

## 6-Pyruvoyltetrahydropterin Synthase Paralogs Replace the Folate Synthesis Enzyme Dihydroneopterin Aldolase in Diverse Bacteria<sup>∇†</sup>

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**Dihydroneopterin aldolase (FolB) catalyzes conversion of dihydroneopterin to 6-hydroxymethyldihydropterin (HMDHP) in the classical folate biosynthesis pathway. However, *folB* genes are missing from the genomes of certain bacteria from the phyla *Chloroflexi*, *Acidobacteria*, *Firmicutes*, *Planctomycetes*, and *Spirochaetes*. Almost all of these *folB*-deficient genomes contain an unusual paralog of the tetrahydrobiopterin synthesis enzyme 6-pyruvoyltetrahydropterin synthase (PTPS) in which a glutamate residue replaces or accompanies the catalytic cysteine. A similar PTPS paralog from the malaria parasite *Plasmodium falciparum* is known to form HMDHP from dihydroneopterin triphosphate in vitro and has been proposed to provide a bypass to the FolB step in vivo. Bacterial genes encoding PTPS-like proteins with active-site glutamate, cysteine, or both residues were accordingly tested together with the *P. falciparum* gene for complementation of the *Escherichia coli folB* mutation. The *P. falciparum* sequence and bacterial sequences with glutamate or glutamate plus cysteine were active; those with cysteine alone were not. These results demonstrate that PTPS paralogs with an active-site glutamate (designated PTPS-III proteins) can functionally replace FolB in vivo. Recombinant bacterial PTPS-III proteins, like the *P. falciparum* enzyme, mediated conversion of dihydroneopterin triphosphate to HMDHP, but other PTPS proteins did not. Neither PTPS-III nor other PTPS proteins exhibited significant dihydroneopterin aldolase activity. Phylogenetic analysis indicated that PTPS-III proteins may have arisen independently in various PTPS lineages. Consistent with this possibility, merely introducing a glutamate residue into the active site of a PTPS protein conferred incipient activity in the growth complementation assay, and replacing glutamate with alanine in a PTPS-III protein abolished complementation.**

The folate biosynthesis pathway is present in most bacteria, as well as plants, fungi, heterokonts, and certain protozoa (8, 15, 19). The classical pathway (Fig. 1A) involves eight specific enzymes, all of whose genes are known (9, 13, 15). This pathway was until recently thought to be the only one. However, the advent of large-scale genome sequencing has revealed numerous cases of “missing” folate synthesis enzymes (“pathway holes”) in which particular enzymes are lacking in bacterial genomes with otherwise complete pathways (9). This situation implies the existence of either different enzymes with sequences unlike the known ones (“nonorthologous displacement”) or alternative routes (16, 29). One such case is dihydroneopterin aldolase (FolB), which mediates the fourth step in the classical folate pathway, the shortening of the side chain of dihydroneopterin to give 6-hydroxymethyldihydropterin (HMDHP) (Fig. 1B). Comparative genomic analysis showed that the *folB* gene is missing from diverse bacteria (9), as well as from heterokonts (diatoms and oomycetes) (19).

Dihydroneopterin aldolase is also missing in the malaria para-

site *Plasmodium falciparum* (14) and was recently proposed from biochemical evidence to be functionally replaced by a paralog of the tetrahydrobiopterin synthesis enzyme 6-pyruvoyltetrahydropterin synthase (PTPS) (10). This paralog forms HMDHP (and a small amount of the normal PTPS product, 6-pyruvoyltetrahydropterin) from the first intermediate of folate synthesis, dihydroneopterin triphosphate (Fig. 1C) and can thus provide a bypass for the missing dihydroneopterin aldolase step (Fig. 1A). Evidence that this bypass operates in vivo was, however, not provided and indeed may be impossible to obtain because disrupting folate synthesis by gene deletion appears to be lethal in *P. falciparum* even when folate is supplied (38). The *P. falciparum* PTPS paralog is distinct in having a glutamate residue in place of a cysteine in the active-site region bounded by the three histidine residues that coordinate the essential Zn<sup>2+</sup> ion. Mutating the glutamate to cysteine abolished the formation of HMDHP but not 6-pyruvoyltetrahydropterin (10). Interestingly, heterokonts also have a PTPS paralog with an active-site glutamate, which could explain their lack of FolB (19).

Tetrahydrobiopterin synthesis is known to occur in only a few bacterial groups such as *Cyanobacteria* and *Chlorobia* (12, 24) and, predictably, these organisms have PTPS enzymes (designated PTPS-II) that are related to mammalian PTPS and absent from other bacteria (24). However, they also have a second PTPS-like protein (designated PTPS-I) that has no in

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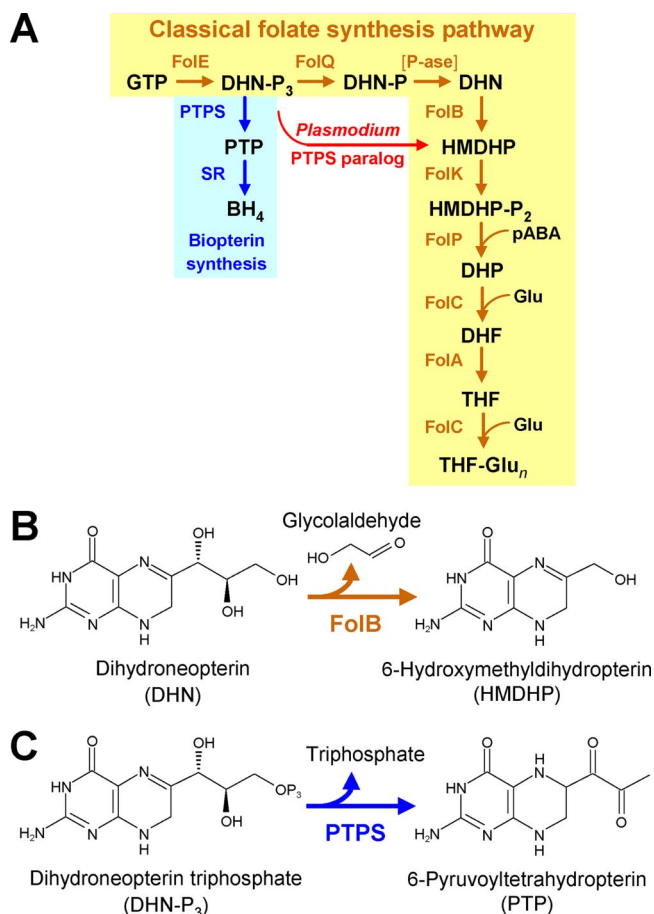


