locate the start codon, or a region with homology to the 5' end of the *P. falciparum* sequence. Assuming that there are still another 10 amino acids to be identified, the coding sequence predicts a mature polypeptide of 683 amino acids, with an approximate molecular mass of 80,305 kDa.

The *P. chabaudi* pppk-dhps sequence was used to screen the Malaria Genome Sequence Tag Project database at the University of Florida (http://parasite.vetmed.ufl.edu/). A partial DNA sequence of 300 bases with significant homology to the *pppk* gene was identified from the *P. vivax* genome sequence survey (GSS) project (UFL 267PvH07) and two sequences, one with significant homology to the *pppk* gene and one with homology to the *dhps* gene, were identified in the *P. berghei* expressed sequence tag (EST) project (UFL 055PbE09, UFL 228PbG09).

4.1.3 Analysis of the predicted dihydropteroate synthase (DHPS) amino acid sequence

As reported previously for other protozoan DHPS enzymes, the *P. chabaudi* DHPS is part of a bifunctional protein, containing both the DHPS and PPPK enzyme domains (Walter and Konigk 1980). Comparison with the *P. falciparum* pppk-dhps gene shows that the region from amino acids 323-673 encodes the *P. chabaudi* DHPS enzyme while residues 1-322 encode the PPPK enzyme. There is no junction region between the two protein domains; the only bifunctional protozoan PPPK-DHPS known to have a junction region is that of *Toxoplasma gondii* (Pashley *et al.* 1997).

Comparison of the predicted *P. chabaudi* DHPS amino acid sequence with other available DHPS sequences (Figure 11) indicated that it has highest homology with a fragment of the *P. berghei* homologue. These sequences were 92% identical and 95% similar at the amino acid level, if conservative substitutions are allowed. Similarity with the *P. falciparum* DHPS was also high; 63% identical and 75% similar. The *P. chabaudi* DHPS has a 29 amino acid insertion (amino acids 476-505) relative to the *P. falciparum* protein. Identity with other DHPS proteins was lower; 30% with the *Pneumocystis carinii* DHPS, 27% with the *T. gondii* DHPS, 24% with
Figure 11. Alignment of five known PPPK-DHPS’s and the predicted P. chabaudi, P. berghei and P. vivax PPPK-DHPSs. The PPPK-DHPS from P. chabaudi, P. falciparum (U07706), P. vivax (University of Florida Gene Sequence Survey Project), P. berghei (University of Florida Gene Sequence Tag Project), T. gondii (U81497), Pn. carinii (M86602), S. cerevisiae (ORF 848 in X96722) and E. coli (L06495 and X68776) were aligned using the Gap and PileUp programmes of the UWGCG. GeneBank accession numbers are shown in brackets. Amino acids are designated by the single letter code. The sequences are numbered with respect to the P. falciparum sequence. A dash indicates a gap introduced to obtain optimal alignment. Conserved residues are shown in blue while positions within the P. falciparum sequence that have been implicated in sulfadoxine resistance are shown in red. The sequences to which degenerate oligonucleotides were made for PCR amplification are marked with arrows. The highly conserved DHPS motif is boxed.
Figure 11 continued.

262

P. chabaudi:  
\[
\begin{align*}
\text{T. gondii:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{Pf. falciparum:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{S. cerevisiae:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{E. coli:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\end{align*}
\]

350  

P. chabaudi:  
\[
\begin{align*}
\text{T. gondii:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{Pf. falciparum:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{S. cerevisiae:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{E. coli:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\end{align*}
\]

351  

P. chabaudi:  
\[
\begin{align*}
\text{T. gondii:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{Pf. falciparum:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{S. cerevisiae:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{E. coli:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\end{align*}
\]

380  

P. chabaudi:  
\[
\begin{align*}
\text{T. gondii:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{Pf. falciparum:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{S. cerevisiae:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{E. coli:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\end{align*}
\]

381  

P. chabaudi:  
\[
\begin{align*}
\text{T. gondii:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{Pf. falciparum:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{S. cerevisiae:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{E. coli:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\end{align*}
\]

423  

P. chabaudi:  
\[
\begin{align*}
\text{T. gondii:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{Pf. falciparum:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{S. cerevisiae:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{E. coli:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\end{align*}
\]

424  

P. chabaudi:  
\[
\begin{align*}
\text{T. gondii:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{Pf. falciparum:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{S. cerevisiae:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{E. coli:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\end{align*}
\]

480  

P. chabaudi:  
\[
\begin{align*}
\text{T. gondii:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{Pf. falciparum:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{S. cerevisiae:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\text{E. coli:} & \quad \text{NLNNNNNIKK} & \quad \text{V VIHPDVRPV} & \quad \text{KQWPLTLIP} & \quad \text{EVLYLKEK} \\
\end{align*}
\]


Figure 11 continued.

P. chabaudi:

IDTMNYDLFK BCVDKINLVDI LANDISACTND PQIIKLKLKK N-KYYSVVLML HKRGERPHTMD MLTQYEDVY -DIKKYLEER --LNFLTL-

P. falciparum:

IDTNINVFK BCVDNDLDVI LANDISACTNN PBIIKLKLKK N-KFYSVVLML HKRNGHPHTMD KLTNYDLVY -DIKKYLEQ--LNFLVL-

P. berghei:

NYDLFK BCVDKINLVDI LANDISACTND PKIIKLKLKK N-KYYSVVLML HKRGERPHTMD MLTQYEDVY -DIKKYLEER --LNFLTL-

T. gondii:

VDTSKAEVAR DAVTAG-ADW VNDDQTGESK GGEGDLESF VGNSTTVVLML HKRGTPTFED GYQGYD-DVY HEVGSWLASM SEALQR---

Pn. carinii:

IDTFRSEVAVE QNVRAG-ASL VNDDISGGRYD K---VPICIM HMRGNFLNMD NLTQGTIDII EQITIELEK--LNSAEK-

S. cerevisiae:

IDTYRNVAK EAIKVGD-VDI LANDISGGLFD SNMFAVIAEN PE---ICYILS HTRGDISTMN RLAHYEN--F ALGSISIQFVE VHNTIQQLD

E. coli:

VDTSKPEVIR ESAVKG-AHI LANDIRSLSEP GALEA-AAT G---LPVCLML HMQGGNPMTQ AEAKYD-DVF AEVNRYFEIR---QIARCEQ-

P. chabaudi:

-------- -------- -----

P. falciparum:

-------- -------- -----

P. berghei:

-------- -------- -----

T. gondii:

-------- -------- -----

Pn. carinii:

-------- -------- -----

S. cerevisiae:

-------- -------- -----

E. coli:

-------- -------- -----

608

P. chabaudi:

RKRFTISHTLA QTCEICPNT IAQNE--NQ N---DESDK-N GPTVRNIRKD KDQYLYQKNI LGLILAASIC --FEKAKWEF IRVHDFETIR

P. falciparum:

RKRFTIACMN DNQVINTQQ KLDHEQNREN KNIKVDSHNN MFOQYNMYRD KDQLLQKNI CGGLIAASYS --YKRDVLK IRVHDVLETK

P. berghei:

-------- -------- -----

T. gondii:

-------- -------- -----

Pn. carinii:

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S. cerevisiae:

-------- -------- -----

E. coli:

-------- -------- -----

694

P. chabaudi:

-------- -------- -----

P. falciparum:

-------- -------- -----

T. gondii:

-------- -------- -----

Pn. carinii:

-------- -------- -----

S. cerevisiae:

-------- -------- -----

E. coli:

-------- -------- -----

695

P. chabaudi:

CVLDMKMKLH QNE*

P. falciparum:

SVDLVLTKID QV*

T. gondii:

LVVDVTERIQ GVKSTINME KEPMAPLYSFW*

Pn. carinii:

KISMSDAIW ---KEIY*

S. cerevisiae:

KSISKLADAY --KGE*

E. coli:

EAMRVVEATL SAKENKRYE*
the *Saccharomyces cerevisiae* DHPS, and 28% to the equivalent *Escherichia coli* protein. The *P. chabaudi* sequence contains the conserved DHPS motif (I/V)R(V/T)HDV, found in all DHPS proteins, at positions 651-656 (Pashley *et al.* 1997).

### 4.1.4 Analysis of the predicted hydroxymethyldihydropterin pyrophosphokinase (PPPK) amino acid sequence

The region from amino acids 1-322 is predicted to encode the *P. chabaudi* PPPK enzyme, although approximately 10 amino acids predicted to occur at the amino-terminus are missing. Alignment of the *P. chabaudi* PPPK sequence with available PPPK sequences (Figure 11) showed that it is much less conserved than the DHPS domain. While a few residues are absolutely conserved, there are no conserved motifs. The predicted *P. chabaudi* PPPK protein had highest homology with the predicted *P. berghei* PPPK; the sequences were 92% identical and 95% similar at the amino acid level. Identity with other *Plasmodium* PPPK proteins was lower; 51% with the *P. falciparum* PPPK and 50% identity with predicted *P. vivax* PPPK. Comparison of the *P. chabaudi* sequence with that from other organisms revealed 38% identity to *T. gondii*, 31% to *Pn. carinii*, 24% to *S. cerevisiae* and 28% to the equivalent *E. coli* protein. The *Plasmodium* PPPK proteins have a large insertion relative to those from other organisms; in *P. falciparum* the PPPK has a 79 amino acid insertion (positions 222-301), while in the *P. chabaudi* PPPK, the insertion is only 34 amino acids.

It is interesting to note that the *P. berghei pppk* sequence also appears to have an additional intron at the 5' end of the gene. As seen in the *P. chabaudi* translated sequence, the absence of a region with homology to the 5' end of the *P. falciparum* sequence, suggests that an additional intron may be present at the 5' end of both rodent malaria genes. All other intron positions in the *Plasmodium pppk-dhps* genes are highly conserved.
4.1.5 Sequencing the *pppk-dhps* alleles of the drug-selected lines

The *dhps* sequences (nucleotides 1270-2432) were determined from all lines that survived drug selection, including AS (50S/P) and AS(75S/P). Sequence analysis of these lines did not reveal any polymorphisms when compared to the *dhps* sequence of AS (PYR); all sequences were identical to that shown in Figure 10. In addition, the entire *pppk-dhps* sequence (nucleotides 25-2432) was sequenced from AS (PYR), AS (50S/P) and AS(75S/P) and a drug-sensitive clone AJ. Again, the sequences were identical to that shown in Figure 10. Taken together, these results suggest that resistance to S/P is not conferred by mutations in *pppk-dhps* in these lines.

4.2 Sequencing the *dihydrofolate reductase-thymidylate synthase* (DHFR-TS) alleles of the drug-selected lines

The gene encoding dihydrofolate reductase (DHFR) has been strongly implicated in the mechanism of S/P resistance in *P. falciparum* (Watkins *et al.* 1997, Basco *et al.* 1998b, Plowe *et al.* 1998). Parasites resistant to S/P in the current work were selected from a *P. chabaudi* clone, AS (PYR), already resistant to pyrimethamine. The pyrimethamine resistance is conferred by the presence of an asparagine rather than a serine at position 106 in its DHFR (Cowman and Lew 1990, Cheng and Saul 1994). To investigate the possibility that additional mutations in the *dhfr* gene were responsible for S/P resistance, the *dhfr* gene was sequenced from AS (PYR) and all the drug-selected lines, including AS (50S/P) and AS(75S/P) (Figure 12). The DHFR is also a bifunctional enzyme, containing both DHFR activity and the thymidylate synthase (TS) domain (Garrett *et al.* 1984). The *ts* domain was sequenced from AS (PYR), AS (50S/P) and AS(75S/P). There were no sequence differences in the respective genes of any of the clones. These results show that resistance to S/P is not conferred by additional mutations in *dhfr-ts* in these lines.

