Dihydropteroate Synthase Mutations in *Pneumocystis jiroveci* Can Affect Sulfamethoxazole Resistance in a *Saccharomyces cerevisiae* Model

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Dihydropteroate synthase (DHPS) mutations in *Pneumocystis jiroveci* have been associated epidemiologically with resistance to sulfamethoxazole (SMX). Since *P. jiroveci* cannot be cultured, inherent drug resistance cannot be measured. This study explores the effects of these mutations in a tractable model organism, *Saccharomyces cerevisiae*. Based on the sequence conservation between the DHPS enzymes of *P. jiroveci* and *S. cerevisiae*, together with the structural conservation of the three known DHPS structures, DHPS substitutions commonly observed in *P. jiroveci* were reverse engineered into the *S. cerevisiae* DHPS. Those mutations, T297A and P599S, can occur singly but are most commonly found together and are associated with SMX treatment failure. Mutations encoding the corresponding changes in the *S. cerevisiae* dhps were made in a yeast centromere vector, p414FYC, which encodes the native yeast DHPS as part of a trifunctional protein that also includes the two enzymes upstream of DHPS in the folic acid synthesis pathway, dihydroneopterin aldolase and 2-amino-4-hydroxymethyl dihydropteridine pyrophosphokinase. A yeast strain with dhps deleted was employed as the host strain, and transformants having DHPS activity were recovered. Mutants having both T297 and P599 substitutions had a requirement for p-aminobenzoic acid (PABA), consistent with resistance being associated with altered substrate binding. These mutants could be adapted for growth in the absence of PABA, which coincided with increased sulfa drug resistance. Upregulated PABA synthesis was thus implicated as a mechanism for sulfa drug resistance for mutants having two DHPS substitutions.

Dihydropteroate synthase (DHPS) is an important target for antifolate compounds, such as sulfofones and sulfonamides, in both prokaryotic and eukaryotic microbes. Organisms treated with sulfa drugs include pathogens, such as *Pneumocystis ji- roveci*, *Mycobacterium leprae*, *Neisseria meningitidis*, and *Plasmodium falciparum*. Numerous studies have reported that mutations in the primary sequence of dhps are associated with sulfa drug resistance (7, 10, 12, 18, 20, 22, 23, 32, 36, 38, 39). The *P. jiroveci* mutant alleles map to a highly conserved sequence in DHPS that is associated with direct substrate contact (1, 3, 14), although there is some controversy over whether these influence drug treatment outcomes (2, 6, 15, 18, 19, 24, 26, 28, 33, 34). In vitro culture of *P. jiroveci* has not yet been possible, nor has heterologous complementation of *P. jiroveci* DHPS.

For other pathogens, the association between the efficacy of antifolate drugs and genetic mutations in the dihydrofolate reductase gene (*dfr*) and dhps has been demonstrated using model systems (11, 29, 31, 40). This would be particularly useful for *P. jiroveci*, since there is no direct evidence that mutant alleles identified in *P. jiroveci* dhps cause sulfa drug resistance.

We have sought to better understand the relationship between DHPS changes and sulfa drug resistance by employing a *Saccharomyces cerevisiae* model system. *S. cerevisiae* is a good model for studying *P. jiroveci* DHPS because (i) both organisms are fungi, (ii) both have trifunctional dihydroneopterin aldolase (DHNA)—2-amino-4-hydroxymethyl dihydropteridine pyrophosphokinase (PPPK)—DHPS enzymes, (iii) the DHPS amino acid sequence alignments show 30% identity and 62% similarity and DHNA-PPPK-DHPS (folic acid synthesis [FAS]) alignments show 37% identity and 64% similarity, (iv) the organisms have identical sequences in the regions associated with sulfamethoxazole (SMX) resistance (T597R, P599E, and P599N in *S. cerevisiae*; T517R, S518N, and P519S in *P. jiroveci*) (Fig. 1); (v) the conservation rates of these residues across 75 published DHPS species are 82, 80, and 99%, respectively, and (vi) the residues map to the same region in the three known structures of DHPS that have been solved to date (1, 3, 14).

