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, TrpFCtL2, which has broad substrate specificity. TrpFCtL2, 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, TrpFCtL2 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 TrpFCtL2 and CT610 in the tetrahydrofolate pathway completes our understanding of folate biosynthesis in Chlamydia. Moreover, the novel roles for TrpFCtL2 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 TrpFCtL2, 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.
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: *folE*Q*B*K 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 *Chlamydia 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 Chlamydia 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 F420. The activity of this alternate folic glutamate synthase was demonstrated by complementation of an Escherichia coli ΔfolC 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 (GCH-I; EC 3.5.4.16), which catalyzes the conversion of GTP to 7,8-dihydroneopterin triphosphate (H2NTP). 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 was the first case of nonorthologous displacement of an nate folylglutamate synthase was demonstrated by complementation of a gene now called folC2 (ct611; see Table S1 in the supplemental material). Here, we refer to folC2—a homolog of 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).

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 Chlamydia 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 ct610) 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 multi-locus operon which includes ct610—see Fig. S1 in the supplemental material). Here, we refer to ct610 with the C. trachomatis D locus name, ct610, for consistency with the literature.
Experimental validation of activity of the *Chlamydia 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 triphosphatase epimerase involved in the biosynthesis of tetrahydromonapterin (18). Since *Chlamydia* species do not have a pathway for tetrahydromonapterin, CT614 is more likely FolB, the dihydronopterin 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* ΔfolB mutant, and demonstrated to complement the *E. coli* ΔfolB mutant on LB in the absence of added thymidine (Fig. 3A). These results are consistent with CT614 functioning as a dihydronopterin aldolase.

cT613, the next gene in the *C. trachomatis* folate gene cluster, is annotated as a folK-folP fusion. To verify that the *Chlamydia 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-folK-folA-folC2-ct610), was used to transform *E. coli* ΔfolK:kan and *E. coli* ΔfolP:kan, neither of which grows on LB without supplemental thymidine. Interestingly, only the *E. coli* ΔfolP mutant was complemented by the *C. trachomatis* folate operon, while *E. coli* ΔfolK was not (Fig. 3B). Based on these complementation experiments, the FolKP, which encodes FolKP, is driven by its native promoter, as shown by lack of growth on M9 minimal medium without thymidine (dT). Over 150 transformants that grew on minimal medium without thymidine were recovered in three independent transformations.

**FIG 3** Activity of the *C. trachomatis* FolB, FolK, and FolP orthologs. (A) FolB complementation by either folB<sub>Ec</sub> (pNEA122) or folX<sub>Ec</sub> (ct614; pNEA127). (B) Complementation of *E. coli* ΔfolK and ΔfolP. (Top) *E. coli* ΔfolK 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* ΔfolP is complemented by the folate cluster of *C. trachomatis*, presumably by ct613, which encodes FolKP.
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 ΔfolE 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 ΔfolE, 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 E. coli trpC domain of the E. coli trpCF gene (pAM238::trpCEc, 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 (trpFEc) domain and the trpF genes of Chlamydiaceae alone fail to complement an E. coli ΔtrpCF mutant, the trpF genes of Chlamydiaceae fully complement the ΔtrpCF 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 ΔtrpCF mutant of E. coli. As expected, the E. coli folE gene (pNEA50) failed to complement the ΔtrpCF 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 ΔtrpF mutant (strain FBG-Wf) which retains the TrpC do-

### Table: Gene (Plasmid) vs. Complementation Activity

| Gene (Plasmid) | Organism | Complementation in E. coli ΔfolE | Complementation in E. coli ΔtrpF |
|---------------|----------|-------------------------------|-------------------------------|
| folE (pNEA50) | E. coli  | 85.5%                         | ND                            |
| trpCF (pNEA59)| E. coli  | ND                            | 77.5%                         |
| trpC (pNEA67) | E. coli  | ND                            | ND                            |
| trpF (pNEA61) | C. trachomatis L2 | 33.8% | 61.8% |
| 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 < 0.0001%

