Mutations in the *Pneumocystis jirovecii* DHPS Gene Confer Cross-Resistance to Sulfa Drugs

Peter Iliades,^{1*} Steven R. Meshnick,² and Ian G. Macreadie¹

CSIRO, Health Sciences and Nutrition, Parkville, Victoria, Australia,¹ and Department of Epidemiology, University of North Carolina, Chapel Hill, North Carolina²

Received 20 August 2004/Returned for modification 23 September 2004/Accepted 4 October 2004

Pneumocystis jirovecii is a major opportunistic pathogen that causes *Pneumocystis* pneumonia (PCP) and results in a high degree of mortality in immunocompromised individuals. The drug of choice for PCP is typically sulfamethoxazole (SMX) or dapsone in conjunction with trimethoprim. Drug treatment failure and sulfa drug resistance have been implicated epidemiologically with point mutations in dihydropteroate synthase (DHPS) of *P. jirovecii*. *P. jirovecii* cannot be cultured in vitro; however, heterologous complementation of the *P. jirovecii* trifunctional folic acid synthesis (PjFAS) genes with an *E. coli* DHPS-disrupted strain was recently achieved. This enabled the evaluation of SMX resistance conferred by DHPS mutations. In this study, we sought to determine whether DHPS mutations conferred sulfa drug cross-resistance to 15 commonly available sulfa drugs. It was established that the presence of amino acid substitutions (T₅₁₇A or P₅₁₉S) in the DHPS domain of PjFAS led to cross-resistance against most sulfa drugs evaluated. The presence of both mutations led to increased sulfa drug resistance, suggesting cooperativity and the incremental evolution of sulfa drug resistance. Two sulfa drugs (sulfachloropyridazine [SCP] and sulfamethoxypyridazine [SMP]) that had a higher inhibitory potential than SMX were identified. In addition, SCP, SMP, and sulfadiazine (SDZ) were found to be capable of inhibiting the clinically observed drug-resistant mutants. We propose that SCP, SMP, and SDZ should be considered for clinical evaluation against PCP or for future development of novel sulfa drug compounds.

Pneumocystis jirovecii is a major opportunistic pathogen that results in *Pneumocystis* pneumonia (PCP) of AIDS patients and immunocompromised individuals. It accounts for 40% of all AIDS-defining conditions and is the major cause of mortality of children with AIDS in Africa (2, 16).

Clinically, PCP has been treated with antifolates including combination therapy with sulfamethoxazole (SMX) and trimethoprim (TM) as the preferred first-line treatment (19). Sulfa drugs are inhibitors of folic acid synthesis (FAS). In fungi, including *Candida albicans*, *Saccharomyces cerevisiae*, and *P. jirovecii*, the FAS genes are part of a single open reading frame that encodes a trifunctional, multidomain enzyme that includes dihydroneopterin aldolase, hydroxymethyl-dihydropterin pyrophosphokinase, and dihydropteroate synthase (DHPS) (15, 34). The FAS genes, including dihydrofolate synthase, are essential to prokaryotes and lower eukaryotes since they are dependent on de novo folate biosynthesis and do not possess the capability to actively sequester exogenous folate. Sulfa drugs such as SMX are competitive inhibitors of DHPS and work synergistically with TM, which inhibits microbial dihydrofolate reductase. In *P. jirovecii*, however, there is some evidence to suggest that TM is ineffective and that such treatment is actually sulfamethoxazole monotherapy (26, 35). Drug treatment failure of SMX-TM has been associated with point mutations in the *P. jirovecii* DHPS gene in a large number of epidemiological studies (3, 8, 11, 17–23, 27–29, 31–33, 36).

In the absence of a functional *P. jirovecii* trifunctional FAS

(PjFAS) gene, previous studies of *P. jirovecii* sulfa drug resistance relied on model systems to determine whether DHPS mutations conferred SMX resistance (13, 14, 24, 25). These studies employed the *S. cerevisiae* FAS (ScFAS) gene as a surrogate for PjFAS due to their high degree of similarity. Resistance was analyzed by using homologous complementation in a DHPS-deleted *S. cerevisiae* host strain (14). Mutations were engineered at the analogous ScFAS residues (T₅₉₇A and P₅₉₉S). This model indicated that mutants having two amino acid substitutions were initially compromised for growth due to an increased requirement for *p*-aminobenzoic acid (pABA). Prototrophs that could grow in the absence of pABA were isolated. Following adaptation (via continual passage on low-pABA medium), these double mutants were found to be capable of improved growth vigor and consequently increased sulfa drugs resistance. This implicated pABA up-regulation with sulfamethoxazole resistance. Increased pABA synthesis probably reflects an adaptive response that compensates for the reduced pABA binding affinity by the double amino acid substitutions at the catalytic site of DHPS.

The second study employed heterologous complementation using the same mutant constructs in a DHPS-disrupted *E. coli* strain (13). The *E. coli* model proved to be more robust than the *S. cerevisiae* model. The *E. coli* model did not have the pABA-dependent phenotype observed in the *S. cerevisiae* model. This indicated that endogenous pABA levels were much higher in *E. coli* than in *S. cerevisiae* as would be expected with a faster-growing strain. The data from these two studies indicated that (i) mutants having two amino acid substitutions, T₅₉₇A and P₅₉₉S, had increased sulfa drug resistance relative to the wild type (WT), (ii) mutants having the single amino acid substitution T₅₉₇A were more sensitive than the WT, and (iii)

* Corresponding author. Mailing address: CSIRO Health Sciences and Nutrition, 343 Royal Parade, Parkville, Victoria 3052, Australia. Phone: (613) 9662 7259. Fax: (613) 9662 7266. E-mail: peter.ilides@csiro.au.

there was cooperativity between individual mutations that led to the increased sulfa drug resistance of the double mutants.

