Variation among Plasmodium falciparum Strains in Their Reliance on Mitochondrial Electron Transport Chain Function†‡

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With greater than 3 billion people at risk, ~240 million people infected, and nearly 1 million deaths in 2008, malaria remains one of the world’s leading killers (38). Plasmodium falciparum is the most lethal among the species causing human infections. Asexual blood-stage parasites of P. falciparum contain a single mitochondrion with minimal, but essential, physiological functions (22, 34). The mitochondrial electron transport chain (mtETC) is the primary generator of the proton electrochemical gradient (Δp) across the mitochondrial inner membrane. While Δp does not appear to power mitochondrial ATP synthesis in blood stages of P. falciparum, its establishment is vital to the parasite, and it is required to transport metabolites and proteins in and out of the mitochondrion (20, 25, 30). Another critical function of the malaria mtETC is the reoxidation of dihydroorotobiquinone (CoQH₂) to ubiquinone (CoQ), which is the electron acceptor for several mitochondrial dehydrogenases, such as dihydroorotatodehydrogenase (DHODH), glycerol 3-phosphate dehydrogenase (GPDH), succinate dehydrogenase (SDH), type 2 NADH dehydrogenase (NDH2), and malate-quinone oxidoreductase (MQO) (10, 12, 20, 21, 25). DHODH catalyzes the fourth step and the sole redox reaction of the de novo pyrimidine biosynthesis pathway, which is the only source of pyrimidines in malaria parasites (17). Atovaquone, one of the two drugs composing the antimalarial product Malarone, poisons the parasites by inhibiting the cytchrome bc₁ complex, thereby blocking the mtETC (13, 19, 30). P. falciparum DHODH itself is also a promising drug target, with recent reports of inhibitors with low-nanomolar 50% effective concentrations (EC₅₀), such as several aryI-substituted triazolopyrimidines (2, 16, 26).

Recent studies in our laboratory revealed that in erythrocytic stages of the parasite, ubiquinone-dependent dehydrogenase activities other than those of DHODH are dispensable in some strains but are essential in others.
enced the level of transgene expression and subsequent degree of mtETC independence as judged by resistance to atovaquone. To circumvent this potentially confounding effect of episomal expression, we have integrated a single copy of the yDHODH gene into the genomes of four different parasite lines using the nucortiophasterie Bxb1 attB×attP recombination system (23). The growth responses of these transgenic lines to the mtETC inhibitor atovaquone and the Plasmodium-specific DHODH inhibitor DSM1 showed strain-specific differences in the requirement for a functional mtETC among different P. falciparum strains, suggesting variations in the potentially critical role played by the enzymatic activities of other mitochondrial dehydrogenases.

MATERIALS AND METHODS

Plasmid construction. The yeast DHODH gene was amplified from the plasmid pHpyDHODH-GFP (25) using primers DHODHAvrII 

(GACGTA CGAATGCTGTTCAACTTCCCAC). The plasmid pHpyDHODH-GFP (25) was obtained from the Malaria Research and Reference Reagent Resource (MR4). This plasmid was digested by AvrII and BsiWl and ligated with the yDHODH PCR product digested by the same enzymes to generate pLN-yDHODH-GFP. The yeast DHODH gene was also amplified from the plasmid pHpyDHODH-GFP using the same forward primer (shown above) and a different reverse primer, DHODHmped 3′(GACTTAGTTAATGCTGTTCAACTTCCCAC). This PCR product was cloned into pLN-ENR-GFP digested with AvrII and MspcI to yield pLN-yDHODH.