This is the first report of the sequence of *ts* from the *P. chabaudi chabaudi* subspecies. As previously reported, the TS domain is more highly conserved than the DHFR domain (Hyde 1990). The DHFR domains from the two *P. chabaudi* subspecies, *P. c chabaudi* and *P. c. adami*, are 98% identical to one another, while the
Results: The *pppk-dhps* and *dhfr-ts* genes of *P. chabaudi*

Chapter IV

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<td>AS:</td>
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<td>AS:</td>
<td>GNAHVYNNHVESLKVLNRTFYPFPTLKLNPQIKNIDFTISDFT</td>
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| 581 | **MAA** | 73 |

Figure 12. Amino acid sequence alignment of the *P. chabaudi* DHFR-TS. The complete amino acid sequence of *P. chabaudi adami* clone DS (accession number M30834) and the *P. chabaudi chabaudi* clone AS (SENS) are shown. The differences are shown in blue and the site of the serine to asparagine mutation associated with pyrimethamine resistance in AS (PYR), AS (50S/P) and AS (75S/P) is shown in red. The arrows indicated the boundaries of the oligonucleotides used in the PCR to amplify the *dhfr* gene for sequencing.
TS domains show 100% identity. All polymorphisms within the gene are in the regions encoding the DHFR domain or the junction region.

4.3 Summary of sequencing results

The analysis of the \textit{dhfr} and \textit{dhrs} sequences from the S/P-selected lines did not reveal any polymorphisms when compared to the respective sequences of the AS (PYR) genes. The \textit{pppk} and \textit{ts} sequences were also determined from AS (PYR), AS (50S/P) and AS (75S/P). There were no sequence differences in the respective genes of any of the clones. Taken together, these data suggest that S/P resistance in \textit{P. chabaudi} is not conferred by additional mutations in the \textit{dhfr} or \textit{dhrs} genes.
5. Results: The Crosses and Linkage Analysis

5.1 The crosses

In order to determine the genetic basis of resistance to S/P, the resistant clones were crossed with the drug sensitive clone, AJ. The crosses were repeated to increase the numbers of different meiotic products analysed and the crosses performed are shown in Table 8. Rats were infected with AJ mixed with either AS (50S/P) or AS (75S/P) and mosquitoes for each cross permitted to feed on the rats on day 3 or 4, when mature gametocytes were present (described in section 2.9). Ten days after mosquito infection, a sample of mosquitoes from each cage were dissected and examined for the presence of oocysts. The mosquito infection rates are shown in Table 9. Oocysts were found on the midguts of mosquitoes that had been fed on rats infected with AJ, AS (50S/P) and AJ x AS (50S/P). No oocysts were seen on midguts of mosquitoes that had been fed on rats infected with AS (75S/P) alone. Sporozoite-induced infections were established in mice either by allowing infected mosquitoes to feed on mice on days 15, 17 and 21 (crosses 1 and 3) or by removing salivary glands from mosquitoes by dissection and injecting the contents into mice (ip) (crosses 2 and 4). Approximately 100 mosquitoes were used for each feed or injection.

Table 8. The crosses performed in this work

<table>
<thead>
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<th>Cross Number</th>
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<th>Sporozoites</th>
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<tr>
<td>1</td>
<td>AJ x AS (50S/P)</td>
<td>Feed</td>
</tr>
<tr>
<td>2</td>
<td>AJ x AS (50S/P)</td>
<td>Injection</td>
</tr>
<tr>
<td>3</td>
<td>AJ x AS (75S/P)</td>
<td>Feed</td>
</tr>
<tr>
<td>4</td>
<td>AJ x AS (75S/P)</td>
<td>Injection</td>
</tr>
</tbody>
</table>

*Sporozoite-induced infections were established in mice by mosquito feeds, permitting infected mosquitoes to feed on mice, or by injection by removing salivary glands from mosquitoes and injecting (ip) the contents directly.
Table 9. Oocyst rates of mosquitoes from crosses

<table>
<thead>
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<th>Line</th>
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<th>Oocystsa</th>
<th>Line</th>
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<td></td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td>AJ x AS (50S/P)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td>2</td>
<td>7</td>
</tr>
<tr>
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<td>3</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>AJ x AS (75S/P)</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*aNumber of oocysts per mosquito midgut are shown. ND, not determined.
5.2 The progeny of the AJ x AS (50S/P) crosses

5.2.1 Identification of recombinants in the cross progeny

The uncloned progeny of AJ x AS (50S/P) and AJ x AS (75S/P) crosses were examined for the presence of the two parent alleles of \textit{msp1} by allele-specific PCR, and for \textit{dhfr} by mutation-specific PCR (described in section 5.3.3). The \textit{msp1} marker was chosen as it is unlikely that there would be selection against these alleles, while the \textit{dhfr} was chosen in order to identify recombinant forms following pyrimethamine treatment. For \textit{msp1}, a band of approximately 300 bp was observed in AS (50S/P), while in AJ it was approximately 30 bp larger; the two alleles could easily be distinguished after electrophoresis. The results for the crosses are given in Table 10.

Both parental \textit{msp1} and \textit{dhfr} genes were found in the uncloned AJ x AS (50S/P) progeny from crosses 1 and 2, even though transmission of AS (50S/P) alone was unsuccessful in cross 1. The uncloned progeny from both crosses 1 and 2 were treated with pyrimethamine and examined for \textit{msp1} and \textit{dhfr}. Both parental \textit{msp1} alleles were found in the progeny that survived treatment, showing that recombination between the \textit{dhfr} and \textit{msp1} had occurred, confirming that AJ and AS (50S/P) gametes had undergone crossing in mosquitoes. In the absence of crossing, only the \textit{msp1} allele of the drug-resistant parent would have been present in the drug-treated progeny. In contrast, progeny that had inherited the AJ \textit{dhfr} allele were eliminated by drug treatment as no AJ alleles were present in the progeny that survived drug treatment. This confirmed the role of this gene in resistance to pyrimethamine.

Only the AJ alleles of \textit{msp1} and \textit{dhfr} were found in the progeny of the AJ x AS (75S/P) from both crosses 3 and 4. This suggested that AS (75S/P) had not been transmitted through mosquitoes, which was consistent with the absence of oocysts on midguts of mosquitoes fed AS (75S/P)-infected rats (Table 9).
## Results: The Crosses and Linkage Analysis

### Table 10. Analysis of the crosses with \textit{dhfr} and \textit{msp1}

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Cross No.</th>
<th>Treatment</th>
<th>Markera\textsuperscript{b}</th>
<th>\textit{dhfr} (14)</th>
<th>\textit{msp1} (16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent Clones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJ</td>
<td></td>
<td>None</td>
<td></td>
<td>AJ</td>
<td>AJ</td>
</tr>
<tr>
<td>AS (50S/P)</td>
<td></td>
<td>None</td>
<td></td>
<td>AS</td>
<td>AS</td>
</tr>
<tr>
<td>Uncloned Progeny</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJ x AS (50S/P)</td>
<td>1</td>
<td>None</td>
<td>AS &amp; AJ</td>
<td>AS &amp; AJ</td>
<td></td>
</tr>
<tr>
<td>uncloned progeny</td>
<td></td>
<td>10 mg/kg pyrimethamine for 4 days</td>
<td>AS</td>
<td>AS &amp; AJ</td>
<td></td>
</tr>
<tr>
<td>AJ x AS (50S/P)</td>
<td>2</td>
<td>None</td>
<td>AS &amp; AJ</td>
<td>AS &amp; AJ</td>
<td></td>
</tr>
<tr>
<td>uncloned progeny</td>
<td></td>
<td>10 mg/kg pyrimethamine for 4 days</td>
<td>AS</td>
<td>AS &amp; AJ</td>
<td></td>
</tr>
<tr>
<td>AJ x AS (75S/P)</td>
<td>3</td>
<td>None</td>
<td>AJ</td>
<td>AJ</td>
<td></td>
</tr>
<tr>
<td>uncloned progeny</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJ x AS (75 S/P)</td>
<td>4</td>
<td>None</td>
<td>AJ</td>
<td>AJ</td>
<td></td>
</tr>
<tr>
<td>uncloned progeny</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Numbers in brackets refer to the probe number as shown in Table 3.
\textsuperscript{b} AJ, AJ-type allele; AS, AS-type allele.

### 5.2.2 Cloning from the progeny of the cross

In order to perform further genetic analysis, clones were made from the progeny of the AJ x AS (50S/P) crosses. In total, 33 clones were isolated by limiting dilution from the uncloned progeny of both crosses; 14 from cross 1 and 19 from cross 2. Each clone was tested for its response to \textit{S/P} and pyrimethamine as previously described. DNA was purified from each clone for analysis of the inheritance of markers that distinguished the parent clones. No analyses of the AJ x
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AS (75S/P) crosses were carried out, in view of the absence of the AS alleles of *msp1* and *dhfr* which indicated that, at least in these crosses, AS (75S/P) was infertile.

5.3 Genome markers

5.3.1 Chromosome-specific markers

Thirty chromosome-specific DNA markers were used to detect polymorphisms between parent clones AS (50S/P) and AJ. Twenty-six of the polymorphisms were detected by restriction fragment length polymorphism (RFLP) on Southern blots of genomic DNA. Twenty of the RFLP markers were known genes from *Plasmodium* species (Carlton *et al.* 1998a), and the remaining six were anonymous markers isolated from a *P. chabaudi* DNA library (Carlton *et al.* 1998a). Four polymorphisms were detected either by allele-specific PCR (*msp1, dhfr* and *mdrl*) or PCR amplification followed by digestion of the product to produce an RFLP (*cg10*).

5.3.2 Development of mutation-specific PCR for *Pcdhfr*

Two allele-specific amplification assays were designed that were able to detect either the wild-type *dhfr* allele, or the presence of the allele containing the mutation at nucleotide 319. The AS (50S/P) allele has a G to A mutation, resulting in the change from serine to asparagine at position 106, which confers resistance to pyrimethamine (Cowman and Lew 1990, Cheng and Saul 1994). Following the method of Wang *et al.* (1995), mutation-specific primers were designed by incorporating a deliberate mismatched base at the 3' end of the primer. Wang *et al.* (1995) found that this single mismatch was not always sufficient to prevent mutation-specific primers annealing to and amplifying both alleles, and so a deliberate T/T mismatch was introduced at the penultimate base at the 3' end of the primer. This resulted in the destabilisation of the 3' end of the primer on the non-target sequence such that amplification did not occur. A mismatch at the penultimate base was also incorporated into the 3' end of the *P. chabaudi dhfr* mutation-specific primers.
5.3.3 Genotyping the \( dhfr \) alleles of the cloned progeny of the cross using mutation-specific PCR

The mutation-specific PCR was used to genotype the \( dhfr \) alleles of the cloned progeny of the cross. Figure 13 shows the results of genotyping the progeny from cross 1. The asparagine-specific primers amplified a product of 281 bp from DNA extracted from \( \text{AS (50S/P)} \), while no amplification product was seen when \( \text{AJ} \) DNA was used as the template. The serine-specific primers amplified a product of 314 bp from \( \text{AJ} \) DNA, while no product was generated with DNA from \( \text{AS (50S/P)} \).

![Figure 13. Genotyping the \( dhfr \) alleles of the progeny of cross 1 using mutation-specific PCR.](image)

Two allele-specific amplification assays were designed, serine-specific and asparagine-specific, that were able to detect either the wild-type \( \text{AJ} \) \( dhfr \) allele, or the mutant \( \text{AS (50S/P)} \) allele respectively.
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Progeny clones 1108/8, 1108/11, 1110/6, 1110/8 and 1110/13 AS/AJ were determined to have the AJ-allele of \(dhfr\) due to the presence of a band when amplification was performed with the serine-specific primers, but not with the asparagine-specific primers (Figure 13). Only a very faint band was produced with the serine-specific primers from 1110/10 AS/AJ DNA. Further MS-PCR amplifications confirmed that this clone had inherited the AJ-allele of \(dhfr\). It was concluded that clones 1107/10, 1107/14, 1108/10, 1109/2, 1109/11 and 1110/4 AS/AJ had inherited the AS-allele of \(dhfr\) following the production of a band with the asparagine-specific primers, but not the serine-specific primers, (Figure 13).

