Catalytic Mechanism of *Chlamydia trachomatis* Flavin Dependent Thymidylate Synthase

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Summary

Here we report on a Chlamydia trachomatis gene that complements the growth defect of a thymidylate synthase deficient strain of Escherichia coli. The complementing gene encodes a 60.9 kDa protein which shows low level primary sequence homology to a new class of thymidylate synthesizing enzymes, termed flavin dependent thymidylate synthases (FDTS). Purified recombinant chlamydial FDTS (CTThyX) contains bound flavin. Results with site directed mutants indicate that highly conserved arginine residues are required for flavin binding. Kinetic characterization indicates that CTThyX is active as a tetramer with NADPH, CH₂H₄folate, and dUMP required as substrates, serving as source of reducing equivalents, methyl donor, and methyl acceptor, respectively. dTMP and H₄folate are products of the reaction. Production of H₄folate rather than H₂folate, as in the classical thymidylate synthase reaction, eliminates the need for dihydrofolate reductase explaining the trimethoprim resistant phenotype displayed by thyA E.coli expressing CTThyX. In contrast to the extensively characterized thyA encoded thymidylate synthases, which form a ternary complex with substrates dUMP and CH₂H₄folate and follow an ordered sequential mechanism, CTThyX follows a ping-pong kinetic mechanism involving a methyl enzyme intermediate. Mass spectrometry was used to localize the methyl group to a highly conserved arginine and site directed mutagenesis showed this arginine to be critical for thymidylate synthesizing activity. These differentiating characteristics clearly distinguish FDTS from ThyA, making this class of enzymes attractive targets for rational drug design.
Introduction

Chlamydiae are obligate eubacterial intracellular parasites consisting of four species (1-4). *Chlamydia trachomatis* is primarily a human pathogen. There are numerous serovars with serovars D-K being a leading cause of bacterial sexually transmitted disease, and serovars A-C responsible for trachoma, which is the number one cause of infectious blindness (3). *C. pneumoniae* is a common human pathogen causing acute infection of the respiratory tract and has been identified as a potential risk factor for cardiovascular disease (5). *C. psittaci* and *C. pecorum*, are responsible for a variety of diseases in avian and animal species (6,7). Chlamydiae are extremely successful intracellular pathogens, in part due to their unique biphasic developmental cycle consisting of two morphologically and biochemically distinct forms. The elementary body (EB) is the metabolically inactive extracellular form capable of initiating infection. The reticulate body (RB) is the metabolically active intracellular form, which divides by binary fission within the confines of a membrane bound vacuole, termed an inclusion (1).

Chlamydiae are capable of transporting NTPs but not dNTPs directly from the host cell (8-10). Chlamydiae contain *nrdA* and *nrdB*, encoding the two subunits of ribonucleotide reductase required for the conversion of ribonucleotides to deoxyribonucleotides. Ribonucleotide reductase accounts for the acquisition of three (dCTP, dGTP and dATP) of the four deoxyribonucleotides needed for DNA biosynthesis. The fourth nucleotide, thymidine triphosphate (dTTP), is produced by two well-known processes. Exogenous thymidine can be directly salvaged by thymidine kinase (TK) or dTMP can be synthesized de novo from dUMP, a reaction catalyzed by thymidylate synthase (ThyA) (11,12).

In an earlier study, using mutant cell lines with deficiencies in TK and dihydrofolate reductase (DHFR) as host, we reported that *C. trachomatis* was capable of incorporating...
exogenously added uridine into thymidine nucleotides, a result implying the existence of a thymidylate synthase and a dihydrofolate reductase (13). Interestingly, subsequent in silico analyses of whole genome sequence data indicated that chlamydiae encode a DHFR homologue, however, there was no homologue for TK or ThyA (14-18). This left open the question of how chlamydiae obtain thymidine nucleotides.

Until recently, ThyA was thought to represent the only enzyme capable of catalyzing the de novo formation of dTMP in vivo. ThyA carries out the reductive methylation of deoxyuridine 5’-monophosphate (dUMP), using methylenetetrahydrofolate (CH$_2$H$_4$folate) as a one-carbon donor and source of reducing equivalents, generating dTMP and dihydrofolate (H$_2$folate) as products. Since reduced folates are essential for many biochemical processes, H$_2$folate is rapidly reduced to H$_4$folate by DHFR with subsequent regeneration of CH$_2$H$_4$folate being catalyzed by serine hydroxymethyltransferase (11,19). Together these reactions are known as the thymidylate cycle.

Recently the existence of a novel family of thymidylate synthesizing enzymes, called thymidylate synthase complementing proteins or flavin dependent thymidylate synthases (FDTS), encoded by $\text{thyX}$, has been described (19-24). All members of the family contain a conserved ThyX motif consisting of (T/RHRX$_7$-8S) (19-21,23). The first discovered member of this family was a gene encoding a protein shown to complement a thymidine-requiring mutant of Dictyostelium discoideum (25). in silico analyses indicate that homologues of FDTS are present in upwards of 30% of sequenced microbial genomes (19,20,26,27). Interestingly, many of the genomes containing ThyX lack DHFR (19,26). This information coupled with the observation that NADH or NADPH are required for Helicobacter pylori FDTS dTMP synthesis activity has led to the suggestion that CH$_2$H$_4$folate acts solely as a one-carbon donor producing H$_4$folate as
the product, allowing for the conservation of reduced folates (19-21, 24, 26, 28). It has recently been demonstrated that the reduced pyridine nucleotide required in the FDTS reaction is involved in reducing the enzyme associated FAD molecule prior to substrate interaction with the enzyme (24). This involvement of an alternative reducing agent in FDTS reactions could account for the lack of a DHFR homologue in many organisms encoding thyX.

Several years ago we isolated a clone from a C. trachomatis genomic DNA library that complemented an E. coli thyA mutant to thymidine prototrophy (29). The complementing open reading frame (ORF) showed no homology to proteins deposited in the public databases at that time. Results from the C. trachomatis serovar D genome sequencing project (14) indicated that the complementing ORF is encoded by CT632 and subsequent sequencing projects showed that CT632 is highly conserved in all chlamydiae species (14-18). Cross species in silico analyses have shown that, while CT632 (60.9 kDa) is twice as large as typical FDTS (≈26-30 kDa) proteins and shows very low primary sequence homology to them, it does contain a partial sequence motif characteristic of FDTS proteins (19, 20). In the current study we report the kinetic characteristics of chlamydial FDTS.