Recently, cloning of the trifunctional PjFAS genes and their heterologous complementation in a *DHPS*-disrupted *E. coli* host strain was achieved (15). This provided an assay method that permitted a direct assessment of sulfa drug resistance conferred by mutations observed clinically ($T_{517}A$ and $P_{519}S$) in the PjFAS genes. This work endorsed the prediction that the double mutant ($T_{517}A$ and $P_{519}S$) had increased sulfamethoxazole resistance (threefold) relative to the WT clone. These data provided some explanation for the epidemiological evidence that identified the predominance of the double mutants clinically (18).

While *S. cerevisiae* is taxonomically more closely related to *P. jirovecii* than *E. coli*, the *E. coli* model system proved to be more robust in evaluating sulfa drug resistance because it avoided the above-mentioned complicating parameters of the *S. cerevisiae* model system. In this study, we utilized heterologous complementation of PjFAS in the *DHPS*-disrupted *E. coli* host strain to evaluate sulfa drug cross-resistance of PjFAS mutants ($T_{517}A$ and $P_{519}S$) against 15 sulfa drugs. We report the sulfa drugs that were more effective than SMX and dapsone (DAP), which are currently the drugs of choice to treat PCP.

MATERIALS AND METHODS

Cells, 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× YT medium (0.5% [wt/vol] yeast extract, 0.8% [wt/vol] tryptone, 0.5% [wt/vol] NaCl).

The *DHPS*-disrupted *E. coli* strain was C600 [Δ *folP*: Km^r F^- $e14^-$ (McrA[−]) *thr-1* *leuB6* *thi-1* Δ *lacY1* *glnV44* *rfdB1* *fluA21*] (9). This strain required 1× YT medium supplemented with thymidine or genetic complementation with a functional *DHPS* gene for growth. Cells were made competent by calcium chloride treatment (7), and transformants were selected by using 1× YT medium supplemented with 50 μ g of ampicillin/ml and 30 μ g of kanamycin/ml.

Sulfa drugs. Sulfa drugs used in this study were as follows. DAP, sulfacetamide (SAM), sulfadimethoxine (SDM), sulfamethoxypyridazine (SMP), sulfamoxole (SMO), sulfanilamide (SIA), and sulfapyridine (SPD) were purchased from Sigma Chemical Company. Sulfachloropyridazine (SCP), sulfadiazine (SDZ), sulfamerazine (SMR), SMX, sulfaquinoxaline (SQX), sulfathiazole (STZ), and sulfisoxazole (SSA) were purchased from ICN Biomedicals. Dapsone (diaminodiphenylsulfone) was purchased from Aldrich Chemical Company. Sulfadoxine (SDX) was a gift from Roche.

Construction of vectors. (i) **pGEX.PjFAS and pET28a.PjFAS.** PjFAS expression constructs in pGEX 4T-2 (glutathione *S*-transferase tagged) or in pET28a (six-His tagged) have been described previously (15). In this study, we employed a pET28a.PjFAS mutant construct ($M_{596}T$) that permitted heterologous complementation in *E. coli* C600 Δ *folP*: Km^r and was identified following random chemical mutagenesis with ethane methyl sulfonate. It is believed to confer increased DHPS activity, although the precise mechanism has not been dissected. We refer to this clone as PjFAS $M_{596}T$.

(ii) **pET28a.EcDHPS.** *E. coli* DHPS (EcDHPS) was amplified from *E. coli* genomic DNA by using PCR primers 224155 (5'-ggctcgccgatccATGAACTCTTGCCAGGGTACTTCACTGGACCTTAGCC) and 224156 (5'-aagcttgctgacTTACTCATAGCGTTTGTTCCTTTGAGACAGAGTGGC) having BamHI sites upstream and SalI sites downstream (lowercase letters signify restriction sites). *E. coli* C600 cells (the parental line of C600 Δ *folP*: Km^r) were picked from a 1× YT plate, resuspended in 20 μ l of water, and boiled for 12 min at 99°C. The cellular debris was then pelleted by centrifugation for 10 min at 20,000 × *g*, and 1 μ l of the supernatant containing DNA was used in a 100- μ l PCR with Vent polymerase (NEB) to yield a PCR fragment of 872 bp. The PCR fragment was partially digested with BamHI and SalI and cloned into a similarly digested pET28a vector. This clone was confirmed by diagnostic restriction digests and by DNA sequencing. The pET28a constructs were used to compare intrinsic sulfa drug resistance between PjFAS, ScFAS, and EcDHPS.