Based on these high levels of identity and similarity, we employed an allelic replacement strategy that utilized an *S. cerevisiae* dhps knockout strain that was complemented by a vector-encoded FAS, the product of the *S. cerevisiae* FOL1 gene. Residues in the wild-type FAS were mutated to give the changes associated with SMX resistance. We report here the phenotypic analysis and SMX resistance of various dhps alleles implicated from epidemiological studies with SMX resistance.

**MATERIALS AND METHODS**

*E. coli* strains, growth media, and transformation. The bacterial strain employed for molecular cloning and plasmid amplification was *Escherichia coli* strain MC1061 [araD139 Δ(araABC-leu)7679 galU galK Δ(pucX74) rpmL hsdR (rg- mcrB - mcrB ) merB]. The growth medium utilized was 1× YT (0.5% [wt/vol] yeast extract, 0.5% [wt/vol] tryptone, 0.5% [wt/vol] NaCl). Cells were made competent by calcium chloride treatment, and transformants were selected on 1× YT + 100 μg of ampicillin/ml.
Yeast strains, growth media, and transformation. The *S. cerevisiae* dhps knockout strain used in this study, EHY1, has been described previously (4, 5, 16). EHY1 (MATa leu2-3,112 trp1 ura3-52 tup1 DHPS::LEU2) is a genomic dhps knockout strain. The authenticity of the EHY1 knockout strain was confirmed by molecular approaches and phenotypic analyses and was consistent with a specific dhps deletion. The auxotrophic requirements and complementation of EHY1 were previously described (4). Media for the growth of yeast cells included YEPD (1% (wt/vol) yeast extract [Difco], 2% (wt/vol) peptone, 2% (wt/vol) D-glucose). Synthetic minimal medium (MM 0) was 0.67% yeast nitrogen base containing ammonium sulfate minus folic acid minus *p*-aminobenzoic acid (PABA) (Bio 101) supplemented with 1% (wt/vol) D-glucose and 0.2% (wt/vol) uracil.

Yeast transformants were obtained by the lithium acetate transformation procedure (13), followed by growth and selection on complete synthetic medium minus tryptophan. Complete synthetic medium was 0.67% yeast nitrogen base (Difco) containing ammonium sulfate supplemented with 2% (wt/vol) uracil, amino acids, and adenine as described in the Cold Spring Harbor manual on yeast genetics (8). This medium contained PABA (200 ng/ml) and folate (2 ng/ml). Before being tested for SMX susceptibility, transformants were passaged on MM0 six times.

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**Synthesis of mutant alleles implicated with sulfa drug resistance.** The Quick-change XL kit (Stratagene) was used to generate four mutant constructs from the wild-type DHPS using oligonucleotides listed in Table 1. The wild-type construct was designated TRP (T597, P599). The mutants were A597, P599 (designated ARP); T597, S599 (designated TRS); and a double mutant, A597, S599 (designated ARS). The fourth allele has not been reported clinically and was derived from the ARS allele by a conservative change of A597 to V597. This yielded V597, S599 (designated VRS). This clone arose spontaneously during the mutagenesis procedure.

**TABLE 1. Oligonucleotides used for site-directed mutagenesis**

| Clone | Substitution | Oligonucleotide no. | Direction of priming | Sequence (5' → 3')a |
|-------|--------------|---------------------|----------------------|---------------------|
| ARS   | A597, S599   | 146337              | ←                    | GGAGGGTGTCTCAGGGCAATCTAATGTTACGCC |
|       |              | 146338              | ←                    | GCTCTGAGATTAGTGATAGCCTGACGAGACACCTCC |
| TRS   | T597, S599   | 146339              | ←                    | GGAGGGTGTCTCAGGGCAATCTAATGTTACGCC |
|       |              | 146340              | ←                    | GCTCTGAGATTAGTGATAGCCTGACGAGACACCTCC |
| ARP   | A597, P599   | 146341              | ←                    | CGTGGCAAGAACTTGGATCAGTTGCAATCTACGTTT |
|       |              | 146342              | ←                    | GATAGAGTTTGGGGCAGGGGAGACACCTCC |

*a* Nucleotide substitutions are in boldface.
cEDURE and was kept for its intrinsic interest. In order to validate the synthesis of the mutant allele sequence, each construct was analyzed by DNA sequencing using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). Each of the mutant constructs generated was transformed into EHY1 and plated onto complete synthetic medium minus tryptophan, and complementing clones were selected.