Experimental Procedures

Chemicals, bacterial strains and plasmids

[6-^3^H]dUMP (22 Ci/mmol), and [5-^3^H]dUMP(15 Ci/mmol) were purchased from Moravek Biochemicals, Brea, CA. H$_2$folate, H$_4$folate, dTMP, dUMP, NADPH, NADH, FMN, FAD and thymidine were purchased from Sigma Chemical Co. CH$_2$H$_4$folate was donated by Merck Eprova (Schaffhausen, Switzerland). For H$_4$folate and CH$_2$H$_4$folate the biologically relevant R stereoisomers were used in all assays. All other chemicals were of reagent grade or
better. *E.coli* DH5α [*supE44 lacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] is routinely maintained in our laboratory and *E. coli* MH2707 (*LAM* e14* thi-1 relA1 thyA114(Stable)::Mu) was obtained from the *Escherichia coli* Genetic Stock Center (New Haven, Ct.). The pQE80L plasmid was purchased from Qiagen.

**Cloning of C. trachomatis ThyX and E.coli ThyA**

*C. trachomatis* thyX was PCR amplified from purified chromosomal DNA as described previously (30). The PCR primer sequences for CTThyX are (forward-5’-CCCAGTACCAGTGGAGCAAGAG-3’ and reverse-5’-CCCAAGCAGTTAAGACTTACG-3’) and were designed to include unique KpnI and HindIII restriction sites (underlined) for cloning. The PCR products were gel-purified, restricted then ligated into the expression vector pQE80-L, which had been cut with the same restriction enzymes. Constructs were transformed into DH5α for screening, purified by miniprep and then used to transform MH2720 *E. coli* for complementation and expression of recombinant his-tagged CTThyX.

**Expression and purification of recombinant C. trachomatis FDTS**

*E. coli* strains DH5α and MH2720 transformed with wild type or various mutant CTThyX plasmid constructs were grown in 1 L of LB media containing 100 µg/ml of ampicillin at 37°C to an O.D of 0.6 at 595 nm. The plasmids were induced by the addition of IPTG to a concentration of 1 mM and further incubated at 37°C for 3 hours. Cultures were harvested by centrifugation and the pellet was resuspended in 20 ml of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9) and frozen at -80°C. After thawing on ice the cells were lysed by
sonication in the presence of lysozyme (at a final concentration of 350 µg/ml). Cell lysates were clarified by ultracentrifugation at 45 000 rpm in a Beckman Ti60 rotor for 2 hrs at 4°C. All remaining steps were carried out at 4°C. Recombinant his-tagged protein was purified from the supernatant by metal chelation chromatography. Briefly, clarified lysates were passed through a 3 ml activated nickel metal chelation column followed by washing with 15 ml of binding buffer, and 15 ml of wash buffer (60 mM imidazole, 500 mM NaCl, 20 ml Tris-HCl pH 7.9). The bound recombinant protein was then eluted off the column with elution buffer (200 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9). Glycerol was added to the purified protein at a final concentration of 10% to prevent precipitation. Purified CTThyX was dialyzed overnight at 4°C in the dark against 50 mM Tris-HCl pH 7.2 containing 5% glycerol. Samples were then aliquoted and stored at –80°C.

**Measurement of protein concentration**

Protein concentrations were estimated using the Bio-Rad protein assay based on the dye-binding procedure of Bradford using bovine serum albumin as the standard (31).

**Spectroscopic analysis**

The absorption spectrum of FAD bound to wild type and mutant CTThyX was determined. FAD was extracted from purified CTThyX by incubating at 95°C for 10 min in the dark. Precipitated protein was pelleted by centrifugation at 10,000 rpm (Eppendorf centrifuge 5417C) for 10 min. The absorption spectra of the released flavin present in the supernatant was determined spectrophotometrically (Beckman Du62 Spectrophotometer) from 250 nm to 750 nm in a 1 cm quartz cuvette (19).
Site-directed mutagenesis

The pQE80L plasmid containing wild type *C. trachomatis* thyX was used as a template for site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Primers containing the desired mutations were designed based on the consensus nucleotide sequence of the *C. trachomatis* serovar D CT632 gene (thyX) (GenBank accession number AE001334) and are shown in Table 1. All plasmid constructs were confirmed for appropriate mutations by DNA sequencing.

Complementation

MH2720 cells transformed with pQE80L-CTThyX, pQE80L-CTThyXR124A, pQE80L-CTThyXS133A, pQE80L-CTThyXR397A, pQE80L-CTThyXR477A or pQE80L (no insert, vector control) were grown overnight at 37°C in LB containing 100 µg/ml ampicillin and 40 µg/ml thymidine. One ml of the overnight culture was centrifuged at 6000 rpm for 5 min (Eppendorf centrifuge 5417C), and the supernatant removed. The pellet was then washed three times in ice cold sterile phosphate buffered saline (PBS). The cells were streaked onto minimal medium plates containing IPTG (200 µl of 100 mM IPTG added to the surface and allowed to dry) and either no thymidine or 40 µg/ml thymidine. The plates were incubated at 37°C overnight and then photographed.

High performance liquid chromatography

Nucleotides were separated by high performance liquid chromatography (HPLC) using a 12.5 cm C18 reverse phase column under isocratic conditions with a flow rate of 1 ml/min, the mobile phase consisted of 5 mM potassium phosphate buffer, pH 7.0, 5 mM tetra-butyl-
ammonium dihydrogen phosphate and 5% (v/v) acetonitrile (32). Radioactive peaks were detected by monitoring in-line radioactive flow (171 detector; Beckman Instruments). The identities of the radioactive peaks were confirmed by simultaneously monitoring the A$_{260}$ (1066 UV detector; Beckman Instruments) of dTMP and dUMP standards. Folates were separated using a 25 cm C$_{18}$ column under isocratic conditions with a mobile phase consisting of 10 mM ammonium phosphate, pH 7.0, 5 mM tetrabutyl ammonium phosphate, 5% acetonitrile, 20% methanol and a flow rate of 1 ml/min. The buffer was extensively degassed then maintained in a nitrogen gas environment in order to enhance the stability of the reduced folates. Peaks were detected by monitoring the A$_{295}$. The identities of the individual peaks were determined by comparison with known H$_2$folate, H$_4$folate and CH$_2$H$_4$folate standards.