Cell culture and transfection. P. falciparum parasites were cultured according to the methods published by Trager and Jensen, with modifications (33). Parasites were propagated in 5% hematocrit in human O+ erythrocytes in RPMI 1640 medium containing 0.5% Albumax and incubated at 37°C in a low-oxygen atmosphere (90% N2, 5% CO2, 5% O2). Transfections of P. falciparum parasites were carried out by standard methods (11). Briefly, ring-stage parasites at 5% parasitemia were electroporated with 50 µg plasmid DNA isolated using a Qiagen plasmid maxikit. Electroporation was done using a Bio-Rad GenePulser set at 0.31 kV and 960 µF. Two aliquots of DNA were electroporated for each transgene. Transfected parasites were maintained under drug pressure: 5 nM WR99210 for HDHFR (human dihydrofolate reductase), 2.5 µg/ml blasticidin for the neomycin-selectable plasmid pHHyDHODH-GFP (25) using primers DHODHAvrII5′/H11032GFP using the same forward primer (shown above) and a different reverse primer, DHODHmped 3′(GACTTAGTTAATGCTGTTCAACTTCCCAC). This PCR product was cloned into pLN-ENR-GFP digested with AvrII and MspcI to yield pLN-yDHODH.

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Parasite lines. Dd2attB and 3D7attB, originally generated by Nkromah et al. (23), were obtained from MR4. In both strains, a 44-bp attB fragment was integrated into the nonessential csg gene encoding a glutaredoxin-like protein (23). D10attB and HB3attB lines were created in our laboratory by transfecting D10 and HB3 parasites with the plasmid pCG-attB. After transfection, both cultures were maintained under 5 nM WR99210 for the positive selection of transfecants. Subsequently, WR99210 was cycled off and on to select parasites with the integrated transgene by single-crossover recombination. The integration of the attB site into the parasite genome was analyzed by PCR and Southern blot analysis.

Western blot analysis. Southern blot analysis was performed according to standard methods. Three micrograms of DNA isolated from parasites was digested by appropriate restriction enzymes overnight and run on a 0.8% agarose gel. DNA then was transferred to a GeneScreen Plus (Perkin Elmer) membrane by capillary transfer in high-salt buffer. 32P-labeled probe was hybridized with the blot, which was washed and exposed to film at −80°C for at least 12 h.

Northern blot analysis. Northern blot analysis was performed by standard methods. Twenty micrograms of total RNA isolated from parasites was run on a formaldehyde-agarose gel and transferred to GeneScreen Plus membrane. 32P-labeled probes were hybridized to blots, which were washed and exposed to film at −80°C for at least 12 h.

RESULTS

Generation of attB+ strains in D10 and HB3 lines. Four distinct P. falciparum lines were chosen for this study: D10 (7), frequently used for transfection; 3D7 (35), the genomic sequence reference line; Dd2 (24, 37), a multiple drug-resistant line; and HB3 (3), a chloroquine-sensitive strain used in the HB3×Dd2 genetic cross (36). Dd2attB and 3D7attB lines were previously constructed by Nkromah et al (23). In addition, we generated D10attB and HB3attB strains by the transfection of D10 and HB3 strains with the plasmid pCG-attB plasmid, followed by selection off-and-on cycling with the compound WR99210 as described previously (23). In the D10attB line, the pCG-attB plasmid was integrated into the csg6 site by single-crossover recombination (see Fig. 1A in the supplemental material) after one WR99210 off-and-on cycle. Diagnostic PCR showed
a specific product of the expected size in the D10attB parasites but not in the parental D10 line (see Fig. S1C). There is an alternative recombination site at the hrp3 5′ untranslated region (UTR), since the pCG6-attB plasmid contains a 1.5-kb fragment of the hrp3 5′ UTR region; insertion into this site was not detected in the D10attB parasites (see Fig. S1C). Southern blot analysis confirmed the D10attB genotype, as a diagnostic 3.2-kb EcoRI band was detected in addition to the signature of the wild-type DNA (see Fig. S1D). In the HB3 transfectants, the pCG6-attB plasmid did not integrate into the cg6 locus but did integrate into the hrp3 5′ UTR after one off-and-on WR99210 treatment (see Fig. S1B). Diagnostic PCR detected a product specific for hrp3 integration in the HB3attB parasites, while no product specific for cg6 integration was evident (see Fig. S1E). Southern blot analysis detected two specific bands, 7.2 and 6.0 kb, in the HB3attB DNA that was not found in the HB3 wild-type DNA after digestion with EcoRI and AvrII (see Fig. S1F). These results indicate that we have created D10attB and HB3attB strains by the integration of the pCG6-attB plasmid into two different loci in the parasite genomes, thus completing our desired set of four attB-positive parasites to allow transgene integration (e.g., of the yDHODH gene in this study) in four different parasite genetic backgrounds.

