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Functional characterization of the *Pneumocystis jirovecii* potential drug targets *dhfs* and *abz2* involved in folate biosynthesis

Running title: *P. jirovecii* potential drug targets *dhfs* and *abz2*

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Abstract

Pneumocystis species are fungal parasites colonizing mammal lungs with strict host specificity. *Pneumocystis jirovecii* is the human specific species and can turn into an opportunistic pathogen causing severe pneumonia in immuno-compromised individuals. This disease is nowadays the second most frequent life-threatening invasive fungal infection worldwide. The most efficient drug co-trimoxazole presents important side effects, and resistance towards this drug is emerging. The search of new targets for the development of new drugs is thus of utmost importance. The recent release of the *P. jirovecii* genome sequence opens a new era for this task. It can now be carried out on the actual targets to inhibit, and no more on those of the relatively distant model *Pneumocystis carinii*, the species infecting rats. We focused on the folic acid biosynthesis pathway because (i) it is widely used for efficient therapeutic intervention, and (ii) it involves several enzymes which are essential for the pathogen and which have no human counterparts. In this study, we report the identification of two such potential targets within the genome of *P. jirovecii*, the dihydrofolate synthase (*dhfs*) and the aminodeoxychorismate lyase (*abz2*). The function of these enzymes was demonstrated by the rescue of the null allele of the orthologous gene of *Saccharomyces cerevisiae.*

Introduction

Pneumocystis organisms are extracellular fungi that colonize the lungs of mammals (1, 2). Each species displays strict host specificity for a given mammalian species. These fungi are thought to be obligate biotrophic parasites whose evolution has been marked by gene losses (3, 4, 5, 6, 7). *Pneumocystis jirovecii* is the human specific species whose reservoir would be only humans (8). *P. jirovecii* can turn into an opportunistic pathogen that causes severe pneumonia in immuno-compromised individuals (*Pneumocystis jirovecii* pneumonia, PCP). This disease is nowadays the second most frequent life-threatening invasive fungal infection worldwide with above 400'000 annual cases (9).

The drug of choice for prophylaxis and treatment of PCP is currently co-trimoxazole, a combination of sulfamethoxazole and trimethoprim. The two latter drugs are inhibitors of the dihydropteroate synthase (DHPS) and the dihydrofolate reductase (DHFR), respectively. These two enzymes are involved in the biosynthesis of folic acid, a metabolite which is required for the biosynthesis of crucial cellular components. Organisms such as *Pneumocystis* and other lower eukaryotes can synthesize their own folic acid, whereas this compound is a vitamin obtained from food for mammals. Experiments in the rat animal model suggested that the anti-*Pneumocystis* activity of co-trimoxazole might be mainly due to sulfamethoxazole (10). The widespread use of co-trimoxazole for prevention of PCP since 1989 has been found to be correlated with an increase of the prevalence of specific mutations within the putative active site of DHPS, similar to those observed in other pathogens resistant to co-trimoxazole. These mutations were found to be associated with breakthrough of prophylaxis for PCP (11, 12, 13). The impact of these mutations on PCP treatment remains controversial, but a strong effect seems unlikely because it would have been detected even in

studies with small cohorts (14). However, isolates resistant to the high doses of co-trimoxazole used for treatment may emerge in the future. Co-trimoxazole presents also the disadvantage that it is associated with adverse effects in patients, such as intolerance and toxicity. Because of these drawbacks of the most efficient drug available, the development of new drugs against *P. jirovecii* is presently of utmost importance.

Although *P. jirovecii* is an important cause of mortality of immuno-compromised patients, there is still no *in vitro* long term culture method available for this pathogen. A novel system of co-culture on human pseudostratified airway epithelial cells has been recently described (15), but it remains to be widely established. The lack of a culture method complicates the identification of new drug targets in *P. jirovecii*. The strategy used so far has been to identify potential drug targets in the genome of *Pneumocystis carinii*, the species infecting rats, which was used as model (16, 17, 18, 19, 20). The existing antifungal agents and their targets in *P. carinii* have been recently reviewed (21). The function of the potential targets was then characterized by complementation of the deletion mutant of the orthologous gene in the model yeasts *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. This strategy proved useful but presents the drawback that *P. carinii* is relatively distant from *P. jirovecii*, with a mean divergence at the nucleotide level of ca. 20% (22). Although active sites are generally more conserved than the rest of the proteins, which may ensure development of drugs across species, the sensibility to drugs of the targets may vary between the two species. However, the recent release of the *P. jirovecii* genome sequence (23) opens a new era for the search of new drug targets against this pathogen. Indeed, it offers the opportunity to identify the actual targets to inhibit within the *P. jirovecii* genome, and no more those of the model *P. carinii.*

