Life without dihydrofolate reductase FolA

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Reduced folate derivatives participate in numerous reactions of bacterial intermediary metabolism. Consequently, the well-characterized enzyme implicated in the formation of tetrahydrofolate – dihydrofolate reductase FolA – was considered to be essential for bacterial growth. However, comparative genomics has revealed several bacterial genome sequences that appear to lack the *folA* gene. Here, we provide *in silico* evidence indicating that *folA*-lacking bacteria use a recently discovered class of flavin-dependent thymidylate synthases for deoxythymidine-5′-monophosphate synthesis, and propose that many bacteria must contain uncharacterized sources for reduced folate molecules that are still waiting to be discovered.

One-carbon units linked to tetrahydrofolate (H_4folate) are required for RNA-, DNA- and protein-synthesis (Fig. 1). In actively dividing cells, a large quantity of reduced folates is required for synthesis of deoxythymidine-5′-monophosphate (dTMP or thymidylate), a unique nucleotide component of DNA. Consequently, dTMP synthesis has the potential to diminish the pool-size of reduced folates, thus having indirect consequences for other branches of the intermediary metabolism. Until recently, the only known pathway for *de novo* synthesis of thymidylate was by thymidylate synthase ThyA, an enzyme methylating deoxyuridine-5′-monophosphate (dUMP). Uniquely for a biological reaction, the reductive methylation of dUMP by ThyA is intrinsically linked to formation of dihydrofolate (H_2folate), because ThyA uses methylenetetrahydrofolate (CH_2H_4folate) as both carbon source and reductant [1]. H_2folate formed through oxidation of tetrahydrofolate (H_4folate) by ThyA is rapidly reduced by FolA, as only reduced folate derivatives are functional in intermediary metabolism (Fig. 1). Taking into account the pivotal functional importance of ThyA and FolA, both of these enzymes have been used widely as targets for compounds that inhibit cellular proliferation. For instance, bacterial FolA is specifically inhibited by trimethoprim, a clinically relevant antibacterial agent [2].

Folate metabolism in bacteria carrying a novel thymidylate synthase

In contrast to the earlier prediction that functional coupling of ThyA and FolA should be vital for all bacteria, comparative genomics has revealed a large number of microbial species apparently lacking genes encoding either one of these enzymes (see Table 1). The absence of *thyA* in these genomes was explained by our recent finding that a large family of previously uncharacterized ThyX (also known as Thy1) proteins corresponds to a novel class of flavin-dependent thymidylate synthases [3]. As *thyA* and *thyX* genes have, with few exceptions, mutually exclusive phylogenetic distributions [4], and the novel class of thymidylate synthases is present in up to 30% of completed microbial genome sequences [3], these data unequivocally demonstrate that two major pathways for dTMP formation operate in the microbial world.

Although both ThyA and ThyX are CH_2H_4folate-dependent enzymes, the two distinct classes of thymidylate synthases appear to differ markedly regarding their reductive mechanisms (Fig. 2). Our data indicate that, unlike ThyA proteins, *Helicobacter pylori* ThyX uses CH_2H_4folate as only a one-carbon donor, whereas the

