Promiscuous and Adaptable Enzymes Fill "Holes" in the Tetrahydrofolate Pathway in *Chlamydia* Species

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ABSTRACT **Folates are tripartite molecules comprising pterin, *para*-aminobenzoate (PABA), and glutamate moieties, which are essential cofactors involved in DNA and amino acid synthesis. The obligately intracellular *Chlamydia* species have lost several biosynthetic pathways for essential nutrients which they can obtain from their host but have retained the capacity to synthesize folate. In most bacteria, synthesis of the pterin moiety of folate requires the FolEQBK enzymes, while synthesis of the PABA moiety is carried out by the PabABC enzymes. Bioinformatic analyses reveal that while members of *Chlamydia* are missing the genes for FolE (GTP cyclohydrolase) and FolQ, which catalyze the initial steps in *de novo* synthesis of the pterin moiety, they have genes for the rest of the pterin pathway. We screened a chlamydial genomic library in deletion mutants of *Escherichia coli* to identify the "missing genes" and identified a novel enzyme, $TrpF_{CtL2}$, which has broad substrate specificity. $TrpF_{CtL2}$, in combination with GTP cyclohydrolase II (RibA), the first enzyme of riboflavin synthesis, provides a bypass of the first two canonical steps in folate synthesis catalyzed by FolE and FolQ. Notably, $TrpF_{CtL2}$ retains the phosphoribosyl anthranilate isomerase activity of the original annotation. Additionally, we independently confirmed the recent discovery of a novel enzyme, CT610, which uses an unknown precursor to synthesize PABA and complements *E. coli* mutants with deletions of *pabA*, *pabB*, or *pabC*. Thus, *Chlamydia* species have evolved a variant folate synthesis pathway that employs a patchwork of promiscuous and adaptable enzymes recruited from other biosynthetic pathways.**

IMPORTANCE **Collectively, the involvement of $TrpF_{CtL2}$ and CT610 in the tetrahydrofolate pathway completes our understanding of folate biosynthesis in *Chlamydia*. Moreover, the novel roles for $TrpF_{CtL2}$ and CT610 in the tetrahydrofolate pathway are sophisticated examples of how enzyme evolution plays a vital role in the adaptation of obligately intracellular organisms to host-specific niches. Enzymes like $TrpF_{CtL2}$ which possess an enzyme fold common to many other enzymes are highly versatile and possess the capacity to evolve to catalyze related reactions in two different metabolic pathways. The continued identification of unique enzymes such as these in bacterial pathogens is important for development of antimicrobial compounds, as drugs that inhibit such enzymes would likely not have any targets in the host or the host's normal microbial flora.**

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Chlamydiae are obligately intracellular bacteria that cause a wide variety of infectious diseases in humans and animals. The bacteria infect epithelial cells and cause diseases ranging from pneumonia to pelvic inflammatory disease to blinding trachoma (1). *Chlamydia trachomatis* infection is the most commonly reported bacterial sexually transmitted disease in the United States, with a total of 1,422,976 cases of genital chlamydial infection reported during 2012 (2). In addition to being an important sexually transmitted pathogen, *C. trachomatis* is the causative agent of trachoma, a chronic follicular keratoconjunctivitis, characterized by scarring of the eyelid and corneal opacities. The most recent estimates (from 2008) suggest that there are about 40 million people with active trachoma and that 1.3 million people are blinded as a result of this disease (3). The obligately intracellular organism *Chlamydia* is notable for its unusual dimorphic life cycle. The infectious form of the organism, the elementary body (EB), is small and displays limited metabolic activity (4). Once inside the host cell, the EB begins a developmental cycle and undergoes conversion into the metabolically active noninfectious reticulate body (RB) within 2 to 4 h. RBs replicate by binary fission and then convert back into the EB form after about 24 to 36 h. Lysis of the infected host cell occurs after 48 to 72 h, depending on the *Chlamydia* species, and released EBs go on to infect neighboring cells.

Bacterial evolution that leads to obligately intracellular para-

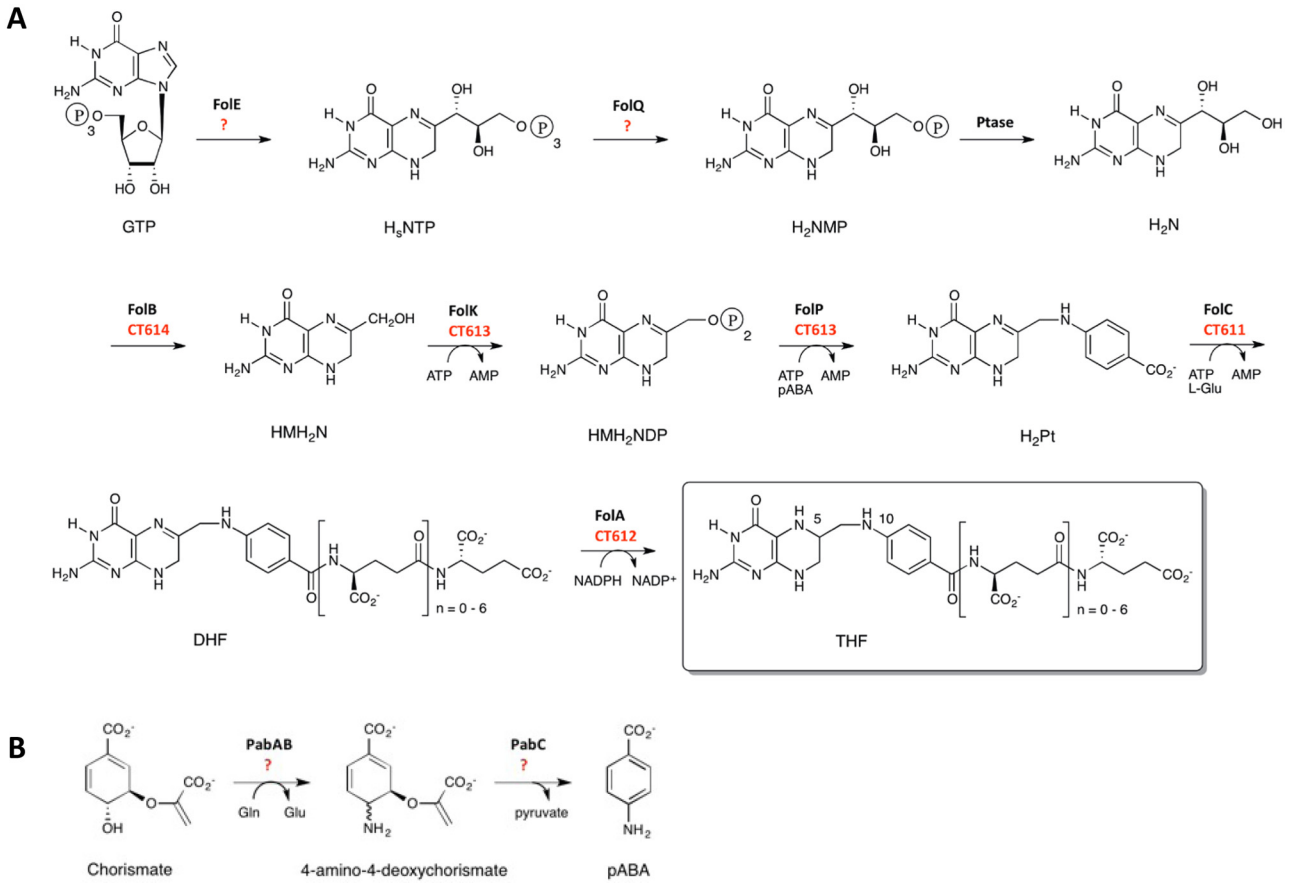


FIG 1 The canonical tetrahydrofolate biosynthetic pathway. Abbreviated enzyme names are in bold, with the homologs in *C. trachomatis* serovar D listed in red. A question mark is present if the *Chlamydia* homolog is not known. (A) GTP to tetrahydrofolate. FolE, GTP cyclohydrolase I; FolQ, dihydroneopterin triphosphate pyrophosphohydrolase; Ptase, nonspecific phosphohydrolase; FolB, dihydroneopterin aldolase; FolK, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase; FolP, dihydropteroate synthase; FolC, dihydrofolate synthetase; FolA, dihydrofolate reductase. Full metabolite names: 7,8-dihydroneopterin triphosphate (H_2NTP), 7,8-dihydroneopterin monophosphate (H_2NMP), 7,8-dihydroneopterin (H_2N), 6-hydroxymethyl-7,8-dihydroneopterin (HMH_2N), 6-hydroxymethyl-7,8-dihydroneopterin diphosphate (HMH_2NDP), 7,8-dihydropteroate (H_2Pt), 7,8-dihydrofolate (DHF), tetrahydrofolate (THF). (B) PABA biosynthesis. PabA, aminodeoxychorismate synthase component II; PabB, aminodeoxychorismate component I; PabC, aminodeoxychorismate lyase.

sitism in a eukaryotic host is thought to be associated with loss of genetic information, especially for genes that become redundant within the host niche (5). These genetic losses are not deleterious for the organism provided that the missing biosynthetic functions can be compensated for by increased transport functions that allow the organism access to essential nutrients found in the intracellular environment. Conversely, metabolic pathways which are either uniquely found in these bacteria or are critical to their intracellular growth are expected to persist despite reductive evolution. Obligately intracellular parasites, like *Chlamydia*, are dependent on their host cell for nutrients, as they have eliminated many redundant biosynthesis genes.

Folates are tripartite molecules comprising pterin, *para*-aminobenzoate (PABA), and glutamate moieties to which one-carbon units at various oxidation levels can be attached at the N5 and N10 positions (Fig. 1A). Tetrahydrofolates (THFs) are essential cofactors required by all organisms for DNA and amino acid synthesis and are obtained through either *de novo* synthesis or transport. Most bacteria, plants, and fungi and some protozoa possess a pathway for *de novo* synthesis of THF (6, 7), whereas vertebrates do not have a pathway for synthesis of folates and must obtain them through their diet. The pathway for THF synthesis involves 10 enzymes: *folEQBK* encode enzymes required for formation of the pterin moiety; *pabA*, *pabB*, and *pabC* encode enzymes required for formation of the PABA moiety; and *folPCA* encode enzymes required for the ligation of the pterin and PABA precursors and the glutamylation and reduction steps (see Fig. 1 for enzyme names and abbreviations and reactions catalyzed) (6).

