Isolation of the *Pneumocystis carinii* dihydrofolate synthase gene and functional complementation in *Saccharomyces cerevisiae*

Philippe M. Hauser[1,*] & Ian G. Macreadie[2]

[1]Institute of Microbiology, University Hospital of Lausanne, Lausanne, Switzerland and [2]CSIRO, Molecular and Health Technologies, Parkville, Victoria, Australia

Correspondence: Philippe M. Hauser, Institute of Microbiology, University Hospital of Lausanne, 1011 Lausanne, Switzerland. Tel.: +41 21 314 40 84; fax +41 21 314 40 60; e-mail: Philippe.Hauser@chuv.ch

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Abstract

The *Pneumocystis carinii* gene encoding the enzyme dihydrofolate synthase (DHFS), which is involved in the essential biosynthesis of folates, was isolated from clones of the *Pneumocystis* genome project, and sequenced. The deduced *P. carinii* DHFS protein shares 38% and 35% identity with DHFS of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively. *P. carinii* DHFS expressed from a plasmid functionally complemented a *S. cerevisiae* mutant with no DHFS. Comparison of available DHFSs with highly similar folylpolyglutamate synthases allowed the identification of potential signatures responsible for the specificities of these two classes of enzymes. The results open the way to experimentally analyse the structure and function of *P. carinii* mono-functional enzyme DHFS, to investigate a possible role of DHFS in the resistance to antifolates of *P. jirovecii*, the species infecting specifically humans, and to develop a new class of antifolates.

Introduction

The utilization of folate is essential in all living organisms for the biosynthesis of crucial cellular components. For some organisms, folate is obtained in the diet, while for others (microbes and plants) folate is obtained through biosynthesis (Fig. 1). The final step in biosynthesis, the enzymatic addition of glutamate to dihydropteroate to make dihydrofolate, is carried out by dihydrofolate synthase (DHFS). A highly similar enzyme, folylpolyglutamate synthase (FPGS), carries out the addition of multiple glutamates onto folate to make polyglutamated folate, a highly stable form of folate.

The specificity of these enzymes for particular or multiple reactions is remarkable. For example, in humans there is only one enzyme of this class, hFPGS, and it has no DHFS activity since humans do not synthesize their own folate (Garrow *et al.*, 1992). Likewise, the bacterium *Lactobacillus casei* obtains its folate from milk and only possesses a FPGS (Cody *et al.*, 1992). However, Salcedo *et al.* (2001) found that the protozoan malaria parasite *Plasmodium falciparum* is like the bacterium *Escherichia coli*, whose *FOLC* gene encodes a single polypeptide enzyme that is bifunctional in being able to be both a FPGS and a DHFS (Bognar *et al.*, 1985). *Saccharomyces cerevisiae* on the other hand is different again. It requires both activities but is unique in having them encoded on two different genes, *FOL3*, which encodes DHFS, and *MET7* which encodes FPGS (Cherest *et al.*, 2000). Interestingly, there is yet another similar protein in yeast, encoded by YKL132c, whose function remains unknown (*Saccharomyces* genome database, http://www.yeastgenome.org). The high degree of similarity of these three subgroups of enzymes is seen in their alignment shown in Fig. 2.

How does such a protein have such narrow or broad specificities? Ways to answer this question may come from detailed structure function studies, mutagenesis and from the knowledge of enzymes from more species. In this study, we have sought to isolate the DHFS gene of *Pneumocystis carinii*, the *Pneumocystis* species infecting specifically rats. Of further interest in *P. carinii* DHFS is the fact that it lies in the folate biosynthesis pathway between dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR), the major targets of chemotherapy for pneumonia caused by *P. jirovecii*, the *Pneumocystis* species infecting specifically humans. Recent work has identified roles of polymorphisms of DHPS (Lane *et al.*, 1997; Nahimana *et al.*, 2003; Iliades *et al.*, 2004, 2005; Meneau *et al.*, 2004) and of DHFR (Nahimana *et al.*, 2004) in sulfa drug resistance of *Pneumocystis* but the possible role of DHFS has not been investigated