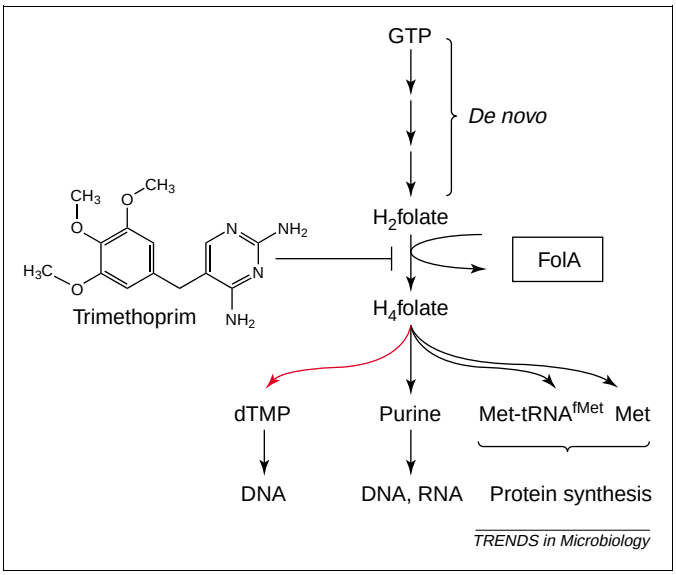

Fig. 1. All cells need reduced folate cofactors for the biosyntheses of many compounds. The *de novo* pathway for folate compounds directly synthesizes dihydrofolate, although only tetrahydrofolate can serve as a donor of one-carbon units for DNA-, RNA-, co-factor- and protein-syntheses [14]. Trimethoprim acts as a specific inhibitor of bacterial dihydrofolate reductase (FolA). The red arrow indicates that, in actively dividing cells, the majority of tetrahydrofolate derivatives are used for dTMP synthesis. Abbreviations: dTMP, deoxythymidine monophosphate; GTP, guanosine triphosphate; H_2folate, dihydrofolate; H_4folate, tetrahydrofolate; Met, methionine; Met-tRNA^{fMet}, a tRNA bearing N-formyl methionine that is required for initiation of protein synthesis in bacteria.

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Table 1. Bacterial species using thymidylate synthase ThyX for thymidylate synthesis[a]

Bacterial species[b]	Classification	ThyX[c]	ThyA	FolA	Tdk	Refs[d]
ThyX without folA						
Rickettsia prowazekii	α-proteobacteria	+	−	−	−	[3]
Rhodobacter capsulatus	α-proteobacteria	+	−	−	+	
Anaplasma phagocytophilia	α-proteobacteria	+	−	−	−	
Campylobacter jejuni	ε-proteobacteria	+	−	−	−	[3,9]
Helicobacter pylori 26695	ε-proteobacteria	+	−	−	−	[3]
Helicobacter pylori J99	ε-proteobacteria	+	−	−	−	[3]
Borrelia burgdorferi	Spirochete	+	−	−	+	[3]
Treponema pallidum	Spirochete	+	−	−	−	[3]
Thermoanaerobacter tengcongensis	Firmicutes	+	−	−	+	[3]
Ruminococcus albus	Firmicutes	+	−	−	−	
Desulfitobacterium hafniense	Firmicutes	+	−	−	−	
Gemmata obscuriglobus	Planctomycetes	+	−	−	−	
Nostoc sp. PCC7120	Cyanobacteria	+	−	−	−	
Synechocystis sp. PCC6803	Cyanobacteria	+	−	−	−	
Aquifex aeolicus	Thermophile	+	−	−	−	
Chlorobium tepidum	Green sulfur bacteria	+	−	−	−	
ThyX with folA[e]						
Rickettsia conorii	α-proteobacteria	+	−	+	−	
Chlamydia sp.	Chlamydiales	+	−	+	−	[3]
Clostridium sp.	Firmicutes	+	−	+	+	
Thermotoga maritima	Thermotogales	+	−	+	+	[13,15]

[a]Note that the majority of species included in this non-comprehensive list seemingly lack FolA. Bacterial species relevant for human health are indicated in bold. Abbreviations: FolA, bacterial dihydrofolate reductase; Tdk, thymidine kinase required for a salvage of extracellular thymidine; ThyA and ThyX, flavin-dependent and 'canonical' thymidylate synthase, respectively.
[b]Archaea containing chemically modified folates were excluded from the list.
[c]Phylogenetic distributions were determined using COG [16] and STRING [17] databases. The symbols + and − refer to the presence and absence of a given gene in a genome, respectively.
[d]If not otherwise indicated, data were collected using the publicly available sequence data accessible at http://www.ncbi.nih.gov, http://www.sanger.ac.uk and http://www.integratedgenomics.com.
[e]See Fig. 3 for phylogenetic analysis of these FolA sequences.

electrons required for formation of the methyl moiety are transferred from reduced pyridine nucleotides via an enzyme-bound flavin cofactor to form dTMP [3]. This reaction mechanism for dTMP formation maintains the folate in its reduced form (as H_4folate) at the end of the catalytic cycle. Therefore, the proposed difference in the reductive mechanisms of ThyA and ThyX offers a plausible explanation as to why all *thyA*-containing bacteria contain *folA*, despite that this gene is often absent from *thyX*-containing organisms. Surprisingly, this observation also indicates that *thyX*-containing organisms do not have an absolute requirement for FolA in their folate metabolism. However, bacteria lacking *folA* must still contain reduced folates for RNA and protein syntheses to take place, although their source for reduced folates remains mysterious.