Therapeutic intervention inhibiting the biosynthesis of folic acid is used successfully against a number of human pathogens. Seven enzyme activities involved in this pathway are ideal drug targets for antimicrobial therapy because (i) they are essential for the life of the pathogen, and (ii) they have no mammalian ortholog, which favours drug specificity and thus reduction of secondary effects in patients. These enzymes are the following: GTP cyclohydrolase (GTP-CH), dihydroneopterin aldolase (DHNA), dihydropterin pyrophosphokinase (HPPK), DHPS, dihydrofolate synthase (DHFS), para-aminobenzoate synthase (ABZ1), and aminodeoxychorismate lyase (ABZ2) (Fig. 1; modified from 20). Only two enzymes have been targeted in this pathway so far: DHFR which has a human ortholog, and DHPS which does not. GTP-CH may not be a good candidate because it includes a pterin binding site which is very well conserved across all living species (24). The other five enzymes remain to be evaluated as drug targets. The DHNA, HPPK, and DHPS activities are encoded by a single trifunctional enzyme in fungi so that their study is complicated. On the other hand, DHFS, ABZ1, and ABZ2 are single enzymes.

In the present study, we report the identification of the *dhfs* and *abz2* genes encoding DHFS and ABZ2 within the *P. jirovecii* genome sequence, as well as the assessment of their function by the successful complementation of the deleted orthologous gene of *S. cerevisiae*.

Antimicrobial Agents and Chemotherapy

Materials and methods

Strains and growth conditions

LCY1 is a *S. cerevisiae* haploid strain that has a disruption of the *FOL3* gene which encodes Dhfs protein (*Mata leu2-3,112 trp1 tup1 ura3-52 FOL3::URA3*; 25). This strain is thereafter named "*dhfs* deletant". In absence of folate synthesis, this strain requires methionine, adenine, histidine, and thymidine monophosphate (TMP). It was grown on complete medium YEPD (1% w/v Difco yeast extract, 2% Difco peptone, 2% glucose) supplemented with TMP (100μg/mL) at 30 °C.

Y00875 is a *S. cerevisiae* haploid strain with a deletion of the *ABZ2* gene which encodes Abz2 protein (*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YMR289w::kanMX4*). This strain is thereafter named "*abz2* deletant". It was obtained from Euroscarf (EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis, http://web.uni-frankfurt.de/fb15/mikro/euroscarf). The deletion of *ABZ2* induces a para-aminobenzoate (PABA) auxotrophy (26). The parental strain of the *abz2* deletant, strain BY4741 from Euroscarf (*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), was used as control in the complementation tests.

Escherichia coli DH5α (Life Technologies, Basel, Switzerland) was used for gene cloning. Cells were made competent using the method of Chung & Miller (27), stored at -80°C, and transformed for resistance to 50 μg/mL ampicillin on solid LB medium (1% w/v Difco tryptone, 0.5% Difco yeast extract, 1% NaCl, 2% Gibco agar).

Source of *P. jirovecii* gene sequences

The *P. carinii* Dhfs protein (NCBI accession number DQ128176; 20), or the *S. cerevisiae* Abz2 protein (NP_014016), was used as query sequence in BLASTp search against the *P. jirovecii* proteome at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The *P. jirovecii* gene sequences encoding the proteins identified were then retrieved from the European Nucleotide Archive (http://www.ebi.ac.uk/ena; 28). The *dhfs* and *abz2* genes correspond respectively to PNEJI1_000945 and PNEJI1_000496 loci in the *P. jirovecii* genome assembly version ASM33397v2 published previously (23). Protein multiple sequence alignments were generated using T-Coffee (29).

Cloning of *P. jirovecii* genes

Since no introns are present in the *P. jirovecii dhfs* gene, this 1269 bps gene was amplified by PCR directly from DNA extracted from a bronchoalveolar lavage fluid sample (BAL) of a patient with PCP using QIAamp DNA Blood KIT (Qiagen). PCR was carried out using the proofreading High Fidelity Expand Polymerase (Roche Diagnostics), a final concentration of 3mM $MgCl_2$, and primers 5'-GCG GGG GAT CCA TGT CGC TAA GAC TAG GTT TAT C-3' and 5'- CCC CCC CGT CGA CTT ATA TTA TTT TTT TAT CAA AAC-3'. These primers created unique *BamHI* and *SalI* restriction sites in the PCR product (restriction sites are underlined in primers). Primers were synthesized by Microsynth (Baglach, Switzerland). The PCR program included an initial denaturation for 3 min at 94°C, followed by 35 cycles consisting of 30 sec at 94°C, 30 sec at 52°C, and 90 sec at 72°C. The reaction ended with a 10 min of extension at 72°C. The PCR product was extracted using QIAquick gel extraction KIT (Qiagen), digested with *BamHI* and *SalI* restriction enzymes, and then ligated using T4 ligase (New England Biolabs) into the p414GPD expression vector (30) previously digested

with the same two restriction enzymes. After ligation, the plasmids were introduced into *E. coli* DH5α competent cells. Minipreparation of plasmid DNA was carried out according to Birnboim & Doly (31).