**Phenotypic analysis of S. cerevisiae transformants.** Each transformant was precultured in YEPD broth at 30°C. The cultures were harvested in log-phase growth, washed with phosphate-buffered saline (PBS; 8% [wt/vol] NaCl, 0.2% [wt/vol] KCl, 1.44% [wt/vol] Na2HPO4, 0.24% [wt/vol] KH2PO4, pH 7.4), and normalized to a cell density of 6 × 10^6 CFU/ml (A600 = 0.1). A series of twofold dilutions was made in a 96-well microtiter plate. One microtiter of the dilution series for each clone was spotted on solidified MM0. Alternatively, plates were supplemented with various combinations of the folate biosynthetic pathway end products, including methionine (20 μg/ml), adenine (20 μg/ml), histidine (20 μg/ml), TMP (20 μg/ml), and formyl-tetrahydrofolate (20 μg/ml). In addition, there was a detailed analysis of the affects of PABA that included supplementing MM0 with PABA to achieve a final concentration of 0 to 10,000 ng/ml. The plates were then incubated at 30°C for 3 to 5 days.

**Determination of SMX resistances of prototrophs by drug diffusion assays.** In order to evaluate the relative sensitivity or resistance of each DHPS allele to SMX, drug diffusion assays were performed with PABA supplementing the molten medium at a final concentration ranging from 0 to 10,000 ng/ml. The assays were performed in petri dishes 86 mm in diameter containing 25-ml MM0 with PABA to achieve a final concentration of 0 to 10,000 ng/ml. The plates were then incubated at 30°C for 3 to 5 days.

**Determination of SMX resistances of prototrophs in broth cultures.** In order to accurately compare the growth rates (generation times) of the clones, the growth of broth cultures in MM0 supplemented with 200 ng of pABA/μl (MM0pA) was monitored periodically over 180 h by turbidity measurements at A595. Each clone was precultured in YEPD broth at 30°C. The cultures were harvested in log-phase growth, washed with PBS, and normalized to an A600 of 0.1. A stainless-steel wire (2-mm diameter) was used to inoculate each clone radially from the center of the agar plate. The plates were then incubated at 30°C for 6 days and counted after 3 to 5 days.

**Determination of SMX resistance of naive transformants in broth cultures.** In order to investigate whether elevated PABA levels could account for the increased resistance observed in the adapted strains (prototrophs), the MICs of the naive transformants were determined in broth cultures. The medium for determination of the MIC was supplemented with PABA at levels up to 1,000 ng/ml. SMX was then added in a range of concentrations (0 to 2,000 μg/ml). After the addition of PABA and SMX to the medium, the pH was adjusted to 7.1 to permit the solubilization of SMX up to 2,000 μg/ml and to eliminate pH differences caused by the SMX and PABA. The upper limit of solubility of SMX is 2,000 μg/ml in MM0, pH 7.1. The DMSO concentration was constant at (0.5%). Fresh (naive) transformants were precultured in MM0 containing 2% glucose. Cells were harvested in mid-log-phase growth, washed twice with 50 ml of PBS, and normalized to an A600 of 1.0. Each strain (5 μl of cells) was then seeded into 100 μl of medium in a 96-well plate (Costar 3596). The cultures were grown at 30°C for up to 2 weeks. The seeding density for this experiment was 2.5-fold higher than that of the liquid growth assays, which permitted limited growth of ARS in MM0 (data not shown). The experiments were set up using a Qiagen Rapidplate liquid-handling robot. Each experiment was performed in triplicate.