Alternative pathway(s) for H_4folate formation?

One explanation for the absence of *folA* in a wide phylogenetic range of bacteria would be the presence of alternative bacterial pathways and/or enzymes forming H_4folate. The existence of such alternative pathways was

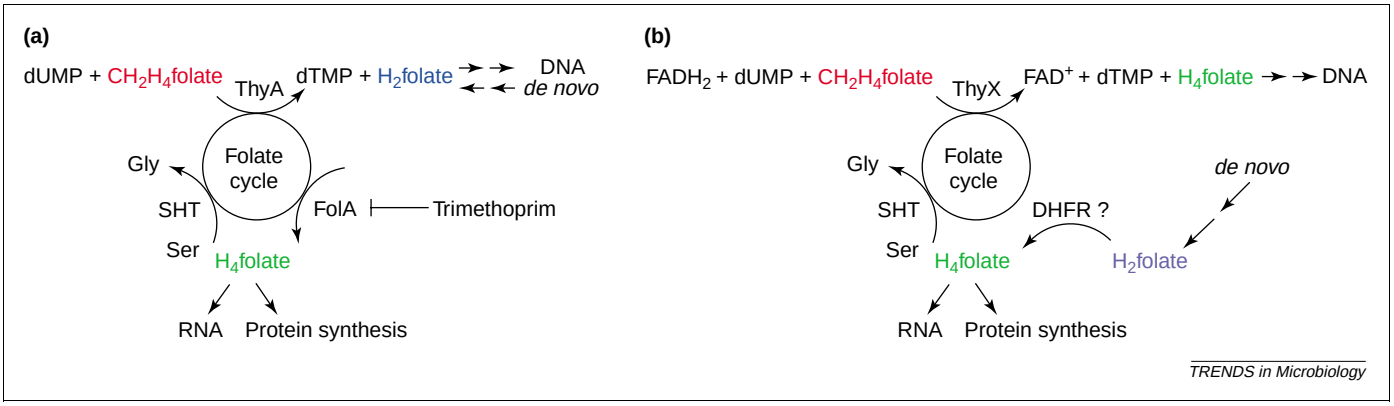

Fig. 2. The different reductive mechanisms of thymidylate synthases (a) ThyA and (b) ThyX. ThyA proteins use methylenetetrahydrofolate (CH_2H_4folate) both as carbon and electron source, thus resulting in the formation of H_2folate. Reduced flavin nucleotides ($FADH_2$) have an obligatory role in ThyX catalysis [3], thus strongly indicating that dTMP catalysis is linked – differently from ThyA proteins – to formation of H_4folate. Strikingly, although all *thyA*-carrying bacteria also contain dihydrofolate reductase *folA*, the known pathways for formation of H_4folate are absent in several *thyX*-containing bacteria (Table 1), suggesting the presence of an alternative dihydrofolate reductase in many bacterial species. Note also that an enzyme implicated in generation of CH_2H_4folate – serine transhydroxymethylase (SHT) – has a universal phylogenetic distribution. Abbreviations: CH_2H_4folate, methylenetetrahydrofolate; DHFR?, a postulated alternative dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; FAD^+, flavin adenine dinucleotide (oxidized form); $FADH_2$, flavin adenine dinucleotide (reduced form); Gly, glycine; H_2folate, dihydrofolate; H_4folate, tetrahydrofolate; Ser, serine; ThyA and ThyX, thymidylate synthase A and X, respectively.

