Role of dihydrofolate reductase in tetrahydrobiopterin biosynthesis and lipid metabolism in the oleaginous fungus Mortierella alpina

Hongchao Wang,1 Chen Zhang,1 Jinghan Feng,1 Yuan Liu,1 Qin Yang,1 Haiqin Chen,1 Zhennan Gu,1 Hao Zhang,1 Wei Chen1 and Yong Q. Chen1,2

Correspondence
Yong Q. Chen
yqchen@jiangnan.edu.cn

Mortierella alpina is a well-known polyunsaturated fatty acid-producing oleaginous fungus. Analysis of the Mort. alpina genome suggests that there is a putative dihydrofolate reductase (DHFR) gene playing a role in the salvage pathway of tetrahydrobiopterin (BH₄), which has never been explored in fungi before. DHFR is the sole source of tetrahydrofolate and plays a key role in maintaining BH₄ levels. Transcriptome data analysis revealed that DHFR was up-regulated by nitrogen exhaustion, when Mort. alpina starts to accumulate lipids. Significant changes were found in the fatty acid profile in Mort. alpina grown on medium containing DHFR inhibitors compared to Mort. alpina grown on medium without inhibitors. To explore the role of DHFR in folate/BH₄ metabolism and its relationship to lipid biosynthesis, we expressed heterologously the gene encoding DHFR from Mort. alpina in Escherichia coli and we purified the recombinant enzyme to homogeneity. The enzymatic activity was investigated by liquid chromatography and MS and VIS–UV spectroscopy. The kinetic parameters and the effects of temperature, pH, metal ions and inhibitors on the activity of DHFR were also investigated. The transcript level of cytosolic NADPH-producing gene involved in folate metabolism is down-regulated by DHFR inhibitors, which highlights the functional significance of DHFR in lipid biosynthesis. The relationship between DHFR and lipid metabolism is thus of major importance, and folate metabolism may be an alternative NADPH source in fatty acid synthesis. To our knowledge, this study is the first to report the comprehensive characterization of a BH₄ salvage pathway in a fungus.

INTRODUCTION

Dihydrofolate reductase (DHFR; EC 1.5.1.3) is an enzyme that reduces dihydrofolate (FH₂) to tetrahydrofolate (FH₄) and is the sole source of FH₄ (Fig. 1), thus having a central role in the maintenance of cellular pools of FH₄ and its derivatives (Schnell et al., 2004). One-carbon transfers mediated by folate coenzymes play essential roles in many major cellular processes, including nucleic acid biosynthesis, mitochondrial and chloroplast protein biosynthesis, amino acid metabolism, methyl group biogenesis and vitamin metabolism (Nilsson et al., 2014). The enzyme is the target of several important anticancer and antibiotic drugs, including methotrexate (MTX), trimethoprim (TRI) and pyrimethamine (PYR) (Schnell et al., 2004). In addition to key roles in folate metabolism, DHFR can also reduce 7,8-

Abbreviations: BH₂, 7,8-dihydrobiopterin; BH₄, tetrahydrobiopterin; DHFR, dihydrofolate reductase; FH₂, dihydrofolate; FH₄, tetrahydrofolate; LC–MS, liquid chromatography and mass spectrometry; LCPUFA, long-chain polyunsaturated fatty acid; MTX, methotrexate; MTHFD, methylene tetrahydrofolate dehydrogenase/cyclohydrolase; PCE, phylogenetically coherent event; PUFA, polyunsaturated fatty acid; PYR, pyrimethamine; qPCR, quantitative PCR; TRI, trimethoprim; XIC, extracted ion chromatogram.

The GenBank accession number for the gene sequence of dihydrofolate reductase is KU517669.

Three supplementary figures and three supplementary tables are available with the online Supplementary Material.
Recent research results demonstrated that the activity of various enzymes in higher animals, including phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, alkylglycerol monooxygenase and nitric oxide synthase (He & Rosazza, 2003; Nagatsu & Ichinose, 1999; Werner-Felmayer et al., 2002). Since BH₄ levels may also be influenced by oxidation, forming BH₂, DHFR plays a key role in BH₄ levels by maintaining the BH₄/BH₂ ratio, particularly under conditions of low total biotinier availability (Crabtree et al., 2009).

