

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, T₅₉₇A 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 T₅₉₇ 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 sulfones and sulfonamides, in both prokaryotic and eukaryotic microbes. Organisms treated with sulfa drugs include pathogens, such as *Pneumocystis jiroveci*, *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 (*dhfr*) 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 (T₅₉₇, R₅₉₈, and P₅₉₉ in *S. cerevisiae*; T₅₁₇, R₅₁₈, and P₅₁₉ 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 *lacX74 rpsL hsdR* (*r_K⁻ m_K⁻*) *mcrB*]. The growth medium utilized was 1 \times YT (0.5% [wt/vol] yeast extract, 0.8% [wt/vol] tryptone, 0.5% [wt/vol] NaCl). Cells were made competent by calcium chloride treatment, and transformants were selected on 1 \times YT + 100 μ g of ampicillin/ml.

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FIG. 1. Alignment of DHPS sequences by using ClustalW. Displayed are sequences from *E. coli* (Ec), *Mycobacterium tuberculosis* (Mt), and *Staphylococcus aureus* (Sa), whose structures have been solved, aligned to those of *S. cerevisiae* (Sc) and *P. jiroveci* (Pj). The asterisks indicate totally conserved amino acids, the colons indicate very high conservation, and the dots indicate weak conservation. Amino acids associated with SMX resistance in *P. jiroveci* DHPS (T→A and P→S) are highlighted and in boldface. Dashes represent gaps introduced by the alignment.

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 (MM₀) 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% D-glucose, 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.

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 pro-

TABLE 1. Oligonucleotides used for site-directed mutagenesis

Clone	Substitution	Oligonucleotide no.	Direction of priming	Sequence (5' → 3') ^a
ARS	A ₅₉₇ , S ₅₉₉	146337	→	GGAGGGTGTTCCTGCCAGGTCTAACTCTATTTCAGGC
		146338	←	GCCTGAATAGAGTTAGACCTGGCAGAACACCCTCC
TRS	T ₅₉₇ , S ₅₉₉	146339	→	GGAGGGTGTTCCTACCAGGTCTAACTCTATTTCAGGC
		146340	←	GCCTGAATAGAGTTAGACCTGGCAGAACACCCTCC
ARP	A ₅₉₇ , P ₅₉₉	146341	→	CGTTGGAGGGTGTTCCTGCCAGGCCTAACTCTATTTC
		146342	←	GAATAGAGTTAGGCCTGGCAGAACACCCTCCAACG

^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 $\sim 6 \times 10^6$ CFU/ml ($A_{595} = 0.1$). A series of twofold dilutions was made in a 96-well microtiter plate. One microliter 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 aliquots of MM₀ solidified with 1.5% agar (Merck). A 6.5-mm-diameter hole was made in the center of each agar plate. SMX (25 µl at 100 mg/ml dissolved in dimethyl sulfoxide [DMSO]) was loaded into the center and allowed to diffuse through the plate for >2 h. In control plates, 25 µl of DMSO was also loaded into the center and allowed to diffuse. Each experiment was performed in triplicate. 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 A_{595} 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. Growth was evaluated after 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/ml (wt/vol) (MM₂₀₀) was monitored periodically over 180 h by turbidity measurements at A_{595} . 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 A_{595} of 0.1. These cells were then used to seed the growth cultures at an A_{595} of 0.02. The MM₂₀₀ broth was supplemented with SMX at 0 to 1,200 µg/ml. Replicate experiments were prepared in 96-well plates (2.2 ml deep), and the cultures were finally aliquoted into four standard-size microtiter plates (Costar 3603) to a final volume of 150 µl. The plates were then incubated in a 30°C humidified incubator and shaken at 1,000 rpm (with a 3-mm displacement). The turbidity was measured using a Multiskan Ascent microplate reader (Thermo Labsystems), and the data were plotted using Graphpad PRISM software (version 3).

Determination of SMX resistance of naïve 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 naïve 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 at pH 7.1. The DMSO concentration was constant at (0.5%). Fresh (naïve) transformants were precultured in MM₃₀₀ containing 2% glucose. Cells were harvested in mid-log-phase growth, washed twice with 50 ml of PBS, and normalized to an A_{595} 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-PPP-K-DHPS trifunctional enzyme (FAS) under the control of its native promoter (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 (H₂PtPP).