**RESULTS**

**Construction of yeast strains with altered DHPS.** The yeast strain EHY1 had its chromosomal sequences encoding DHPS deleted so that it no longer synthesized folates. However, EHY1 could grow in the absence of added folate when transformed with p414FYC, a centromere plasmid that encoded the yeast DHNA-PPPK-DHPS trifunctional enzyme (FAS) under the control of its native promotor (16), p414FYC derivatives, altered only by mutations that produced amino acid substitutions at T597 and P599 within DHPS to T597 and S599, A597, and P599, A597 and S599 or V597 and S599 were prepared using site-directed mutagenesis, confirmed by nucleotide sequence analysis, and transformed into the EHY1 strain. Transformants were selected on yeast minimal medium in the absence of added folate and tryptophan. Transformants were obtained for each of the five constructs, indicating functional complementation by each altered DHPS enzyme. For convenience, we shall refer to the five respective transformants as TRP, TRS, ARP, ARS, and VRS.

**PABA requirement of mutant transformants.** The generation times of transformants expressing each mutant DHPS gene were compared to determine if there were defects caused by the mutant DHPS enzymes. Since SMX resistance assays must be performed in the presence of low levels of PABA (27), the transformants were adapted to low-PABA-folate medium by serial passaging six times on MM0. The growth rates of the strains were compared on minimal medium supplemented with various levels of PABA ranging from 0 to 10,000 ng/ml (Fig. 2). A significant defect was noted with respect to a requirement for PABA by the mutants ARS and VRS. The TRP (wild-type), ARP, and TRS transformants exhibited no significant PABA requirement, while the double mutants, ARS and VRS, exhibited significant PABA requirements, with VRS having less PABA dependence. This indicated that the combined changes of T597 and P599 caused a change in PABA binding or condensation with 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine-pyrophosphate (H4PPT).

**SMX resistance of prototrophs by drug diffusion assays.** We evaluated the relative resistance of each transformant to SMX, using drug diffusion assays following adaptation to PABA-free minimal medium (MM0). MM0 was used initially to avoid the PABA requirement of mutant transformants. MM0 supplemented with PABA at levels up to 1,000 ng/ml. The plates were then incubated at 30°C for 6 days and counted after 3 to 5 days.

**SMX resistances of prototrophs in broth cultures.** In order to determine whether elevated PABA levels could account for the increased resistance observed in the adapted strains (prototrophs), the MICs of the naive transformants were determined in broth cultures. The medium for determination of the MIC was supplemented with PABA at levels up to 1,000 ng/ml. SMX was then added in a range of concentrations (0 to 2,000 μg/ml). After the addition of PABA and SMX to the medium, the pH was adjusted to 7.1 to permit the solubilization of SMX up to 2,000 μg/ml and to eliminate pH differences caused by the SMX and PABA. The upper limit of solubility of SMX is 2,000 μg/ml in MM0, pH 7.1. The DMSO concentration was constant at (0.5%). Fresh (naive) transformants were precultured in MM0 containing 2% glucose. Cells were harvested in mid-log-phase growth, washed twice with 50 ml of PBS, and normalized to an A600 of 1.0. Each strain (5 μl of cells) was then seeded into 100 μl of medium in a 96-well plate (Costar 3596). The cultures were grown at 30°C for up to 2 weeks. The seeding density for this experiment was 2.5-fold higher than that of the liquid growth assays, which permitted limited growth of ARS in MM0 (data not shown). The experiments were set up using a Qiagen Rapidplate liquid-handling robot. Each experiment was performed in triplicate.

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**RESULTS**

**Construction of yeast strains with altered DHPS.** The yeast strain EHY1 had its chromosomal sequences encoding DHPS deleted so that it no longer synthesized folates. However,
resistance, growth assays were performed in MM200 broth supplemented with increasing concentrations of SMX. This analysis produced MICs (Table 2) that agreed with those in the drug diffusion assays (Fig. 3). VRS was highly resistant to SMX, as strong growth was observed at the highest SMX concentration tested, suggesting that the MIC was significantly greater than 1,200 μg/ml (i.e., >1,200 μg/ml). TRP and ARS were significantly (but not completely) inhibited at 1,200 μg/ml, suggesting that the MICs were slightly greater than 1,200 μg of SMX/ml (i.e., >1,200 μg/ml). By contrast, the single mutants appeared to be considerably more sensitive (Table 2).

