Moonlighting Glutamate Formiminotransferases Can Functionally Replace 5-Formyltetrahydrofolate Cycloligase*[s]

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5-Formyltetrahydrofolate (5-CHO-THF) is formed by a side reaction of serine hydroxymethyltransferase. Unlike other folates, it is not a one-carbon donor but a potent inhibitor of folate enzymes and must therefore be metabolized. Only 5-CHO-THF cycloligase (5-FCL) is generally considered to do this. However, comparative genomic analysis indicated (i) that certain prokaryotes lack 5-FCL, implying that they have an alternative 5-CHO-THF-metabolizing enzyme, and (ii) that the histidine breakdown enzyme glutamate formiminotransferase (FT) might moonlight in this role. A functional complementation assay for 5-CHO-THF metabolism was developed in *Escherichia coli*, based on deleting the gene encoding 5-FCL (*ygfA*). The deletion mutant accumulated 5-CHO-THF and, with glycine as sole nitrogen source, showed a growth defect; both phenotypes were complemented by bacterial or archaeal genes encoding FT. Furthermore, utilization of supplied 5-CHO-THF by *Streptococcus pyogenes* was shown to require expression of the native FT. Recombinant bacterial and archaeal FTs catalyzed formyl transfer from 5-CHO-THF to glutamate, with k_{cat} values of 0.1–1.2 min^{-1} and K_m values for 5-CHO-THF and glutamate of 0.4–5 μM and 0.03–1 mM, respectively. Although the formyltransferase activities of these proteins were far lower than their formiminotransferase activities, the K_m values for both substrates relative to their intracellular levels in prokaryotes are consistent with significant *in vivo* flux through the formyltransferase reaction. Collectively, these data indicate that FTs functionally replace 5-FCL in certain prokaryotes.

5-Formyltetrahydrofolate (5-CHO-THF)[3] is exceptional among folates in two respects. First, it is not made by a dedi- cated enzyme. Rather, it is formed almost exclusively by a side reaction of serine hydroxymethyltransferase (SHMT) in which 5,10-methenyltetrahydrofolate (5,10-CH=THF) is hydrolyzed to 5-CHO-THF in the presence of bound glycine (Fig. 1A) (1–3). Other possible enzymatic or chemical sources (4, 5) appear to be minor. Second, 5-CHO-THF is not an active donor of one carbon (C_1) units but a potent inhibitor of various folate-dependent enzymes in the C_1 metabolism network (6). 5-CHO-THF must consequently be removed. The only known removal mechanism for 5-CHO-THF is a salvage reaction in which it is reconverted to 5,10-CH=THF by the enzyme 5-CHO-THF cycloligase (5-FCL, EC 6.3.3.2) (6–9). This reaction requires MgATP and is essentially irreversible (Fig. 1B) (10). The 5-FCL re- action is medically significant because, by recycling 5-CHO-THF back to the active C_1-folate pool, it allows use of 5-CHO-THF as an antifolate rescue agent in cancer chemotherapy (6).

The main reaction mediated by SHMT, the reversible conversion of serine to glycine and 5,10-methylene-THF, is a key source of C_1 units and SHMT is an almost ubiquitous enzyme (11–13). The capacity to form 5-CHO-THF is thus also almost universal, and consequently so is the need for 5-CHO-THF removal. It might therefore be expected that all organisms would have 5-FCL, except those few that lack SHMT (14) or folates (15). However, in the course of a comparative genomic analysis of folate synthesis and metabolism involving hundreds of genomes (16) we were surprised to note that diverse prokaryotes had genes encoding SHMT (*glyA*) and other folate-dependent enzymes but no gene for 5-FCL (*ygfA*).

This lack of the *ygfA* gene appeared to signal a case of a missing enzyme, or pathway hole, *i.e.* where there is either a completely different enzyme (nonorthologous displacement) or an alternative pathway (16, 17). Here, we report an investi- gation of these possibilities using comparative genomics anal- ysis. This analysis, together with literature mining, predicted that the histidine degradation enzyme glutamate formimino- transferase (FT, EC 2.1.2.5) could functionally replace 5-FCL in organisms that lack this enzyme. This prediction was ex- perimentally verified by functional complementation of an *Escherichia coli ygfA* deletant, by antifolate rescue experi-

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[3] The abbreviations used are: 5-CHO-THF, 5-formyltetrahydrofolate; 5,10-CH=THF, 5,10-methenyltetrahydrofolate; 5-NH=CH-THF, 5-formim- inotetrahydrofolate; 5-FCL, 5-formyltetrahydrofolate cycloligase; FIGLU,

N-formimino-L-glutamate; FT, glutamate formiminotransferase; FT-CD, glutamate formiminotransferase-cyclodeaminase; IPTG, isopropyl-β-D- thiogalactopyranoside; SHMT, serine hydroxymethyltransferase.
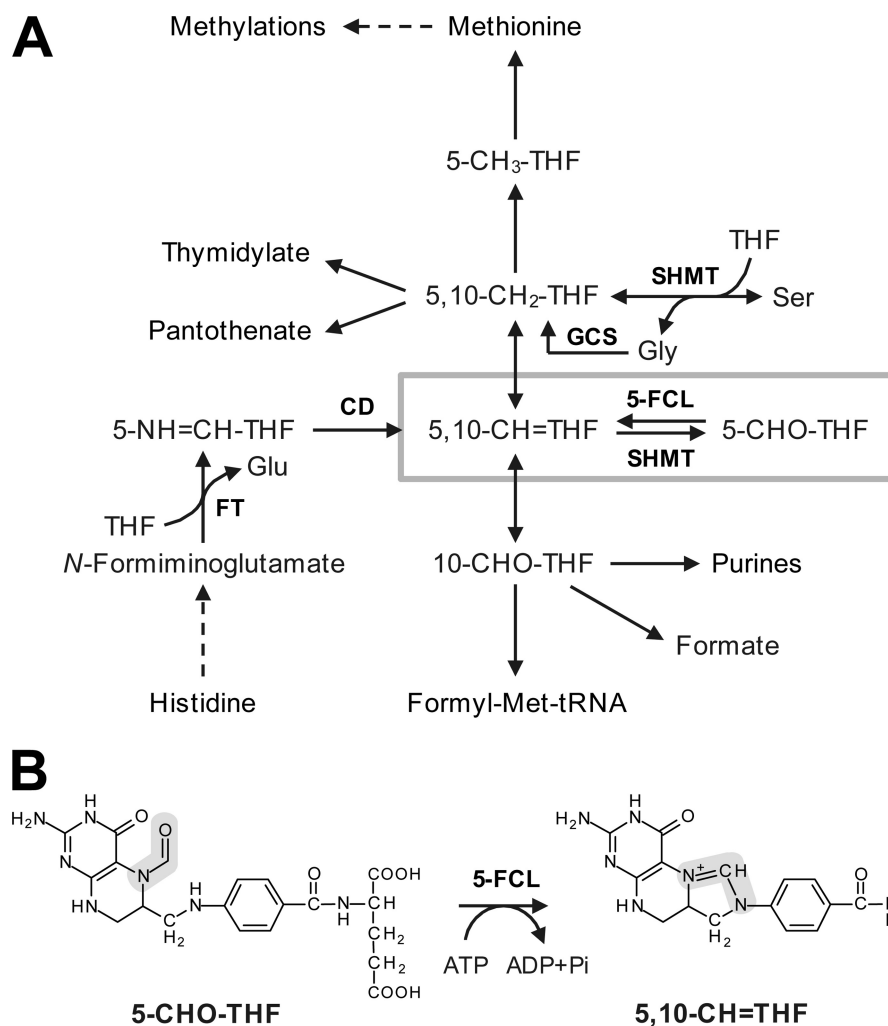
FIGURE 1. **5-CHO-THF in the context of C$_1$ metabolism.** *A*, overview of the C$_1$ folate reaction network. SHMT mediates the reversible conversion of THF and serine to 5,10-CH$_2$-THF and glycine. This reaction is a major source of C$_1$ units for use in other reactions. SHMT also mediates a side reaction that converts 5,10-CH=THF to 5-CHO-THF, which inhibits various folate-dependent enzymes and must therefore be metabolized. 5-FCL is the only enzyme known to do this. *Dashed arrows* depict multiple steps. Abbreviations: *5-CH$_3$-THF*, 5-methyltetrahydrofolate; *5,10-CH$_2$-THF*, 5,10-methylenetetrahydrofolate; *10-CHO-THF*, 10-formyltetrahydrofolate; *GCS*, glycine cleavage system. *B*, the reaction catalyzed by 5-FCL. The C$_1$ moiety is highlighted in *gray*.