Unlike other intracellular bacteria, which salvage THF or precursors from their hosts, many *Chlamydia* species are not folate auxotrophs. *C. trachomatis* L2, *Chlamydia psittaci* 6BC, and *C. psittaci* Cal10 synthesize folates *de novo* and possess FolA and FolP activity (8). Furthermore, *C. trachomatis* is sensitive to antibiotics that target enzymes in the folate biosynthetic pathway (8). We previously conducted a comparative genomic analysis of tetrahydrofolate biosynthesis genes in 500 microbial genomes (with only 10 *Chlamydiaceae* genomes) and reported that the *Chlamydia* species contained homologs of the *folBKPA* genes in a physical cluster (9). Proteomic analyses detected FolP in *C. trachomatis* (10, 11) and *Chlamydia pneumoniae* (12). However, genes encoding orthologs of FolE and FolQ, the first two enzymes of the pterin branch, as well as PabABC, the three PABA pathway enzymes, and

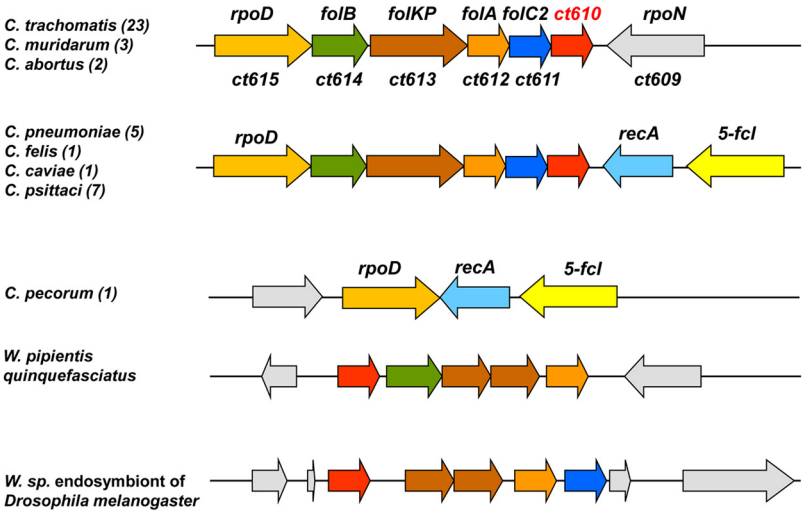


FIG 2 Clustering of folate pathway genes in *Chlamydiae* and other intracellular bacteria. The gene organization of *Chlamydia* spp. and the phylogenetically distant *Wolbachia* is shown. Numbers in parentheses are numbers of genomes analyzed. *ct609* to *ct615* are locus tags for *C. trachomatis* serovar D. They are included for clarity, as several of these loci are cited in previous publications.

FolC, the glutamylation enzyme, could not be identified in any of the 10 genomes analyzed at the time.

The mystery of the missing *folC* gene was solved by the identification of a gene now called *folC2* (*ct611* in *C. trachomatis* D/UW-3/CX) located in the chlamydial folate biosynthesis cluster. FolC2 is not a member of the FolC/COG0285 family but part of the COG1478 family, which contains enzymes involved in the glutamylation of the archaeal cofactor F_{420}. The activity of this alternate folylglutamate synthase was demonstrated by complementation of an *Escherichia coli* Δ*folC* mutant by a plasmid harboring *ct611* (9). This was the first case of nonorthologous displacement in the folate pathway discovered in *Chlamydia* species.

In *E. coli*, the *folE* gene encodes GTP cyclohydrolase I (GCYH-I; EC 3.5.4.16), which catalyzes the conversion of GTP to 7,8-dihydroneopterin triphosphate (H_2NTP). In some *Bacteria* and *Archaea*, the same reaction is catalyzed by another family of GTP cyclohydrolase I enzymes, the IB family (13), but no members of this second family are found in chlamydiae (9). This suggests that chlamydiae either recruit another enzyme family to perform this reaction or that these organisms scavenge H_2NTP from the human host; H_2NTP is the only intermediate of the folate pathway found in humans, where it serves as the first intermediate in biopterin biosynthesis (14). Similarly, the source of PABA in chlamydiae remained unidentified until recently, as the absence of the PABA synthesis genes suggests either a *de novo* PABA synthesis pathway in these organisms or import of PABA from the host. It was found that two members of the COG5424 family, NE1434 from *Nitrosomonas europaea* and CT610 from *C. trachomatis* D/UW-3/CX, are involved in a novel PABA synthesis pathway (15). While the precursor used to make PABA remains unknown, NE1434 and CT610 can complement an *E. coli* Δ*pabABC* mutant.

In this work, we show an example of *Chlamydia*'s metabolic plasticity with the discovery of a novel route to initiate folate synthesis that combines the first enzyme of riboflavin biosynthesis with an enzyme of tryptophan synthesis to make the folate pathway intermediate 7,8-dihydroneopterin monophosphate (H_2NMP) rather than using the first two canonical enzymes, FolE and FolQ. Additionally, we independently show that CT610 is involved in PABA synthesis, confirming another example of the folate pathway variation found in *Chlamydia*. Collectively, these findings complete our understanding of folate biosynthesis in these human pathogens, confirm their ability to synthesize folates, and provide an elegant example of enzyme evolution to create new vitamin pathways (16).

RESULTS

Comparative genomic analysis of THF biosynthesis genes in chlamydiae. The distribution and physical location of folate biosynthesis genes were analyzed with the 43 *Chlamydia* genomes in the SEED database (17) and is available in the "Folate Tryp Chlamydia" subsystem. Strict conservation of the folate gene cluster *folBPKA*, *folC2*, and *ct610* was observed in all genomes analyzed and includes no unrelated gene, with the exception of *Chlamydia pecorum*, which is missing the whole cluster and corresponding genes (Fig. 2). The clusters can be separated into two subgroups: one downstream of *rpoN* in the *C. trachomatis* clade and one downstream of *recA* in the *Chlamydophila* clade. *folK* and *folP* are fused into a single gene in all chlamydiae analyzed. The intergenic region between *ct610* and the gene immediately upstream differed slightly between *C. trachomatis* serovar L2 (3 bp) and *C. trachomatis* serovar D and *Chlamydia muridarum* (21 bp), suggesting that *ct610* (and its orthologs) might be part of a single transcript of the folate gene cluster. Primers (see Table S1 in the supplemental material) designed to amplify the intergenic region between open reading frames (ORFs) in the folate cluster (*folBPKA*, *folC2*, and *ctl0874*—a homolog of *ct610* in *C. trachomatis* D) of *C. trachomatis* L2 434/Bu were used to determine transcriptional organization. Reverse transcription-PCR (RT-PCR) analysis revealed that the genes in the folate cluster are transcribed as a single multigenic message which includes *ctl0874* (see Fig. S1 in the supplemental material). Here, we refer to *ctl0874* with the *C. trachomatis* D locus name, *ct610*, for consistency with the literature.

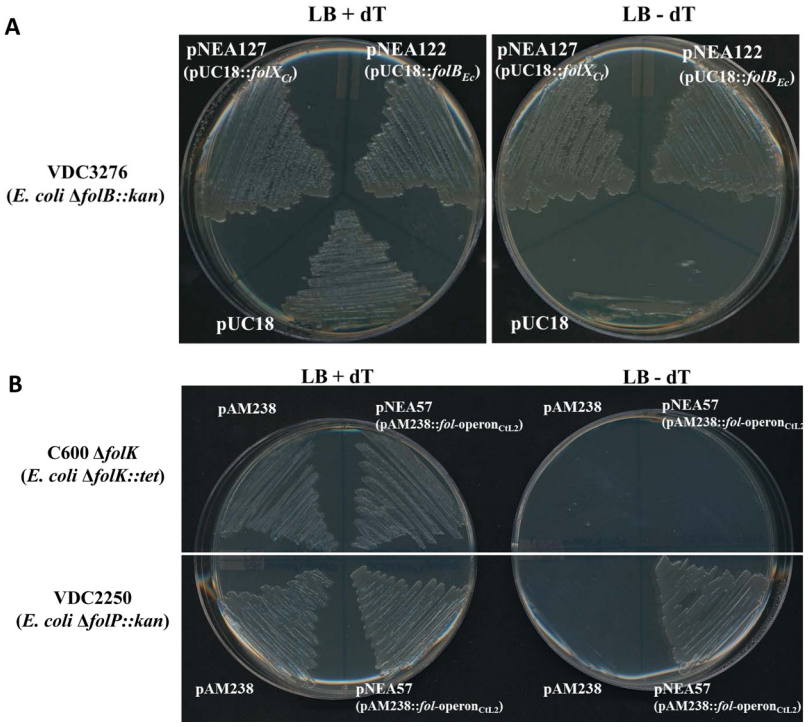


FIG 3 Activity of the *C. trachomatis* FolB, FolK, and FolP orthologs. (A) FolB complementation by either $folB_{Ec}$ (pNEA122) or $folX_{Ct}$ (*ct614*; pNEA127). (B) Complementation of *E. coli* Δ*folK* and Δ*folP*. (Top) *E. coli* Δ*folK* is not complemented by any genes in the entire folate cluster of *C. trachomatis*, as shown by lack of growth on M9 minimal medium without thymidine (dT). (Bottom) *E. coli* Δ*folP* is complemented by the folate cluster of *C. trachomatis*, presumably by *ct613*, which encodes FolKP.