TABLE 1. Oligonucleotides used to synthesize the mutant alleles

Allele	Clone name	Primer no.	Sequence (5'–3') ^a
$T_{517} R_{518} P_{519}$ $V_{517} R_{518} S_{519}$	TRP	259705	GGTGGGCAGTCTGTACGG
	VRS	259706	TCTGGTTACATGTTG CAACATGTGAACCAGaCC GTacAGACTGCCACC
$A_{517} R_{518} S_{519}$	ARS	109216	GGAGGGTGTCTGCCCAGG TCTAACTCTATTTCAGGC
		109217	GCCTGAATAGAGTTAGAC CTGGCAGAACACCCTCC
$T_{517} R_{518} S_{519}$	TRS	109214	GGAGGGTGTCTACCAGG TCTAACTCTATTTCAGGC
		109215	GCCTGAATAGAGTTAGAC CTGGCAGAACACCCTCC
$A_{517} R_{518} P_{519}$	ARP	109212	CGTTGGAGGGTGTCTGTC CAGGCCTAACTCTATTC
		109213	GAATAGAGTTAGGCCTGG CAGAACCCTCCAACG

^a Lowercase letters signify noncoding sequence.

Synthesis of mutant alleles implicated with sulfa drug resistance in pGEX.PjFAS. Three alleles having mutations at $T_{517}A$ and $P_{519}S$ (designated ARS, TRS, and ARP) are found in PCP patients who have been treated with sulfa drugs (3, 17, 18). Their synthesis was achieved by using a Quikchange XL sited-directed mutagenesis kit (15) and has been described previously. The oligonucleotides used and alleles generated are summarized in Table 1. A fourth allele, which has not been reported clinically, was synthesized by using oligonucleotides 259705 and 259706 (Table 1). This synthesis yielded $T_{517}V$ and $P_{519}S$ for PjFAS and is designated VRS herein. The DNA sequence of mutants was confirmed by DNA sequencing analysis using a Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). Functional complementation was confirmed by transforming the clones into the *E. coli* C600 Δ *folP*: Km^r strain and plated onto 1× YT medium minus thymidine.

Analysis of sulfa drug resistance by agar drug diffusion assays. In order to evaluate the sensitivity or resistance of each DHPS allele to sulfa drugs, drug diffusion assays were performed as described previously (13). These assays were performed in 86-mm-diameter petri dishes containing 22.1-ml ($\pm$ 0.1-ml) aliquots of 1× YT medium solidified with 1.5% (wt/vol) agar and supplemented with the appropriate selective antibiotic. A 6.5-mm hole was made in the center of each agar plate. Sulfa drugs were prepared immediately before use and dissolved in dimethyl sulfoxide to 100 mg/ml, and 30 μ l was loaded onto the center of each plate and allowed to diffuse through the agar for at least 5 h. In control plates, 30 μ l of dimethyl sulfoxide was also loaded onto the center of each plate and allowed to diffuse through the agar. Each experiment was done in quadruplicate. Clones were precultured in 2× YT broth at 37°C. The *E. coli* cultures were harvested while in log-phase growth, washed with 1× phosphate-buffered saline (0.8% [wt/vol] NaCl, 0.2% [wt/vol] KCl, 1.44% [wt/vol] Na_2HPO_4 , 0.24% [wt/vol] KH_2PO_4 [pH 7.4]) and normalized to an A_{595} of 0.100. Clones were then diluted 500-fold in 1× phosphate-buffered saline prior to inoculation. A six-spoke inoculation tool (13) was used to inoculate six individual clones radially from the center of the agar plate. Plates were then incubated at 37°C for 24 h and then cooled to 4°C until the zones of inhibition were measured. Zones of inhibition were measured to the nearest half-millimeter and then analyzed by using PRISM version 4.00 statistical analysis software (for Windows; GraphPad Software, San Diego, Calif. [http://www.graphpad.com]). Analyses performed were the determinations of means and standard deviations for all data sets. Each data set was normalized to determine resistance or sensitivity relative to the wild-type allele (TRP) according to the following equation: [mean radius (mutant) – mean radius (WT)]/[mean radius (maximum) – mean radius (WT)] × 100. The mean radius (maximum) was the highest measured radius of inhibition (for the mutant VRS) which was arbitrarily defined as 100% inhibition. The wild-type inhibition was defined as the baseline or 0% inhibition.

Analysis of the IC₅₀ in broth cultures. Transformants of pGEX 4T-2.PjFAS were precultured in 1× YT medium containing ampicillin (100 μ g/ml) and kanamycin (30 μ g/ml). Cells were harvested during mid-log-phase growth and normalized to an A_{595} of 0.1. Cells were then diluted 200-fold, and 5 μ l was used to seed 145 μ l of 1× YT medium containing 100 μ g of ampicillin/ml, 30 μ g of kanamycin/ml, and sulfa drugs in a 96-well plate (catalog number 167008; Nunc). SCP was evaluated at 0 to 250 μ g/ml in 5- μ g/ml increments. SMP was evaluated at 0 to 250 μ g/ml in 5- to 200- μ g/ml increments. SMX and SDZ were evaluated

FIG. 1. Inhibition of PjFAS mutants expressed from pGEX 4T-2 determined by using the agar drug diffusion assay. (a) Means and standard deviations of the zone of inhibition. (b) The mean and standard deviation was calculated for each data set which was then normalized against the wild-type allele (TRP) and expressed as percent sensitivity or percent resistance relative to the wild type. Analysis was performed by using GraphPad Prism software, version 4.00.

a **b**

at 0 to 1,200 $\mu\text{g/ml}$ in 50- to 100- $\mu\text{g/ml}$ increments. Cultures were grown at 37°C for 24 h, the turbidity (A_{600}) was measured by using a Multiskan Ascent microplate reader (Thermo Labsystems), and the data were plotted with Graphpad PRISM software (version 4.00). Each experiment was performed in quadruplicate and set up by using a Rapidplate liquid-handling robot (QIAGEN). The 50% inhibitory concentration (IC_{50}) was determined to be the drug concentration required to reduce the turbidity relative to the “no-drug control” by 50%.