FIG. 1. Biosynthesis of tetrahydrofolate and tetrahydrobiopterin. (A) Steps and enzymes of the classical tetrahydrofolate synthesis pathway and the tetrahydrobiopterin synthesis pathway. The bypass reaction catalyzed by the *P. falciparum* PTPS paralog (PTPS-III type) is in red. Abbreviations: BH<sub>4</sub>, 5,6,7,8-tetrahydrobiopterin; DHF, 7,8-dihydrofolate; DHN, 7,8-dihydroneopterin; DHP, 7,8-dihydropterolate; Glu, glutamate; GTP, guanosine-5'-triphosphate; HMDHP, 6-hydroxymethyl-7,8-dihydropterin; -P, phosphate; -P<sub>2</sub>, pyrophosphate; -P<sub>3</sub>, triphosphate; pABA, *p*-aminobenzoate; P-ase, nonspecific phosphatase; PTP, 6-pyruvoyl-5,6,7,8-tetrahydropterin; PTPS, 6-pyruvoyl-5,6,7,8-tetrahydropterin synthase; SR, sepiapterin reductase; THF, 5,6,7,8-tetrahydrofolate; THF-Glu<sub>n</sub>, tetrahydrofolate polyglutamates. (B) Reaction mediated by FolB (dihydroneopterin aldolase). (C) Reaction mediated by PTPS (classical PTPS-II type).

vivo role in tetrahydrobiopterin synthesis and is widespread in other bacteria (24). PTPS-I mediates an early step in the biosynthesis of the modified tRNA base queuosine (the gene encoding PTPS-I has been named *queD*) (32), specifically the conversion of dihydroneopterin triphosphate to 6-carboxytetrahydropterin (27). In vitro, *Synechocystis* and *Escherichia coli* PTPS-I (QueD) proteins also cleave the side chain of the nonphysiological substrate sepiapterin and also show low PTPS activity (39). Both PTPS-I and PTPS-II have cysteine residues in the active-site region (24).

We report here that bacteria lacking *folB* have a PTPS-like protein (PTPS-III) with glutamate alone or glutamate plus cysteine in the active site region. Complementation of an *E. coli folB* deletant, site-directed mutagenesis, and biochemical

assays established that these PTPS-III proteins functionally replace FolB via a bypass reaction, as proposed for the *P. falciparum* protein. We also showed for the first time that the *P. falciparum* protein can mediate this reaction in vivo.

## MATERIALS AND METHODS

**Bioinformatics.** Bacterial genomes were analyzed by using the SEED database and its tools (30) at [anno-3.nmpdr.org/anno/FIG/subsys.cgi](http://anno-3.nmpdr.org/anno/FIG/subsys.cgi). Sequences were aligned with CLUSTAL W, and phylogenetic analyses were made with MEGA 4 (36).

**Pteridines.** Pterin, 6-hydroxymethylpterin, 7,8-dihydroneopterin, and 6-hydroxymethyl-7,8-dihydropterin hydrochloride were obtained from Schircks Laboratories. Near-saturated solutions were freshly prepared in N<sub>2</sub>-sparged potassium phosphate (10 mM, pH 7.5), excluding light, and titers were determined spectrophotometrically using published extinction coefficients (4, 31). Dihydroneopterin triphosphate was prepared from GTP essentially as detailed previously (23) except that recombinant *Synechocystis* GTP cyclohydrolase I, purified as described previously (25), replaced the *E. coli* enzyme. Aliquots of dihydroneopterin triphosphate solution were frozen in liquid N<sub>2</sub> and stored at -80°C until use.

**Strains and growth conditions.** *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C. Growth media were solidified with 15 g of agar/liter for preparation of the plates. Kanamycin (50 μg/ml), carbenicillin (100 μg/ml), thymidine (75 μg/ml), and L-arabinose (0.2% [wt/vol]) were added as required. *E. coli* K-12 MG1655 was used as the wild-type strain in all experiments. The *folB::Kan* JW3030-2 strain was from the Keio collection (1). The *folB::Kan* deletion was transferred to MG1655 by P1 transduction. Clones with the desired deletion were selected on LB plates containing kanamycin and folate-dependent end products, i.e., thymidine (75 μg/ml), methionine (50 μg/ml), glycine (50 μg/ml), adenine (20 μg/ml), and panthothenate (1 μg/ml) (35). Deletion of the *folB* gene was confirmed by PCR and sequencing. Deletant clones were maintained on LB plates containing thymidine and kanamycin.

