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Cloning, Sequencing, and Enhanced Expression of the Dihydropteroate Synthase Gene of *Escherichia coli* MC4100

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The *Escherichia coli* gene coding for dihydropteroate synthase (DHPS) has been cloned and sequenced. The protein has 282 amino acids and a compositional molecular mass of 30,314 daltons. Increased expression of the enzyme was realized by using a T7 expression system. The enzyme was purified and crystallized. A temperature-sensitive mutant was isolated and found to express a DHPS with a lower specific activity and lower affinities for *para*-aminobenzoic acid and sulfathiazole. The allele had a point mutation that changed a phenylalanine codon to a leucine codon, and the mutation was in a codon that is conserved among published DHPS sequences.

Dihydropteroate synthase (DHPS) (EC 2.5.1.15) catalyzes the condensation of *para*-aminobenzoic acid (pAB) with 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate, forming 7,8-dihydropteroate (39, 44). This intermediary metabolite is subsequently converted to tetrahydrofolic acid, essential for the syntheses of purines, thymidylate, glycine, methionine, pantothenic acid, and *N*-formylmethionyl-tRNA. Sulfonamides are pAB analogs that are recognized by DHPS as alternate substrates (4, 7, 45, 57). In the presence of sulfonamides, DHPS forms a sulfa-pterin adduct that is metabolically inert and diffuses from the cell (40). Folate cofactor depletion results in growth inhibition and in the appropriate environment, cell death (55).

DHPS activity was first identified in crude cell extracts of several organisms by Shiota and coworkers (44, 45) and was identified in *Escherichia coli* by Brown et al. (8). The kinetic characteristics of DHPS have been studied by using partially purified extracts. Recently, a purification procedure for DHPS was published which showed that this enzyme constituted less than 0.01% of all the proteins in a cell (54). DHPS was purified to homogeneity, the sequence of the first 28 amino acids was determined, and the protein was shown to be a homodimer of two 30-kDa subunits. However, the purification procedure yielded less than 2 mg of purified protein from 1 kg of starting material, emphasizing the need to clone and overexpress this important chemotherapeutic target.

Recently, the chromosomal gene that codes for DHPS in *Streptococcus pneumoniae* was cloned, sequenced, and shown to code for a protein of 34 kDa (28). A similar sequence was also identified in a *Bacillus subtilis* folic acid biosynthetic operon (48). Two other genes (*sulI* and *sulII*) that code for plasmid-borne sulfonamide-resistant DHPSs have also been sequenced (38, 52). However, there is no information about the *E. coli* DHPS gene. In this communication, we report the cloning, sequencing, and enhanced expression of the *E. coli* DHPS gene, designated *folP*. We also report conditions that allow crystallization of this enzyme.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The relevant genotype of *E. coli* MC4100 is *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR* (9). *E. coli* SF800 is similar to W3110 but carries *polA1* and is Nal^r (19). RSF1010 is a nonconjugative plasmid coding for sulfonamide and streptomycin resistance (16). pBS$^+$ and pBS$^-$ are cloning vectors coding for ampicillin resistance (46). pACYC184 is a cloning vector coding for chloramphenicol and tetracycline resistance (10, 41). pSC101 is a low-copy-number plasmid coding for tetracycline resistance (3, 11). pBR322 is a cloning vector coding for ampicillin and tetracycline resistance (6). pJB8 is a cosmid cloning vector coding for ampicillin resistance (24). pUC4-KIXX is a plasmid coding for kanamycin resistance (Pharmacia LKB Biotechnology, Piscataway, N.J.).

Isolation of mutants. MC4100 was grown in a minimal salts medium supplemented with 0.1% vitamin-free Casamino Acids, 0.2% glycerol, and 0.1 mM adenosine, which will be referred to hereafter as selection medium (18). These conditions have been shown to exert the greatest selection pressure (55). Twenty tubes with 2.5 ml of selection medium were each inoculated with a single colony of MC4100. After each culture reached an optical density at 600 nm of at least 1.0, the culture was diluted 1:1,000 into fresh selection medium with sulfathiazole (STZ). The growth cycle was then repeated each time with an increased concentration of STZ. The STZ concentrations used were 1, 5, 20, and 100 μM. Cultures growing in selection medium with 100 μM STZ were streaked onto plates of selection medium with 20 μM STZ. Eight isolated colonies were picked from each plate and tested for temperature sensitivity at 42°C on selection medium.

Preparation of plasmids and DNA. Plasmids were purified by the Qiagen system (Qiagen, Studio City, Calif.). Restriction enzyme digests, T4 polymerase reactions, and ligations were performed in the buffer described by O'Farrell et al. (36). Plasmids were introduced into bacteria by electroporation using a Gene Pulser Transfection Apparatus (Bio-Rad, Richmond, Calif.). Nucleotide sequences were determined using the dsDNA Cycle Sequencing System (Life Technologies, Inc., Gaithersburg, Md.) (33). Primers were synthesized on a DNA synthesizer (model 8700; Milligen/Biose-

arch, Burlington, Mass.). The oligonucleotides were deprotected, dried, and resuspended in water. The concentration (in micromolar) was estimated to be half of the optical density at 260 nm. Cosmid cloning was performed by using the protocol of Maniatis et al. (30), except that the partial digests of genomic DNA were not fractionated but a reaction condition that yielded mostly DNA fragments in the 45-kb size range was used.

PCR cloning. Primers were made that included restriction enzyme recognition sites 6 bp from the 5′ end. The GeneAmp System (Perkin-Elmer Cetus, Norwalk, Conn.) was used for amplification with 0.1 μM (each) primer and 1 ng of template per ml (32). Polymerase chain reaction (PCR) products were processed with the PCR Purification Spin Kit (Qiagen). The PCR product was mixed with the cloning vector in a fourfold molar excess, cut with the appropriate restriction enzyme(s), and ligated. The primers for cloning *folP3* were 5′ACGTATGAATTCTTATCAATTCATACC AGGGAT3′ and 5′ATACGTGAATTCGGTACCGAAATA TTTACGATT3′, and the PCR product was cloned into pKAN1. The primers for cloning *folP* for enhanced expression were 5′GGTTTCCCTCTAGAAATAATTTTGTTTAA CTTTAAGAAGGAGATATACATATGAAACTCTTTGC CCAGGGTACTT3′ and 5′TTTAAAGAATTCTTACTCA TAGCGTTTGTTTTCCTT3′, and the PCR product was cloned into pTX007.