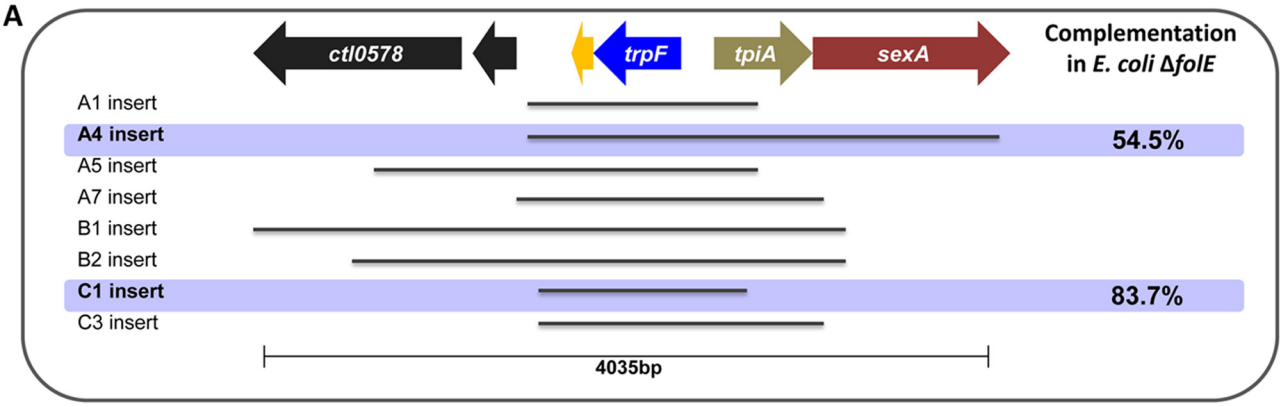
Experimental validation of activity of the *C. trachomatis* FolB, FolK, and FolP orthologs. Aside from the initial steps of pterin synthesis, the remainder of the folate pathway appears to be intact in most *Chlamydia* species (Fig. 2) and encoded on an operon (see above). However, the functions of these enzymes have yet to be verified. CT614 of *C. trachomatis* is currently annotated as a FolX, which suggests that it is a 7,8-dihydroneopterin triphosphate epimerase involved in the biosynthesis of tetrahydromonapterin (18). Since *Chlamydia* species do not have a pathway for tetrahydromonapterin, CT614 is more likely FolB, the dihydroneopterin aldolase involved in tetrahydrofolate biosynthesis, which would be in agreement with the clustering of *ct614* with the other folate synthesis genes (Fig. 2). In order to test this hypothesis, *ct614* was cloned into pUC18 (pNEA127), expressed in *E. coli* Δ*folB*, and demonstrated to complement the *E. coli* Δ*folB* mutant on LB in the absence of added thymidine (Fig. 3A). These results are consistent with CT614 functioning as a dihydroneopterin aldolase.

ct613, the next gene in the *C. trachomatis* folate gene cluster, is annotated as a *folK-folP* fusion. To verify that the *C. trachomatis* FolKP has both 7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase (FolK) and dihydropteroate synthase (FolP) activities, pNEA57, a plasmid expressing the whole folate operon from *C. trachomatis* L2 (i.e., *folB-folKP-folA-folC2-ct610*), was used to transform *E. coli* Δ*folK*::*kan* and *E. coli* Δ*folP*::*kan*, neither of which grows on LB without supplemental thymidine. Interestingly, only the *E. coli* Δ*folP* mutant was complemented by the *C. trachomatis* folate operon, while *E. coli* Δ*folK* was not (Fig. 3B). Based on these complementation experiments, the $FolKP_{CtL2}$ fusion protein as expressed in *E. coli* can carry out FolP activity but not FolK activity, suggesting that CT613 cannot capture the 6-hydroxymethyl-7,8-dihydroneopterin (HMH_2N) precursor from *E. coli* FolB.

Library screen for the *Chlamydia* homolog of the *folE* gene of *E. coli*. *E. coli* Δ*folE* mutants require thymidine, pantothenic acid, adenine, and Casamino Acids for growth on minimal medium. We screened a library of *C. trachomatis* L2 DNA in the *E. coli* Δ*folE*::*kan* mutant P1-7B and selected on minimal medium containing all the required supplements but thymidine. Over 150 transformants that grew on minimal medium without thymidine were recovered in three independent transformations.

Fifteen complementing clones chosen for characterization contained inserts that ranged in size from 3.2 to 9.2 kb. The minimum sequence present in all of the inserts contained a full-length open reading frame annotated as *trpF* (*ctl0581*), encoding *N*′-5′-phosphoribosyl anthranilate (PRA) isomerase (EC 5.3.1.24) with its native promoter as well as the $tRNA^{Met}$ gene just downstream of *trpF* (Fig. 4A). Two transformants, A4 and C1, were chosen for further characterization. We infer that the *trpF* in both clones is driven by its native promoter, as *trpF* in A4 is in the opposite orientation to the plasmid vector promoter, P_{lac}, and *trpF* in C1 is more than 1 kb from the vector promoter. Each clone was retransformed into the Δ*folE*::*kan* mutant, and plating efficiency experiments were carried out to determine phenotypic complementation. The results shown in Fig. 4A and in Table S2 in the supplemental material demonstrate that both inserts complemented the *folE* mutation and that complementation was independent of the vector promoter. The *trpF* genes from *C. tracho-*

A



B

Gene (Plasmid)	Organism	Complementation in *E. coli* Δ*folE*	Complementation in *E. coli* Δ*trpF*
pUC18	-	ND	ND
folE (pNEA50)	*E. coli*	85.5%	ND
trpCF (pNEA59)	*E. coli*	ND	77.5%
trpC (pNEA67)	*E. coli*	ND	ND
trpF (pNEA61)	*E. coli*	ND	61.8%
trpF (pNEA71)	*C. trachomatis* L2	33.8%	91.9%
trpF (pNEA69)	*C. muridarum* Nigg	20.0%	92.9%
trpF (pNEA72)	*C. caviae* GPIC	2.0%	110%

% plating efficiency = #cfu/ml on medium without supplements / #cfu/ml on medium with supplements x 100. ND = no complementation detectable, plating efficiency < 0.00001%

FIG 4 Phylogenomic context and analysis of *trpF* genes in *Chlamydia*. (A) Alignment of library inserts that complemented Δ*folE*. Library clones A4 and C1 (highlighted in blue) were used to test efficiency of complementation on M9 minimal medium containing pantothenic acid (1 μg/ml), adenine (40 μg/ml) and Casamino Acids (0.5%). (B) Complementation of *E. coli* Δ*folE* and Δ*trpF* mutants with *trpF* genes from members of the *Chlamydiaceae*.

matis L2, *C. muridarum*, and *Chlamydia caviae* were cloned into pUC18 and tested for complementation of the *folE* mutation in P1-7B. As shown in Fig. 4B, all *trpF* alleles tested complemented the *folE* mutation, albeit with different efficiencies. In *E. coli* the *trpF* gene is fused to the *trpC* gene, encoding indole-3-glycerol phosphate synthase (EC 4.1.1.48). In contrast to the *Chlamydia trpF* clones, neither the *E. coli trpCF* gene nor the *E. coli trpC* or *trpF* domains expressed alone showed any complementation activity in the Δ*folE* mutant (Fig. 4B).

Sequence and functional analysis of $TrpF_{CtL2}$ as a PRA isomerase. Alignments of TrpF reveal that *C. trachomatis* L2 TrpF is 98 to 100% identical at the amino acid level to TrpF of the other *C. trachomatis* ocular and genital serovars and 78% identical to TrpF of *C. muridarum*, a mouse pathogen, while identity drops to ~48% for the other animal pathogens, *C. caviae*, *C. pecorum*, and *Chlamydia felis*. Moreover, the *Chlamydia trpF* orthologs differ in genomic contexts, including both gene content and neighborhood. *C. pneumoniae*, *C. psittaci*, and *C. abortus* lack all the *trp* genes, including *trpF*, whereas *C. trachomatis* species have lost the *trpC* and *trpD* genes. Thus, the evolutionary history of *trpF* in *Chlamydia* seems to have followed a complex path involving genome decay and rearrangements (Fig. 5) coupled with the acquisition of a novel function to serving as a FolE in the folate biosynthesis pathway. Given the ability of *Chlamydia trpF* orthologs to complement *E. coli* Δ*folE*, we next addressed if evolution of this new enzymatic function was accompanied by a loss of PRA isomerase activity.

To test PRA isomerase (TrpF) activity, a *trpCF* deletion mutant of *E. coli* (ATM932) was transformed with a plasmid containing the *trpF* gene from *Chlamydiaceae* alone or cotransformed with a plasmid containing the *trpC* domain of the *E. coli trpCF* gene (pAM238::$trpC_{Ec}$, pNEA67), as a *trpCF* deletion can be complemented when the *E. coli trpC* and *trpF* domains are expressed from separate plasmids (pNEA67 and pNEA61, respectively). While the *E. coli trp* ($trpF_{Ec}$) domain and the *trpF* genes of *Chlamydiaceae* alone fail to complement an *E. coli* Δ*trpCF* mutant, the *trpF* genes of *Chlamydiaceae* fully complement the Δ*trpCF* mutant when expressed with pNEA67, which contains the *E. coli trpC* domain (Fig. 4B; also, see Table S3 in the supplemental material). Thus, the *Chlamydiaceae trpF* encodes a functional PRA isomerase which can act together with the *E. coli* indole-3-glycerol phosphate synthase (TrpC) to restore tryptophan prototrophy to a Δ*trpCF* mutant of *E. coli*. As expected, the *E. coli folE* gene (pNEA50) failed to complement the Δ*trpCF* mutant even when expressed from pUC19, a high-copy-number plasmid (see Table S3 in the supplemental material).

