One-Carbon Metabolic Pathway Rewiring in *Escherichia coli* Reveals an Evolutionary Advantage of 10-Formyltetrahydrofolate Synthetase (Fhs) in Survival under Hypoxia

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In cells, N^{10} -formyltetrahydrofolate (N^{10} -fTHF) is required for formylation of eubacterial/organellar initiator tRNA and purine nucleotide biosynthesis. Biosynthesis of N^{10} -fTHF is catalyzed by 5,10-methylene-tetrahydrofolate dehydrogenase/cyclohydrolase (FolD) and/or 10-formyltetrahydrofolate synthetase (Fhs). All eubacteria possess FolD, but some possess both FolD and Fhs. However, the reasons for possessing Fhs in addition to FolD have remained unclear. We used *Escherichia coli*, which naturally lacks *fhs*, as our model. We show that in *E. coli*, the essential function of *folD* could be replaced by *Clostridium perfringens fhs* when it was provided on a medium-copy-number plasmid or integrated as a single-copy gene in the chromosome. The *fhs*-supported *folD* deletion ($\Delta folD$) strains grow well in a complex medium. However, these strains require purines and glycine as supplements for growth in M9 minimal medium. The *in vivo* levels of N^{10} -fTHF in the $\Delta folD$ strain (supported by plasmid-borne *fhs*) were limiting despite the high capacity of the available Fhs to synthesize N^{10} -fTHF *in vitro*. Auxotrophy for purines could be alleviated by supplementing formate to the medium, and that for glycine was alleviated by engineering THF import into the cells. The $\Delta folD$ strain (harboring *fhs* on the chromosome) showed a high NADP⁺-to-NADPH ratio and hypersensitivity to trimethoprim. The presence of *fhs* in *E. coli* was disadvantageous for its aerobic growth. However, under hypoxia, *E. coli* strains harboring *fhs* outcompeted those lacking it. The computational analysis revealed a predominant natural occurrence of *fhs* in anaerobic and facultative anaerobic bacteria.

The pathway of one-carbon metabolism is central to the synthesis of purine nucleotides, thymidylate, glycine, and methionine (Fig. 1). The enzymes that catalyze interconversions of the pathway intermediates are highly conserved across the three domains of life (1–6). Serine hydroxymethyltransferase (GlyA) catalyzes the reversible reaction of conversion of serine and tetrahydrofolate (THF) to glycine and 5,10-methylene-tetrahydrofolate (5,10-CH₂-THF) (7). FolD, a bifunctional enzyme, carries out sequential steps of reversible conversions of 5,10-CH₂-THF to 5,10-methenyltetrahydrofolate (5,10-CH⁺-THF), followed by the conversion of the latter to N^{10} -formyltetrahydrofolate (N^{10} -fTHF) by its dehydrogenase and cyclohydrolase activities, respectively (8). Availability of N^{10} -fTHF is crucial for the *de novo* pathway of purine nucleotide biosynthesis and formylation of the initiator tRNA (tRNA^{fMet}) to initiate protein synthesis in eubacteria and eukaryotic organelles (9).

N^{10} -fTHF can also be synthesized by formyltetrahydrofolate synthetase, Fhs (also known as formate-tetrahydrofolate ligase), by utilizing THF, formate, and ATP (Fig. 1). The dual scheme of N^{10} -fTHF synthesis is conserved in eukaryotes and some archaea (6). Many eukaryotic organisms possess FolD with trifunctional activities of dehydrogenase-cyclohydrolase-synthetase (10, 11). Among eubacteria, all organisms possess FolD, but some possess both FolD and Fhs (12). The advantages of possessing Fhs in addition to FolD are unclear. However, in the presence of formate (such as under the anaerobic conditions of growth), predominant synthesis of N^{10} -fTHF may occur via Fhs. N^{10} -fTHF may then be converted to the other one-carbon metabolism intermediates (13).

Furthermore, 5,10-CH⁺-THF is not known to be involved in any other essential processes in the cell. Both of its known functions, i.e., as a cofactor for photolyase (an enzyme responsible for

the direct repair of pyrimidine dimers in DNA) (14) and as an intermediate in the synthesis of N^5 -formyltetrahydrofolate (N^5 -fTHF), are not essential in *E. coli* (15, 16). This raises the question of whether Fhs could replace FolD for its essential function of N^{10} -fTHF production in the cell.

Earlier studies have shown that in *Leishmania*, a protozoan where the *de novo* pathway of purine biosynthesis is not present (*Leishmania* organisms obtain purines from the growth medium), FolD could be deleted when Fhs was provided as an additional copy (17). Thus, while the intracellular availability of N^{10} -fTHF is essential in *Leishmania*, this essentiality appears largely for the formylation of the initiator tRNA (tRNA^{fMet}) for organellar protein synthesis (17). However, at least in the case of *Saccharomyces cerevisiae*, the requirement of tRNA^{fMet} formylation appears non-essential (18). Thus, a long-standing question that remains unanswered is whether FolD function could be replaced with Fhs even

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FIG 1 Schematic of the one-carbon metabolic pathway. Reaction networks of the standard (A) and re-wired (B) one-carbon metabolic pathways are shown. The standard pathway includes dihydrofolate reductase (FoaA), serine hydroxymethyltransferase (GlyA), 5,10-methylenetetrahydrofolate dehydrogenase/cyclohydrolase (FoaD), 5,10-methylenetetrahydrofolate reductase (MetF), thymidylate synthase (ThyA), cobalamin-independent homocysteine transmethylease (MetE), cobalamin-dependent methionine synthase (Meth), and the glycine cleavage system (GCS). In the re-wired pathway, FoaD was replaced with formyl tetrahydrofolate synthetase (Fhs).

in the organisms where Fhs is not naturally present, which (unlike *Leishmania*) possess *de novo* pathways of purine biosynthesis and where formylation of tRNA^{Met} is known to be essential. In this context, we observed that the majority of eubacteria that possess Fhs are either anaerobes or facultative anaerobes. *Escherichia coli*, a facultative anaerobe, lacks Fhs. Thus, *E. coli* presented us with an excellent model to investigate the physiological and functional importance of FoaD and Fhs.