Purification and crystallization of DHPS. BL21[DE3] (pFOL435) was grown overnight in Luria broth with 20 μg of tetracycline hydrochloride per ml and 0.002% Mazu DF 60 P, an antifoaming agent, in a 16-liter fermentor (New Brunswick Scientific, Edison, N.J.) at 37°C. The cells were harvested, washed, and pelleted by centrifugation. The cell pellet (110 g [wet weight]) was suspended in 110 ml of 50 mM Tris-HCl (pH 8.0) containing 50 μg of lysozyme per ml, 5 mM EDTA, and 5 mM 2-mercaptoethanol. The cells were disrupted by one passage through a French pressure cell (Aminco, Urbana, Ill.) at 10,000 lb/in^2. $MgCl_2$ was added to a concentration of 10 mM, and the crude lysate was treated with 5 μg of DNase I per ml for 30 min at 4°C. The crude lysate was centrifuged at 47,000 × *g* for 30 min, and the pellet was discarded. The supernatant (186 ml) was dialyzed against buffer A (20 mM Tris-HCl, pH 8.0) and absorbed onto a Q Sepharose column (5 by 12 cm) that had been equilibrated with buffer A. The column was washed with buffer A until the A_{278} was down to baseline, and the protein was eluted with a 2.5-liter solution with a gradient (0 to 0.4 M NaCl in buffer A). The active DHPS fractions were pooled, and the protein was precipitated with 60% $(NH_4)_2SO_4$. The pellet was dissolved in buffer A, and the solution was dialyzed against buffer A. The volume of the dialyzed solution was reduced to 30 ml and loaded onto a S-300 sieving column (7.5 by 60 cm) that had been equilibrated with buffer A containing 250 mM NaCl. Fractions with the highest DHPS activities were pooled and concentrated to 100 ml with Centriprep 30 filters (Amicon, Beverly, Mass.). The concentrated material was loaded onto a column (5 by 15 cm) of Matrix Gel Green A (Amicon), as described previously (54). The protein was eluted with a 2-liter solution with a gradient (0.25 to 2.0 M NaCl) in buffer A. The most active fractions were pooled, concentrated to 50 ml, dialyzed against buffer A, and stored at −20°C.

DHPS was crystallized by vapor diffusion, using the hanging drop technique. Droplets contained 45 mg of protein per ml with 1 mM $MgCl_2$ and dithiothreitol mixed 1:1 with reservoir buffer. The reservoir consisted of citric acid-sodium phosphate buffer (31), PEG 8000, and 1 M LiCl. Crystals grew at 4°C in 12 to 15% (wt/vol) PEG 8000 at pH 7.6 to 8.0 and took at least 1 month to grow.

Enzyme assays. DHPS activity was determined by measuring the formation of dihydropteroate either in the direct assay or in a coupled assay with 7,8-dihydro-6-hydroxymethylpterin-pyrophoskinase (HPPK). The assays were performed as previously described with minor modifications (14). The reaction mixture for the direct assay consisted of 0.04 M Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 5 mM dithiothreitol, 0.02 mM 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate (H_2ptCH_2OPP), and 0.10 mM [^{14}C]pAB in a total reaction volume of 0.10 ml. The coupled assay was performed in the same reaction mixture supplemented with excess purified HPPK, 20 mM ATP, and 0.16 mM 7,8-dihydro-6-hydroxymethylpterin (H_2ptCH_2OH) replaced H_2ptCH_2OPP (54). The reactions were initiated by the addition of enzyme and the formation of dihydropteroate was measured by paper chromatography (39, 45). H_2ptCH_2OH and H_2ptCH_2OPP were prepared by the methods outlined by Shiota et al. (45). A rapid assay was used to estimate DHPS activity and reactivity with STZ in strains with various plasmids. Culture samples (10 to 50 ml) were rapidly chilled (0°C). The cells were washed twice with assay buffer and then resuspended in assay buffer with 1% toluene for 15 min, and enzyme activity at 30°C was determined by the coupled assay. Purification of DHPS at 37°C was monitored by using the coupled assay. The kinetic properties, specific activity, thermolability, and reactivity with STZ of wild-type and mutant DHPS from crude cell extracts were determined by the direct assay procedure. Specific activity was measured at 30°C with at least five different protein concentrations, chosen so that enzyme activity was a linear function of protein concentration. The specific activity (in picomoles per minute per milligram of total protein) was then calculated by linear regression of enzyme activity versus protein concentration. The standard errors of specific activities were calculated from the error in enzyme measurement, obtained from regression analysis, and from the measured error of protein determination. Analyses of enzyme kinetics were carried out on measurements of initial reaction velocities. Data on enzyme reaction velocity as a function of substrate concentration were fitted to the Michaelis-Menten equation by the nonlinear regression procedure of Spector and Hajian (49). To estimate the relative maximum velocities (V_{max}) of the wild-type and mutant enzymes in the crude extracts, it was assumed that the amounts of total DHPS present in a fraction of total protein were identical for both strains. Heat stability of DHPS in crude extracts from wild-type and mutant strains was determined at 50°C in the presence of H_2ptCH_2OPP (0.025 mM) by the methods of Swedberg and Sköld (53). Protein concentrations were determined by the method of Lowry et al. (29) with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Ames (1).

