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* (C600Δ*folP::Km*). Expression of *M. leprae* *folP* in C600Δ*folP::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 C600Δ*folP::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* of *M. leprae* encodes a functional DHPS and that mutations within this gene are associated with drug resistance in clinical isolates of *M. leprae*. Transformants created with *M. leprae* *folP2* did not confer growth on the C600Δ*folP::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). Because 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-dihydropteroylglutamic acid. The key bacterial enzyme in this step is DHPS, encoded by *folP* (8, 13). The subsequent conversion of 7,8-dihdropteroylglutamic acid to dihydrofolate 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

\[
\begin{align*}
\text{GUANOSINE TRIPHOSPHATE} & \quad \downarrow 1 \\
\text{DIHYDROFOLATE} & \quad \downarrow 2 \\
\text{DIHYDROFOLATE SYNTHASE}^* & \quad (\text{folP}) \\
\text{PARA-AMINOBENZOATE} & \quad + \\
\text{DIHYDROFOLATE} & \quad \downarrow 3 \\
\text{TETRAHYDROFOLATE} & \quad \text{7,8-DIHYDROFOLATE} \quad \text{PHOSPHATE} \\
\end{align*}
\]

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

TETRAHYDROFOLATE

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

**Materials and Methods**

**Genomic Annotation of \textit{folP} and \textit{folD}**

\textit{M. leprae} \textit{folP1} and \textit{folD}. \textit{M. leprae} \textit{folP1} is found on cosmid MLCB2548 (AL023093) and is accessible through the Sanger Centre (Cambridge, England) website (www.sanger.ac.uk). \textit{M. leprae} \textit{folP2} is found on cosmid B1912 (U15180) and is accessible through Genome Therapeutics Corp. (Waltham, Mass.) website (www.genomelc.com).

**Bacterial Strains.** Dapsone-resistant and -susceptible strains of \textit{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 propagated thereafter in the footpads of BALB/c mice fed appropriate concentrations of dapsone ad libitum. SA26 was 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. These dapsone concentrations are 10- and 100-fold, respectively, above the minimal effective doses (MEDs) for susceptible strains of \textit{M. leprae}. Thai-53, a dapsone-susceptible strain of \textit{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 \textit{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.

Bacteria were harvested from ethanol-fixed tissues following a 60-min rehydration in 10 mM Tris-1 mM EDTA buffer, pH 7.4 (TE). Rehydrated tissue was minced with scissors to a gelatinous consistency, resuspended in 0.3 ml of TE, and frozen in liquid nitrogen. The specimen was thawed at 95°C, and the freeze-thaw treatment was repeated twice. The tissue was digested for 18 h at 60°C with proteinase K (2.5 mg/ml) in 100 mM Tris-150 mM NaCl-10 mM EDTA (pH 7.4) digestion buffer. Proteinase K was heat inactivated at 95°C for 10 min, and DNA was extracted with phenol-chloroform-isooamyl alcohol as described previously (34). The precipitated DNA was resuspended in 30 μl of TE buffer.

**Escherichia coli** strain C600, the \textit{folP} knockout C600Δ\textit{folP}:Km' (obtained from G. Swedberg, Uppsala University, Uppsala, Sweden [9]), and C600Δ\textit{folD}:Km' recombinants were propagated in either 2X Luria-Bertani (LB) medium (Sigma Chemical, St. Louis, Mo.) or Mueller-Hinton medium (Difco, Detroit, Mich.). \textit{E. coli} XL-1 Blue cells (Strategene, La Jolla, Calif.) were grown in standard-concentration LB medium.