Generation of yDHODH±GFP integrated strains in four attB-positive lines. The yeast DHODH gene, with or without DNA encoding GFP added onto the 3′ end (designated yDHODH±GFP), was cloned into the pLN plasmid that bears attB sites (Fig. 1A) and cotransfected into Dd2attB, 3D7attB, D10attB, and HB3attB strains along with the pINT plasmid, which contains a gene encoding the mycobacteriophage Bxb1 integrase. Two to 3 weeks after transfection, parasites were observed in all of the transfected cultures, and diagnostic PCR using specific primers (Fig. 1A, short arrows; also see Fig. S2A in the supplemental material) revealed evidence of integration in each of these strains. Subsequently, the selective drugs G418 and blasticidin were removed for at least 1 month to allow the elimination of episomal plasmids in the transfected cultures. Genomic DNA then was isolated from each attB transgenic line and analyzed by Southern blotting hybridized with a yDHODH gene probe (Fig. 1A; also see Fig. S2A). Two specific EcoRV bands, 1.36 and 13.6 kb (or 14.37 kb in GFP-containing strains), were detected in cg6-integrated strains (Fig. 1B and C). The bands around 21 kb in Fig. 1B appear to be due to the partial digestion of the genomic DNAs and are specific, as they have the sizes predicted for genomic EcoRV cuts (Fig. 1A, lower diagram) and were recognized by the yDHODH probe, which had no background in wild-type parasites. In the hrp3-integrated strain HB3attB, a specific 7.25- or 8-kb SacI band was detected (see Fig. S2B). Fluorescence microscopy of live Dd2attB-yDHODH-GFP and HB3attB-yDHODH-GFP parasites showed the strong expression of the yDHODH-GFP fusion protein in the cytosol (Fig. 1D; also see Fig. S2C). Similar results were obtained with the D10attB-yDHODH-GFP and 3D7attB-yDHODH-GFP parasites (data not shown). Western blot analysis using an anti-GFP monoclonal antibody further confirmed the size of the yDHODH-GFP fusion protein in all four GFP-containing strains (data not shown). To detect the expression of yDHODH, total RNAs from the four yDHODH transgenic strains were isolated and tested by Northern blot analysis using yDHODH DNA as the probe. As shown in Fig. 1E and Fig. S2D in the supplemental material, yDHODH was expressed in all yDHODH transgenic strains. Equal amounts of RNAs (20 μg per sample) from transgenic or wild-type parasites were loaded on the formaldehyde gel used for blotting, evidenced by ethidium bromide staining and imaging (data not shown). Before comparing effects of mtETC inhibitors on yDHODH±GFP-integrated strains, it was critical to quantitatively verify gene expression levels in each of these lines. As shown in Fig. 2, real-time PCR analysis employing two pairs of yDHODH-specific primers showed that the yDHODH±GFP transgenes were expressed equally in all eight transgenic lines. Thus, we have integrated the yDHODH gene with or without a GFP tag into four strains, generating eight transgenic parasite lines. All of these transgenic lines possess a single copy of the yDHODH gene, and the transgene was robustly expressed at comparable levels in all lines.