The BH₄ de novo synthesis and regeneration pathways in Mortierella alpina were first purified and characterized in fungi in our laboratory (Wang et al., 2011a; Wang et al., 2013). Though the function of DHFR in folate metabolism has been characterized in the yeast Candida albicans, Candida glabrata and Saccharomyces cerevisiae (Baccanari et al., 1989; Daly et al., 1994; Lagosky et al., 1987; Liu et al., 2008), the role of DHFR in fungal BH₄ salvage pathway has never been explored before. Mort. alpina is a well-known polyunsaturated fatty acid (PUFA)-producing oleaginous fungus commonly found in soil (Sakuradani et al., 2009). After sequencing the whole genome of Mort. alpina (ATCC 32222) (Wang et al., 2011b), our analysis suggested that there is a putative DHFR gene involved in the salvage pathway in the Mort. alpina genome.

Understanding the mechanisms by which highly efficient lipogenesis is achieved may be instrumental in using single-cell oils such as biodiesels and dietary fat. However, the molecular mechanism of efficient lipid biosynthesis is still not well understood in oleaginous fungi in general, as well as in Mort. alpina in particular. NADPH is the critical reducing agent and limiting factor in fatty acid synthesis (Zhang et al., 2007). The cytosolic malic enzyme was considered to be the most important source of reducing power for fatty acid synthesis in oleaginous microbes (Wynn et al., 1999). Our recent research results demonstrated that the pentose phosphate pathway also plays a significant role during fungal lipid synthesis (Chen et al., 2015). However, the role of alternative NADPH sources in fatty acid synthesis is not well understood. BH₄ has been suggested to be essential for lipid metabolism in higher organisms (Forrest & Van Baalen, 1970; Giovannini et al., 1995; Kaufman, 1967, 1993; Moseley et al., 2002; Rudzite et al., 1998). In our previous study, a requirement for BH₄ in phenylalanine hydroxylation has been reported in Mort. alpina, which has been shown to be functionally significant in lipid metabolism by contributing NADPH (Wang et al., 2013). Recently, quantitative flux analysis of DHFR in immortalized baby mouse kidney epithelial cells revealed the crucial function of folate metabolism in generating NADPH, with a majority of this NADPH consumed by fatty acid synthesis (Fan et al., 2014). Taking into account the pivotal functional importance of DHFR in BH₄ and folate metabolism, the functional significance of DHFR in the biosynthesis of lipids and closely related compounds has yet to be fully elucidated.

In this study, we investigated the role of DHFR in lipid metabolism and probed its possible function in NADPH generation. To clarify the molecular mechanism of folate metabolism and the BH₄ salvage pathway in fungi, we characterized the function of the gene encoding DHFR in Mort. alpina in vitro. Kinetic parameters of DHFR and the effects of temperature, pH, metal ions and antifolates on DHFR activity were investigated.

**METHODS**

**Strains and growth conditions.** Mort. alpina (ATCC 32222) was cultured on PDA plates (BD Difco Potato Dextrose Agar) and incubated for 15 days at 28 °C. The cultures were initially cultivated in 50 ml Kendrick medium (Kendrick & Ratledge, 1992) and incubated at 28 °C for 4 days. Mycelia were blended in fresh medium (0.25 g ml⁻¹) using a Braun hand blender for 8 pulses of 5 s each. Two millilitres of mycelial suspension was inoculated into 200 ml of medium in a 1000 ml flask and shaken at 200 r.p.m., 28 °C for 8 days. The mycelia were collected by filtration through sterile cheesecloth and were frozen immediately in liquid nitrogen for RNA extraction.
Gene searching. Predicted genes in the Mort. alpina (ATCC 32222) genome (GenBank accession number ADAGO1000000) were annotated as described previously (Wang et al., 2016).

Transcriptome data analysis. RNA-sequencing data obtained from previous research were analysed as described previously (Chen et al., 2015). Transcript abundance was calculated using Cufflinks version 0.9.3 and the output normalized expression values in FPKM (fragments per kilobase of exon per million fragments mapped) analogous to single-read RPKM (reads per kilobase million) were used for further comparative analysis (Mortazavi et al., 2008; Trapnell et al., 2010).