MATERIALS AND METHODS

Chemicals, plasmids, DNA oligomers, and *E. coli* strains and their growth. (6-*R,S*)-THF and trimethoprim (TMP) were from Sigma-Aldrich. (6-*R,S*)-5,10-CH₂-THF (calcium salt) and (6-*R,S*)-5,10-CH⁺-THF chloride were from Schircks Laboratories (Jona, Switzerland). Stock solutions were prepared in N₂-sparged buffer, with minimal light exposure. *E. coli* strains, plasmids, and DNA oligomers are listed in Tables 1 and 2. Bacteria were grown in Luria-Bertani broth (LB), LB-agar (1.8% agar; Difco), or M9 minimal medium (which includes 0.4% glucose as a carbon source) containing 1 μg ml⁻¹ thiamine (19) at 37°C with shaking at 200 rpm. Ampicillin (Amp; 100 μg ml⁻¹), kanamycin (Kan; 25 μg ml⁻¹), or tetracycline (Tet; 7.5 μg ml⁻¹) was used as needed.

Cloning of *fhs*. Standard recombinant DNA methods were employed. Cpe-fhs-FP and fhs-RP primers were used to amplify *fhs* from *Clostridium perfringens* genomic DNA. The amplicon (~1.9 kb) was cloned into pJET1.2 (ThermoFischer), followed by its subcloning into pTrc-NdeI at NdeI and EcoRI sites to generate pTrc-*fhs* (Amp^r). Subsequently, *fhs* was amplified from pTrc-*fhs* using fhs-pTrc-FP and fhs-RP and subcloned in pACDH at NcoI and EcoRI sites to generate p-*fhs* (Tet^r). In p-*fhs*, transcription of *fhs* was driven by the *lac* promoter.

Cloning of *folD*, *folA*, *purN*, and *purD* and purification of FoaD, PurN, and PurD. Standard genetic engineering techniques were used. Phosphoribosylglycinamide formyltransferase (*purN*), glycinamide ribonucleotide synthetase (*purD*), and dihydrofolate reductase (*dhfr* or *folA*) were PCR amplified using pBAD-pNTR-FP and pBAD-pNTR-RP primers from pNTR-SD-*purN*, -*purD*, and -*folA* plasmids (20), respectively, digested with SfiI and ligated to pBAD-SfiI (Amp^r) at the same sites. *E. coli folD* was amplified using FoaD-FP and FoaD-RP, digested with NcoI and

BglII, and cloned into the same sites of pQE60 to give rise to p-*folD* (Amp^r). PurN and PurD were expressed in *E. coli* BL21 (Rosetta) upon induction with arabinose (0.02%). FoaD expression in *E. coli* TG1/p-*folD* was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 0.5 mM). PurN, PurD, and FoaD were purified using a HisTrap HP column (GE Healthcare).

GlyA, Fhs, and FoaD assays. *E. coli* TG1 (also referred to as TG1, TG1 Δ*folD*::*kan/p-fhs* (Kan^r Tet^r; also referred to as the Δ*folD*/p-*fhs* strain), and TG1 Δ*folD*-*fhs*-*kan* (Kan^r; also referred to as the Δ*folD*-*fhs* strain) were grown in LB medium to an optical density at 600 nm (OD₆₀₀) of ~0.8 to 1.0. The cells were harvested, and the cell extracts were made in 50 mM Tris-HCl (pH 7.5) and 10 mM β-mercaptoethanol by ultrasonication, followed by centrifugation to remove cellular debris. GlyA, Fhs, and FoaD assays were performed as described previously (7, 8, 21). A typical reaction mixture (320 μl) for GlyA assay contained 0.5 mM THF, 20 mM β-mercaptoethanol, 10 mM serine, 1 mM NADP⁺, 50 mM Tris-HCl (pH 8.2), cell extract (40 μg total protein), and *E. coli* FoaD (2.5 μg). The reaction mixture for Fhs assay contained 50 mM sodium formate, 2.5 mM ATP, 0.5 mM THF, 20 mM β-mercaptoethanol, 50 mM KCl, 40 mM MgCl₂, 50 mM Tris-HCl (pH 8.2), and cell extract (10 μg total protein) in 320 μl. FoaD was assayed in a 320-μl reaction mixture containing 5,10-CH₂-THF (1 mM), 20 mM β-mercaptoethanol, 1 mM NADP⁺, 50 mM Tris-HCl (pH 8.2), and cell extract (40 μg total protein). Aliquots (64 μl) were withdrawn at different times and added to 400 μl of 0.5% perchloric acid to stop the reaction, destroy NADPH, and convert the N¹⁰-fTHF to 5,10-CH⁺-THF, which was measured using its extinction coefficient (ε_{350 nm} = 24,900 M⁻¹ cm⁻¹).