**FIG 4** Phylogenomic context and analysis of trpF genes in Chlamydia. (A) Alignment of library inserts that complemented ΔfolE. 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 ΔfolE and ΔtrpF mutants with trpF genes from members of the Chlamydiaceae.
As expected, pASK::trpF::CtL2 complemented the \( \Delta \text{trp} \) 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-(\( \alpha \)-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 \( \Delta \text{folE} \) mutant, in vitro GCYH-I activity was assessed using a standard fluorescence assay in which the H2NPT 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)-pyrimidinimidine 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 H2NTP. Similar ribosyl ring opening and Amadori rearrangements are also catalyzed by PRA isomerase (TrpF), as well as by the N’-[\( 5’ \)-phosphoribosyl]formiminono]-5-aminoimidazole-4-carboxamid 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)-pyrimidinimidine 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 H2NMP. 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 H2NTP by producing H2NMP directly, obviating FolQ.

To test if TrpF::CtL2 catalyzes the conversion of compound V to H2NMP, 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 H2NMP, compound V was produced and purified in large-scale RibA reactions and used...
in TrpF<sub>Col2</sub> reactions as a putative substrate. Again, only the TrpF<sub>Col2</sub> assays revealed a distinct fluorescent signal, whereas controls that contained no protein or BSA in place of TrpF<sub>Col2</sub> did not (Fig. 7B).

Further analysis of the formation of H<sub>2</sub>NMP from compound V by TrpF<sub>Col2</sub> was investigated by liquid chromatography-mass spectrometry (LC-MS) (Fig. 7C). In the total ion chromatogram of the TrpF<sub>Col2</sub> 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 ΔpabA::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 c10874 gene, which is a homolog of ct610 in C. trachomatis serovar D. Here, we refer to c10874 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...
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 /H9004 pabA mutant (VDC9500) (Fig. 8A). More importantly, CT610 was also able to complement the PABA auxotrophy of E. coli /H9004 pabB and /H9004 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 ΔaroA 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 ΔaroA 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 ΔaroD ΔpabA (VDC9510) and E. coli ΔaroB ΔpabA (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,
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* *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* *ubiC*/*H9004 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* *ubiC*/*H9004 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 *folC* (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 chloramphenicol folB gene, encoding dihydroneopterin aldolase, and the dihydropterotate synthase (FolP) activity of the *folK* gene. Our inability to show FolK activity in the folate operon despite an active FolP suggests that the chloramphenicol enzyme cannot accept the HMH2N precursor from *E. coli* FolB. More sophisticated biochemical analyses are required to formally validate the inferred chloramphenicol 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, \textit{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 \textit{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 \textit{TrpFCtL2} initiates a novel route to the formation of a precursor for THF synthesis. While \textit{TrpFCtL2} catalyzed an \textit{E. coli} \textit{folE} mutant, biochemical analysis revealed that purified recombinant \textit{TrpFCtL2} catalyzed the conversion of compound V to \textit{H}$_2$NMP. This observation, plus our inability to demonstrate conversion of GTP to \textit{H}$_2$NTP, leads us to conclude that \textit{TrpFCtL2} functions in \textit{Chlamydia} not as a classic GCYH-I but instead as an isomerase that converts compound V, the product of GCYH-II, into \textit{H}$_2$NMP (Fig. 7A). Thus, in \textit{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 \textit{TrpFCtL2} catalyzes the latter half of the GCYH-I transformation is an elegant solution to the loss of a discrete observation that \textit{TrpFCtL2}, catalyzes the latter half of the GCYH-I reaction to that of the reaction catalyzed by TrpF (Fig. 6), the reaction and the similar chemistry of the latter portion of this (Fig. 7A). Thus, in \textit{Chlamydia}, the GCYH-II enzyme catalyzes the first step in both the flavin and folate pathways.