Effect of DHFR inhibitors on lipid synthesis. For the whole-cell inhibition studies, cultures were grown in 50 ml of Kendrick medium (Kendrick & Ratledge, 1992) with the addition of 5 mM MTX, TRI and PYR after 3 days of incubation. The medium and incubation protocols were as described previously (Wang et al., 2011b). The mycelia were then collected by filtration after 8 days of cultivation and approximately 20 mg pulverized mycelium was used for lipid extraction using the method of Bligh and Dyer (Bligh & Dyer, 1959).

Cloning and plasmid construction. The DHFR gene was amplified by PCR from cDNA using the primer pairs shown in Table 1. The PCR cycling conditions used were as follows: denaturation at 92°C for 30 s, annealing at 50°C for 45 s and extension at 68°C for 45 s (for 25 cycles in total); the final volume in each well was 50 µl. The amplified product was cloned into pET28a to construct pw754 (containing DHFR), which was confirmed by sequencing using an ABI 3730 Sequencer.

Protein expression and purification. Recombinant plasmids carrying the DHFR gene were transformed into the BL21 DE3 gold strain of Escherichia coli. The cells were grown overnight at 37°C with shaking in LB medium containing 50 µg ml⁻¹ kanamycin. The overnight culture (5 ml) was inoculated into 250 ml of fresh medium and grown until the OD₆₀₀ reached 0.6. Expression of DHFR was induced by the addition of 0.01 mM IPTG at 20°C for 48 h. After the IPTG induction, cells were harvested by centrifugation, washed with binding buffer (50 mM Tris/HCl, 300 mM NaCl and 10 mM imidazole; pH 7.4), resuspended in the same buffer (supplemented with 1 mM phenylmethanesulfonyl fluoride and 1 mg ml⁻¹ lysozyme) and then sonicated. Cell debris were removed by centrifugation, and the supernatants (containing soluble proteins) were collected. The His-tagged fusion protein was purified by nickel ion affinity chromatography using a Chelating Sepharose Fast Flow column according to the manufacturer’s instructions (GE Healthcare).

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence*</th>
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<tr>
<td>DHFR (forward)</td>
<td>CTAGCTAGCATGAGCAGCACA-CAACAGG</td>
</tr>
<tr>
<td>DHFR (reverse)</td>
<td>CCCCAGCTTCTATTGCTATTCTGAGGTTTGC</td>
</tr>
<tr>
<td>DHFR (qPCR)</td>
<td>AACACCGACTGCTGTCCTACCACTACC</td>
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<tr>
<td>DHFR (qPCR)</td>
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<td>CGAGAAAAAGCTACAGGTTT</td>
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<tr>
<td>MTHFD1 (qPCR)</td>
<td>GAGACCCAGTGAATCAGATG</td>
</tr>
<tr>
<td>MTHFD2 (qPCR)</td>
<td>GAAGGCTGCTACAAGCAGCA</td>
</tr>
<tr>
<td>MTHFD2 (qPCR)</td>
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<td>IBS rDNA</td>
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</tr>
<tr>
<td>IBS rDNA</td>
<td>GCAGGCATCGGATAATTG</td>
</tr>
</tbody>
</table>

*Restriction cleavage sites are underlined.

Unbound proteins were washed through with 50 ml of wash buffer (50 mM Tris/HCl, 300 mM NaCl and 25 mM imidazole; pH 7.4). Fusion proteins were eluted with 5 ml of elution buffer (50 mM Tris/HCl, 300 mM NaCl and 500 mM imidazole; pH 7.4) and dialysed overnight at 4°C against 1 M Tris/HCl buffer containing 20% glycerol (pH 7.4). The BL21 DE3 gold strain of E. coli carrying blank pET28a was induced and purified under the same condition. The protein concentration was determined using the Bradford method and proteins were stored at -80°C.