Generation of Δ*folD* strains supported by a medium-copy-number or a single-copy *fhs*. The *folD* gene was deleted using an established method (22). Briefly, a Kan^r cassette was amplified from pKD4 by *Pfu* DNA polymerase using *folD*-KO-FP and *folD*-KO-RP primers. The DNA of interest (~1.4 kb) was electroporated into *E. coli* DY330 harboring p-*fhs* (DY330/p-*fhs*). Colonies that appeared were screened for the *folD* locus using *folD*-C-FP and *folD*-C-RP. Later, the Δ*folD*::*kan* allele was moved into *E. coli* TG1 harboring p-*fhs* (TG1/p-*fhs*) by P1-mediated transduction to generate TG1 Δ*folD*::*kan/p-fhs* (also referred to as the Δ*folD*/p-*fhs* strain). Transformation of TG1 Δ*folD*::*kan/p-fhs* with pCP20

TABLE 1 Description of *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype, description, or purpose	Reference or source
Strains		
TG1	<i>E. coli</i> K-12 <i>supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5</i> ($r_K^- m_K^-$) (<i>traD36 proAB⁺ lacI^qΔM15</i>)	19
DY378	<i>E. coli</i> W3110 λ c1857 $\Delta(cro-bioA)$	24
KL16	<i>E. coli</i> K-12 <i>thi1 relA1 spoT1</i>	50
MG1655	<i>E. coli</i> K-12 F ⁻ λ mbda ⁻ <i>rph-1</i>	51
DY330	<i>E. coli</i> W3110 $\Delta lacU169 gal490$ [λ c1857 $\Delta(cro-bioA)$]	24
DY330/p- <i>fhs</i>	DY330 harboring pACDH- <i>fhs</i> (Tet ^r)	This study
TG1/p- <i>fhs</i>	TG1 harboring p- <i>fhs</i> (Tet ^r)	This study
TG1 $\Delta folD$ /p- <i>fhs</i>	TG1 with a deletion of <i>folD</i> harboring p- <i>fhs</i> (Kan ^r Tet ^r)	This study
TG1 $\Delta folD$ - <i>fhs</i>	TG1 with a deletion of <i>folD</i> harboring a single copy of <i>fhs</i> in the chromosome (Kan ^r)	This study
TG1 $\Delta folD$ /p- <i>fhs</i> /pQE60	TG1 $\Delta folD$ /p- <i>fhs</i> strain harboring pQE60 (Kan ^r Tet ^r Amp ^r)	This study
TG1 $\Delta folD$ - <i>fhs</i> /pQE60	TG1 $\Delta folD$ - <i>fhs</i> harboring pQE60 (Kan ^r Amp ^r)	This study
TG1 $\Delta folD$ - <i>fhs</i> /p- <i>folD</i>	TG1 $\Delta folD$ - <i>fhs</i> harboring p- <i>folD</i> (Kan ^r Amp ^r)	This study
TG1 $\Delta folD$ /p- <i>fhs</i> /p- <i>folD</i>	TG1 $\Delta folD$ /p- <i>fhs</i> harboring p- <i>folD</i> (Kan ^r Tet ^r Amp ^r)	This study
TG1 $\Delta folD$ /p- <i>fhs</i> /p- <i>folA</i>	TG1 $\Delta folD$ /p- <i>fhs</i> harboring p- <i>folA</i> (Kan ^r Tet ^r Amp ^r)	This study
TG1 $\Delta folD$ - <i>fhs</i> /p- <i>FBT</i>	TG1 $\Delta folD$ - <i>fhs</i> harboring p- <i>FBT</i> (Kan ^r Tet ^r)	This study
Plasmids		
pNTR-SD	Library genes cloned at SfiI sites (Amp ^r)	20
pBAD-SfiI	pBAD-SfiI is a derivative of pBAD-HisB with two SfiI restriction sites following the His ₆ tag (Amp ^r)	This study
p- <i>purN</i>	<i>purN</i> cloned at the SfiI site of pBAD-SfiI (Amp ^r)	This study
p- <i>purD</i>	<i>purD</i> cloned at the SfiI site of pBAD-SfiI (Amp ^r)	This study
p- <i>folA</i>	<i>folA</i> cloned at the SfiI site of pBAD-SfiI (Amp ^r)	This study
pCP20	FLP ⁺ λ c1857 ⁺ λ_{PR} Rep ^{ts} Ap ^r Cm ^r	23.
pACDH	Plasmid with ACYC <i>ori</i> of replication, which is compatible with ColE1 <i>ori</i> of replication plasmids (Tet ^r)	52
p- <i>fhs</i>	<i>Fhs</i> cloned between NcoI and EcoRI sites of pACDH (Tet ^r)	This study
p- <i>fhs-kan</i>	Derivative of p- <i>fhs</i> with both <i>kan</i> and <i>tet</i> markers	This study
pQE60	Expression vector harboring T5 promoter (Amp ^r)	Qiagen
p- <i>folD</i>	<i>folD</i> cloned at NcoI and BglII sites in pQE60 (Amp ^r)	This study
p- <i>FBT</i>	The pLOI707HE vector containing the <i>slr0642</i> cDNA of folate-biopterin transporter (FBT) family (Tet ^r)	30
pTrc99C	<i>E. coli</i> expression vector (Amp ^r)	Amersham
pTrc-NdeI	Derivative of pTrc99C	53

(23) resulted in excision of the Kan^r cassette and generation of TG1 $\Delta folD$ /p-*fhs* (Kan^r).