The PRA isomerase activity was further confirmed using an *E. coli* Δ*trpF* mutant (strain FBG-Wf) which retains the TrpC do-

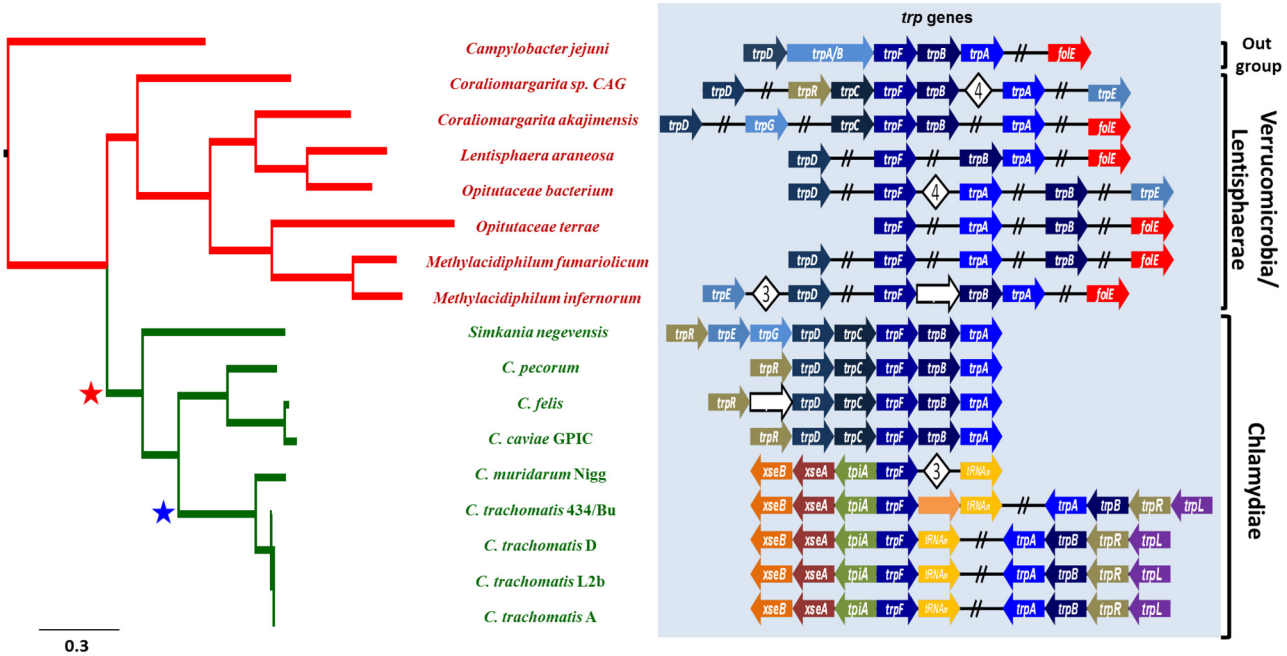


FIG 5 Phylogenetic tree of TrpF homologs and comparative genomics of *trpABCDEFGRL* and *folE* genes. The phylogenetic tree was constructed using the WAG model (42). The *Verrucomicrobia* and *Lentisphaera* group conserved *folE* (red clade), while the *Chlamydiae* group lost *folE* (green clade and red star). In *Chlamydiae*, a subclade consisting of *C. trachomatis* and *C. muridarum* possess the *trpF* in a different genomic context (blue star). Genes and their positions are shown in arrows, genes of unknown function are marked in white, multiple genes occurring in both directions are marked with diamonds, and the number inside the diamond indicates how many genes are predicted in this category. Two diagonal black lines indicate that the distance between *trp* genes and *folE* spans >15 genes.

main (19, 20). As expected, pASK::$trpF_{CtL2}$ complemented the Δ*trpF* mutation and rescued the tryptophan auxotrophy *in vivo* (data not shown). *In vitro* assays were hindered by difficulty in obtaining sufficient amounts of pure $TrpF_{CtL2}$ protein and a poor signal-to-noise ratio of the enzyme assay, which prevented us from obtaining enzyme kinetics for the conversion of PRA to 1-(*o*-carboxyphenylamino)-1′-deoxyribulose 5′-phosphate (CdRP). Alternatively, use of cell-free extracts revealed that $TrpF_{CtL2}$ did indeed catalyze the conversion of PRA to CdRP, but at a level lower than that of the PRA isomerase activity of PriA from *Streptomyces coelicolor*, which was used as a positive control (see Fig. S2 in the supplemental material).

Biochemical assays for GTP cyclohydrolase I (GCYH-I) activity. After it was observed that $TrpF_{CtL2}$ complemented an *E. coli* Δ*folE* mutant, *in vitro* GCYH-I activity was assessed using a standard fluorescence assay in which the H_2NPT produced from GTP is first oxidized to neopterin (13). We observed a small but consistent enhancement of the fluorescent signal (data not shown) in cell-free extracts of *E. coli* Rosetta BL21(DE3) that expressed $TrpF_{CtL2}$ as a fusion with the *E. coli* maltose-binding protein (pMAL-c4x::$trpF_{CtL2}$, pNEA83). However, attempts to measure activity with either the semipurified (affinity) or purified (HiTrap Q; GE Healthcare) fusion protein were unsuccessful, suggesting that the enzyme lost activity or that an essential cofactor during purification or that the enzyme was not functioning as a GCYH-I and may instead catalyze a different but related reaction.

The GCYH-I reaction is especially complex and requires multiple steps (Fig. 6A) (21). The first half of the reaction comprises two sequential hydrolysis reactions that result in purine ring opening and release of formic acid to give 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-triphosphate (compound II) (Fig. 6A), which subsequently undergoes ribosyl ring opening, an Amadori rearrangement, and ring closure in the second half of the reaction to give H_2NTP. Similar ribosyl ring opening and Amadori rearrangements are also catalyzed by PRA isomerase (TrpF), as well as by the *N*′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) isomerase (HisA; EC 5.3.1.16) (22) (Fig. 6B).

Notably, while compound II is an intermediate in the GCYH-I reaction, the monophosphate derivative of compound II, 2,5-diamino-6-ribosylamino-4(3 H)-pyrimidinone 5′-phosphate (compound V) (Fig. 7A), is the product of the GCYH-II enzyme, which in *E. coli* is designated RibA and is involved in flavin biosynthesis. Since all sequenced chlamydial species possess a RibA ortholog (CT731), we reasoned that the failure to observe GCYH-I activity with $TrpF_{CtL2}$ might be because it instead functions in the chlamydial folate pathway by converting compound V to H_2NMP. The absence of an identifiable *folQ* gene that clusters with other folate biosynthetic genes in chlamydial genomes is also consistent with this hypothesis, as a pathway that utilized compound V as the precursor to the pterin system would bypass H_2NTP by producing H_2NMP directly, obviating FolQ.

To test if $TrpF_{CtL2}$ catalyzes the conversion of compound V to H_2NMP, we carried out coupled assays with purified recombinant *E. coli* RibA (22, 23) and recombinant $TrpF_{CtL2}$. In contrast to the assays containing only GTP, a distinct fluorescent signal was observed in the RibA-coupled assays that was both time and $TrpF_{CtL2}$ dependent (data not shown). To confirm that the signal was in fact due to the conversion of compound V to H_2NMP, compound V was produced and purified in large-scale RibA reactions and used

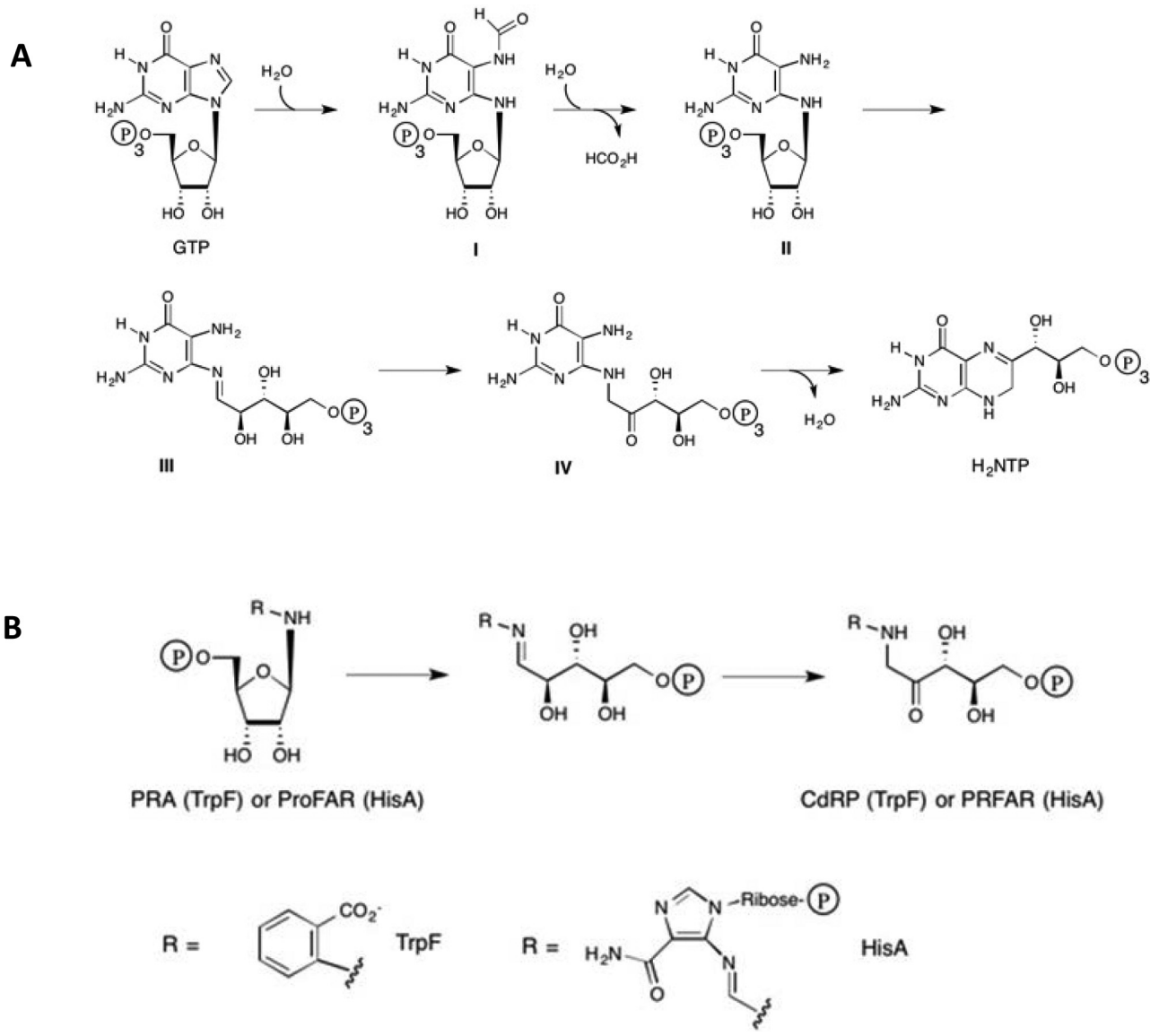


FIG 6 Biochemical reactions catalyzed by FolE and GTP cyclohydrolase I (A) and by TrpF and HisA (B).

in $TrpF_{CtL2}$ reactions as a putative substrate. Again, only the $TrpF_{CtL2}$ assays revealed a distinct fluorescent signal, whereas controls that contained no protein or BSA in place of $TrpF_{CtL2}$ did not (Fig. 7B).