To check that mixtures could be easily detected, amplification was performed on template containing both AS (50S/P) and AJ DNA with both sets of primers. As expected, a product was generated each primer pair. Subsequently, progeny 'clones' 1111/3 and 1111/10 AS/AJ were found to be mixtures containing both the AS and AJ-alleles of \(dhfr\), and were thus excluded from further analysis. Similar amplifications were performed using DNA from the clones of the second cross (cross 2).

5.3.4 Identification of independent recombinants

Thirty-three clones were made from the progeny of the AJ x AS (50S/P) crosses. Six of the 33 clones examined were found to be mixtures, two were AJ-parental type, and one AS-parental type. Eight recombinants had genotypes identical to at least one other clone as determined from inheritance of data of 20-30 markers. These were considered to derive from duplicate cloning events, and thus excluded from further analysis. In total sixteen of the original 33 clones were thus found to be independent recombinants; eight from cross 1 (1107/10, 1107/14, 1108/8, 1109/11, 1110/4, 1110/8, 1110/10 and 1110/13 AS/AJ) and 8 from cross 2 (1351/2, 1384/10, 1385/2, 1396/1, 1398/14, 1538/12, 1538/14 and 1539/1 AS/AJ). These were used in the linkage analysis studies.
5.4 Phenotyping the parents and progeny of the crosses

5.4.1 Growth rates of the parental clones

In the absence of treatment, the parental clones AJ and AS (50S/P) grew rapidly in CBA/Ca mice. Figure 14 shows a typical growth curve for the clones AJ, AS (PYR) and AS (50S/P) following inoculation of $10^6$ parasitised red blood cells. In this experiment, AJ reached an average parasitaemia of 49.2% on day 6, while AS (PYR) and AS (50S/P) reached average parasitaemias of 33.8% and 36.2% respectively. The difference in day 6 parasitaemias between AJ and AS (50S/P) is significant ($P<0.01$), as is the difference between AJ and AS (PYR) ($P<0.01$). No significant difference was seen between the AS (50S/P) and AS (PYR) parasitaemias. The importance of the different growth characteristics of the parent clones, regarding the progeny, is analysed further in section 5.5.3.

![Figure 14. The course of infection of clones AJ, AS (PYR) and AS (50S/P). Each data point represents the average parasitaemia of two mice taken from a representative experiment $±$ 1 S.E. ■ = AJ, ● = AS (PYR), ○ = AS (50S/P)](image)
5.4.2 Sulfadoxine/pyrimethamine susceptibility tests

The growth rates of each progeny clone treated with two different doses of S/P were compared with each of the two parents and with AS (PYR) (Table 11). Each clone was tested in at least two separate experiments. Blood smears were taken on days 6, 8 and 10 and examined for the presence of parasites.

Ten progeny clones had growth rates similar to AS (50S/IP) and were classified as S/P resistant (R). These clones reached peak parasitaemias by day 8 after treatment with 25/1.25 mg/kg S/P. Following treatment with 50/2.5 mg/kg S/P, the resistant progeny clones were usually found at detectable levels in the bloodstream on day 6, reaching peak parasitaemias between days 9-11. Some resistant progeny (1110/4 and 1539/1 AS/AJ) grew faster than the resistant parent following treatment with 25/1.25 mg/kg S/P, reaching higher peak parasitaemias, two days earlier than AS (50S/P). The difference in day 6 parasitaemias between AS (50S/P) and 1110/4 AS/AJ and AS (50S/P) and 1539/1 AS/AJ were highly significant (P<0.01). 1110/4 AS/AJ also reached higher parasitaemias than either parent in the undrugged controls.

Five clones showed a drug response similar to AJ. These clones were cleared by treatment with both doses of S/P and were typed as S/P sensitive (S). One clone showed a growth pattern similar to AS (PYR). 1385/2 AS/AJ recrudesced after treatment with 25/1.25 mg/kg S/P but was cleared by treatment with the higher dose of S/P. It was classified as having low-level S/P resistance (LR).

5.4.3 Pyrimethamine susceptibility tests

The growth rates of each progeny clone treated with 10 mg/kg pyrimethamine were compared with each parent and with AS (PYR) treated similarly (Table 12). Blood smears were examined on days 6, 8 and 10. Each clone was tested in at least two independent experiments.

Five progeny clones showed a pyrimethamine response similar to AJ. These clones were cleared by treatment with pyrimethamine and typed as pyrimethamine sensitive (S). Eleven progeny clones had growth rates similar to AS (50S/P) under
Table 11. Sulfadoxine/pyrimethamine phenotypes of 16 progeny clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>Day 6 Undrugged</th>
<th>Day 6 Drugged</th>
<th>Day 8 Drugged</th>
<th>Day 10 Drugged</th>
<th>Day 6 Drugged</th>
<th>Day 8 Drugged</th>
<th>Day 10 Drugged</th>
<th>S/P Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 6 25/1.25 mg/kg S/P</td>
<td>Day 6 25/1.25 mg/kg S/P</td>
<td>Day 10 25/1.25 mg/kg S/P</td>
<td>Day 6 50/2.5 mg/kg S/P</td>
<td>Day 8 50/2.5 mg/kg S/P</td>
<td>Day 10 50/2.5 mg/kg S/P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AJ</td>
<td>40.2 (±3.3)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>AS (PYR)</td>
<td>29.6 (±3.3)</td>
<td>0.3 (±0.2)</td>
<td>4.0 (±2.4)</td>
<td>11.6 (±3.2)</td>
<td>0.0 (±0.0)</td>
<td>0.1 (±0.1)</td>
<td>0.9 (±0.7)</td>
<td>LR</td>
</tr>
<tr>
<td>AS (50S/P)</td>
<td>31.4 (±3.3)</td>
<td>14.3 (±3.4)</td>
<td>34.2 (±4.2)</td>
<td>pp</td>
<td>3.5 (±1.3)</td>
<td>16.1 (±4.2)</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>1107/10</td>
<td>44 (±9.8)</td>
<td>1.9 (±1.9)</td>
<td>20.2 (±20.2)</td>
<td>pp</td>
<td>0.0 (±0.0)</td>
<td>1.6 (±1.6)</td>
<td>8.5 (±8.5)</td>
<td>R</td>
</tr>
<tr>
<td>1107/14</td>
<td>27.8 (±5.4)</td>
<td>9.6 (±3.2)</td>
<td>37.8 (±6.4)</td>
<td>pp</td>
<td>0.8 (±0.8)</td>
<td>12.8 (±12.6)</td>
<td>24.1 (±15.9)</td>
<td>R</td>
</tr>
<tr>
<td>1108/8</td>
<td>38.3 (±8.5)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>1109/11</td>
<td>46.2 (±3.7)</td>
<td>23.0 (±22.3)</td>
<td>pp</td>
<td>pp</td>
<td>0.2 (±0.2)</td>
<td>4.6 (±4.6)</td>
<td>7.3 (±7.0)</td>
<td>R</td>
</tr>
<tr>
<td>1110/4</td>
<td>58.6 (±1.3)</td>
<td>40.9 (±2.1)</td>
<td>pp</td>
<td>pp</td>
<td>7.8 (±7.8)</td>
<td>8.9 (±6.8)</td>
<td>pp</td>
<td>R</td>
</tr>
<tr>
<td>1110/8</td>
<td>52.6 (±0.5)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>S</td>
</tr>
<tr>
<td>1110/10</td>
<td>36.7 (±15.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>S</td>
</tr>
<tr>
<td>1110/13</td>
<td>35.4 (±11.6)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>S</td>
</tr>
<tr>
<td>1351/2</td>
<td>18.5 (±7.8)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.3 (±0.3)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>S</td>
</tr>
<tr>
<td>Clone</td>
<td>Day 6 Undrugged</td>
<td>Day 6 25/1.25 mg/kg S/P Drugged</td>
<td>Day 8 25/1.25 mg/kg S/P Drugged</td>
<td>Day 10 25/1.25 mg/kg S/P Drugged</td>
<td>Day 6 50/2.5 mg/kg S/P Drugged</td>
<td>Day 8 50/2.5 mg/kg S/P Drugged</td>
<td>Day 10 50/2.5 mg/kg S/P Drugged</td>
<td>Phenotype</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
<td>----------------------------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
<td>---------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>1384/10</td>
<td>17.7 (±9.3)</td>
<td>10.0 (±6.6)</td>
<td>31.5 (±9.6)</td>
<td>pp</td>
<td>0.0 (±0.0)</td>
<td>1.6 (±1.6)</td>
<td>8.3 (±6.3)</td>
<td>R</td>
</tr>
<tr>
<td>1385/2</td>
<td>37.1 (±11.1)</td>
<td>0.2 (±0.2)</td>
<td>5.8 (±4.6)</td>
<td>25.9 (±12.2)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>LR</td>
</tr>
<tr>
<td>1396/1</td>
<td>43.3 (±9.0)</td>
<td>19.9 (±1.4)</td>
<td>51.1 (±0.3)</td>
<td>pp</td>
<td>0.0 (±0.0)</td>
<td>12.9 (±12.9)</td>
<td>19.6 (±19.1)</td>
<td>R</td>
</tr>
<tr>
<td>1398/14</td>
<td>39.5 (±7.6)</td>
<td>14.6 (±10.0)</td>
<td>pp</td>
<td>pp</td>
<td>0.9 (±0.9)</td>
<td>14.1 (±14.1)</td>
<td>20.7 (±18.5)</td>
<td>R</td>
</tr>
<tr>
<td>1538/12</td>
<td>28.2 (±2.4)</td>
<td>17.7 (±1.1)</td>
<td>pp</td>
<td>pp</td>
<td>1.7 (±1.7)</td>
<td>17.4 (±15.1)</td>
<td>31.6 (±8.4)</td>
<td>R</td>
</tr>
<tr>
<td>1538/14</td>
<td>26.2 (±7.0)</td>
<td>24.5 (±1.5)</td>
<td>pp</td>
<td>pp</td>
<td>2.0 (±2.0)</td>
<td>20.9 (±20.9)</td>
<td>pp</td>
<td>R</td>
</tr>
<tr>
<td>1539/1</td>
<td>40.9 (±7.4)</td>
<td>39.2 (±0.9)</td>
<td>pp</td>
<td>pp</td>
<td>2.9 (±2.9)</td>
<td>18.5 (±18.5)</td>
<td>28.3 (±8.7)</td>
<td>R</td>
</tr>
</tbody>
</table>

Figures shown are mean parasitaemias from at least two separate experiments and figures in brackets are standard errors. Infections that have already peaked are indicated by pp. S/P, sulfadoxine/pyrimethamine; R, resistant; S, sensitive; LR, low level resistance.
### Table 12. Pyrimethamine phenotypes of 16 progeny clones