Short-term response of yDHODH transgenic lines to mtETC inhibitors. In our previous study, we found that the D10-yDHODH-GFP parasites expressing a plasmid-encoded yDHODH-GFP fusion gene were able to grow normally in the presence of atovaquone and other mtETC inhibitors, as measured by the standard 48-h [3H]hypoxanthine incorporation assay (25). These parasites could be cultured for longer periods in the presence of atovaquone as well. We performed the 48-h [3H]hypoxanthine incorporation assays with all eight yDHODH integrated lines, exposing them to various concentrations of atovaquone. Data from these assays indicated that parasites with or without the GFP tag on yDHODH behaved similarly in response to atovaquone (data not shown), suggesting that tagging yDHODH with GFP did not affect the phenotype imparted on the transgenic parasites of four different genetic backgrounds. Therefore, for all subsequent studies (except for those shown in Fig. S3 in the supplemental material), we used the four transgenic strains expressing untagged yDHODH.

As shown in Fig. 3A, while all parental attB lines were fully susceptible to atovaquone, the yDHODH-integrated lines were highly resistant to atovaquone. Under increasing concentrations of atovaquone up to 2,250 nM, the D10attB-yDHODH and 3D7attB-yDHODH strains showed almost 100% hypoxanthine incorporation relative to that in the absence of the drug, indicating that these two transgenic lines were highly resistant to atovaquone. The Dd2attB-yDHODH and HB3attB-yDHODH strains also were resistant to atovaquone, although their resistance did decline gradually to around 75% at the highest concentration of atovaquone (2,250 nM) (Fig. 3A). DSM1, a selective inhibitor of P. falciparum DHODH (26), also was tested. All of the yDHODH-integrated strains were highly resistant to DSM1, even at the highest concentration (11.1 μM), as shown in Fig. 3B. Overall, in the short-term (48 h) growth inhibition assays, all of the yDHODH-integrated strains showed strong resistance to the mtETC inhibitor atovaquone and the P. falciparum-specific DHODH inhibitor DSM1. These results are consistent with our previous findings for episomally expressed yDHODH-GFP (25).

Long-term response of yDHODH transgenic lines to mtETC and DHODH inhibitors. To examine the possible mtETC in-
dependence of yDHODH-integrated strains during long-term growth, we cultured the parasites in medium supplemented with either 100 nM atovaquone or 1.5 μM DSM1. The high concentration of atovaquone (100 nM) was chosen to avoid the emergence of cytochrome b mutations, which frequently occur at lower concentrations after a long exposure time (3 weeks or more). As shown in Fig. 4, D10attB-yDHODH and 3D7attB-yDHODH showed similar growth rates in the presence or absence of atovaquone during the 15-day culture period. These parasites were able to grow in 100 nM atovaquone for months (data not shown). However, although the parasitemia was comparable to that of the untreated controls during the first 3 to 4 days, the majority of Dd2attB-yDHODH and HB3attB-yDHODH parasites stopped growing after 3 to 4 days of atovaquone treatment. The parasitemia dropped down to 0 to 0.1% after 6 to 7 days of 100 nM atovaquone treatment. The Dd2attB-yDHODH and HB3attB-yDHODH responses to long-term atovaquone exposure were reproducible (five independent experiments). On the other hand, all four yDHODH-integrated attB strains exhibited complete resistance to 1.5 μM atovaquone.