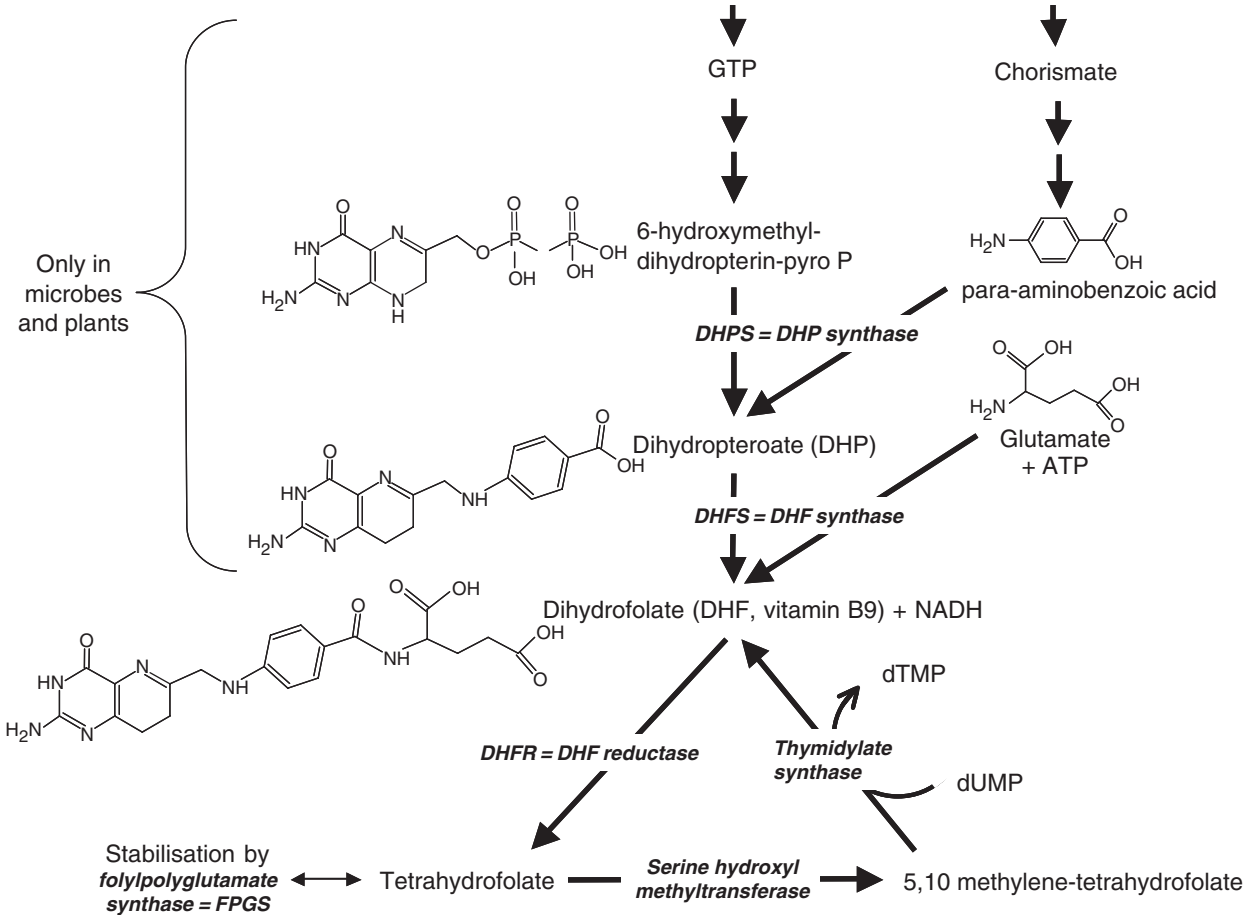

Fig. 1. Folate biosynthesis and utilization pathways. Tetrahydrofolate and derivatives are involved in synthesis of adenine, histidine, methionine, as well as DNA metabolism.

because the gene was not available. Moreover, DHFS may become a target for a new class of antifolate drugs. Thus this study lays the foundation for much more work.

Materials and methods

Pneumocystis carinii genomic DNA library

Information on sequences and clones of the *Pneumocystis* genome project were obtained from http://pgp.cchmc.org. Relevant clones LUCc35p62 and LUCc2p5c have been obtained from M.T. Cushion (University of Cincinnati). Theses clones had been derived from amplified *Pneumocystis carinii* DNA and cloned into pSMART© vectors (www.Lucigen.com).

Strains, growth, transformation and complementation tests

Escherichia coli DH5α (Life Technologies, Basel, Switzerland) was employed for sub-cloning. It was made competent using the method of Chung & Miller (1988), stored as frozen aliquots at −80 °C, and transformed for resistance to 35 μg mL^{-1} ampicillin on solid LB medium (1% weight in volume (w/v) Difco tryptone, 0.5% Difco yeast extract, 1% NaCl, 2% Gibco agar).

