Dihydropteroate Synthase of Mycobacterium leprae and Dapsone Resistance

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Two Mycobacterium leprae genes, folP1 and folP2, encoding putative dihydropteroate synthases (DHPS), were studied for enzymatic activity and for the presence of mutations associated with dapsone resistance. Each gene was cloned and expressed in a folP knockout mutant of Escherichia coli (C600ΔfolP::Km). Expression of M. leprae folP1 in C600ΔfolP::Km conferred growth on a folate-deficient medium, and bacterial lysates exhibited DHPS activity. This recombinant displayed a 256-fold greater sensitivity to dapsone (measured by the MIC) than wild-type E. coli C600, and 50-fold less dapsone was required to block (expressed as the 50% inhibitory concentration [IC50]) the DHPS activity of this recombinant. When the folP1 genes of several dapsone-resistant M. leprae clinical isolates were sequenced, two missense mutations were identified. One mutation occurred at codon 53, substituting an isoleucine for a threonine residue (T53I) in the DHPS-1, and a second mutation occurred in codon 55, substituting an arginine for a proline residue (P55R). Transformation of the C600ΔfolP::Km knockout with plasmids carrying either the T53I or the P55R mutant allele did not substantially alter the DHPS activity compared to levels produced by recombinants containing wild-type M. leprae folP1. However, both mutations increased dapsone resistance, with P55R having the greatest affect on dapsone resistance by increasing the MIC 64-fold and the IC50 68-fold. These results prove that the folP1 gene of M. leprae encodes a functional DHPS and that mutations within this gene facilitate the development of dapsone resistance in clinical isolates of M. leprae. Transformants created with M. leprae folP2 did not confer growth on the C600ΔfolP::Km knockout strain, and DNA sequences of folP2 from dapsone-susceptible and -resistant M. leprae strains were identical, indicating that this gene does not encode a functional DHPS and is not involved in dapsone resistance in M. leprae.

Prior to the development and implementation of multidrug therapy (MDT) for leprosy using dapsone, rifampin, and clofazamine, most patients were treated with dapsone monotherapy. During this period dapsone-resistant strains of Mycobacterium leprae were identified and dapsone-resistant leprosy became a significant problem for leprosy control programs (15, 17, 28). Currently recommended control measures for treating leprosy with MDT should control the spread of drug-resistant strains; however, dapsone resistance continues to be reported even in areas of the world with successful implementation of MDT (1, 6).

Comprehensive estimates of drug resistance in leprosy are difficult to obtain because of the cumbersome nature of the drug screening method (30). Advances in the elucidation of molecular events responsible for drug resistance in mycobacteria have allowed the development of new tools for drug resistance screening (3, 7, 21, 35, 36). Application of these tools has revealed the presence of both monoresistant (18, 35) and multidrug-resistant strains of M. leprae (18). Recently, point mutations in the putative M. leprae gene for dihydropteroate synthase (folP) have been identified in dapsone-resistant strains of M. leprae (19, 38); however, definitive evidence linking these mutations with dapsone resistance and proof of enzymatic activity of the putative dihydropteroate synthase (DHPS) of M. leprae have not been found. A fuller understanding of the mechanism of action of dapsone and modes of resistance present in M. leprae should facilitate the development of new tools for monitoring dapsone resistance and lead to investigations into new strategies to circumvent dapsone resistance.

Dapsone, 4,4-diaminodiphenylsulfone, is a synthetic sulfone with effective antileprosy activity (16). The antibacterial activity of dapsone is inhibited by para-aminobenzoate (PABA), it is thought that dapsone has a mechanism of action similar to that of the sulfonamides, which involves inhibition of folic acid synthesis. Sulfonamides block the condensation of PABA and 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate to form 7,8-dihydropteroate (Fig. 1). The key bacterial enzyme in this step is DHPS, encoded by folP (8, 13). The subsequent conversion of 7,8-dihydropteroate to tetrahydrofolate by dihydrofolate synthase and dihydrofolate reductase is critical to the formation of various cellular cofactors including thymidylate, glycine, methionine, pantothenic acid, and n-formylmethionyl-tRNA. The mechanism of dapsone resistance in M. leprae is thought to be associated with DHPS in a manner similar to the mechanism of resistance developed in other bacteria to the sulfonamides (22, 24, 29). Genome analysis of M. leprae has identified two folP homologs (folP1 and folP2), both of which contain significant sequence homology with other bacterial folP genes, as well as conserved regions found in many DHPS molecules (see Fig. 2).