FIG. 1. Site-specific integration of yeast DHODH into P. falciparum Dd2attB, 3D7attB, and D10attB lines mediated by Bxb1 integrase. (A) Schematic of the dual-plasmid attB/attP recombination approach according to the method reported by Nkrumah et al. (23). Bxb1 integrase expressed in trans from the pINT plasmid facilitates the integration of the pLN-yDHODH+GFP plasmid into the cg6-attB site by single-crossover recombination. The attB/attP recombination generates two sites, attL (left) and attR (right). EcoRV (EV) digestion sites and the sizes of resulting DNA fragments are shown. Short arrows indicate primers used to check integration by diagnostic PCR. The forward primer (P2) is 417 bp upstream of the cg6 coding sequence, and the reverse primer (P4) is from hpt+3 in the bsd cassette. The primer sequences were described by Nkrumah et al. (23). (B) Southern blot analysis confirmed the integration of the yDHODH+GFP gene into Dd2attB and 3D7attB strains. EcoRV-digested genomic DNA was separated, blotted, and hybridized with the indicated yDHODH probe. Lane 1, Dd2attB-yDHODH-GFP; lane 2, Dd2attB-yDHODH; lane 3, Dd2attB; lane 4, 3D7attB-yDHODH-GFP; lane 5, 3D7attB-yDHODH; lane 6, 3D7attB. Upper bands (matching the predicted size of 21.63 or 20.86 kb [A]) likely resulted from incomplete digestion by EcoRV. (C) Southern blot analysis confirmed the integration of the yDHODH+GFP gene into the D10attB line. Genomic DNA from D10attB-yDHODH+GFP was digested by EcoRV and hybridized with yDHODH probe. Lane 1, D10attB-yDHODH-GFP; lane 2, D10attB-yDHODH; lane 3, D10attB. The near absence of upper bands (20.86 and 21.63 kb) indicated more-complete digestion by EcoRV. (D) Fluorescence microscopy of Dd2attB-yDHODH-GFP parasites showed the presence of the yDHODH-GFP protein in the parasite cytosol. The same results were obtained from 3D7attB-yDHODH-GFP and D10attB-yDHODH-GFP parasites (data not shown). (E) Northern blot analysis showed the expression of yDHODH in Dd2attB-yDHODH, 3D7attB-yDHODH, and D10attB-yDHODH parasites. Lane 1, Dd2attB-yDHODH; lane 3, 3D7attB-yDHODH; lane 5, D10attB-yDHODH. Lanes 2, 4, and 6 are the corresponding wild-type controls.
DSM1 during 15 days of culture (Fig. 5). These parasites also were able to grow in 1.5 µM DSM1 for months (data not shown). The resistance to the *P. falciparum*-specific DHODH inhibitor indicates that the yDHODH expressed in each of these strains is able to produce sufficient orotate to supply parasite pyrimidine biosynthesis.

We also assessed the possibility that atovaquone treatment of Dd2attB-yDHODH and HB3attB-yDHODH transgenic parasites resulted in the downregulation of yDHODH gene expression. We used flow cytometry to monitor the expression of the yDHODH-GFP fusion protein in each of the four yDHODH-GFP transgenic lines under 100 nM atovaquone treatment for several days. As shown in Fig. S3 in the supplemental material, D10attB-yDHODH-GFP and 3D7attB-yDHODH-GFP transgenic parasites showed similar percentages of GFP-positive cells in the presence or absence of 100 nM atovaquone during the course of 7 days. In Dd2attB-yDHODH-GFP and HB3attB-yDHODH-GFP transgenic parasites, the percentages of GFP-positive cells were similar in the presence or the absence of 100 nM atovaquone treatment for the first 3 days. However, with 100 nM atovaquone treatment, GFP-positive percentages started to decline on day 4 and eventually dropped down to around 0.2% on day 7. With medium alone, GFP-positive percentages kept increasing as the parasites grew. These data suggest that the yDHODH gene continues to be expressed upon atovaquone treatment in the Dd2attB-yDHODH-GFP and HB3attB-yDHODH-GFP transgenic parasites until the parasites start to die. The reason for their sensitivity to atovaquone after several days was not due to the abolishment of yDHODH gene expression or the lack of pyrimidines.

Decylubiquinone rescues Dd2attB-yDHODH and HB3attB-yDHODH growth inhibition by atovaquone. Since fumarate is depleted upon atovaquone treatment in the long term, which in turn would cause Dd2attB-yDHODH-GFP and HB3attB-yDHODH-GFP transgenic parasites to die. To assess this, we supplemented the Dd2attB-yDHODH-GFP and HB3attB-yDHODH-GFP transgenic parasites with 5 mM fumarate along with treatment with 100 nM atovaquone for the long term, but we failed to rescue the parasites (data not shown). To test whether the overproduction of reactive oxygen species (ROS) could be a cause of Dd2attB-yDHODH and HB3attB-yDHODH parasite growth inhibition under extended atovaquone treatment, we supplemented these parasites with ROS scavengers. N-acetyl-cysteine, a widely used ROS scavenger (18), at 50 µM failed to rescue parasite growth in the presence of atovaquone (data not shown). Vitamin E, a lipid-soluble antioxidant that maintains the integrity of cell membranes by acting as a peroxyl radical scavenger (32), at 50 µM failed to rescue these parasites under extended atovaquone treatment (data not shown).