Thymidylate synthesis assay

Thymidylate synthesizing activity was determined by monitoring the amount of tritium transferred to water using [5-$^3$H]dUMP as substrate similar to that described for assaying thymidylate synthase activity and H. pylori FDTS activity (20,33). All reactions were performed at 37°C in a nitrogen gas environment to limit reduced folate decomposition. The optimized standard reaction mixture contained, in a total volume of 100 µl, 50 mM Tris-HCl pH 7.2, 200 µM 5, 10-CH$_2$H$_4$folate, 2 mM NADPH, 200 µM [5-$^3$H]dUMP (15 µCi/ml), 5% glycerol. The reaction was initiated by the addition of 2.5 µg of purified CTThyX. After 3 minutes the reaction was terminated by the addition of 300 µl of a 100 mg/ml activated charcoal suspension containing 2% TCA, to remove the unused radiolabelled substrate [5-$^3$H]dUMP. The samples were mixed at room temperature for 1 hr and then centrifuged at 14000 rpm (Eppendorf centrifuge 5417C) for 5 min to pellet the charcoal. Radioactivity in the supernatant was
quantitated in a Beckman LS 5000 liquid scintillation counter after the addition of 5 ml of scintillation fluid (Universol, ICN Biomedical). One unit of enzyme activity corresponds to the production of 1 $\eta$mol of dTMP synthesized per min under optimum assay conditions. For the thymidylate synthesizing half reaction a standard reaction mixture was used except that the dUMP was omitted. *E. coli* thymidylate synthase reaction was carried out as previously described (11).

**Kinetic analysis**

Substrate saturation kinetics were determined for dUMP, $\text{CH}_2\text{H}_4\text{folate}$ and NADPH. The kinetic reaction for each substrate was carried out at fixed saturating concentrations of the other two substrates. In all cases, at least 11 different concentrations of the variable substrate were used in each set of experimental assays.

Multiple substrate kinetics were determined by varying the concentration of dUMP (0, 2.5, 10, 20, 40 and 75 $\mu$M) in the presence of various fixed concentrations of $\text{CH}_2\text{H}_4\text{folate}$ (10, 25, 50, 100 and 200 $\mu$M). Product inhibition kinetics for dUMP were determined by varying the concentration of dUMP (0, 2.5, 10, 20, 40 and 75 $\mu$M) in the presence of varying fixed concentrations of either dTMP (0, 10, 25, 50 and 100 $\mu$M) or $\text{H}_4\text{folate}$ (0, 10, 25, 50 and 100 $\mu$M). $\text{CH}_2\text{H}_4\text{folate}$ (200 $\mu$M) and NADPH (2 mM) were present at saturating concentrations. Product inhibition kinetics for $\text{CH}_2\text{H}_4\text{folate}$ were determined by varying the concentration of $\text{CH}_2\text{H}_4\text{folate}$ (0, 10, 20, 50, 100 and 200 $\mu$M) in the presence of varying fixed concentrations of dTMP (0, 25, 50, 100 and 200 $\mu$M) or $\text{H}_4\text{folate}$ (0, 25, 50, 100 and 200 $\mu$M). dUMP (200 $\mu$M) and NADPH (2 mM) were present at saturating concentrations.
All the measurements were made in triplicate, and the mean and standard error of the mean (SEM) were determined using GraphPad PRISM 3.0 software. The Michaelis-Menten equation was used when hyperbolic kinetics were obtained; $K_m = \text{the substrate concentration giving one-half the maximal velocity (} V_{max} \text{)}$ (34). Lineweaver-Burk transformations were used for the determination of the inhibition mechanism. These calculations were fitted using a non-linear least-squares regression and linear least-squares regression computer kinetics program supplied by GraphPad PRISM 3.0 software.

**Mass Spec Analysis**

To determine where chlamydial FDTS was methylated highly purified CTThyX was used as a source of enzyme in a thymidylate synthesizing half reaction. Following incubation for 5 minutes at 37°C SDS sample buffer was added and the sample was run on a 10% SDS-PAGE gel. The FDTS band was excised and sent to the Scripps Center for Mass Spectrometry (La Jolla, CA, [http://masspec.scripps.edu](http://masspec.scripps.edu)) for analysis.

**Results**

*Identification of a *C. trachomatis* thymidylate synthase complementing protein*

We employed functional complementation to clone a *C. trachomatis* gene which rescues the thymidine auxotrophy of an *E. coli thyA* mutant, MH$_{2720}$ (Figure 1a) (29). Subsequent sequencing and *in silico* analyses indicated that the encoded open reading frame corresponds to *C. trachomatis* CT632, a highly conserved 60.9 kDa chlamydial protein. The amino acid identity between CT632 homologues identified from all sequenced chlamydial genomes is between 71% and 91% (14-18). CT632 shows very low primary sequence homology with the newly identified
ThyX family of folate dependent thymidylate synthases (19,20) (Figure 2). CTThyX (60.9 kDa) is also approximately twice the molecular weight of most other FDTS proteins (≈26-30 kDa). From *in silico* analyses, it has been suggested that the CTThyX is a fusion of two individual subunits (20). While much less common than the smaller FDTS proteins, which occur in upwards of 30% of all sequenced bacterial genomes, there are several homologues to CTThyX in the protein database and all contain at least a partial ThyX motif (T/RHRX7-8S) (19-21,23,27) (Figure 2b). FDTS from *C. trachomatis* does not contain a fully conserved ThyX motif, as indicated in the amino acid sequence alignment (Figure 2). There are two regions, one in the N-terminal region and one in the C-terminal region that have a partial ThyX motif, and that may complement each other in the tertiary structure to form the complete motif. The N-terminal region contains DARX₈S, while the C-terminal region contains RHRX₇/₈L/L.

In contrast with the phenotype displayed by recombinant *E. coli* expressing *E. coli* ThyA, CTThyX confers trimethoprim resistance to an *E. coli* thyA mutant strain (Figure 1b), a similar result was reported for *H. pylori* ThyX (19,24). Similar to ThyA expressing recombinant *E.coli*, CTThyX recombinants were still sensitive to sulphonamide and sulphamethoxazol-trimethoprim (Figure 1b) and 5-fluorodeoxyuridine (Figure 1c), characteristics typical of classical thymidylate synthases (11).

