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# Yeast dihydroorotate dehydrogenase as a new selectable marker for *Plasmodium falciparum* transfection

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ABSTRACT

Genetic manipulation of *Plasmodium falciparum* in culture through transfection has provided numerous insights into the molecular and cell biology of this parasite. The procedure is rather cumbersome, and is limited by the number of drug-resistant markers that can be used for selecting transfected parasites. Here we report a new selectable marker that could allow multiple transfections. We have taken advantage of our finding that a critical function of the mitochondrial electron transport chain (mtETC) in the erythrocytic stages of *P. falciparum* is the regeneration of ubiquinone as co-substrate of dihydroorotate dehydrogenase (DHODH), and that transgenic *P. falciparum* expressing ubiquinone-independent DHODH from yeast (y*DHODH*) are resistant to all mtETC inhibitors. We assessed the possibility of using y*DHODH* as a positive selectable marker for transfections of *P. falciparum*, including its use in gene disruption strategies. We constructed a transfection vector designed for gene disruption, termed pUF-1, containing the y*DHODH* gene as the positive selection marker in combination with a previously described fused yeast cytosine deaminase-uracil phosphoribosyl transferase gene as a negative selection marker. Transfection of the D10 strain followed by selection with atovaquone yielded positively selected parasites containing the plasmid, demonstrating that y*DHODH* can be used as a selective marker. Atovaquone, however, could not be used for such selection with the Dd2 strain of *P. falciparum*. On the other hand, we demonstrated that y*DHODH* transgenic parasites could be selected in both strains by *Plasmodium* DHODH-specific triazolopyrimidine-based inhibitors. Thus, selection with DHODH inhibitors was superior in that it successfully selected transgenic Dd2 parasites, as well as yielded transgenic parasites after a shorter period of selection. As a proof of concept, we have successfully disrupted the type II vacuolar proton-pumping pyrophosphatase gene (*PfVP2*) in *P. falciparum* by double crossover recombination, showing that this gene is not essential for the survival of blood stage parasites.


## 1. Introduction

Molecular genetic investigations of malaria parasites hold promise for a deeper understanding of parasite biology to guide treatment and control of malaria. Although genome sequences of several parasite species have been completed [1–4], application of these advances is often hampered by difficulties in carrying out biochemical and genetic studies. Successful transfection of *Plasmodium falciparum* has provided an invaluable means to study a number of important biological properties of this parasite such as drug resistance and cytoadherence. The technique can also be used to gain insight into metabolic pathways, protein trafficking and parasite differentiation. Transfection is, however, a highly demanding technique in *P. falciparum*, with a very low efficiency. The technique has also been limited by the number of selectable markers. For genetic transformations of *P. falciparum* only a few positive

*Abbreviations:* *bsd*, blasticidin S deaminase; *DHFR*, dihydrofolate reductase; DHODH, dihydroorotate dehydrogenase; DSM1, 5-methyl[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)naphthalen-2-ylamine; DSM74, 5-methyl[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)(4-trifluoromethylphenyl)amine; *FCU*, yeast (*Saccharomyces cerevisiae*) cytosine deaminase-uracil phosphoribosyl transferase fusion gene; mtETC, mitochondrial electron transport chain; PfDHODH, *Plasmodium falciparum* DHODH; UTR, untranslated region (of DNA); V-PPase, proton-pumping vacuolar-type pyrophosphatase; y*DHODH*, yeast DHODH (Saccharomyces Genome Database: Ura1p).

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selection markers are available—primarily human dihydrofolate reductase (*DHFR*), fungal blasticidin S deaminase (*bsd*) and bacterial neomycin phosphotransferase (*neo*) [5,6]. The majority of selections in *P. falciparum* have been carried out using human *DHFR* selected with WR99210, which has a large selection window (an $IC_{50}$ increase of about 4000 fold was reported upon transfection with a plasmid containing human *DHFR* [5]). *bsd* plus blasticidin and *neo* plus G418 were reported to have much narrower selection windows, with a 10 fold or less change in $IC_{90}$ (for low copy number results) [6]. In addition, it was recently reported that growth under blasticidin pressure results in the selection of resistant transport mutants in at least one strain of *P. falciparum* [7]. Nevertheless, reported use of *bsd* has been increasing, reflecting the mounting need for multi-step molecular genetic manipulations. The dearth of selectable markers limits our ability to carry out gene disruptions, complementation of mutants, and allelic replacement experiments to select specific mutants in important biological pathways. In addition, availability of additional selectable markers would be essential for the generation of double and triple gene knockouts to study the role of multiple proteins or pathways that may have redundant or alternate functions. Such markers would also be important for strain constructions requiring multiple genomic alterations.