The growth rates of the adapted strains were also measured in the presence of SMX and are shown in Fig. 4. In the absence of SMX, VRS grew faster than all the other transformants, with a generation time of 4 h. TRP, TRS, and ARP had similar generation times of ~5 h, and ARS had the longest generation time, almost 6 h. With increasing concentrations of SMX, the generation times of all transformants increased (Fig. 4), but the effect on the generation time varied for each transformant. The single mutant ARP became inhibited most quickly, followed by the other single mutant, TRS. The resistance pattern in Fig. 4 precisely matched that shown in Table 2. Likewise, the consistency of profiles held with the other transformants. At SMX concentrations of ≤600 μg/ml, TRP had a shorter gen-

![FIG. 2. Growth of transformants on minimal medium supplemented with various levels of PABA.](image)

![FIG. 3. Determination of SMX resistance by agar drug diffusion assay. SMX was solubilized in DMSO (100 mg/ml), and 25 μl was applied to the centers of agar plates. Additional plates were supplemented with increasing concentrations of PABA. Transformants were harvested in log-phase growth, washed in PBS, and normalized to 6 x 10⁶ CFU/ml. Each strain was then streaked radially from the center of the plate. The plates were grown for 3 to 5 days, and the zones of inhibition for each isolate, TRP, VRS, ARS, TRS, and ARP, were then scored. The results are averages of triplicate experiments; error bars represent the standard deviations of the means.](image)

![TABLE 2. MICs of SMX for yeast isolates grown in MM200](image)

| DHPS sequence | Isolate | MIC (μg/ml) |
|---------------|---------|-------------|
| A₉₉₇, P₉₉₉ | ARP     | 150         |
| T₉₉₇, S₉₉₉ | TRS     | 600         |
| A₉₉₇, S₉₉₉ | ARS     | >1,200      |
| T₉₉₇, P₉₉₉ | TRP     | >1,200      |
| V₉₉₇, S₉₉₉ | VRS     | >1,200      |
eration time than the double mutant ARS. However, at SMX concentrations of >800 µg/ml, the double mutant ARS grew faster than TRP (Fig. 4). Therefore, ARS was more resistant than TRP, as it had a shorter generation time and the MIC for it was higher than that of TRP at high SMX concentrations (data deduced from Fig. 4; cf. Table 2).

VRS, by comparison to all other transformants tested, was the most resistant to SMX in MM<sub>100</sub> (data not shown) and MM<sub>200</sub>. The MIC was measured as &gt;1,200 µg/ml (Table 2), but it is obvious from Fig. 4 that at that level of SMX, it had a generation time of ~7 h.

**SMX resistances of naive transformants in broth cultures.** The MIC of SMX for each allele was plotted against the PABA concentration. The data indicated that the resistance of the single mutant ARP did not change significantly when the PABA concentration was elevated. By contrast, the MICs of all other mutants increased significantly with added PABA. The rate of change differed for each clone, indicating that the resistance of each allele was modulated by PABA to various degrees. Specifically, the MIC for naive ARS was lower than those for TRS and the wild type (TRP) for all conditions tested. This indicated that PABA was not the only factor that led to the resistance of the passaged ARS prototroph (Fig. 3). The MIC for VRS was observed to be highest, 2,000 µg/ml, when the PABA concentration was 100 ng/ml, reflecting the lower requirement of VRS for PABA relative to the ARS mutant. The MIC for TRP was 2,000 µg/ml when the PABA concentration was 150 µg/ml.