LCY1 (*Mat***a** *leu2-3,112 trp1 tup1 ura3-52 FOL3::URA3*) is a *Saccharomyces cerevisiae* strain that has a disruption of *FOL3* which encodes DHFS (Bayly *et al.*, 2001). In the absence of folate synthesis, strain LCY1 requires the pathway end products methionine, adenine, histidine, and thymidine monophosphate. It was grown in rich medium YEPD (1% w/v Difco yeast extract, 2% w/v Difco peptone, 2% glucose) supplemented with thymidine monophosphate (100 μg/mL). Yeast transformations utilized the one-step method described by Chen *et al.* (1992). TRP+ transformants of LCY1 were selected on solid yeast nitrogen base medium (YNB, 0.67% w/v yeast nitrogen base, 2% glucose, 2% Gibco agar) supplemented with leucine (20 μg mL^{-1}), methionine (20 μg mL^{-1}), adenine (20 μg mL^{-1}), histidine (20 μg mL^{-1}), and thymidine monophosphate (100 μg mL^{-1}). Transformants were then streaked on YEPD lacking thymidine monophosphate to check complementation of the disrupted *FOL3*.

DNA amplification and cloning

The DHFS gene from 109 nt upstream of the start codon to 66 nt downstream of the stop codon was amplified from *P. carinii* genomic DNA (rat 876/4–1997, kindly provided by the late A.E. Wakefield, University of Oxford) using proof-reading Expand Taq polymerase (Boeringher), 3mM $MgCl_2$, and primers 5′-CAA GTG CTT GAT AAT TCC-3′ and 5′-GTA TGT ACT TAA ACA GCG TTA TCA C-3′ which were synthesized by Microsynth (Baglach, Switzerland). PCR conditions included a denaturation for 3 min at 94 °C, followed by 40 cycles consisting of 30 s at 94 °C, 30 s at 48 °C, and 1 min 72 °C. The reaction ended with a 5 min of extension at 72 °C. The PCR yielded a 1467 bp fragment, which was analyzed by electrophoresis in a 1.0% agarose gel containing ethidium bromide (0.5 μg mL^{-1}).

For cloning, the full-length coding region of the *P. carinii* DHFS (1284 nt) was amplified from *P. carinii* genomic DNA using proof-reading Expand Taq polymerase (Boehringer, Basel, Switzerland), a final concentration of 3mM $MgCl_2$, and primers 5′-CGC GGA TCC AAA AAT GTT AGT AAA GTT GGG-3′ and 5′-CCC CCC CCG TCG ACT TAT TAT TAT TAT TTT CTA-3′, creating unique *Bam*HI and *Sal*I restriction sites in the PCR product (underlined in primers). The PCR yielded a 1311 bp fragment which was digested with *BamH*I and *Sal*I, cloned under the control of the strong promoter of *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase isozyme 3 gene (*TDH3*) into the expression vector p414GPD (Mumberg *et al.*, 1995). Minipreparation of plasmid DNA was performed according to Del Sal *et al.* (1988) and the plasmid was selected in *S. cerevisiae* LCY1 by transformation for tryptophan prototrophy.

DNA sequencing

Both strands of the PCR products were directly sequenced using a Big Dye Terminator DNA sequencing kit and an ABI PRISM 3100 automated sequencer (both from Perkin-Elmer Biosystems, Rotkreuz, Switzerland). The following primers within the *P. carinii* DHFS gene were used: 5′-CAA GTG CTT GAT AAT TCC-3′ (nt 109–127 upstream of the start codon), 5′-AGC AAG GGA AAA ATC CGG-3′ (nt 486–504 downstream of the start codon), and 5′-TGT GGA TTC CCA AGA TAC-3′ (nt 62–44 downstream of the start codon).