Plasmid construction. Several plasmids were constructed for the cloning and expression of the DHPS gene. A vector coding for kanamycin resistance was formed by replacing *bla* in pBS$^+$ by a DNA fragment with a kanamycin resistance gene from Tn*5* (2). An *Aat*II site was created in pBS$^+$ just 3′ to *bla* by site-directed mutagenesis which incorporated 2 additional bp into the plasmid using the oligonucleotide 5′AAGAAGATCCTTTGACGTCTTTTCTACGGGG3′ (underlining shows the 2 bp inserted into the plasmid) (23, 58). Since pBS$^+$ had an *Aat*II site 5′ to *bla*, cutting with *Aat*II released a *bla*-containing DNA fragment of 1,160 bp. After

the mutagenized plasmid was cut with *Aat*II, the unpaired ends were made double stranded with T4 DNA polymerase and the kanamycin resistance gene was inserted as a blunt-ended DNA fragment, forming pKAN1 (5, 36). The source of the resistance gene was the pUC4-KIXX plasmid. The gene was excised from this plasmid on a *Hin*dIII-*Sma*I fragment, and the ends of the fragment were made double stranded with T4 DNA polymerase prior to ligation.

A low-copy-number cosmid cloning vector conferring chloramphenicol resistance was constructed in a seven-step procedure (Fig. 1). First, a double-stranded oligonucleotide was subcloned into the pBS$^-$ vector at the *Bam*HI site (46). The complementary oligonucleotides were 5'GATCTAGC TGGTGGGCGGCCGCGGATCCGCGGCCGCA3' and 5'GATCTGCGGCCGCGGATCCGCGGCCGCCCACC AGCTA3'. The small DNA fragment would anneal to the unpaired *Bam*HI ends of the vector but would not restore the *Bam*HI recognition site. Within the oligonucleotide was another *Bam*HI site that was bracketed by *Not*I sites. Also included was a chi sequence to increase the likelihood of interplasmid recombination and thereby increase the efficiency of in vivo packaging of cosmids into lambda phage heads (this was never experimentally tested) (13). The new plasmid was called pBSN. The chloramphenicol acetyltransferase gene (*cat*) from pACYC184 was subcloned into the new plasmid at the *Sma*I site as an *Asu*II-*Hha*I fragment that had first been reacted with T4 DNA polymerase to make the ends of the fragment base paired. The new plasmid was designated pNC3. Next, a *Hin*cII fragment with the replication region from pSC101 was subcloned into the *Hin*cII site of pNC3, forming pNC101. The new plasmid could be selected for directly by transforming the ligation mixture into SF800 and selecting for chloramphenicol resistance. SF800 has a *polA* mutation which does not allow replication of pBS$^-$. The pBS$^-$ replication region and flanking sequences were deleted from pNC101 by cutting with *Sac*I and *Nde*I, treating with T4 DNA polymerase, and religating. This new plasmid was designated pNC102.

A plasmid with two copies of a *cos* region from bacteriophage lambda was constructed in a separate set of experiments. First, a 223-bp *Hae*III fragment from pJB8 was cloned into the *Hin*cII site of pBS$^+$, forming pCOS137. Next, the *cos* fragment was excised as a *Pst*I-*Xba*I fragment, the ends were made double stranded with T4 DNA polymerase, and the fragment was inserted back into pCOS137 but this time at the *Sma*I site. Sequencing enabled the identification of a plasmid with a tandem duplication of the fragment in a head-to-tail arrangement. This plasmid was designated pCBC5. A unique *Bam*HI site was between the two *cos* regions. This site was destroyed and replaced by a *Hpa*I site by cutting the plasmid with *Bam*HI, making the ends double stranded with T4 DNA polymerase, and ligating in the presence of an *Hpa*I linker (GTTAAC; Pharmacia LKB Biotechnology, Pleasant Hill, Calif.), forming pCHC6. Finally, the *cos-Hpa*I-*cos* region was inserted as an *Eco*RI-*Hin*dIII fragment with base-paired ends into *Xba*I-cut pNC102 that had also been treated with T4 DNA polymerase. The new plasmid was designated pNC202.

An expression vector coding for tetracycline resistance and having a T7 promoter and transcription termination sequence was constructed for enhanced expression from *folP*. The pAR3038 vector had the ϕ10 promoter and transcription terminator region from bacteriophage T7 inserted at the *Bam*HI site of pBR322 in the orientation that would transcribe the ampicillin resistance gene (42). First, the unique *Eco*RI site was destroyed by cutting the plasmid with *Eco*RI, treating with T4 DNA polymerase, and religating. Next, a synthetic double-stranded oligonucleotide was cloned between the unique *Xba*I site and *Bam*HI sites, resulting in the replacement of the plasmid fragment with the sequence CTAGAAATAATTTTGTTTAACTTTAAGAAG GAGAAGAATTCGTTAACGAGCTCGGATCCTAGCTA ACTAGGGCCCT. The modification did not alter the ϕ10 5' untranslated sequence until 3 bp prior to the initiation ATG codon. These 3' sequences were replaced with recognition sequences for the enzymes *Eco*RI, *Hpa*I, *Sac*I, *Bam*HI, and *Apa*I. Stop codons for all three reading frames were between the last two restriction enzyme recognition sequences. The new plasmid had two *Eco*RV sites close together and on the same side of the T7 insert. The sites were reduced to a single *Pst*I site by cutting the plasmid with *Eco*RV, ligating in the presence of *Pst*I linkers, and transforming. A plasmid was isolated with no *Eco*RV sites and two *Pst*I sites (the second site was in *bla*). The tetracycline resistance gene was restored in this plasmid by fragment substitution. First, the *Eco*RI site in pBR322 was converted to a *Bgl*II site with T4 DNA polymerase and *Bgl*II linkers. Next, the *Sal*I-*Bgl*II fragment that contained the 5' coding sequences for the tetracycline resistance gene was isolated from the modified pBR322. This fragment was then ligated to *Bgl*II-*Sal*I-cleaved T7 plasmid. A new plasmid was isolated that coded for tetracycline resistance. Finally, *tet* and the T7 expression regions from this plasmid were isolated as an *Ava*I-*Pst*I fragment and ligated to the larger *Pvu*II fragment of pBS$^-$ that had also been isolated on gels. The resulting plasmid was designated pTX007. DNA sequence analysis of the T7 promoter region of this plasmid revealed that the sequence was not exactly what had been planned. Substitution of one nucleotide ruined the *Hpa*I recognition site (position 42 of the oligonucleotide was a G instead of an A), while a missing base (the C at position 63) put one termination codon in frame with one other termination codon.