The *P. jirovecii abz2* gene without its two introns is 750 bps and was synthesized by GeneCust Europe (Dudelange, Luxembourg). It was cloned into p416GPD (27) as described here above for the *dhfs* gene.

Transformation of *S. cerevisiae* deletants

Recombinant plasmids p414GPD.*Pjdhfs* and p416GPD.*Pjabz2* were introduced into their corresponding *S. cerevisiae* deletant by transformation for tryptophan or uracile prototrophy, respectively. Yeast transformations utilized the one-step method described by Chen et al (32). Transformants were selected on solid yeast nitrogen base medium (YNB, 0.67% w/v yeast nitrogen base, 2% glucose, 2% Gibco agar) supplemented with CSM lacking tryptophan or uracile (MPbiomedicals). Four randomly chosen isolated colonies of transformants were purified by streaking and growth on the same selective medium.

Complementation tests

Functional complementation of the *S. cerevisiae dhfs* deletant with the *P. jirovecii dhfs* gene was assessed by growth on YEPD lacking TMP. As a further validation of functional complementation, the presence or absence of the *P. jirovecii dhfs* gene in the different strains was confirmed by PCR. The PCR conditions described here above were used. *S. cerevisiae* genomic DNA was extracted as described previously (33).

Functional complementation of the *S. cerevisiae abz2* deletant with the *P. jirovecii abz2* gene was assessed by the growth rate at 30°C in YNB lacking PABA and folic acid which was

supplemented with CSM. Overnight cultures were diluted at an absorbance at 540 nm of 0.1 (ca. $1.5x10^6$ cells/ml), and growth was followed by the optical density at 540 nm. In order to express its auxotrophy phenotype, the deletant was subcultured twice overnight in the medium lacking PABA and folic acid before the experiment. To confirm the presence or absence of the *P. jirovecii abz2* gene, primers 5'-GCG ATG AAA AAA ACA GAA AAG C-3' and 5'- CCC CTA TTC GAA GAA TGC CTG -3' were used to amplify the complete gene (GCG or CCC were added at the 5' end of the primers before the start and stop codons of the ORF in order to obtain similar melting temperatures). The PCR conditions were as described above for the *dhfs* gene except that the final concentration of $MgCl_2$ was 4.5 mM, the temperature of hybridization 58°C, and the elongation 1 min at 72°C.

Assessment of the extracted DNAs was done by amplification of the unrelated *S. cerevisiae BRL1* gene encoding an essential nuclear membrane protein (18). The primers used were 5'- GAA ACT CTT GGT ACA GAG G -3' and 5'- TGA TCT GTC CCA GTT GTG -3'. The PCR conditions were as described above for the *P. jirovecii dhfs* gene except that the temperature of hybridization was 52°C and the elongation time was 2 min at 72°C. The PCR product was 2008 bps.

Antimicrobial Agents and Chemotherapy

Results

Identification and cloning of the *P. jirovecii* dhfs gene

The Dhfs protein was identified within the *P. jirovecii* proteome by homology search using the Dhfs protein of *P. carinii* as query sequence. The gene encompasses no introns. The translation product of the ORF bears the highest degree of amino acid similarity with the Dhfs protein of *P. carinii* (72%), and a lower degree with those of *S. cerevisiae* (36%) and *S. pombe* (40%) (Fig. 2A). Because of the absence of introns, the *P. jirovecii dhfs* gene was directly amplified by PCR from the genomic DNA extracted from a BAL of patient with PCP, and cloned into the expression vector p414GPD.