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 competitive effect of PABA with the sulfa drugs. The results of the replicates of drug diffusion assays in the presence of various levels of PABA are shown in Fig. 3. In agreement with previous studies (9), PABA and SMX were shown to compete so that the degree of inhibition by SMX for each strain decreased with increasing concentrations of PABA. The smallest inhibitory zones were observed with VRS and ARS, indicating that the double substitutions confer the greatest SMX resistance, and ARP and TRS, with single-amino-acid substitutions, were more sensitive to growth inhibition than the wild type.

All transformants retained at least some SMX sensitivity, probably reflecting the fact that DHPS is essential, and it might not be possible to diverge to retain DHPS function and have no sensitivity to SMX. It was possible to show that the inhibition of VRS and ARS, as well as TRP, converged to zero, or no inhibition (complete resistance), when the PABA concentration was >300 ng/ml, while the single-mutant alleles (ARP and TRS) were still inhibited with PABA concentrations as high as 500 ng/ml (Fig. 3). By drug diffusion assays on solidified media, the pattern of resistance to the action of SMX was VRS > ARS > TRP > TRS > ARP (most resistant to least resistant).

SMX resistances of prototrophs in broth cultures. In order to precisely analyze whether the growth rate influenced drug

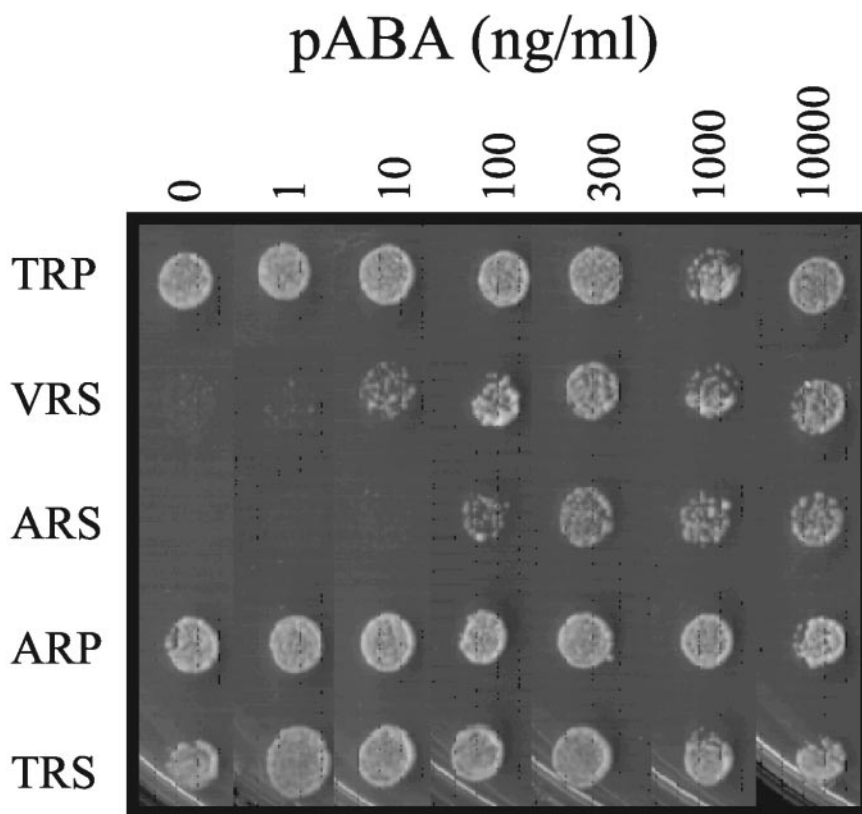


FIG. 2. Growth of transformants on minimal medium supplemented with various levels of PABA.

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

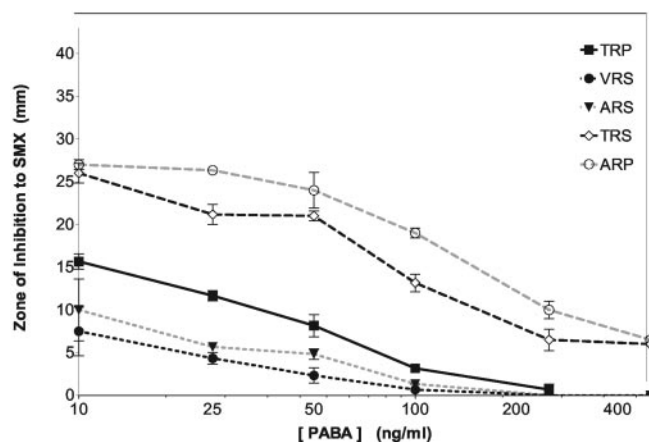


FIG. 3. Determination of SMX resistance by agar drug diffusion assay. SMX was solubilized in DMSO (100 mg/ml), and 25 μ l was applied to the centers of agar plates. Additional plates were supplemented with increasing concentrations of PABA. Transformants were harvested in log-phase growth, washed in PBS, and normalized to 6×10^6 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.