**Cloning of \textit{folP} Homologs and Complementation of \textit{folP} Knockout Mutants.** The \textit{folP1} and \textit{folP2} genes were amplified from Thai-53 by PCR with primers \textit{folP1}-7 and -8 and \textit{folP2}-1 and -2 (Table 2), respectively. These primers incorporated BamHI ends, and \textit{folP1} and \textit{folP2} were ligated into the multiple cloning site of pUC18 to create in-frame \textit{folP} fragment. The resultant PCR fragments were digested with BamHI and ligated into the multiple cloning site of pUC18 to create in-frame lacZ translational fusions with \textit{folP1} (pML101) and \textit{folP2} (pML201). \textit{E. coli} XL-1 Blue cells were transformed with these plasmids, and recombinant clones were selected on LB agar containing 100 μg of ampicillin/ml. Clones containing \textit{M. leprae} \textit{folP1} or \textit{folP2} were identified by PCR amplification of the respective gene from crude cell lysates of selected bacterial colonies using \textit{folP1}-7 and -8 and \textit{folP2}-1 and -2. The resultant PCR fragments were purified and concentrated using a QIAquick PCR Purification Kit (QIAGEN, Valencia, Calif.). DNA sequences were obtained by automated sequencing on a PE Biosystems 377 automated DNA sequencer (Perkin-Elmer, Gaithersburg, Md.). Plasmids from appropriate clones were purified using a QIAprep Spin Miniprep Kit (QIAGEN), and competent \textit{E. coli} C600Δ\textit{folP}:Km' cells were transformed. Recombinants were selected on 2X LB agar containing 100 μg of ampicillin/ml, 50 μg of kanamycin/ml, and 1 mM isopropylthiogalactoside (IPTG). The presence of \textit{M. leprae} \textit{folP1} or \textit{folP2} in recombinant clones was confirmed by PCR and DNA sequencing as described above. Several clones from each transformation were then streaked on Mueller-Hinton agar containing 100 μg of ampicillin/ml, 50 μg of kanamycin/ml, and 1 mM IPTG to select for growth complementation. Mueller-Hinton medium is a PABA- and thymine/thymidine-deficient medium, which will not support the growth of \textit{E. coli} C600Δ\textit{folP}:Km'. The presence of an

### TABLE 1. \textit{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>2898</td>
<td>Resistant (0.01)</td>
<td>United States</td>
</tr>
<tr>
<td>591</td>
<td>Resistant (0.01)</td>
<td>Nepal</td>
</tr>
<tr>
<td>SA26</td>
<td>Resistant (0.001)</td>
<td>United States</td>
</tr>
<tr>
<td>569</td>
<td>Resistant (0.001)</td>
<td>Nepal</td>
</tr>
</tbody>
</table>

*a Measured in the mouse footpad assay. Values in parentheses represent the maximum doses of dapsone (as percentages of the weight of food) at which \textit{M. leprae} grew. Susceptible strains did not grow at 0.0001% dapsone.
inactivated chromosomal copy of E. coli folP in E. coli C600ΔfolP::Km' recombinant clones was verified using PCR with EC2 and EC3 primers (Table 2) to ensure that growth was a result of the cloned folP 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 folP1-1 and folP1-2 or folP2-1 and folP2-2 (Table 2). The resultant plasmids, pML102 (containing the folP1 T53I mutant allele) and pML103 (containing the folP2 P55R mutant allele), were transformed separately into E. coli XL-1 Blue. Recombinant clones were selected on 2× LB medium containing kanamycin and ampicillin. Plasmid DNA was purified from selected clones, and C600ΔfolP::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 dihydropteroic acid as described previously (9). Briefly, E. coli C600, C600ΔfolP::Km', and recombinant strains of C600ΔfolP::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 660 nm (OD660) 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 (2), disrupted by sonication at 4°C, and centrifuged at 100,000 × g for 1 h, and the protein concentration of the supernatant fraction (cell lysate) was determined (26). One hundred microliters of cell lysate (100 μg of protein) was combined with 14C-labeled PABA, dihydrophosphic acid, and monol(2-amino-1,4,7,10-tetrahydro-4-oxo-6-pyrimidinylmethylene)glycerol (a gift from M. Nast, Division of AIDS, National Institute of Allergy and Infectious Diseases, Rockville, Md.) in Tris buffer, pH 8.3, containing dithiothreitol and MgCl2 in a 200-μl reaction volume, and the mixture was held at 37°C for 15 min. One hundred microliters of each reaction volume was spotted onto 3MM paper (Whatman) and radioactivity was measured using an LS 6000IC Liquid Scintillation System (Beckman 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 (2× 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 2× LB medium with 5% ethanol. Ethanol was included in the controls to mimic soluble concentrations used for dapsone-containing samples.