Enzyme activity assays. The DHFR activity for FH₂ was assayed spectrophotometrically by monitoring the decrease in absorbance at 340 nm using the method described earlier with minor modifications (Sirawaraporn et al., 1993b). The standard assay for DHFR contained 50 mM Tris/HCl (pH 7.0), 100 mM FH₂, 100 µM NADPH, 75 µM 2-mercaptoethanol, 1 mg ml⁻¹ bovine serum albumin and 1 µM of enzyme in a total volume of 100 µl. Unless otherwise specified, the reaction was initiated with the addition of FH₂. The reactions were incubated at 37°C for 10 min and an equal volume of methanol was added to the mixture to terminate the DHFR reaction. This assay method was defined as the standard assay condition for DHFR activity. The molar extinction coefficient for FH₂ at 340 nm is 12 270 M⁻¹cm⁻¹ (Hilicoat et al., 1967). The DHFR reaction for BH₄ contained 50 mM Tris/HCl (pH 7.0), 100 µM BH₄, 1 mM NADPH, 5 µM 2-mercaptoethanol and 1 µM of enzyme in a total volume of 100 µl. The disappearance of NADPH at 25°C was monitored continuously at 340 nm (Abelson et al., 1978). The reaction without the addition of enzyme was used as a control.

Liquid chromatography and MS analysis. The DHFR reaction products were also purified by liquid chromatography and MS (LC-MS) using a Thermo Scientific Dionex UltiMate 3000 RSLC system as described previously (Wang et al., 2016). Chromatographic separation was carried out on a TSK gel Amide-80 column (150 mm × 2.0 mm, 2.0 µm), which was maintained at 35°C. Aliquots of reaction mixtures were injected into the column in 5 mM CH₃COONH₄ and acetonitrile following the gradient elution program shown in Table 3 (available in the online Supplementary Material). A heated electrospray ionization source was operated in negative mode for FH₂ or positive mode for BH₄.

The effects of temperature, pH, metal ions and inhibitors on the activity of DHFR. For determinations of optimal temperature, DHFR reactions were carried out at 4–55°C for 10 min at pH 7.0 under the abovementioned standard assay conditions. In order to determine the optimal pH, we used three different buffers over a range of pH 3–12, including 100 mM sodium acetate-acetic acid (pH 3–6), 100 mM Tris/HCl (pH 7–8) and 100 mM glycine-NaOH (pH 10–12) under the abovementioned standard assay conditions. The thermal stability of DHFR was assayed by incubating the enzyme in Tris/HCl buffer (100 mM, pH 8.0) at various temperatures. At given time intervals (0–2 h), samples were withdrawn and the residual activity was measured under the abovementioned standard assay conditions. For determinations of pH stability, DHFR was incubated at pH 3–12 at 4°C for up to 2 h, and the remaining enzyme activity was measured under standard assay conditions. A range of metal ion concentrations (from 1 µM to 10 mM) was used to test the effect of metal ions on the DHFR activity, including KCl, NaCl, MgCl₂, MnCl₂, FeCl₃, CuCl₂ and CoCl₂. These reactions were carried out under standard assay conditions and replicated 3 times. A range of inhibitors concentrations (from 1 µM to 1 mM) was used to test the effects of MTX, TRI and PYR on the DHFR activity under abovementioned standard assay conditions.

Measurement of kinetic parameters. To measure the Km and Vmax values for DHFR using FH₂, we carried out reactions using various concentrations of FH₂ (0.2–2 mM) in conjunction with a fixed concentration of NADPH (1 mM). The DHFR reactions were performed in Tris/
HCl, pH 7.0 at 30 °C for 10 min in a final volume of 100 µl. The kinetic parameters were obtained at various concentrations of one substrate at a fixed concentration of the other based on the Lineweaver–Burk equation. This experiment was replicated three times.

**Real-time quantitative PCR (qPCR).** Total RNA was isolated from *Mort. alpina* using Trizol Reagent (Invitrogen) and was reverse-transcribed with the PrimeScript RT reagent kit (Takara) according to the manufacturer’s instructions. qPCR was performed using the method used before (Wang *et al.*, 2016).

**RESULTS**

**Gene searching**

The whole-genome shotgun sequence of *Mort. alpina* ATCC 32222 has been recently completed and deposited as GenBank accession no. ADAG01000000 (Wang *et al.*, 2011b). Based on a search of the genomic sequence, a gene with ID MA-00120-337 was identified as the putative DHFR gene (Table S1). The GenBank accession number for the gene sequence of DHFR is KU517669. This revealed the likely presence of a salvage pathway of BH4 in this fungus.