RESULTS

Sulfonamide cross-resistance of mutant PjFAS alleles. In order to assess whether the mutations that conferred SMX resistance (as previously demonstrated [15]) also conferred cross-resistance to other sulfa drugs, the growth inhibition of four mutant clones (VRS, ARS, TRS, and ARP) by 15 sulfa drugs was evaluated by using an optimized agar drug diffusion assay described previously (13). The growth inhibition was analyzed as described in Materials and Methods. The absolute inhibition of each allele to each sulfa drug is presented in Fig. 1a. The WT (normalized to 0% inhibition) was compared against each mutant allele (with the maximum inhibition [for VRS] normalized to 100%) (Fig. 1b). Each sulfa drug evaluated in this study inhibited the mutants by various degrees. No consistent resistance profile was observed between the mutants across the range of sulfa drugs. The magnitude of resistance of each allele varied depending on the identity of the sulfa drug. These data were indicative of cross-resistance and suggested that the resistance conferred by an amino acid substitution (in the catalytic site of DHPS) does not establish unequivocal or comparable resistance to all sulfonamides.

While it was observed that the mutant alleles were more resistant than the WT, there were a number of exceptions where the mutant alleles were more sensitive than the WT. Specifically, these exceptions were noted: (i) VRS was more sensitive than the WT to the sulfa drugs SCP, SMP, and SDZ; (ii) TRS was more sensitive than the WT to the sulfa drugs SCP, SMP, SDZ, and SDM; and (iii) ARP was more sensitive than the WT to SCP. This was also the case in medium that was prepared by using tryptone which was free of sulfonamide antagonists (catalog number 1.02239; Merck). Evidently, the two sulfa drugs (SCP and SMP) had the highest inhibitory activity overall and appeared to be more effective against the mutant alleles relative to the WT. SDZ also showed greater inhibitory potential against the mutants VRS, ARS, and TRS than the WT, but ARP was more resistant.

The 15 sulfa drugs evaluated against PjFAS were ranked from highest to lowest inhibitory potential as follows: SCP > SMP > SMX > STZ > SAM > SDZ > SDM > SMO > SMR > SIA > SSA > DAP > SQX > SPD > SDX. The drugs SMO to SDX, which had the lowest inhibitory potential, were also the least effective against the mutant alleles. The sulfa drugs SCP to SDM (with the exception of SMX and STZ) showed some capacity to inhibit the mutant alleles relative to the inhibition of the WT. Comparison of the R groups of the “top-10-ranked” sulfa drugs revealed some common elements (Fig. 2). With the exception of SAM and SSA, the sulfa drugs that had the highest inhibitory potential have an R group that consists of an aromatic ring with an N substitution at the *ortho*

FIG. 2. (a) Structure of pABA and sulfonamides. (b) Three-dimensional structural alignment of SCP and SMP.

FIG. 3. Inhibition of EcDHPS, PfFAS, ScFAS, and PjFAS $M_{596}T$ constructs (means and standard deviations) expressed from pET28a determined by using the agar drug diffusion assay.

position or adjacent to the amide group. SAM and SSA have an O substitution at the *ortho* position. Using this model, it was evident that DAP, which has been utilized for the treatment of PCP, had a significantly lower inhibitory potential than SMX. In fact, DAP was among the least effective inhibitors in this assay system.

The three-dimensional alignment of SCP and SMP (Fig. 2b) done by ChemSketch/3D Viewer (version 5.07; ACDlabs software, Advanced Chemistry Development Inc., Toronto, Ontario, Canada) revealed that they maintain an identical three-dimensional spatial orientation that is unique among the sulfa drugs evaluated in this study. This result may be an indicator of a more specific fit of these two drugs into the pABA binding site of DHPS of PjFAS. Alternatively, these drugs may achieve higher intracellular concentrations either through a higher diffusion rate constant into *E. coli* or, conversely, a lower efflux rate.

Sulfa drug inhibition of DHPS from various species. We sought to investigate whether (i) the sulfa drug resistance pattern observed using this assay system was a function of the permeability of specific sulfa drugs through the membrane of the *folP* host strain, (ii) the antifolate activity of the 15 sulfa drugs was comparable between different species of FAS and DHPS, and (iii) the DHPS mutations were significant variables in sulfa drug resistance profiles. We therefore compared the resistance profile of the WT and mutant alleles expressed from pET28a of PjFAS $M_{596}T$ with those from previous comparable studies performed with ScFAS (13), Pf-PPPK.DHPS (PfFAS) (6), and EcDHPS (this study).