PRA isomerase (TrpF) is well known as a promiscuous and evolvable enzyme (26) and is the paradigm of an adaptable enzyme (27). (βα)$_5$-barrel fold enzymes like PRA isomerase are catalytically versatile and excellent candidates for evolutionary selection of diverse activities. For example, a HisA homolog in \textit{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 \textit{Chlamydia} TrpF, PRA isomerase activity is retained, allowing \textit{C. caviae}, \textit{C. felis}, and \textit{C. pecorum} to carry out the full tryptophan synthesis pathway starting with the anthranilic acid 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 \textit{trp} operon expression when tryptophan is plentiful. In \textit{C. trachomatis} L2, \textit{trpF} is not regulated by TrpR (30), but \textit{trpF} in \textit{C. caviae}, \textit{C. pecorum}, and \textit{C. felis} is in the \textit{trp} operon and under TrpR control. It is possible that under tryptophan-replete conditions, \textit{trpF} expression is repressed in these strains. However, it is likely that the level of \textit{trpF} expression needed for folate synthesis is lower than what is needed for tryptophan synthesis and basal (uninduced) levels of \textit{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 \textit{Nitrosomonas europaea} function as PABA synthesis enzymes capable of replacing PabA, PabB, and PabC in \textit{E. coli} (15). Like the chlamydial, \textit{N. europaea} does not contain homologs of \textit{pabA}, \textit{pabB}, or \textit{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 mutants (\textit{ΔaroA}, \textit{ΔaroD}, \textit{ΔaroB}, and \textit{ΔubiC}) in either wild-type \textit{E. coli} or an \textit{E. coli} \textit{ΔpabA} 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 \textit{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 \textit{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 \textit{Chlamydia} species, but the pathway is a patchwork of recruited enzymes. FolC2, an enzyme of archaeal origin associated with glutamylation of the \textit{F}$_{420}$ cofactor, has been recruited to perform the folate glutamylation reactions (9). The biosynthesis of \textit{H}$_2$NMP, an early intermediate in the pathway, is performed by the first enzyme of riboflavin biosynthesis (RibA) together with a PRA isomerase (\textit{TrpFCtL2}) 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 \textit{Chlamydia}. Clearly, folate biosynthesis in \textit{Chlamydia} provides another example of the remarkable metabolic versatility and ingenuity of the species. The unique nature of the \textit{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 Multalin (36). Protein domain
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.

**Library screen.** Freshly prepared P1-7B (E. coli ΔfolE::kan) and ATM825 (E. coli ΔpabA::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. Transformants were single-colony purified twice before characterization of phenotype.

**Efficiency of plating.** Cultures of P1-7B carrying plasmids of interest were single-colony purified twice before characterization of phenotype (efficiency of plating) and genotype (DNA sequence of library clone).
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of M9 minimal medium without tryptophan, except for the negative control (28). The cells were grown at 37°C for 72 h in 500 ml of Streptomyces coelicolor® strain SP6; Cm, C. muridarum Nigg; CtL2, C. trachomatis L2; Ec, E. coli MC4100 or MG1655; Scoe, Streptomyces coelicolor; Spc, spectinomycin.

