

# The genomics of malaria infection

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**Malaria research is now dominated by information flowing from the genome sequencing projects and the associated transcriptome- and proteome-mapping projects. As more species are sequenced, comparative and phylogenetic comparisons are improving the quality of gene finding, and are providing various approaches to the identification of genes important to parasite biology and the pathogenesis of disease. We are still in the early days of exploiting these data in a systematic way and the sheer volume of data presents daunting challenges. This article reviews the progress in using this genomic information and discusses opportunities for other approaches.**

The malaria genome has completely permeated our thinking about malaria and it is a shock to realize that the first draft of the genome has been available only since October 2002. Malaria parasites contain 14 chromosomes and, at the time of the release of the *Plasmodium falciparum* genome, the sequence of seven of these chromosomes were complete and the remaining seven contained between three and 27 gaps [1]. The total genome size of the published first draft was 22.81 Mb which was smaller than the size derived from optical-mapping measurements (24.15 Mb) [2]. The remaining unmapped sequences include several well-characterized malaria proteins such as histidine-rich protein 2 (HRP-2) and rhoptry-associated membrane antigen (RAMA) [3,4]. There are >5300 predicted open-reading frames (ORFs), and ~60% of these code for proteins of unknown function. The initial *P. falciparum* genome sequencing was performed by a consortium of laboratories at the Wellcome Trust Sanger Institute (WTSI; <http://www.sanger.ac.uk>), The Institute for Genomics Research (TIGR; <http://www.tigr.org>) and Stanford University (<http://www.stanford.edu>). TIGR and the WTSI have continued efforts to close remaining gaps in the *P. falciparum* sequence, and to sequence other malaria parasites including the commonly used rodent malaria parasites and primate malaria parasites. The regions that remain are those that are relatively difficult to work with using current cloning and sequencing methodologies, and we might well be reaching a point of diminishing returns. A snapshot of the current state of *Plasmodium* genome projects is provided in Table 1 and shows that sequences providing coverage of varying degrees is available for several parasites species,

predominantly human or rodent hosts. The completed *P. falciparum* sequence will be useful as a scaffold on which the partial sequences of other species are assembled. In turn, comparison of the genome sequences from other species will be useful in refining gene models of *P. falciparum* genes and this together with the information from full-length complementary DNA (cDNA) sequences of the full-malaria project [5] will improve the quality of the current *falciparum* genome annotation. Cross-species analysis reveals that ~60% of genes are shared across all *Plasmodium* species. These common genes appear to occur in arrangements where synteny among species is largely conserved. As might be expected, the sites where synteny breaks down corresponds to the location of species-specific genes but, more importantly, the genes at such sites are often involved in host-parasite interactions. Examples include genes that encode merozoite surface protein-2 (MSP-2) [6] and a putative erythrocyte-binding protein-1 (EBL-1). The previously known site of synteny breakdown and species-specific gene clustering was the telomeres, where genes involved in antigenic variation, immune evasion and host cell adhesion had been identified, including the *P. falciparum* *var*, *rifin* and *stevor* genes [1], the *Plasmodium vivax* *vir* genes [7] and the *Plasmodium yoelii* *yir* genes [8]. The first attempt to build a genome sequence for another parasite based on syntenic approaches was that for *P. yoelii yoelii* [8] and, more recently, this approach has been extended by Taco Kooij *et al.* (Leiden University Medical Centre, Leiden, The Netherlands) to map the genomes of all three rodent parasites of malaria, *Plasmodium chabaudi*, *P. yoelii* and *P. berghei*. The data presented at the 2003 Woods Hole Molecular Parasitology Conference described maps that revealed a complex mosaic of syntenic regions reshuffled among chromosomes and separated by breaks in which species-specific genes were frequently found\*

The initial application of genome information to malaria research has been in assisting various researchers to identify genes similar to sequences of interest or for which they already possessed partial information. Previous examples included the identification of: (i) 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DOXPR) gene, which is involved in isoprenoid biosynthesis and whose product is inhibited by the antibiotic fosmidomycin, thus leading to death of malaria parasites *in vitro* and *in vivo* [9]; (ii) the enoyl-acyl-carrier protein (ACP) reductase

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Available online 18 October 2004

\* Molecular Parasitology Meeting XIV held 14–18 September 2003, at Marine Biological Laboratory, Woods Hole, MA, USA.