To generate a $\Delta folD$ strain supported by a single copy of *fhs*, the *kan* marker obtained from pUC4K by EcoRI treatment was ligated to the p-*fhs* plasmid at the EcoRI site to construct p-*fhs-kan*. The *fhs-kan* region from p-*fhs-kan* was then PCR amplified using primers pACDH-KI-Ch-FP and

pACDH-KI-Ch-RP. The amplicon was electroporated into *E. coli* DY378 (24). The transformants were selected on plates containing Kan. Colonies were screened for integration of *fhs* at the *chb* locus (chitobiose operon) by colony PCR using primers Ch-KI-C-FP and Ch-KI-C-RP. P1 phage raised on DY378 $\Delta chb::fhs-kan$ was used to transduce TG1 $\Delta folD$ /p-*fhs* (Kan^r Tet^r) to generate TG1 $\Delta folD \Delta chb::fhs-kan$ /p-*fhs* (Kan^r Tet^r), which was

TABLE 2 List of primers

Primer	Sequence (5'–3')
pACDH-KI-Ch-FP	GCCATGTTTGTATTAATTAACAACGTTTTTCTAAGCTTTGGGTCTCGGCTGGCAGCAGAC
pACDH-KI-Ch-RP	AATGCTTCGCGGCGATCCAGCGTTTTATAAAGCTTCATCAGACGGTATCGATAAGCTTGA
Ch-KI-C-FP	TGGTTTAGGCGTGCTTAAGG
Ch-KI-C-RP	AAAGCGTTTAAATCCGGTATGA
folD-KO-FP	ACGTGCGGATGTTTACCTAAAGAAAATGTCGCCATGGTGTAGGCTGGAGCTGCTTCG
folD-KO-RP	CCTTCGATCTACGTAACAGATGGAATCCTCTCTCTGCATATGAATATCCTCCTT
pBAD-pNTR-FP	CATCATCATCATCATGGGGCCATGAGGGCC
pBAD-pNTR-RP	CCAGGCAAATTCTGTTTTATGGCCTCATAGGCC
Fhs-pTrc-FP	CCATGGGACATATGAAAAATGATA
Cpe-fhs-FP	TGCGGACATATGAAAAATG
Cpe-fhs-RP	TATCATGAATTCCTAAAAAC
fhs-RP	TATCATGAATTCCTAAAAAC
folD-C-FP	ACGTAACCCATGGAATCCTCTC
folD-C-RP	AGCAAGCTTCACAGCTCAACGTG
FolD-FP	GTAACCATGGCAGCAAAGATTATTGACGGT
FolD-RP	TCGAAGATCTCTCATCTGTGGATCATGAT

then subjected to curing of *p-fhs*. During curing of *p-fhs*, the *F'* plasmid (*traD36 proAB⁺ lacI^qΔM15*) was also lost from this strain. This resulted in an additional requirement of proline in M9 minimal medium for this particular strain (referred to as TG1 Δ *folD*-*fhs* Kan^r).

Growth curves. *E. coli* (three to four biological replicates) were grown in 2 ml of LB medium at 37°C with or without the desired antibiotic(s) as overnight cultures. The cultures were diluted (10^{-3}) in M9 minimal medium, and 200 μ l of these diluted cultures was added to the wells of a honeycomb plate. The plate was placed in automated Bioscreen C growth reader (Oy Growth, Helsinki, Finland). The culture growth was measured at the OD₆₀₀ at 1-h intervals. Mean values with standard errors of the means (SEM) were plotted against time.

Estimation of N^{10} -fTHF in cell extracts. *E. coli* TG1 was grown in M9 minimal medium (300 ml) to an OD₆₀₀ of 0.5 ± 0.1 . The Δ *folD*/p-*fhs* strain was grown in M9 minimal medium supplemented with formate (10 mM)/adenine (0.1 mg ml⁻¹) and glycine (0.3 mg ml⁻¹). The cultures were harvested, and the pellets were resuspended in N₂-sparged buffer containing 50 mM Tris-HCl (pH 7.5) and 100 mM β -mercaptoethanol. The cells were lysed by sonication and centrifuged at $13,000 \times g$ at 4°C for 30 min. The amount of N^{10} -fTHF was quantified by an enzymatic method using PurN. In brief, the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM glycine, 12 mM MgCl₂, cell extract (5 mg total protein) or N^{10} -fTHF (100 μ M synthesized from 5,10-CH⁺-THF for the control reaction), 1 mM ATP, PurD (3 μ g), and PurN (6 μ g) in a volume of 500 μ l. The reaction was started using 900 μ M phosphoribosylamine (PRA) and monitored for 5 min at room temperature (25°C). PRA was synthesized and quantified as described before (25). The conversion of N^{10} -fTHF to THF was measured at 312 nm using the molar absorption coefficient 12,000 M⁻¹ cm⁻¹ (26).

Estimation of the NADP⁺-to-NADPH ratio. *E. coli* TG1 was grown to exponential phase in M9 minimal medium containing sodium formate (10 mM), whereas TG1 Δ *folD*-*fhs* was grown in M9 minimal medium containing both sodium formate (10 mM) and glycine (0.3 mg ml⁻¹). NADP⁺/NADPH quantification was done using a BioVision colorimetric kit (catalog number K347-100), according to the manufacturer's protocol. Each experiment was performed with at least two replicates.

Computational analysis of the occurrence of *fhs*. Genomes were analyzed using SEED (27) and UniProt databases. The genomes of 104 eubacterial strains positive for the presence of *fhs* were used for the analysis. The aerobic, anaerobic, and facultative anaerobic nature of the strains was gathered from published reports. Data were represented in a pie diagram.