**Construction of expression vectors.** Genomic DNA of *Leptospira interrogans* L1-130, *Desulfovibrio vulgaris*, *Acidobacteria bacterium*, *Dehalococcoides ethenogenes*, and *Aquifex aeolicus*, were obtained from M. Picardeau (Institut Pasteur), D. Stahl (University of Washington), C. Kuske (Los Alamos National Laboratory), S. Zinder (Cornell University), and P. Schimmel (Scripps Research Institute), respectively. Genomic DNA of *Legionella pneumophila* and *Pirellula* sp. strain 1 was purchased from the American Type Culture Collection and the German Resource Centre for Biological Material, respectively. The *Thermotoga maritima* PTPS-III gene was amplified using genomic clone TM0038 (from S. Lesley, Novartis Research Foundation). The *E. coli folB* gene was amplified from *E. coli* genomic DNA (strain MG1655). PTPS-pET46 (10) was used as a template to amplify the *P. falciparum* PTPS gene. *Clostridium botulinum* PTPS-I and PTPS-III, *Synthrophobacter fumaroxidans* PTPS-I, and *Synthrophus aciditrophicus* PTPS-III sequences were synthesized by GenScript after optimizing codon usage for expression in *E. coli* (see Table S1 in the supplemental material). PCR amplifications were carried out using *Pfu* Turbo DNA polymerase (Stratagene). For functional complementation experiments, all sequences were PCR amplified with a forward primer harboring an NcoI, BspHI, or PciI site and a reverse primer with an XbaI site (see Table S2 in the supplemental material). The amplicons were cloned between the NcoI and XbaI sites of pBAD24 (18) and then introduced into *E. coli* strain DH5α and sequenced. The expression of PTPS genes in the pBAD24 vector was induced with 0.2% (wt/vol) L-arabinose. For recombinant protein production, PTPS-I and PTPS-III sequences from *C. botulinum* (Cb) and *L. interrogans* (Li) were amplified using a forward primer containing an NdeI site and a reverse primer containing a BamHI site (see Table S2 in the supplemental material). The amplicons were cloned into the corresponding sites in pET28b (Novagen) and the resulting constructs (Cb PTPSI-pET28b, Cb PTPSIII-pET28b, Li PTPSI-pET28b, and Li PTPSIII-pET28b) were confirmed by sequencing.

**Site-directed mutagenesis.** The *Synthrophobacter fumaroxidans* PTPS-I and *Synthrophus aciditrophicus* PTPS-III sequences were mutagenized by using overlap extension PCR (7). In each case, two specific, complementary oligonucleotides were designed (see Table S2 in the supplemental material). The PCR products obtained were cloned into pBAD24 as described above, and the resulting constructs were verified by sequencing.

**Functional complementation experiments.** *E. coli folB* deletant cells were transformed with pBAD24 alone (negative control) or with pBAD24 containing *E. coli folB* (positive control) or various PTPS genes. Complementation tests were made by streaking transformed cells on LB plates containing appropriate

antibiotics and L-arabinose, with or without thymidine. Four independent clones were used for each construct. Plates were incubated for 2 days at 37°C.

**Folate analysis.** Wild-type and *folB* deletant strains harboring pBAD24 alone or containing PTPS-I or PTPS-III genes from *L. interrogans* were grown at 37°C in 50 ml of LB medium containing thymidine, L-arabinose, and appropriate antibiotics. Cells were pelleted when  $A_{600}$  reached  $1.0 \pm 0.1$  and stored at  $-80^\circ\text{C}$  until use. Pellets were resuspended in 10 ml of 50 mM HEPES-CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid] (pH 7.8) containing 2% (wt/vol) ascorbic acid and 10 mM  $\beta$ -mercaptoethanol and then sonicated, boiled for 10 min, and centrifuged (13,000  $\times$  g, 10 min). The pellets were reextracted the same way, and the combined extracts were treated for 2 h at 37°C with 2 ml of dialyzed rat plasma to deglutamylate folates. Samples were then boiled for 10 min, centrifuged as described above, and filtered. Folates were isolated by using 2-ml folate affinity columns (17). Samples of the eluate were analyzed by high-pressure liquid chromatography (HPLC) with electrochemical detection (2). Detector response was calibrated with standards from Merck Eprova. The folate content of wild-type cells was in the normal range for *E. coli* (34); note that methionine, present at a high level in LB medium, represses 5,10-methylenetetrahydrofolate reductase (*metF*) so that little, if any, 5-methyltetrahydrofolate is formed (21).

**Expression and purification of recombinant proteins.** *E. coli* BL21-CodonPlus (DE3)-RIPL competent cells (Stratagene) were transformed with hsPTPS-pET28a, PPTPS-pET46 (10), CbPTPSI-pET28b, CbPTPSIII-pET28b, LiPTPSI-pET28b, or LiPTPSIII-pET28b constructs, all of which introduced an N-terminal His tag. Cells were grown at 37°C in LB medium containing appropriate antibiotics. When  $A_{600}$  reached 0.7, IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) was added to a final concentration of 1 mM, and growth was continued for 3 h. Subsequent steps were carried out at 4°C. The cells were harvested by centrifugation, resuspended in 50 mM Tris-HCl (pH 8.0), and disrupted by sonication. The cleared supernatant was loaded onto a  $\text{Ni}^{2+}$ -nitrilotriacetic acid agarose resin column (Qiagen) equilibrated with the sonication buffer. After the column was washed with 50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, and 20 mM imidazole (pH 8.0), the recombinant protein was eluted with the same buffer containing 250 mM imidazole and dialyzed overnight against 100 mM Tris-HCl (pH 8.0) containing 2 mM dithiothreitol. Protein was estimated by the Bradford method (5) using bovine serum albumin as a standard. Purified proteins were frozen in liquid  $\text{N}_2$  and stored at  $-80^\circ\text{C}$  after the addition of 10% (vol/vol) glycerol. Freezing was shown not to affect activity.