The work described in this study provides evidence that M. leprae folP1, but not folP2, encodes a functional DHPS enzyme which is effectively inhibited by low levels of dapsone. We have also identified mutations in folP1 associated with dapsone resistance. Characterization of these mutations indicated that dapsone resistance in M. leprae was related to missense muta-
Folate Biosynthesis Pathway

1. Guanosine triphosphate
2. Dihydronicotinamide triphosphate
3. 2-Amino-4-hydroxy-6-hydroxymethyl-dihydropteridine
4. Dihydropteridine synthase
5. Dihydrofolate reductase
6. Para-aminobenzoate
7,8-Dihydrodopterinate

![Dihydrodopterinate and 7,8-Dihydrodopterinate](Image)

FIG. 1. Folate biosynthetic pathway with proposed sites for dapsone and sulfonamide inhibitory action (11). Numbers represent enzymes in the pathway: 1, guanosine triphosphate hydrolase; 2, dihydronicotinamide triphosphate; 3, dihydropteridine pyrophosphokinase; 4, dihydrofolate synthase; 5, dihydrofolate reductase. The asterisk indicates the step in which sulfonamides and dapsone compete with PABA in the DHPS reaction.

### MATERIALS AND METHODS

**Genomic annotation of folP1 and folP2.** *M. leprae* folP1 is found on cosmid MLCB2548 (AL023093) and is accessible through the Sanger Centre (Cambridge, England) website (www.sanger.ac.uk). *M. leprae* folP2 is found on cosmid B1912 (U15180) and is accessible through Genome Therapeutics Corp. (Waltham, Mass.) website (www.genomemcp.com).

**Bacterial strains.** Dapsone-resistant and -susceptible strains of *M. leprae* were originally obtained from leprosy patients from the Anandaban Leprosy Hospital, Kathmandu, Nepal, and from G. W. Long Hansen’s Disease Center, Carville, La. (Table 1). Resistance to dapsone was determined in the mouse footpad system by Shepard’s kinetic method (30), and dapsone-resistant strains, except SA26, were analyzed directly from a patient’s skin biopsy specimen. Dapsone-resistant strains grew in footpads of mice receiving either 0.001 or 0.01% dapsone as a percentage of the weight of mouse chow. Dapsone-resistant strains grew in footpads of mice receiving either 0.001 or 0.01% dapsone as a percentage of the weight of mouse chow. These dapsone concentrations are 10- and 100-fold, respectively, above the minimal effective dose (MED) for susceptible strains of *M. leprae*. Thai-53, a dapsone-susceptible strain of *M. leprae*, was the kind gift of M. Matsuoka, Leprosy Research Center, National Institute of Infectious Disease, Higashimurayama, Tokyo, Japan. The WHO DNA was purified from armadillo-grown *M. leprae* which originated from pooled biopsies of lepromatous leprosy patients from India and was kindly provided by M. J. Colston, National Institute for Medical Research, Mill Hill, London, United Kingdom.

**Cloning of folP homologs and complementation of folP knockout mutants.** The folP1 and folP2 genes were amplified from Thai-53 by PCR with primers folP1-7 and -8 and folP2-1 and -2 (Table 2), respectively. These primers incorporated BamHI tails on the 5′ ends, and HindIII and EcoRI ends, of the PCR products. The resultant PCR fragments were propagated in either 2× Luria-Bertani (LB) medium (Sigma Chemical, St. Louis, Mo.) or Mueller-Hinton medium (Difco, Detroit, Mich.). *E. coli* XL-1 Blue cells (Strategene, La Jolla, Calif.) were grown in standard-concentration LB medium.

**TABLE 1. M. leprae strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dapsone susceptibility</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thai-53</td>
<td>Susceptible</td>
<td>Thailand</td>
</tr>
<tr>
<td>WHO</td>
<td>Susceptible (0.01)</td>
<td>India</td>
</tr>
<tr>
<td>591</td>
<td>Resistant (0.01)</td>
<td>United States</td>
</tr>
<tr>
<td>SA26</td>
<td>Resistant (0.001)</td>
<td>Nepal</td>
</tr>
<tr>
<td>569</td>
<td>Resistant (0.001)</td>
<td>Nepal</td>
</tr>
</tbody>
</table>

a Measured in the mouse foot pad assay. Values in parentheses represent the maximum doses of dapsone (as percentages of weight of food) at which *M. leprae* grew. Susceptible strains did not grow at 0.0001% dapsone.