Functional complementation of the *S. cerevisiae dhfs* deletant with the *P. jirovecii dhfs* gene

The recombinant plasmid p414GPD.*Pjdhfs* and the empty p414GPD vector were introduced into the *S. cerevisiae dhfs* deletant. Transformant isolates were then grown on rich medium supplemented with or without TMP. Growth occurred on the medium lacking TMP only in the presence of p414GPD.*Pjdhfs,* but not of p414GPD (Fig. 3). This proved that expression of the *P. jirovecii* gene rescued the function of the deleted *FOL3* gene encoding Dhfs protein. However, the growth rate of the rescued deletant proved to be lower than that of the wild-type strain (results not shown; notably, the deletant rescued with the *P. carinii* Dhfs protein constructed in reference 20 also showed a similar reduced growth rate).The presence or absence of the *P. jirovecii dhfs* gene in the different strains was assessed by PCR analysis. As expected, the *P. jirovecii dhfs* was present in the functionally complemented strains, but

not in the deletant (Fig. 4A). To confirm that the DNA from which the *P. jirovecii dhfs* gene could not be amplified was valid, the unrelated *S. cerevisiae* gene *BRL1* was amplified (Fig. 4A).

Identification and cloning of the *P. jirovecii abz2* gene

The *P. jirovecii abz2* gene was retrieved as described above for the *dhfs* gene, except that the *S. cerevisiae* Abz2 protein was used as the initial query sequence. The gene encompasses two introns. The translation product of the ORF bears the highest degree of similarity with the Abz2 protein of *S. pombe* (33%), and a lower degree with that of *S. cerevisiae* (20%) (Fig. 2 A). We identified only a truncated *P. carinii abz2* gene (locus PNECA1_004600), possibly because of the known incompleteness of the genome sequence; this truncated gene was 240 bps long and its translation product consistently shared 59% identity with the corresponding region of the *P. jirovecii abz2* gene. Because *S. cerevisiae* does not process *Pneumocystis* introns, a synthetic *P. jirovecii abz2* gene without introns was cloned into p416GPD.

Functional complementation of the *S. cerevisiae abz2* deletant with the *P. jirovecii abz2* gene

The recombinant plasmid p416GPD.*Pjabz2* and the empty p416GPD vector were introduced into the *S. cerevisiae abz2* deletant. Transformant isolates, the parental wild type strain of the *abz2* deletant, and the *abz2* deletant were grown in minimal medium lacking PABA and folic acid. A growth rate similar to the parental wild type strain was observed in the presence of p416GPD.*Pjabz2,* but not of p416GPD (Fig. 5). This proved that the *P. jirovecii* gene rescued the function of the deleted *ABZ2* gene. The presence or absence of the *P.*

jirovecii abz2 gene in the different strains was assessed by PCR analysis. As expected, the *P. jirovecii abz2* was present in the functionally complemented strains, but not in the deletant (Fig. 4B). To confirm that the DNA from which the *P. jirovecii abz2* could not be amplified was valid, the unrelated *S. cerevisiae* gene *BRL1* was amplified (Fig. 4B).

Discussion

Because of the emergence of drug resistance in *P. jirovecii* towards the most efficient drug available and because of the side effects of this drug, the development of new drugs against this fungal pathogen is crucial. The publication of the *P. jirovecii* genome sequence opens a new era for the search of potential new drug targets because the actual genes to inhibit can be studied and no more those of models. Most enzymes involved in the biosynthesis of folic acid are ideal drug targets because of their essentiality and absence in humans. Accordingly, there are many drugs inhibiting this pathway which are currently used against many human pathogens. We focused on two enzymes involved in this pathway which were poorly investigated so far, the DHFS (dihydrofolate synthase) and the ABZ2 (deoxychorismate lyase). In this study, we identified the two *P. jirovecii* genes encoding these enzymes and demonstrated their function by their ability to rescue the null allele of their respective *S. cerevisiae* orthologous gene. These are steps required in the search of new targets. The *P. jirovecii* enzymes identified bear a higher homology with the *S. pombe* orthologs than with those of *S. cerevisiae*. This is consistent with the fact that *P. jirovecii* and *S. pombe* are members of the Taphrinomycotina subphylum, but no *S. cerevisiae*.

The DHFS enzyme carries out the final step of the folic acid biosynthesis, namely the addition of a glutamate to dihydropteroate to make folic acid (*i.e.* dihydrofolate; Fig. 1). The DHFS enzyme shares a high degree of similarity with the enzyme folypolyglutamate synthase (FPGS), which stabilize folic acid by the addition of several glutamates (Fig. 1). The specificity of these two enzymes for the addition of single or multiple glutamates is noteworthy. Humans have only a FPGS that has no DHFS activity (34), while *S. cerevisiae* and other fungi have both FPGS and DHFS activities encoded by two different genes (35). Other organisms such