**Table 1. Current and planned genome sequencing efforts in malaria<sup>a</sup>**

Organism	Status
<i>Plasmodium falciparum</i> 3D7 isolate	First draft published October 2002
<i>P. falciparum</i> virulent field isolate	3x sequencing in progress
<i>Plasmodium vivax</i> Salvador I isolate	Full sequencing in progress
<i>Plasmodium berghei</i>	8x shotgun coverage
<i>Plasmodium yoelii yoelii</i> 17XNL line	5.6x coverage published October 2002
<i>Plasmodium chabaudi</i>	8x shotgun complete
<i>Plasmodium knowlesi</i>	8x shotgun in progress
<i>Plasmodium reichenowi</i>	3x shotgun in progress
<i>Plasmodium gallinaceum</i>	3x shotgun in progress

<sup>a</sup>In addition to whole genome sequencing, various full-length complementary DNA, expressed-sequence tag and GSS (genome survey sequences) sequencing surveys have been carried out.

(FABI), whose gene product is inhibited by the antiseptic triclosan *in vitro* and *in vivo* [10]; and (iii) several paralogs of genes involved in red blood cell invasion, including erythrocyte-binding antigen 175 (EBA-175) and serine-rich antigen (SERA), and proteins containing epidermal-growth factor (EGF)-like domains similar to these found in merozoite surface protein 1 (MSP-1), MSP-4 and MSP-5, and genes similar to the *P. vivax* reticulocyte-binding protein (EBP) [11]. Each of these additional genes identified with the assistance of genome information have, in turn, proved of biological interest, and most seem also to share involvement in various phases of invasion. The continued use of the genome in this way was evident at the Molecular Approaches to Malaria (MAM) meeting held 1–5 February 2004 in Lorne, Australia, where work of this nature included: (i) identification and characterization of the function of falcipain paralogs, a cysteine protease family involved in hemoglobin degradation (Phil Rosenthal, University of California San Francisco, CA, USA); (ii) characterization of a *Plasmodium* gene with similarity to the red blood cell protein stomatin that is released from rhoptries during invasion and inserted into raft-rich vacuoles (Kasturi Haldar, Northwestern University, Chicago, IL, USA); (iii) identification of asexual-stage-expressed genes containing specific domains similar to those seen in the adhesive protein of sporozoites thrombospondin-related adhesive protein (TRAP) (Lawrence Bergman, Drexel University, Philadelphia, PA, USA; Belinda Morahan, Monash University, Melbourne, Australia). Bergman *et al.* showed that this TRAP-like protein expressed in mature stages of parasites contained a cytoplasmic tail with motifs that interacted with aldolase, suggesting it might be involved in the function of the molecular motor that drives the merozoite into the red blood cell.

However, there have been few published studies that examine genome sequences as a whole to identify general properties of the genome. Ouzounis *et al.* have searched for proteins involved in the control of gene expression in *P. falciparum* [12]. Although the basic machinery of transcription initiation is present and similar to that found in the crown eukaryotic group, there is a surprising paucity of transcriptional regulators and transcriptional-

associated proteins. Such proteins in other crown eukaryotes are often encoded by large families of paralogs, but these transcription factors are virtually absent in *P. falciparum* and a much lower proportion of the genome is devoted to encoding these types of proteins. One striking exception to this picture is the CCCH-type zinc-finger proteins, which function in regulating the stability and localization of messenger RNA (mRNA). These proteins are twice as common as in other eukaryotes and this might mean that post-transcriptional removal of mRNA is a prominent method of gene regulation [12]. Sequence polymorphism has been a subject of interest because of its implications for immune evasion and one study looked at single nucleotide polymorphisms (SNP) across chromosome two of *P. falciparum* using hybridization to an array of synthetic oligonucleotide 25-mers [13]. Genomic DNA from five well-known laboratory isolates originating from different geographical areas was compared and revealed that genetic variation was not spread randomly across gene-coding regions, but was concentrated in genes encoding membrane proteins such as MSP-2 and MSP-4. This suggests selection for variation in these genes, with the host immune system being the most likely selection pressure. Richard Carter (University of Edinburgh, UK) described an ambitious program using linkage approaches to map various phenotypic characteristics in *P. chabaudi* infection of mice, such as the targets of strain-specific immunity and drug-resistance markers. The method, called linkage group selection, involves the use of two parasite strains that differ in a phenotype under study, conventional crossing in mice to produce a large number of progeny and then selection for a phenotype of interest. Random molecular markers are used to map characteristics by reference to the genome sequence and the markers that are selected for or against are identified. This method demonstrates that MSP-1 is the major target of infection-induced strain-specific immunity and accords with previous work demonstrating that reactivity to MSP-1 is a major component of the inhibitory action of sera from immune humans [14]. The method has been further validated by examining crosses of pyrimethamine-resistant and -susceptible lines, which demonstrated that not only the known locus dihydrofolate reductase is linked to resistance, but that it is also strongly selected against in the absence of drug pressure. This suggests that removal of this drug from areas where resistance is common for a period of time should see reappearance of susceptible strains and might be part of the explanation for the reappearance of susceptible parasites in areas that use insecticide-treated bednets [15].