*Expression, purification and spectroscopic characterization of C. trachomatis FDTS*

Recombinant *C. trachomatis* FDTS was over expressed in *E. coli* MH₂⁷₂₀, using the pQE80L expression system. Subsequent purification by nickel affinity chromatography typically yielded 5 to 10 mg/liter of soluble purified CTThyX. On SDS-polyacrylamide gel electrophoresis recombinant CTThyX showed one predominant band with an apparent molecular
mass of ≈ 61 kDa (Figure 3a). The purified protein was bright yellow in color, suggesting the presence of an enzyme bound flavin molecule, similar to that reported for *H. pylori* and *T. maritima* FDTS (19,21). Spectroscopic studies on the flavin extracted from the enzyme indicated that it was in fact an oxidized flavin, with the characteristic flavin peak at 260 nm, and peaks at 375 and 450 nm indicating that it is in the oxidized form (Figure 3b) (19,21,24). These results are analogous to those obtained for the *H. pylori* and *T. maritima* ThyX where an oxidized flavin is bound to the ThyX protein (19,24). Further HPLC analysis comparing the isolated flavin to FAD and FMN standards demonstrated that the enzyme bound flavin is in fact FAD (data not shown). Native polyacrylamide gel electrophoresis revealed a native molecular mass of ≈ 250 kDa, indicating that the native protein is in the form of a tetramer (data not shown). This is similar to the *H. pylori* FDTS which has also been shown to exist as a tetramer, in contrast *E. coli* ThyA forms dimers (11,19,21,35).

*Optimization of the dTMP synthesizing activity of C. trachomatis FDTS*

To conclusively demonstrate that purified recombinant CTThyX was capable of catalyzing the formation of dTMP from dUMP an in vitro assay was developed and optimized. CTThyX dTMP formation activity was absolutely dependent on dUMP, CH$_2$H$_4$folate, and a reduced pyridine nucleotide (NADH or NADPH). With regards to pyridine nucleotides, the maximal velocity with NADH ($V_{\text{max}} = 88.57$) is less than half that with NADPH ($V_{\text{max}} = 192.84$). Since NADPH is the preferred substrate it was used in all subsequent assays. CTThyX showed maximal activity at pH 7.2 and activity was linear with respect to time and enzyme concentration (data not shown).
Folate oxidation of C. trachomatis FDTS

It has been proposed that like FADH₂-dependent ribothymidyl synthase (36), FDTS uses CH₂H₄folate solely as a one-carbon donor with enzyme bound flavin serving as the source of reducing equivalents (19-22,24,26,28). To directly determine if H₄folate is a product of the CTThyX catalyzed reaction we analyzed the folate products by HPLC. We compared the activity of CTThyX with that of E. coli ThyA, to demonstrate the difference between the two families of proteins. In contrast to the ThyA (Figure 4a), which produces H₂folate, CTThyX produces H₄folate as a product of the thymidylate synthesizing reaction (Figure 4b).

Saturation kinetics

CTThyX displayed Michaelis-Menten kinetics with respect to dUMP, CH₂H₄folate and NADPH. Figure 5 shows that CTThyX exhibits hyperbolic kinetics with respect to varying concentrations of dUMP, CH₂H₄folate or NADPH, respectively under saturating conditions of the other two substrates. Apparent Kₘ values for dUMP, CH₂H₄folate and NADPH are 5.99 ± 0.54 µM, 22.66 ± 2.02 µM, and 60.80 ± 4.86 µM, respectively. Vₘₐₓ is approximately 165 ± 3.3 7 n mol dTMP produced/min/mg. The kₜₐₜ was determined to be 39.98 ± 1.77 min⁻¹.

Two-substrate kinetics

The ThyA catalyzed reaction follows an ordered sequential mechanism (11,37). To determine the kinetic mechanism of CTThyX dTMP synthesis activity was measured at several fixed concentrations of CH₂H₄folate while varying the concentration of dUMP. The results shown in the double reciprocal plot is that of several parallel lines indicating that CTThyX follows a ping-pong kinetic mechanism (Figure 6a). For comparison the two-substrate kinetics
of *E. coli* ThyA was carried out at several fixed concentrations of CH$_2$H$_4$folate while varying dUMP concentrations. The results presented in Figure 6b show lines intersecting to the left of the Y-axis, indicating a sequential mechanism.

*Product inhibition*

To deduce the order of substrate binding and product exit from the active site, enzyme kinetics were carried out in the presence of varying concentrations of dTMP and H$_4$folate. With dUMP as the variable substrate, hyperbolic saturation curves were obtained when CTThyX was assayed in the presence of various fixed concentrations of dTMP. Increasing the concentration of dTMP increased the K$_m$ for dUMP and decreased the V$_{max}$. Double reciprocal plots of the saturation data for the different dTMP concentrations gave a series of straight lines, which intersect at a point to the left of the y-axis indicating that dTMP is a mixed non-competitive inhibitor with respect to dUMP (Figure 7a). The inhibition constant (K$_i$) was determined to be 10 µM. Hyperbolic saturation curves were also obtained for CTThyX when dUMP was the variable substrate in the presence of several fixed concentrations of H$_4$folate having a K$_i$ of 170 µM. Increasing the concentration of H$_4$folate increased the K$_m$ of CTThyX for dUMP but had no effect on the V$_{max}$. The double reciprocal plots of the saturation data for the different concentrations of H$_4$folate gave a series of straight lines, which intersect at a point on the y-axis (Figure 7b) indicating competitive inhibition between H$_4$folate and dUMP.

With CH$_2$H$_4$folate as the variable substrate, hyperbolic saturation curves were obtained when CTThyX was assayed in the presence of fixed dTMP or H$_4$folate concentrations. Increasing the concentration of dTMP increased the K$_m$ of CTThyX for CH$_2$H$_4$folate and had no effect on the V$_{max}$ of the enzyme, giving a K$_i$ of 290 µM. A double reciprocal plot of the
saturation data for the different concentrations of dTMP show a series of straight lines intersecting at the y-axis (Figure 7c). These results show that dTMP is a competitive inhibitor with respect to CH₂H₄folate. Increasing concentrations of H₄folate increased the Kₘ for CH₂H₄folate but decreased the Vₘₐₓ of CTThyX, giving a Kᵢ of 115 µM. The double reciprocal plot again produced a series of straight lines but the lines intersect the x-axis to the left of the y-axis (Figure 7d) indicating that H₄folate follows classical non-competitive inhibition with respect to CH₂H₄folate. The pattern of product inhibition for CTThyX is again consistent with a ping-pong kinetic mechanism.

*C. trachomatis* FDTS half reaction

A characteristic feature of ping-pong kinetic mechanisms is that a secondary product is released from the enzyme, prior to the final substrate entering the active site. In the case of the CTThyX, H₄folate would be generated and released from the enzyme active site, prior to dUMP entering. To verify that CTThyX does follow a ping-pong mechanism as demonstrated by the enzyme kinetics, a standard CTThyX assay was carried out in the absence of dUMP the methyl acceptor. The production of H₄folate was followed by HPLC. As shown in Figure 8, H₄folate is produced in an enzyme dependent manner in the absence of dUMP.