**DHPS susceptibility testing.** The MICs for E. coli 600 and E. coli C600ΔfolP::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 108 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 U 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.

**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 70°C in a thermal cycler. The reaction mix was kept on ice; then 6.5 μl of PCR Master Mix (Advantage RT for PCR kit; Clontech) containing 4.0 μl of 5× reaction buffer, 1.0 μl of a deoxyadenosine 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 reaction mixture was incubated at 42°C for 60 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. M. leprae folP1 and folP2 were amplified by PCR using 5 μl of cDNA and primer pairs folP1-7–folP1-8 and folP2-1–folP2-2, respectively (Table 2).

![Table 2. Primers used to study M. leprae folP1 and folP2](http://aac.asm.org/ Downloaded from http://aac.asm.org)
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 (Fig. 2) of another high-level (0.01%) dapsone-resistant strain, *M. leprae* 591 (Fig. 3). The missense mutation in *M. leprae* 591 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 MEDs 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 folP* 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 insertionally 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*::*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 folP* gene) 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* and *folP2* from dapsone-resistant strains were tested for growth on Mueller-Hinton agar and lysates produced low 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).

**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
DNase-treated RNA using either set of primers yielded no amplification products, indicating that no detectable chromosomal DNA was present in the total-RNA preparation (Fig. 4, lanes 2 and 6).

**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 lufu*, a mycobacterium known to exhibit dapsone susceptibility similar to that of *M. leprae*. *M. lufu* 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 MLCB2548, 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 into 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, we 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 activity 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*.

Resistance 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 dihydropterin 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 mutation of the DHPS gene in *E. coli* by insertion of a specific mutation into the DHPS gene (6). Mutations were introduced into the folP gene of *E. coli* to introduce the folP1 mutations found in the dapsone-resistant *M. leprae* strain 569. At the amino acid 72, the folP1 mutation in *E. coli* replaced the isoleucine in wild-type DHPS with a lysine. The folP1 mutation at amino acid 53 replaced the histidine in wild-type DHPS with a glutamine. This mutation was introduced into *E. coli* DHPS-1 and DHPS-2. Both DHPSs associated with DHPS enzymes were identified from the Prosite database (14) and were PS00792 (bases 28 to 41) and PS00793 (bases 65 to 77). Arrowheads mark the start of the gene and the stop codon. The multiple sequence alignments were done on OMIGA 2.0, Oxford Molecular Group, Inc., Campbell, Calif.

**FIG. 2.** Alignment of the deduced amino acid sequences of DHPSs from *B. subtilis* (31), *E. coli* (5), *Neisseria meningitidis* (8), and *Staphylococcus haemolyticus* (20) with those of *M. leprae* DHPS-1 and DHPS-2. Domains associated with DHPS enzymes were identified from the Prosite database (14) and were PS00792 (bases 28 to 41) and PS00793 (bases 65 to 77). Arrowheads mark the start of the gene and the stop codon. The multiple sequence alignments were done on OMIGA 2.0, Oxford Molecular Group, Inc., Campbell, Calif.
directed mutagenesis of wild-type \textit{folP1}. Recombinant mutants were constructed with either the T53I 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 pML101, pML102, or pML103 and wild-type \textit{E. coli} C600 were compared.

Recently, Kai et al. (19) identified mutations within codons 53 and 55 of \textit{folP1} in six dapsone-resistant \textit{M. leprae} strains. Three of these mutants contained a threonine-to-isoleucine mutation, and one strain showed a threonine-to-alanine mutation, at codon 53. The other three strains contained mutations at codon 55, with a proline-to-leucine mutation in \textit{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 \textit{folP1}. Taken together, these results strongly suggest that mutations in this region (designated the sulfone resistance-determining region [SRDR]) of \textit{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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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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