ments with *Streptococcus pyogenes*, and by biochemical characterization of the formyltransferase activity of recombinant FT proteins.

EXPERIMENTAL PROCEDURES

Bioinformatics—Genomes were analyzed using the SEED data base and its tools (18). Archaea lacking folates were excluded from the analysis. Genes with anticorrelated distributions to that of *ygfA* were sought using the Phylogenetic Profiler tool at JGI and the Signature Genes tool at NMPDR. Sequences were aligned using ClustalW or Multalin. Phylogenetic analyses were made using MEGA4 (19).

Chemicals—(6S)-Tetrahydrofolic acid (THF), (6S)-5-formyltetrahydrofolic acid, calcium salt (5-CHO-THF), and (R,S)-5-formyltetrahydrofolate triglutamate were from Schircks Laboratories (Jona, Switzerland). Saturated stock solutions were prepared under N$_2$, minimizing light exposure, by dissolving 2–10 mg of these folates in N$_2$-sparged K-phosphate buffer (100 mM, pH 7.0) containing 10 mM

β-mercaptoethanol. Concentrations of THF and 5-CHO-THF were determined spectrophotometrically using published extinction coefficients, $\epsilon_{298 \text{ nm}} = 28,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$ and $\epsilon_{287 \text{ nm}} = 31,500 \text{ M}^{-1}\cdot\text{cm}^{-1}$, respectively (20). *N*-Formyl-L-glutamate was prepared as described (21) except that acetone was used in place of benzene to promote crystallization. The product was separated from glutamate by passage through a 15-ml column of AG-50 X8 (H$^+$) (Bio-Rad) and washing with 15 ml of water. The eluate was neutralized with 1 M NH$_4$OH and frozen. *N*-Formimino-L-glutamate (FIGLU) was synthesized and purified as described (22) except that FIGLU was separated from glutamate on a 25-ml column of AG-1 X8 (acetate) (Bio-Rad) that was eluted with a linear gradient of acetic acid (0–0.5 M) at a rate of 1 ml min^{-1}, collecting 5-ml fractions. Fractions were analyzed by thin layer chromatography on 0.1 mm cellulose plates (Merck, Darmstadt, Germany) developed with *n*-butanol:acetic acid:water, 60:15:25 (v/v/v). Detection was with ninhydrin; to detect FIGLU, plates were first

exposed to NH_3 vapor for 2 h. Fractions containing FIGLU were pooled and freeze-dried.

Strains and Media—*E. coli* Top10 (Invitrogen, Carlsbad, CA) and BL21-CodonPlus (DE3)-RIPL strains (Stratagene, La Jolla, CA) were used for plasmid amplification and protein overexpression, respectively. The Δ*ygfA* deletion was made in *E. coli* K12 strain MG1655 (see below). All *E. coli* strains were grown at 37 °C with agitation in LB medium (23) or, for complementation tests, in M9 medium (23) containing 0.2% (w/v) glucose, micronutrients, and $FeSO_4$ (24). *S. pyogenes* JRS4, a streptomycin-resistant derivative of serotype M6 strain D471 (25), was from I. Biswas (University of Kansas Medical Center). FT knockouts were made in this background (see below). *S. pyogenes* cultures were grown statically at 37 °C with 5% CO_2 supply on TH-HS medium (Todd-Hewitt medium (Difco, Detroit, MI) supplemented with 5% (v/v) horse serum (Sigma-Aldrich) (26)) and on CDM chemically defined medium (27) from which *p*-aminobenzoic acid and folic acid were omitted. Antibiotic concentrations (μg ml^{-1}) were: for *E. coli*, carbenicillin 100, chloramphenicol 34, erythromycin 500, kanamycin 50, streptomycin 50; for *S. pyogenes*, erythromycin 1.5, streptomycin 200.

Mutant Construction—Primers are listed in supplemental Table S1. The *ygfA*::Kan deletion from Keio collection strain JW2879 (28) was transferred to *E. coli* strain MG1655 by P1 transduction. Clones harboring the deletion were selected on LB containing kanamycin, and verified by sequencing. The *S. pyogenes* FT knock-out was obtained by single crossover recombination. A 421-bp fragment of the *S. pyogenes FT* gene was cloned in pSK-Erm, a pBluescript vector modified to confer erythromycin resistance (29). *S. pyogenes* JRS4 competent cells were obtained as described (26). Briefly, cells were grown in 20 ml of TH-HS medium containing streptomycin until A_{560} reached 0.3 and harvested by centrifugation at 4 °C (4500 × *g*, 5 min). The washed cell pellet was resuspended in 100 μl of ice-cold 0.5 M sucrose. Electroporation was performed by adding 2 μg of the Sp421bp-pSK-Erm construct to 40 μl of fresh competent cells; a Bio-Rad gene pulser was used (peak voltage 2.5 kV; capacitance 25 μF; pulse controller 200 Ω). Cells were incubated for 2 h at 37 °C in 1 ml TH-HS, followed by plating on TH-HS containing erythromycin. Colonies appeared after 2 days. Mutants were screened by PCR using gene- and vector-specific primers (SpKOver-F and M13rev-R; SpKOver-R and M13fwd-F). The *queT* gene was used as a positive control (primers QueT-F and QueT-R). FT knock-out strains were electroporated as above with the *S. pyogenes FT* gene under the control of the strong P23 promoter (30, 31) in the shuttle vector pAT28 (32). pAT28 has the *spc* spectinomycin resistance gene and the pAMβ1 origin of replication, which confers high copy number in Gram+ bacteria (33). The *FT* gene was first subcloned into BamHI-SalI digested pOri23 (30). The P23-*FT* fragment was then excised with EcoRI-SalI and cloned into EcoRI-SalI-digested pAT28.