**Expression levels of the DHFR gene during the course of lipid accumulation**

Our previous transcriptome analyses were carried out at various time points prior to and after nitrogen exhaustion (Chen *et al.*, 2015). The mycelia samples were collected during the cell multiplication stage (18.5 h), just before nitrogen was used up completely (20 h and 22 h) and once lipids had accumulated for a long period of time (33 h and 69 h). The DHFR transcript level was investigated during the course of lipid accumulation (Table S1), which was induced by nitrogen exhaustion (Wynn *et al.*, 1999). The DHFR gene was up-regulated in cells collected at 22 h when nitrogen was completely exhausted and lipids started to accumulate (Fig. 2a). Thus, DHFR may have a potential relationship with lipid accumulation.

**Effects of MTX, PYR and TRI on PUFA synthesis**

To explore the relationship between the DHFR and lipid synthesis, we investigated the effects of 5 mM MTX, PYR and TRI on the fatty acid status (Table S2). The *Mort. alpina* cultures grown in medium plus MTX, PYR and TRI showed reduced total fatty acid accumulation levels by about 33 %, 25 % and 27 %, respectively (Fig. 3). Of all the fatty acids, arachidonic acid – the main commercial product of *Mort. alpina* (Sakuradani *et al.*, 2009) – showed the most pronounced change.

**Expression and purification of DHFR**

To explore the role of DHFR in lipid biosynthesis, we expressed the recombinant enzyme as a His-tagged fusion protein in the BL21 DE3 gold strain of *E. coli* under the control of the T7 RNA polymerase/promoter system. With the use of a one-step purification process by nickel affinity column chromatography, DHFR was purified to electrophoretic homogeneity, with a single band on SDS-PAGE. DHFR encodes a polypeptide of 227 amino acids with a theoretical isoelectric point of 5.81. The molecular mass of DHFR was estimated to be about 30 kDa by SDS-PAGE (Fig. S1), which corresponds well with the calculated mass.

**Activity of DHFR**

To examine whether the purified protein showed DHFR activity, we determined the initial velocity of its FH2 consumption. The initial rate of FH2 consumption was dramatically increased by the addition of the recombinant protein,
indicating that the purified protein possesses DHFR activity (Fig. 4a). When BH$_2$ was used instead of FH$_2$, the initial rate of NADPH consumption was dramatically decreased by the addition of the recombinant protein, indicating that the purified protein can also convert BH$_2$ into BH$_4$ (Fig. 4b). The reaction products were also confirmed by LC–MS, as shown in Fig. 4. Extracted ion chromatogram (XIC) extraction (444.16 m/z, FH$_4$) of the DHFR reaction product presented a peak at 9.0 min (Fig. 4e), corresponding well to the position of the FH$_4$ standard (Fig. 4c). XIC extraction (242.12 m/z, BH$_4$) of the DHFR reaction product presented a peak at 8.2 min (Fig. 4f), corresponding well to the position of the BH$_4$ standard (Fig. 4d). These results confirm that the recombinant protein shows DHFR activity.

**Effects of temperature, pH and metal ions on the activity of DHFR**

DHFR was active at each of the temperatures tested (in the range 12–55 °C), with the greatest activity detected at 37 °C (Fig. 5a). The DHFR activity seemed to be relatively sensitive to temperature. It showed high relative activity with >50% of maximum activity in the range 20–37 °C; however, the relative activity was significantly outside of this range. DHFR retained 60% of its initial activity after 1.5 h of exposure at 20 °C, retained 70% of its initial activity after 0.5 h of exposure at 37 °C and lost more than 90% of its initial activity after 0.5 h of exposure at 45 °C (Fig. 5c). DHFR lost nearly 100% of its activity after 0.5 h of exposure above 55 °C (Fig. 5c). This indicated that DHFR was unstable at high temperature.