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inactivated chromosomal copy of E. coli folD in E. coli C600::folD::Km' recombinant clones was verified using PCR with EC2 and EC3 primers (Table 2) to ensure that growth was a result of the cloned folD gene (8).

DNA sequencing of dapsone-resistant and -susceptible strains of M. leprae. The entire folP1 and folP2 genes were amplified separately by PCR from DNA preparations of dapsone-susceptible and -resistant strains of M. leprae using primer set folP1-1 and -2 or folP2-1 and -2, respectively (Table 2). PCR fragments were purified, and the DNA sequence of folP1 was obtained using primers folP1-1, folP1-2, folP1-9, and folP1-20. The folP2 sequence was obtained using primers folP2-1, folP2-2, folP2-3, and folP2-4. The folP1 and folP2 sequences from each strain were compared to those of dapsone-susceptible M. leprae strains found in the Sanger Centre and Genome Therapeutics M. leprae genome databases.

Site-directed mutagenesis. Mutant sequences found in folP1 of M. leprae dapsone-resistant strains were substituted for wild-type sequences in the folP1 contained on pML101 by PCR site-directed mutagenesis (37) using either folP1-7–folP1-8 and folP2-1–folP2-2, respectively (Table 2). The resultant plasmids, pML102 (containing the folP1 T53I mutant allele) and pML103 (containing the folP1 P55R mutant allele), were transformed separately into E. coli XL1-Blue. Recombinant clones were selected on 2% LB medium containing kanamycin and ampicillin. Plasmid DNA was purified from selected clones, and C600::folD::Km' cells were transformed with either pML102 and pML103. Recombinants were selected on 2% LB medium containing kanamycin and ampicillin, and mutations were identified by DNA sequencing as described above. The entire folP1 gene was sequenced from recombinant clones found to contain the desired mutant alleles.

DHPS assay. DHPS activity was measured by incorporation of radioactivity from [14C]-labeled PABA into dihydropteroate as described previously (9). Briefly, E. coli C600, C600::folP::Km' were grown in 2% LB medium containing 1 mM IPTG, 100 μg of ampicillin/ml, and 50 μg of kanamycin/ml until cultures reached an optical density at 600 nm (OD600) of 1.0. Cells were collected by centrifugation and washed three times in phosphate-buffered saline, pH 7.6. Cells were resuspended in sonication buffer containing dithiothreitol and MgCl2 in a 200-μl aliquot. The purified cDNA samples were stored at −70°C.

Reverse transcription-PCR (RT-PCR). cDNA was prepared by adding 1 μl of RNA, 11.5 μl of RNase-free water, and 1 μl of primer (20 pmol), either folP1-7 or folP2-1, in a sterile 0.5-ml PCR tube and heating for 2 min at 97°C in a thermal cycle. The reagents were quenched rapidly on ice; then 6.5 μl of PCR Master Mix (Advantage RT for PCR kit; Clontech) containing 4.0 μl of 5X reaction buffer, 1.0 μl of a deoxynucleoside triphosphate mixture (10 mM each), 0.5 μl of recombinant RNase inhibitor, and 1.0 μl of Moloney murine leukemia virus reverse transcriptase was added. The reagents were incubated at 42°C for 5 min, then at 94°C for 5 min, and were cooled to 25°C, and 80 μl of RNase-free water was added. The contents of the tube were mixed using a vortex mixer and centrifuged briefly in a microcentrifuge. The purified cDNA samples were stored at −70°C.