Results

Isolation and analysis of the *Pneumocystis carinii* DHFS gene

Clones expected to contain portions of *Pneumocystis carinii* DHFS gene were identified from annotations containing

```
N.g DHFS-FPGS       ------------------------------------------------------------
E.c DHFS-FPGS       ------------------------------------------------------------
M.t DHFS-FPGS       ------------------------------------------------------MNS
Pf  DHFS-FPGS       -------------------------MEKNQNDKSNKNDIIHMNDKSGNYDKNNINNFID
L.c FPGS            ------------------------------------------------------------
Human FPGS          ------------------------------------------------------------
S.c FPGS            MHKGKKNYPNLITSFRMNLKKIILNHDRFSHPERWKTNALLRFTFVYIKFLFDLMIIKNP
S.c DHFS            ------------------------------------------------------------


N.g DHFS-FPGS       --------------MKTLQDWLSHLETAHSGG------LIDMGLERVSEVKKRMNLTPQC
E.c DHFS-FPGS       -----MIIKRTPQAASPLASWLSYLENLHS-K------TIDLGLERVSLVAARLGVLKPA
M.t DHFS-FPGS       TNSGPPDSGSATGVVPTPDEIASLLQVEHLLDQRWPETRIDPSLTRISALMDLLGSPQRS
P.f DHFS-FPGS       KNDEHDMSDILHKINNEEKKYEEIKSYSECLELLYKTHALKLGLDNPKKLNESFGHPCDK
L.c FPGS            -----------MNYTETVAYIHSFPRLAKTGDHRR-----------ILTLLHALGNPQQQ
Human FPGS          -----MEYQDAVRMLNTLQTNAGYLEQVKRQRGDPQ--TQLEAMELYLARSGLQVEDLDR
S.c FPGS            LRMVGKTYRDAVTALNSLQSNYANIMAIR-QTGDRKNTMTLLEMHEWSRRIGYSASDFNK
S.c DHFS            ------------------------------------MAIELGLSRITKLLEHLGNPQNS

                           ---P---                        ----Ω----
N.g DHFS-FPGS       P-VVVVAGTNGKGSVCAYLTQIYKQAG---FKTGTLTSPHLLHYNERIAINAEPVSDDTI
E.c DHFS-FPGS       PFVFTVAGTNGKGTTCRTLESILMAAG---YKVGVYSSPHLVRYTERVRVQGQELPESAH
M.t DHFS-FPGS       YPSIHIAGTNGKTSVARMVDALVTALH---RRTGRTTSPHLQSPVERISIDGKPISPAQY
P.f DHFS-FPGS       YKTIHIAGTNGKGSVCYKIYTCLKIKK---FKVGLFSSPHIFSLRERIIVNDEPISEKEL
L.c FPGS            GRYIHVTGTNGKGSAANAIAHVLEASG---LTVGLYTSPFIMRFNERIMIDHEPIPDAAL
Human FPGS          LNIIHVTGTKGKGSTCAFTECILRSYG---LKTGFFSSPHLVQVRERIRINGQPISPELF
S.c FPGS            LNIVHITGTKGKGSTAAFTSSILGQYKEQLPRIGLYTSPHLKSVRERIRINGEPISEEKF
S.c DHFS            LRVLHIAGTNGKGSVCTYLSSVLQQKS---YQIGKFTTPHLVHVTDSITINNKPIPLERY
                         ::** ** :                  *  ::* :    : : :: : :

N.g DHFS-FPGS       IASFERIEAARGEISLT--------------YFEFNALAAVDIFIREQVDVMILEVGLGG
E.c DHFS-FPGS       TASFAEIESARGDISLT--------------YFEYGTLSALWLFKQAQLDVVILEVGLGG
M.t DHFS-FPGS       VATYREIEPLVALIDQQ-SQASAGKGGPAMSKFEVLTAMAFAAFADAPVDVAVVEVGMGG
P.f DHFS-FPGS       IHLVNEVLNKAKKLYIN------------PSFFEIITLVAFLHFLNKKVDYAIIETGIGG
L.c FPGS            VNAVAFVRAALERLQQQ-------QADFNVTEFEFITALGYWYFRQRQVDVAVIEVGIGG
Human FPGS          TKYFWRLYHRLEETKD--GSCVSMPP-----YFRFLTLMAFHVFLQEKVDLAVVEVGIGG
S.c FPGS            AKYFFEVWDRLDSTTSSLDKFPHMIPGSKPGYFKFLTLLSFHTFIQEDCKSCVYEVGVGG
S.c DHFS            QNIRLQLEALNKSHSLK------------CTEFELLTCTAFKYFYDVQCQWCVIEVGLGG
                          :                        *   :      *       : * *:**

N.g DHFS-FPGS       RLDAVNGFD-G--DCAVVTSVDLDHQAFLGDTVEQVGFEKAGVFRSG------KPAICGQ
E.c DHFS-FPGS       RLDATNIVD-A--DVAVVTSIALDHTDWLGPDRESIGREKAGIFRSE------KPAIVGE
M.t DHFS-FPGS       RWDATNVIN-A--PVAVITPISIDHVDYLGADIAGIAGEKAGIITRAPDGSPDTVAVIGR
P.f DHFS-FPGS       RLDATNILTKP--EVIVITSIGYDHLNILGDNLPIICNEKIGIFKKD-----------A