Growth competition experiments. Exponential-phase cultures of *E. coli* TG1 and TG1/p-*fhs* (Tet^r) were grown in M9 minimal medium (2 ml) supplemented with sodium formate (10 mM), whereas cultures of TG1 Δ *folD*-*fhs* (Kan^r) were obtained in M9 minimal medium (2 ml) supplemented with both sodium formate (10 mM) and glycine (0.3 mg ml⁻¹). Growth competitions between the mixed cells of *E. coli* TG1 and TG1/p-*fhs* (Tet^r) were done in M9 minimal medium containing sodium formate (10 mM), whereas those between *E. coli* TG1 and TG1 Δ *folD*-*fhs* (Kan^r) were done in M9 minimal medium containing both sodium formate (10 mM) and glycine (0.3 mg ml⁻¹). Vitamin C (10 mM) was added to screw-cap flat-bottom tubes containing medium for creating a hypoxic/microaerophilic environment (28). Hypoxic cultures were grown with slow stirring in a multipoint magnetic board at 37°C, and methylene blue was used as an oxygen indicator. Aliquots were taken at various times and dilution plated on LB plates. The colonies were picked randomly and patched on LB plates lacking any antibiotic or containing Tet or Kan to distinguish between *E. coli* TG1 (Kan^s Tet^s), TG1/p-*fhs* (Kan^s Tet^r), and TG1 Δ *folD*-*fhs* (Kan^r Tet^s).

RESULTS

Generation of *E. coli* Δ *folD*/p-*fhs*. Both Fhs and FolD activities lead to the formation of N^{10} -fTHF (Fig. 1) used in purine biosynthesis as well as in formylation of tRNA^{fMet}. FolD is a highly conserved and an essential enzyme. To understand the physiological

importance of FolD, we carried out deletion of the *folD* gene (by replacing it with a *kan* marker) from an *E. coli* strain harboring the *Clostridium perfringens fhs* gene on a medium-copy-number plasmid (p-*fhs*). Figure 2A shows organization of the *folD* locus in the *E. coli* genome. Both the wild-type and the deletion (Δ *folD*::*kan*/p-*fhs*, also referred to as the Δ *folD*/p-*fhs* strain) strains showed the expected amplicons of 0.9 kb and 1.4 kb, respectively, using folD-C-FP and folD-C-RP primers (Fig. 2B). As expected for an essential gene, we were unable to obtain *folD* deletion in the strain harboring an empty vector (lacking *fhs*). The Δ *folD*/p-*fhs* strain grew well in LB medium (Fig. 2C). To further verify that the deletion strain lacked FolD, biochemical assays using cell extracts were performed. As shown in Fig. 2D, while the wild-type strain showed FolD dehydrogenase activity, the deletion strains supported by p-*fhs* did not.

Deletion of *folD* in *E. coli* results in auxotrophy for purines and glycine. The Δ *folD*/p-*fhs* strain did not grow in M9 minimal medium. However, it showed growth when the medium was supplemented with purines (adenine, guanine, or inosine) and glycine (Table 3). Introduction of p-*folD* (Amp^r) into the Δ *folD*/p-*fhs* strain allowed its growth in M9 minimal medium without any supplementation of purines/glycine (Table 3), suggesting that the observed auxotrophies in the strain were primarily a consequence of *folD* deletion and not due to any polar effects of *folD* deletion.

Generation of a Δ *folD* strain supported by single-copy *fhs* insertion. To rule out the possibility that excess expression of Fhs from a medium-copy-number vector was responsible for the phenotypes listed in Table 3, we carried out single-copy integration of *fhs* (linked to a *kan* resistance marker) at the *chb* locus (Δ *chbBCARF*, chitobiose operon) (see Fig. S1A in the supplemental material). The *chb* locus was chosen to target *fhs* because the whole locus, including the promoter and operator, could be deleted without a consequence for our investigation (29). The single-copy integration of *fhs* (linked to the *kan* marker) in the genome of *E. coli* TG1 Δ *folD*-*fhs*-*kan* (referred to as the Δ *folD*-*fhs* strain) was also confirmed by PCR, resulting in a 3.9-kb amplicon (as opposed to 3.3-kb amplicon for the wild-type locus) using Ch-KI-C-FP and Ch-KI-C-RP primers (see Fig. S1B). Like the Δ *folD*/p-*fhs* strain, this strain (Δ *folD*-*fhs*) also lacked FolD activity (see Fig. S1C) and was auxotrophic for purines and glycine (see Table S1 in the supplemental material). Furthermore, these deficiencies could be rescued by complementation of the strains with p-*folD* (see Table S1 in the supplemental material). Such a similarity of the phenotypes of the two strains allowed us to use either or both of the strains in the following studies to suit the experimental design.