as *E. coli* and *Plasmodium falciparum* have only one gene which encodes a single bifunctional polypeptide enzyme (36). The molecular basis for the mono- versus bifunctional activity remains to be elucidated. DHFS enzymes act in the cytoplasm of eukaryotes and do not include an N-terminal targeting signal sequence in order to be transferred into other cell compartments. On the other hand, eukaryotic FPGS enzymes are working in the cytoplasm as well as in the mitochondrion and possess a mitochondrial targeting signal sequence. The comparison of the *P. carinii* DHFS to the *S. cerevisiae* DHFS and FPGS showed that *P. carinii* DHFS is devoid of mitochondrial targeting signal sequence and thus has probably no FPGS activity (20). The *P. jirovecii* DHFS isolated in the present study is close to that of *P. carinii* without a supplementary N-terminal sequence (72% identity; Fig. 2A), strongly suggesting that it has also no FPGS activity. Consistently, the *P. carinii* and *P. jirovecii* DHFSs share more identity with the *S. cerevisiae* DHFS (35% and 36%, respectively) than with the *S. cerevisiae* FPGS (20% and 19%). The essentiality of its activity in many organisms together with its absence in humans suggests that the DHFS enzyme is a good candidate drug target against *P. jirovecii*.

The aminodeoxychorismate lyase encoded by the *abz2* gene is required for the biosynthesis of PABA, which in turn is necessary to produce folic acid (Fig. 1). The *S. cerevisiae abz2* deletant has a reduced growth rate in a minimal medium lacking PABA and folate (26; Fig. 5). This is probably due to a cellular pool of PABA sufficient to allow survival for several generations. Subculturing the *abz2* deletant in absence of PABA leads to exhaustion of this pool of PABA, allowing expression of PABA auxotrophy (26). Although an external source of PABA by scavenging from the host is possible, the product of the *P. jirovecii abz2* gene might be required to allow survival of the pathogen during infection, rendering this gene

a potential new drug target. This is plausible because antifolate drugs are effective against *P. falciparum* despite that this pathogen can scavenge folic acid from its human host (37, 38). This is also supported by the fact that the PABA synthase of *Aspergillus fumigatus* is essential for pathogenicity (39).

In conclusion, we characterized two new potential drug targets in *P. jirovecii*. They deserve future investigations. They could be involved in a strategy taking advantage of the synergism provided by combination therapy, a strategy which is widely and successfully used against important human pathogens.

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417 **Legends**

418

419 **Fig. 1. Folate biosynthesis and utilization pathway.**

420

421 **Fig. 2**. **Multiple sequence alignment of Dhfs (A) and Abz2 (B) proteins**. T-Coffee (29) was
422 used. The identical, strongly, and weakly conserved residues are indicated respectively by
423 asterisks, double points, and single points. Dashes indicate gaps. **A:** Alignment of Dhfs
424 proteins of *P. jirovecii* (locus tag PNEJI1_000945), *P. carinii* (accession number DQ128176),
425 *S. cerevisiae* (NP_013831), and *S. pombe* (NM_001018363.2). Also shown is the P loop
426 (phosphate binding), the Ω loop (involved in the folate binding site), and the linker that
427 connects the N- and C-domains. **B:** Alignment of Abz2 proteins of *P. jirovecii* (locus tag
428 PNEJI1_000496), *S. cerevisiae* (NP_014016.1), and *S. pombe* (NM_001021876.2). 200593).
429 Also shown is the pyridoxal-binding (Py) site located at the interface of N- and C-domains of
430 the enzyme which is a hallmark of aminotransferase-like enzyme. Conserved residues of both
431 domains which form the active site are underlined.