**Enzyme assays.** Reactions (50  $\mu\text{l}$ ) were performed in 100 mM Tris-HCl (pH 8.0), containing 2 mM dithiothreitol, 9  $\mu\text{M}$  dihydroneopterin triphosphate, 10 mM  $\text{MgCl}_2$ , and 75  $\mu\text{g}$  of PTPS enzyme as described previously (10). After incubation for 1 h at 37°C, the reaction products were oxidized for 1 h in the dark at 4°C by the addition of 10  $\mu\text{l}$  of 1%  $\text{I}_2$ -2% KI (wt/vol) in 0.1 M HCl, after which the excess iodine was oxidized by adding 10  $\mu\text{l}$  of 10% (wt/vol) sodium ascorbate. Pterins were separated on a 4- $\mu\text{m}$ , 250-by-4.6-mm Synergi Fusion-RP 80 column (Phenomenex) that was eluted isocratically with 10 mM sodium phosphate (pH 6.0) at 1.5 ml/min. Peaks were detected by fluorescence (350-nm excitation, 450-nm emission) and identified relative to standards.

## RESULTS

**Bacteria lacking FolB have unusual PTPS-like proteins.** We first extended a comparative genomics analysis of the folate biosynthesis pathway (9) to hundreds of newly available bacterial genomes. The results are summarized in Fig. 2A and are available in full at the SEED server (theseed.uchicago.edu) in the subsystem titled "Experimental-PTPS." We found 40 highly diverse genomes that lack FolB but have the other enzymes of de novo folate synthesis (represented for simplicity in Fig. 2A by those on either side of the FolB step, namely, FolE, FolK, and FolP). Thirty-eight of these genomes (95%) specified, in addition to a typical PTPS-I, a PTPS-like protein with an active-site glutamate but no cysteine or, alternatively, a PTPS-like protein with both glutamate and cysteine (Fig. 2A). Both types of glutamate-containing proteins were designated PTPS-III. In sharp contrast, of 355 genomes having FolB as well as a PTPS-like protein, only 10 (3%) were of the PTPS-III type, i.e., containing an active-site glutamate. The

occurrence of PTPS-III is thus strongly anticorrelated with that of FolB, constituting a strong a priori case that PTPS-III functionally replaces FolB in the folate pathway. Moreover, PTPS-III genes are often situated in folate synthesis gene clusters, whereas PTPS-I genes are frequently in queuosine synthesis gene clusters (Fig. 2B).

**Genes encoding PTPS-III proteins complement the deletion of *E. coli folB*.** A *folB* deletion was transferred by P1 transduction from Keio collection strain JW3030-2 to the K-12 strain MG1655. Like other folate synthesis mutants (20, 22), the resulting deletant strain showed little or no growth on rich medium unless it was supplied with thymidine. The deletant was transformed with the arabinose-inducible plasmid vector pBAD24 containing PTPS-I or PTPS-III genes from diverse bacteria or the PTPS gene of *P. falciparum*. The native *E. coli folB* gene served as a positive control. The transformants were plated on LB medium supplemented with arabinose or arabinose plus thymidine. As expected from the biochemical evidence (10), the *P. falciparum* gene conferred thymidine prototrophy, thereby validating the complementation assay (Fig. 3A).

In the absence of thymidine, five of the six PTPS-I genes showed no growth, and the sixth (from *Pirellula* sp.) showed very little. In contrast, 8 of the 10 bacterial PTPS-III genes allowed vigorous growth, as did the *folB* control (Fig. 3A). Of the two inactive PTPS-III genes, one came from a hyperthermophile (*Thermotoga maritima*) whose proteins may not be active in *E. coli* at 37°C (28). The other was from *Legionella pneumophila*, which—unlike the sources of the other PTPS-III genes—has a *folB* gene and so presumably needs no functional alternative. All strains grew in the presence of thymidine, confirming that none of the PTPS genes is toxic.

To substantiate the results from complementation of the growth phenotype, we analyzed folates in the wild-type strain and the *folB* deletant harboring either vector alone or vector plus representative PTPS-I or PTPS-III sequences (from *L. interrogans*) (Fig. 3B). As expected, no folates were detectable in the *folB* deletant. Consistent with the growth complementation data, expression of PTPS-III substantially restored folate levels, whereas the expression of PTPS-I did not.

**PTPS-III proteins catalyze the conversion of dihydroneopterin triphosphate to HMDHP.** To reinforce the complementation results, we tested the ability of bacterial PTPS-III and PTPS-I proteins to mediate formation of HMDHP from dihydroneopterin triphosphate in vitro. PTPS-III and PTPS-I from the representative species *C. botulinum* and *L. interrogans* were overexpressed as N-terminally His-tagged proteins in *E. coli* and affinity purified; the products were judged to be  $\geq 90\%$  homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4A). The purified proteins were incubated with dihydroneopterin triphosphate generated from GTP using recombinant GTP cyclohydrolase I. Purified *P. falciparum* PTPS and human PTPS were included as controls (10). The reaction mixtures were oxidized by treatment with acidic iodine before HPLC separation. The oxidation step converts HMDHP to 6-hydroxymethylpterin and 6-pyruvoyltetrahydropterin to pterin, allowing sensitive fluorescence detection of the reaction products. As reported by Dittrich et al. (10), 6-hydroxymethylpterin and pterin were, respectively, the major and minor products of the *P. falciparum* enzyme, and pterin