Supplementation of growth medium with formate alleviates purine auxotrophy. To understand the mechanism of the purine auxotrophy of the *fhs*-supported Δ *folD* strains, we performed biochemical assays for Fhs in cell extracts to determine their capacities to synthesize N^{10} -fTHF, a metabolite required for purine biosynthesis. As shown in Fig. 3, the levels of Fhs produced in the Δ *folD*/p-*fhs* and the Δ *folD*-*fhs* strains, when supplied with sufficient substrate, were capable of synthesizing of at least four times higher levels of N^{10} -fTHF than the original strain (compare with Fig. 2D). This observation suggests that the activity of Fhs produced in the cell was not limiting. When M9 minimal medium was supplemented with formate and glycine, it alleviated auxotrophy for purines in both the Δ *folD*/p-*fhs* and Δ *folD*-*fhs* strains (Fig. 4A and B). However, as assayed in the extracts of the cells grown in

FIG 2 Confirmation of the *folD* deletion. (A) Organization of *folD* locus in *E. coli*. Lines with a single arrowhead indicate the location of the primers used for PCR, and those with double arrowheads represent the expected sizes of amplicons obtained with the primers (folD-C-FP and folD-C-RP) from the wild-type or $\Delta folD$ strain. (B) Agarose gel electrophoresis. Lane M, lambda DNA digested with HindII and HindIII; lane 1, amplicon of *folD* (~0.9 kb); and lane 2, amplicon of $\Delta folD::kan$ region (~1.4 kb). (C) Growth profile of TG1 $\Delta folD/p-fhs$ in Luria-Bertani (LB) medium. (D) FolD activity was measured from aliquots drawn at different time intervals (0 to 20 min) from a reaction setup with cell extracts of TG1 and TG1 $\Delta folD/p-fhs$. The product N^{10} -fTHF was measured as 5,10-CH⁺-THF (see Materials and Methods). Error bars represent SEM of at least two replicates. Data shown are representative of three such independent experiments performed at different times.

M9 medium containing formate/adenine and glycine, even in the strain harboring *fhs* on a plasmid ($\Delta folD/p-fhs$ strain), deletion of *folD* resulted in a significantly lower steady-state accumulation of N^{10} -fTHF (Fig. 5). Since glucose was provided as a carbon source, ATP was not limiting (see Fig. S2 in the supplemental material).

Engineering THF uptake alleviates glycine auxotrophy in a $\Delta folD$ strain. To understand the mechanism of glycine auxotrophy in the *folD* deletion strains, we assayed the cell extracts for GlyA activities and observed no significant differences between the *fhs*-supported $\Delta folD$ and the wild-type strains (Fig. 6A). The

$\Delta folD/p-fhs$ strain failed to grow in M9 minimal medium supplemented with serine alone or in combination with either adenine or formate (see Fig. S3 in the supplemental material). Thus, a more likely possibility for glycine auxotrophy of the *folD* deletion strains might be their deficiency for THF synthesis. To test for this, we decided to make use of a plasmid-borne gene of folate transporter (on p-FBT, Tet^r) to enable *E. coli* to utilize folates from the medium (30). As shown in Fig. 6B, upon introduction of p-FBT into the $\Delta folD-fhs$ strain, glycine auxotrophy of the strain could be

TABLE 3 Effect of various supplements on growth of *E. coli* TG1 $\Delta folD$ derivatives in M9 minimal medium^a

Strain and supplement(s)	Growth
$\Delta folD/p-fhs/pQE60$ strain	
None	—
Adenine	—
Guanine	—
Inosine	—
Glycine	—
Adenine + glycine	+++
Guanine + glycine	+
Inosine + glycine	++
$\Delta folD/p-fhs/p-folD$ strain with no supplements	++++

^a *E. coli* TG1 $\Delta folD/p-fhs$ was transformed with pQE60 (empty vector) or p-*folD*. Growth of the transformants was monitored in M9 minimal medium supplemented with various metabolites (see Materials and Methods).

FIG 3 *In vitro* Fhs activities of cell extracts to support formation of N^{10} -fTHF. Fhs activity was measured at different time intervals (0 to 20 min) by withdrawing aliquots from the reaction setup with the cell extracts of TG1 and the $\Delta folD$ strain harboring multiple copies of *fhs* (TG1 $\Delta folD/p-fhs$) and a single copy of *fhs* (TG1 $\Delta folD-fhs$). The product N^{10} -fTHF was measured as 5,10-CH⁺-THF (Materials and Methods). Error bars represent SEM of at least two replicates. Data shown are representative of three such independent experiments performed at different times.

FIG 4 Alleviation of purine auxotrophy in ΔfolD strains (supported by Fhs) by formate supplementation. TG1 and ΔfolD strains harboring multiple copies of *fhs* ($\Delta\text{folD}/\text{p-fhs}$) (A) or a single copy of *fhs* ($\Delta\text{folD-fhs}$) (B) were inoculated in M9 minimal medium supplemented with formate (10 mM) and/or glycine (0.3 mg ml⁻¹) and monitored for their growth. wt, wild type.

alleviated by supplying THF in the growth medium. This observation indicated a THF deficiency in the *fhs*-supported ΔfolD strains, and it provided the basis for glycine auxotrophy.

$\Delta\text{folD-fhs}$ strain reveals an elevated NADP⁺-to-NADPH ratio. A major role of FolD is to synthesize N¹⁰-fTHF, needed by PurN and PurH for purine synthesis and by formyl-Met-tRNA^{fMet} transferase (Fmt) to formylate aminoacylated tRNA^{fMet}. However, the FolD-mediated forward reaction also leads to production of NADPH. Other steps, for example, MetF-mediated conversion of 5,10-CH₂-THF to 5-methyltetrahydrofolate (5-CH₃-THF) and Fola-mediated conversion of dihydrofolate (DHF) to THF, consume NADH and NADPH, respectively. Hence, to understand the basis of THF deficiency, we estimated the NADP⁺-to-NADPH ratios in cell extracts of the $\Delta\text{folD-fhs}$ strain. As shown in Fig. 7, the strains revealed an elevated ratio of NADP⁺ to NADPH, suggestive of a deficiency of NADH and/or NADPH. Importantly, such a deficiency of NADH and/or NADPH provided a rationale for THF deficiency in the ΔfolD strains (see below).