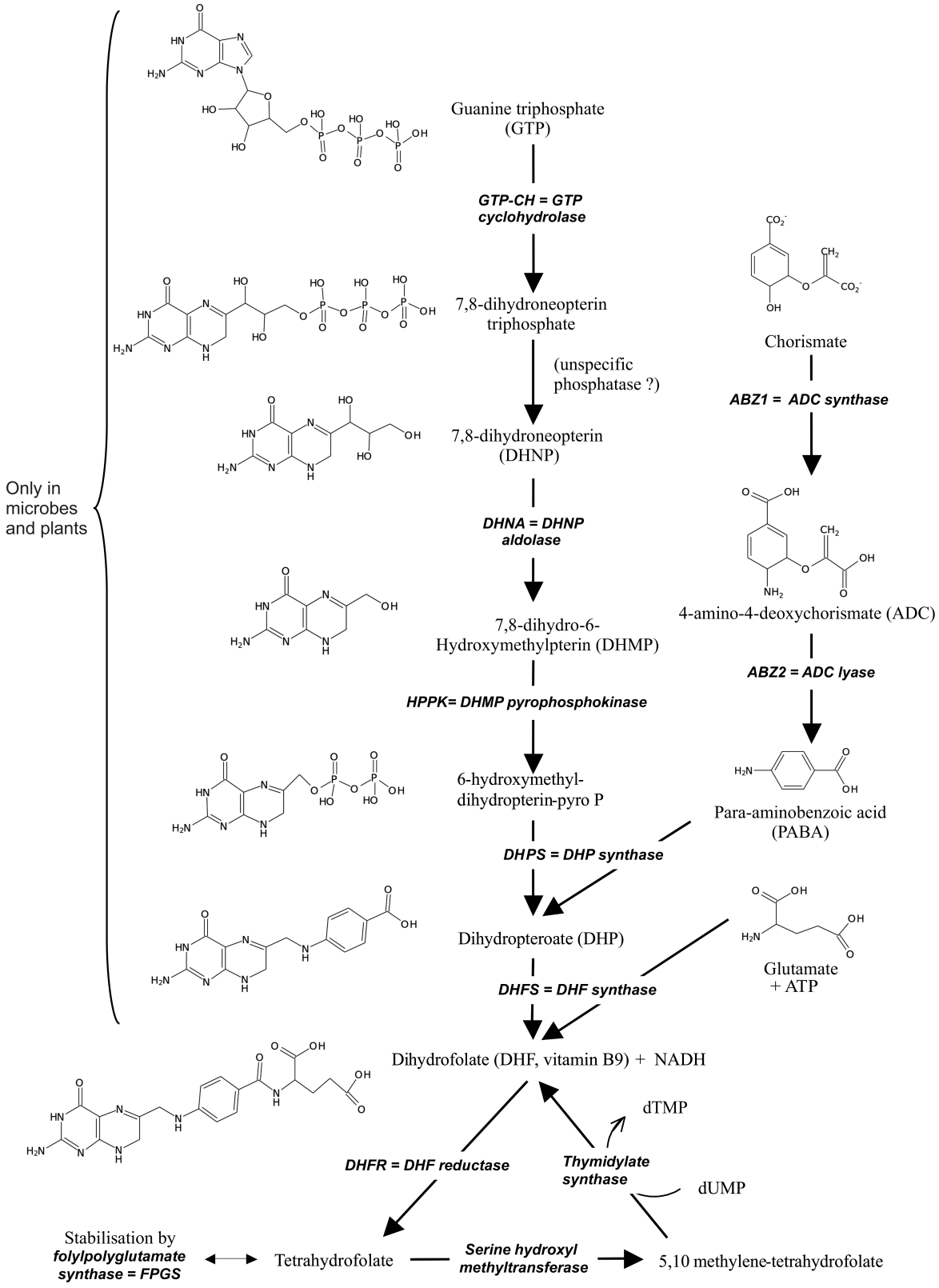
432

433

434 **Fig. 3. Complementation of the *S. cerevisiae dhfs* deletant by expression of *P. jirovecii***
435 ***dhfs* gene on plasmid**. Four single colonies were isolated from the original transformation
436 Petri dish, purified by streaking on the same selection medium, and grown on rich medium
437 YEPD with TMP (**A**) or without TMP (**B**) for 3 days at 30°C. Number 1 corresponds to the
438 control strain bearing the empty p414GPD vector. Numbers 2 to 5 correspond to the four
439 isolates bearing p414GPD.*Pjdhfs*.

Fig. 4. PCR assessment of the presence or absence of the *P. jirovecii dhfs* and *abz2* genes. **A:** The presence of the *P. jirovecii dhfs* gene (PCR product of 1293 bps) was confirmed in the DNA from the BAL of a patient with PCP (lane 1) and in one isolate of *S. cerevisiae dhfs* deletant bearing p414GPD.*Pjdhfs* (lane 3), whereas the gene was absent in the *dhfs* deletant without plasmid (lane 2). As a control, the unrelated *S. cerevisiae BRL1* gene was amplified (PCR product of 2008 bps) from the *dhfs* deletant bearing p414GPD.*Pjdhfs* (lane 5) or without plasmid (lane 4). **B:** The presence of the *P. jirovecii abz2* gene was confirmed in the DNA from the BAL of a patient with PCP (lane 1; PCR product with introns of 829 bps) and in one isolate of *S. cerevisiae abz2* deletant bearing p416GPD.*Pjabz2* (lane 3; PCR product without introns of 756 bps), whereas the gene was absent in the DNA of the *abz2* deletant (lane 2). The unrelated *S. cerevisiae BRL1* gene was amplified from the *abz2* deletant bearing p416GPD.*Pjabz2* (lane 5) or without plasmid (lane 4).