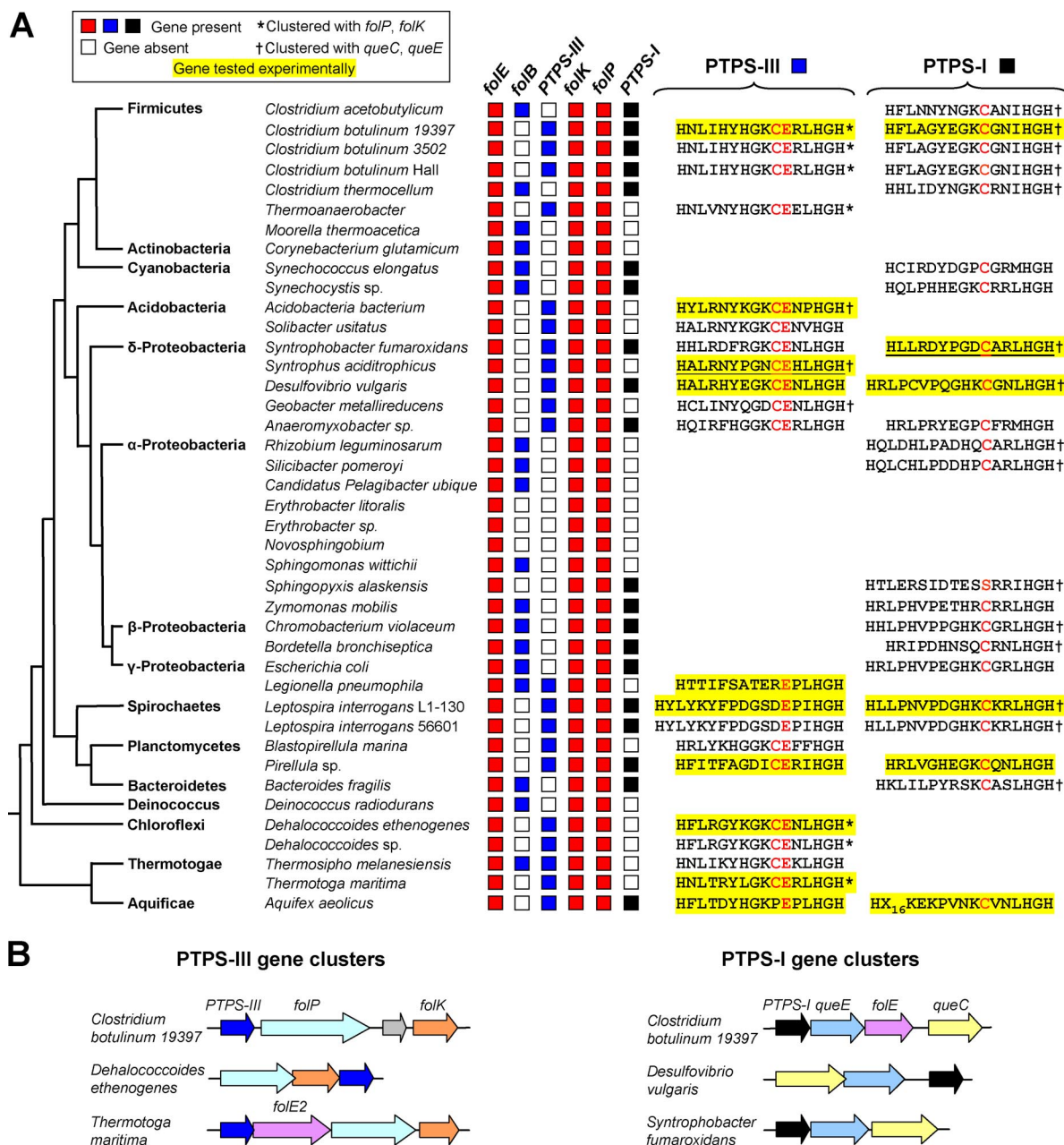


FIG. 2. Comparative genomic evidence implying that PTPS-III can replace FolB in bacteria. (A) Phylogenetic distribution of genes encoding FolB, PTPS-III, and PTPS-I among representative bacteria. The tree shows the standard branching order for each group and is adapted from Barrick and Breaker (3), with the addition of *Planctomycetes* and *Aquificae* (6). Apart from *folB*, which is missing in some cases, all species shown have complete sets of folate synthesis genes as exemplified by *folE*, *folK*, and *folP*. The active-site regions of PTPS-III and PTPS-I proteins, bounded by three His residues coordinating the essential Zn<sup>2+</sup> ion, are shown on the right. Asterisks indicate that the corresponding gene is clustered on the chromosome with *folK* and *folP*; daggers indicate clustering with the queuosine synthesis genes *queC* and *queE*. Yellow highlighting indicates that the corresponding gene was tested experimentally. Underlining denotes the two sequences selected for mutagenesis. (B) Clustering of genes specifying PTPS-III with folate synthesis genes, and clustering of genes specifying PTPS-I with queuosine synthesis genes. Matching colors correspond to orthologous genes; gray arrows are unrelated genes. Arrows show transcriptional direction; overlap denotes translational coupling.

was the sole product of the human enzyme (Fig. 4B). Both bacterial PTPS-I proteins gave pterin, as reported for *E. coli* PTPS-I (10, 39) (Fig. 4C), whereas the bacterial PTPS-III proteins gave 6-hydroxymethylpterin, as well as pterin (Fig. 4D). Collectively, these data are consistent with the view that PTPS-III proteins catalyze HMDHP formation from dihydro-

neopterin triphosphate and that PTPS-I proteins do not. It should be noted that the bacterial proteins gave rise to other fluorescent peaks besides pterin and 6-hydroxymethylpterin; these presumably corresponded, for PTPS-I proteins at least, to 6-carboxytetrahydropterin or its breakdown products (27). The bacterial, *P. falciparum*, and human proteins all appeared