Primer 

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location (bp)</th>
<th>Application(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>f0P1-1</td>
<td>5′ TGTAGCTCCTCTCTCGGC 3′</td>
<td>28–45 upstream of GTG start</td>
<td>PCR, sequencing of folP1</td>
</tr>
<tr>
<td>f0P1-2</td>
<td>5′ AAGCCTTTGCGGGGCGG 3′</td>
<td>61–78 downstream of TAG stop</td>
<td>PCR, sequencing of folP1</td>
</tr>
<tr>
<td>f0P1-9</td>
<td>5′ AGGCCGCTGACACGACG 3′</td>
<td>91–108</td>
<td>Sequencing of folP1</td>
</tr>
<tr>
<td>f0P1-20</td>
<td>5′ CTGCAAGATCCTGCTT 3′</td>
<td>308–323</td>
<td></td>
</tr>
<tr>
<td>f0P1-7</td>
<td>5′ CCCTTTCCGCCGTTGCGG 3′</td>
<td>1–12 + EcoRI site</td>
<td>PCR, RT-PCR, cloning of folP1</td>
</tr>
<tr>
<td>f0P1-8</td>
<td>5′ GCCGATCTGCACCTCATCAC 3′</td>
<td>842–856 + BamHI site</td>
<td></td>
</tr>
<tr>
<td>f0P1-21</td>
<td>5′ CGGGTGAAAGGAAGACCA 3′</td>
<td>164–182</td>
<td>Site-directed mutagenesis of folP1 (T53I)</td>
</tr>
<tr>
<td>f0P1-22</td>
<td>5′ GCCGGATCTGAGCTCCACGG 3′</td>
<td>147–163</td>
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</tr>
<tr>
<td>f0P1-23</td>
<td>5′ GCACCGCCGGCCCTTAC 3′</td>
<td>155–173</td>
<td>Site-directed mutagenesis of folP1 (P55R)</td>
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<td>f0P1-24</td>
<td>5′ ATTCCGCCGGACTGCG 3′</td>
<td>137–154</td>
<td></td>
</tr>
<tr>
<td>f0P2-1</td>
<td>5′ CGCGATCTTCCGGCTGATCAATTT 3′</td>
<td>1–11 + EcoRI site</td>
<td>PCR, RT-PCR, cloning of folP2</td>
</tr>
<tr>
<td>f0P2-2</td>
<td>5′ GCCGGATCTTGCTGCTGAG 3′</td>
<td>866–876 + BamHI site</td>
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<tr>
<td>f0P2-3</td>
<td>5′ CAGAACACTGCTCCACAC 3′</td>
<td>492–507</td>
<td>Sequencing of folP2</td>
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<tr>
<td>f0P2-4</td>
<td>5′ CAGAACACTGCTCCACAC 3′</td>
<td>483–502</td>
<td></td>
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<tr>
<td>EC2</td>
<td>5′ TGGATTCTTATCAATCACCAGGG 3′</td>
<td>35–59 upstream of ATG start</td>
<td>PCR of E. coli folP</td>
</tr>
<tr>
<td>EC3</td>
<td>5′ GAGAACCTGCAAGGAAGACCAATCCTACT 3′</td>
<td>20–47 downstream of TAA stop</td>
<td></td>
</tr>
</tbody>
</table>

* Mutated nucleotides are underlined.

TABLE 2. Primers used to study M. leprae folP1 and folP2.

(Confocal Instruments, Fullerton, Calif.). Results were expressed as the mean and standard deviation of triplicate samples in picomoles of product formed per milligram of total protein. Buffer and medium (2X LB) were substituted in the assay for cell lysate and served as negative controls.

Dapsone inhibition studies. The effect of dapsone on DHPS activity was determined using the DHPS assay as described above. Briefly, increasing concentrations of dapsone (0.002 to 20 μg) were added to cell lysates (100 μg of protein) and DHPS activity assay reagents in a final volume of 200 μl and were incubated at 37°C for 15 min. One hundred microliters of each reaction volume was spotted onto Whatman 3 MM paper, ascending chromatography was performed, and radioactivity measurements were determined. Results were expressed as the concentration of dapsone which inhibited product formation by 50% (IC50) compared to that in untreated cell lysates. All values were corrected by subtracting background counts observed with negative controls containing DHPS assay reagents with either 5% ethanol or 2X LB medium with 5% ethanol. Ethanol was included in the controls to mimic solvent concentrations used for dapsone-containing samples.

Dapsone susceptibility testing. The MICs for E. coli 600 and E. coli C600::folP::Km' recombinant clones were determined by culture on Mueller-Hinton agar plates containing twofold serial dilutions of dapsone (0.025 to 256 μg/ml) and 1 mM IPTG. The MIC for each strain was defined as the lowest concentration of dapsone needed to inhibit bacterial growth.