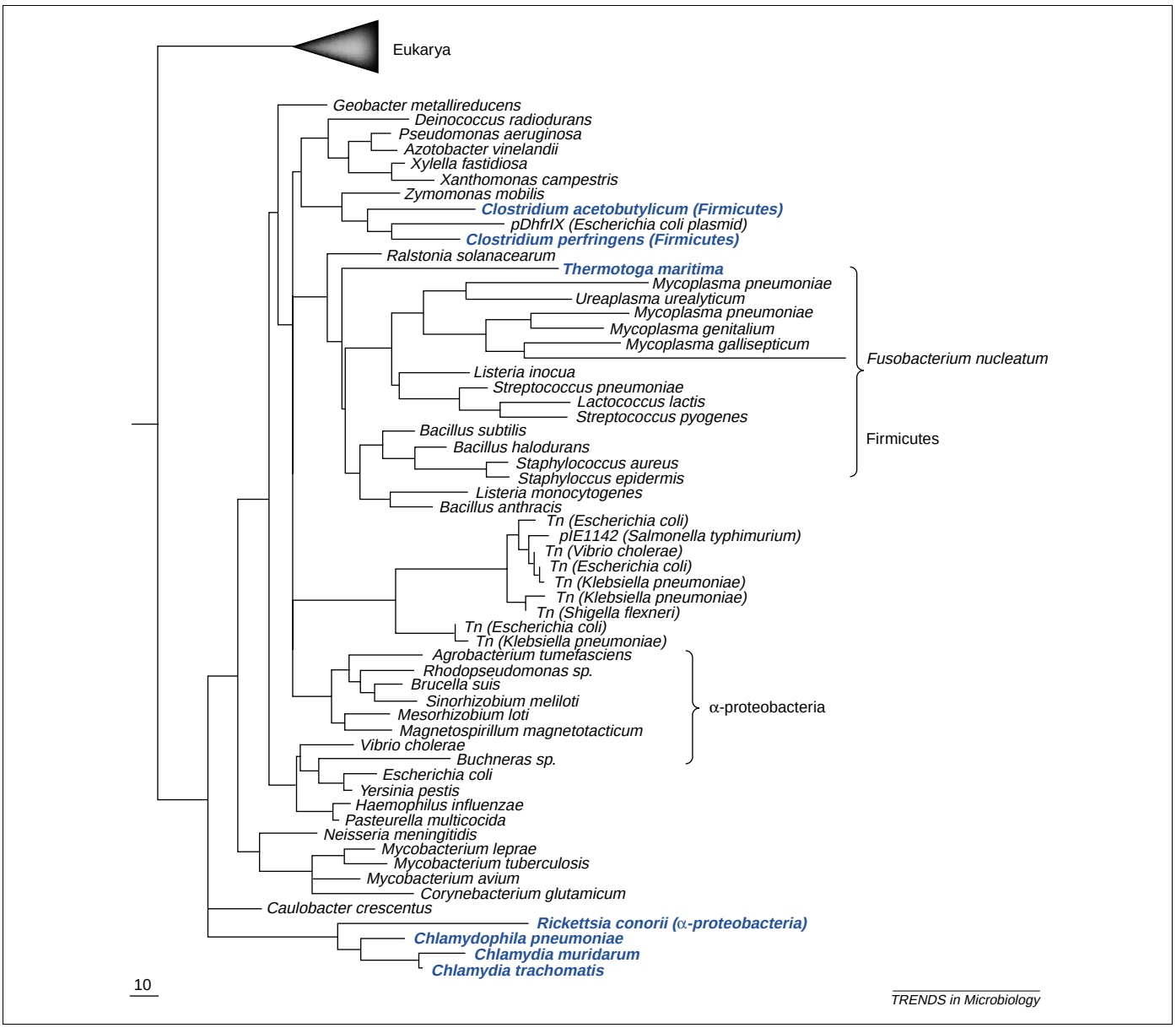

Fig. 3. Phylogenetic analysis of various FolA sequences. The maximum likelihood analysis was performed as described in the text [3]. Bacterial species indicated in red correspond to species containing FolA and ThyX proteins. The presence of FolA in ThyX-containing *Rickettsia conorii* and *Clostridium* spp. could result from a lateral gene transfer event. The tree was rooted using eukaryotic sequences. Abbreviation: Tn, transposon.