We have previously shown that a critical role of the mitochondrial electron transport chain (mtETC) in blood-stage *P. falciparum* is to regenerate ubiquinone, an oxidative co-substrate of dihydroorotate dehydrogenase (DHODH). DHODH, the fourth enzyme in the pyrimidine biosynthetic pathway, is essential for malaria parasites' survival because they cannot salvage pyrimidines [8]. Transgenic *P. falciparum* parasites expressing *Saccharomyces cerevisiae* DHODH (y*DHODH*), which utilizes fumarate as an electron acceptor, were completely resistant to inhibitors of the mtETC, including the antimalarial drug atovaquone, which inhibits the cytochrome $bc_1$ complex (Complex III) [9]. In this prior work, y*DHODH* was expressed from an extrachromosomal plasmid that had been selected using the human *DHFR* marker and the inhibitor WR99210. Further investigation of these transgenic parasites revealed that they could be propagated in the presence of atovaquone during long-term culture (without any further requirement for WR99210). In this study, we report the use of y*DHODH* as a new selectable marker, which confers resistance to atovaquone in specific strains and, more broadly, to triazolopyrimidine-based, parasite-specific DHODH inhibitors [10]. As a proof of concept, we used the new plasmid vector, designated pUF-1, for successful double cross-over disruption of the gene encoding type II vacuolar proton-pumping pyrophosphatase (*PfVP2*) [11], thereby showing its non-essentiality in blood stages of the parasite.

## 2. Materials and methods

### 2.1. Parasite lines, culture, transfection and selection methods

*P. falciparum* D10 strain parasites were used for most experiments in this study, unless mentioned otherwise. Parasites were cultured in RPMI 1640 medium supplemented with 300 mg/L L-glutamine, 10 mg/L hypoxanthine (Sigma), 15 mM HEPES (Hyclone), 0.225% $NaHCO_3$ (Cellgro), 0.5% Albumax II (InVitrogen), and 50 μg/ml gentamicin (Cellgro), under a gas mixture of 5% $O_2$, 5% $CO_2$, and 90% $N_2$. Parasites were transfected as previously described [5]. Briefly, 0.2 cm electroporation cuvettes were loaded with 0.3 ml of 50% parasitized erythrocytes and 50 μg of plasmid DNA in incomplete cytomix solution. Electroporation conditions were 0.31 kV and 960 μFd. For stable transfections, parasites were cultured in media containing 100 nM atovaquone, or 1.5 μM compound DSM1 [10] for positive selection. For comparison of selection efficiency, the transfection was done as described above, the resulting culture was divided, and each half was selected with either atovaquone or DSM1.

### 2.2. Knockout vector (pUF-1) construction

The DNA fragment containing *bsd* and *P. falciparum* calmodulin promoter (CAM 5′ UTR) was excised from pCC4 [12] with the restriction enzymes *Spe*I and *Hin*dIII and cloned into the pBluescript plasmid, producing pBluescript-*bsd*. The *S. cerevisiae* DHODH gene (y*DHODH*) was amplified from the pHHyDHOD-GFP vector [9] by forward and reverse primers containing *Bam*HI and *Hin*dIII restriction sites, respectively. The PCR product was digested with *Bam*HI and *Hin*dIII and cloned into pBluescript-*bsd* digested with the same set of enzymes, yielding pBluescript-y*DHODH*. The sequence of the y*DHODH* gene was confirmed by automated sequencing. Since *Eco*RI and *Nco*I restriction sites were present both in the y*DHODH* gene and also in one of the multiple cloning sites of pCC4, we eliminated these restriction sites in the y*DHODH* gene by silent mutagenesis using the QuikChange protocol (Stratagene). The primer sequences used for PCR amplification and restriction site mutations are listed in Table 1. The DNA fragment of pBluescript-y*DHODH** containing mutated y*DHODH* along with the CAM 5′ UTR was excised with *Spe*I and *Hin*dIII restriction enzymes and cloned into the pCC4 plasmid digested with the same restriction sites, yielding the new plasmid pUF-1 (Fig. 1).

### 2.3. Construction of a specific PfVP2 knockout vector

The type II vacuolar pyrophosphatase gene knockout plasmid, pUF1ΔVP2, was constructed by amplifying 5′ and 3′ regions from the *PfVP2* gene, each ~1 kb in size, from *P. falciparum* genomic DNA using specific primers listed in Table 1. Both these regions originated within the gene's coding region and would result in a deletion of 417 bp within the gene under a double crossover recombination event (Fig. 1B). The amplicons were cloned into the pBluescript SK plasmid, sequence-verified, and inserted into the pUF-1 cloning sites surrounding the y*DHODH* cassette. The presence of the inserts was confirmed by restriction enzyme digestion with *Eco*RI and *Nco*I for the 5′ insert and *Spe*I and *Sac*II for the 3′ insert.

### 2.4. Southern blot

DNA obtained from the wild type and knock-out parasites was digested with the enzymes *Eco*RI and *Hin*dIII, and electrophoresed in a 0.8% agarose gel. Southern blot transfer and hybridization was carried out using a standard protocol [13]. Briefly, the DNA was transferred onto GeneScreen Plus membrane (Perkin Elmer) by capillary blotting. One of the homologous regions, the 3′ insert, was amplified from *P. falciparum* D10 genomic DNA and labeled with dATP-$\alpha^{32}$P using Prime It II random primer labeling kit (Stratagene). The probe was hybridized to the membrane overnight (42 °C) and washed with 2× SSC containing 1% SDS (60 °C). X-ray film was exposed to the blot for 24 h, then developed and analyzed.

### 2.5. Chemical synthesis

Triazolopyrimidine inhibitors of *P. falciparum* DHODH, 5-methyl[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)naphthalen-2-ylamine (DSM1), and 5-methyl[1,2,4]triazolo[1,5-*a*]pyrimidin-7-yl)(4-trifluoromethylphenyl)amine (DSM74) were synthesized as described previously [10,14].