L.c FPGS            DTDSTNVIT-P--VVSVLTEVALDHQKLLGHTITAIAKHKAGIIKRG------IPVVTGN
Human FPGS          AYDCTNIIRKP--VVCGVSSLGIDHTSLLGDTVEKIAWQKGGIFKQG------VPAFTVL
S.c FPGS            ELDSTNIIEKP--IVCGVTLLGIDHTFMLGDTIEEIAWNKGGIFKSG------APAFTVE
S.c DHFS            RLDATNVIPGANKACCGITKISLDHESFLGNTLSEISKEKAGIITEG------VPFTVID
                      *  *           :: :  **   **     :   * * ::
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Fig. 2. Multiple sequence alignment of *Saccharomyces cerevisiae* dihydrofolate synthase (DHFS) against other DHFS, folylpolyglutamate synthase (FPGS), and DHFS–FPGS enzymes. The *S. cerevisiae* DHFS protein (accession number NP_013831) sequence is aligned with *Neisseria gonorrhoeae* DHFS–FPGS (CAA92428), *Escherichia coli* DHFS–FPGS (AAC75375), *Mycobacterium tuberculosis* DHFS–FPGS (AAK46822), *Plasmodium falciparum* DHFS–FPGS (CAD52396), *Lactobacillus casei* FPGS (P15925), human FPGS (Q05932), and *S. cerevisiae* FPGS protein (AAT92837) sequences using the Biology WorkBench program (http://workbench.sdsc.edu). The identical and strongly conserved residues are indicated respectively by asterisks and double points. Dashes indicate missing residues. Also shown is the P loop, the W loop and the linker that connects the N- and the C-domain.

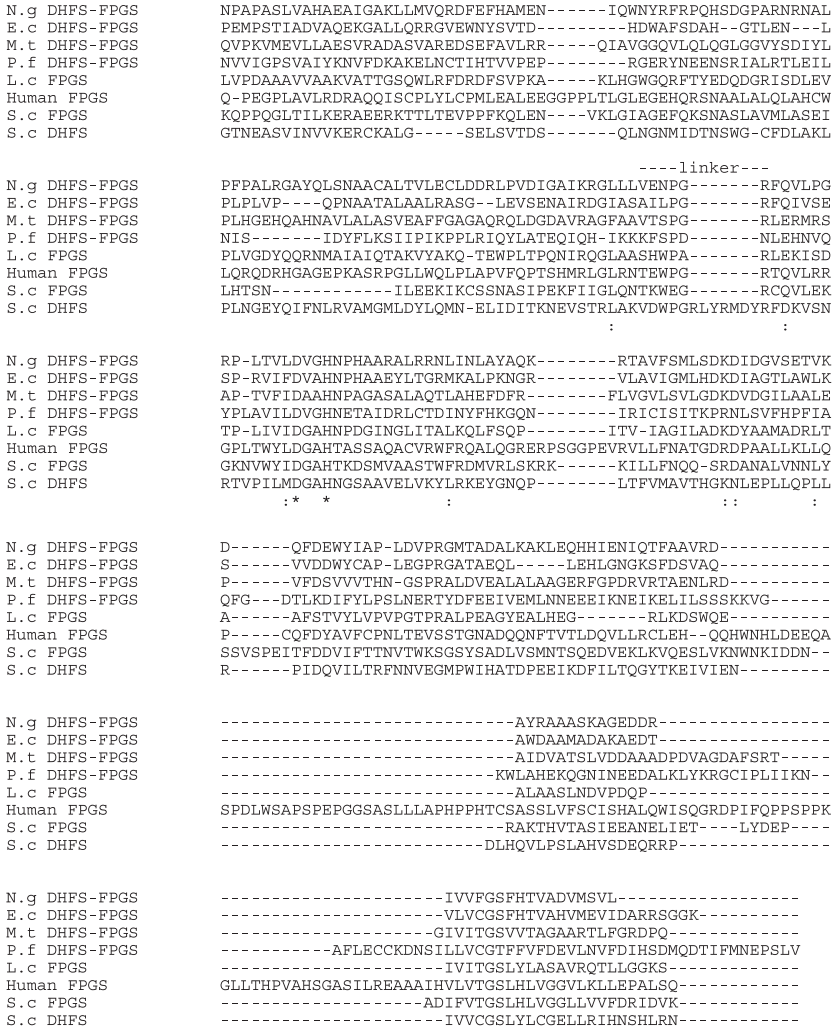

Fig. 2. Continued.

'FPGS' or 'DHFS' in the *Pneumocystis* genomic database of the genome project (http://pgp.cchmc.org). In order to identify those most likely to include the true *P. carinii* DHFS gene, the sequences of these clones were subjected to BLAST searches against databases of NCBI, as well as against *Saccharomyces cerevisiae* (http://www.yeastgenome.org) and the *Schizosaccharomyces pombe* (http://www.genedb.org/genedb/pombe/index.jsp) genomic databases. Some clones appeared to be more similar to bacterial sequences and were excluded from further analyses. Two clones, LUCc35p62 and LUCc2p5c, contained sequences that were considered to be portions of the *P. carinii* DHFS gene. The sequences in these plasmids overlapped and were estimated to encode the first half of the DHFS. Further detailed mapping by restriction digests and PCR amplifications indicated that the insert in LUCc35p62 was likely to encode the entire DHFS sequence. The sequence of the second half of DHFS was obtained by 'walking' through the insert of LUCc35p62.

The clones from the genome sequencing project potentially carried PCR-induced errors because they were obtained by amplification using a DNA polymerase without a proofreading activity (M.T. Cushion, pers. commun.). To avoid errors, we amplified the DHFS gene using primers derived from the sequence of clone LUCc35p62 from purified *P. carinii* genomic DNA using a proofreading polymerase and sequenced the gene present on the PCR product. The genomic sequence gave six nonsynonymous differences when compared with the sequences present in the *Pneumocystis* genome project clones. The *P. carinii* DHFS sequence from genomic DNA has been deposited in GenBank of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), accession number DQ128176. The putative translation product, *P. carinii* DHFS of 424 amino acids, is shown in Fig. 3. At position 149–146 nt upstream of the putative start codon, is a putative TATA box