RESULTS

Isolation and characterization of a Ts mutant. Our strategy to clone the DHPS gene was to complement the growth of a temperature-sensitive (Ts) mutant. To isolate the mutant, we used a multistep procedure to select for STZ resistance and then tested the resistant strains for growth at 42°C (37). Twenty independent cultures were subjected to the mutant selection procedure, and one yielded a temperature-sensitive mutant. MC4100ts3 was an isolate that was both resistant to STZ (MIC of 1,250 μM compared with 20 μM for MC4100) and unable to grow at 42°C in minimal medium. The temperature sensitivity was reversed by the addition of the metabolic end products whose syntheses require folic acid cofactors (thymidine, glycine, methionine, pantothenic acid, and either adenosine or guanosine) just prior to the temperature shift. The association of the temperature-sensitive phenotype with DHPS was confirmed by genetic complementation. RSF1010 carries a STZ resistance DHPS gene (*sulII*) that has been sequenced (38). The DHPS gene from RSF1010 was subcloned as an *Ssp*I fragment and inserted into the *Hin*cII site of the pKAN1 vector. The new plasmid, pTZ11, conferred STZ and kanamycin resistance to its host. Introduction of pTZ11 into MC4100ts3 enabled the strain to grow at 42°C in selection medium, but the vector alone did not allow the mutant to grow under these conditions.

The mutant DHPS was found to have approximately 5% the specific activity of native enzyme (Table 1). It was also found that the K_i for STZ was at least 2,000-fold higher for

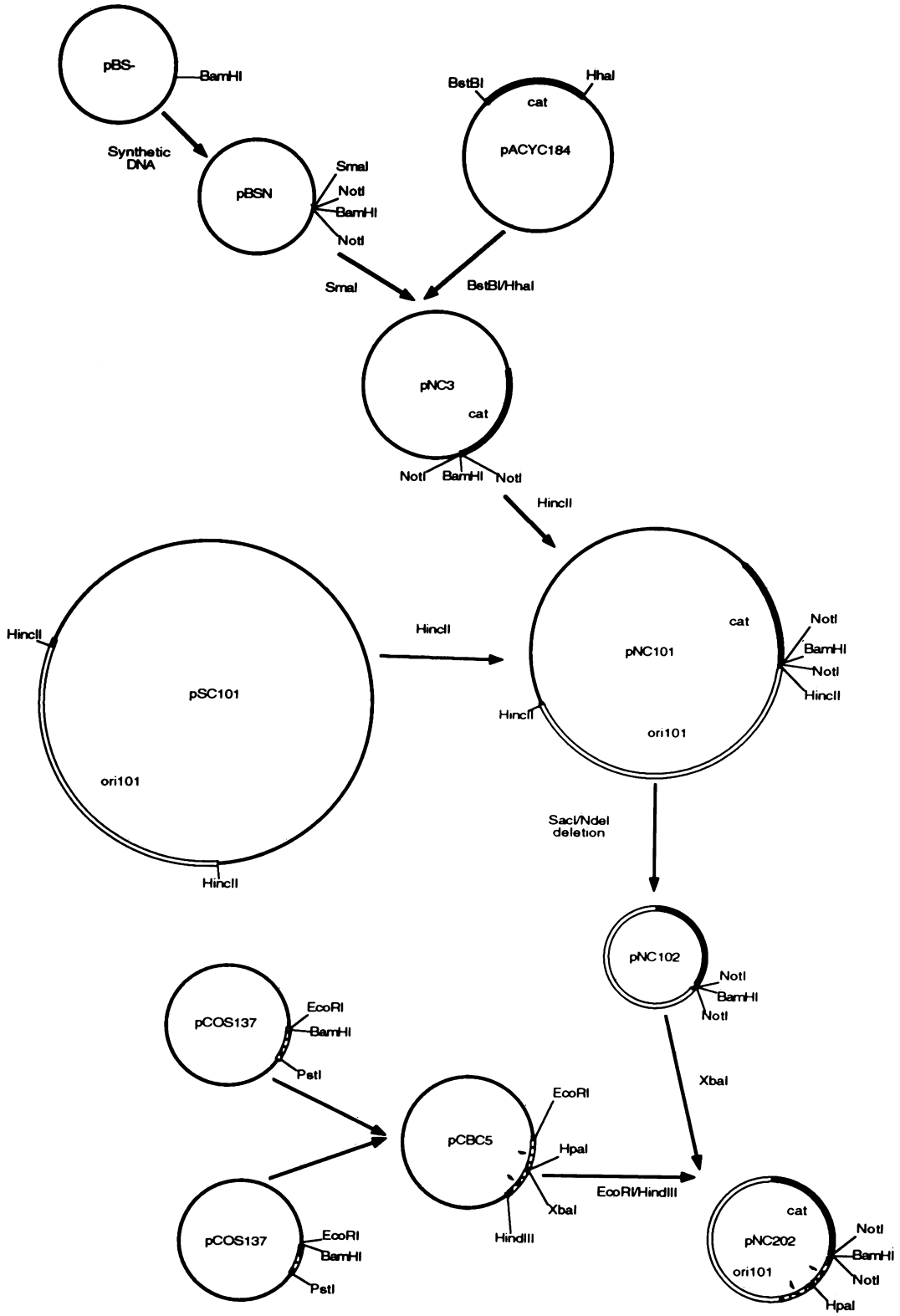

FIG. 1. Schematic diagram of the various steps in the construction of the cosmid vector pNC202. Only restriction enzyme recognition sites relevant to the manipulations are shown. An explanation of the steps in the construction is given in Materials and Methods.