TABLE 2 Plasmids used in this studya

| Name                      | Genotype               | Description                                                                 | Source or reference |
|---------------------------|------------------------|-----------------------------------------------------------------------------|---------------------|
| pGEM-T                    |                         | Cloning vector for PCR products                                             | Promega             |
| pUC18                     |                         | Cloning vector with P<sub>stu</sub>, ColE1 ori, Ap<sup>+</sup>               | 53                  |
| pUC19                     |                         | Cloning vector with P<sub>stu</sub>, ColE1 ori, Ap<sup>+</sup>               | 53                  |
| pAM238                    |                         | N-terminal maltose binding protein tag fusion expression vector; P<sub>lac</sub> Ap<sup>+</sup> | New England Biolabs |
| pMAL-c4x                  |                         | Expression vector for C-terminal strep tag fusion; P<sub>lac</sub>, Ap<sup>+</sup> | IBA GmbH            |
| pASK::IBA3plus            |                         |                               |                     |
| pAIM94                    | pUC18:araA<sub>Ec</sub> | arA<sub>Ec</sub> from E. coli MG1655 with P<sub>ara</sub>, Ap<sup>+</sup>     | This work           |
| pAIM95                    | pUC19::ct610           | ct610 (ct0874) from CtL2 with genomics-predicted start                     | This work           |
| pAIM96                    | pUC19::ct610           | ct610 (ct0874) from CtL2 with proteomics-predicted start                    | This work           |
| pASK                      |                         | Ndel site of pASK::IBA3plus deleted, His tag from pET15b inserted into the EcoRI and HindIII sites | This work           |
| pASK::ctl0581             | pASK::ctl0581           | trpF<sub>CtL2</sub> (ct0581) with P<sub>stu</sub>, Ap<sup>+</sup>            | This work           |
| pASK::priA_Scoe           | pASK::priA_Scoe         | priA from Scoe with P<sub>stu</sub> Ap<sup>+</sup>                         | (28)                |
| pJT722                    | pUC19::pab<sub>Ec</sub> | pab<sub>E</sub> from E. coli MG1655 with P<sub>pab</sub>, Ap<sup>+</sup>     | This work           |
| pJT723                    | pUC19::pab<sub>Bg</sub>| pab<sub>Bg</sub> from E. coli MG1655 with P<sub>pab</sub>, Ap<sup>+</sup>    | This work           |
| pJT770                    | pBAD24::pab<sub>CtL2</sub>| pab<sub>CtL2</sub> from E. coli MG1655 with P<sub>bad</sub>, Ap<sup>+</sup>  | This work           |
| pNEA50                    | pUC19::folE<sub>Ec</sub> | folE<sub>Ec</sub> with native promoter, Ap<sup>+</sup>                     | This work           |
| pNEA57                    | pAM238::folE<sub>CtL2</sub> | Folate gene cluster (folX-folKP-fola-folC2-ct610) from CtL2 with native promoter, Sp<sup>+</sup> | This work           |
| pNEA59                    | pUC18::trpCF<sub>Ec</sub> | trpCF<sub>Ec</sub> with P<sub>trp</sub>, Ap<sup>+</sup>                      | This work           |
| pNEA61                    | pUC18::trpCF<sub>Bg</sub> | trpCF<sub>Bg</sub> domain with P<sub>trp</sub>, Ap<sup>+</sup>               | This work           |
| pNEA64                    | pAM238::trpCF<sub>CtL2</sub> | trpCF<sub>CtL2</sub> with P<sub>trp</sub>, Ap<sup>+</sup>        | This work           |
| pNEA65                    | pUC18::trpCF<sub>CtL2</sub> | trpCF<sub>CtL2</sub> with P<sub>trp</sub>, Ap<sup>+</sup>       | This work           |
| pNEA67                    | pAM238::trpCF<sub>CtL2</sub> | trpCF<sub>CtL2</sub> domain with P<sub>trp</sub>, Ap<sup>+</sup> | This work           |
| pNEA69                    | pUC18::trpCF<sub>Bg</sub> | trpCF<sub>Bg</sub> with P<sub>trp</sub>, Ap<sup>+</sup>               | This work           |
| pNEA71                    | pUC18::trpCF<sub>CtL2</sub> | trpCF<sub>CtL2</sub> (ct0581) with P<sub>trp</sub>, Ap<sup>+</sup>    | This work           |
| pNEA72                    | pUC18::trpCF<sub>Bg</sub> | trpCF<sub>Bg</sub> with P<sub>trp</sub>, Ap<sup>+</sup>               | This work           |
| pNEA79                    | pAM238::trpCF<sub>Ec</sub> | trpCF<sub>Ec</sub> from MC4100 with P<sub>trp</sub>, Spc<sup>+</sup> | This work           |
| pNEA83                    | pMAL-c4x::trpCF<sub>CtL2</sub> | N-terminal maltose binding protein-CtL0581 fusion with P<sub>stu</sub>, Ap<sup>+</sup> | This work           |
| pNEA122                   | pUC18::folB<sub>Ec</sub>| folB<sub>Ec</sub> from MC4100 with P<sub>stu</sub>, Ap<sup>+</sup> | This work           |
| pNEA127                   | pUC18::folB<sub>CtL2</sub>| folB<sub>CtL2</sub> (ct0878) from CtL2 with P<sub>stu</sub>, Ap<sup>+</sup> | 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.
LC-MS analysis of type I GTP cyclohydrolase activity of TrpFCtL2: GCYH assays were carried out as described above for HPLC analysis of TrpFCtL2 assays along with control assays containing BSA in place of the TrpFCtL2 fusion protein. After protein removal by an Amicon centrifugal filter, the filtrate was concentrated by evaporation in a speed-vac. The samples were analyzed by an LCQ-Orbitrap 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 dihydropterin was analyzed under the same conditions.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.orglookup 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.

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