The optimum pH for this enzyme was about pH 10.0 (Fig. 5b), which is similar to the optimum pH for recombinant human DHFR (White et al., 2004). DHFR was most active at the higher pH values, and the activity at pH 5 was 40% of that at pH 10. The enzyme displayed a relatively wide pH spectrum and the pH effect was less dramatic. In addition, less than 45% activity of DHFR was retained after incubation at pH 3 at 4 °C for 2 h (Fig. 5d), indicating that DHFR was not stable under acidic conditions.

To investigate the effect of metal ions, we measured the relative activity of DHFR in the presence of different metal ions. As shown in Fig. 4(e) no metal ions were found to be necessary for the activity of DHFR. DHFR activity was inhibited by 52%, 46%, 58% and 57% by 10 mM Mg$^{2+}$, Cu$^{2+}$, Mn$^{2+}$ and K$^+$, respectively (Fig. 5e). The enzyme activity was completely inhibited in the presence of Co$^{2+}$ at 10 µM. When Fe$^{2+}$ or Na$^+$ was added at 10 mM, they completely inhibited DHFR activity. As with the enzymes from *Cand. albicans*, *Cryptococcus neoformans* and *E. coli* (Trapnell et al., 2010; Wright et al., 2002), both KCl and NaCl inhibit *Mort. alpina* DHFR, with 50% and 100% inhibition at 10 mM, respectively. This is in contrast to the DHFRs from *Mycobacterium tuberculosis*, *Halofax volanii* and *Plasmodium vivax* (Leartsakulpanich et al., 2002; White et al., 2004; Wright et al., 2002).

**Effects of inhibitors on the activity of DHFR**

The inhibitory properties of several clinically used antifolates against purified DHFR were shown in Fig. 5(f). DHFR activity was inhibited by 10 µM concentrations of a variety of inhibitors, including MTX (27% inhibition), TRI (31% inhibition) and PYR (27% inhibition) (Fig. 5f). MTX and PYR (0.1 mM) were more effective in reducing DHFR activity (both 100% inhibition) than TRI (75% inhibition). DHFR activity was completely inhibited by TRI at 1 mM. The inhibitory effects of MTX, TRI and PYR were also observed for DHFR in humans, *P. vivax*, *Myco. tuberculosis* and *Crypt. neoformans* and this inhibition is one of the hallmarks of DHFR activity (Leartsakulpanich et al., 2002; Ledley et al., 1987; Sirawaraporn et al., 1993a; White et al., 2004).

**Kinetic parameters of DHFR**

The kinetics of the reaction catalysed by DHFR present a good fit with the Lineweaver and Burk model (unpublished data). The value of $K_{m}$ and $V_{max}$ for FH$_2$ was determined to be 0.14 mM and 0.053 mM min$^{-1}$.

**Amino acid sequence alignment of Mort. alpina DHFR protein and other homologous proteins**

The sequence of *Mort. alpina* DHFR shares low but significant similarities with DHFR from *Mus musculus* (29.06%), *Homo sapiens* (28.26%), *E. coli* (25.41%), *Bombyx mori* (28.43%), *Drosophila melanogaster* (27.50%), *Heliothis vir-escens* (28.84%), *Oryctolagus cuniculus* (25.90%) and *P. vivax* (26.88%) at the amino acid level. Multiple sequence alignment of the *Mort. alpina* DHFR and other
characterized DHFR proteins revealed the presence of certain conserved motifs and residues (Fig. S2), including a motif Ile-Val-Ala(7–9) involved in contact with the 6-methylpteridine moiety of MTX; residue Trp24 that interacts with the carboxamide of NADPH; two residues (Ala31 and Arg81) involved in the MTX binary complex; residue Asp29 involved in the hydride transfer of NADPH; residue Leu26 in regions that form hydrogen bonds between Met20 and the F–G or G–H loops; three residues (Ile16, Ile22 and Ile145) that would overlap partially with atoms of the nicotinamide rings of NADPH (Liu et al., 2008; Schnell et al., 2004); a NADPH binding residue Asp175 that is involved in a critical hydrogen bonding interaction with the Met20 loop that stabilizes the closed active-site conformation (Schnell et al., 2004); four residues (Ile6, Met30, Phe33 and Tyr151) interacting with the PYR ring of the inhibitor (Liu et al., 2008; Paulsen et al., 2011); a dimethoxyphenyl ring fits nicely in a hydrophobic pocket composed of Met30, Thr67, Ile71, Pro72, Phe75 and Leu78 (Liu et al., 2008); and loop residues Thr67–Phe75 critical for ligand potency and selectivity (Paulsen et al., 2011). Previous evolutionary analysis of DHFRs from 233 species identified three prominent phylogenetically coherent events (PCEs), with strong implications regarding their importance in the preservation and divergence of enzyme functions through time (Liu et al., 2013). The most recent PCE region (23–36) represents a unique evolutionary hotspot with a well-defined deletional/insertional history (Liu et al., 2013). The timing for the development of this region in Mort. alpina is very clear: after tunicate and before E. coli. The second strong PCE found is the transition from Phe32 in human into Met30 in Mort. alpina, which occurred after fish and urchin and has persisted as phenylalanine ever since (Liu et al., 2013). The most ancient PCE identified occurs around 72–75 in Mort. alpina. This sequence differs in length across the main species, suggesting that this is a very ancient divergence (Liu et al., 2013). The phylogenetic analysis (Fig. S3) revealed that Mort. alpina DHFR was most closely related to the