### Functional genomic methodologies

The availability of a genome sequence opens the way for deployment of several functional genomics methodologies including transcriptome, proteome, metabolome and structural biological analyses. Advances in proteomic analysis of *Plasmodium* are detailed elsewhere in this Special Issue by D. Carucci, whereas the difficulties in expressing *Plasmodium* proteins in heterologous systems has delayed the progress of structural genomics projects. Microarray methods have been more amenable to study

[16,17], and updates were presented at the meeting by the two major groups in the area. Although the two groups used different oligonucleotide arrays for their experiments, a panel of 367 000 25-mer probes from both coding and non-coding sequence (E. Winzeler, Scripps Research Institute, San Diego, USA) and 7642 70-mers from the 5400 predicted ORFs (Z. Bozdech), and different analysis methods, the overall conclusions of the experiments are similar. There appears to be a fairly rigid program for transcription employed by the parasites, and genes with similar functional roles are transcribed at similar times. This provides important predictions about the more than 3000 ORFs for which no particular function has been ascribed. Over 87% of predicted genes have been shown to be transcribed in the life cycle stages so far examined including the asexual stages, salivary gland sporozoites and mature gametocyte stages [16,17]. These analyses will be extended using more up to date gene models and the remaining genes that will appear in the finished genome. Building on these initial studies, further microarray studies were reported at MAM 2004. Francesco Silvestrini (University of California at San Francisco) compared two parasite clones that differed in their ability to develop gametocytes. mRNA extracted from these two lines cultured under conditions that induced gametocytogenesis were compared on the DeRisi array, and several genes were found to be upregulated in the line capable of forming gametocytes including *Pfs16* and *Pfg27*. Further analysis of upregulated genes of unknown function could reveal their role in this process. Further data on stage-specific transcription was provided by Qian Wang (New York University, NY, USA) who obtained mRNA from purified exo-erythrocytic stages of *P. yoelii*, constructed a cDNA library and performed random expressed-sequencing tag (EST) sequencing of >1700 clones that corresponded to >650 ORFs. Their analysis revealed a shift in transcriptional pattern from sporozoites-like to asexual-stage-like with few (if any) genes expressed that are not found in either stage.

A complementary approach to the definition of the transcriptome is provided by serial analysis of gene expression (SAGE), which provides information on both mRNA repertoire and transcript abundance. Abundant transcripts in asexual-stage parasites included genes on the mitochondria, enzymes involved in glucose metabolism and several invasion-related genes including MSP-3, MSP-4 and several rhoptry proteins [18]. SAGE analysis revealed that 12–17% of nuclear genes gave rise to abundant and readily detectable anti-sense transcripts [18,19], and that sense and anti-sense abundance from single loci were inversely related [19]. This raises the possibility that anti-sense transcripts might be involved in gene regulation but, at present, no experimental proof supports this idea.

The availability of these large datasets from genomic and functional genomics studies provides major informatics challenges to all areas of biology including malaria. Database projects including Flybase, Wormbase and EcoCyc (Table 2) service the data derived from studies of *Drosophila*, *Caenorhabditis elegans* and *Escherichia coli*, respectively. Within the malaria field, the major database

is PlasmoDB, a project initiated with the support of the Burroughs Wellcome Fund. A summary of other malaria information resources are listed in Table 2. David Roos (University of Pennsylvania) presented a summary of the current state of the *Plasmodium* genome resource, and a roadmap for future progress at MAM 2004. Contents include the 'first-draft' *P. falciparum* genome sequence, the partial genome sequences of additional *Plasmodium* species, the microarray results listed above and proteomics analysis of several life cycle stages. The major challenges for PlasmoDB, as for all the genome databases, are improved methods of integrating diverse data types together with better user interfaces and visualization tools to assist users in searching and data browsing. PlasmoDB was built using semi-automated methods of data capture and the quality of annotation reflects this origin. Other views of the genome are accessible on the web including GeneDB, an alternative approach to that provided by PlasmoDB. However, GeneDB is dependent on the quality of primary annotation and gene models. In this issue, A.E. Berry *et al.* outlines plans to improve annotation and curation of *Plasmodium* data by recruitment of the malaria community to this task. It is an important goal that deserves our best efforts.