Further analysis of the formation of H_2NMP from compound V by $TrpF_{CtL2}$ was investigated by liquid chromatography-mass spectrometry (LC-MS) (Fig. 7C). In the total ion chromatogram of the $TrpF_{CtL2}$ assay, a peak with the same retention time as authentic dihydroneopterin was observed, which was absent from the negative control (Fig. 7C), and both peaks possessed a molecular ion (*m/z* 254.08892 versus *m/z* 254.08903 for the authentic standard) consistent with dihydroneopterin. Furthermore, tandem MS (MS/MS) analysis of the *m/z* 254 ion produced an identical fragment ion at *m/z* 194 in both samples (Fig. 7D).

CT610 functionally replaces PabA, PabB, and PabC in *E. coli*. Because of the strict linkage of *ct610* and its orthologs with the folate biosynthesis genes in both *Chlamydia* and the phylogenetically distant *Wolbachia* (Fig. 2), it was highly probable that this enzyme family was involved in folate biosynthesis; however, its role was a mystery when we began this study.

We looked for a gene which encoded the missing PABA synthesis enzyme by screening a library of *C. trachomatis* L2 DNA in ATM825, an *E. coli* Δ*pabA*::*kan* mutant, which requires PABA supplementation for growth on minimal medium. Transformants were selected for growth on minimal medium without PABA supplementation. The complementing clone that was isolated contained nucleotides 28 to 696 of the *ctl0874* gene, which is a homolog of *ct610* in *C. trachomatis* serovar D. Here, we refer to *ctl0874* with the *C. trachomatis* D locus tag *ct610* for consistency with the literature. There is some discrepancy concerning the actual start site of *ct610*. Mass spectrometry (MS) analysis determined the N-terminal amino acid sequence to be MNFLDQLDLI, indicating a translation start 15 nucleotides downstream of the start site predicted by genomics data at STDgen (http://stdgen

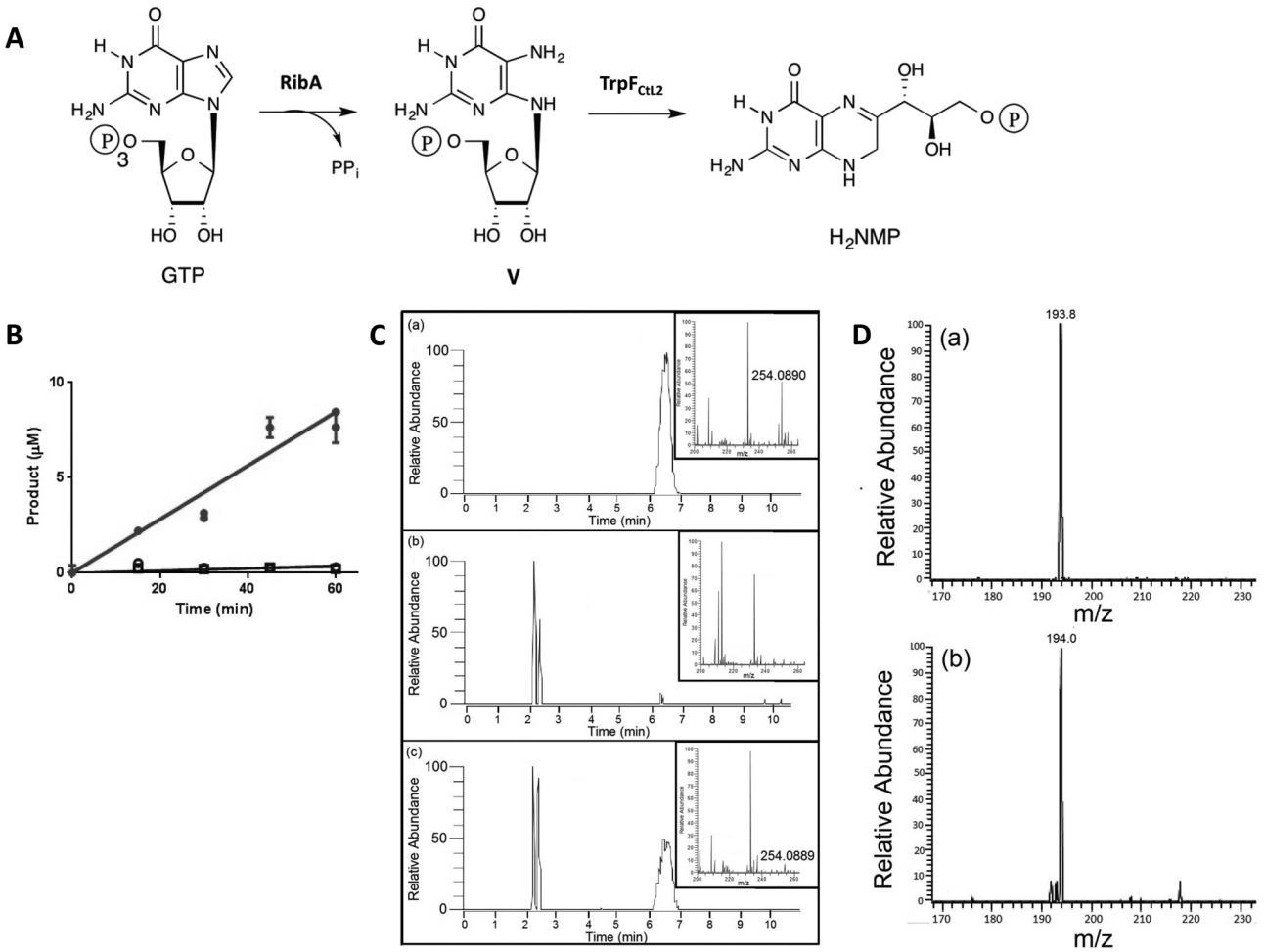


FIG 7 Formation of H_2NMP from GTP by RibA and $TrpF_{CtL2}$. (A) Reactions mediated by RibA and $TrpF_{CtL2}$. (B) Fluorescence assay for H_2NMP formation from compound V by $TrpF_{CtL2}$. Assays were carried out as described in Materials and Methods. ●, $TrpF_{CtL2}$; ○, BSA; □, no added protein. (C) LC-MS analysis of $TrpF_{CtL2}$ activity. Total ion chromatograms with mass spectra of peaks at 6.5 min (insets) of authentic 7,8-dihydroneopterin (a), a control assay with no $TrpF_{CtL2}$ (b), and an assay with $TrpF_{CtL2}$ (c) are shown. (D) MS/MS analysis of the *m/z* 254 ion produced in the LC-MS experiments whose results are shown in panel C. (a) Authentic 7,8-dihydroneopterin assay; (b) assay with $TrpF_{CtL2}$.

.northwestern.edu/) and NCBI (http://www.ncbi.nlm.nih.gov). We cloned the *ct610* gene containing the MS-predicted start site into pUC19 (yielding pAJM96) and with the genomics-predicted translation start site into pUC19 (yielding pAJM95). Plasmids containing either of the predicted starts for CT610 complemented the PABA auxotrophy of the *E. coli* Δ*pabA* mutant (VDC9500) (Fig. 8A). More importantly, CT610 was also able to complement the PABA auxotrophy of *E. coli* Δ*pabB* and Δ*pabC* mutants (VDC9502 and VDC9504) as well (Fig. 8B), indicating that CT610 is able to make PABA by an alternative route that does not involve the PabA, PabB, or PabC enzymes. Thus, chlamydiae possess a novel pathway for the synthesis of PABA.

Search for the potential CT610 substrate. Chorismate is the precursor for the PABA moiety of folate in the canonical pathway (Fig. 1B). To test if chorismate is also the precursor for PABA via the CT610-mediated reaction, an *E. coli* Δ*aroA* mutant was constructed (ATM816). AroA carries out the penultimate step of chorismate biosynthesis (24); thus, *aroA* mutants are unable to produce chorismate, and growth in minimal medium requires supplementation with the aromatic amino acids, 4-hydroxybenzoate, and PABA. CT610 was still able to complement an *E. coli* Δ*aroA* mutant with 72.6% efficiency, allowing growth on M9 medium supplemented with tryptophan, tyrosine, phenylalanine, and 4-hydroxybenzoate but lacking PABA (see Fig. S3 in the supplemental material).