Purification of RNA: RNA was isolated from 10^7 M. leprae bacteria which were propagated in and purified from the hind footpads of athymic nude mice (Hsd:athymic Nude-nu; Harlan Sprague-Dawley, Indianapolis, Ind.). The bacteria were resuspended in LETS buffer (200 mM LiCl, 10 mM Tris [pH 7.5], 1% sodium dodecyl sulfate), frozen in liquid nitrogen, and stored at −70°C. Bacteria were thawed on ice, and total RNA was purified as previously described (27). Chromosomal DNA was removed from RNA extracts by adding 1 unit of RNase-free DNase I (Clontech, Palo Alto, Calif.) in 0.025 M Tris, pH 7.5, containing 0.025 M MgCl2, and incubating the mixture at 25°C for 15 min. DNase I was inactivated by adding 5 μl of 0.2 M EDTA and incubating the mixture at 65°C for 10 min. RNA was quantified using a GeneQuant RNA/DNA Calculator Spectrophotometer (Pharmacia Biotech, Cambridge, England), and RNA samples were stored at −70°C in 500-ng/ml aliquots.

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RESULTS

*M. leprae* *folP* homologs. Deduced amino acid sequence alignments of putative DHPS enzymes from *M. leprae* (DHPS-1 and -2) and four representative bacterial species showed that both homologs of *M. leprae* were similar in size and contained consensus patterns associated with this class of enzyme (Fig. 2). A total-sequence comparison of *M. leprae* DHPS-1 with DHPS-2 showed only 45% identity. Comparison of the two *M. leprae* polypeptides through the first highly homologous region, PS00792 of *Bacillus subtilis* (bases 28 to 41) (Fig. 2), showed a relatively low degree of relatedness, with only 5 of 14 amino acids shared. In contrast, comparisons between DHPS-1 or -2 and the consensus sequence of this region showed a higher percentage of identity (64%). The second region of homology, PS00793, which is located between residues 65 and 77 of *B. subtilis* (Fig. 2), showed strong homology (92.3%) between *M. leprae* DHPS-1 and the consensus sequence, based on the other bacterial DHPS proteins in the alignment. The only change in the *M. leprae* DHPS-1 sequence was at residue 66, where valine was found in place of either leucine or isoleucine in at least one other bacterium. In contrast, *M. leprae* DHPS-2 shared only 6 of 13 (46.2%) residues in this region, with major differences in amino acids from residue 71 to 77. The latter amino acid residues encompass the region where mutations in dapsone-resistant *M. leprae* were identified and linked to dapsone resistance in this study.

Mutations associated with dapsone resistance. Full-length DNA sequencing of *folP1* and *folP2* from four dapsone-resistant strains of *M. leprae* was carried out (Table 1). All dapsone-resistant strains produced *folP2* DNA sequences identical to those of the *folP2* from the dapsone-susceptible *M. leprae* strain Thai-53 (data not shown). Two strains (2898 and 591) revealed missense mutations associated with *folP1* when compared to the *folP1* of *M. leprae* Thai-53 (Fig. 3). A single-base mutation (ACC → ATC) was found in codon 53 (*M. leprae* numbering) (Fig. 2) of strain 2898 substituting an isoleucine residue for a threonine (T53I) in DHPS (Fig. 3). A separate and distinct *folP1* mutation (CCC → CGC) was found in codon 55 (*M. leprae* numbering) (Fig. 2) of another dapsone-resistant strain, *M. leprae* strain *M. leprae* folP2 (data not shown). The mutation in *M. leprae* 591I substituted an arginine for proline (P55R) in DHPS-1. Each of the above two mutations was created in *M. leprae* folP1 by site-directed mutagenesis, and the mutant sequences were cloned into *C600*folP::Km′ for subsequent expression and characterization of mutant enzymes. Mutation T53I was in *pML102*, and mutation P55R was in *pML103* (Table 3).

No mutations in *folP1* were observed in two dapsone-susceptible strains of *M. leprae* with MES of 0.0001% dapsone (Table 1). In addition, no mutations in *folP1* were seen in two dapsone-resistant strains of *M. leprae* (SA26, resistant at 0.01% dapsone, and 569, resistant at 0.001% dapsone), suggesting that there are other mechanisms of resistance to dapsone in *M. leprae* not associated with mutations in *folP1*.