```
S. c DHFS          MAIELGLSRITKLLEHLGNPQNSLRVLHIAGTNGKGSVCTYLSSVLQQKSYQIGKFTTPH
S. p DHFS          MPIQLGLQRMLQLLKHLGNPQESFCAVQIAGTNGKGSICSYIYTSLLQAAIKTGRYTSPH
P. c DHFS          MLVKLGLLRIRQLLKYLGNPQNSFQAVHVAGTNGKGSVCAYLSSCLALSGIRVGQYCSPH
                   * ::*** *: :**::*****:*:  :::********:*:*: : *     : *:: :**

S. c DHFS          LVHVTDSITINNKPIPLERYQNIRLQLEALNKSHSLKCTEFELLTCTAFKYFYDVQCQWC
S. p DHFS          FLEPRDTISINGQIASEEIFNTCWKQVIEVDRRFRTKATEFELLTATAFQCFHHSGVRVA
P. c DHFS          LIDRWDCVKVIGRDIDKHQFLEIESKIKNLNQRCNIGATEFEIMTAVAFEILSKNNVELA
                   ::   * : :  :      :     ::  :::      ****::*  **: :

S. c DHFS          VIEVGLGGRLDATNVIPGANKACCGITKISLDHESFLGNTLSEISKEKAGIITEGVPFTV
S. p DHFS          VIETGMGGRLDATNVFEEP--VLSIISRICLDHQAFLGNTLEAIAKEKAGIFKKNVP-CV
P. c DHFS          VIETGVVGRLDATNVLSQV--LLTIITKISMDHQELLGNTIQKIAREKSGIMKKNIP-CI
                   *** *: ********:         *::* :**: :****:  *::**:**  : :*  :

S. c DHFS          IDGTNEASVINVVK---ERCKALGSELSVTDSQLNGN--MIDTNSWGCFDLAKLPLNGEY
S. p DHFS          VDGLNEVNVLNQLKLSAEETRAHPFYLAKGKSGENKNEWIINTPNWGTNTFS-TPLKGDY
P. c DHFS          VDGTNEDSVLKVIK--EESIKSGSSRVILTPMDLDKS---LYIQEWKKHEFK-TSLYRTY
                   :** **  *:: :*   *  ::    :       :     :    *    :    *   *