At least three groups have tried to take the genome sequence and construct metabolic descriptions of the organism. The group at the Kyoto Encyclopedia of Genes and Genomes (KEGG) have attempted to reconstruct the metabolic pathways of the parasite by identifying homologs of genes with known enzymatic functions in other organisms. Yeh *et al.* have taken this approach further in PlasmoCyc [20], a pathway and genome database based on EcoCyc. The authors have attempted to place *Plasmodium* genes in pathways and to identify chokepoints, which are unique enzymes involved in production or consumption of substrates. They found 216 chokepoints that might represent new drug targets and, of these, 30 were proteins that did not have a homolog in the human genome. Finally, there are unique parasite processes that are important for activities such as invasion, protein trafficking and cytoadherence that do not map well into databases that focus on metabolism. The malaria parasite metabolic database attempts to collate information on these processes but, as yet, there are no good methods to make this information searchable or predictive.

At MAM 2004, two groups presented bioinformatics analyses that tried to identify gene sets that shared some sequence feature indicative of either a common location or a common function. Geoff McFadden (University of Melbourne, Australia) identified proteins exported to the apicoplast, the non-photosynthesizing chloroplast-like organelle essential for growth of apicomplexan parasites. Using features of homologues of known chloroplast proteins, which had been confirmed to target to the apicoplast, motifs including the presence of a conventional signal sequence and a positively charged sequence element present in each transit peptide was identified. This signature identifies ~500 nuclear-encoded proteins from *Plasmodium* spp. as potential apicoplast proteins. These candidates were used to construct a metabolic pathway map for the apicoplast and identified this

**Table 2. *Plasmodium* genomics, genetics and biology information resources<sup>a</sup>**

Information resource	URL
PlasmoDB	<a href="http://www.PlasmoDB.org">http://www.PlasmoDB.org</a>
<i>P. falciparum</i> GeneDB site	<a href="http://www.genedb.org/genedb/malaria/index.jsp">http://www.genedb.org/genedb/malaria/index.jsp</a>
TIGR parasites database	<a href="http://www.tigr.org/tdb/parasites/">http://www.tigr.org/tdb/parasites/</a>
Sanger Institute parasite genomes project	<a href="http://www.sanger.ac.uk/Projects/Protozoa/">http://www.sanger.ac.uk/Projects/Protozoa/</a>
Sanger institute <i>P. falciparum</i> genome project	<a href="http://www.sanger.ac.uk/Projects/P_falciparum/">http://www.sanger.ac.uk/Projects/P_falciparum/</a>
Malaria full-length complementary DNA project	<a href="http://fullmal.ims.u-tokyo.ac.jp/">http://fullmal.ims.u-tokyo.ac.jp/</a>
KEGG implementation of the <i>P. falciparum</i> metabolic pathways	<a href="http://www.genome.ad.jp/dbget-bin/www_bfind?p.falciparum">http://www.genome.ad.jp/dbget-bin/www_bfind?p.falciparum</a>
PlasmoCyc genome pathway database	<a href="http://plasmocyc.stanford.edu/">http://plasmocyc.stanford.edu/</a>
Malaria parasite metabolic pathways	<a href="http://sites.huji.ac.il/malaria/">http://sites.huji.ac.il/malaria/</a>
WHO/TDR malaria database	<a href="http://www.who.int/maldb/">http://www.who.int/maldb/</a>
NCBI malaria genetics and genomics	<a href="http://www.ncbi.nlm.nih.gov/projects/Malaria/">http://www.ncbi.nlm.nih.gov/projects/Malaria/</a>
DeRisis laboratory malaria transcriptome database	<a href="http://malaria.ucsf.edu/index.php">http://malaria.ucsf.edu/index.php</a>
Structural genomics of parasitic protozoa consortium	<a href="http://depts.washington.edu/sgrp">http://depts.washington.edu/sgrp</a>

<sup>a</sup>Further links can be viewed at <http://www.plasmodb.org/links.shtml>. Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; TIGR, The Genomic Institute for Research.

organelle as the site of synthesis of heme, fatty acids and isoprene. Kieran Kirk (Australian National University, Canberra, Australia) set out to identify the repertoire of transport molecules present in the genome. Using homology alone, only a limited number of transporters had been identified. By using the arrangement of hydrophobic regions within transporter families as the search terms, Kirk *et al.* were able to identify >70 proteins with characteristics of transporters including transporters of amino acids and vitamins. A family of novel transporters with unknown specificity was also revealed. In addition, analysis of the *P. falciparum* chloroquine-resistance transporter (*PfCRT*) gene [21] showed that it belonged to a previously described subfamily of drug and metabolite transporters.