<table>
<thead>
<tr>
<th>Clones</th>
<th>Day 6 Undrugged</th>
<th>Day 6 10 mg/kg PYR Drugged</th>
<th>Day 8 10 mg/kg PYR Drugged</th>
<th>Day 10 10 mg/kg PYR Drugged</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ</td>
<td>40.2 (±3.3)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>S</td>
</tr>
<tr>
<td>AS (PYR)</td>
<td>29.6 (±3.3)</td>
<td>3.8 (±2.7)</td>
<td>6.0 (±4.7)</td>
<td>14.3 (±4.1)</td>
<td>R</td>
</tr>
<tr>
<td>AS (50SP)</td>
<td>31.4 (±3.3)</td>
<td>15.0 (±3.1)</td>
<td>46.2 (±3.8)</td>
<td>pp</td>
<td>R</td>
</tr>
<tr>
<td>1107/10</td>
<td>44 (±9.8)</td>
<td>10.3 (±1.65)</td>
<td>54.4 (±9.7)</td>
<td>pp</td>
<td>R</td>
</tr>
<tr>
<td>1107/14</td>
<td>27.8 (±5.4)</td>
<td>18.4 (±15.9)</td>
<td>pp</td>
<td>pp</td>
<td>R</td>
</tr>
<tr>
<td>1108/8</td>
<td>38.3 (±8.5)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>(0.0) (±0.0)</td>
<td>S</td>
</tr>
<tr>
<td>1109/11</td>
<td>46.2 (±3.7)</td>
<td>24.0 (±21.4)</td>
<td>36.6 (±0.4)</td>
<td>pp</td>
<td>R</td>
</tr>
<tr>
<td>1110/4</td>
<td>58.6 (±1.3)</td>
<td>13.3 (±4.6)</td>
<td>pp</td>
<td>pp</td>
<td>R</td>
</tr>
<tr>
<td>1110/8</td>
<td>52.6 (±0.5)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>(0.0) (±0.0)</td>
<td>S</td>
</tr>
<tr>
<td>1110/10</td>
<td>36.7 (±15.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.1 (±0.1)</td>
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</tr>
<tr>
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<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
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</tr>
<tr>
<td>1351/2</td>
<td>18.5 (±7.8)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
<td>0.0 (±0.0)</td>
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</tr>
<tr>
<td>1384/10</td>
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<td>5.3 (±0.5)</td>
<td>36.9 (±2.5)</td>
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<td>R</td>
</tr>
<tr>
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<td>29.8 (±23.6)</td>
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<td>pp</td>
<td>R</td>
</tr>
<tr>
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<tr>
<td>1398/14</td>
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<td>32.3 (±1.7)</td>
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<td>pp</td>
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<tr>
<td>1538/12</td>
<td>28.2 (±2.4)</td>
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<tr>
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</tr>
<tr>
<td>1539/1</td>
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<td>32.8 (±1.0)</td>
<td>pp</td>
<td>pp</td>
<td>R</td>
</tr>
</tbody>
</table>

Figures shown are mean parasitaemias from at least two separate experiments and figures in brackets are standard errors. Infections that had already peaked are indicated by pp. PYR, pyrimethamine; R, resistant; S, sensitive.
pyrimethamine pressure and were classified as pyrimethamine resistant (R). These clones reached peak parasitaemias by day 8 following treatment. As with S/P, some progeny clones (e.g. 1396/1 and 1539/1 AS/AJ) grew faster than the resistant parent following treatment with pyrimethamine, reaching peak parasitaemias two days earlier than AS (50S/P). The difference in day 6 parasitaemias between AS (50S/P) and 1396/1 AS/AJ, and between AS (50S/P) and 1539/1 AS/AJ, were highly significant (P<0.01). No intermediate phenotypes were observed.

5.5 Linkage Analysis

5.5.1 Inheritance of chromosome-specific markers

A total of 361 polymorphic sites were analysed among the progeny clones, most of which exhibited an approximately even distribution. 211 (58%) of all loci were inherited from the AJ parent and 150 (42%) from the AS (50S/P) parent (180.5 expected), compared to the 50:50 ratio expected if there is an equal chance of inheriting markers from either parent. There was a skewed inheritance of markers associated with chromosome 9. Out of 28 polymorphic sites for this chromosome, 25 (14 expected) of these were inherited from the AJ parent. In addition, no AS (50S/P) type alleles of marker 18 (Ag 3027) on chromosome 9 were detected among the progeny.

5.5.2 Simple analysis for linkage between markers and S/P resistance

The inheritance patterns of the 30 chromosomal markers and the S/P and pyrimethamine responses among the 16 progeny are shown in Figure 15. Linkage was assessed by comparing the inheritance pattern of each chromosome-specific marker with inheritance of the S/P phenotype. Linkage ratios were determined as the ratio of the number of progeny showing linkage of S/P susceptibility with an individual marker to the total number of progeny analysed. For this analysis, sensitive and low-level resistant progeny were pooled. Markers showing a linkage ratio of 12/16 or greater were crudely taken as showing linkage to resistance; due to the small number of markers, the very high probability that they are not evenly
Figure 15a. Inheritance of 15 chromosomal markers (chromosomes I-VII) among 16 progeny clones from two AJ x AS (50S/P) crosses. Progeny clones are listed vertically, Roman numerals refer to chromosome number and each chromosome marker has been allocated the same probe number as in Table 3. Red boxes indicate inheritance of AS (50S/P)-type alleles, blue boxes indicate inheritance of AJ-type allele, grey boxes indicate data not available.
### Figure 15b. Inheritance of 15 chromosomal markers (chromosomes VIII-XIV) among 16 progeny clones from two AJ x AS (50S/P) crosses.

Progeny clones are listed vertically, Roman numerals refer to chromosome number and each chromosome marker has been allocated the same probe number as in Table 3. **Red** boxes indicate inheritance of AS (50S/P)-type alleles, **blue** boxes indicate inheritance of AJ-type allele, **grey** boxes indicate data not available.

<table>
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<th>XIII</th>
<th>XIV</th>
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</table>

**Links**  
- **S/P and pyrimethamine resistant**
- **Low-level S/P resistance, pyrimethamine resistant**
- **S/P and pyrimethamine sensitive**
distributed among the genome and that little is known of recombination rates in \textit{P. chabaudi}, it was difficult to apply statistical analyses to these linkage ratios. Therefore, the only statistical analysis of linkage was that performed in the quantitative trait analysis (section 5.5.3).

The \textit{dhfr} marker (marker 14) on chromosome 7 showed strongest co-segregation with S/P resistance, with a linkage ratio of 15/16. Figure 15 shows that \textit{dhfr} is almost perfectly linked to the S/P resistance phenotype in both crosses; all the sensitive progeny clones have inherited the AJ \textit{dhfr} allele, while all the resistant progeny clones have the AS \textit{dhfr} allele. The \textit{dhfr} would show perfect linkage (16/16) had clone 1385/2 AS/AJ been classified with the resistant progeny. 1385/2 AS/AJ, which shows intermediate S/P resistance, was grouped with the sensitive progeny since it was the resistant S/P phenotype that was under investigation. This intermediate S/P resistance was similar to that conferred by mutant \textit{dhfr} alone in AS (PYR). It appears therefore, that while 1385/2 AS/AJ had inherited the mutant \textit{dhfr} from AS (50S/P), it had not inherited the additional gene(s) required for resistance to S/P. As expected, \textit{dhfr} (marker 14) is perfectly linked to pyrimethamine resistance in both crosses (Figure 15).

Other markers showing segregation with S/P resistance were \textit{pBS110} (marker 3) on chromosome 3 with a linkage ratio of 11/15, P.1 (marker 5) on chromosome 4 with a linkage ratio of 7/10, \textit{Ag3024} (marker 11, 10/13) on chromosome 5 and P.22 (marker 23) on chromosome 11 with a linkage ratio of 11/14. Markers on chromosomes 13, the location of \textit{dhps} (Carlton \textit{et al}, 1998b), showed linkage ratios of 10/16 or less. These ratios do not differ from markers showing a random distribution, indicating that \textit{dhps} is not tightly linked to resistance.

Unexpectedly, five markers showed a linkage ratio indicative of linkage with inheritance of the AJ-allele and S/P resistance. These were \textit{Ag3035} (marker 8, 3/15) and \textit{DNA polδ} (marker 9, 3/16) on chromosome 5, and \textit{aldo1} (marker 25, 3/10), \textit{G6PD} (marker 27, 2/15) and \textit{Pfcrk3} (marker 28, 3/15) on chromosome 13. Markers on all other chromosomes showed a random distribution expected for normal linkage equilibrium.
5.5.3 Quantitative trait loci analysis

Since resistance to S/P may be a complex trait, involving the additive effects of two or more genes, the data were analysed in order to map the trait to discrete quantitative trait loci (QTL). Details of the analysis are given in Appendix II. The results of the QTL analysis are shown in Table 13. The data were normalised by taking \( \log_{10} \) transformations. The differences between the mean log parasitaemias of grouped progeny clones inheriting the AS (50S/P) versus AJ allele of each marker are shown for day 6 parasitaemias for progeny treated with either 25/1.25 mg/kg S/P, 50/2.5 mg/kg S/P or 10 mg/kg pyrimethamine. The difference in mean log parasitaemias of progeny treated with 50/2.5 mg/kg S/P on day 8 are also shown. Days 8 and 10 for the remaining drug treatments could not be included as some of the resistant progeny reached peak parasitaemias on day 7, and so were difficult to assess accurately on subsequent days. Positive integers indicate that the AS (50S/P) marker allele is segregating with resistance, while negative integers indicate that the AJ allele is segregating with resistance.

The strongest linkage of the S/P resistance phenotype was to \( dhfr \) (marker 14) on chromosome 7. On days 6 and 8, the differences between the mean log-parasitaemias of the two groups of progeny clones inheriting either the AJ or AS (50S/P) \( dhfr \) allele were highly significant (25/1.25 mg/kg S/P and 10 mg/kg pyrimethamine, \( P<0.001 \); 50/2.5 mg/kg S/P (day 6), 0.01\( <P<0.05 \); 50/2.5 mg/kg S/P (day 8) \( P<0.001 \)). Other possible loci with linkage to S/P resistance were \( P.1 \) (marker 5) on chromosome 4 (25/1.25 mg/kg S/P, 0.001\( <P<0.01 \)), \( AMA-1 \) (marker 19) on chromosome 9 (50 mg/kg S/P (day 8), 0.001\( <P<0.01 \)) and \( Ag3024 \) (marker 11) on chromosome 5 (50 mg/kg S/P (day 8), 0.001\( <P<0.01 \)). The same marker on chromosome 5 also segregated with pyrimethamine resistance (0.01\( <P<0.05 \)).

The inheritance of AJ-type alleles on chromosomes 5 and 13 showed a significant linkage with both S/P and pyrimethamine resistance. AJ-type alleles segregating with resistance to 25/1.25 mg/kg S/P were \( Ag3035 \) (marker 8) (0.01\( <P<0.05 \)) and \( DNA \) \( pol \) \( \delta \) (marker 9) (0.001\( <P<0.01 \)) on chromosome 5 and
Table 13. Analysis for linkage between inheritance of markers and sulfadoxine/pyrimethamine and pyrimethamine resistance.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Marker</th>
<th>No. of Progeny Analysed (n)</th>
<th>Day 6 25/1.25 mg/kg S/P Treated</th>
<th>Day 6 50/2.5 mg/kg S/P Treated</th>
<th>Day 8 50/2.5 mg/kg S/P Treated</th>
<th>Day 6 10 mg/kg PYR treated</th>
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</table>
Figures represent the mean difference in log parasitaemia between progeny clones inheriting the AS (50S/P) versus the AJ allele of each marker. The analysis could not be carried out for marker 7 and marker 18 because no progeny inherited the AS (50S/P) allele of this locus. SIP, sulfadoxine/pyrimethamine; PYR, pyrimethamine.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

**G6PD** (marker 27) ($0.001 < P < 0.01$) on chromosome 13. AJ-type alleles showing linkage to 50/2.5 mg/kg S/P were *Pfcrk3* (marker 28) (day 6; $0.001 < P < 0.01$) and **G6PD** (marker 27) (day 8; $0.001 < P < 0.01$) on chromosome 13 and **DNA pol δ** (marker 9) (day 8, $0.001 < P < 0.01$) on chromosome 5. **Ag3035** (marker 8) ($0.001 < P < 0.01$) on chromosome 5 and **G6PD** (marker 27) ($P < 0.001$) and *Pfcrk3* (marker 28) ($P < 0.001$) also showed highly significant segregation with resistance to pyrimethamine. Figure 16 shows the mean day 6 log parasitaemias, for four markers, for each drug treatment; one representative marker (*msp1*) showing no linkage with resistance and three markers (*dhfr*, *P.1* and **G6PD**) that showed significant between-

<table>
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<th>Day 6</th>
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Figure 16. Marker contrasts for four loci. Graphs show the mean day 6 log parasitaemia between progeny clones inheriting the AS (50S/P) (grey bars) versus the AJ allele (white bars) of four markers. Progeny were treated with either 25/1.25 mg/kg S/P (25S/P), 50/2.5 mg/kg S/P (50S/P) or 10 mg/kg pyrimethamine (10 PYR). Inheritance of *msp1* (marker 16) shows random distribution indicative of normal linkage equilibrium, the AS (50S/P)-type *dhfr* (marker 14) is strongly linked to S/P and pyrimethamine resistance, while P.1 (marker 5) shows linkage with resistance to 25/1.25 mg/kg S/P. Inheritance of the AJ-type allele of marker 27 shows significant linkage with 25/1.25 mg/kg S/P and pyrimethamine resistance. Error bars are ± 1 S.E. * P < 0.05, ** P < 0.01, ***P < 0.001.
allele difference in parasitaemia, showing that these markers are segregating with the drug-resistant phenotype.