S. c DHFS          QIFNLRVAMGMLDYLQMNELIDITKNEVSTRLAKVDWPGRLYRMDYRFDKVSNRTVPILM
S. p DHFS          QGQNLACAVTALDILSS--SFSIMLPHVQNGVKNTSWPG---RLDIRSVPSLG---DILF
P. c DHFS          QRTNLACVSASLEILSKY-YPKITPDILSKGLLETYWPGRLEWIDLSQIAFGAN--KILL
                   *  **      *: *       *    :   : :  ***    :*            **:

S. c DHFS          DGAHNGSAAVELVKYLRKEYG--NQPLTFVMAVTHGKNLEPLLQPLLRPIDQVILTRFNN
S. p DHFS          DGAHNKEAAIELAKFVNSQRREHNKSVSWVVAFTNTKDVTGIMKILLRKGDTVIATNFSS
P. c DHFS          DGAHNIDGINSLSEYINSIRNG-VQSVSWLTAFTQGKDVDSLLSILLKPYDKIHSVEFEP
                   *****      * :::        : :::: * *: *::  ::  **:  * :    *

S. c DHFS          VEGMPWIHATDPEEIKDFILTQGYTKEIVIENDLHQVLPSLAHVSDEQRRPIVVCGSLYL
S. p DHFS          VSGMPWIKSMEPEVIKNSISSESSVECYTADN---LTISEILRLAKEKNSSVIVCGSLYL
P. c DHFS          VDGMQWIKPVNSSEIAKIARKYLYEENVKQHG--TDLLSAIRSISQDK-GLQVICGSLYL
                   * ** **:  :   *          :           :  :  :: ::    ::******

S. c DHFS          CGELLRIHNSHLRN------
S. p DHFS          LGDMYRYLKLDV--------
P. c DHFS          IGQVHRLLHKRILLQKGSRK
                    *:: *  :  :
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Fig. 3. Alignment of *Pneumocystis carinii* dihydrofolate synthase (DHFS) with *Saccharomyces cerevisiae* DHFS and putative *Schizosaccharomyces pombe* DHFS.

and a series of four stop codons is present at the end of the ORF.

The predicted translation product of the *P. carinii* ORF bears a high degree of similarity with the DHFS of *S. cerevisiae*, as well as with the putative DHFS of the closest known existing relative of *P. carinii*, *S. pombe*, which is present in the genomic database (Fig. 3). Those two proteins exhibit 39% identity with each other. The *P. carinii* product shares 35 and 38% identity with DHFS of *S. cerevisiae* and *S. pombe*, respectively. By contrast, the identity with *S. cerevisiae* FPGS is only 20%.

Functional complementation of a yeast fol3 deletant with the *Pneumocystis carinii* DHFS

The *P. carinii* DHFS ORF was cloned into the centromeric expression vector p414GPD under the control of the strong promoter of the gene encoding *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase isozyme 3 (*TDH3*). This construct, p414GPD.PcDHFS, as well as p414GPD were transferred into the yeast *fol3* deletant, LCY1. LCY1 [p414GPD.PcDHFS] but not LCY1 [p414GPD] was capable of growth in the absence of thymidine monophosphate on rich medium (Fig. 4), as well as on minimal medium lacking folates or lacking folate pathway end products (methionine, adenine, histidine, and thymidine monophosphate). This result indicated that the *P. carinii* DHFS encoded by p414GPD.PcDHFS was able to functionally replace the *S. cerevisiae* DHFS.

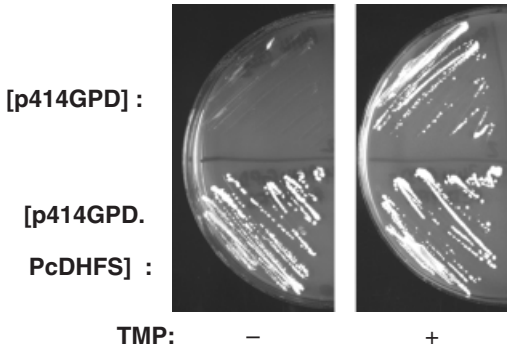

Fig. 4. Complementation of *Saccharomyces cerevisiae* LCY1 with disrupted dihydrofolate synthase (DHFS) by expression of *Pneumocystis carinii* DHFS gene on p414GPD plasmid. Cells were grown on rich YEPD medium with or without TMP supplementation for 5 days at 30 °C.