Shikimate and 3-dehydroquinate were investigated next as possible substrates. Both are intermediates in the biosynthesis of chorismate and could serve as precursors for PABA in an alternative pathway that branches earlier than chorismate. In the canonical pathway, 3-dehydroquinate synthase (AroB) catalyzes the cyclization of 3-deoxy-D-arabino-heptulosonate-7-phosphate to 3-dehydroquinate, which is then converted to 3-dehydroshikimate by 3-dehydroquinate dehydratase (AroD). Reduction of 3-dehydroshikimate to shikimate is catalyzed by shikimate dehydrogenase (AroE). CT610 was able to complement both *E. coli* Δ*aroD* Δ*pabA* (VDC9510) and *E. coli* Δ*aroB* Δ*pabA* (VDC9598) mutants (see Fig. S3 in the supplemental material), allowing growth on M9 supplemented with either shikimic acid or just the aromatic amino acids but lacking PABA. CT610 therefore appears to utilize a molecule other than shikimate,

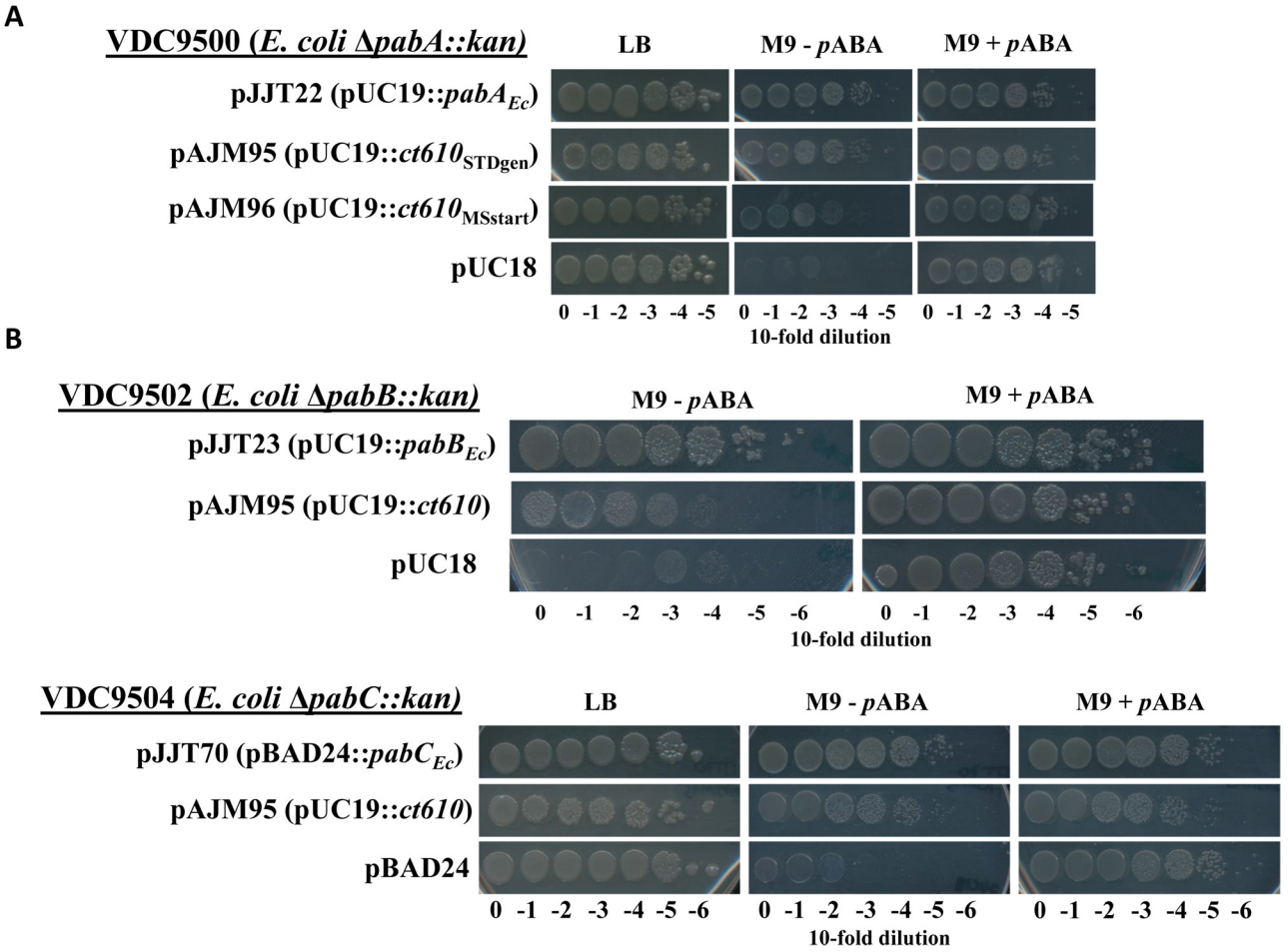


FIG 8 CT610 complements PABA auxotrophy of *E. coli* PABA mutants. (A) Complementation of VDC9500 (Δ*pabA*::*kan*) with either $pabA_{Ec}$ or *ct610*. (B) Complementation of VDC9502 (Δ*pabB*::*kan*) and VDC9504 (Δ*pabC*::*kan*) with CT610. All cultures were washed twice in M9 medium without PABA and normalized to an OD_{600} of 1 in M9 medium without PABA, and 10 μl of 10-fold serial dilutions was spotted onto LB agar, M9 glucose agar, and M9 glucose agar with PABA. Arabinose (0.1% [wt/vol]) was added to plates for complementation of VDC9504 for induction of pBAD24::$pabC_{Ec}$. All plates contained 100 μg/ml ampicillin.

3-dehydroquinate, chorismate, or any intermediate in this pathway as a precursor for PABA.

Since intermediates in the chorismate biosynthetic pathway are not the substrates for CT610, we tested the aromatic compound ubiquinone and its precursor, 4-hydroxybenzoate. Chorismate pyruvate lyase (UbiC) catalyzes the first step of ubiquinone biosynthesis, the conversion of chorismate to 4-hydroxybenzoate (25). An *E. coli* Δ*ubiC* mutant can grow on glucose as a sole carbon source without 4-hydroxybenzoate or ubiquinone supplementation. To test if CT610 can utilize ubiquinone or 4-hydroxybenzoate as a precursor for PABA, an *E. coli* Δ*ubiC* Δ*pabA* mutant was constructed which does not make ubiquinone or PABA (ATM851). Similar to the observations with the chorismate pathway mutants, expression of CT610 in *E. coli* Δ*ubiC* Δ*pabA* allowed the strain to grow on M9 with glucose in the absence of PABA, indicating that CT610 does not use ubiquinone or any of the intermediates from chorismate to ubiquinone as a precursor for PABA (see Fig. S3 in the supplemental material).

DISCUSSION

Folate synthesis has long been a target for antimicrobial drug development because the pathway is found in bacteria but not in mammalian cells. *C. trachomatis* and *C. psittaci* synthesize folates *de novo* and are sensitive to sulfonamides, which target enzymes in the folate synthesis pathway (8). However, metabolic reconstruction of the folate pathway in sequenced *Chlamydia* strains revealed many pathway holes (9). In this study, we performed a comprehensive examination of pathways for synthesis of pterin and PABA, two critical moieties that form folate, and solved the remaining mysteries in this area of metabolism in these pathogenic bacteria.

RT-PCR analysis of the *C. trachomatis* L2 folate gene cluster showed that the five contiguous genes are expressed as an operon (see Fig. S1 in the supplemental material). *folA* (8) and *folC2* (9) had previously been shown to encode functional enzymes in the THF pathway. Genetic complementation experiments with *E. coli* mutants allowed us to demonstrate functionality of the chlamydial *folB* gene, encoding dihydroneopterin aldolase, and the dihydropteroate synthase (FolP) activity of the *folKP* gene. Our inability to show FolK activity in the folate operon despite an active FolP suggests that the chlamydial enzyme cannot accept the HMH_2N precursor from *E. coli* FolB. More sophisticated biochemical analyses are required to formally validate the inferred chlamydial FolP

activity. Thus, four of the five genes in the folate operon have now been assigned a role in THF synthesis. The results obtained by Satoh et al. (15) and our own data demonstrate that the last gene in the folate operon, *ct610*, is involved not in the pterin biosynthesis pathway but in the synthesis of PABA, the other component of THF.

One outstanding question was how *Chlamydia* initiates the pathway for THF biosynthesis in the absence of any ortholog of GCYH-I (FolE), the first enzyme in the canonical pathway. We used a combined genetic and biochemical strategy to reveal that $TrpF_{CtL2}$ initiates a novel route to the formation of a precursor for THF synthesis. While $TrpF_{CtL2}$ complemented an *E. coli* Δ*folE* mutant, biochemical analysis revealed that purified recombinant $TrpF_{CtL2}$ catalyzed the conversion of compound V to H_2NMP. This observation, plus our inability to demonstrate conversion of GTP to H_2NTP, leads us to conclude that $TrpF_{CtL2}$ functions in *Chlamydia* not as a classic GCYH-I but instead as an isomerase that converts compound V, the product of GCYH-II, into H_2NMP (Fig. 7A). Thus, in *Chlamydia*, the GCYH-II enzyme catalyzes the first step in both the flavin and folate pathways.

Given the mechanistic complexity of the GCYH-I-catalyzed reaction and the similar chemistry of the latter portion of this reaction to that of the reaction catalyzed by TrpF (Fig. 6), the observation that $TrpF_{CtL2}$, catalyzes the latter half of the GCYH-I transformation is an elegant solution to the loss of a discrete GCYH-I activity in *Chlamydia*. Indeed, evolving the ability to convert available compound V to H_2NMP in the context of TrpF catalysis is a far more likely process than evolving this ability together with purine ring hydrolysis and deformylation.

PRA isomerase (TrpF) is well known as a promiscuous and evolvable enzyme (26) and is the paradigm of an adaptable enzyme (27). $(\beta\alpha)_8$-barrel fold enzymes like PRA isomerase are catalytically versatile and excellent candidates for evolutionary selection of diverse activities. For example, a HisA homolog in *Actinobacteria* possesses dual-substrate specificity of both HisA and TrpF (28, 29). This new isomerization reaction is another example of the plasticity of the TrpF fold that has already been recruited to perform different types of isomerization reactions (Fig. 6) (29). In the case of *Chlamydia* TrpF, PRA isomerase activity is retained, allowing *C. caviae*, *C. felis*, and *C. pecorum* to carry out the full tryptophan synthesis pathway starting with the anthranilate precursor. Thus, promiscuous enzymes with broad substrate specificity may play a significant role in reductive evolution with far-reaching metabolic implications.