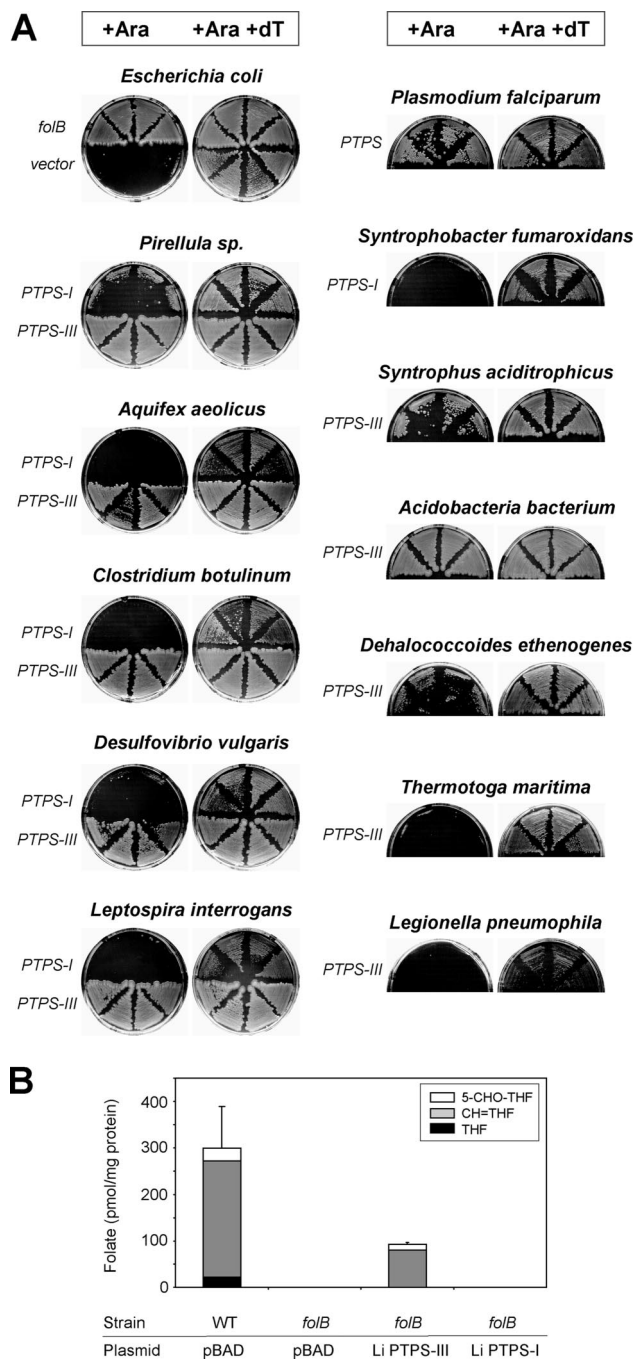


FIG. 3. Functional complementation of the *E. coli folB* deletant by genes specifying PTPS-III but not PTPS-I. (A) The *E. coli folB* deletant strain harboring pBAD24 alone or containing PTPS genes from various sources was plated on LB medium containing 0.2% L-arabinose (Ara) with or without 75  $\mu$ g of thymidine (dT)/ml, 100  $\mu$ g of carbenicillin/ml, and 50  $\mu$ g of kanamycin/ml. Plates were incubated at 37°C for 2 days. (B) Folate analysis from 50-ml liquid cultures of wild-type (WT) and *folB* deletant strains harboring pBAD24 alone or containing PTPS-I or PTPS-III genes from *L. interrogans* (Li). The values are expressed as the means and standard error from three replicates. The medium was LB plus 75  $\mu$ g of thymidine/ml, 0.2% L-arabinose, and 100  $\mu$ g of carbenicillin/ml. Cells were harvested when the  $A_{600}$  reached  $1.0 \pm 0.1$ . No folates were detected in *folB* deletant cells or in deletant cells expressing *L. interrogans* PTPS-I; detection limits for individual folates were 0.2 to 0.5 pmol/mg of protein. THF, tetrahydrofolate; CH=THF, 5,10-methenyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate.

to lack significant dihydroneopterin aldolase activity since they formed only traces of HMDHP (detected after oxidation as 6-hydroxymethylpterin) when dihydroneopterin was used as the substrate (data not shown). This trace HMDHP formation was no greater in PTPS-III reactions than in PTPS-I reactions, supporting its physiological insignificance.

**Phylogenetic and mutational analyses of the origin of PTPS-III proteins.** The evolutionary origin of PTPS-III proteins was investigated first by phylogenetic analysis of the representative PTPS-III and PTPS-I proteins that had been tested experimentally. The resulting tree (Fig. 5A) showed an intermingling of PTPS-III and PTPS-I sequences. The absence of distinct PTPS-III and PTPS-I clades implies that these proteins diverged at various times in various lineages. This inference is corroborated by the strong bootstrap support for the nodes linking *C. botulinum* PTPS-III with two PTPS-I proteins, and *Syntrophus aciditrophicus* PTPS-III with *Syntrophobacter fumaroxidans* PTPS-I.

The high overall sequence similarity (75%) of the *Syntrophus aciditrophicus* PTPS-III and *Syntrophobacter fumaroxidans* PTPS-I sequences, especially that of the active site region (Fig. 2A), prompted us to use site-directed mutagenesis to investigate the role of the active-site glutamate residue in determining enzyme activity (Fig. 5B). This residue in *Syntrophus aciditrophicus* PTPS-III (E27) was accordingly changed to alanine, and the equivalent residue in *Syntrophobacter fumaroxidans* PTPS-I (A27) was changed to glutamate. Replacing the glutamate residue abolished the ability to complement the *E. coli folB* mutation, confirming that this amino acid is necessary for PTPS-III activity, as is the case for the *P. falciparum* enzyme (10). Conversely, adding a glutamate residue conferred incipient complementation activity in the plate assay (Fig. 5B). To corroborate this result, we measured the growth in liquid medium (LB plus 0.2% L-arabinose, 100  $\mu$ g of carbenicillin/ml, and 50  $\mu$ g of kanamycin/ml) of 10 independent clones expressing mutant (A27E) or wild-type *Syntrophobacter fumaroxidans* PTPS-I. The former grew significantly ( $P < 0.05$ ) more than the latter, yielding a difference in mean  $A_{600}$  values of 1.2 after 20.5 h.