Comparison of the ratio of inhibition between the six-His tagged constructs of EcDHPS, ScFAS, PfFAS, and PjFAS ($M_{596}T$, which enabled complementation) revealed no consis-

tent trend between individual drugs across DHPS species (Fig. 3). We therefore ruled out permeability as a significant factor in the resistance profile of the FAS/DHPS constructs. This did not, however, discount permeability as a factor in the overall ranking of inhibitory activity of the sulfa drugs. It is quite possible that some sulfa drugs may have improved permeability through the membrane of *E. coli* compared to those of others. Each sulfa drug inhibited the DHPS from different species with various degrees. The inhibition of EcDHPS was consistently the lowest, and the inhibition of PjFAS $M_{596}T$ was consistently the highest. ScFAS and PfFAS showed an intermediate level of resistance relative to EcDHPS and PjFAS $M_{596}T$. Clearly, EcDHPS had a significantly higher intrinsic sulfa drug resistance relative to ScFAS and PjFAS $M_{596}T$. This finding indicated that the DHPS sequence, and therefore structure, from different species had a significant impact on sulfa drug resistance. This was also evident by the observation that the ranking of inhibitory potential of sulfa drugs was different for each DHPS sequence (DHPS from four different species). (Table 2). These data indicated that SCP was consistently the most

TABLE 2. Ranking of inhibitory potential of sulfa drugs for DHPS from various species in the *E. coli* C600Δ*FolP* strain

DHPS species	Sulfa drug by rank					
	1	2	3	4	5	6
<i>S. cerevisiae</i>	SCP	STZ	SMX	SAM	SSA	SMP
<i>P. jirovecii</i>	SCP	SMP	SMX	STZ	SAM	SDZ
<i>P. falciparum</i>	SCP	SAM	SMO	STZ	SIA	SMX
<i>E. coli</i> ^a	SCP	SMX	SSA	SMP	STZ	SAM

^a WT only.

FIG. 4. Liquid growth inhibition assays of each PjFAS mutant against four sulfonamide drugs (SCP, SMP, SMX, and SDZ) performed in 96-well microtiter plates; the percent inhibition relative to the no-drug control is shown. Each point represents the mean of quadruplicate experiments, and the standard errors are shown by the error bars.

inhibitory sulfa drug. However, the ranking of the remaining 14 sulfa drugs differed from species to species. Furthermore, a different pattern of resistance was noted between PjFAS (T₅₁₇A and P₅₁₉S) and ScFAS (T₅₉₇A and P₅₉₉S) mutants (10). This result indicated that identical amino acid substitutions at comparable sites can have significantly different resistance outcomes, implicating subtle structural differences between the individual DHPS superstructures.

Determination of IC₅₀s of PjFAS alleles to SCP, SMP, SMX, and SDZ. In order to quantitatively determine the resistance of the four drugs found to be most inhibitory to the drug-resistant alleles, liquid growth inhibition assays were performed with 96-well microtiter plates. In agreement with the drug diffusion assays, these data indicated that SCP and SMP had significantly higher inhibitory potential than SMX (Fig. 4). The MIC (the concentration required to inhibit 100% of growth) of SCP was in the order of 60 µg/ml (0.2 mM), and the MIC of SMP was in the order of 150 µg/ml (0.5 mM), while the MIC of SMX and SDZ was closer to 700 µg/ml (2.8 mM).

A finer discrimination of resistance between each allele could be discerned by determining the IC₅₀ (Fig. 4 and Table 3). It was determined that the resistance pattern of each allele relative to the WT was consistent with the results of the agar drug diffusion assay (with one exception). In agreement with the SDZ drug diffusion assays, the mutants TRS, VRS, and ARS were more sensitive than the WT, while ARP was more resistant than the WT in the liquid growth inhibition experiment. Also in agreement with the SMX drug diffusion assays, it was found that all mutant alleles were more resistant than the WT in the liquid growth inhibition experiment. Furthermore, as predicted by the SMP drug diffusion assay, VRS and TRS were more sensitive than the WT in the liquid growth inhibition experiment. ARS was slightly more resistant than the WT (but was within experimental error), and ARP was found to be more resistant than the WT. Finally, as predicted by the SCP drug diffusion assay, TRS and VRS were more sensitive than the WT, while ARS was more resistant. ARP was found to be more resistant than the WT in the liquid

growth inhibition assay, which was contrary to the agar drug diffusion data. Despite this latter incongruous result, it was evident that like SDZ, SMP was capable of inhibiting the naturally occurring double mutant ARS and single mutant TRS to a greater degree than the WT.

The discordant data between the agar drug diffusion assay and the liquid growth inhibition assay for SCP remains unresolved. However, it was not surprising that sulfa drug resistance results do not always translate from agar to liquid media. A case in point is the sulfamethoxazole resistance of the PjFAS mutants determined previously (15) by using the validated Etest methodology. The MIC of 1 to 3 µg/ml determined in that study does not correlate to the MIC determined with liquid medium, which was in the order of 600 µg/ml. Clearly, the dynamics of nutrient, sulfa drug, and sulfa adduct diffusion (30) in and out of the cell are significantly different between agar and liquid.

DISCUSSION

A large amount of epidemiological data has linked the emergence of mutations in PjFAS to the exposure to sulfa drugs, but direct evidence demonstrating that such mutations confer resistance in *P. jirovecii* has only recently emerged (15). That previous work demonstrated that amino acid substitutions in PjFAS T₅₁₇A and P₅₁₉S (ARS) conferred a threefold-increased level of SMX resistance relative to the WT and that the indi-

TABLE 3. IC₅₀ of *E. coli* C600Δ*folP*::Km^r transformed with mutant or WT PjFAS

Drug	IC ₅₀ (µg/ml)				
	TRP	ARP	TRS	ARS	VRS
SCP	11.7	15.1	6.4	16.3	11.4
SMP	28	37	15	27	18
SMX	38	58	53	121	74
SDZ	171	275	48	98	60

vidual amino acid substitutions functioned cooperatively, resulting in the elevated resistance of double mutant ARS.