Fig. 5. Complementation of the *S. cerevisiae abz2* deletant by expression of *P. jirovecii abz2* gene on plasmid. Strains were grown overnight in YNB lacking PABA and folic acid which was supplemented with CSM. The cultures were diluted in the same medium at an optical density of 0.1 (time 0) and incubated at 30°C. The optical density at time 0 was normalized at 0.1 for each strain. Standard deviations of triplicate optical density measurements were small (below 0.005). The four complemented isolates were analyzed with similar results, one representative experiment of one complemented isolate is shown. The labels of the curves are explicated on the up left side of the figure.



Antimicrobial Agents and Chemotherapy

A

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                                        ---P---                       -----Q-----
P.jirovecii     MSLRLGLSRIRQLLDYLGNPQNYFQAIHVAGTNGKGSVCAYLSSYLNFSGIRVGLYCSPHLMDRWDCIKVAG
P.carinii       MLVKLGLLRIRQLLKYLGNPQNSFQAVHVAGTNGKGSVCAYLSSCLALSGIRVGQYCSPHLIDRWDCVKVIG
S.cerevisiae    MAIELGLSRITKLLEHLGNPQNSLRVLHIAGTNGKGSVCTYLSSVLQQKSYQIGKFTTPHLVHVTDSITINN
S.pombe         MPIQLGLQRMLQLLKHLGNPQESFCAVQIAGTNGKGSICSYIYTSLLQAAIKTGRYTSPHFLEPRDTISING
                * :.*** *: :**.:*****: : .:::********:*:*: : *   . : * : :**::.  * :.: .

P.jirovecii     EVVDKDIFFRIENKIKILNQEHNVGATEFEIMTAVAFEIFYRSKIELAVIETGVGGRLDATNVLSRV--LLT
P.carinii       RDIDKHQFLEIESKIKNLNQRCNIGATEFEIMTAVAFEILSKNNVELAVIETGVGGRLDATNVLSQV--LLT
S.cerevisiae    KPIPLERYQNIRLQLEALNKSHSLKCTEFELLTCTAFKYFYDVQCQWCVIEVGLGGRLDATNVIPGANKACC
S.pombe         QIASEEIFNTCWKQVIEVDRRFRTKATEFELLTATAFQCFHHSGVRVAVIETGMGGRLDATNVFEEP--VLS
                .    . :     ::  :::     .****::*..**: :     . .***.*:*********:

P.jirovecii     IITKISTDHQELLGNTLEDIAKEKSGIMKNNVPC-VVDGANEDSVLKVIKDESIKCESGQIILATM--DLDK
P.carinii       IITKISMDHQELLGNTIQKIAREKSGIMKKNIPC-IVDGTNEDSVLKVIKEESIKSGSSRVILTPM--DLDK
S.cerevisiae    GITKISLDHESFLGNTLSEISKEKAGIITEGVPFTVIDGTNEASVINVVKERCK-ALGSELSVTDS--QLNG
S.pombe         IISRICLDHQAFLGNTLEAIAKEKAGIFKKNVPC-VVDGLNEVNVLNQLKLSAEETRAHPFYLAKGKSGENK
                 *::*. **: :****:. *::**:**:.:.:*  ::** ** .*:: :*  .    .  . ::      :

                                                                        ----linker-----
P.jirovecii     S---IYIQQWKKSEI-KTILDISYQRNNLACVLVSLEVLSKYY-SVITPKFFSEGFLRTYWPGRLEWIDL--
P.carinii       S---LYIQEWKKHEF-KTSLYRTYQRTNLACVSASLEILSKYY-PKITPDILSKGLLETYWPGRLEWIDL--
S.cerevisiae    N--MIDTNSWGCFDLAKLPLNGEYQIFNLRVAMGMLDYLQMNELIDITKNEVSTRLAKVDWPGRLYRMDYRF
S.pombe         NEWIINTPNWGTNTF-STPLKGDYQGQNLACAVTALDILSSSF-SIMLPH-VQNGVKNTSWPGRLDIRSV--
                .   :   .*    : .  *   **  **  .   *: *.      :  . ..  . .. *****   .