Growth complementation and DHPS activity in crude bacterial lysates. Having shown the similarity between potential DHPS proteins of *M. leprae* and those of other bacteria, we cloned the two putative *M. leprae* DHPS genes into plasmids and transformed them into an *E. coli* *folP1* knockout mutant for further characterization. To ensure that enzymatic activity was a function of cloned *M. leprae* *folP* genes, all recombinants were tested by PCR and showed the predicted 1.8-kb amplification product corresponding to the kanamycin-disrupted *E. coli* *folP* gene (data not shown) (8).

Bacterial lysates prepared from each strain were normalized for protein content (100 μg) and tested for DHPS activity. *E. coli* C600 produced the highest enzymatic activity at 370 pmol/mg; inactivation of the native *folP* by insertion mutagenesis (*C600*folP::Km′) reduced this activity approximately 38-fold, to 9.8 mol/mg, and was a lethal mutation when bacteria were plated on Mueller-Hinton agar (Table 3). Transformation of *C600*Δ*folP::Km′ with pUC18 did not confer growth competence on the knockout strain on Mueller-Hinton agar, nor did it change the level of DHPS activity significantly. Transformation of the *E. coli* *folP* knockout with pML101 (containing wild-type *M. leprae* *folP1*) did confer growth competence on Mueller-Hinton agar, and lysates from this strain produced 48.7 pmol of DHPS activity/mg. Recombinants carrying mutations in *M. leprae* *folP1* (pML102 and pML103) conferred growth competence on the knockout strain and produced levels of DHPS activity similar to that of the pML101 strain (Table 3). In contrast to *M. leprae* folP1 transformants, transformants carrying *M. leprae* folP2 were unable to complement growth on Mueller-Hinton agar and lysates produced low levels of DHPS activity similar to that of the knockout strain, even though DNA sequencing confirmed in-frame cloning of *folP2* (Table 3).

Inhibition of DHPS activity and of growth of bacteria by dapsone. Dapsone was tested for its ability to inhibit the DHPS activity of bacterial lysates from each strain. In addition, *E. coli* C600 and each recombinant strain were tested for growth on Mueller-Hinton agar containing various concentrations of dapsone. The IC50 of dapsone in a DHPS activity assay for *E. coli* C600 was 3.0 μg/ml. In contrast, for the recombinant strain carrying *M. leprae* folP1 (pML101), dapsone showed an IC50 of 0.06 μg/ml and a MIC of 1 μg/ml. This represents approximately a 50-fold-greater sensitivity to dapsone of *M. leprae* DHPS compared to *E. coli* DHPS; when measured by the MIC, the difference in sensitivity was even greater (>256-fold for *M. leprae* DHPS compared to *E. coli* DHPS). Growth of *E. coli* C600 was not inhibited at 256 μg of dapsone/ml, which was the highest concentration that could be tested due to the solubility properties of dapsone at concentrations above this level (Table 3).

*follP1* and *folP2* mutants (pML102 and pML103), encoding single-aminocacid changes in DHPS-1, affected both the IC50 of dapsone in the DHPS assay and the respective dapsone MICs (Table 3) compared to those for *E. coli* carrying the *M. leprae* *folP1* (pML101). For example, for recombinant strain *C600*Δ*folP::Km′ (pML102) carrying the T53I mutation, the dapsone MIC showed a 10-fold increase and the IC50 showed almost a 2-fold increase. The P55R mutation in *C600*Δ*folP::Km′ (pML103) had a much greater effect on sensitivity to dapsone, as measured by the IC50 and MIC (Table 3). The IC50 for this mutant was increased 68-fold over that for the wild type, and the MIC for the mutant was 64 μg/ml compared to 1 μg/ml observed with the recombinant strain expressing the wild-type *M. leprae* folP1. Taken together, these findings provide evidence that *M. leprae* folP1 mutations P55R and T53I affect the inhibitory action of dapsone on *M. leprae*, thereby accounting for the resistance to dapsone observed in *M. leprae* strains 591 and 2898, respectively.