Since one of the functions of the mETC is to recycle reduced ubiquinone to serve CoQ-dependent dehydrogenases within the mitochondrion, we tested whether a CoQ analog could rescue Dd2attB-yDHODH and HB3attB-yDHODH parasites. Decylubiquinone (Q₉), a relatively soluble CoQ analog, was used to avoid solubility problems caused by the strong hydrophobicity of CoQ. As shown in Fig. 6, Q₉ dramatically reduced the toxicity of atovaquone in Dd2attB-
yDHODH and HB3attB-yDHODH parasites. Increasing concentrations of Q\textsubscript{d} (0, 0.1, 10, and 50 \textmu M) were tested in combination with 100 nM atovaquone. In Dd2attB-yDHODH parasites, both 10 and 50 \textmu M Q\textsubscript{d} dramatically reversed the toxicity of 100 nM atovaquone. At these concentrations of Q\textsubscript{d}, parasites showed healthy morphology at all stages (data not shown) and maintained a parasitemia comparable to that of the parasites in the untreated control. Similarly in HB3attB-yDHODH parasites, both 10 and 50 \textmu M Q\textsubscript{d} successfully rescued growth in the presence of atovaquone.

**DISCUSSION**

Of the five CoQ-requiring mitochondrial dehydrogenases, DHODH is clearly the most important for the survival of erythrocytic stages of *P. falciparum*. Because yDHODH transgenic *P. falciparum* became resistant to all cytochrome bc\textsubscript{1} complex inhibitors, and indeed because yDHODH could be used as a marker for positive selection with atovaquone in transfection experiments (14), it appeared logical to conclude that enzymatic functions of the other four mitochondrial electron transport chain dehydrogenases other than DHODH were not essential for blood-stage *P. falciparum*. This has become a subject of debate, as it applies to the validity of dehydrogenases other than DHODH as potential targets for antimalarial drugs (11a, 34a). We have attempted to address this debate by examining the role of parasite genetic background as it relates to mtETC and its role in serving mitochondrial dehydrogenases. We generated D10attB and HB3attB lines, doubling the number of *P. falciparum* lines available for Bxb1 integrase-mediated site-specific transgene insertions. Using the four *attB* lines, we generated eight strains expressing essentially equal levels of yDHODH (with or without GFP tags) in four genetic backgrounds: D10, 3D7, HB3, and Dd2. The D10attB-yDHODH and 3D7attB-yDHODH parasites exhibited resistance to the mtETC inhibitor atovaquone in short- and long-term growth experiments. The Dd2attB-yDHODH and HB3attB-yDHODH parasites, however, were unable to maintain long-term growth in the presence of 100 nM atovaquone, even though they showed high-level resistance in short-term growth inhibition assays. In contrast to the differences in response to atovaquone, all four yDHODH-integrated strains exhibited strong resistance to the *P. falciparum*-specific DHODH inhibitor DSM1 in both short- and long-term experiments. The equal degree of resistance to DSM1 in all strains argues against the possibility that the differences in strain response to atovaquone were due to the malfunction of the yDHODH or other steps within the pyrimidine biosynthesis pathway in the Dd2attB-yDHODH and HB3attB-yDHODH parasites. Thus, the variations in resistance to atovaquone seem to reveal potential functional differences associated with the mtETC functions in parasites with different genetic backgrounds. This also implies that, besides the essential DHODH, the other four CoQ-dependent dehydrogenases in the mitochondrial electron transport chain (SDH, MQO, NAD2, and GPDH) are not essential in some parasite isolates (such as