Hypersensitivity of the ΔfolD strains to trimethoprim. A major source of THF synthesis in cells is via Fola (dihydrofolate reductase [DHFR])-mediated reduction of DHF in the presence of NADPH (Fig. 1). Decrease in cellular levels of NADPH upon deletion of *folD* (Fig. 7) provided a mechanism of THF deficiency. To further test the impact of THF deficiency on the $\Delta\text{folD}/\text{p-fhs}$ strain, we made use of trimethoprim. Trimethoprim (TMP) is a well-known inhibitor of Fola, which leads to inhibition of THF

synthesis. Thus, a cell already deficient in THF would be expected to show hypersensitivity to TMP. As shown in Fig. 8A and B, the $\Delta\text{folD}/\text{p-fhs}$ strain was found to be hypersensitive to TMP. Consistent with this observation, overexpression of Fola from a plasmid-borne copy of *folA* resulted in a better growth yield of the $\Delta\text{folD}/\text{p-fhs}$ strain (Fig. 8C).

Fhs provides a fitness advantage to *E. coli* under hypoxic conditions. While the presence of *folD* is absolutely conserved in all organisms, *fhs* is not. It is intriguing, therefore, why some organisms continue to retain *fhs*. To understand this phenomenon better, we carried out bioinformatics analysis for the presence of *fhs* in aerobic, anaerobic, and facultative anaerobic organisms (see Table S2 in the supplemental material). Our analysis indicates that *fhs* is more predominantly present in the strict and facultative anaerobes, accounting for about 76% of the organisms analyzed (Fig. 9A). To analyze if the organisms gain any fitness advantage by the presence of *fhs* during hypoxia, we carried out growth competition experiments between the strains expressing *fhs* and those not expressing *fhs* under hypoxic and aerobic conditions. As shown in Fig. 9B (panel i), while under aerobic conditions, *E. coli* TG1 harboring p-*fhs* was outcompeted by the strain not expressing *fhs*, under hypoxia (Fig. 9B, panel ii) the strain harboring p-*fhs* gained the upper hand. The advantage of p-*fhs* harboring *E. coli* TG1 was retained even when the starting inoculum was biased in favor of the wild-type strain (Fig. 9B, panel iii). A similar observation was made when *fhs* was present within the ΔfolD background in single copy (Fig. 9C, panels i and ii). The advantage of *fhs* under hypoxia, particularly in the presence of formate in the medium, was also observed in *E. coli* KL16 (see Fig. S4A in the supplemental material). However, in the case of *E. coli* MG1655, the hypoxia growth advantage (in the presence of *fhs*) was observed only when formate was also present in the medium (see Fig. S4B).

FIG 5 Steady-state levels of N¹⁰-fTHF in *E. coli* TG1 and ΔfolD strains. N¹⁰-fTHF was determined in cell extracts by a coupled enzyme assay (see Materials and Methods). *E. coli* TG1 was grown in M9 minimal medium, and TG1 $\Delta\text{folD}/\text{p-fhs}$ was grown in M9 minimal medium supplemented with formate (F; 10 mM) plus glycine (G; 0.3 mg ml⁻¹) or with adenine (A; 0.1 mg ml⁻¹) plus glycine. Error bars represent SEM of two independent experiments. Each experiment was performed with three replicates. ***, $P < 0.001$.

DISCUSSION

The one-carbon metabolic pathway has been extensively explored in both the prokaryotes and eukaryotes (31) and is a well-known target for both antimicrobial and anticancer drugs (32–36). In this study, we have shown that in *E. coli*, which naturally lacks *fhs* and possesses a *de novo* pathway of purine biosynthesis, the essential function of FolD could be compensated for by expression of a heterologous Fhs. While the strain with such a rewiring of one-carbon metabolism grows well in complex medium (Fig. 2C and 8A), in the minimal medium, it triggered requirements of formate (or purines) and glycine. Both of these auxotrophies allowed us to

FIG 6 Mechanism of glycine auxotrophy in the $\Delta folD$ strains. (A) Assay of GlyA activity. GlyA activity was measured at different time intervals (0 to 30 min) from aliquots drawn from a reaction setup with the cell extracts of *E. coli* TG1, TG1 $\Delta folD$ /p-fhs, and TG1 $\Delta folD$ -fhs. The product 5,10-CH₂-THF was measured as 5,10-CH⁺-THF (see Materials and Methods). (B) Effect of engineering THF import on glycine auxotrophy. TG1 $\Delta folD$ -fhs harboring p-FBT (TG1 $\Delta folD$ -fhs/p-FBT) was grown in M9 minimal medium supplemented with formate (10 mM) and glycine (0.3 mg ml⁻¹) or formate (10 mM) and THF (15 μ M).

investigate the physiological importance of Fhs and FolD. Of the intracellular formate-generating reactions in *E. coli*, hydrolysis of N¹⁰-fTHF by PurU offers an unlikely means for any net generation of formate in the $\Delta folD$ strain as the production of N¹⁰-fTHF by Fhs itself requires formate (37). Another pathway for generation of formate from pyruvate (catalyzed by pyruvate formate lyase [Pfl]) operates primarily under anaerobic conditions (38). Nonetheless, the observation that the $\Delta folD$ /p-fhs strain sustains itself in minimal medium supplemented with glycine and purines suggests that, even under aerobic conditions, this pathway might be able to provide sufficient formate to support N¹⁰-fTHF synthesis (via Fhs) to at least meet the requirement of the formylation of tRNA^{fMet} for initiation of protein synthesis. However, it may also be that low levels of formate arise from guanine nucleotides (39).