Expression Constructs for Functional Complementation and Protein Production—Genomic DNA of Acidobacteria bacterium Ellin345, *Elusimicrobium minutum*, *Herpetosiphon aurantiacus*, *Syntrophus aciditrophicus*, *Picrophilus torridus*,

and *Thermoplasma acidophilum* were kindly provided by C. R. Kuske (Los Alamos National Laboratory), A. Brune (Max Planck Institute for Terrestrial Microbiology), D. A. Bryant (Penn State University), M. J. McInerney (University of Oklahoma), W. Liebl (Technische Universität München), and W. Baumeister (Max Planck Institute of Biochemistry), respectively. Genomic DNA of *S. pyogenes* M1 was from the American Type Culture Collection (Manassas, VA). FT sequences were amplified with *PfuTurbo* DNA Polymerase (Stratagene) using genomic DNA as template and primers (supplemental Table S1) designed with restriction sites to subclone the amplified product into pBluescript II SK (Stratagene) for complementation assays, or pET28b or pET21a (Novagen) for overexpression of proteins carrying a C-terminal His tag. Constructs were verified by sequencing.

Functional Complementation—*E. coli* Δ*ygfA* cells harboring pACYC-RP and pSC101-RIL (Stratagene) encoding rare tRNAs were transformed with pBS II SK alone (negative control) or harboring *E. coli ygfA* (positive control) or a *FT* gene, plated on LB containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), kanamycin, chloramphenicol, streptomycin, and carbenicillin, and incubated at 37 °C. The next day, independent clones were streaked on the M9 medium above or a modified version in which 50 mM glycine replaced 20 mM NH_4Cl as nitrogen source. Plates were incubated for 4 days at 37 °C.

Production and Isolation of Recombinant Proteins—Porcine (*Sus scrofa*) glutamate formiminotransferase-cyclodeaminase (FT-CD) was produced as described (34) using an expression construct in pBke obtained from R. E. MacKenzie (McGill University). FTs from Acidobacteria bacterium Ellin345 (Ab), *S. pyogenes* (Sp), *P. torridus* (Pt), and *T. acidophilum* (Ta) were prepared as follows: BL21-CodonPlus (DE3)-RIPL cells were transformed with AbFT-CD-pET21a, SpFT-pET28b, PtFT-pET28b, or TaFT-pET28b. Cultures were induced with 1 mM IPTG when A_{600} reached 0.7, then held for 3 h at room temperature. Cells were pelleted (6000 × *g*, 20 min, 4 °C), resuspended in 100 mM K-phosphate, pH 7.3, and disrupted by sonication (15 s × 12). The proteins were purified on 0.5-ml Ni^{2+}-affinity columns (Qiagen, Valencia, CA) equilibrated with sonication buffer. Columns were washed with 100 mM K-phosphate, pH 7.3 containing 300 mM NaCl and 20 mM imidazole, eluted by raising the imidazole concentration to 250 mM, and desalted on PD-10 columns (GE Healthcare, Piscataway, NJ) equilibrated with 100 mM K-phosphate, pH 7.3 containing 10% (v/v) glycerol. The desalted samples were flash frozen in liquid N_2 and stored at −80 °C; all proteins were stable in these conditions except Acidobacteria bacterium FT-CD, which lost 80% of activity after storage for 8 weeks. Protein concentration and purity were determined by the Bradford method (35) and SDS-PAGE, respectively.

Enzyme Assays—Formiminotransferase and reverse direction formyltransferase assays were slightly modified from those of Bortoluzzi and MacKenzie (36). Standard reactions (100 μl) contained 100 mM K-phosphate, pH 7.3, 10 mM β-mercaptoethanol, 0.5 mM THF, 5 mM FIGLU or *N*-formylglutamate (which were shown to be saturating concentrations), and 0.001–1 μg of enzyme. After incubation at 30 °C
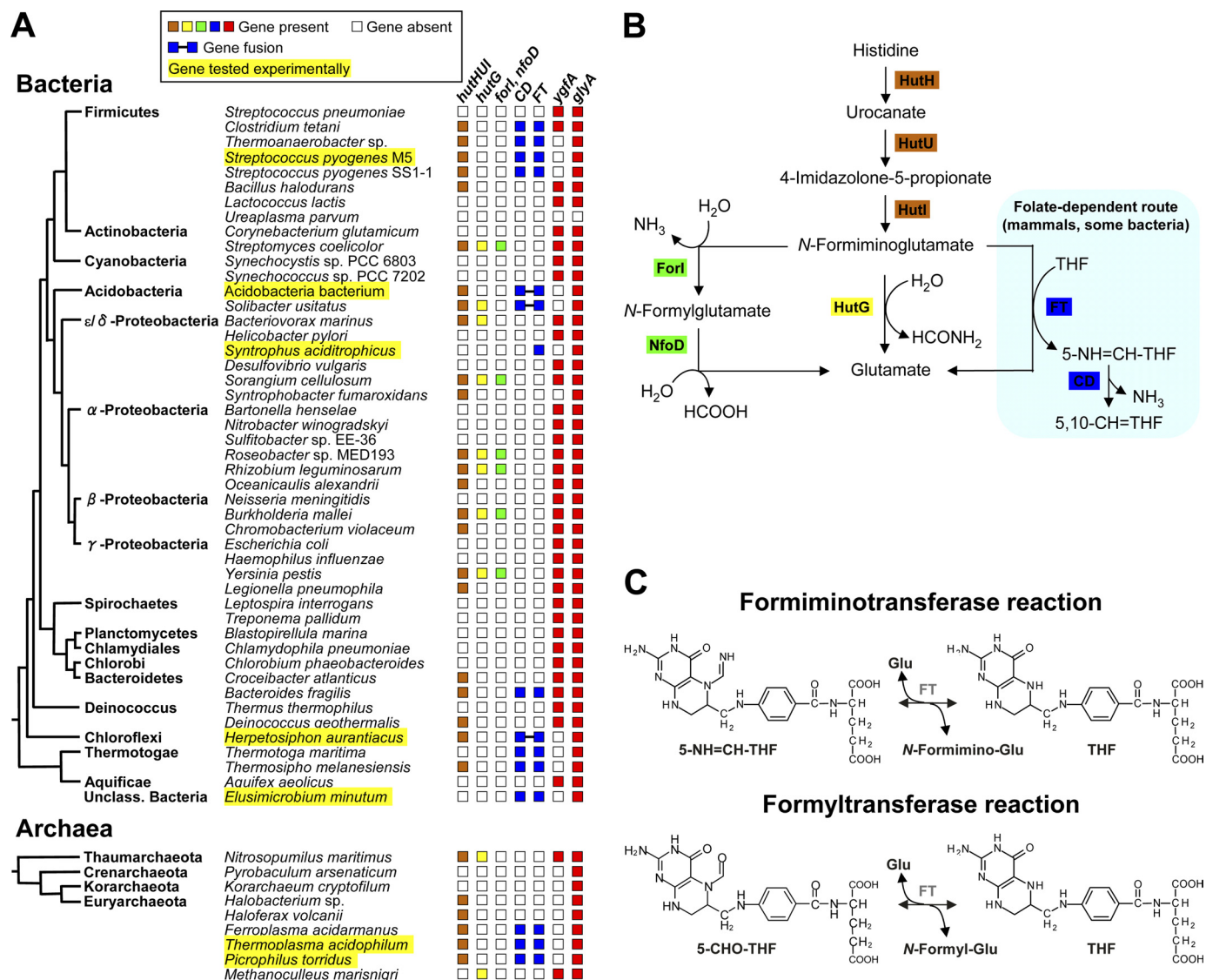
5-Formyltetrahydrofolate Metabolism