**FIG. 4.** Effect of mtETC inhibitor atovaquone on yDHODH-integrated Dd2attB, 3D7attB, D10attB, and HB3attB parasites during long-term growth in culture. Growth (y axis) was calculated as parasitemia times splitting factors. The splitting factor is 5 when the culture is split 1:5. (A) D10attB-yDHODH in the presence and absence of 100 nM atovaquone. (B) 3D7attB-yDHODH in the presence and absence of 100 nM atovaquone. (C) Dd2attB-yDHODH in the presence and absence of 100 nM atovaquone. (The parasitemia of the culture under atovaquone pressure declined to a negligible level after 7 days of drug treatment, hence that is the last date point recorded for this condition.) (D) HB3attB-yDHODH in the presence and absence of 100 nM atovaquone. (The parasitemia of the culture under atovaquone pressure declined to a negligible level after 6 days of drug treatment, hence that is the last data point recorded for this condition.) Atv, atovaquone. For all strains at each time point, parasitemia was determined manually by counting the number of parasites in 1,000 red blood cells. These growth experiments were repeated twice for D10attB-yDHODH and 3D7attB-yDHODH and five times for Dd2attB-yDHODH and HB3attB-yDHODH.
D10 and 3D7 strains) but are essential in others (such as Dd2 and HB3 strains).

What could be other reasons for the Dd2attB-yDHODH and HB3attB-yDHODH parasites to die under long-term 100 nM atovaquone exposure? Since the inhibition of certain active sites within the mtETC is known to promote the formation of deleterious ROS in other organisms (4, 6), one possible explanation for the strain-specific differences seen here is the

![Graphs and images showing growth curves for different strains under various conditions.](image)

FIG. 5. Effect of *Plasmodium* DHODH-specific inhibitor DSM1 (a triazolopyrimidine) on yDHODH-integrated strains during longer term growth in culture. The DSM1 concentration used was 1.5 μM (EC50 ~ 100 nM). (A) Growth of D10attB-yDHODH in the presence and absence of 1.5 μM DSM1. (B) Growth of 3D7attB-yDHODH in the presence and absence of 1.5 μM DSM1. (C) Growth of Dd2attB-yDHODH in the presence and absence of 1.5 μM DSM1. (D) Growth of HB3attB-yDHODH in the presence and absence of 1.5 μM DSM1. For all strains at each time point, parasitemia was manually counted as the number of parasites per 1,000 red blood cells. These growth experiments were repeated twice for each strain.

![Graphs and images showing growth curves for different strains under various conditions.](image)

FIG. 6. Decylubiquinone (Qd) reversed the long-term susceptibility of Dd2attB-yDHODH and HB3attB-yDHODH parasites to atovaquone. (A) The structures of Qd (top) and ubiquinone (CoQ10; bottom) are shown. (B) Growth of Dd2attB-yDHODH parasites in the presence of various concentrations of Qd: 0, 0.1, 10, and 50 μM in combination with 100 nM atovaquone. Parasites grown in regular RPMI 1640 served as a positive control. (C) Growth of HB3attB-yDHODH parasites in the presence of various concentrations of Qd in combination with 100 nM atovaquone. Parasites grown in regular RPMI 1640 served as a positive control.
differences in the ability to handle ROS by distinct parasite strains. However, ROS scavengers N-acetyl-cysteine and vitamin E failed to overcome the toxicity of atovaquone, suggesting that differences in the processing of ROS are unlikely to explain the observed strain differences. The yDHODH gene not only needs to be expressed but also needs to function properly to provide the pyrimidine synthesis bypass. The yDHODH gene was integrated into the genomes and driven by the strong promoter calmodulin 5' UTR. We have confirmed that the yDHODH gene was expressed in Dd2attB-yDHODH and HB3attB-yDHODH parasites (Fig. 1 and 2; also see Fig. S2 in the supplemental material). Furthermore, yDHODH gene expression was not downregulated upon atovaquone treatment (see Fig. S3 in the supplemental material). Fumarate is the cosubstrate (as the electron acceptor) for the yDHODH enzyme, so the depletion of fumarate could be an underlying cause of parasite demise. However, the provision of 5 mM fumarate failed to rescue the Dd2attB-yDHODH and HB3attB-yDHODH parasites under the extended atovaquone treatment (data not shown). These data suggest that the sensitivity of Dd2attB-yDHODH and HB3attB-yDHODH parasites to atovaquone in the long term is not due to the malfunctioning of the yDHODH enzyme.