*Site-directed mutagenesis*

As indicated in Figure 2, CTThyX has two regions each containing a portion of the conserved ThyX motif. To determine the functional role of these two regions in FDTS catalysis, we employed site-directed mutagenesis. Several site-specific mutations were constructed as summarized in Table 2 and shown in Figure 2. R124 and S133 of the N-terminal region (*T.
*T. maritima* R80 and S88) as well as R397 (equivalent to *T. maritima* R80) in the COOH terminal ThyX motif were all mutated to alanine.

The ability of the various mutant CTThyX proteins to functionally complement the growth defect of an *E. coli* thyA mutant was assessed. The results presented in Figure 9 indicate that all of the mutant ThyX proteins lost their ability to complement the thymidine auxotrophy of a thyA⁺ strain of *E. coli*. To explore the loss of activity in more detail, we over expressed and purified the CTThyX mutant proteins. Two of the mutant proteins, (R124A and R397A) lacked the yellow color characteristic of flavin containing enzymes. Absorption spectroscopy confirmed that mutation of these conserved arginine residues resulted in a loss of enzyme bound flavin (data not shown). *In vitro* dTMP synthesizing activity was assessed and as shown in Table 2, R124A and R397A CTThyX proteins were inactive, confirming the essential role of FAD for CTThyX activity. The S133A mutant protein retained its ability to bind flavin, however, the protein was inactive *in vitro*.

From the kinetic analysis CTThyX follows a ping-pong kinetic mechanism, which implies that there is a methyl-enzyme intermediate formed during the reaction. Mass spectrometry was employed to elucidate the peptide fragment that harbors the methyl group. MALDI-TOF was carried out on trypsin-digested CTThyX after the thymidylate synthesizing protein half reaction. Results from the analysis revealed a peptide corresponding to amino acid residues 469-477 (GLQWLCEL), which showed a mass shift of 14 amu following the methyl donating half reaction (Figure 10, peak 1188.6). The only residue within the identified peptide that is appropriately located in the active site to be a methyl donor, conserved in all FDTS proteins deposited in the public databases and that can accept a methyl group is R477. R477 was changed to alanine by site-directed mutagenesis. Figure 9 shows that the R477A CTThyX
protein lost its ability to complement an *E. coli thyA* mutant to thymidine prototrophy. The mutant protein was over expressed and purified, and was shown to retain the ability to bind FAD. In agreement with the complementation results, the mutant protein lacked *in vitro* dTMP synthesizing activity (Table 2).

**Discussion**

*in silico* analyses indicate that *C. trachomatis* open reading frame CT632 encodes a protein which is highly conserved (66% identity, 88.9% similarity) in all five chlamydial genomes (*C. trachomatis* serovar D, *C. muridarum*, *C. caviae*, *C. abortus*, *C. pneumoniae*) sequenced to date (14-16,18), including the more distantly related parachlamydia, a chlamydial-like symbiont of free living amoeba (38) and that it is distantly related to the FDTS family of proteins. Here we show that CT632 can complement a *thyA* mutant strain of *E. coli* and *in vitro* assays confirmed that the protein possesses thymidylate-synthesizing activity. Purified recombinant CT632 contains bound flavin. Thus taken together it is clear that CT632 encodes a chlamydial FDTS homologue. We demonstrated that CTThyX uses CH$_2$H$_4$folate strictly as a one-carbon donor, and employs an enzyme bound FAD molecule as source of reducing equivalents. Results from *in vitro* mutagenesis studies indicate that the highly conserved arginines (R124 and R397) are essential for flavin binding and FDTS activity. This is in agreement with structural studies with the *T. maritima* FDTS which show that the counterpart arginine (R80) interacts with FAD (21). Mutagenesis studies with *H. pylori* FDTS indicate that the earlier arginine in the ThyX motif (R74) is also essential for FAD binding and thymidylate synthesizing activity (20). For CTThyX the counterpart of *H. pylori* R74 is conserved in the COOH (R395) but not in the NH$_2$ (D122) terminal half of the enzyme. In addition, similar to
studies with H. pylori FDTS (20) our in vitro mutagenesis results indicate that CTThyX S133, the conserved serine in the NH2 terminal ThyX motif [(T/RHRX7-8S) H. pylori S84, T. maritima S88] is essential for thymidylate synthesizing activity. It has been proposed that this serine activates dUMP by nucleophilic attack at its C-6 position (20,24). Interestingly, a leucine occupies the seventh and eighth amino acid positions downstream of the COOH terminal ThyX motif in chlamydial FDTS.

In using CH2H4folate solely as a one-carbon donor, H4folate is produced and the need for reduced folate recycling by DHFR is eliminated (Figure 11a). This would account for the absence of folA in many of the thyX containing genomes (19) and the trimethoprim resistant phenotype displayed by thyA mutant E. coli strains expressing recombinant H. pylori FDTS and CTThyX. Interestingly all chlamydiae encode a DHFR homologue (14,15,18). This is not surprising as C. trachomatis can carry out de novo folate synthesis with DHFR being an essential enzyme in this pathway (39). Presumably other organisms that contain a FDTS homologue, but lack DHFR are auxotrophic for reduced folates or use an alternate enzyme, with little or no homology to DHFR, for reducing folate.

Substrate saturation kinetics demonstrated that CTThyX follows standard Michaelis-Menten kinetics with respect to dUMP, CH2H4folate and NADPH. The apparent K_m values for CTThyX value for dUMP was determined to be 5.99 µM± 0.54 µM, which is comparable to FDTS of Chlorella virus-1 (K_m = 15 µM) (40) and ThyA of E. coli (K_m = 10 µM) (41) and L. casei (K_m = 0.7 µM) (37). The CTThyX K_m value for CH2H4folate was determined to be 22.66± 2.02 µM, which is similar to that reported for Chlorella virus-1 (K_m = 20 µM) FDTS and L casei (K_m = 14 µM) ThyA (37) but much lower than that reported for T. maritima FDTS (K_m = 4mM) (24) and ThyA of E. coli (K_m = 14 mM) (41). The CTThyX V_max was estimated to be 165 ± 3.3 7
ηmol dTMP produced/min/mg, which is significantly less than in other organisms, with that of *E. coli* being 1.45 µmole dTMP produced/min/mg and that of *L. casei* being 4.39 µmole dTMP produced/min/mg (37,41). The *kcat* of CTThyX was determined from the saturation kinetics to be approximately 40.0 min⁻¹ (0.67 s⁻¹), similar to that of Chlorella virus-1 (40) whereas; the *kcat* for ThyA has been reported to be approximately 300 min⁻¹ (5 s⁻¹) (11). For comparison, with another slow growing organism, the *kcat* for the *H. pylori* ThyX has been reported to be 0.46 min⁻¹ (20). The difference in the specific activity may be attributed to the growth rate of the individual organisms. Faster growth rates such as that for *E. coli*, with a doubling time of 20 min, would require more efficient thymidylate synthesis to prevent a shortage of dTTP during DNA replication. However, *C. trachomatis* has a much slower growth rate with a doubling time of approximately 2 hours. The slower growth rate could account for the reduced need for rapid dTMP synthesis, allowing for an enzyme with much lower specific activity.