![Graphs and diagrams showing spectrophotometry and LC-MS chromatographs of DHFR reaction products.](http://mic.microbiologyresearch.org)

**Fig. 4.** The spectrophotometry of DHFR reaction product at 340 nm for FH\textsubscript{2} (a) and BH\textsubscript{2} (b). LC–MS chromatographs: (c) XIC of the FH\textsubscript{4} standard (m/z 444.16); (d) XIC of the BH\textsubscript{4} standard (m/z 242.12); (e) XIC of the DHFR reaction product (m/z 444.16); (f) XIC of the DHFR reaction product (m/z 242.12).
corresponding gene in yeast *C. albicans*. The bacteria DHFR genes were located in a separate branch from other organisms. This suggests that DHFR genes from fungi and plasmodium may have evolved from the same ancestor.

**Effects of MTX, TRI and PYR on the expression levels of genes involved in folate metabolism**

In one-carbon folate metabolism, the FH$_4$ derivative methylene tetrahydrofolate is oxidized to 10-formyl-tetrahydrofolate by the bifunctional methylene tetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD) with the reduction of NADP$^+$ to NADPH. Real-time qPCR was performed to investigate the changes in the transcript levels of DHFR, MTHFD1 and MTHFD2 genes, which are involved in folate metabolism. Total RNA samples were extracted from the mycelia after 8 days of cultivation in modified Kendrick medium with the addition of MTX, TRI and PYR at 5 mM. Unexpectedly, none of these inhibitors had an impact on the transcript levels of DHFR (Fig. 2b). This may simply due to the fact that these

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*Fig. 5. Effect of temperature, pH, metal ions and GDP-L-fucose on the activity of DHFR.* (a) and (b) show the effect of temperature and pH on the activity of DHFR. The effects of temperature and pH on the stability of DHFR are represented in (c) and (d), respectively. The pH stability was analysed by preincubating the enzyme in buffers of different pH values at 4°C for 2 h and measuring the remaining activity at 37°C and pH 8.0. The effects of metal ions and GDP-L-fucose on DHFR activity are represented in (e) and (f), respectively. Values are means of three replicates ±SD. One enzyme unit is defined as the amount of purified enzyme (mg) required for the conversion of 75 µmol FH$_2$ into FH$_4$; 100% GMD2 activity represents 1.22 (a), 0.89 (b), 1.32 (c), 1.21 (d), 1.11 (e) or 1.33 (f) enzyme unit.
antifolates inhibit DHFR activity by binding to the enzyme both on the substrate and cofactor sites (Schnell et al., 2004). Compared to the samples grown in inhibitor-free medium, the expression of MTHFD2 was significantly down-regulated (Fig. 2b). By contrast, the addition of exogenous inhibitors leads to an up-regulation in the expression levels of MTHFD1 (Fig. 2b). Subcellular localization analysis (Yu et al., 2006) indicated that MTHFD1 is located in mitochondria and that MTHFD2 is located in cytoplasm. The two isoforms may be involved in different aspects of NADPH generation depending on their intracellular localization.