## DISCUSSION

Our findings solve the enigma (9) of how numerous bacteria dispense with the essential folate synthesis enzyme FolB and show that they do this by an expedient adopted by *P. falciparum* and perhaps heterokonts: the use of a variant form of PTPS. FolB (dihydroneopterin aldolase) thus joins a growing list of steps in the classical folate synthesis pathway for which there exist alternative, completely different enzymes. Other examples include FolE (11), FolA (26), and FolC (9). In fact, the FolB case is more radical than the others since it involves a bypass that replaces three steps, the other two being those mediated by FolQ (dihydroneopterin triphosphate pyrophosphatase) and a nonspecific phosphatase (Fig. 1A). We can therefore predict that FolQ is also missing from genomes in which PTPS-III replaces FolB, but this cannot be verified from genome sequences alone because FolQ proteins are heterogeneous and belong to the large and almost omnipresent Nudix family (13, 23).

Our functional complementation and folate analysis data

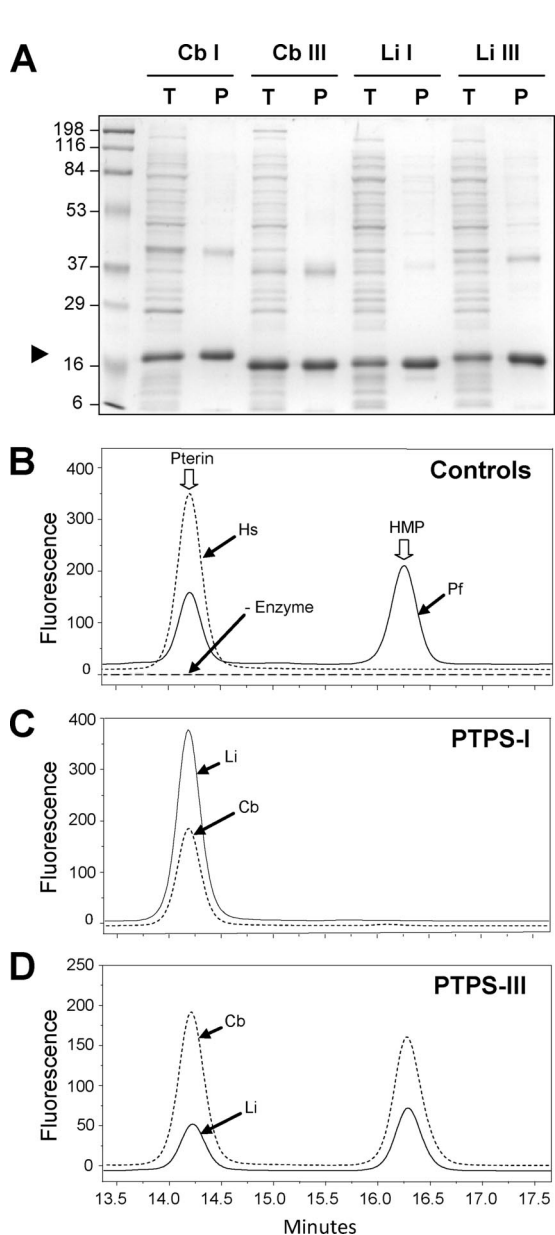


FIG. 4. Purification of PTPS proteins and HPLC analysis of the products they form from dihydroneopterin triphosphate. (A) SDS-polyacrylamide gel electrophoresis of 15  $\mu$ g of total proteins of IPTG-induced *E. coli* cells (T lanes), and 5  $\mu$ g of Ni<sup>2+</sup>-affinity-purified PTPS-I or PTPS-III proteins (P lanes). Staining was performed with Coomassie blue. The positions of molecular markers (in kilodaltons) are indicated. The PTPS monomer bands are indicated with an arrowhead. Li, *L. interrogans*; Cb, *C. botulinum*. (B to D) HPLC analyses of reaction products. No products were formed in blank reactions without dihydroneopterin triphosphate substrate. Reaction products were subjected before chromatography to iodine oxidation, which converts 6-pyruvoyltetrahydropterin to pterin and HMDHP to 6-hydroxymethylpterin, and products were detected fluorometrically. The normalized retention times of pterin and 6-hydroxymethylpterin (HMP) standards added to each sample are marked with arrows. Frame B shows data for controls using human (Hs) PTPS-II (which forms only 6-pyruvoyltetrahydropterin) and *P. falciparum* PTPS (Pf) (which forms mainly HMDHP with some 6-pyruvoyltetrahydropterin). Frames C and D show data for PTPS-I or PTPS-III proteins from *L. interrogans* and *C. botulinum*.