Glycine auxotrophy of the fhs-supported $\Delta folD$ strains was primarily a consequence of THF deficiency, which most likely results from deficient production of NADPH in the absence of FolD (Fig. 7). However, it may be argued that the glycine cleavage system (GCS) (Fig. 1) also contributes to THF and glycine deficiencies to meet the cellular requirement of 5,10-CH₂-THF. While it may seem surprising that FolD served as a major source of NADPH under the conditions used here, a more recent study using a hu-

FIG 7 Determination of NADP⁺-to-NADPH ratios. NADP⁺-to-NADPH ratios in cell extracts of the strains were estimated using a BioVision colorimetric kit. Error bars represent SEM of two independent experiments. Each experiment was performed with at least two replicates (**, $P < 0.0001$). WT, wild type.

man cell line has also shown that oxidation of 5,10-CH₂-THF to N¹⁰-fTHF makes a major contribution to NADPH production (40). A consequence of the THF deficiency of the $\Delta folD$ strain was its hypersensitivity to trimethoprim (TMP), a known inhibitor of dihydrofolate reductase (41). TMP binds with high affinity to bacterial DHFRs compared to vertebrate DHFRs (41). Importantly, the hypersensitivity of the $\Delta folD$ strain suggests that the absence of FolD potentiates the role of TMP and may even overcome the resistance to TMP that results from *dhfr* gene amplification (e.g., in the experiment shown in Fig. 8C, overexpression of FolA from a multicopy-number plasmid led to only partial growth rescue). Therefore, we propose that inclusion of inhibitors that target FolD (42, 43) along with TMP treatment could make for effective antibacterials.

Bacteria living in anaerobic environments produce mixed acids through fermentation, and one-third of sugar carbon is converted to formate (44). Also, production of formate is pronounced during the shift from aerobic to anaerobic/microaerophilic growth conditions. Pyruvate formate lyase (Pfl) is activated under the latter growth conditions and converts pyruvate to acetyl coenzyme A (acetyl-CoA) and formate (45). These observations raise the question of whether Fhs could be offering a fitness advantage under anaerobic/microaerophilic growth conditions. As shown in Fig. 9, Fhs indeed provides a fitness advantage to *E. coli* TG1 under anaerobic conditions. The growth advantage is so striking that the Fhs-containing strain outcompetes the wild-type strain even when the strain is in the minority (Fig. 9B). Such a growth advantage of fhs under hypoxia, particularly in the presence of formate in the medium, was also observed in *E. coli* KL16. However, in the case of *E. coli* MG1655, the hypoxia growth advantage due to the presence of fhs was observed only when formate was also present in the medium (see Fig. S4 in the supplemental material). While the mechanism of the strain-dependent variations in the phenotypes is unclear, the two strains (TG1 and KL16) showing stronger phenotypes of growth advantages are *relA* mutants. Nevertheless, the experiments reveal that under hypoxic conditions, the presence of Fhs function offers a growth advantage. Fhs is known to be upregulated under anaerobic conditions in *Staphylococcus aureus* (46). Thus, the role of Fhs extends beyond providing N¹⁰-fTHF. It appears important to also avoid cellular toxicity caused by excess formate (47). However, in *E. coli*, formate could also be utilized by PurT as a substrate for purine biosynthesis (48). Other enzymes such as formate dehydrogenases

FIG 8 Hypersensitivity of the $\Delta folD/p-fhs$ strain to trimethoprim (TMP). TG1/p-*fhs* and TG1 $\Delta folD/p-fhs$ strains were inoculated in LB medium (A) and in LB medium containing $1.8 \mu\text{g ml}^{-1}$ TMP (B) and monitored for their growth. (C) Impact of p-*folA* on the growth of TG1 $\Delta folD/p-fhs$ in M9 minimal medium containing adenine (0.1 mg ml^{-1}) and glycine (0.3 mg ml^{-1}).

(e.g., FDH-O and FDH-N) also metabolize formate to CO_2 . FDH-O and FDH-N have their catalytic domains pointed toward periplasmic space and thus require a formate transporter to oxidize formate. FocA in *E. coli* transports formate in and out of the cell to facilitate formate oxidation by FDH-O and FDH-N. Interestingly, *focA* and *pflB* are cotranscribed, thus facilitating delivery of formate to FDH-O and FDH-N (49). Under anaerobic conditions, FocA is constitutively active to export formate out of the

cell. Notwithstanding these mechanisms of formate detoxification, formate utilization by Fhs might also contribute to the removal/detoxification of excess formate. Interestingly, it was also reported that Fhs constitutes up to 3 to 4% of dry weight of clostridial species (7). Furthermore, it may be speculated that under anaerobic conditions, N^{10} -fTHF/purine production may be favored via Fhs, which may also explain why anaerobic organisms retain Fhs.

FIG 9 Growth advantage by Fhs under hypoxia. (A) Distribution of *fhs* in different bacteria. Genomes were analyzed using SEED and UniProt databases. Genomes positive for the presence of *fhs* were used to prepare the pie diagram. (B) Growth competition between *E. coli* TG1 and TG1/p-*fhs*. (C) Growth competition between *E. coli* TG1 and TG1 $\Delta folD-fhs$. Cultures were mixed in different proportions as shown and grown under aerobic or hypoxic conditions. Total viable counts were determined at the different time points, and the percent abundance of each strain was plotted.

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