The cross-resistance data presented herein provide a similar overall resistance profile and support the previous findings of cooperativity between individual mutations that resulted in increased resistance of the double mutant ARS. This point was not absolutely true nor was it without exception in this cross-resistance study. The single mutant ARP was more resistant than the double mutant ARS for five drugs (SCP, SMP, SAM, SDZ, and SMR). Clearly, cooperativity between individual amino acid substitutions that lead to higher resistance is dependent on the drug and its fit into the pABA binding site of DHPS.

Of particular interest was the finding that the mutant ARS, which is the clinical isolate observed most frequently, was considerably more resistant than the synthetic double mutant VRS and the single mutants ARP and TRS. Liquid growth inhibition studies confirmed previous findings (15) which showed a threefold-increased level of SMX resistance (IC_{50}) of ARS compared to that of the WT. However, the absolute drug concentration required was significantly higher in liquid growth inhibition assays than in agar drug diffusion assays. This may be explained by the significant differences in drug and nutrient diffusion in the two medium types.

This finding is in contrast with previous findings with *S. cerevisiae* model systems (13, 14) that showed VRS to be more resistant than ARS. This finding highlights that the role of residues T₅₁₇ and P₅₁₉ of PjFAS (or T₅₉₇A and P₅₉₉S of ScFAS) is highly conserved in terms of conferring sensitivity or resistance to sulfa drugs between homologs of FAS, the tertiary context of these residues in the overall DHPS sequence and structure is critically important. That is, subtle differences in the catalytic site can have profound effects on drug resistance. Clearly, VRS is significantly more resistant than ARS in the *S. cerevisiae* FAS structure, but ARS is significantly more resistant than VRS in the *P. jirovecii* FAS structure.

The data presented in this study demonstrated that the mutations T₅₁₇A and P₅₁₉S in PjFAS led to cross-resistance for most sulfa drugs evaluated. Clearly, mutations that lead to resistance against one drug can have broad implications for resistance against an entire class of drug. This finding would be consistent with a highly conserved drug binding site and is supported by the consistent resistance trend observed from previous model studies (13). Clearly, subtle structural differences in the pABA binding site dictate drug specificity and resistance for different species as evidenced by the different intrinsic resistance-susceptibility pattern to sulfa drugs (Table 2). Four DHPS structures have been solved, *E. coli* (1), *Staphylococcus aureus* (10), *Mycobacterium tuberculosis* (4), and *S. cerevisiae* (M. C. Lawrence et al., unpublished data). It is evident that the pterin binding site is exquisitely conserved, while the pABA binding site shows significant structural variation (despite sequence conservation) and is reflected by the observed variation in sulfa susceptibility between species. SCP consistently showed the highest inhibitory potential for all DHPS enzymes, indicating that some sulfonamides may have broad-spectrum activity against DHPS from various species while some sulfonamides can act with greater species specificity. On this last point, there seemed considerable overlap in the efficacy of the top-6-ranked sulfa drugs across four different

species of DHPS (Table 2). Selective diffusion through the *E. coli* membrane may be a significant contributor to the higher activity of SCP. If so, drug diffusion would be a key criterion to investigate for the selection of a species-specific inhibitor.

PjFAS M₅₉₆T was observed to have higher susceptibility to sulfa drugs than EcDHPS and ScFAS. Based on the ranking of inhibitory activity for various sulfa drugs, it is possible that PCP therapy could be more efficacious by the choice of a sulfa drug (such as SCP or SMP) which had higher activity relative to SMX. It would seem that these data can be interpreted to suggest that antifolate compounds (sulfonamides or otherwise) can be selected through screening to have greater drug specificity to individual DHPS species. Furthermore, antifolates such as SCP that had the highest activity against the four DHPS species (including Pf-PPPK.DHPS, as described by Berglez et al. [6]) indicates that antifolates that have broad-spectrum activity can be identified.

Of the 15 drugs evaluated, 2 drugs were noted to be conspicuously superior to SMX in their inhibitory potential: SCP and SMP, which were ranked first and second. SMX, which has been the sulfa drug of choice to treat or prevent PCP infections, was ranked third. More importantly, it was observed that SDZ (ranked sixth), was superior to all other drugs due to its ability to effectively inhibit the mutant alleles (ARS, VRS, and TRS) to a greater degree than the WT. SDZ was the only drug capable of inhibiting ARS. These features make these three sulfa drugs worthy candidates for further evaluation against PCP. These data support previous findings which demonstrated that SMP was effective against a mouse model of PCP (5, 12).

ACKNOWLEDGMENTS

We thank Doris Clarke and Ashton Clarke for preparation of the media and plates used in this study and Athena Iliades for scoring the zones of inhibition. We appreciate the efforts of Kate Griffiths, Anthony Roberts, and Onisha Patel for their critical review of the manuscript.