Expression of *folP* homologs in *M. leprae*. RT-PCR analysis of purified *M. leprae* RNA was performed to determine whether *folP1* and *folP2* were transcribed in *M. leprae*. Analysis of cDNA showed that an 863-bp fragment, corresponding to the correct length for *M. leprae* *folP2* mRNA, was obtained when amplification was carried out with *M. leprae* folP2-specific primers (Fig. 4, lane 1). Similarly, an 856-bp fragment, corresponding to *M. leprae* *folP1* mRNA, was obtained with *M. leprae* folP1-specific primers (Fig. 4, lane 5). PCR analysis of...
DISCUSSION

Seminal work by Kulkarni and Seydel showed that dapsone inhibited folate synthesis in cell extracts of *M. leprae* (22). Their work suggested that the heightened sensitivity of *M. leprae* DHPS was due to the high affinity of the enzyme for dapsone. Much of this work focused on analysis of extracts from *Mycobacterium luteus*, a mycobacterium known to exhibit dapsone susceptibility similar to that of *M. leprae*. *M. luteus* provided a surrogate for *M. leprae* in which studies could be performed on dapsone’s effect on folate biosynthesis and dapsone analogs could be screened for new, more effective sulfones.

Recent developments in the *M. leprae* genome project have fostered a more direct approach to studying the biochemical and genetic basis of metabolism and cellular physiology of *M. leprae*. Newly annotated open reading frames have provided insight into the genetic potential of *M. leprae*, facilitating studies on the modes of action of antimycobacterial drugs and mechanisms of drug resistance. We utilized genomic information to identify potential *folP* homologs of *M. leprae* with the idea of studying both the effect of dapsone on DHPS activity and the nature of resistance to dapsone in *M. leprae*.

Annotation of the *M. leprae* genome identified two *folP* homologs, *folP1* and *folP2*, which appeared to encode DHPS enzymes with conserved regions found in DHPS enzymes of other microorganisms (31, 33). Within cosmid MLCC2548, *folP1* is located in what appears to be an operon containing other genes encoding enzymes related to folate biosynthesis, including *folK*, *folB*, and *folE*. *folP2* is located within cosmid B1912 with two small, undefined open reading frames upstream of *folP2*, which apparently are not involved in folate biosynthesis. *Streptococcus pneumoniae* and *B. subtilis* provide examples of bacteria where *folP* is located in a folate operon (23, 31), whereas *E. coli* *folP* gives an example of the non-operon-associated genomic configuration (4). It is interesting that *Mycobacterium tuberculosis* displays an organization similar to that seen in *M. leprae*. For example, the *M. tuberculosis* homolog of *M. leprae* *folP1* (MT 3712; 80% amino acid identity) is found in association with other folate genes, while a second *M. tuberculosis* *folP* homolog (MT1245; 86% amino acid homology to *M. leprae* *folP2*) is not.

Genetic and biochemical studies were done in *E. coli* because direct manipulation of *M. leprae*’s enzymes and genes is encumbered primarily by our inability to cultivate the organism in vitro. To provide evidence that one or both *M. leprae* *folP* homologs produced a functional DHPS, each gene was cloned and expressed in the *folP* knockout mutant of *E. coli*. Only *folP1* complemented the mutant for growth on Mueller-Hinton medium, indicating that *folP1*, and not *folP2*, encoded a functional DHPS enzyme. DNA sequencing of the recombinant plasmid pML201 (containing *folP2* of *M. leprae* Thai-53) confirmed that *folP2* was in the proper orientation and frame for expression as a lacZ fusion protein in *E. coli* (data not shown). It is important to remember that based on sequence similarity, *M. leprae* *folP2* was a putative *folP* homolog (10). In addition, the provided evidence that *folP2* was expressed in *M. leprae* based on RT-PCR of RNA extracts from viable organisms. Unfortunately, annotation built on generic similarities of various proteins is not always as sophisticated as intended, reminding us that laboratory confirmation of such hypotheses is imperative.

Analysis of DHPS activity from recombinants supported the findings of the growth complementation studies, showing that bacterial lysates from recombinants carrying pML101, and not pML201, contained a functional enzyme. DHPS activities levels for all growth-competent recombinants were approximately 15% of that seen with *E. coli* C600, possibly reflecting a difference in the functional efficiency of *M. leprae*’s DHPS-1 expressed in *E. coli*. Enzyme kinetic studies on purified DHPS-1 from *M. leprae* should help define functional efficiencies of the wild-type and mutant forms of *folP* and better define the growth potential of *M. leprae*.