**DISCUSSION**

DHFRs from various sources can differ markedly in their affinities for DHFR inhibitors. DHFR inhibitors are in wide use as antibacterial and antiprotozoal agents. Inhibition studies revealed that DHFRs from human, bacterium, and protozoa were highly susceptible to MTX, TRI, and PYR (Table 2) (Leartsakulpanich et al., 2002; Ledley et al., 1987; Sirawaraporn et al., 1993a; White et al., 2004). However, as previously reported for DHFRs from yeast *Cand. albicans* (Baccanari et al., 1989), *Mort. alpina* DHFR was less susceptible to MTX, TRI and PYR inhibitors than human, bacterium, and protozoa DHFRs, with IC$_{50}$ values in the micromolar (µM) range (Table 2). Ser58 and Ser117 are responsible for antifolates resistance in *P. vivax* DHFR, and the mutation in these residues increased resistance to DHFR inhibitors (Leartsakulpanich et al., 2002). Neither of these residues is conserved in *Mort. alpina* DHFR, which may account for the fact that MTX, TRI and PYR were not effective inhibitors of DHFR from *Mort. alpina*. Leu22, Met30, Thr67, Phe75, Ser71, Pro72, Ile73 and Ile145 involved in TYR interactions were conserved in both *Mort. alpina* and *Cand. glabrata* DHFRs, which may explain that these two enzymes have similar IC$_{50}$ values of TYR (Liu et al., 2008). Moreover, these inhibitors do not show selectivity in inhibition of *Mort. alpina* DHFR versus the human, bacterium, protozoa and yeast enzyme. The lack of potency and selectivity of these commonly used antifolates points to the need for new inhibitors. Since numerous DHFR inhibitors have so far been synthesized, it is possible that such agents already exist and would be uncovered by an appropriate screening program. With the characterization of *Mort. alpina* DHFR, it is now feasible to pursue such studies.

**Table 2.** IC$_{50}$ values of DHFR inhibitors to various DHFR

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th><em>Mort. alpina</em></th>
<th><em>Human</em>†</th>
<th><em>P. vivax</em>†</th>
<th><em>Crypt. neoformans</em>†</th>
<th><em>Cand. albicans</em>†</th>
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<tr>
<td>MTX</td>
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<td>ND</td>
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<tr>
<td>TRI</td>
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<td>3.02 µM</td>
<td>50 µM</td>
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<tr>
<td>PYR</td>
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<td>1.68 µM</td>
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</table>

*ND*, Not determined.
†Baccanari et al. (1989), Leartsakulpanich et al. (2002) and Sirawaraporn et al. (1993a).
generated by MTHFD1 cannot be transported to the cytosol for fatty acid synthesis. The down-regulation of MTHFD2 by DHFR inhibitors suggests that cytosolic NADPH produced by folate metabolism was decreased. As the cytoplasm has considered to be the major site of fatty acid synthesis for a long time been (Rous, 1971), these results highlight the functional significance of folate metabolism in lipid metabolism. The relationship between DHFR and lipid metabolism is thus of major importance, and folate metabolism may be an alternative NADPH source in oleaginous fungi. Our identification of a DHFR enzyme involved in the BH₄ salvage pathway and folate metabolism extends the range of target candidates for genetic manipulation in Mort. alpina for obtaining strains with increased amounts of lipids.

In conclusion, the BH₄ salvage pathway has been characterized at the molecular level in a fungus, Mort. alpina. The relationship between DHFR and lipid metabolism is of major importance, and folate metabolism may be an alternative NADPH source in fatty acid synthesis. RNAi of the DHFR gene should be utilized in future work to explore the relationship between BH₄/folate metabolism and lipid biosynthesis.

ACKNOWLEDGEMENTS

This study was supported in part by the National Science Foundation of China (31400038, 31530056 and 31471128), the Program for New Century Excellent Talents (NCET-13-0831) and the Program for Changjiang Scholars and Innovative Research Team in University (IRT1249). This study is supported by program of ‘Collaborative innovation center of food safety and quality control in Jiangsu Province’.

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Edited by: A. H. Herrera-Estrella and V. J. Cid

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