SMX, as strong growth was observed at the highest SMX concentration tested, suggesting that the MIC was significantly greater than 1,200 μ g/ml (i.e., $\gg 1,200$ μ g/ml). TRP and ARS were significantly (but not completely) inhibited at 1,200 μ g/ml, suggesting that the MICs were slightly greater than 1,200 μ g of SMX/ml (i.e., $>1,200$ μ g/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 μ g/ml, TRP had a shorter gen-

TABLE 2. MICs of SMX for yeast isolates grown in MM₂₀₀

DHPS sequence	Isolate	MIC (μ g/ml)
A ₅₉₇ , P ₅₉₉	ARP	150
T ₅₉₇ , S ₅₉₉	TRS	600
A ₅₉₇ , S ₅₉₉	ARS	>1,200
T ₅₉₇ , P ₅₉₉	TRP	>1,200
V ₅₉₇ , S ₅₉₉	VRS	>>1,200

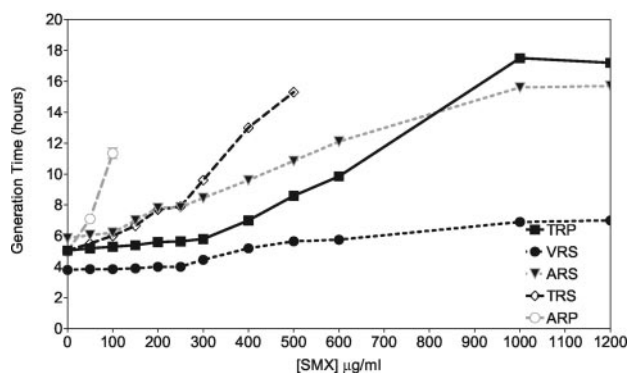


FIG. 4. Generation time of adapted transformants supplemented with increasing concentrations of SMX in MM_{200} . The results are averages of four growth experiments.

eration time than the double mutant ARS. However, at SMX concentrations of $>800 \mu\text{g/ml}$, 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_{100} (data not shown) and MM_{200} . The MIC was measured as $\gg 1,200 \mu\text{g/ml}$ (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 naïve 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 naïve 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, $2,000 \mu\text{g/ml}$, 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 $2,000 \mu\text{g/ml}$ when the PABA concentration was $150 \mu\text{g/ml}$.

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 sulfa prophylaxis failed 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

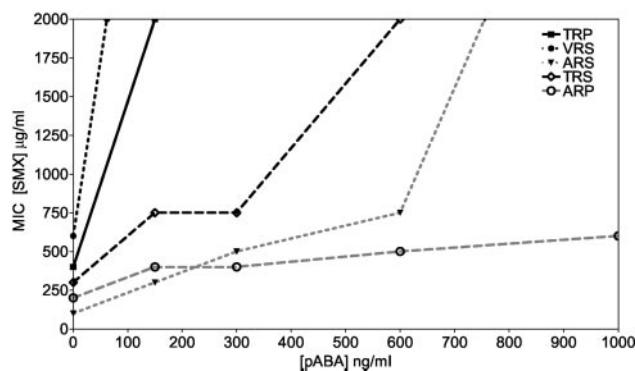


FIG. 5. MICs for naïve transformants growing in MM_0 supplemented with increasing concentrations of SMX and PABA. The results are averages of three experiments. Note that the higher seeding density for this experiment permitted the growth of all naïve transformants.

for sulfa drugs and for PABA or by affecting the catalysis of condensation of sulfa drugs and PABA with H_2PtPP . 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 naïve 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_{597V} and P_{599S} 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

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 a model system that may have implications for understanding 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 sulfadihydropteroate (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 preference to sulfa-DHP, and the ability of the double mutants to do this is due to their discrimination between PABA and sulfa drugs based on the binding affinity of DHPS. In addition, there may be other factors affecting sulfa drug resistance, such as the scavenging of nutrients (25, 35), PABA levels (9), and folic acid levels (4).

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