suspected earlier, but before the discovery of ThyX, their physiological relevance was largely ignored. For instance, *E. coli* strains carrying inactive *folA* are still viable [5] and contain reduced folates [6]. Direct evidence for this unexpected source of H_4folate in *E. coli* Δ*folA* strains is lacking, although it has been proposed to result from the presence of an alternative dihydrofolate reductase in this species. The identity of this putative enzyme is unclear, although it could correspond to an *E. coli* enzyme that was originally described as 'dihydropteridine reductase', which is able to reduce *in vitro* H_2folate to H_4folate, albeit very inefficiently [7]. In our opinion, this low level of dihydrofolate reductase activity is not sufficient to explain the surprisingly high amount of reduced folates in *E. coli* Δ*folA* strains (60–80% compared with that found for wild-type strains [6]). In addition, we have now noticed that the N-terminal protein sequence of *E. coli* dihydropteridine reductase (accession number gi:78392) identifies this protein as oxygen-insensitive NAD(P)H nitroreductase (NfsB). As genetic data have revealed a role for NfsB in mediating microbial resistance to several nitro-substituted compounds [8], it is possible that this enzyme also plays a role in folate metabolism, although this is awaiting confirmation.

Helicobacter spp. and *Campylobacter* spp. that lack *folA* provide particularly interesting cases in the experimental identification of new, physiologically relevant pathways leading to the formation of H_4folate. Not only do *H. pylori* [3] and *C. jejuni* [9] use thymidylate synthase ThyX for dTMP synthesis, but they are also considered endogenously resistant to low levels of trimethoprim, a classical inhibitor of bacterial FolA [10]. The molecular basis for this chromosomally encoded trimethoprim resistance of ε-proteobacteria is poorly understood. However, salvage of

thymidine compounds from the growth medium is unlikely to contribute to trimethoprim resistance, and genomic data (not shown) indicate the absence of the trimethoprim-insensitive plasmid-encoded family 2 dihydrofolate reductase [2] in these species. Taken together, these observations suggest that the uncharacterized enzyme, which is only poorly inhibited by trimethoprim, synthesizes H_4folate in ε-proteobacteria and possibly in many other bacterial species.

Interestingly, a subgroup of *thyX*-containing organisms also contains the *folA* gene (Table 1), revealing that parallel pathways for H_4folate synthesis are not necessarily mutually exclusive, assuming that FolA has not functionally replaced the predicted alternative dihydrofolate reductase. The simultaneous presence of *thyX* and *folA* in a given genome could result either from the non-orthologous replacement of *thyA* by *thyX*, or, alternatively, from transfer of *folA* into a *thyX*-containing organism. Phylogenetic analysis of FolA (Fig. 3) supports this latter possibility. In particular, our data indicate that the position of *Rickettsia conorii* FolA is the result of lateral gene transfer, because its position is far away from all the other α-proteobacteria. They further indicate that FolA in *Clostridium* spp. is closely related to a FolA variant encoded by an *E. coli* plasmid (Fig. 3). Unfortunately, the phylogenetic positions of *Chlamydia* spp. and *Thermotoga maritima* have not yet been firmly established, thus preventing a firm conclusion regarding the origin of their *folA* genes. It is also worth noting that the transfer of a transposon-coded *folA* into clinical isolates of *C. jejuni* has been demonstrated previously [10].

ThyX and alternative dihydrofolate reductase as ideal drug targets

For several reasons, ThyX proteins and the postulated alternative dihydrofolate reductase are ideal targets for compounds specifically inhibiting microbial growth. Not only is *thyX* present in several pathogenic bacteria lacking *folA* genes and absent in humans, but also the *de novo* synthesis of pyrimidine compounds is required for the growth and/or virulence of pathogenic microorganisms that often reside in pyrimidine-limited environments [11,12]. The development of ThyX inhibitors will be facilitated by the recently solved structure of the *Thermotoga maritima* ThyX protein [13], which has already revealed that the two classes of thymidylate synthases are completely unrelated structurally. Therefore, the design of ThyX inhibitors does not have to rely on small structural differences between ThyX and human ThyA proteins. Similarly, it is tempting to speculate that yet-to-be-identified alternative pathways and/or enzymes participating in bacterial metabolism of H_4folate will turn out to be unrelated to their functional counterparts in Eukarya.

Concluding remarks

The discovery of an alternative flavin-dependent mechanism for thymidylate synthesis has revealed a plausible explanation for the unexpected observation that life without two 'essential' enzymes – ThyA and FolA – is still possible. Further understanding of the hitherto poorly characterized folate metabolism in bacteria using ThyX for thymidylate synthesis will undoubtedly aid in understanding the evolution of intermediary metabolism, as well as in designing new compounds for inhibiting microbial growth.

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