FIGURE 2. **Identification of FT as a potential candidate for 5-CHO-THF removal.** *A*, distribution among representative prokaryotes of the *FT* gene, of other histidine degradation genes (*hutHUI*, *hutG*, *forI*, *nfoD*, *CD*), and of the genes involved in 5-CHO-THF formation (*glyA*) and removal (*ygfA*). The tree shows the standard branching order for each group and is adapted from (60), with the addition of archaea (61) for which chemical, biochemical, or genomic evidence supports the presence of folates (15, 16, 62, 63). Note the anticorrelated distributions of *ygfA* and *FT*. Organisms whose FTs were tested experimentally are highlighted in *yellow*. *B*, histidine degradation pathways. Folate-dependent reactions are highlighted in *pale blue*. *C*, reactions mediated by mammalian FT, *i.e.* formimino or formyl group transfer between glutamate and THF. Abbreviations: *hutH*, histidine ammonia-lyase; *hutU*, urocanate hydratase; *hutI*, imidazolone propionase; *hutF*, formiminoglutamate iminohydrolase; *hutG*, formiminoglutamase; *nfoD*, *N*-formylglutamate deformylase; *forI*, formiminoglutamate iminohydrolase.

for 5–60 min, reactions were stopped by adding 100 μl of 0.36 M HCl followed by 55 s boiling, which converts 5-formimino-THF (5-NH=CH-THF) to 5,10-CH=THF. After cooling and centrifuging to clear (15,800 × *g*, 5 min), 5,10-CH=THF was estimated spectrophotometrically (ϵ_{350} = 24,900 $M^{-1} \cdot cm^{-1}$) using a blank containing no FIGLU or *N*-formylglutamate. Standard forward-direction formyltransferase assays (100 μl) contained 100 mM K-phosphate, pH 7.3, 10 mM β-mercaptoethanol, 0.1 mM 5-CHO-THF, 5 mM L-glutamate, and 0.1–1 μg of enzyme. After incubation at 30 °C for 5–30 min, reactions were stopped by adding 10 μl of 100 mM β-mercaptoethanol containing 20% (w/v) Na-ascorbate and boiling for 60 s, then cooled on ice and centrifuged as above. Folates were separated by high-pressure liquid chromatography (HPLC) on a 150 × 3.2 mm Prodigy 5 μm

ODS(2) column (Phenomenex, Torrance, CA) with a 2.5–25% (v/v) acetonitrile gradient in 87 mM phosphoric acid, pH 2.3 (flow rate 1.5 ml min^{-1}) and detected by fluorescence (excitation 295 nm, emission 356 nm) (37). THF and 5-CHO-THF standards were used to quantify substrate consumption and product formation. K_m and k_{cat} values were obtained by curve fitting to a rectangular hyperbola using SigmaPlot11 (SYSTAT Software, Chicago, IL).

Folate Analysis—*E. coli* wild-type or $\Delta ygfA$ strains harboring pBS II SK alone or containing *E. coli* *ygfA* or *S. pyogenes* or *S. aciditrophicus* *FT* genes were grown at 37 °C in 120 ml of M9 medium containing 0.2% glucose, 1 mM IPTG, 7 mM glycine, and appropriate antibiotics. When A_{600} reached 1.0 ± 0.1, cells were pelleted, frozen in liquid N$_2$, and stored at −80 °C. Folates were then extracted as described (38). Briefly,

pellets were resuspended in 10 ml of 50 mM HEPES-CHES, pH 7.8, containing 2% (w/v) Na-ascorbate and 10 mM β-mercaptoethanol, sonicated, boiled for 10 min, and centrifuged (13,000 $\times$ g, 10 min). The extraction step was repeated once. The combined extracts were treated for 2 h at 37 °C with rat plasma conjugase to deglutamylate folates. Samples were then boiled for 10 min, centrifuged as above, and filtered. Folates were isolated using 2-ml folate affinity columns, which were eluted with 5 ml of 87 mM phosphoric acid containing 1% (w/v) ascorbic acid (adjusted to pH 2.3 with KOH). Samples (400 μl) were analyzed by HPLC with electrochemical detection (39). Detector response was calibrated with standards from Merck Eprova (Schaffhausen, Switzerland). Tests with *E. coli* extracts spiked with 1.2 nmol of 5,10-CH=THF showed 4.9 ± 0.5% conversion to 5-CHO-THF; experimental values for endogenous 5-CHO-THF and 5,10-CH=THF were corrected accordingly.

RESULTS

A Candidate for 5-CHO-THF Removal Predicted by Comparative Genomics—A systematic analysis of 621 bacterial and 19 archaeal genomes using the SEED data base (18) revealed that 618 have *glyA* genes encoding SHMT. Of these 618, 555 have *ygfA* genes specifying 5-FCL, and 63 (a significant minority) do not. The *ygfA*-deficient group includes taxonomically diverse bacteria and archaea (Fig. 2*A*). As some other gene is expected to replace *ygfA* when it is absent, we sought genes whose phylogenetic distribution is anticorrelated with that of *ygfA*. The gene encoding the histidine degradation enzyme FT emerged from this analysis as the strongest candidate. The *FT* gene is frequently present when *ygfA* is absent and almost always absent when *ygfA* is present (Fig. 2*A*); globally, *FT* occurs in 46% of the genomes without *ygfA*, but in only 2% of the genomes with it.

Classical biochemical data on FT add to its plausibility as a substitute for 5-FCL. FT is a folate-dependent enzyme that mediates a late step in histidine degradation in mammals and certain bacteria; other bacteria use alternative, folate-independent routes (Fig. 2*B*) (18, 36). Specifically, FT catalyzes the transfer of a formimino group from FIGLU to THF, yielding 5-formimino-THF (5-NH=CH-THF), which is then converted to 5,10-CH=THF by the action of formiminotetrahydrofolate cyclodeaminase (CD) (Fig. 2*C*). In mammals and some bacteria, FT and CD are fused together in a single protein (Fig. 2*A*). Besides formimino transfer, mammalian FT is known to mediate an analogous formyl transfer between *N*-formylglutamate and THF, and to do so reversibly (Fig. 2*C*) (36, 40). The formyltransferase activity of mammalian FT is far lower than its formiminotransferase activity and has been viewed as physiologically insignificant (6, 36). Nevertheless, formyltransferase activity in the 5-CHO-THF → THF direction (henceforth termed the forward direction) would in principle enable 5-CHO-THF removal.