TABLE 1. Strain characteristics

Strain	Growth[a]			STZ MIC (μM)	Mean DHPS sp act ± SE (pmol/min/mg of total protein)	Mean K_m ± SE (μM)		Mean V_{max} ± SE[b]	Mean STZ K_i ± SE (μM)
	30 or 37°C	42°C	42°C with pTZ11			pAB	H_2ptCH_2OPP		
MC4100	+	+	+	20	107 ± 15	0.7 ± 0.2	1.2 ± 0.4	100 ± 12	0.15 ± 0.03
MC4100ts3	+	−	+	1,250	7 ± 1	220 ± 15	1.9 ± 0.2	23 ± 4	392 ± 45

[a] Bacteria were grown in minimal salts medium supplemented with 0.1% vitamin-free Casamino Acids, 0.2% glycerol, and 0.1 mM adenosine.
[b] Crude extracts were used. V_{max} in arbitrary units.

the mutant DHPS than for the native DHPS (Table 1). When substrate affinities were determined, no difference in K_m for pterin pyrophosphate was apparent. However, the K_m for pAB was 300-fold higher for the mutant and the relative V_{max} was about 4-fold lower (Table 1). In the absence of substrates, both enzymes lost about 50% of the activity at 50°C in 10 min. However, in the presence of 0.025 mM H_2ptCH_2OPP, neither enzyme lost any appreciable activity after 30 min at 50°C.

Cloning the DHPS gene. A cosmid library was made by *Sau*3A partial digestion of MC4100 genomic DNA, ligation into the *Bam*HI site of pNC202, and amplification in χ2819 (26). The lysate was used to infect MC4100ts3, and the mixture was plated on selection medium with chloramphenicol (10 μg/ml) and incubated at 42°C. Eighteen transfectants were recovered. Restriction analysis of plasmids prepared from the transfectants indicated that each had an insert of about 40 kb, 17 plasmids were identical, and all plasmids had common DNA fragments. DHPS activities were determined for several of the transfectants, and each was found to have elevated levels of DHPS (fourfold) compared with that of the mutant. The additional DHPS activity was found to be sensitive to STZ.

The DHPS gene was localized to a smaller region of the cloned DNA by deletion analysis of one of the cosmids, pFOL12. Restriction enzymes that cut pFOL12 twice were identified, and deletion derivatives were isolated by cutting the plasmid with one of the enzymes, religating, electroporating, plating MC4100ts3 under permissive conditions, identifying deletion plasmids, and testing for growth complementation under nonpermissive conditions. The DHPS activity was then characterized for each complemented isolate. The DHPS activity profile of MC4100ts3 carrying pFOL122, the *Stu*I deletion plasmid (21 kb in size), was more similar to the DHPS activity profile of MC4100 than that of MC4100ts3. This plasmid therefore had retained the DHPS gene. The plasmid was used to make further deletions by using the same protocol. It was found that an *Fsp*I deletion derivative of pFOL122 (pFOL122F) in MC4100ts3 restored growth at 42°C and that the DHPS activity profile of MC4100ts3 (pFOL122F) was similar to that of MC4100ts3(pFOL12). The new plasmid was 15 kb in size.

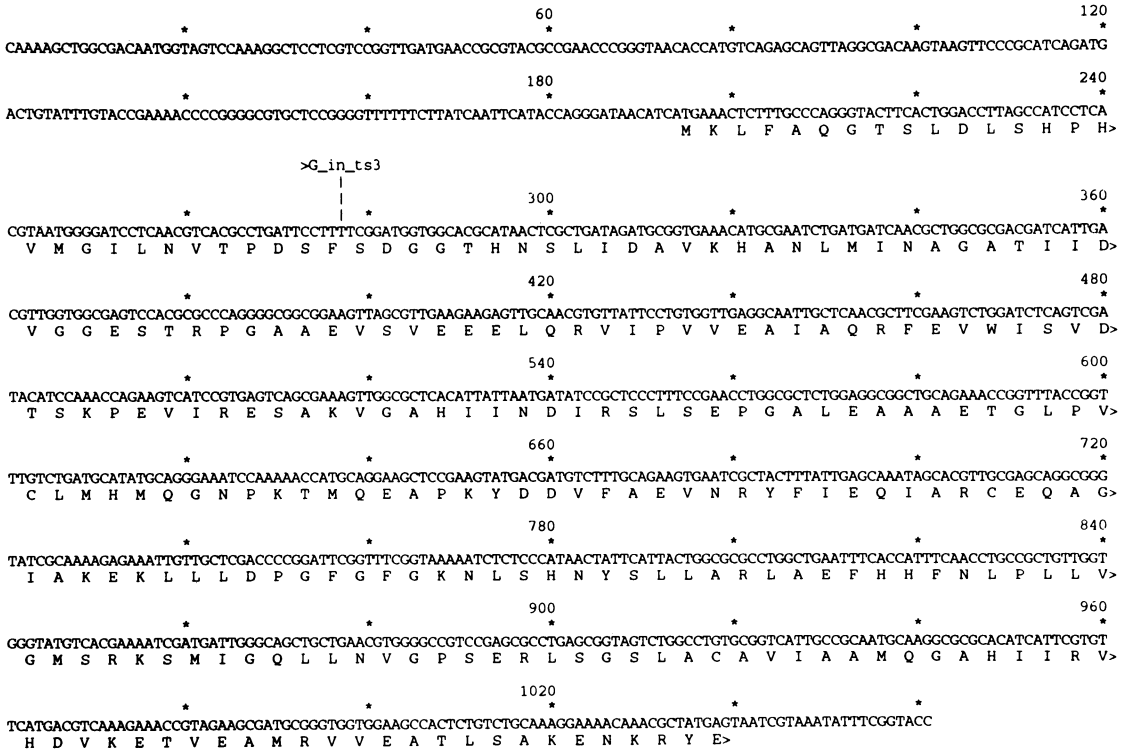

FIG. 2. Nucleotide sequence of *folP* with flanking regions. The amino acid sequence of DHPS is shown beneath the corresponding codons. The point mutation in the *folP3* allele of MC4100ts3 is also shown.