P.jirovecii     SQIAFGADKILLDGAHNIEGMHSLSKYVNSIRS-GTHSVSWLIAFSQTKDADSLLSILLRPYDKVYSVEFET
P.carinii       SQIAFGANKILLDGAHNIDGINSLSEYINSIRN-GVQSVSWLTAFTQGKDVDSLLSILLKPYDKIHSVEFEP
S.cerevisiae    DKVSNRTVPILMDGAHNGSAAVELVKYLRK-EY-GNQPLTFVMAVTHGKNLEPLLQPLLRPIDQVILTRFNN
S.pombe         PS----LGDILFDGAHNKEAAIELAKFVNSQRREHNKSVSWVVAFTNTKDVTGIMKILLRKGDTVIATNFSS
                 .       **:***** ..  .* :::.. .    :.:::: *.:: *:   ::. **:  * :  ..*.

P.jirovecii     VDGMPWVKAMSSHDIAKKALKYVYKENIIQYSTD---LFSAIKSISQDKGL-RIICGSLYLIGQVHRLLRKC
P.carinii       VDGMQWIKPVNSSEIAKIARKYLYEENVKQHGTD---LLSAIRSISQDKGL-QVICGSLYLIGQVHRLLHKR
S.cerevisiae    VEGMPWIHATDPEEIKDFILTQGYTKEIVI-ENDLHQVLPSLAHVSDEQRRPIVVCGSLYLCGELLRIHNSH
S.pombe         VSGMPWIKSMEPEVIKNSISSE---SSVECYTADNL-TISEILRLAKEKNSSVIVCGSLYLLGDMYRYLKLD
                *.** *::. ..  * .   .    ..:     *    :. :  ::.::    ::****** *:: *  .

P.jirovecii     CFDKKII--
P.carinii       ILLQKGSRK
S.cerevisiae    LR------N
S.pombe         V--------
```

B

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P.jirovecii     MKKTEKL---------NNIIQGRTWELLETILYDG-----------------------------------
S.cerevisiae    MSLMDNWKTDMESYDEGGLVANPNFEVLATFRYDPGFARQSASKKEIFETPDPRLGLRDEDIRQQIINEDYS
S.pombe         MEES----------------------NLFETTLYDG-----------------------------------
                *.                      ::: *  **

P.jirovecii     ---------------------------------------------KDFFLLEKHMQRLVKSSMDF--GWKTV-
S.cerevisiae    SYLRVREVNSGGDLLENIQHPDAWKHDCKTIVCQRVEDMLQVIYERFFLLDEQYQRIRIALSYFKIDFSTSL
S.pombe         ---------------------------------------------ELFLLPSHLQRMKASAKSL--GYSWP-
                                                            :*** .: **:  :   :  .:.

                                                                      -----Py-----
P.jirovecii     ----------------DIEIVKKELWNSVTRCKSSKVRLTIAQNGTINIEISPFILPKNL------------
S.cerevisiae    NDLLKLLVENLINCKEGNSEYHEKIQKMINERQCYKMRVLVSKTGDIRIEAIPMPMEPILKLTTDYDSVSTY
S.pombe         ----------------GEQYIENKLREAVQDTSMARVRWELSKAGDVTVQIVPIQT----------------
                             . .  .::: : :   .  ::*  ::: * : ::  *:

P.jirovecii     -----FGVFSKNEQQETKPWKVYLDTIPMNDALRPFFCHKTTYRDPYETSRKR--LKIG-------------
S.cerevisiae    FIKTMLNGF---LIDSTINWDVVVSSEPLNAS--AFTSFKTTSRDHYARARVR--MQTAINNLRGSEPTSSV
S.pombe         --------------LEKAPYTLILDKQPSSTEKNPSCINKMTNRAIYIEAMNRNDAQYS-------------
                           ..  : : :.. * .    .    * * *  *  :  *   : .

P.jirovecii     EAMEVLLYNQHGYVMEGSICNVAFFR-----DHQWITPSLKEGCLPGVMRETLLERGHIVERP---IQVSEL
S.cerevisiae    SQCEILFSNKSGLLMEGSITNVAVIQKDPNGSKKYVTPRLATGCLCGTMRHYLLRLGLIEEGD---IDIGSL
S.pombe         KAQDVLLYNHQGFVTEATIFNVAFHR-----NGQWITPSLKHGLLSGTMRKNLLENGSIHEDDKGLLQKDNL
                .  ::*: *: * : *.:* ***. :     . :::** *  * * *.**. **. * * *     :: ..*

P.jirovecii     VNGERLLLFNSLRGCFNGILYFKPMSRNNFKK---YQAFFE
S.cerevisiae    TVGNEVLLFNGVMGCIKGTVKTK----------------Y
S.pombe         KNGEQVLLFNSFRKVCKGVLIIQPEKACELLKKKDSSEKLS
                  *:.:****..    :* :  :
```