### Three species challenge

Much is made of the fact that for malaria infection of humans, genome sequences are available not only for the parasite, but also for the vertebrate and invertebrate hosts. Although, in theory, this is a major opportunity to glean insights into the biology and co-evolution of these organisms, at present, there is no consensus as to what analytical methods will be worth deploying. The identification of similar sequences, and the presence or absence of orthologs across all three species have not been particularly informative, and the sheer volume of data when combined with functional genomics results are daunting. Several gene variants are well known to confer resistance to malaria on the human host including hemoglobin S, hemoglobin C, and deletions in band 3 in Southeast Asian ovalocytosis and glucose-6 phosphate dehydrogenase. Previous studies have identified polymorphism in several host genes, including receptors for cytoadherent cells present on endothelial cells and red cells and promoter regions of cytokine genes, and correlated these with receptor binding or clinical outcome to malaria [22–25]. These studies have focused on genes already identified as partaking in the host–parasite relationship. An interesting study might be the performance of SNP surveys of humans living in areas of high malaria endemicity to identify genes showing types of polymorphism not seen in areas where malaria is absent. This might lead to identification of novel genes not currently implicated in host responses to malaria or in malaria pathogenesis. Our

understanding of the biology of mosquitoes will be enhanced by access to the *Anopheles gambiae* genome [26] and we are already able to identify genes important for survival of the parasite within the vector [27]. Perhaps in turn this will lead to identification of parasite genes that interact with the mosquito host factors and these parasite genes might be amenable to dysregulation by drugs or vaccines.

### Transcriptional analysis of the infected host

Another area where genomic analysis of the vertebrate host has been informative has been in microarray examination of the changes involved during infection, and the genes associated with resistance to infection. The most extensive reported study is that of the *P. berghei* ANKA infection of laboratory mice, a model of cerebral malaria. Serial microarray comparisons were performed on mRNA extracted from brains, spleen and bone marrow of infected mice taken at Days 1, 3 and 5 post-infection [28], and revealed changed regulation of 600 genes in the spleen and 400 genes in the brain. These changes were indicative of suppression of erythropoiesis and upregulation of genes that controlled glycolysis, particularly lactate dehydrogenase. The inflammatory response to infection was dominated by changes to interferon- $\gamma$  regulated genes, cytokines and marked upregulation of serum amyloid A. These changes provide possible mechanisms for the appearance in malaria infection of anemia and hypoglycaemia. These studies have now been extended to malaria-infected humans. Chris Ockenhouse (Walter Reed Army Institute of Research, Silver Spring, USA) described his work with microarray using blood samples taken from subjects during early stages of *P. falciparum* infection. Pathways with altered transcription included those involved in macrophage activation, immune responsiveness, signaling molecules and genes involved in metabolism of heavy metals. In related studies using blood taken from children with mild, moderate or severe malaria, Jürgen Kun (Institute for Tropical Medicine, Tübingen, Germany) identified 300 genes with altered patterns of transcription. Pathways with altered regulation included those involved in myeloid proliferation indicative of a T helper cell type 1 (Th1) response, the innate immune response and reticulocyte formation. Different patterns could be detected in children with

different types of disease, and a group of 20 genes could be used to differentiate between mild and severe malaria. Further work is needed to determine whether this difference in pattern of transcription occurs before clinical syndromes are established, allowing identification of the high-risk patient. Comparisons between these three studies have not yet been performed to determine how consistent these gene changes are between hosts and epidemiological situations. Finally, linkage studies have been performed to identify genes involved in resistance to *P. chabaudi* infection [29,30]. Mapping experiments had identified the char 1 locus, a region on the distal end of chromosome nine in the mouse that controlled resistance to infection and peak parasitaemia levels. Originally mapped to a region of 3.7 Mb encompassing 60 genes, Simon Foote (Walter and Eliza Hall Institute, Melbourne, Australia) have bred congenic mice that enable mapping of the site of char1 to 100 kb. The effect of char1 appears to be to reduce the capacity of parasites to invade but it might also be involved in capacity to ameliorate the severe anaemia that develops in infected mice.

### Perspective

Clearly, we are still in the early phase of functional genomic analysis of malaria. It is notable that many of these studies have confirmed previous conclusions by workers striving in the Stygian darkness that characterized the pre-genomic era. Future results will provide important new insights into malaria biology and, hopefully, new targets that might allow new approaches to the control of malaria.

### Acknowledgements

We acknowledge support of the National Institutes of Health DK-32094, the Australian National Health and Medical Research Council and the Burroughs Wellcome Fund. R.L.C. is an international fellow of the Howard Hughes Medical Institute.

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