Further comparative genomic evidence of an alternative function for FT comes from its distribution pattern relative to those of other histidine degradation enzymes. Most prokaryotic genomes having *FT* also have *CD* and the other genes (*hutHUI*) needed for a complete histidine degradation path-
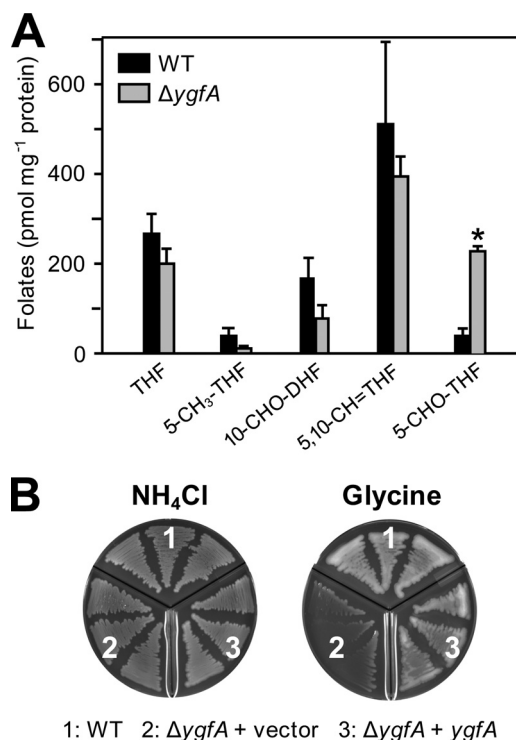
FIGURE 3. **Characterization of the *E. coli* Δ*ygfA* mutant.** *A*, deletion of *ygfA* causes a 6-fold increase in cellular 5-CHO-THF content, significant at $p < 0.05$ by *t* test (*asterisk*). Folate analysis was performed on *E. coli* wild-type (*WT*) and deletant (Δ*ygfA*) cells grown on M9 minimal medium supplemented with 0.2% glucose and 7 mM glycine. Data are means and S.E. from three replicates. *10-CHO-DHF*, 10-formyldihydrofolate; other folate abbreviations are as in the legend to Fig. 1 and the abbreviations footnote. *B*, growth phenotype of the Δ*ygfA* deletant. *E. coli* wild type or Δ*ygfA* cells transformed with pBS II SK alone or harboring *ygfA* were plated on minimal medium containing either 20 mM NH₄Cl or 50 mM glycine as sole nitrogen source. Plates were incubated for 3 days.

way (Fig. 2*A*), and some (*e.g. S. pyogenes*) have these genes in an operonic arrangement (18). In these cases, the FT enzyme is predicted to retain a function in histidine breakdown. However, certain genomes such as *E. minutum* and *S. aciditrophicus* lack the *hutHUI* genes, and the latter lacks *CD* as well (Fig. 2*A*). Such conservation of *FT* in the absence of other histidine utilization genes implies a second function.

Phenotype of the E. coli ygfA Deletant—In order to develop a functional complementation test for alternatives to 5-FCL, we first determined the effects on folate metabolism and growth of deleting the *ygfA* gene in *E. coli* (which has no *FT* gene). The deletion was made in the standard K12 strain MG1655 by P1-transduction from a Keio collection strain (28). The deletant grew normally on LB rich medium or M9 minimal medium, and folate analysis detected little or no intracellular accumulation of 5-CHO-THF (not shown). However, supplementing M9 medium with glycine caused a 6-fold accumulation of 5-CHO-THF (Fig. 3*A*), presumably because the SHMT side-reaction that generates 5-CHO-THF is promoted by bound glycine (2). Glycine supplementation did not affect growth of the deletant but completely replacing the NH₄Cl nitrogen source in M9 medium with 50 mM glycine led to a severe growth defect (Fig. 3*B*). This defect is likely due to a buildup of 5-CHO-THF inhibiting SHMT (6) and glycine cleavage (41), both of which *E. coli* needs to use glycine as
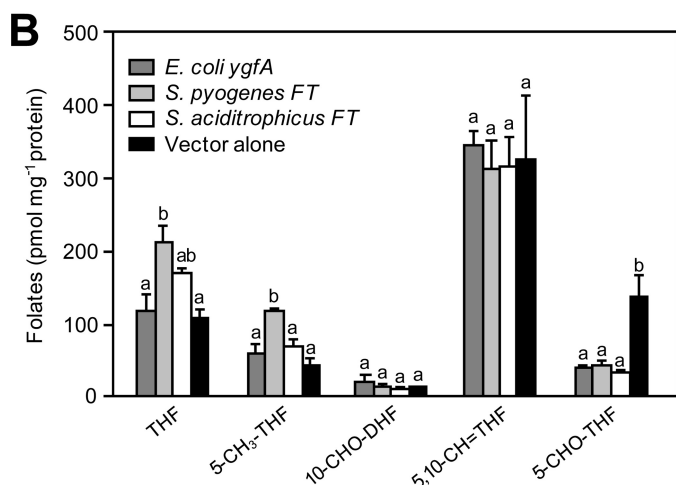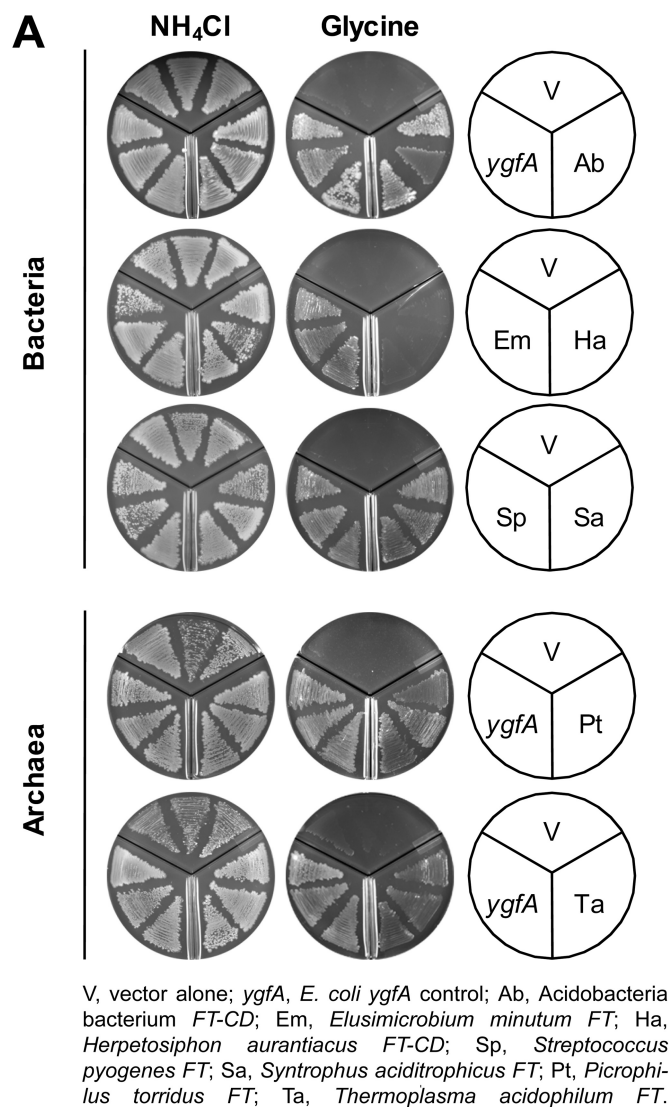
A

V, vector alone; *ygfA*, *E. coli ygfA* control; Ab, Acidobacteria bacterium *FT-CD*; Em, *Elusimicrobium minutum FT*; Ha, *Herpetosiphon aurantiacus FT-CD*; Sp, *Streptococcus pyogenes FT*; Sa, *Syntrophus aciditrophicus FT*; Pt, *Picrophilus torridus FT*; Ta, *Thermoplasma acidophilum FT*.