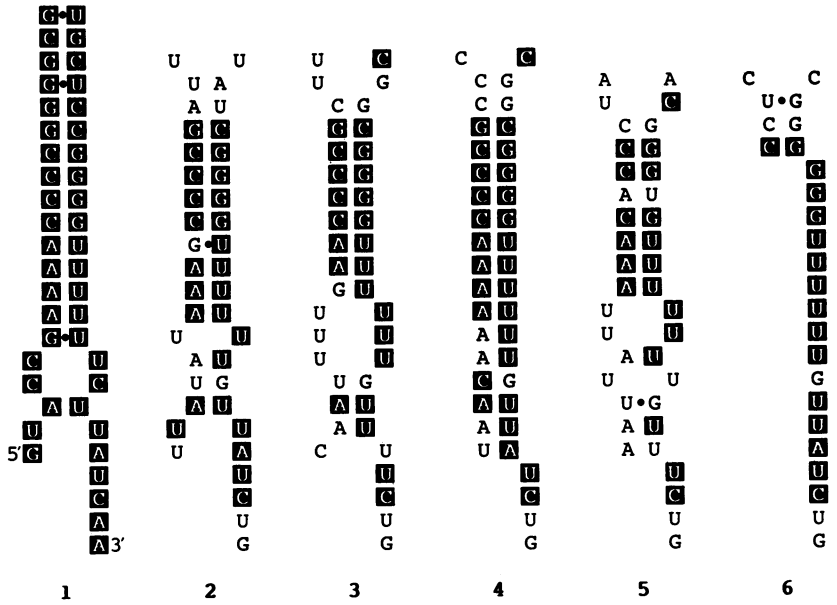

FIG. 3. Potential RNA stem-loop structures formed from sequences 5′ to *folP* (structure 1) and five other regions that show sequence similarity. Structures: 2, sequences between *metY* and *nusA*; 3, sequences after tRNA genes for Arg, His, Leu, and Pro; 4, sequences after *rpbM* and *rpmG*; 5, sequences after *metZ*; 6, sequences 5′ to *ilvB*. The boxed bases are identical to those in sequences near *folP*.

The *Kpn*I enzyme cut pFOL122F six times, generating fragments ranging in size from 1.8 to 3.8 kb. The *Kpn*I fragments were ligated to pKAN1, the ligation mixture was introduced into MC4100ts3, and the bacteria were plated at 42°C on selection medium. Eight colonies grew, and all were found to have a 2.3-kb fragment inserted into pKAN1. DHPS activity profiles confirmed the presence of the DHPS gene on this *Kpn*I fragment. Plasmids with the two possible insert orientations were identified: one plasmid was designated pFOL308, and the other was designated pFOL309.

Sequence of the DHPS gene. Nested deletions were made for both pFOL308 and pFOL309, using exonuclease III and S1 nuclease (20). The plasmids were introduced into MC4100ts3, and complementation was determined. By this method, we were able to delineate the DHPS locus. From this analysis, appropriate plasmids were chosen as templates for DNA sequence determination. The sequences of both strands were determined.

An 846-bp open reading frame was identified (Fig. 2). Translation of the region predicted a 282-amino-acid protein. The amino-terminal sequence of the predicted protein matched the sequence that had been determined for purified DHPS (54). The compositional molecular mass was 30,314 Da, which was equivalent to the empirically determined value (54). The gene coding for DHPS was designated *folP*.

Examination of the nucleotide sequence proximal to *folP* did not reveal a region with extensive sequence similarity to a canonical *E. coli* promoter (17). A potential ribosome binding site was identified beginning 12 bp from the initiation codon and could base pair with 5 of the last 6 bases of the 16S rRNA (43). Another region proximal to *folP* that shared features with a rho-independent transcription termination signal was also identified. A transcript from this region could form a 13-bp stem (with a GU mismatch) and a 2-base loop (Fig. 3).

PCR primers were synthesized that enabled the amplification and cloning of the mutated *folP* (*folP3*) from MC4100ts3. The primers had *Eco*RI sites at the 5′ ends, allowing the insertion of the PCR product into pKAN1 at the *Eco*RI site. The insert was sequenced by the cycle sequencing method. A single mutation was found at bp 277 where a transversion from a TA bp to a GC bp had occurred. The mutation would result in the substitution of a leucine codon (TTG) in place of a phenylalanine codon (TTT).

Enhanced expression and purification of DHPS. The expression of DHPS was increased by using a T7 expression system (51). The *folP* sequences were amplified by PCR with a primer that included the 5′ expression sequences (from an *Xba*I site to the ATG initiation codon) of T7 gene ϕ10. The 3′ PCR primer included an *Eco*RI site following the TAA termination codon of *folP*. The PCR product was cloned into the vector pTX007, forming pFOL435. The authenticity of the insert was confirmed by DNA sequence determination of the entire insert, and the plasmid was electroporated into BL21[DE3]. This strain was found to express significant amounts of DHPS. The expression was approximated to be 7,000-fold higher than the wild-type strain by comparison of specific activities from crude extracts (0.0005 compared with 3.4). A summary of the purification steps and yields of DHPS is presented in Table 2. Pure DHPS (2.7 g) was obtained from 110 g (wet weight) of cells using a three-step procedure. An SDS-PAGE profile of samples from various steps in the purification protocol is presented in Fig. 4. It should be noted that the specific activity of the purified protein is twofold higher than that reported for the wild-type enzyme (54).

Sufficient protein was isolated to proceed with crystallization experiments. We were successful in obtaining crystals that are rectangular plates of various lengths and thicknesses (Fig. 5).