Some markers showed a large difference in mean parasitaemias between progeny clones inheriting the AS (50S/P) versus the AJ allele, but the difference was not found to be significant. This is due to a large variance about the mean parasitaemia and possibly small sample size. Therefore, these loci cannot be ruled out as analysis of more progeny clones may reveal linkage to S/P resistance.

5.5.4 Quantitative trait loci analysis of the fast growth phenotype

The growth characteristics of the parent clones differed significantly (Figure 14), AJ growing faster than AS (50S/P) and reaching a higher peak parasitaemia. QTL analysis was carried out for the day 6 undrugged controls to see if growth rate was linked to any of the loci examined. The differences between the mean log-parasitaemias of grouped progeny clones inheriting the AS (50S/P) versus the AJ allele of each marker are shown in Table 12. The inheritance of two AS-type alleles were weakly associated with fast growth rate; Ag3020 (marker 1) on chromosome 1 (0.01<P<0.05) and DNA pol δ (marker 9) on chromosome 5 (0.01<P<0.05). No AJ-type alleles segregated with fast growth rate.

This finding showed that the inheritance of genes determining fast growth rate from the AJ parent, does not account for the linkage between drug-resistance and the inheritance of AJ-type alleles on chromosomes 5 and 13.

5.5.5 Summary of linkage analysis

Taken together, these results suggest that mutant dhfr is a major determinant of S/P resistance in P. chabaudi. However, at least one other gene is required, and these results suggest that other loci for S/P resistance are present on chromosomes 4, 5 and 9. In addition, inheritance of loci on chromosomes 5 and 13 from the drug-sensitive parent appear to contribute to the level of resistance observed.
Table 14.
Analysis for linkage between inheritance of markers and growth rate.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Marker</th>
<th>No of Progeny Analysed</th>
<th>Day 6 Undrugged</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>12</td>
<td>0.18*</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>13</td>
<td>0.13</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>15</td>
<td>-0.06</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>12</td>
<td>-0.03</td>
</tr>
<tr>
<td>4</td>
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</tr>
<tr>
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</tr>
<tr>
<td>4/5</td>
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<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>15</td>
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<tr>
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</tr>
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<tr>
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<td>9</td>
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</table>
### Chromosome Marker No. of Progeny Day 6 Analysed Undrugged

<table>
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<tr>
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<th>Marker</th>
<th>No. of Progeny Analysed</th>
<th>Day 6 Undrugged</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td></td>
<td>30</td>
<td>16</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Figures represent the mean difference in log parasitaemia between progeny clones inheriting the AS (50S/P) versus the AJ allele of each marker. The analysis could not be carried out for marker 7 and marker 18 because no progeny inherited the AS (50S/P) allele of this locus. * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$
6. Discussion

6.1 Summary of results

In order to identify the genes conferring resistance to sulfadoxine/pyrimethamine (S/P), resistant mutants of *P. chabaudi* were derived from AS (PYR) by treating infected mice with high doses of S/P. Two representative S/P resistant lines were cloned and chosen for further analysis. AS (PYR) parasites were eliminated by S/P treatment, whereas AS (50S/P) and AS (75S/P) recrudesced at different rates following S/P pressure. Parasites of the AS (75S/P) clone always recrudesced earlier than those of the AS (50S/P) clone. However, AS (50S/P) always recrudesced before AS (75S/P) when treated with the individual components, sulfadoxine or pyrimethamine, alone. The resistance of AS (50S/P) and AS (75S/P) was stable after multiple blood passages in the absence of drug, and stable in AS (50S/P) following mosquito transmission.

The *P. chabaudi pppk-dhps* gene was cloned by homology and sequenced. Analysis of this gene and the *dhfr-ts* of AS (75S/P) and AS(50S/P) did not reveal any sequence changes when compared to the respective sequences of the AS (PYR) genes. Therefore, the mechanism of resistance to S/P in these drug-selected lines does not involve additional mutations in *dhfr-ts* and *pppk-dhps*.

To determine the genetic basis of S/P resistance in *P. chabaudi*, AS (50S/P) was crossed with an unrelated, drug sensitive clone, AJ. Sixteen independent recombinant progeny clones were phenotyped for their susceptibility to S/P and pyrimethamine and genotyped for the inheritance of 30 chromosome-specific markers. Linkage analysis showed that mutant *dhfr* is a major determinant of S/P resistance in *P. chabaudi*. However at least one other gene is involved. Quantitative trait analysis suggested that other loci for S/P resistance could be present on chromosomes 4, 5 and 9. In addition, loci on chromosomes 5 and 13 appear to contribute to the level of resistance observed. No association was found between S/P susceptibility and the inheritance of the *dhps* gene.
6.2 Selection for resistant parasites

S/P resistant mutants of *P. chabaudi* were obtained following single-step selection by the drug combination on a clone already resistant to pyrimethamine, AS (PYR). Pyrimethamine resistance in AS (PYR) is conferred by the presence of asparagine at position 106 in its DHFR, equivalent to the serine to asparagine change at position 108 conferring resistance to pyrimethamine in *P. falciparum* (Peterson et al. 1988, Cowman and Lew 1990, Cheng and Saul 1994). A clone already resistant to pyrimethamine was chosen as the starting material because in *P. falciparum* pyrimethamine resistance and hence mutations in *dhfr*, were already widespread when S/P was introduced in the field (Peters 1987).

The drug responses of the S/P selected lines were of two types; intermediate and high resistance, suggesting that the resistant phenotypes are conferred by different genetic mechanisms. Furthermore, the ease with which resistance was selected (single step, high-pressure selection) suggests that single point mutations could have caused each type of resistance. Interestingly, the clone with high resistance to S/P, AS (75S/P), showed lower resistance to the individual components sulfadoxine and pyrimethamine, than AS (50S/P). This suggests that the mechanism of resistance to the individual drugs may be different from the mechanism of resistance to the combination. If the same is true of *P. falciparum*, *in vitro* resistance assays for pyrimethamine and sulfadoxine individually may be of little relevance in predicting S/P response.

This is the first report of S/P resistance in malaria parasites, selected by single-step drug treatment in the laboratory, which shows stable resistance in the absence of drug. Stable pyrimethamine-resistant mutants of *P. chabaudi* have been produced by exposure of sensitive forms to this drug alone (Walliker et al. 1975, Cowman and Lew 1990), and MacLeod (1977) produced sulfadiazine resistant mutants of *P. chabaudi* in a similar way. With regard to other rodent malaria species, S/P resistant parasites have been selected using a continuous low-dose method in *P. berghei* and *P. yoelii* (Merkli and Richle 1980, Merkli and Richle 1983, Peters and Robinson 1984). The resistant phenotypes of these parasites were not stable in the
absence of drug; once drug pressure was removed, parasites reverted to the sensitive phenotype. No sequence data of the \textit{dhfr} and \textit{dhps} genes of these parasites are available, but as resistance was not stable, resistance was probably due to physiological adaptations, where genes that are not normally expressed become active in the presence of the drugs. Parasites then reverted to their normal sensitive phenotype once drug pressure was removed.

There are two methods of selecting drug resistant mutants in the laboratory; i) high-dose, single-step selection or ii) continuous, low-drug pressure selection. The single step selection method was used in this study because in \textit{P. falciparum}, resistance to S/P arose quickly and independently at various geographical locations. The ease with which resistance developed is indicative of a simple mutation being responsible for conferring resistance, as seen in pyrimethamine resistance. This is unlike the evolution of chloroquine resistance. Resistance to chloroquine emerged from two locations in the late 1960's, 20 years after it was first introduced and has gradually been spreading from these two sites (Payne 1987). This suggests that chloroquine resistance is a multigenic trait, the result of an additive effect of mutations in many genes (Cowman and Foote 1990). Selection for stable chloroquine resistance in rodent malarias was only successful using the continuous, low drug pressure selection procedure, over many passages (Powers \textit{et al.} 1969, Rosario 1976a, Padua 1980). However, work done by Merkli and Richle (1980) and Peters and Robinson (1984) has shown that the low-dose, continuous pressure method does not produce S/P mutants with stable resistance, suggesting that this method only selects for physiological adaptations to S/P.

While resistance to S/P is likely to be a multigenic trait, and therefore resistance in \textit{P. falciparum} might have been expected to have taken a long time to emerge, this work and that of others, has shown that mutant \textit{dhfr} is a major determinant (Watkins \textit{et al.} 1997). Since resistance to pyrimethamine, and therefore mutant \textit{dhfr} alleles, was already widespread in \textit{P. falciparum} when S/P was introduced, resistance required only one (or very few) more mutation(s) at other loci,
which were easily selected for. Whether or not the second locus is dhps is still a much debated topic, but this work points to the role of other loci.

Selection on high numbers of parasites parallels the situation encountered in the therapeutic use of S/P in man. The 20:1 ratio of sulfadoxine to pyrimethamine has also been maintained, and is the only study on rodent malaria to do so. Both Merkli and Richle (1980) and Peters and Robinson (1984) used ratios of 3:1. However, one major drawback is that no pharmacological data are available for the half-life of sulfadoxine in mice, so it is not clear if the same ratio was appropriate. In general, mice metabolise drugs much faster than humans; the half-life of pyrimethamine is approximately 95 hours in man, but only 24 hours in mice (Weidekamm et al. 1982). Sulfadoxine has an estimated half-life of 184 hours in humans (Weidekamm et al. 1982) and because sulfadoxine and pyrimethamine have similar half-lives in humans, this work has assumed that sulfadoxine also has a half-life similar to that of pyrimethamine in mice. S/P was administered to infected mice daily and appropriate doses for P. chabaudi were chosen by identifying a dose which eradicated all sensitive parasites. However, without pharmacological data, it is not clear whether this exposure of P. chabaudi to S/P is analogous to the exposure of P. falciparum parasites in treated humans.

In selecting for S/P resistance, 100 mice were infected with 10⁶ parasites and the first drug dose was administered four days later. It is estimated that there were approximately 10⁹ parasites per mouse at the time of the first drug administration. Therefore, approximately 10¹ⁱ parasites were subjected to S/P selection. Eight of the selected lines showed an increase in resistance, giving an approximate mutation rate of 0.8 x 10⁻⁴. This is comparable to the mutation rates of 2.4 - 4.3 x 10⁻¹⁰ observed during selection for pyrimethamine resistance from rodent malaria parasites (Morgan 1974), but is lower than that calculated for other organisms such as S. cerevisiae which has an estimated mutation rate of between 2 x 10⁻⁶ and 1 x 10⁻⁷ (Drake 1991). This is probably because other parasites that were selected for mutations for resistance to S/P either harboured deleterious mutations and/or were eliminated by the immune system before they were able to replicate.
If a higher dose had been used in the selection, or selection pressure applied for a longer period of time, parasites with even higher resistance to S/P might have been obtained. This has important implications in the linkage analysis. A parasite with higher resistance to S/P would have been easier to distinguish from parasites with pyrimethamine resistance alone and the progeny phenotypes may have been more discrete. However, transmission through mosquitoes of the clone with high-level resistance to S/P, AS (75S/P), proved difficult. The parasite was eventually transmitted, but it was not characterised further in this work. In addition, the AS (50S/P) clone was found to have a reduced infectivity to mosquitoes. It was transmitted on its own through mosquitoes on the second attempt, but it produced fewer oocysts in mosquito midguts that the sensitive clone, AJ. It may be that the AS-type parasites have reduced infectivity to mosquitoes as a result of multiple blood passages, or it may be that drug-resistance confers a fitness cost in these parasites. This may be particularly relevant to pyrimethamine and S/P resistant mutants, since these parasites have one or more mutations in the genes encoding enzymes in the folate biosynthesis pathway. Changes in DHFR resulting in decreased pyrimethamine binding also result in a decrease in catalytic activity (Siriwaraporn and Yuthavong 1984). Therefore, the parasite may be less efficient at pyrimidine synthesis, resulting in slower DNA synthesis and replication. When this hypothesis was tested, pyrimethamine-resistant *P. berghei* were found to undergo slower sporogonic development in the mosquito than the pyrimethamine-sensitive clone from which they were derived, leading to the conclusion that, in the absence of drug, pyrimethamine-resistant parasites are less fit than sensitive parasites, although the mechanism of resistance in these parasites was not determined (Shinondo *et al.* 1994). An additional mutation in the folate pathway to overcome S/P inhibition might result in an even greater fitness cost. However, selection for S/P resistance in AS (50S/P) also resulted in increased resistance to pyrimethamine and the loss of the PABA-sensitivity phenotype, suggesting that in selecting for resistance to S/P, the parasites have overcome the cost of having a DHFR with reduced catalytic activity. The potential mechanisms for this are discussed in section 6.6.
Attempts to select for sulfadoxine-resistant parasites were unsuccessful. While some parasites emerged after selection, they were not resistant to the drug and the reverted to the normal sensitive phenotype once drug pressure was removed. Like the previously obtained S/P resistant parasites (section 6.2), the ability to survive in high levels of drug was probably due to physiological adaptations.