One distinguishing characteristic of ThyA is its ability to form a stable ternary complex between the enzyme, CH₂H₄folate and 5-fluorodUMP (FdUMP), a mechanism based inhibitor that prevents catalysis. This observation is consistent with the ordered sequential catalytic mechanism of ThyA (11,33,37,42). A Cleland plot depicting the ordered reaction sequence for ThyA is shown in Figure 11b (33,37). In contrast, our kinetic analyses demonstrate that the dTMP synthesis reaction catalyzed by CTThyX follows a ping-pong (double displacement) mechanism, which differs markedly from that of ThyA. From the studies of Agrawal *et al* with *T. maritima* FDTS it has been determined that the first substrate to bind to the enzyme is NADPH (see below) (24). From our studies with CTThyX we propose that the next substrate to enter the enzyme active site is CH₂H₄folate, at which point the methyl group is transferred from CH₂H₄folate to the enzyme, producing H₄folate and a methyl enzyme intermediate. Through the
use of MALDI-TOF we have demonstrated that R477 is the most likely methyl group acceptor. *in silico* analysis indicates that the counterpart of CTThyX R477 is absolutely conserved in all FDTS sequences deposited in the protein databases. Furthermore, our *in vitro* mutagenesis results indicate that R477 is essential for thymidylate synthesizing activity as assessed by complementation and *in vitro* enzyme assay.

The crystal structure of *T. maritima* FDTS indicates that R477 (*T. maritima* R174) is located in the active site of the enzyme (21). The only rearrangement that has to take place is a 180 degree rotation of the dUMP base to put R174 (Chlamydia R477) within 2.8 Å of the carbon (dUMP C-5) that is going to be methylated. Following the transfer of the methyl group from CH$_2$H$_4$folate, H$_4$folate exits the active site, allowing dUMP to enter with subsequent transfer of the methyl group from R477 to the C-5 position of dUMP producing dTMP. dTMP then exits the active site. A ping-pong mechanism is consistent with our inability to detect a ternary complex when purified recombinant CTThyX was incubated with CH$_2$H$_4$folate and 5FdUMP (data not shown), since both substrates are never in the active site at the same time during catalysis. The ping-pong mechanism has also been confirmed by carrying out the enzyme assay in the absence of dUMP (half reaction) and following the formation of H$_4$folate. With the half reaction the formation of H$_4$folate was absolutely dependent on the presence of enzyme and NADPH (data not shown). The unique situation that arises with the ping-pong kinetic mechanism is that there is a methyl enzyme intermediate being formed.

In a recent study it was shown that Chlorella virus-1 FDTS catalyzed reaction occurs by a different mechanism (40). While the reaction requires the same three substrates (dUMP, CH$_2$H$_4$folate and NADPH) the order of binding is different and the mechanism is reported to be sequential rather than ping pong, as shown here for CTThyX. In the Chlorella virus FDTS
reaction, dUMP is the first substrate to bind and its binding is required for NADPH oxidation and subsequent reduction of the enzyme bound FAD. Following NADP⁺ release CH₂H₄folate binds to the active site, allowing for transmethylation of dUMP. In contrast, with *T. maritima* FDTS it has recently been shown that NADPH binding occurs first, enzyme bound FAD is reduced, NADP exists (first half reaction) then dUMP and CH₂H₄folate bind in a sequential fashion (random or ordered), followed by ordered release of H₄folate then dTMP (second half reaction), thus the overall reaction is ping-pong (24). The first half reaction was supported by experimental evidence while the second half reaction was proposed based on analogy to the classical ThyA reaction (24). Therefore unlike the CTThyX reaction, there is no methyl-enzyme intermediate and dUMP and CH₂H₄folate are proposed to bind in the active site at the same time. Interestingly, despite different proposed reaction mechanisms and low overall primary sequence homology all the FDTS enzymes have conserved the counterpart of the CTThyX methyl accepting R477 (*H. pylori* R174, *T. maritima* R174, Chlorella virus R182).

In summary, the results presented indicate that *C. trachomatis* contains an active thymidylate synthesizing enzyme that has been grouped into a novel family of proteins termed FDTS (19-22,24,26,28,40). We have demonstrated CTThyX employs CH₂H₄folate solely as a one-carbon donor, and use an enzyme bound FAD molecule and an external NADPH as the reducing agents required for reduction of the methyl moiety needed for dTMP biosynthesis. CTThyX catalytic reaction follows a ping-pong mechanism involving a methyl enzyme intermediate whereby R477 accepts the methyl group. Taking all the experimental data into consideration we propose the order of substrate binding and product release for CTThyX as depicted in the Cleland plot presented in Figure 11c.
Acknowledgements

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Figure Legends

Figure 1. Characteristics of C. trachomatis open reading frame CT632.

a) Analysis of CT632 protein function by genetic complementation. The gene encoding ORF CT632 was cloned into E. coli expression vector pQE-80L. Vector without insert was used as a control. Constructs were transformed into E. coli MH2720 (thyA−) and growth of the transformants was assessed on M9 minimal agar plus IPTG with or without added thymidine. b) Sensitivity of E. coli MH2720 transformed with C. trachomatis CT632 or E. coli thyA, as a control, to trimethoprim (5 µg), sulphonamide (300 µg) or trimethoprim (1.25 µg)/sulphamethoxazole (3.75 µg). Cultures were grown overnight in LB medium, spun down washed 3 times in PBS then spread on Mueller-Hinton agar plates containing 1mM IPTG and the indicated antibiotic discs. c) Sensitivity of E. coli MH2720 transformed with C. trachomatis CT632 or E. coli thyA to 5-fluorodeoxyuridine. Cultures were grown overnight in LB broth then diluted to an OD of approximately 0.2 in Mueller-Hinton broth plus 0.2 mM IPTG. The indicated concentrations of 5-fluorodeoxyuridine were added and growth was monitored by measuring the OD. (●) 0 µM, ( ) 0.62 µM, (■) 1.25 µM, (◇) 2.5 µM, (▲) 5 µM and (○) 10 µM FdUMP.