REFERENCES

1. Achari, A., D. O. Somers, J. N. Champness, P. K. Bryant, J. Rosemond, and D. K. Stammers. 1997. Crystal structure of the anti-bacterial sulfonamide drug target dihydropteroate synthase. *Nat. Struct. Biol.* 4:490-497.
2. Ansari, N. A., A. H. Kombe, T. A. Kenyon, L. Mazhani, N. Binkin, J. W. Tappero, T. Gebrekristos, S. Nyirenda, and S. B. Lucas. 2003. Pathology and causes of death in a series of human immunodeficiency virus-positive and -negative pediatric referral hospital admissions in Botswana. *Pediatr. Infect. Dis. J.* 22:43-47.
3. Armstrong, W., S. Meshnick, and P. Kazanjian. 2000. *Pneumocystis carinii* mutations associated with sulfa and sulfone prophylaxis failures in immunocompromised patients. *Microbes Infect.* 2:61-67.
4. Baca, A. M., R. Sirawaraporn, S. Turley, W. Sirawaraporn, and W. G. Hol. 2000. Crystal structure of *Mycobacterium tuberculosis* 7,8-dihydropteroate synthase in complex with pterin monophosphate: new insight into the enzymatic mechanism and sulfa-drug action. *J. Mol. Biol.* 302:1193-1212.
5. Bartlett, M. S., M. M. Shaw, J. W. Smith, and S. R. Meshnick. 1998. Efficacy of sulfamethoxypyridazine in a murine model of *Pneumocystis carinii* pneumonia. *Antimicrob. Agents Chemother.* 42:934-935.
6. Berglez, J., P. Iliades, W. Sirawaraporn, P. Coloe, and I. Macreadie. 2004. Analysis in *Escherichia coli* of *Plasmodium falciparum* dihydropteroate synthase (DHPS) alleles implicated in resistance to sulfadoxine. *Int. J. Parasitol.* 34:95-100.
7. Cohen, S. N., A. C. Chang, and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* 69:2110-2114.
8. Demanche, C., J. Guillot, M. Berthelemy, T. Petitt, P. Roux, and A. E. Wakefield. 2002. Absence of mutations associated with sulfa resistance in *Pneumocystis carinii* dihydropteroate synthase gene from non-human primates. *Med. Mycol.* 40:315-318.
9. Fermer, C., and G. Swedberg. 1997. Adaptation to sulfonamide resistance in