**DISCUSSION**

Mutations in the DHPS gene that are implicated in sulfa drug resistance were reverse engineered into vector-encoded *S. cerevisiae* FAS (DHNA-PPPK-DHPS) and then transformed into an *S. cerevisiae* strain having a *dhps* deleted, effectively resulting in allelic replacement of the sequences encoding DHPS. The two mutations found in the *P. jiroveci* DHPS gene from patients for whom sulfonamides were used were sufficient to cause a PABA auxotrophic requirement, as well as SMX resistance, when engineered into *S. cerevisiae* DHPS (Fig. 1). We conclude from this that such amino acid substitutions probably exert their effects either by reducing the affinity of DHPS for sulfa drugs and for PABA or by affecting the catalysis of condensation of sulfa drugs and PABA with H<sub>2</sub>PtPP. We have also determined that the requirement for exogenous PABA is much greater in primary transformants. After six passages, yeast cells adapted to abrogate this PABA requirement. The mechanism of this adaptation will be the subject of a future publication (P. Iliades, M. M. Barraclough, S. R. Meshnick, and I. G. Macreadie, unpublished data). This adaptive mechanism may involve increased biosynthesis of PABA. Upregulated PABA synthesis has been suggested as a mechanism for drug-resistant isolates of *N. meningitidis* (17, 21, 37). In a yeast model, it has been established that overexpression of the yeast PABA synthase gene, *ABZ1*, results in resistance to sulfa drugs (9). In order to ascertain whether adaptation (which possibly alters PABA synthesis) was the only parameter affecting resistance in our model, we determined the MICs for PABA-resistant strains in a range of PABA concentrations. These data indicated that PABA can confer increased SMX resistance on mutant strains. However, the most striking observation was the divergent resistance pattern among alleles (Fig. 5). If PABA was the only factor effecting resistance, the change in resistance would be expected to be uniform for each mutant. The divergent resistance profiles observed demonstrate that PABA levels can modulate the resistance of each mutant to various degrees. The ARS mutant showed significantly lower resistance than the ARS prototroph relative to the wild type, indicating that PABA may not be the only parameter implicated in the increased resistance of the adapted strains.

Importantly, our findings that the double-mutant constructs were more resistant than the wild-type and single-mutant alleles are consistent with the epidemiological data that indicate that double-mutant alleles predominate in frequency over the single mutants (6, 18). If the same is true in *P. jiroveci*, then the evolution of the double mutation occurred despite negative selection for the first mutation.

Finally, we have found a mutant that was more resistant to SMX than constructs with changes found naturally. The VRS mutant, with the novel T<sub>597</sub>V and P<sub>599</sub>S DHPS substitutions, was robust, having the lowest PABA dependence (of the double mutants), the shortest generation times, and the highest SMX resistance. Clearly there is an important interplay that

**FIG. 4.** Generation time of adapted transformants supplemented with increasing concentrations of SMX in MM<sub>200</sub>. The results are averages of four growth experiments.

**FIG. 5.** MICs for naive transformants growing in MM<sub>0</sub> supplemented with increasing concentrations of SMX and PABA. The results are averages of three experiments. Note that the higher seeding density for this experiment permitted the growth of all naive transformants.
appears to operate between PABA requirements and SMX resistance. This mutant has fortunately not been observed clinically, suggesting that in _P. jiroveci_ it is disadvantageous or, conversely, it might be just a matter of time before it appears.

The construction of _S. cerevisiae_ strains with altered DHPS sequences opens many new approaches to examining resistance to sulfa drugs. It will now be possible to more precisely assay the whole process of the emergence of sulfa drug resistance in infectious organisms, using sophisticated tools associated with _S. cerevisiae_ genetics and molecular biology. For example, using _S. cerevisiae_ FAS mutants (30) showed that sulfa drugs are converted to sulfadihydropterate (DHP) adducts that are growth inhibitory. Thus, sulfa drugs exert an effect by more than one mechanism. It will be interesting to compare the roles of SMX-DHP and the depletion of folate production with different DHPS enzymes. We predict that VRS and ARS synthesize DHP in _S. cerevisiae_ in yeast:

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