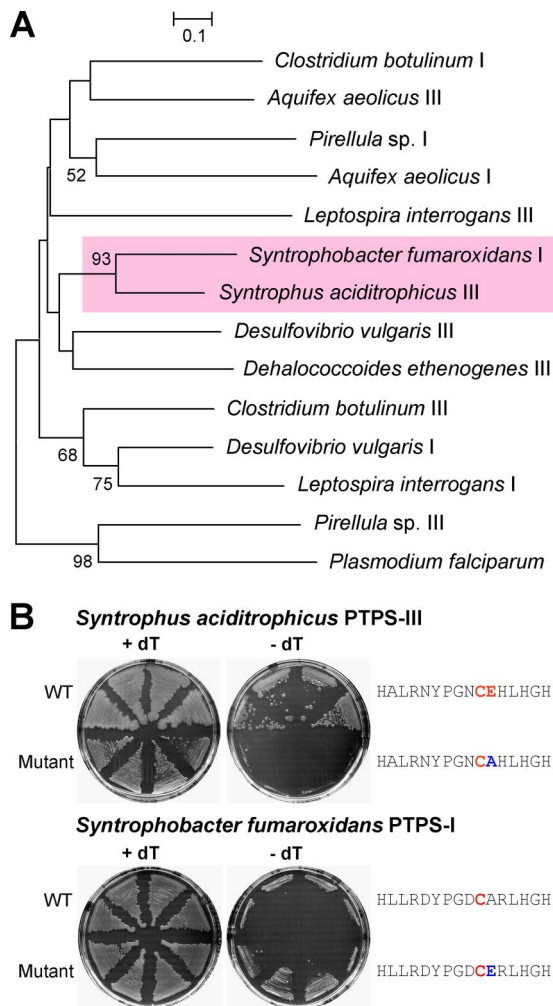


FIG. 5. Phylogenetic and mutational evidence on the origin of PTPS-III proteins. (A) Phylogenetic tree of PTPS proteins from representative bacteria and *P. falciparum*. Sequences were taken from the SEED database. The tree was constructed by the neighbor-joining method; bootstrap values are indicated only for nodes with >50% support. The tree is drawn to scale; evolutionary distances are in units of number of amino acid substitutions per site. The two sequences selected for mutagenesis are highlighted in rose. (B) Effects of mutations in the active-site region of *Syntrophus aciditrophicus* PTPS-III or *Syntrophobacter fumaroxidans* PTPS-I on functional complementation of the *E. coli folB* deletant. The deletant strain harboring pBAD24 containing wild-type (WT) or mutated PTPS genes was plated on LB medium containing 0.2% L-arabinose with or without 75  $\mu$ g of thymidine (dT)/ml, and appropriate antibiotics. Plates were incubated at 37°C for 2 days. Active-site regions are shown on the right; the predicted catalytic cysteine and glutamate residues in the wild type are colored red, and the mutated residues are colored blue.

provide the first genetic evidence for replacement of FolB by PTPS-III type proteins, since prior evidence on this point—for the *P. falciparum* protein—was solely biochemical (10). Our biochemical data indicate that bacterial PTPS-III proteins, like their *P. falciparum* counterpart (10), mediate the formation of HMDHP from dihydroneopterin triphosphate. Note, however, that since HMDHP was not detected directly, the data do not exclude the possibility that the native PTPS-III product is hydroxymethyltetrahydropterin or another pterin that yields



6-hydroxymethylpterin after acidic iodine treatment (33). Our biochemical data also leave open the question of the other product(s) of the reaction and consequently of the mechanism involved. A two-carbon fragment must be released from the pterin side chain, as in the FolB reaction (Fig. 1B), and it will be interesting to determine whether this is accompanied by the unusual elimination of triphosphate that characterizes the normal PTPS reaction leading to 6-pyruvoyltetrahydropterin (Fig. 1C) (37).

Our results bear also on the evolutionary origin of bacterial PTPS-III proteins. Their relative rarity and sporadic taxonomic distribution compared to the far more common PTPS-I proteins suggest a likely origin from PTPS-I proteins. Phylogenetic analysis further suggests that PTPS-III proteins may have arisen multiple times in various PTPS-I lineages, several PTPS-III proteins being more similar to various PTPS-I proteins than to each other. The idea of multiple origins of PTPS-III proteins is supported by the finding that, for closely related PTPS proteins, a single glutamate residue immediately downstream of the catalytic cysteine can determine the presence or absence of PTPS-III activity. It is thus reasonable to suppose that evolution of PTPS-III proteins began with a mutation that introduced a glutamate residue into a PTPS-I protein. Such mutations could in some instances have been a single base change, as in the case of the alanine-to-glutamate conversion (GCG→GAG) in *Syntrophobacter fumaroxidans* PTPS-I (Fig. 5B). Subsequent mutations could have increased the efficiency of HMDHP production, eventually making FolB redundant. It is also reasonable to suppose that adding a glutamate residue does not abolish PTPS-I activity. *Syntrophus aciditrophicus* and several other bacteria that lack FolB have a single glutamate- and cysteine-containing PTPS (which in the case of *Syntrophus aciditrophicus* has PTPS-III activity) whose gene is clustered with genes of queuosine biosynthesis (Fig. 2A), the clustering implying that the protein remains a functional PTPS-I (QueD) that participates in queuosine biosynthesis (32). In contrast to these cases of putatively bifunctional PTPS-III/PTPS-I proteins, there are several genomes (e.g., *Aquifex aeolicus*) that specify a PTPS-III protein with no active-site cysteine, plus a PTPS-I protein with cysteine only. In such cases the PTPS-III protein may have become specialized and lost the ancestral PTPS-I function. A final point about the acquisition of PTPS-III proteins is that the phylogenetic tree (Fig. 5A) suggests the possibility of horizontal gene transfer in the clade containing the planctomycete bacterium *Pirellula* and eukaryotic *P. falciparum* proteins.

Although the details of PTPS-III evolution necessarily remain conjectural, there is no mistaking the overall pattern. It is seemingly facile for a PTPS-I protein to acquire PTPS-III activity, and this probably occurred independently in various lineages so that PTPS-III proteins provide striking instances of convergent evolution. Their occurrence in pathogenic bacteria (*Clostridia*, *Spirochaetes*), and probably also in the oomycete pathogen *Phytophthora* (19), could make PTPS-III proteins targets for novel antifolate compounds.

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