6.3 Sequencing the dhps and dhfr genes

6.3.1 Isolation of the *P. chabaudi pppk-dhps*

Mutations in the gene coding for dihydropteroate synthase (DHPS) have been implicated in the mechanism of S/P resistance in *P. falciparum* (Triglia et al. 1998). Therefore, to identify any possible mutations associated with resistance to S/P in *P. chabaudi*, the homologue was isolated using degenerate oligonucleotide primers. The full length sequence coding for the *P. chabaudi* PPPK-DHPS was subsequently obtained through the technique of 'PCR-walking' using Vectorette genomic DNA libraries.

The approximate \( M_r \) calculated from the predicted amino acid sequence (80,305 kDa) is half that of the measured \( M_r \) of the native bifunctional protein (190,000 kDa) (Walter and Konigk 1980). This suggests that the *P. chabaudi* PPPK-DHPS forms a dimer in vivo, as proposed for the *P. falciparum* and *T. gondii* proteins (Brooks et al. 1994, Pashley et al. 1997). Comparison with the PPPK and DHPS amino acid sequences of other organisms showed that the *P. chabaudi* PPPK-DHPS has highest homology with the *P. berghei* homologue, identified in the *P. berghei* EST project. Residues that are absolutely conserved in all PPPK and DHPS enzymes are those that have been shown to be involved in substrate binding and catalysis in DHPS enzymes of other organisms (Achari et al. 1997, Hampele et al. 1997, Hennig et al. 1999).
6.3.2 Sequencing the pppk-dhps and dhfr-ts genes from AS (50S/P) and AS (75S/P)

The dhps and dhfr genes were sequenced from all the lines that survived S/P selection. Furthermore, the full length sequences of dhps-pppk and dhfr-ts were determined from AS (50S/P) and AS (75S/P). Sequence analysis did not reveal any polymorphisms when compared to the pppk-dhps and dhfr-ts sequences of AS (PYR). The mechanism of resistance to S/P in these drug-selected lines is not therefore conferred by additional mutations in the dhfr or dhps genes.

Due the presence of an intron at the 5' end of the gene coding for the P. chabaudi PPPK-DHPS, the first 10 amino acids of the PPPK domain are still to be determined. While it is possible that an amino acid change in the amino terminus region of the PPPK could result in structural changes in the DHPS, resulting in sulfa drug resistance, such a mechanism has never been described. Additionally, the pppk-dhps gene was not linked to resistance in the AJ x AS (50S/P) cross.

Increased expression of dhfr has been shown to confer low-level resistance to pyrimethamine (Inselberg et al. 1987, Cowman and Lew 1989, Cowman and Lew 1990, van Dijk et al. 1994). Stable resistance would require a permanent increase in expression, caused by a mutation in the promoter region, or a gene duplication event. While expression studies were not completed in this work, again the pppk-dhps gene was not linked to resistance in the cross, indicating that increased expression is not the mechanism of S/P resistance in the P. chabaudi mutants described here.

6.4 The AJ x AS (50S/P) crosses

6.4.1 The genetic basis of resistance to S/P

The inheritance of S/P susceptibility was examined among 16 progeny clones from two AJ x AS (50S/P) crosses. Five progeny clones were clearly sensitive to S/P and ten clearly resistant. One clone was classified as having low-level S/P resistance, similar to the S/P response of AS (PYR). This clone had also inherited the mutant dhfr allele, suggesting that mutant dhfr alone is able to confer low-level resistance. This is supported by the quantitative trait loci (QTL) analysis that shows mutant dhfr
to be a major determinant of S/P resistance. However, at least one other gene is required, which is present in the ten S/P resistant progeny, but absent from the progeny clone with low-level resistance. The quantitative trait analysis suggest that other loci for S/P resistance are present on chromosomes 4, 5 and 9.

The inheritance of the loci conferring resistance can be explained by a variety of mechanisms. As a progeny clone with low-level resistance is present among the progeny, a simple genetic model can be predicted:

1) **Two genes confer resistance to S/P in AS (50S/P)**

   If two genes confer resistance to S/P, one mutant *dhfr* and the other present on either chromosome 4, 5 or 9, each mutant gene may confer a low-level of resistance, but show an additive effect together, resulting in the high level of resistance in AS (50S/P). The progeny of the cross between AS (50S/P) (DHFR R) and AJ (dhfr +) would show three S/P phenotypes; (i) sensitive, (ii) low-level resistance and (iii) resistant (Figure 19). This would suggest a ratio of 1:2:1 of sensitive: low-level resistant: resistant progeny. This does not fit with the 16 progeny characterised where the ratio of sensitive: low-level resistance: resistant was 5:1:10. However, it is difficult to draw conclusions from these ratios due to the small number of progeny analysed. The isolation of more progeny clones may reveal an increased proportion of progeny with low-level resistance, or alternatively, these progeny may have been selected against in either the mosquito or in mice.

2) **Two genes confer resistance, but mutant *dhfr* is epistatic to the unknown gene**

   A variation of the two-gene hypothesis is that both are involved in resistance, but that *dhfr* is epistatic to the unknown gene, i.e. the latter has no effect in the absence of mutant *dhfr*, but increases the level of resistance to S/P when it is present (Figure 18). This is similar to the proposed role of the *mdr* gene in chloroquine resistance in *P. falciparum* (Foote *et al.* 1990). Since the unknown gene does not confer resistance on its own, the ratio of sensitive: low-level resistance: resistant in
Figure 17. Diagram illustrating the two-gene hypothesis. Upper case letters indicate that the allele has been inherited from the resistant parent AS (50S/P), lower case letters or + indicate that the allele was inherited from the sensitive parent. S/P, sulfadoxine/pyrimethamine; PYR; pyrimethamine.
Figure 18. Diagram illustrating inheritance of resistance if $dhfr$ is epistatic to $R$. Upper case letters indicate that the allele has been inherited from the resistant parent AS (50S/P), lower case letters or + indicate the allele was inherited from the sensitive parent. S/P, sulfadoxine/pyrimethamine; PYR, pyrimethamine.
the progeny clones is 2:1:1. This is more in agreement with the proportions of the progeny phenotypes found in this study.

3) Two genes confer resistance in AS (50S/P), but mutant dhfr is epistatic to the unknown gene and the inheritance of an additional gene from AJ contributes to the level of resistance

The association between resistance and the inheritance of loci on chromosomes 5 and 13 from the sensitive parent can be explained by the inheritance of genes that contribute to the level of resistance observed. This model can be combined with either the two-gene hypothesis, or the two-gene epistasis hypothesis. The latter is shown in Figure 19. Here, dhfr is epistatic to the unknown gene, both of which are inherited from the AS (50S/P) parent and a third gene (A), inherited from the AJ parent contributes to the level of resistance, for example by influencing growth rate under drug pressure. This results in a ratio of 2:1:1 of sensitive: low-level resistance: resistant, with one resistant progeny genotype also inheriting a fast growth rate, contributing to the level of resistance observed. It is not possible to draw conclusions from the observed numbers of clones of each phenotype because of the small number of progeny analysed, but an excess of resistant parasites may have been selected for due to their fast growth rate.
Figure 19. Diagram illustrating inheritance of resistance if dhfr is epistatic to R, and an additional gene, A inherited from AJ contributes to the level of resistance observed. S/P, sulfadoxine/pyrimethamine; PYR; pyrimethamine.
6.4.2 The quantitative trait loci analysis

The QTL analysis assumes that the drug resistance phenotype results from the sum of the effects of the individual QTLs. It also relies on the hypothesis that the S/P resistant genes are inherited from the AS (50S/P) parent. However, the inheritance of multiple loci on chromosomes 5 and 13 from the sensitive parent AJ are significantly linked to the resistant phenotype in the progeny. It is unlikely that these are false positives as linkage is observed with both the resistance to S/P and pyrimethamine and with different doses of S/P. Markers 27 and 28 on chromosome 13 show highly significant linkage to pyrimethamine resistance. The reason for this is not clear; AJ is sensitive to both pyrimethamine and S/P.

The \textit{pppk-dhps} gene has been mapped to chromosome 13 in \textit{P. chabaudi} (Carlton et al. 1998b). No sequence polymorphisms were observed between the AS (50S/P) and AJ alleles, but it is estimated that approximately 30 bp are missing from the 5' end of the \textit{pppk} sequence. One explanation for linkage of drug-resistance to loci inherited from chromosome 13 of the sensitive parent, is that the AJ DHPS may confer innate resistance to sulfonamides. This could be due to amino acid changes in the amino terminus of PPPK that were not detected, resulting in structural changes in the DHPS. Therefore, when the AJ \textit{dhps} is inherited along with the AS (50S/P) \textit{dhfr} allele and the other gene(s) conferring resistance, a level of resistance higher than that of the resistant parent is observed. However, attempts to develop an RFLP marker to distinguish the AS and AJ \textit{dhps} alleles have been unsuccessful (Carlton 1995), suggesting that the \textit{pppk-dhps} sequences in the two parasite clones are identical.

If the inheritance of loci from AJ contributes to the level of resistance observed, it is unclear why all the resistant progeny have inherited these loci from the AJ parent and the majority of the sensitive progeny have inherited the corresponding alleles from the resistant parent. This association may be due to chance, and analysis of more progeny may break down this association. Alternatively, resistance may confer a fitness cost that is compensated by the inheritance of genes from the sensitive parent, e.g. determining growth rate. Selection on the progeny may have
then occurred either in mosquitoes, during the exo-erythrocytic stage in the mice, or during growth in the blood, such that only the resistant progeny that had inherited the loci on chromosomes 5 and 13 from the AJ parent survived to be cloned. Analysis of more progeny may break down this association.

The parasitaemias from the day 6 undrugged control were analysed to see if growth rate was linked to the loci on chromosomes 5 and 13. No AJ-type alleles segregated with fast growth rate. Therefore, the inheritance of genes determining fast growth rate from the AJ parent do not account for the linkage between drug-resistance and the inheritance of AJ-type alleles on chromosomes 5 and 13. Carlton et al. (1998a) found a weak association between growth rate between days 4 and 6 and the inheritance of loci on chromosomes 11 and 14 in the AJ x AS (3CQ) cross. No such association was found in the AJ x AS (50S/P) crosses, but the analysis used in this work looked at parasitaemia on day 6 only, so loci controlling growth between days 4 and 6 may have been overlooked.

QTL analysis also assumes that each QTL locus has an additive effect to the phenotype under investigation. For example, S/P resistance may have been caused by an additive effect of mutant genes at two loci, each able to confer low S/P resistance (Figure 17). Alternatively, one gene may be epistatic to the other, i.e. one locus has no effect on drug resistance in the absence of the other, but increases the resistance level when it is present (Figure 18). Genes with epistatic effects are much harder to map as they do not confer any low-level of resistance on their own.