B

FIGURE 4. **Functional complementation of the *E. coli* Δ*ygfA* deletant with various formiminotransferase genes.** *A*, complementation of the growth phenotype. The Δ*ygfA* strain harboring pBS II SK alone or containing *E. coli ygfA,* or various *FT* or *FT-CD* genes, was plated on M9 medium containing 0.2% glucose, 1 mM IPTG, and either 20 mM NH$_4$Cl or 50 mM glycine as nitrogen source. Plates were incubated for 3 days. *B*, folate contents of Δ*ygfA* cells expressing pBS II SK alone or containing *E. coli ygfA*, *S. pyogenes FT*, or *S. aciditrophicus FT*. Cells were grown in M9 medium containing 0.2%

nitrogen source (42). The growth defect was reversed by expressing the *E. coli ygfA* gene from a plasmid (Fig. 3*B*). These observations establish the *ygfA* deletant as a suitable platform for complementation tests of possible alternatives to 5-FCL.

Complementation of the Δ*ygfA Strain by Prokaryotic Formiminotransferase Genes*—The Δ*ygfA* deletant was transformed with pBS II SK alone or containing *FT* genes from five bacteria and two archaea; two of the genes were *FT-CD* fusions (Fig. 2*A*). *E. coli ygfA* served as a positive control. Transformants were plated on M9 medium containing NH$_4$Cl or 50 mM glycine as nitrogen source. All strains grew well on NH$_4$Cl, indicating that none of the *FT* genes was toxic. Six out of the seven *FT* genes tested permitted good growth on glycine (Fig. 4*A*), indicating that they can functionally replace 5-FCL. The exception, *H. aurantiacus FT-CD*, may be a pseudogene; uniquely, the histidine utilization (*hut*) operon in *H. aurantiacus* is entirely broken up, suggesting that this pathway no longer functions. Also, related bacteria (*e.g. Roseiflexus* spp.) entirely lack *FT* and most other histidine utilization genes. Folates were analyzed in Δ*ygfA* cells harboring vector alone, or containing representative *FT* genes (from *S. pyogenes* and *S. aciditrophicus*) or *E. coli ygfA* as a control. The data confirmed that FTs can replace 5-FCL: both *FT* genes reversed the accumulation of 5-CHO-THF as effectively as *E. coli ygf*A (Fig. 4*B*); the effects of *FT* expression on other folates were minor.

Function of S. pyogenes FT in Its Native Host—To reinforce the above evidence from heterologous expression that FT functions *in vivo* in 5-CHO-THF metabolism, we investigated the function of the FT from *S. pyogenes* in its native host. *S. pyogenes* was selected because it is genetically manipulable and a model human pathogen (43). We first ablated the *FT* gene by single crossover integration (Fig. 5*A*); qRT-PCR tests confirmed lack of *FT* expression in the disruptant and detected weak expression in wild-type cells (not shown). The disruption had no impact on the growth or the folate profile of cells cultured on TH-HS or CDM medium (not shown). This may reflect the low level of *FT* expression in wild-type cells, itself due to the *FT* gene being in a histidine operon (Fig. 5*A*) that is repressed by the culture conditions (44, 45). We therefore compared the *FT* disruptant strain with or without the native gene expressed from a plasmid. In this comparison, folate synthesis was blocked by the antifolate drug sulfathiazole, resulting in inhibition of growth, and 5-CHO-THF was supplied to attempt to reverse the inhibition. For reversal to occur, cells must take up 5-CHO-THF and convert it to other folates. Expression of *S. pyogenes FT* effectively reversed sulfathiazole inhibition (Fig. 5*B*), showing that this gene confers the capacity to metabolize 5-CHO-THF when expressed in its native host. This result also shows that *S. pyogenes* has a folate transport system, as do other Firmicutes (46).

In Vitro Activities of Formiminotransferase Proteins—To show directly that prokaryote FT and FT-CD proteins have

glucose, 1 mM IPTG, and 7 mM glycine, and harvested when A$_{600}$ reached 1.0. Data are means and S.E. from three replicates. For each folate, means marked with different letters are significantly different ($p < 0.01$, one-way ANOVA followed by Holm-Sidak test). Folate abbreviations are as in the legend to Fig. 3.
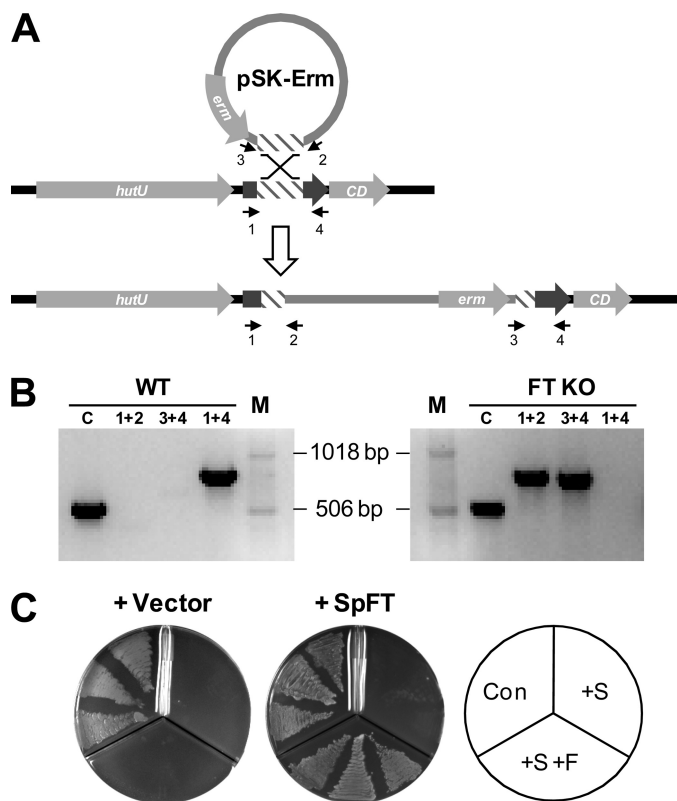
FIGURE 5. **Evidence that the *S. pyogenes* formiminotransferase gene mediates 5-CHO-THF metabolism in its native host.** *A*, construction of a null *FT* mutant by single crossover recombination (not drawn to scale). A 0.4-kb *FT* fragment (*hatched box*) was cloned into pSK-Erm, which contains the *erm* erythromycin resistance gene. Recombination between vector-borne and target *FT* sequences led to insertional inactivation of *FT*. *B*, PCR using pairs of vector and *FT* primers (*numbers 1–4*) verified the mutant; the *queT* gene served as a positive control (*lane C*). *Lane M* shows molecular size markers. Note that PCR with *FT*-specific primers (*1 + 4*) failed in the disruptant (*FT KO*), whereas a 0.8-kb band was amplified in the wild type (*WT*). *C*, rescue of *S. pyogenes* from antifolate blockade by 5-CHO-THF when the *FT* gene is expressed. The *S. pyogenes* disruptant harboring pAT28 alone or containing the *S. pyogenes* FT gene was plated on CDM containing 1% glucose alone (*Con*), or supplemented with 200 μM sulfathiazole (*+S*) or 200 μM sulfathiazole plus 50 μM 5-CHO-THF (*+S +F*). Plates were incubated for 2 days.