- Neisseria meningitidis* may have required compensatory changes to retain enzyme function: kinetic analysis of dihydropteroate synthases from *N. meningitidis* expressed in a knockout mutant of *Escherichia coli*. *J. Bacteriol.* **179**:831–837.
10. Hampele, I. C., A. D'Arcy, G. E. Dale, D. Kostrewa, J. Nielsen, C. Oefner, M. G. Page, H. J. Schonfeld, D. Stuber, and R. L. Then. 1997. Structure and function of the dihydropteroate synthase from *Staphylococcus aureus*. *J. Mol. Biol.* **268**:21–30.
 11. Helweg-Larsen, J., T. L. Benfield, J. Eugen-Olsen, J. D. Lundgren, and B. Lundgren. 1999. Effects of mutations in *Pneumocystis carinii* dihydropteroate synthase gene on outcome of AIDS-associated *P. carinii* pneumonia. *Lancet* **354**:1347–1351.
 12. Hughes, W. T., and J. Killmar. 1996. Monodrug efficacies of sulfonamides in prophylaxis for *Pneumocystis carinii* pneumonia. *Antimicrob. Agents Chemother.* **40**:962–965.
 13. Iliades, P., S. R. Meshnick, and I. G. Macreadie. Analysis of *Pneumocystis jirovecii* DHPS alleles implicated in sulfamethoxazole resistance using an *Escherichia coli* model system. *Microb. Drug Resist.*, in press.
 14. Iliades, P., S. R. Meshnick, and I. G. Macreadie. 2004. Dihydropteroate synthase mutations in *Pneumocystis jirovecii* can affect sulfamethoxazole resistance in a *Saccharomyces cerevisiae* model. *Antimicrob. Agents Chemother.* **48**:2617–2623.
 15. Iliades, P., D. J. Walker, L. Castelli, J. Satchell, S. R. Meshnick, and I. G. Macreadie. 2004. Cloning of the *Pneumocystis jirovecii* trifunctional FAS gene and complementation of its DHPS activity in *Escherichia coli*. *Fung. Genet. Biol.* **41**:1053–1062.
 16. Kaplan, J. E., D. L. Hanson, T. R. Navin, and J. L. Jones. 1998. Risk factors for primary *Pneumocystis carinii* pneumonia in human immunodeficiency virus-infected adolescents and adults in the United States: reassessment of indications for chemoprophylaxis. *J. Infect. Dis.* **178**:1126–1132.
 17. Kazanjian, P., W. Armstrong, P. A. Hossler, W. Burman, J. Richardson, C. H. Lee, L. Crane, J. Katz, and S. R. Meshnick. 2000. *Pneumocystis carinii* mutations are associated with duration of sulfa or sulfone prophylaxis exposure in AIDS patients. *J. Infect. Dis.* **182**:551–557.
 18. Kazanjian, P., A. B. Locke, P. A. Hossler, B. R. Lane, M. S. Bartlett, J. W. Smith, M. Cannon, and S. R. Meshnick. 1998. *Pneumocystis carinii* mutations associated with sulfa and sulfone prophylaxis failures in AIDS patients. *AIDS* **12**:873–878.
 19. Kovacs, J. A., V. J. Gill, S. Meshnick, and H. Masur. 2001. New insights into transmission, diagnosis, and drug treatment of *Pneumocystis carinii* pneumonia. *JAMA* **286**:2450–2460.
 20. Lane, B. R., J. C. Ast, P. A. Hossler, D. P. Mindell, M. S. Bartlett, J. W. Smith, and S. R. Meshnick. 1997. Dihydropteroate synthase polymorphisms in *Pneumocystis carinii*. *J. Infect. Dis.* **175**:482–485.
 21. Ma, L., L. Borio, H. Masur, and J. A. Kovacs. 1999. *Pneumocystis carinii* dihydropteroate synthase but not dihydrofolate reductase gene mutations correlate with prior trimethoprim-sulfamethoxazole or dapsone use. *J. Infect. Dis.* **180**:1969–1978.
 22. Ma, L., J. A. Kovacs, A. Cargnel, A. Valerio, G. Fantoni, and C. Atzori. 2002. Mutations in the dihydropteroate synthase gene of human-derived *Pneumocystis carinii* isolates from Italy are infrequent but correlate with prior sulfa prophylaxis. *J. Infect. Dis.* **185**:1530–1532.
 23. Mei, Q., S. Gurunathan, H. Masur, and J. A. Kovacs. 1998. Failure of co-trimoxazole in *Pneumocystis carinii* infection and mutations in dihydropteroate synthase gene. *Lancet* **351**:1631–1632.
 24. Meneau, I., D. Sanglard, J. Bille, and P. M. Hauser. 2003. Site-directed mutagenesis of the *Saccharomyces cerevisiae* dihydropteroate synthase FOL1 gene to study *Pneumocystis jirovecii* mutations in the orthologue gene FAS. *J. Eukaryot. Microbiol.* **50**(Suppl.):652–653.
 25. Meneau, I., D. Sanglard, J. Bille, and P. M. Hauser. 2004. *Pneumocystis jirovecii* dihydropteroate synthase polymorphisms confer resistance to sulfadoxine and sulfanilamide in *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* **48**:2610–2616.
 26. Meshnick, S. R. 1999. Drug-resistant *Pneumocystis carinii*. *Lancet* **354**:1318–1319.
 27. Nahimana, A., M. Rabodonirina, J. Helweg-Larsen, I. Meneau, P. Francioli, J. Bille, and P. M. Hauser. 2003. Sulfa resistance and dihydropteroate synthase mutants in recurrent *Pneumocystis carinii* pneumonia. *Emerg. Infect. Dis.* **9**:864–867.
 28. Nahimana, A., M. Rabodonirina, G. Zanetti, I. Meneau, P. Francioli, J. Bille, and P. M. Hauser. 2003. Association between a specific *Pneumocystis jirovecii* dihydropteroate synthase mutation and failure of pyrimethamine/sulfadoxine prophylaxis in human immunodeficiency virus-positive and -negative patients. *J. Infect. Dis.* **188**:1017–1023.
 29. Navin, T. R., C. B. Beard, L. Huang, C. del Rio, S. Lee, N. J. Pieniazek, J. L. Carter, T. Le, A. Hightower, and D. Rimland. 2001. Effect of mutations in *Pneumocystis carinii* dihydropteroate synthase gene on outcome of *P. carinii* pneumonia in patients with HIV-1: a prospective study. *Lancet* **358**:545–549.
 30. Patel, O., J. Satchell, J. Baell, R. Fernley, P. Coloe, and I. Macreadie. 2003. Inhibition studies of sulfonamide-containing folate analogs in yeast. *Microb. Drug Resist.* **9**:139–146.
 31. Robberts, F. J., L. J. Chalkley, and L. D. Liebowitz. 2002. *Pneumocystis* pneumonia. *SADJ* **57**:451–453.
 32. Takahashi, T., N. Hosoya, T. Endo, T. Nakamura, H. Sakashita, K. Kimura, K. Ohnishi, Y. Nakamura, and A. Iwamoto. 2000. Relationship between mutations in dihydropteroate synthase of *Pneumocystis carinii* f. sp. *hominis* isolates in Japan and resistance to sulfonamide therapy. *J. Clin. Microbiol.* **38**:3161–3164.
 33. Visconti, E., E. Ortona, P. Mencarini, P. Margutti, S. Marinaci, M. Zolfo, A. Siracusano, and E. Tamburrini. 2001. Mutations in dihydropteroate synthase gene of *Pneumocystis carinii* in HIV patients with *Pneumocystis carinii* pneumonia. *Int. J. Antimicrob. Agents* **18**:547–551.
 34. Volpe, F., S. P. Ballantine, and C. J. Delves. 1993. The multifunctional folic acid synthesis fas gene of *Pneumocystis carinii* encodes dihydroneopterin aldolase, hydroxymethyldihydropterin pyrophosphokinase and dihydropterate synthase. *Eur. J. Biochem.* **216**:449–458.
 35. Walzer, P. D., J. Foy, P. Steele, C. K. Kim, M. White, R. S. Klein, B. A. Otter, and C. Allegra. 1992. Activities of antifolate, antiviral, and other drugs in an immunosuppressed rat model of *Pneumocystis carinii* pneumonia. *Antimicrob. Agents Chemother.* **36**:1935–1942.
 36. Zingale, A., P. Carrera, A. Lazzarin, and P. Scarpellini. 2003. Detection of *Pneumocystis carinii* and characterization of mutations associated with sulfa resistance in bronchoalveolar lavage samples from human immunodeficiency virus-infected subjects. *J. Clin. Microbiol.* **41**:2709–2712.