The progeny clones that grow faster than the resistant parent under S/P and pyrimethamine pressure, due to the inheritance of loci from the sensitive as well as the resistant parent, are of interest as this has important implications in the field. Recombination between drug-sensitive and drug-resistant *P. falciparum* may result in drug-resistant parasites with increased virulence. Therefore, clinical failures must be treated with an effective drug, if available, as soon as possible. The AJ x AS (50S/P) cross provides the opportunity to map the loci determining growth rate/virulence. The genes *aldo-1* and *G6PD* are found in conserved syntenic groups on chromosome 13 in *P. chabaudi* and chromosome 14 in *P. falciparum* (Carlton et al. 1998b). On
completion of the genome project, it will clearly be of interest to see what other
genomes are present on chromosome 14 in *P. falciparum*.

The inheritance patterns of the markers show that extensive recombination
and reassortment has occurred among the chromosomes during meiosis. The markers
are numbered according to Carlton (1995), such that the number of crossovers per
chromosome in the AJ x AS (3CQ) cross were minimal. Su *et al.* (1999) estimate a
rate of one crossover per $1.67 \times 10^6$ bp per meiosis in the *P. falciparum* Dd2 x HB3
cross. As marker coverage is less than optimal in this work, if the gene conferring
resistance and the marker are far apart in the AJ x AS(50S/P) cross, there is a high
probability that a crossover event will separate them. As seen in the inheritance of
markers 14 and 15 on chromosome 7, recombination breaks down the association
of the AS-allele of P.23 and the AS-allele of dhfr; no linkage is seen between P.23 and
pyrimethamine or S/P resistance. Therefore, QTLs on chromosomes with few
markers may easily be overlooked. In addition, due to the number of markers tested,
false positives can be expected. The inheritance of 30 markers was analysed for
linkage to four different drug-resistant phenotypes, resulting in 120 marker contrast
analyses. With an estimated false positive rate of 5%, i.e. that there is a 5% chance
that a marker may be linked to resistance just by chance, it can be estimated that 1 in
24 QTL are false positives. Alternatively, it can be assumed that only QTLs that are
highly significant ($P < 0.001$) are genuinely linked to a quantitative trait.

A major problem with genome mapping in *P. chabaudi* is the current lack of
chromosome-specific markers. It is likely that an insufficient number of progeny
from the AJ x AS (50 S/P) cross were phenotyped and genotyped in order to identify
real QTLs. Carlton *et al.* (1998a) estimate that for a single marker 9, 17 or 32
progeny clones are required if the nearest marker is 0, 10 or 20 centiMorgans (cM)
respectively away from the gene (genetic distance between two loci is measured in
cM, with one cM equivalent to a crossover rate of 1%; a recombination rate of 1%
indicates tight linkage, whereas a frequency of 50% indicates that the loci are far
apart). Although the recombination rate in *P. chabaudi* is unknown, based on that
calculated for *P. falciparum* (Su *et al.* 1999), 16 progeny should be sufficient to
identify a single gene effect. However, when 25 markers are used, the increased probability of obtaining a false positive increases the number of progeny required to 14, 27 or 52. Therefore the ability to detect loci other than those closely linked to a genetic marker was not optimal, in particular for any loci which may be present on the larger chromosomes that only have one or two markers. Only one locus was examined on chromosome 12 and while two loci were examined on chromosome 14, they appear to be very tightly linked. Therefore, if a QTL is present on chromosome 14, recombination may have broken down the linkage between the QTL and the chromosome 14 markers and hence linkage to resistance was not detected. In addition, there are no markers for the extra-chromosomal DNA; the 6 kb mitochondrial element and the 35 kb apicoplast genome. Therefore, if resistance is linked to changes in genes present on the extra-chromosomal DNA, they will not have been detected.

Only 16 progeny were phenotyped due to the time and expense of obtaining progeny clones, phenotyping and RFLP genotyping. Lander and Botstein (1989) suggest that growing a large number of progeny, but only genotyping those with the most extreme phenotypes (selective genotyping) can minimise the number of progeny that need to be scored with genetic markers. Such methods can be applied to QTL mapping in *P. chabaudi* in future work.

### 6.4.3 The inheritance of chromosome-specific markers

The inheritance data indicates that certain genotypes were indeed selected during the crosses. Out of the polymorphic loci analysed, 58% of all loci were inherited from the AJ parent, compared to the 50:50 ratio expected if there is an equal chance of inheriting markers from either parent. The advantage that the AJ clone appears to have over AS-type parasites has been reported in other work. An excess of AJ-parental type clones was found among the progeny of the AJ x AS (30CQ) crosses (Rosario 1976a, Rosario 1976b, Carlton 1995 and the AJ x AS (30CQ) cross (Padua 1980). In the AJ x AS (3CQ) and AJ x AS (30CQ) crosses,
there was also evidence that recombinant progeny with an AJ genetic background were at an advantage (Padua 1980, Carlton 1995).

There was no excess of AJ-parentals in the progeny of the AJ x AS (50S/P) crosses in this work, but while only a relatively small number of progeny have been characterised, it is interesting to note that once again, the AJ genetic background appears to be favoured among the progeny. It was notable that all progeny inherited the AJ-type allele of $Ag_{3027}$ on chromosome 9. A possible explanation for this is that the AJ-type allele of $Ag_{3027}$ may confer a growth advantage in the mosquito or in mice. However, the AS-type allele of $Ag_{3027}$ is present in the progeny of the AJ x AS(3CQ) cross (Carlton et al. 1998a), so the reasons for the skewed inheritance in the current cross are unclear. Carlton et al. (1998a) also noted a bias towards the inheritance of AJ-type markers associated with chromosome 5 and skewed RFLP distributions have also been observed in the $P. falciparum$ crosses (Walker-Jonah et al. 1992). In the $P. falciparum$ HB3 x Dd2 cross, where 35 progeny were mapped for the inheritance of 901 markers, biased inheritance was found in seven marker groups (Su et al. 1999); most of chromosome 2 and terminal regions of chromosomes 9 and 13 showed significant excesses of Dd2 markers, while regions on chromosomes 3 and 8 carried an excess of HB3 markers. Therefore, skewed inheritance is not unusual in $Plasmodium$ genetics, at least in the comparatively few crosses carried out so far.

6.4.4 The sulfadoxine/pyrimethamine susceptibility tests

The pyrimethamine and S/P drug tests used in this work were based upon parasite recrudescence following drug treatment of 25/1.25 mg/kg S/P, 50/2.5 mg/kg S/P or 10 mg/kg pyrimethamine for 4 days. Resistant parasites appeared on or before day 8, whereas sensitive parasites were completely eliminated by these doses. All tests were repeated on at least two independent occasions to minimise the effect of environmental noise, but there were variations in parasitaemias and the day of recrudescence between repeated tests of the same clone. In particular, variations were seen in clones following treatment with 50/2.5 mg/kg S/P. In the QTL analysis,
loci showing linkage to 50/2.5 mg/kg S/P did not always show linkage to 25/1.25 mg/kg S/P and vice versa. It may be that different genes are involved in resistance to 50/2.5 mg/kg from those conferring resistance to 25/1.25 mg/kg S/P, but a more likely explanation is that the phenotyping is not optimal for the 50/2.5 mg/kg drug dose. This is apparent in the size of the variance of the mean parasitaemias on days 6, 8 and 10 following treatment with 50/2.5 mg/kg S/P. In some experiments, resistant progeny reached peak parasitaemias on day 8, while during other tests the same clones reached peak parasitaemias between days 9 and 12. This 'noise' could be overcome by improving the test, perhaps by altering the dose given, the time between treatments or the duration of the drug treatment. Alternatively, analysis could take into account only the presence or absence of parasites. For example, if three mice have day 8 parasitaemias of 0, 1 and 40% respectively, in the QTL analysis, the difference between 1 and 40% is highly significant, where as the difference between 0 and 1% may not be significant. However, *in vivo* the difference between a 0 and 1% parasitaemia is much more important. The mouse with no parasites may never develop an infection, while the mouse with a 1% parasitaemia will have a peak parasitaemia within 2 or 3 days. If the quantitative trait analysis could be modified to look at the presence or absence of parasites following drug treatment, environmental noise may be reduced, resulting in more of the variation being due to the individual QTLs.

Environmental noise has been noted by others analysing *P. chabaudi* genetic crosses (Rosario 1976a, Padua 1981, Carlton 1995). The age, sex and strain of the mouse used, as well as the host diet have all been shown to have an effect on the outcome of infection (Stevenson *et al.* 1982). To minimise the effect of these variables, all drug tests were carried out using 6-9 week old male inbred CBA/Ca mice, maintained on a constant diet with drinking water supplemented with 0.05% PABA, except where described for sulfadoxine susceptibility tests. Variation can also be due to the immune status of the individual mice (Carlton 1995). This is particularly relevant for sulfadoxine as it is a 'static' drug, i.e. it does not actually kill the parasite, but it inhibits DNA synthesis and hence replication. Its activity relies on
either the spleen or immune system to clear the developmentally arrested parasites. In addition, the antifolates and sulfonamides also act on a wide range of microorganisms. Therefore, any concurrent infection in the mice may also metabolise the drugs, decreasing the concentration of drug acting on *P. chabaudi*. While unwanted infections were kept to a minimum, their presence cannot be ruled out.

6.5 Mechanisms of sulfadoxine/pyrimethamine resistance

Which genes are conferring resistance to S/P in *P. chabaudi*? As a rule, microbial resistance to a single drug can be expected to be due to a variety of genetic mechanisms. Such mechanisms include variation in the target (modification, amplification or bypassing of the pathway) or variations in drug levels by decreased uptake, increased expulsion, compartmentalisation or failure to activate a prodrug. Any of these mechanisms could be acting independently, or in conjunction with mutations in *dhfr* and *dhps*. While pyrimethamine-resistant parasites are known have an increased requirement for PABA (Jacobs 1964), early work on sulfonamide selected *P. berghei* showed that sulfa-resistant parasites have a reduced requirement for PABA (Jaswant-Singh *et al.* 1954, Ramakrishnan *et al.* 1956). The authors suggest that sulfa-resistant parasites may have acquired the ability to synthesise PABA *de novo*. While AS (PYR) has an increased requirement for PABA and is more sensitive to sulfadoxine on a PABA-deficient diet, after selection with S/P, the S/P resistant parasites have a reduced requirement for PABA, confirming that sulfa-resistant parasites may have either acquired the ability to synthesise PABA or are able to by-pass the pathway completely. Uptake of PABA and hence sulfadoxine has been shown to be reduced in sulfadoxine resistant parasites, suggesting that resistant parasites are able to use an alternative pathway (Tan-Ariya and Brockelman 1983, Dieckmann and Jung 1986). The presence of the shikimate pathway, necessary for PABA biosynthesis, has recently been confirmed in malaria parasites (Roberts *et al.* 1998). Therefore, sulfadoxine-resistant parasites may have the ability to obtain all of their required PABA for growth from this pathway, while sensitive parasites also need to take up exogenous PABA from their host. The polymorphisms observed in
the *P. falciparum dhps* gene may be due to natural variation, as seen in *Pneumocystis carinii* (Lane et al. 1997) before sulfa-resistant *Pn. carinii* were identified (Helweg-Larsen et al. 1999) or may have been selected for by widespread use of sulfa drugs for other infections.

### 6.5.1 Folate uptake

Wang *et al.* (1999) explain the synergy between sulfadoxine and pyrimethamine by showing that pyrimethamine has a second site of action, in addition to inhibiting DHFR. Pyrimethamine blocks the utilisation of exogenous folate, which forces the parasite to use the endogenous folate pathway, rendering it extremely susceptible to sulfadoxine inhibition. The mechanism by which this occurs is unknown, but it is likely that since pyrimethamine is a structural analogue of folic acid, it inhibits either the uptake or processing of exogenous folate (Wang *et al.* 1999). A mechanism to overcome pyrimethamine inhibition of folate uptake would allow the parasite to process exogenous folate in the presence of pyrimethamine and when combined with a mutant *dhfr*, would reverse inhibition of the folate pathway. Since the parasite no longer relies on the endogenous folate pathway, and the reaction catalysed by DHPS precedes DHFR in the folate pathway, inhibition of DHPS by sulfadoxine becomes irrelevant, resulting in resistance to S/P. Therefore, while mutations in *dhps* cause resistance to sulfadoxine *in vitro* (Triglia *et al.* 1998), they may not contribute to resistance to S/P *in vivo*.