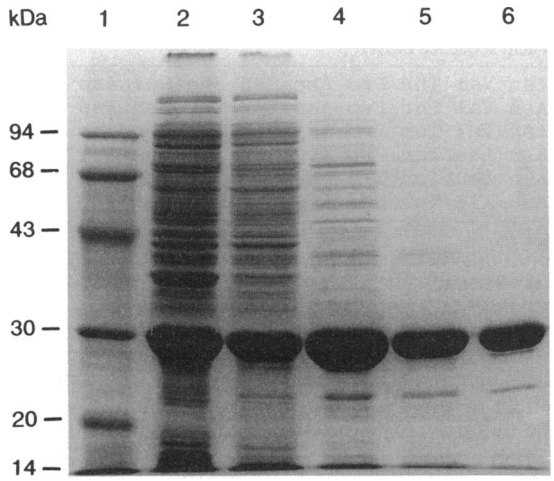

FIG. 4. SDS-PAGE profile of DHPS purification. Aliquots from each fraction in the purification regimen (Table 2) were analyzed by SDS–12% polyacrylamide gels stained with Coomassie blue G250. Lane 1 has molecular mass markers (expressed in kilodaltons); lanes 2 to 6 are aliquots from fractions 1 to 5, respectively, identified in Table 1.

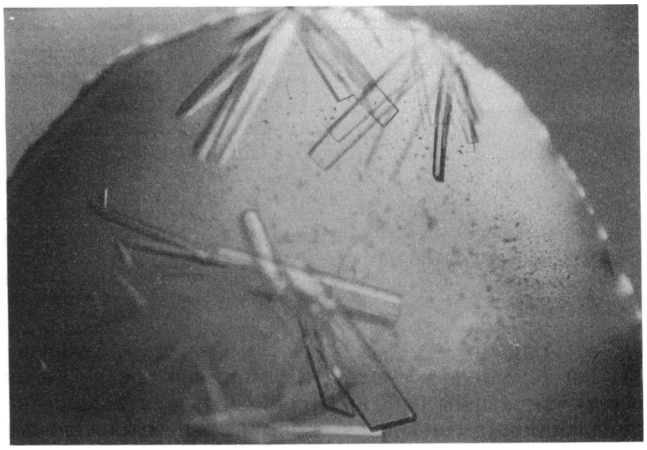

FIG. 5. Photomicrograph of DHPS crystals. This picture shows a section that was magnified 35-fold.

DISCUSSION

Stassi et al. (50) were successful in cloning a mutant DHPS gene from *S. pneumoniae* by selecting for sulfonamide resistance. However, this approach had yielded fortuitous results, because the resistance phenotype was later found to be associated with another gene which mapped close to the DHPS gene. Nichols and Guay (35) tried the same method to clone the DHPS gene from *E. coli* but were unsuccessful. Our strategy for cloning the DHPS gene was based on the approach described by Sköld (47) in which DHPS genes on R factors were identified by complementation of a temperature-sensitive (Ts) mutant. Pato and Brown (37) isolated the first *E. coli* with a Ts DHPS by selecting for resistance to STZ. They reasoned that a mutation in the DHPS gene could lead to sulfa resistance and that changing the amino acid(s) might also increase the thermolability of the enzyme. Interestingly, mutants that were selected on the basis of resistance to other sulfonamides (sulfadiazine, sulfabenzamide, and sulfacetamide) did not express an altered DHPS. We, too, were successful in isolating a mutant that was resistant to STZ, expressed a DHPS with a lower affinity for STZ, and was temperature sensitive. However, we could find no evidence for increased thermal lability of the mutant DHPS: that is, we did not see any difference in percent enzyme inactivation with increasing temperature. One explanation that accounts for the Ts phenotype of the mutant is contingent upon the reduced DHPS activity of the mutant. Since both mutant and wild-type enzymes show decreasing activity with increasing temperatures, it may be that at 42°C the total DHPS activity in the mutant (which is already lower than in the wild-type strain) has been reduced to a level that cannot support growth. While the total DHPS activity from MC4100 also decreased at 42°C, sufficient dihydropteroate is still made, enabling growth. The association of the Ts phenotype with DHPS was confirmed by showing that the phenotype was masked by introduction of an R-factor DHPS gene (*sulII*).

We chose cosmids as the vehicle to introduce MC4100 genomic fragments into the mutant for cloning by complementation. First, we constructed a vector with a low copy number in the event that the DHPS gene or a nearby gene was lethal when amplified. The vector carried a gene coding for chloramphenicol resistance and two copies of the *cos* region separated by a unique *Hpa*I site, which simplified the manipulations necessary for cosmid cloning (24). A cosmid library was made, amplified, and used to infect MC4100ts3. We were successful in isolating transfectants that grew under nonpermissive conditions. The initial cosmids were about 45 kb in size. After deletion analysis and subcloning, the DNA region associated with the complementing phenotype was localized to a 2.3-kb *Kpn*I fragment. Through DNA sequence analysis, an open reading frame was identified and the amino terminus coded by this gene was found to match the sequence that had been determined for DHPS. The gene was designated *folP*.

The DHPS gene was used as a hybridization probe for both plasmid and chromosomal Southern hybridization analyses. We found that the various cosmid clones were not colinear with the chromosome (data not shown). This implied that either noncontiguous DNA fragments were ligated together during the cosmid cloning or a rearrangement within the cosmids occurred in vivo during the library amplification step. However, a 2.3-kb *Kpn*I fragment was present in chromosomal Southern blots, implying that the DNA sequence of this fragment is colinear with the chromosome (data not shown). The DHPS gene was originally cloned as a *Sau*3A partial digest, and sequencing revealed four *Sau*3A sites in *folP* and none in the flanking regions.

TABLE 2. Purification of DHPS from BL21[DE3](pFOL435)

Fraction no. and description	Fraction vol (ml)	Total protein (g)	Total U (10^3)	DHPS sp act[a]
1. Total crude extract	220	17.5	61	3.4
2. Supernatant	186	14.5	52	3.5
3. Q Sepharose	250	5.7	30	4.8
4. S-300 Sephacryl	235	4.4	26	6.0
5. Matrix Gel Green A	175[b]	2.7	17	6.3

[a] In micromoles per minute per milligram of protein.
[b] Pool concentrated to 50 ml, dialyzed against buffer A, and stored at −20°C.