Discussion

The major outcome of this study is the cloning of the DHFS gene of *Pneumocystis carinii*. Sequences encoding portions of DHFS were identified in the database of the *Pneumocystis* genome project and the appropriate clones were sequenced. Because these clones were obtained by amplification without a proofreading enzyme, we re-amplified the gene from *P. carinii* genomic DNA and re-sequenced the DHFS gene. The new sequence had six nonsynonymous polymorphisms when compared to the sequences in the clones of the genome project. We consider it unlikely that such a large

number of sequence changes are due only to PCR-induced errors and that some may correspond to real polymorphisms between *P. carinii* isolates. Comparison to other DHFS sequences suggested that three of these six polymorphisms may have been PCR-induced errors because they affected highly conserved amino acids.

The sequence encoding *P. carinii* DHFS is typical of other eukaryotic sequences in having a TATA box 149–146 nt upstream of the start codon. However, it is unlike most *Pneumocystis* genes in that introns are absent. To our knowledge, this is the first *Pneumocystis* gene to have four consecutive and identical stop codons (TAA) at the end of the ORF. Such tandem stop codons have been reported in *S. cerevisiae* where they are considered to be maintained by selection and to constitute a backup to terminate protein translation if the first stop codon is read by a wrong tRNA (Liang *et al*., 2005).

To examine its functionality, we cloned the *P. carinii* DHFS gene into a vector for expression in a *Saccharomyces cerevisiae* DHFS deletant. The mutant was complemented, showing that the *P. carinii* gene encodes the DHFS function. So far, only three other *P. carinii* genes have been shown to functionally complement *S. cerevisiae*; they are *MAPK* (Fox & Smulian, 1999), *DHFR* (Brophy *et al*., 2000), and *CBK1* (Kottom & Limper, 2004). DHFS enzymes are required in the cytoplasm of eukaryotes and do not require an N-terminal targeting signal to migrate into another compartment of the cell. In contrast, eukaryotic FPGS enzymes are found both in the cytoplasm and in the mitochondrion and require a mitochondrial targeting signal (an amphipathic helix; Appling, 1991). Comparison of the *P. carinii* DHFS protein to *S. cerevisiae* DHFS and FPGS proteins (Figs 2 and 3) shows that *P. carinii* DHFS lacks the mitochondrial targeting signal, strongly suggesting that *P. carinii* DHFS has no FPGS activity. To further assess this assumption, we have tried to complement a *S. cerevisiae* FPGS deletant. However, several attempts with various vectors and promoters have not yielded FPGS complementation (data not shown). Although this negative result is not conclusive, it is consistent with *P. carinii* DHFS having no FPGS function. Furthermore, the *P. carinii* DHFS shares much more identity with DHFS than FPGS of *S. cerevisiae* (35 vs. 20%). Thus, it seems likely that *P. carinii* sequence encodes a mono-functional DHFS enzyme.

Does the *P. carinii* DHFS help in understanding what gives specificity to a DHFS versus an FPGS, or a DHFS-FPGS ? By performing multiple protein sequence alignments of enzymes of the two sub-groups DHFS and FPGS we can now start to elucidate a little more about the specificities. The first mono-functional DHFS which has been clearly identified in functional studies is that of *S. cerevisiae* (Patel *et al*., 2002, 2003), the second member of this subgroup is the *P. carinii* DHFS isolated here. The DHFS from at least 10 other fungi are present in the databases, mainly from ongoing genome sequencing projects, but they remain putative DHFS because they are only inferred by sequence similarity. These include those from *Schizosaccharomyces pombe*, *Aspergillus fumigatus*, *Ashbya gossypii*, *Aspergillus niger*, *Candida albicans*, *Candida glabrata*, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Neurospora crassa*, and *Yarrowia lipolytica*. In order to identify the conserved amino acids or block of amino acids responsible for the specificity of DHFSs and FPGSs, we have aligned the 12 fungal DHFSs with the 15 FPGSs from the same 12 fungi as well as from *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Homo sapiens* (figure available from the authors). These analyses revealed amino acids changes within blocks conserved among both DHFSs and FPGSs, as well as amino acids and blocks of amino acids conserved only in DHFSs or in FPGSs. Site-directed mutagenesis will help understanding which residues are the most important for DHFS and FPGS specificities.

Full elucidation of the specificity of a DHFS will require the determination of its three-dimensional structure, an analysis, which is in progress. This will also allow the design of specific inhibitors. The recently released three-dimensional structure of *E. coli* DHFS-FPGS (Mathieu *et al*., 2005) will also prove helpful. The investigation of a possible role of DHFS in drug resistance of *P. jirovecii* will require the isolation of the gene of this *Pneumocystis* species from clinical specimens of patients.

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