Figure 2. Comparison of various FDTS amino acid sequences. a) Helicobacter pylori, Thermotoga maritima, Chlorella virus-1 and Chlamydia trachomatis FDTS (Accession numbers 026061, Q9WYT0, 041156 and NP220149, respectively) were aligned using ClustalW. C. trachomatis CT632 was divided into NH2 and COOH terminal domains based on in silico analyses suggesting that a duplication event has occurred (28) b) C. trachomatis,
Parachlamydia, Thermoplasma acidophilum, and Thermoanaerobacter tengcongensis (Accession numbers NP220149, YP007154, NP623772, CAC12549, respectively) were aligned using ClustalW. The residues mutagenized in this study are bolded and marked with a # sign. The methyl accepting arginine is bolded and underlined. Putative thyX motifs (T/RHRX7-8S) are bolded and italicized.

Figure 3. **Biochemical analysis of recombinant C. trachomatis FDTS.** a) A purified sample of ≈ 5 µg of C. trachomatis recombinant FDTS was run on a 10% SDS-polyacrylamide gel along with the molecular weight standards. Sizes are indicated in kilodaltons (kDa). b) Spectroscopic analysis of flavin released from C. trachomatis FDTS. FAD was extracted from purified CTThyX by incubating at 95°C for 10 min in the dark. The absorption spectrum of the released flavin present in the supernatant was determined spectrophotometrically.

Figure 4. **Analysis of folate products following thymidylate synthesizing reaction.** a) *E. coli* ThyA and b) C. trachomatis FDTS thymidylate synthesizing reactions were run with and without added enzyme as described in “Experimental Procedures”. Folate products were separated by HPLC using a 25 cm C18 reverse phase column under isocratic conditions with a flow rate of 1 ml/min. The identity of the peaks was confirmed by comparison to known standard CH₂H₄folate (17.5 min) H₂folate (14.2 min) and H₄folate (8.2 min). The reaction with enzyme is indicated by the solid line and the dotted line represents the run in the absence of enzyme.
Figure 5. Michaelis-Menten saturation kinetics of *C. trachomatis* FDTS. Kinetics were determined for a) dUMP, b) CH₂-H₄folate and c) NADPH. Enzyme activity was determined at various concentrations of the indicated substrates, with all other substrates at fixed saturating concentrations as described in “Experimental Procedures”. The assays were run in triplicate and the mean ± S.E.M are shown.

Figure 6. Double reciprocal plot of initial velocity data for *C. trachomatis* FDTS and *E. coli* ThyA with dUMP as the variable substrate. Enzyme activity was determined with various concentrations of CH₂H₄folate and dUMP, under saturating conditions of NADPH as described in “Experimental Procedures”. a) *C. trachomatis* FDTS- the concentrations of CH₂H₄folate were (▼) 10 µM, ( ) 25 µM, ( ) 50 µM, (▲) 100 µM and (●) 200 µM. b) *E. coli* ThyA- the concentrations of CH₂H₄folate were (▼) 5 µM, ( ) 10 µM, ( ) 20 µM, (▲) 50 µM and (●) 100 µM. The assays were run in triplicate and the mean ± S.E.M are shown.

Figure 7. Double reciprocal plot of product inhibition data for *C. trachomatis* FDTS. a) and b) dUMP and c) and d) CH₂-H₄folate as the variable substrates and a) and c) dTMP or b) and d) H₄folate the product inhibitors. The reactions were conducted as described in “Experimental Procedures”. a) and b) The concentrations of dTMP or H₄folate were (▼) 0 µM, ( ) 10 µM, ( ) 25 µM, (▲) 50 µM and (●) 100 µM. c) and d) The concentrations of dTMP or H₄folate were (▼) 0 µM, ( ) 25 µM, ( ) 50 µM, (▲) 100 µM and (●) 200 µM. The assay was run in triplicate and the mean ± S.E.M are shown.
Figure 8. *Chlamydia trachomatis* FDTS half reaction. The standard *C. trachomatis* FDTS assay reaction was carried out in the absence of dUMP with saturating amounts of NADPH (2mM) and CH$_2$H$_4$folate (200 µM). Folate products were separated by HPLC using a 25 cm C$_{18}$ reverse phase column under isocratic conditions with a flow rate of 1 ml/min. Positions of peaks were determined by comparison with standard CH$_2$H$_4$folate (12.5 min) and H$_4$folate (6.5min). The dotted line indicates the reaction incubated with *C. trachomatis* FDTS and the solid line represents a reaction incubated without enzyme. *Inset*: blow up of the CH$_2$H$_4$folate peak spanning 12-14 minutes.

Figure 9. Analysis of mutant *C. trachomatis* FDTS enzymatic function by genetic by complementation. *In vitro* mutagenized *C. trachomatis* FDTS constructs pQE80L-CTThyXR124A, pQE80L-CTThyXS133A, pQE80L-CTThyXR397A, pQE80L-CTThyXR477A and pQE80L vector control were transformed into *E. coli* MH2720 (*thy*A) and growth of the transformants was assessed on M9 minimal agar plus IPTG with or without added thymidine.

Figure 10. MALDI-TOF mass spectrometric analysis of purified recombinant *C. trachomatis* FDTS following the thymidylate synthesizing half reaction. The thymidylate synthesizing half reaction was carried out as described in “Experimental Procedures”. MALDI-TOF was carried out on trypsin-digested CTThyX after the thymidylate synthesizing protein half reaction. Only those peaks with a mass between 1000 and 2400 are displayed. Peptide 1188.6 represents residues 469-477 (GLQWLCELR) and shows an increase of 14 amu in calculated mass as compared to the expected mass.
Figure 11. Thymidylate synthase cycle and Cleland plots of the reaction sequence of *C. trachomatis* FDTS and *E. coli* ThyA. a) Schematic representation of proposed *C. trachomatis* thymidylate synthesis cycle. Schematic representation of the order of substrate binding and product release during  b) *E. coli* ThyA (11) and  c) *C. trachomatis* FDTS catalytic cycle.
<table>
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<th>Mutated Amino Acid</th>
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<td>5’-GAAGATGCTGCGATTGGAGGGTCCCCC-3’</td>
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<tr>
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<td>3’CTThyXR477A</td>
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*a Codons that are mutated are bolded.
Table 2. Functional complementation and *in vitro* activity of CTThyX mutant proteins