FIGURE 6. **Purification of bacterial and archaeal formiminotransferases and analysis of their formyltransferase activity.** *A*, Ni^{2+}-affinity purification of FTs from *P. torridus* (*PtFT*), *S. pyogenes* (*SpFT*), *T. acidophilum* (*TaFT*), and the FT-CD from Acidobacteria bacterium Ellin 345 (*AbFT-CD*). Total soluble proteins (*S*) (15 μg) and purified enzymes (*P*) (5–10 μg) were separated by SDS-PAGE and stained with Coomassie Blue. The positions of molecular markers (*kDa*) are indicated. *B*, HPLC analysis of forward direction FT reactions (complete or minus glutamate) with *P. torridus* FT (0.1 μg), *S. pyogenes* FT (0.2 μg), or porcine FT-CD (1 μg) as a positive control. Reactions were run for 10 min (prokaryotic FTs) or 30 min (porcine FT-CD). The positions of THF and 5-CHO-THF standards are indicated. Similar results were obtained for *T. acidophilum* FT and Acidobacteria bacterium FT-CD (not shown). Note that no THF was formed in the absence of glutamate, nor was THF formed when enzyme was omitted or preboiled (not shown).

formyltransferase activity, His-tagged recombinant proteins were purified and assayed for formyltransferase activity in both directions. Formiminotransferase activity in the physiological direction (FIGLU + THF → Glu + 5-NH=CH-THF) was measured for comparison. The proteins analyzed were the FTs from *S. pyogenes*, *P. torridus*, and *T. acidophilum*, the FT-CD from Acidobacteria bacterium Ellin 345, and (as a benchmark) porcine FT-CD. The prokaryote protein preparations were all ≥95% homogeneous as judged by SDS-PAGE (Fig. 6*A*).

All the prokaryote enzymes catalyzed glutamate-dependent THF formation from 5-CHO-THF (Fig. 6*B*); the fact that no THF was formed in the absence of glutamate demonstrates that the reaction is a formyl transfer, not simply a deformylation. The porcine FT-CD control mediated the same reaction, as expected (40). None of the prokaryote enzymes catalyzed detectable THF formation when glutamate was replaced by any of the other 19 protein amino acids, indicating high specificity for glutamate as formyl acceptor. Kinetic characteriza-
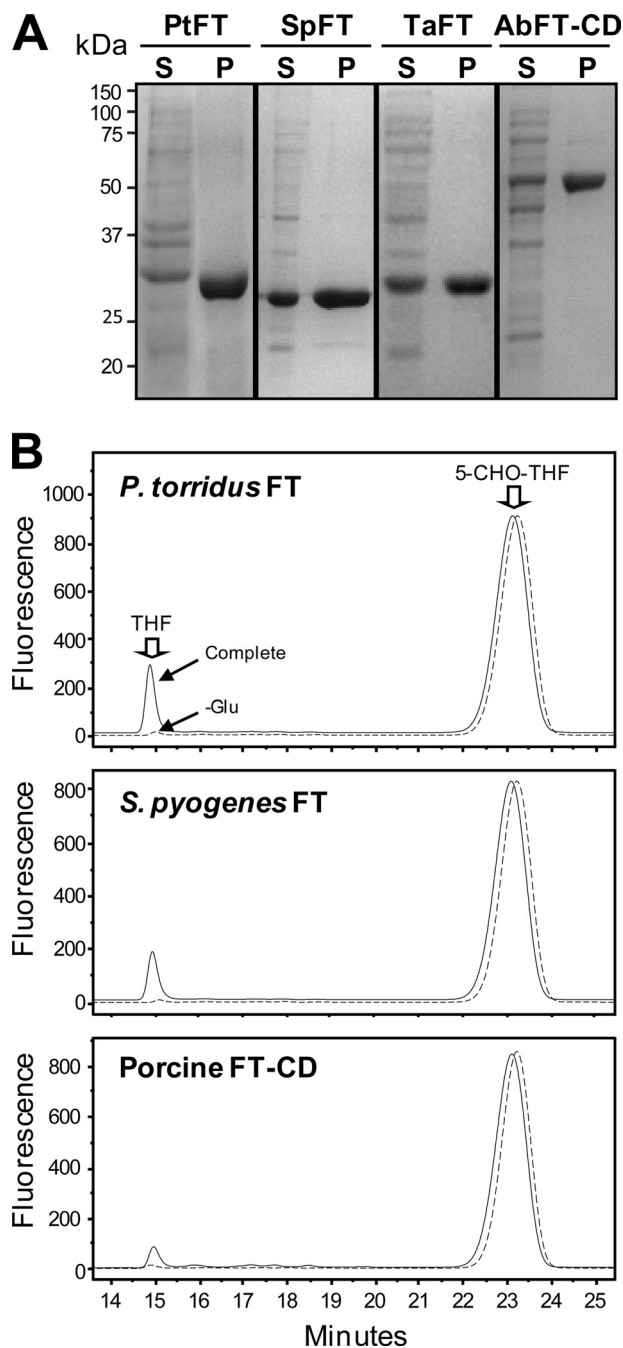
tion of the formyltransferase activities (Table 1) showed K_m values in the micromolar range for 5-CHO-THF and for its triglutamate form; as triglutamates have been found to be the predominant folates in growing cells of various bacteria (47)

TABLE 1

Kinetic constants for formyltransferase activities measured in the forward direction

Data are means of triplicate determinations ± S.E.

Enzyme	K_m (5-CHO-THF)[a]	K_m (5-CHO-THF-Glu$_3$)[a]	K_m (L-glutamate)	k_{cat}[b]
	μM	μM	μM	min^{-1}
S. pyogenes FT	5.4 ± 0.7	4.8 ± 1.3	1030 ± 30	0.13 ± 0.01
P. torridus FT	0.40 ± 0.04	0.70 ± 0.2	34 ± 6	1.16 ± 0.05
T. acidophilum FT	3.7 ± 0.5	3.8 ± 0.5	113 ± 30	0.27 ± 0.03
Acidobacteria bacterium FT-CD	1.8 ± 0.1	0.5 ± 0.1	276 ± 45	0.55 ± 0.03
Porcine FT-CD	7.2 ± 0.3	5.8 ± 0.4	206 ± 7	0.11 ± 0.01

[a] The cofactors used were the natural (*S*) form of 5-CHO-THF *versus* mixed natural and unnatural forms (*R,S*) of 5-CHO-THF-Glu$_3$, so that K_m values for 5-CHO-THF-Glu$_3$ should be halved for comparison with those for 5-CHO-THF.

[b] Values for k_{cat} were determined with 5-CHO-THF as cofactor; those determined with 5-CHO-THF-Glu$_3$ were similar.

the latter result confirms the effectiveness of the physiological form of the cofactor. The K_m values for glutamate were two orders of magnitude greater. The k_{cat} values for the prokaryote enzymes were equal to or greater than that for the porcine enzyme, and in one case (*P. torridus*) the difference was 10-fold. Because *P. torridus* FT also had the lowest K_m value for 5-CHO-THF (Table 1), this result suggests that the *P. torridus* enzyme may have become more efficient in formyl transfer relative to formimino transfer.