Bulusu et al. recently reported on the metabolic fate of fumarate in P. falciparum (5). Per these investigators, fumarate, derived from tricarboxylic acid (TCA) metabolism and the purine salvage pathway, is converted to malate by the mitochondrial fumarate hydratase (FH). Malate then is converted to oxaloacetate by the mitochondrial malate quinone oxidoreductase (MQO). Oxaloacetate also can be generated by phosphoenolpyruvate carboxylase (PEPC) from phosphoenolpyruvate and CO₂ (31). Oxaloacetate is converted to aspartate by the cytosolic aspartate aminotransferase (AAT). Aspartate is converted back to fumarate via the purine salvage pathway to complete the fumarate cycle, in addition to being incorporated into proteins and pyrimidines. This fumarate cycle requires the functioning of FH, MQO, AAT, and the purine salvage enzymes. Bulusu et al. stated that 1 μM atovaquone (1,000-fold above the EC₅₀) could abolish the conversion of fumarate to aspartate, probably by inhibiting MQO (5). They did these experiments using free parasites. However, it is not clear whether this fumarate cycle is significant in intact parasitized red blood cells, where aspartate also can arise from hemoglobin digestion and from the growth medium (the medium has 0.15 mM aspartate). D10attB-yDHODH and 3D7attB-yDHODH parasites are resistant to atovaquone indefinitely. In these lines, the inhibition of MQO by atovaquone in parasites abolishing the conversion of fumarate to aspartate clearly is not deleterious. Dd2attB-yDHODH and HB3attB-yDHODH parasites are sensitive to atovaquone in the long term. If the fumarate cycle is critical for these two lines, leading to parasite demise due to a shortage of aspartate, pyrimidine starvation, or purine starvation when treated with atovaquone, we should be able to rescue them by the provision of additional aspartate. However, excess aspartate (1 to 5 mM) failed to reverse the toxicity of atovaquone in Dd2attB-yDHODH and HB3attB-yDHODH parasites in the long term (data not shown). These data suggest that the diminishment of fumarate conversion to aspartate by atovaquone through inhibiting MQO likely is not the reason for Dd2attB-yDHODH and HB3attB-yDHODH parasites becoming sensitive to atovaquone in the long term.

In contrast, an analog of CoQ, Q₉, which can act as an electron acceptor for DHODH and other dehydrogenases (1, 21), successfully reversed atovaquone growth arrest in Dd2attB-yDHODH and HB3attB-yDHODH parasites (Fig. 6). The Q₉ rescuing effect suggests that the differential production, utilization, and/or turnover of CoQ in these strains could play an important role in parasite survival. In malaria parasites, CoQ differs from the human counterpart in the length of the side chain: parasites have 8 or 9 isoprenoid groups, while humans have 10 (8, 27, 29). Parasites do not salvage CoQ from their hosts but synthesize CoQ₈ and CoQ₉ de novo. CoQ is the cosubstrate electron acceptor for five mitochondrial dehydrogenases in P. falciparum: DHODH, SDH, NDH2, GPDH, and MQO. In parasites where mitochondrial DHODH has been made superfluous, exogenously supplied Q₉ is capable of serving the other four dehydrogenases in all strains. For the D10 and 3D7 strains, this is not necessary, whereas it is for the Dd2 and HB3 strains. It remains to be determined whether the functions served by these four enzymes differ in different genetic backgrounds or whether alternative electron acceptors are present to serve these enzymes in some of the parasite strains. The resolution of these questions would require ascertaining the effects of the direct inhibition of individual dehydrogenases and/or the knockout of the genes encoding the enzymes in distinct parasite genetic backgrounds.

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