Resistances to dapsone in *M. leprae* is thought to be associated with DHPS in a manner similar to that of resistance developed in other bacteria to the sulfonamides (22, 29). Sulfonamide resistance in various bacterial species has been shown to be associated with mutations in *folP* (5, 9, 12, 25, 32). Some resistant mutants occur as a result of spontaneous mutations within the chromosomal copy of *folP*, while others appear to result from translocation events (8). In most cases, resistant organisms produce altered DHPS enzymes which continue to catalyze the condensation reaction to form dihydropyrimidine but are refractory to inhibition by sulfonamides.

Our sequencing results for *folP1* from four dapsone-resistant strains of *M. leprae* identified two separate mutations associated with the mutant phenotype. The two mutations were localized to a highly conserved region of *folP* where mutations have been shown to affect susceptibility to sulfonamides in other bacteria (8, 12). The missense mutations were found only in two of the three high-level-resistant strains, 2898 and 591. The latter two strains were characterized for resistance in the mouse footpad assay and then propagated in mice under dapsone selection prior to DNA sequencing. The other high-level-resistant strain (SA26) was analyzed directly from a biopsy specimen taken from a patient who tested positive for dapsone resistance at the 0.01% level. Since strain SA26 and the low-level-resistant strain 569 showed the wild-type *folP* genotype, it is possible that other mechanisms may be responsible for dapsone resistance. In the case of SA26, an alternative explanation could be considered. Since SA26 originated from a patient’s biopsy, the specimen may have contained a mixed culture of dapsone-resistant and dapsone-susceptible bacteria. If that was the case, then sequencing the mixed culture following PCR amplification of *folP* would produce the dominant species of DNA, which in this case would represent the wild-type, dapsone-susceptible *folP* gene.

Supportive evidence that the two missense mutations were responsible for dapsone resistance was acquired through site-specific PCR of RNA extracts from viable organisms. Unfortunately,
directed mutagenesis of wild-type folP1. Recombinant mutants were constructed with either the TS31 or P55R mutation. In both cases DHPS activity was slightly increased compared to that of \textit{M. leprae} wild-type DHPS-1; however, significant changes were observed in the susceptibility of the mutated DHPS-1 enzymes to dapsone as measured by the IC$_{50}$. Moreover, significant increases in dapsone resistance were observed when MICs obtained with strains carrying either plasmid \textit{D}$_{C600}::$Kmr(pML101) or \textit{D}$_{C600}::$Kmr(pML103) and wild-type \textit{E. coli} C600 were compared.

Recently, Kai et al. (19) identified mutations within codons 53 and 55 of folP1 in six dapsone-resistant \textit{M. leprae} strains. Three of these mutants contained a threonyne-to-isoleucine mutation, and one strain showed a threonyne-to-alanine mutation, at codon 53. The other three strains contained mutations at codon 55, with a proline-to-leucine mutation in folP1. Combining these results with ours shows that 8 of 10 (80%) dapsone-resistant clinical strains analyzed contained missense mutations within either codon 53 or 55 of folP1. Taken together, these results strongly suggest that mutations in this region (designated the sulfone resistance-determining region [SRDR]) of folP1 are responsible for the majority of the dapsone resistance found in \textit{M. leprae}. Furthermore, should future studies confirm the association of these mutations as markers for dapsone resistance, a simple and rapid test could be developed that would detect the susceptible or resistant genotype. This test could be implemented as a leprosy control tool to survey populations thought to harbor dapsone-resistant strains and, thereby, to tailor treatment regimens appropriately.

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\section*{REFERENCES}


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Dihydropteroate Synthase of *Mycobacterium leprae* and Dapsone Resistance

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Volume 44, no. 6, p. 1530–1537, 2000. Page 1534: The arrowheads in Fig. 2 should appear as shown below.

![Fig. 2](image)

Page 1535: Figure 3 should appear as shown below.

![Fig. 3](image)

High-Level Fluoroquinolone-Resistant Clinical Isolates of *Escherichia coli* Overproduce Multidrug Efflux Protein AcrA

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