To investigate this possibility, we estimated k_{cat} values for formiminotransferase activity (in the physiological direction) and compared them to the above values for formyltransferase in the forward direction (Fig. 7). We also measured formyltransferase k_{cat} values for the reverse reaction, to juxtapose with the literature on the porcine FT-CD (Fig. 7); our values for this enzyme agreed with published ones (36). The forward formyltransferase/formiminotransferase ratio (in parentheses, Fig. 7, *bottom panel*) for *P. torridus* FT was 330-fold higher than that of the porcine enzyme, and 170–660-fold higher than those of the other prokaryote enzymes. The high ratio for *P. torridus* FT reflects a high formyltransferase activity combined with an exceptionally low formiminotransferase activity (Fig. 7). The *P. torridus* enzyme is thus a markedly more efficient formyltransferase than the others in terms of (i) the k_{cat}/K_m ratio for this activity and (ii) the relative magnitudes of the formyltransferase and formiminotransferase k_{cat} values. However, even for this enzyme, the formyltransferase k_{cat} value is 30-fold less than the formiminotransferase k_{cat} value; for the other prokaryote enzymes, and for porcine FT, the formyltransferase k_{cat} values are ~5,000–20,000-fold less than the formiminotransferase k_{cat} values. Formyltransferase activity is thus not the major activity in any of the enzymes tested.

DISCUSSION

The comparative genomic, genetic, and biochemical data presented above support the conclusion that the formyltransferase activity of the histidine degradation enzyme FT replaces 5-FCL in diverse prokaryotes. Most of these enzymes presumably retain their principal function in histidine catabolism because they have high formiminotransferase activity *in vitro* and their genes co-occur with the other genes of histidine breakdown, sometimes in operonic arrangements. Such FTs can therefore be viewed as moonlighting enzymes, *i.e.* as metabolic enzymes with an additional functional activity (48), in this case 5-CHO-THF salvage. As previously noted, how-
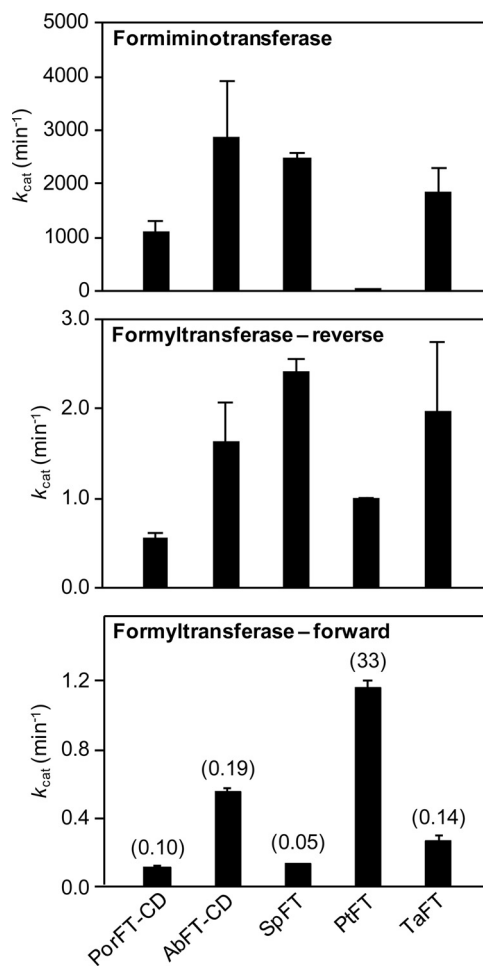

FIGURE 7. **Comparison of k_{cat} values for the formimino- and formyltransferase activities of glutamate formiminotransferases.** Data are mean values ± S.E. from three replicates. *Top panel*, formiminotransferase activity in the physiological direction (FIGLU + THF → glutamate + 5-NH=CH-THF). *Middle panel*, formyltransferase activity in the reverse direction (*N*-formylglutamate + THF → glutamate + 5-CHO-THF). *Bottom panel*, formyltransferase activity in the forward direction (glutamate + 5-CHO-THF → *N*-formylglutamate + THF); the ratio of each forward k_{cat} to the corresponding formiminotransferase k_{cat} in per mil units (‰) is given in *parentheses*. *Por*, porcine; *Ab*, Acidobacteria bacterium; *Sp*, *S. pyogenes*; *Pt*, *P. torridus*; *Ta*, *T. acidophilum*.

ever, some FTs occur in genomes that lack most or all other histidine degradation genes; in these cases the moonlighting activity may have become the only physiological one.

Although the *in vitro* formyltransferase activity of FTs is much lower than their formiminotransferase activity, three factors may enable the formyltransferase activity to function effectively in 5-CHO-THF removal. First, prokaryote FTs

have low K_m values for 5-CHO-THF (0.4 to 5.4 μM; Table 1). Second, the *in vivo* concentration of the glutamate substrate would be saturating, because K_m values for glutamate are ≤ 1 mM (Table 1) and intracellular glutamate concentrations in prokaryotes are typically 30–100 mM (49–52). Third, no more than a low formyl transfer flux is needed to remove 5-CHO-THF, which is formed only by a minor side reaction (6) and which does not come to dominate the folate pool even if normal removal is blocked (Fig. 3*A*).

If FTs function simultaneously in histidine degradation and 5-CHO-THF salvage in the same cell, then they must carry flux in both directions because the formiminotransferase reaction consumes THF and produces glutamate and the formyltransferase salvage reaction does the opposite (Fig. 2*C*). Given that moonlighting functions can depend on differences in protein-protein interactions (48), it seems possible that running these opposing reactions concurrently is facilitated by FT joining/not joining a protein complex related to histidine degradation. In this connection, it is relevant that mammalian FT-CD is an octamer that can dissociate into two types of monofunctional dimers, and that while the FT and CD activities of the octamer function separately with monoglutamylated folates, polyglutamates are channeled from the FT to the CD active site (34, 53). Analogous behavior of prokaryote FT oligomers, or perhaps FT-CD complexes, together with polyglutamate-mediated channeling between FT and CD reactions, could result in the functional partitioning, *i.e.* mutual non-interference, of formyl- and formiminotransferase activities.

One of the FTs examined (that from the archaeon *P. torridus*) was far more efficient as a formyltransferase than the other prokaryote enzymes, which were themselves comparable in efficiency to porcine FT-CD. These findings are consistent with natural selection of the *P. torridus* protein for increased formyltransferase activity. They also imply that mammalian FT-CD cannot be ruled out *a priori* as a contributor to 5-CHO-THF salvage, particularly because glutamate levels in liver(to which FT-CD is confined) far exceed the K_m for glutamate (206 μM; Table 1) (54). However, any contribution from FT-CD is probably small relative to that from 5-FCL because 5-FCL is highly expressed in liver (55) and its specific activity appears to be at least one order of magnitude greater than that of FT-CD formyltransferase (36, 56).

In removing 5-CHO-THF, the formyltransferase reaction generates *N*-formylglutamate (Fig. 2*C*), which is a metabolic dead-end that can apparently be metabolized only via hydrolysis to glutamate and formate. Although a dedicated *N*-formylglutamate hydrolase (Fig. 2, *NfoD*) participates in histidine degradation in certain bacteria (18, 57), this protein is absent from prokaryotes in which FT replaces 5-FCL (Fig. 2). However, deacylation of amino acids is a common reaction and nonspecific enzymes that hydrolyze *N*-formylglutamate have been reported from bacteria and other organisms (58, 59).

Finally, because our comparative genomic analysis indicated that *FT* occurs in only 46% of the genomes with SHMT (*glyA*) and without 5-FCL (*ygfA*), it is likely that at least one other gene for 5-CHO removal remains to be discovered in prokaryotes. Such a hypothetical missing gene might also be present in plants. Ablating the single gene encoding 5-FCL in *Arabidopsis* results in only a 2-fold increase in leaf 5-CHO-THF content, suggesting that 5-FCL is not the sole route for 5-CHO-THF disposal (9).

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