TABLE 3. DHPS amino acid comparisons

Source of DHPS	Amino acid sequence of DHPS[a]
E. coli	15-Pro His Val Met Gly Ile Leu Asn Val Thr Pro Asp Ser Phe Ser Asp Gly Gly
E. coli ts3[b]	15-Pro His Val Met Gly Ile Leu Asn Val Thr Pro Asp Ser Leu Ser Asp Gly Gly
B. subtilis	25-Thr Leu Val Met Gly Ile Leu Asn Val Thr Pro Asp Ser Phe Ser Asp Gly Gly
S. pneumoniae	10-Thr Val Ile Cys Gly Ile Leu Asn Leu Thr Pro Asp Ser Phe Phe Asp Gly Gly
sulI[c]	3-Thr Val Phe Gly Ile Leu Asn Leu Thr Glu Asp Ser Phe Phe Asp Glu Ser
sulII[c]	5-Leu Ile Ile Phe Gly Ile Val Asn Ile Thr Ser Asp Ser Phe Ser Asp Gly Gly

[a] The numbers in front of the amino acid sequences are the positions of the first residues shown.
[b] The temperature-sensitive mutant MC4100ts3.
[c] Isolated from strains with R factors and their DHPSs can discriminate between pAB and STZ.

Therefore, the DNA sequences surrounding the cloned DHPS gene must be colinear with chromosomal *folP*.

Inspection of the DNA sequence immediately 5′ to *folP* did not identify a region that resembled a promoter. Surprisingly, we did identify a region 5′ to *folP* that had many features of a transcription termination site (12). Transcripts from this region had the potential to form a 13-bp stem with one GU mismatch and a 2-base loop. A search for this sequence among other *E. coli* sequences identified five similar regions, some of which have been shown to function as transcription terminators (15, 22, 25, 27, 34) (Fig. 3). The presence of the terminator and absence of a promoterlike sequence suggested that the amount of *folP* transcript is dependent upon extension of a transcript through a transcription terminator. One mechanism limiting the abundance of DHPS (about 0.01% of the total soluble protein) would be efficient transcription termination at this site and thereby synthesis of few *folP* transcripts. This type of transcriptional control has been demonstrated for *nusA* (25).

Given the relative paucity of DHPS in normal cells, we were interested in developing a strain that would express more enzyme. We constructed an expression vector that was a modification of a plasmid described by Rosenberg and coworkers (42). The new plasmid (pTX007) was smaller, had different restriction sites for cloning, and coded for tetracycline resistance. The most important elements for enhanced expression (the T7 promoter, 5′ untranslated region of the ϕ10 gene, and T7 transcription termination region) were retained. The DHPS gene was amplified by PCR with appropriate primers for expression and cloning and inserted between the *Xba*I and *Eco*RI sites of pTX007. The insert was sequenced, and the plasmid was then introduced into BL21[DE3] for estimation of DHPS levels. We found that active DHPS accumulated in this strain, and we estimated that at least 30 to 40% of the total cell protein was DHPS. Lopez et al. (28) had successfully used a similar method for enhanced expression of the DHPS gene from *S. pneumoniae*.

The enhanced expression enabled us to develop a three-step purification procedure and to purify gram quantities of the enzyme. Using the purified protein, we were successful in establishing conditions for crystallization of the enzyme. Preliminary X-ray crystallography experiments have shown that the crystals diffract to sufficient resolution (2.6 Å [0.26 nm]) to continue data collection (data not shown). The solution of the three-dimensional structure of DHPS should be valuable in helping to identify the active site and to determine the catalytic mechanism of the enzyme, which could aid in the development of novel inhibitors.

Lopez et al. (28) subcloned and sequenced the DHPS gene, *sul-d*, from *S. pneumoniae*. They also sequenced the allele that expressed a DHPS with altered affinities for pAB (twofold-higher K_m) and sulfanilamide (sevenfold-higher K_i). The effect of the mutation on substrate affinity was small, and the mutation was a two-codon duplication that was not in a region of conservation among DHPSs (data not shown). In contrast, the point mutation in DHPS we have identified has dramatic effects on both DHPS activity and affinities for one substrate (300-fold-higher K_m for pAB and 2,000-fold-higher K_i for STZ), but the pterin PP_i substrate affinity did not change. The mutation was a single-base change that would result in an amino acid substitution (Phe to Leu) in a region that is highly conserved among the five DHPSs whose amino acid sequences have been derived (Table 3). Interestingly, two of these proteins are R-factor DHPSs that can discriminate between STZ and pAB. It is not possible to extrapolate much from these two observations on mutant enzymes, especially considering that the proteins were from two different organisms and two different sulfa compounds were evaluated. However, since the mutation in *folP3* is in a conserved region and at a conserved amino acid, it could be valuable to apply site-directed mutagenesis to other codons in this region and evaluate substrate binding. This approach could identify a role for this region in pAB binding.

The relationship between resistance to sulfonamides and mutations in DHPS is not totally understood. R factors conferring resistance have been isolated and shown to carry DHPS genes coding for enzymes which discriminate between pAB and sulfonamides (47, 56). It is also possible to isolate spontaneous sulfa-resistant mutants (*E. coli* or *S. pneumoniae*) using procedures which could lead to multiple mutations (21, 37). The gene(s) conferring sulfa resistance in the mutants are apparently distinct from the DHPS gene, even though DHPS in these strains can have an altered K_m for sulfonamides (28, 35). The Ts mutant we have isolated undoubtably has mutations in addition to the base change identified in *folP*. We have initiated homologous recombination experiments using a mutated cloned *folP* to make mutations in the chromosomal gene that would result in enzyme inactivation. A mutant without DHPS would be useful for beginning an analysis of the role that spontaneous mutations in DHPS have in resistance to STZ. These mutants would serve as recipients for DHPS genes with altered sulfonamide affinities.

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