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<th>Complement <em>E. coli</em> MH2720</th>
<th>FAD binding</th>
<th>Enzyme activity&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
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<tr>
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<td>-</td>
<td>0.34 ± 0.29</td>
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<sup>a</sup> Results are means ± standard deviations for two determinations. Enzyme assays were conducted at 37°C, with 200 µM CH$_2$H$_4$folate and 200 µM dUMP under standard assay conditions as described in “Experimental Procedures”. Activity expressed in ηmoles dTMP produced/min/mg of protein. N/A – not applicable.
strain MH2720 mutation thyA expressed gene CT thyX

strain MH2720 mutation thyA expressed gene CT thyX or EC thyA

+Thymidine

E. coli thyA

-Thymidine

C. trachomatis thyX

Figure 1a
Figure 1b
C. trachomatis 1 MLSKE-GGFSEEQARLHSTFVTLNSPIFAKLNEPVKALFSDKYRST-LGLRALLLK 58
Parachlamydia 1 MLDDYEETESIQQLRKYRTTSSHVFALRNLEPVKGLALFSDYRSLSS-RGLRLSLLK 59
T. acidophilum 1 --------------------------MFSSNDRVFVITDSDMIDRGMARSRASPDIDIVFRRH 42
T. tengcongensis 1 ---MQMRKLTPEEKLQNFVTDVYFGFVHSLPFIPTPPNSKVRKD-TSWSNLNie 56

C. trachomatis 59 EFLDGE---------GGNFLDDDQQ--DCELGIQKAADFYRRVLDNFGDDSVGELGGAHL 107
Parachlamydia 60 EFISNNDETAFEDIVGVAFKEEKQQTHQSEAIRKAQAFYDRILDGYGDDSIGELGGAHL 119
T. acidophilum 43 EFEGNQ---------------------------KRSEDFYRRIFLEYGDESIAELVTAQV 75
T. tengcongensis 57 SLTDGD---------LDITEYLPHISIPMERAIEKAREFHEKWVEKFHSSIAEQLMLH 107

C. trachomatis 167 TCFDNLNTYSDLIPQVRSHFEKLYPKD-------------------------PEVSQSAYTVSRL 206
Parachlamydia 179 TCNMLFDTYSLRPIFIEQFVFKFD-------------------PSIKTAYTAALRL 218
T. acidophilum 196 TLANLDARFILPASTLTNMGVSGNGRSFIHLQKLMSVEPESERLHVLKEFQFQ 235
T. tengcongensis 202 KIAFEDARYALATRTSFAVALNASLDQDIVRKLFAHPTEEAKVLAQKSLIPS 262

C. trachomatis 207 AKVLDCDLRGGLPAAATLNLGFPGFNGRFQWQLLHRQDNSLVVEVRFNIEQGSLSLELMKIIPS 266
Parachlamydia 219 AKVLDCDLRGGLPAGTTLNMTGYNGRFQFEHLHRMCQNLALQDIKRQHSQELSLSIKIPS 278
T. acidophilum 196 TLANLDARFILPASTLTNMGVSGNGRSFIHLQKLMSVEPESERLHVLKEFQFQ 235
T. tengcongensis 202 KIAFEDARYALATRTSFAVALNASLDQDIVRKLFAHPTEEAKVLAQKSLIPS 262

C. trachomatis 267 FVSRASESHHYYHQMVDYRRALEKQLSFARHYGEEREIESKEAVGKYLVGDFFPYKIAA 326
Parachlamydia 279 FVRSSPDTHRTQTHQFASYQFLETQMSLERLLIAEQYLGCKDRSLQPGVRVSSDFEAVTKVAA 338
T. acidophilum 256 IADDLSQHGH--QDIYYRKRSLASLFPYTDGGRFKEVRLKIS-------NEREMQKVLAA 307
T. tengcongensis 263 TLRLHLDTPYQMKVERLKLDSIKLGDAISSDDVLLYDYGK----TDSFSPVDTVWA 319

C. trachomatis 327 AYMFYSEHTAEDILDRCRPEDLRMLESAGSFRENRRHKSPFRGECAFADFITAD 386
Parachlamydia 339 ALFLSGLDKGFADINQQRKSLSELRADILDRCAENRFRQQFSSRALEDHAEFFTEIS 398
T. acidophilum 308 LLMMYPFARLE-AQSGIFRRAMELRASELSAILERIRLDRNRMKVRGGFPEAEEYVFTETNE 366
T. tengcongensis 320 HILFTYSGKEFVKEVRKRAMEKEKEEKVINTAVEIEGFDHLLD-AFKSVRFKQKLIS 378

C. trachomatis 387 FGAYRDLQRRHILTQERQLLITTKGLYTMYRPLQGRLQWLCEL-----------PEPQFREMEKADQAYRLIAEEF 445
Parachlamydia 399 FGAYRDLQRRHILTQERQLLITTKGLYTMYRPLQGRLQWLCEL-----------PEPQFREMEKADQAYRLIAEEF 445
T. acidophilum 367 YGAFRDLQRRHILTQERQLLITTKGLYTMYRPLQGRLQWLCEL-----------PEPQFREMEKADQAYRLIAEEF 445
T. tengcongensis 379 EANWQHQLRRHILTQERQLLITTKGLYTMYRPLQGRLQWLCEL-----------PEPQFREMEKADQAYRLIAEEF 445

C. trachomatis 446 -PEEAQYVYPVAYNIRNLFHINARGLWCLALPQFGHESYRIAIDMEAREVQIFHPAY 504
Parachlamydia 458 -PEEAQYVPMAYNNIRNFYHVNLQLALPQFAGAHPYHQAIATMLCEAEFFPAF 516
T. acidophilum 427 GMWIAQYVARFYPFYRPVPTNMLAEATYEFIELSRTQAHFDFLDRIAMYEINKEVSHPS 486
T. tengcongensis 438 -PEVPSYVTVNKRLVVMNADLWAFDFAHLTCTKEAQWIQDRETFSKMGLIREVAQPL 496

C. trachomatis 505 ELFLKFVTDYSETDGLRLQGESRKS------- 529
Parachlamydia 517 ERFKVFVDEYGELRLQDEQFRKDIQQLSIL 548
T. acidophilum 487 AGIIKFVDSYDTRLQGEVSKQNEKAI------- 516
T. tengcongensis 497 AFFFARKRA---------- 506

Figure 2b
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
+ Thymidine

- Thymidine

Figure 9
Figure 10.
Figure 11
Catalytic mechanism of chlamydia trachomatis flavin dependent thymidylate synthase
Jonathon Griffin, Christine Roshick, Emma Iliffe-Lee and Grant McClarty

J. Biol. Chem. published online December 8, 2004

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