The tryptophan repressor, TrpR, represses *trp* operon expression when tryptophan is plentiful. In *C. trachomatis* L2, *trpF* is not regulated by TrpR (30), but *trpF* in *C. caviae*, *C. pecorum*, and *C. felis* is in the *trp* operon and under TrpR control. It is possible that under tryptophan-replete conditions, *trpF* expression is repressed in these strains. However, it is likely that the level of *trpF* expression needed for folate synthesis is lower than what is needed for tryptophan synthesis and basal (uninduced) levels of *trpF* expression are probably sufficient for folate production.

An unresolved question is that of the mechanism of action of CT610. It has been implicated as a type III secreted factor capable of triggering apoptosis in host cells (31). In addition, CT610 is similar to PqqC (32), which catalyzes a ring cyclization and eight-electron oxidation in the final step of pyrroloquinoline quinone (PQQ) biosynthesis. However, the active site residues of PqqC are not conserved in CT610, and we demonstrate here that CT610 can functionally replace PabA, PabB, and PabC, indicating that it acts as a novel PABA synthase. Similarly, Satoh et al. recently reported that both CT610 and its homolog in *Nitrosomonas europaea* function as PABA synthesis enzymes capable of replacing PabA, PabB, and PabC in *E. coli* (15). Like the chlamydiae, *N. europaea* does not contain homologs of *pabA*, *pabB*, or *pabC*, yet it possesses the remainder of the tetrahydrofolate biosynthesis enzyme genes.

Based on the structure of CT610 (32), we predicted that its substrate was likely an aromatic or similar cyclic compound such as chorismate, an intermediate of PABA synthesis, or a downstream product derived from chorismate (i.e., ubiquinone, aromatic amino acids). We tested chorismate, shikimate, 3-dehydroquinate, and ubiquinone as potential precursors to PABA synthesis by genetic complementation of a series of mutations (Δ*aroA*, Δ*aroD*, Δ*aroB*, and Δ*ubiC*) in either wild-type *E. coli* or an *E. coli* Δ*pabA* mutant (a PABA auxotroph), and we found that, in each case, CT610 restored growth in the absence of PABA. Our results are similar to the results with the *N. europaea* CT610 homolog, confirming that CT610 and its homologs are indeed novel PABA synthases. However, the identity of the substrate used by CT610 to form PABA remains unknown.

CT610 is classified in the superfamily of heme oxygenases (32). Other enzymes in this superfamily catalyze a diverse array of reactions, and include those involved in thiamine salvage (TenA), thiazole biosynthesis (Thi4), and PQQ biosynthesis (PqqC). Interestingly, Thi4p of *Saccharomyces cerevisiae* has been shown to be a suicide thiamine thiazole synthase which, in contrast to the five enzymes required by bacteria to produce thiamine thiazole (33), forms the thiazole moiety in a single step. Thi4p acts as a cosubstrate for the formation of thiazole through a complex reaction in which a cysteine residue of Thi4p serves as the sulfur source (33). It is intriguing to speculate that CT610 may similarly be a single-turnover enzyme, serving as a cosubstrate to produce PABA. In such a scenario, the conserved lysine residue at position 152 within the predicted binding pocket of CT610 might serve as the amino source for the production of PABA. However, testing of this hypothesis must await the identification of the PABA precursor.

In summary, folate biosynthesis is fully functional in most *Chlamydia* species, but the pathway is a patchwork of recruited enzymes. FolC2, an enzyme of archaeal origin associated with glutamylation of the F_{420} cofactor, has been recruited to perform the folate glutamylation reactions (9). The biosynthesis of H_2NMP, an early intermediate in the pathway, is performed by the first enzyme of riboflavin biosynthesis (RibA) together with a PRA isomerase ($TrpF_{CtL2}$) showing broad substrate specificity. Finally, the PABA moiety appears to be synthesized via a unique route using CT610, an enzyme homologous to PqqC, an enzyme involved in the biosynthesis of pyrroloquinoline quinone (PQQ). However, CT610 has no validated role in PQQ synthesis in *Chlamydia*. Clearly, folate biosynthesis in *Chlamydia* provides another example of the remarkable metabolic versatility and ingenuity of the species. The unique nature of the *Chlamydia* folate synthesis enzymes makes them ideal targets for development of highly specific antibacterial agents.

MATERIALS AND METHODS

Comparative genomics. The BLAST tools (34) and resources at NCBI (http://www.ncbi.nlm.nih.gov/) were routinely used. Sequence alignments were built using ClustalW (35) or Multialin (36). Protein domain

TABLE 1 *Escherichia coli* K-12 strains used in this study

Strain	Genotype	Relevant auxotrophic requirements	Reference or source
MC4100	F$^-$ *araD139* Δ(*lacZYA-argF*)*U169 rpsL150 relA1 deoC1* pt *F25 rbsR flbB5301*		47
MG1655	F$^-$ *rph-1* λ$^-$ Su$^\circ$		48
BW25113	F$^-$ Δ(*araD-araB*)567 Δ*lacZ4787*(::*rrnB3*) *lacIp-4000*(*lacI*^{q}) λ$^-$ *rph-1* Δ(*rhaD-rhaB*)568 *rrnB3 hsdR514* pKD46: *araBp-gam-bet-exo oriR101 repA101*(Ts) *bla*		49
Rosetta BL21(DE3)	F$^-$ *ompT hsdS*$_B$ (r$_B^-$ m$_B^-$) *gal dcm* (DE3) pRARE (*cat*)		EMD Millipore
C600 Δ*folK*	C600 Δ*folK*::*tet*	Thymidine, pantothenic acid, adenine, glycine, methionine	50
P1-7B	MG1655 Δ*folE*::*kan*	Thymidine, pantothenic acid, adenine, glycine, methionine	51
ATM816	MG1655 Δ*aroA*::*kan*	Phenylalanine, tyrosine, tryptophan, 2,3-dihydroxybenzoate, PABA	This work
ATM825	MG1655 Δ*pabA*::*kan*	PABA	This work
ATM842	MG1655 Δ*ubiC*::*kan*	No auxotrophic requirements on minimal	This work
ATM843	MG1655 Δ*ubiC*	No auxotrophic requirements on minimal	This work
ATM851	MG1655 Δ*ubiC* Δ*pabA*::*kan*	PABA, ubiquinone	This work
ATM932	MG1655 Δ*trpCF*::*cat*	Tryptophan	This work
FBG-Wf	Δ*trpF*	Tryptophan	19
VDC2250	MG1655 Δ*folP*::*kan*	Thymidine, pantothenic acid, adenine, glycine, methionine	This work
VDC3276	MG1655 Δ*folB*::*kan*	Thymidine, pantothenic acid, adenine, glycine, methionine	52
VDC9500	BW25113 Δ*pabA*::*kan*	PABA	This work
VDC9502	BW25113 Δ*pabB*::*kan*	PABA	This work
VDC9504	BW25113 Δ*pabC*::*kan*	PABA	This work
VDC9508	BW25113 Δ*aroD*::*cat*	Phenylalanine, tyrosine, tryptophan, ubiquinone, PABA	This work
VDC9510	BW25113 Δ*aroD*::*cat* Δ*pabA*::*kan*	Phenylalanine, tyrosine, tryptophan, ubiquinone, PABA	This work
VDC9579	MG1655 Δ*aroB*	Phenylalanine, tyrosine, tryptophan, ubiquinone, PABA	This work
VDC9598	MG1655 Δ*aroB* Δ*pabA*::*kan*	Phenylalanine, tyrosine, tryptophan, ubiquinone, PABA	This work

analysis was performed using the Pfam database tools (http://pfam.xfam.org/) (37). Analysis of the phylogenetic distribution was performed on the public SEED server (http://pubseed.theseed.org/SubsysEditor.cgi) (38). Physical clustering was analyzed with the SEED subsystem coloring tool or the Seedviewer Compare region tool (38) as well as on the MicrobesOnline tree-based genome browser (http://www.microbesonline.org/) (39).

Phylogenetic reconstructions. Sequence alignments were done using MUSCLE from within the software SEAVIEW (40). ProtTest (41) was used to define the fittest model for the TrpF phylogenetic reconstruction, and reconstruction was done using a WAG model (42) followed by a Bayesian analysis using MrBayes 3.2.1 (43). Branch supports were estimated using 1,000,000 bootstrap replications.

Bacterial strains and growth conditions. Strains of *E. coli* K-12 used in this study are listed in Table 1, and plasmids are listed in Table 2. Construction of strains and plasmids is described in the supplemental material. Strains were grown in Luria-Bertani (LB) medium or M9 minimal medium with aeration or on agar. Medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), spectinomycin (100 μg/ml), thymidine (75 μg/ml), pantothenate (1 μg/ml), adenine (40 μg/ml), PABA (0.1 μg/ml), 4-hydroxybenzoate (2 μM), phenylalanine (10 or 40 μg/ml), tryptophan (40 μg/ml), tyrosine (40 μg/ml), or Bacto Casamino Acids (0.5%) as necessary, and cultures were incubated at 37°C. Chemicals were purchased from Sigma-Aldrich Company. Phosphate-buffered saline (PBS; Lonza) and buffered saline gelatin (BSG; 150 mM NaCl, 2 mM KH_2PO_4, 4 mM Na_2HPO_4, 0.01% gelatin) (44) were used as washes and diluents for bacterial cultures, respectively.

Library screen. Freshly prepared P1-7B (*E. coli* Δ*folE*::*kan*) and ATM825 (*E. coli* Δ*pabA*::*kan*) electrocompetent cells were transformed with ~300 ng of *C. trachomatis* L2 genomic library (45) and recovered in SOC supplemented with thymidine or PABA overnight at 25°C. After recovery, the culture was washed twice in PBS before plating on M9 medium containing ampicillin, pantothenic acid, adenine, and Casamino Acids for P1-7B and on M9 plates with ampicillin for ATM825. Transformation titers were determined by plating dilutions on M9 medium containing ampicillin, thymidine, pantothenic acid, adenine, and Casamino Acids for P1-7B and on M9 ampicillin PABA for ATM825. Transformants were single-colony purified twice before characterization of phenotype (efficiency of plating) and genotype (DNA sequence of library clone).