A gene influencing the use of exogenous folate has been mapped to chromosome 4 in *P. falciparum*, closely linked to *dhfr* (Wang *et al.* 1997a). The chromosome location of different genes is highly conserved between *P. falciparum* and *P. chabaudi* (Carlton *et al.* 1998b) where the *dhfr* gene is found on chromosome 7 (Cowman and Lew 1989). Therefore, a locus linked to an equivalent folate effect in *P. chabaudi*, which may modulate the response to S/P, may also be present on chromosome 7. Unfortunately, the QTL analysis is unable to detect the presence of another QTL, in addition to *dhfr*, on chromosome 7 due to poor marker coverage.
6.6 Relevance of this work to S/P resistance in *P. falciparum*

Mutant *dhfr* was found to be a major determinant of S/P resistance in *P. chabaudi*. This agrees with pharmacokinetic data showing that inhibition of *P. falciparum* DHFR by S/P treatment is more important than the inhibition of DHPS, leading to the hypothesis that mutations in *dhfr* are sufficient to confer resistance to S/P (Watkins *et al.* 1997). This work shows that *dhfr* is a major determinant, but also points to the presence of other loci. To identify the gene(s) at these loci, it will first be necessary to develop many more chromosome-specific markers. While over 800 polymorphic microsatellite markers have been developed for *P. falciparum* (Su *et al.* 1999), it has been reported that there are a limited number of microsatellites in the genomes of rodent malaria parasites (van Belkum *et al.* 1992). This has yet to be confirmed by extensive genome sequencing, but other methods for detecting polymorphisms, such as amplified fragment length polymorphisms (AFLP), a rapid method for detecting large numbers of DNA polymorphisms (Vos *et al.* 1995), and typing using anchored polyA and polyT oligonucleotides (APADs) (Su and Carlton 2000) can easily be applied to *P. chabaudi* genetic analyses. This would permit the identification of the gene(s) conferring resistance to S/P in *P. chabaudi*. With the completion of the genome project, the *P. falciparum* homologue can be rapidly identified and sequenced from field isolates to see if the mechanism of resistance in *P. falciparum* is similar to that in *P. chabaudi*. Transfection of candidate genes into drug-sensitive clones can then be carried out to confirm a causal relationship with resistance (van Dijk *et al.* 1995, Wu *et al.* 1996). Molecular analysis of several pyrimethamine resistant rodent clones has shown that the mechanisms of pyrimethamine resistance are remarkably similar to those seen in *P. falciparum*, therefore it might be expected that drug-resistant *P. chabaudi* are a good model for studying drug-resistance in *P. falciparum* (Cowman and Lew 1990, Cheng and Saul 1994).

Fine mapping of the loci for S/P resistance present on chromosomes 4, 5 and 9 may take some time, and time is running out for S/P in Africa. It is estimated that at the current rate of spread of resistance, S/P will be ineffective within five years.
(White et al. 1999). In order to develop strategies to slow the spread of resistance, it may be possible to identify these loci in *P. falciparum*, since some genes present on a single chromosome in *P. chabaudi*, were also found to be on a single chromosome in *P. falciparum* (Carlton et al. 1998b). For example, the genes *crk-4* and *csp* were both found to be located on chromosome 4 in *P. chabaudi*, and in *P. falciparum*, they were found together on chromosome 3, while *AMA-I* and *RAN* were found together on chromosome 9 in *P. chabaudi* and chromosome 11 in *P. falciparum* (Carlton et al., 1998b). The complete sequence of chromosome 3 of *P. falciparum* is already available and it is predicted to encode 215 proteins, including three transporters and at least 10 open-reading frames with unknown function (Bowman et al. 1999). To identify the genes at the putative QTL on chromosome 4 in *P. chabaudi*, candidate genes from *P. falciparum* chromosome 3 could be used to probe pulse-field gels, to identify chromosomal location, and Southern blots of genomic DNA to identify RFLPs between AS (50S/P) and AJ. If polymorphic markers are identified, the QTL analysis can be repeated. If this approach is successful, the gene underlying the quantitative trait on chromosome 3 may be identified and the *P. falciparum* homologue can be rapidly sequenced from field isolates to see if the locus also contributes to S/P resistance in *P. falciparum*.

This work suggests that the mechanism of resistance to sulfadoxine alone may be different to the mechanism of resistance to the S/P combination. If the same is true of *P. falciparum*, in vitro assays for sulfadoxine may be of little relevance in predicting S/P response. Taken together, these results suggests that caution must be taken when using polymorphisms in *dhfr* and *dhps* in epidemiological studies to estimate the frequency of S/P-resistant parasites and that all molecular surveys should be validated with some kind of in vitro test for S/P resistance.

6.7 The future of QTL mapping in *Plasmodium*

The sequencing of the *P. falciparum* genome is well underway, with chromosomes 2 and 3 finished and many of the other 12 chromosomes nearing completion (Gardner et al. 1998, Bowman et al. 1999). With the completion of the
Discussion

Chapter VI

genome sequence, positional cloning of genetic determinants, which was previously very expensive and time consuming, will become much easier. Recent advances in microarray technology and proteomics permit rapid identification in changes in gene expression in thousands of genes under different physiological conditions, such as drug exposure (Hayward et al. 2000, Yates 2000). Both methods will detect an increase in expression, such is seen in mdr duplication in mefloquine resistance, while proteomics can detect shifts in molecular weight due to insertions or deletions and in isoelectric point (pI) due to a single amino acid substitution. As the cost of genome sequencing is continually reducing, it looks increasing likely that the rodent malarias will also benefit from a sequencing project, allowing these techniques to be applied to P. chabaudi (van Lin et al. 2000). The rodent malarias have an important advantage over P. falciparum for this type of work due to the ability to select isogenic drug-resistant clones from sensitive parasites, making the detection of changes in a single mRNA or protein much easier. These high throughput technologies will revolutionise candidate gene studies, identify new drug and vaccine targets and aid our understanding of malaria biology and pathology, all of which are essential to malaria control, and may be, eventually, eradication of the disease.
Appendix I: The composition of solutions

All solutions were sterilised by autoclaving or filtration, unless otherwise stated.

Calf serum/Ringer
- 50% (v/v) calf serum
- 50% (v/v) Ringer solution
- 20 units heparin/ml mouse blood
Not sterilised and made fresh.

Citrate saline
- 0.9% (w/v) NaCl
- 1.5% (w/v) sodium citrate

Deep-freeze solution
- 28% (v/v) glycerol
- 3% sorbitol
- 0.65% (v/v) NaCl

DNA lysis solution
- 10 mM Tris
- 50 mM EDTA
- 0.1% SDS

Gel-loading buffer (5X)
- 30% (v/v) glycerol
- 70% (v/v) dH$_2$O
- 0.25% (w/v) bromophenol blue
Not sterilised

Luria-bertani (LB) medium
- 10 g bacto-typtone
- 5 g bacto-yeast extract
- 10 g NaCl
Made up to 800 ml with dH$_2$O, pH adjusted to 7.5 and solution made up to 1000 ml with dH$_2$O.

Phosphate buffered saline (PBS)
- 137 mM NaCl
- 2.7 mM KCl
- 4.3 mM Na$_2$HPO$_4$
- 1.47 mM KH$_2$PO$_4$

PCR dNTP solution (100X)
- 6.25 mM dGTP
- 6.25 mM dATP
- 6.25 mM dTTP
- 6.25 mM dCTP

Pre-hybridisation solution
- 10% (w/v) dextran sulphate
- 10% (v/v) 10% SDS
- 5.8% (w/v) NaCl
Made up to 1000 ml with dH$_2$O
Mixed at 65°C for 40 mins, autoclaved, aliquoted and stored at -20°C.

Ringer solution
- 27 mM CaCl$_2$
- 0.15 M NaCl

Saline-sodium citrate (SSC) (20X)
- 3 M NaCl
- 0.3 M sodium citrate

Sequencing gel loading buffer (5X)
- 83% (v/v) deionised formamide
- 25 mM EDTA pH 8.0
- 50 mg/ml blue dextran
Not sterilised and made fresh.
Tris/borate/EDTA (TBE) buffer (1X)
- 0.09 M Tris
- 0.09 M boric acid
- 0.002 M EDTA

Tris/EDTA (TE) buffer
- 10 mM Tris-HCl pH 8.0
- 1 mM EDTA pH 8.0
Appendix II: The Student's $t$ test

The Student's $t$-test is used for comparing the means of two small samples. The mean difference between the two samples is divided by the standard error of the difference, which takes into account the small sample size. The answer is then compared with the distribution of $t$ at the appropriate degrees of freedom, where $t$ equals:

$$
t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2}{n_1 + n_2 - 2}}} \sqrt{\frac{n_1 + n_2}{n_1n_2}}
$$

where $\bar{x} = \frac{\sum x}{n}$, $s = \sqrt{\frac{\sum (x-\bar{x})^2}{n-1}}$ and the degrees of freedom are $(n_1 + n_2) - 2$.

For example, the value for $t$ for the differences between mean log parasitaemias of grouped progeny clones inheriting the AS (50S/P) versus the AJ allele of marker 1, on day 6 following treatment with 25/1.25 mg/mg S/P, was calculated as follows:

Table 15. Mean parasitaemias of progeny grouped according to the inheritance of marker 1

<table>
<thead>
<tr>
<th>Mean day 6 parasitaemias following 25/1.25 mg/kg S/P</th>
<th>Progeny inheriting the AS (50S/P)-type allele</th>
<th>Log$_{10}$ $^a$ (mean +1)</th>
<th>Progeny inheriting the AJ-type allele</th>
<th>Log$_{10}$ $^a$ (mean +1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9</td>
<td>0.46</td>
<td>0.0</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>9.6</td>
<td>1.02</td>
<td>10.0</td>
<td>1.04</td>
<td>1.04</td>
</tr>
<tr>
<td>0.0</td>
<td>0.00</td>
<td>0.2</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>23.0</td>
<td>1.38</td>
<td>19.9</td>
<td>1.32</td>
<td>1.32</td>
</tr>
<tr>
<td>0.0</td>
<td>0.00</td>
<td>14.6</td>
<td>1.19</td>
<td>1.19</td>
</tr>
<tr>
<td>0.0</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39.2</td>
<td>1.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Sigma 4.47$</td>
<td></td>
<td>$\Sigma 3.62$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\bar{x}_1 = 0.64$</td>
<td></td>
<td>$\bar{x}_2 = 0.72$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$s_1 = 0.69$</td>
<td></td>
<td>$s_2 = 0.64$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$s_1^2 = 0.48$</td>
<td></td>
<td>$s_2^2 = 0.41$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The data were normalised by taking log$_{10}$ transformations
Substituting in the formula for $t$:

$$t = \frac{(0.64 - 0.72)}{\sqrt{\frac{(7-1) 0.48 + (5-1) 0.41}{(7+5-2)} \frac{7+5}{7 \times 5}}}$$

$$= \frac{-0.08}{\sqrt{\frac{2.88 + 1.64}{10}}} \times 0.34$$

$$= \frac{-0.08}{\sqrt{0.45 \times 0.34}} = \frac{-0.08}{0.392}$$

$$= -0.21$$

The values of -0.08 and 0.392 in the final step are the difference between the mean log parasitaemia and the standard error of the difference respectively. Consulting a table of the distribution of $t$, a value of 0.21 is lower than the tabulated value of 1.812 at $P = 0.05$ for 10 (12-2) degrees of freedom. Therefore, it can be concluded that there is no statistically significant difference between the means and that marker 1 is not linked to a locus conferring resistance to 25/1.25 mg/kg S/P.
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