Efficiency of plating. Cultures of P1-7B carrying plasmids of interest were grown overnight in LB containing ampicillin, thymidine, panto-

TABLE 2 Plasmids used in this study[a]

Name	Genotype	Description	Source or reference
pGEM-T		Cloning vector for PCR products	Promega
pUC18		Cloning vector with P_{lac}, ColE1 *ori*, Ap^r	53
pUC19		Cloning vector with P_{lac}, ColE1 *ori*, Ap^r	53
pAM238		Cloning vector with P_{lac}, pSC101 *ori*, Spc^r	54
pMAL-c4x		N-terminal maltose binding protein tag fusion expression vector; P_{tac} Ap^r	New England Biolabs
pASK::IBA3plus		Expression vector for C-terminal strep tag-fusion; P_{tet} Ap^r	IBA GmbH
pAJM94	pUC18::$aroA_{Ec}$	$aroA_{Ec}$ from *E. coli* MG1655 with P_{lac}; Ap^r	This work
pAJM95	pUC19::*ct610*	*ct610* (*ctl0874*) from CtL2 with genomics-predicted start	This work
pAJM96	pUC19::*ct610*	*ct610* (*ctl0874*) from CtL2 with proteomics-predicted start	This work
pASK		NdeI site of pASK::IBA3plus deleted, His tag from pET15b inserted into the EcoRI and HindIII sites	This work
pASK::*ctl0581*	pASK::*ctl0581*	$trpF_{CtL2}$ (*ctl0581*) with P_{tet}, Ap^r	This work
pASK::*priA_Scoe*	pASK::*priA_Scoe*	*priA* from Scoe with P_{tet}, Ap^r	(28)
pJJT22	pUC19::$pabA_{Ec}$	*pabA* from *E. coli* MG1655 with P_{lac}; Ap^r	This work
pJJT23	pUC19::$pabB_{Ec}$	*pabB* from *E. coli* MG1655 with P_{lac}, Ap^r	This work
pJJT70	pBAD24::$pabC_{Ec}$	*pabC* from *E. coli* MG1655 with P_{BAD}, Ap^r	This work
pNEA50	pUC19::$folE_{Ec}$	$folE_{Ec}$ with native promoter, Ap^r	This work
pNEA57	pAM238::fol_{CtL2} genes	Folate gene cluster (*folX-folKP-folA-folC2-ct610*) from CtL2 with native promoter, Spc^r	This work
pNEA59	pUC18::$trpCF_{Ec}$	$trpCF_{Ec}$ with P_{lac}, Ap^r	This work
pNEA61	pUC18::$trpF_{Ec}$	$trpF_{Ec}$ domain with P_{lac}, Ap^r	This work
pNEA64	pAM238::$trpF_{Cc}$	$trpF_{Cc}$ with P_{lac}, Spc^r	This work
pNEA65	pUC18::$trpC_{Cc}$	$trpC_{Cc}$ with P_{lac}, Ap^r	This work
pNEA67	pAM238::$trpC_{Ec}$	$trpC_{Ec}$ domain with P_{lac}, Ap^r	This work
pNEA69	pUC18::$trpF_{Cm}$	$trpF_{Cm}$ with P_{lac}, Ap^r	This work
pNEA71	pUC18::$trpF_{CtL2}$	$trpF_{CtL2}$ (*ctl0581*) with P_{lac}, Ap^r	This work
pNEA72	pUC18::$trpF_{Cc}$	$trpF_{Cc}$ with P_{lac}, Ap^r	This work
pNEA79	pAM238::$trpC_{Ec}$	$trpF_{Ec}$ from MC4100 with P_{lac}, Spc^r	This work
pNEA83	pMAL-c4x::$trpF_{CtL2}$	N-terminal maltose binding protein-CTL0581 fusion with P_{tac}, Ap^r	This work
pNEA122	pUC18::$folB_{Ec}$	$folB_{Ec}$ from MC4100 with P_{lac}, Ap^r	This work
pNEA127	pUC18::$folX_{CtL2}$	*ct614* (*ctl0878*) from CtL2 with P_{lac}, Ap^r	This work

[a]Ap, ampicillin; *Cc*, *C. caviae* GPIC strain SP6; *Cm*, *C. muridarum* Nigg; *CtL2*, *C. trachomatis* L2; *Ec*, *E. coli* MC4100 or MG1655; *Scoe*, *Streptomyces coelicolor*; Spc, spectinomycin.

thenic acid, adenine, and Casamino Acids. All Δ*pab* strains were grown in M9 medium containing ampicillin and PABA. ATM932 (*E. coli* Δ*trpCF*::*kan*) was grown overnight in LB containing tryptophan, ampicillin, or spectinomycin as needed. All strains were diluted into fresh medium and grown to mid-log phase (optical density at 600 nm [OD_{600}] = 0.5 to 0.7), washed in PBS, serially diluted in BSG, and then plated at 37°C on appropriate growth media.

Complementation drops. Strains were grown overnight in LB containing ampicillin or spectinomycin, as needed, and supplemented with thymidine for the *E. coli* Δ*folB* mutants. Strains were diluted into fresh medium, grown to an OD_{600} of ~1.0, washed in PBS, normalized to an OD_{600} of 1.0, and serially diluted in PBS. A 10-μl portion of each serial 10-fold dilution was spotted onto appropriate growth media.

RT-PCR. L2 mouse fibroblasts were infected to a multiplicity of infection of 1 with *C. trachomatis* L2 434/Bu, and total RNA and DNA were harvested using TRIzol (Invitrogen) at 46 h postinfection. RNA was DNase I treated (Invitrogen) and reverse transcribed with random hexamers using the Thermoscript RT-PCR kit (Invitrogen). Total RNA, total DNA, and cDNA samples were analyzed with *Taq* polymerase (Fermentas).

Determination of phosphoribosylanthranilate (PRA) isomerase activity. PRA isomerase activity of $TrpF_{CtL2}$ was confirmed in an *E. coli* Δ*trpF* mutant, FBG-Wf, as previously described (19, 20) using cell-free extracts of *E. coli* FBG-Wf carrying pASK::$trpF_{CtL2}$, empty pASK as a negative control, and pASK::*priA_Scoe*, encoding a phosphoribosyl isomerase A from *Streptomyces coelicolor* which exhibits both TrpF and HisA activity, as a positive control (28). The cells were grown at 37°C for 72 h in 500 ml of M9 minimal medium without tryptophan, except for the negative control, where tryptophan was added, before induction of the P_{tet} promoter of pASK with anhydrotetracycline (20 ng/ml). For the PRA isomerase enzyme assay, 62.5 μM of anthranilic acid and 2.6 μg/ml of total protein were used as previously reported (20).

HPLC assay for type I GTP cyclohydrolase activity. Assays to identify GCYH-I activity of the $TrpF_{CtL2}$ fusion protein were carried out as described previously (46), except that the reaction volume was 200 μl and solutions were degassed with N_2 to minimize postreaction oxidation. Reaction mixtures containing *Neisseria gonorrhoeae* GCYH-1B (0.5 μM) as a positive control or $TrpF_{CtL2}$ fusion protein (~5.0 μM) were incubated in the dark at 37°C for 60 min and then for an additional 60 min after the addition of five units of alkaline phosphatase. Protein was removed from the mixture using an Amicon centrifugal device, and the samples were analyzed by reversed-phase HPLC on a Gemini C_{18} (Phenomenex) column equilibrated in 200 mM ammonium acetate (pH 6.0), at a flow rate of 0.7 ml/min. The following acetonitrile solvent gradient was used to develop the column: 0 min, 0%; 10 min to 30 min, 0 to 30% gradient; 30 min to 40 min, 30 to 0% gradient; and 50 min, 0%.

Fluorescence assay for 7,8-dihydroneopterin formation. Enzyme assays were run at 37°C in 100-μl reaction mixtures containing 1 mM GTP or 500 μM purified compound V (purification details of compound V are in the supplemental material), 100 mM HEPES (pH 8.0), 100 mM KCl, 1 mM dithiothreitol (DTT), 20 mM $MgCl_2$, and either $TrpF_{CtL2}$ fusion protein (400 μg/ml), BSA (400 μg/ml), or no protein. The reactions were run for 0, 15, 30, 45, or 60 min, before 12 μl of 1% I_2 (wt/vol) and 2% KI (wt/vol) in 1 M HCl were added and incubated at room temperature in the dark for 15 min. Excess iodine was reduced by the addition of 6 μl of 2% ascorbic acid (wt/vol), and the samples were analyzed by fluorescence spectroscopy with a Gemini XPS fluorimeter (excitation at 365 nm, emission at 446 nm). A standard curve using authentic neopterin was generated in tandem with the analysis.

LC-MS analysis of type I GTP cyclohydrolase activity of $TrpF_{CtL2}$. GCYH assays were carried out as described above for HPLC analysis of $TrpF_{CtL2}$ assays along with control assays containing BSA in place of the $TrpF_{CtL2}$ fusion protein. After protein removal by an Amicon centrifugal device, the filtrate was concentrated by evaporation in a speed-vac. The samples were analyzed by an LCQ Orbi-trap mass spectrometer (Thermo) interfaced with an Accela HPLC system. The interface was operated in the negative mode scanning the range *m*/*z* 0 to 800, with the following instrument conditions: capillary voltage, −16 V; capillary temperature, 299°C; tube lens, −90 V; spray voltage, 9 V. The separation was carried out using a reversed-phase column (Phenomenex Gemini [5 μm particle] C18; 250 by 2.00 mm column) using a mobile phase of 20 mM ammonium acetate (pH 6.0) with an acetonitrile gradient at a flow rate of 0.3 ml/min. The injection volume was 10 μl. Authentic dihydroneopterin was analyzed under the same conditions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01378-14/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.2 MB.
Figure S4, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.1 MB.
Text S1, PDF file, 0.1 MB.

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