Cloning and Characterization of the Thymidylate Synthase Complementing Protein from Environmental Parachlamydia

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ABSTRACT

Parachlamydiae are intracellular endosymbionts of free living amoeba, first identified in 1997. Parachlamydiae are closely related to chlamydiae, which are obligate intracellular eubacterial parasites responsible for a variety of diseases in humans. Parachlamydiae are potential emerging pathogens and very little is known about their biology. The recent complete genome sequencing of *Parachlamydia* isolate UWE25 indicates that it does not encode homologues of thymidylate synthase or thymidine kinase. Until recently, these two proteins were the only enzymes known capable of synthesizing thymidylate, one of four deoxyribonucleotide precursors of DNA. However, *in silico* analyses have shown that Parachlamydia do contain an open reading frame, encoded by PC0155, which shows low level sequence homology to a newly identified family of proteins called thymidylate synthase complementing proteins. The PC0155 gene was amplified from *Parachlamydia* UWE25 genomic DNA by polymerase chain reaction. The PC0155 amplicon was cloned into an *Escherichia coli* expression plasmid. The recombinant plasmid, expressed a 63.2 kDa protein which complemented the growth defect of a thymidylate synthase deficient strain of *Escherichia coli*. The recombinant protein, pCTThyX, was over expressed and purified to homogeneity by affinity chromatography. pCTThyX was yellow in color and spectroscopic analysis revealed that the enzyme contains bound flavin. An *in vitro* enzyme assay was used to show that purified recombinant pCTThyX was able to synthesize thymidylate using dUMP, NADPH and methylene tetrahydrofolate as substrates.

INTRODUCTION

Chlamydiae are obligate intracellular bacterial pathogens of humans and animals. Presently the genus is divided into four species: *Chlamydia trachomatis, C. pneumoniae, C.pecorum,* and *C.psittaci.* In humans *C.trachomatis* infection is an important cause of preventable blindness and sexually transmitted diseases. Until recently chlamydiae were considered a small unique group of bacteria comprising only a few medically important pathogenic species. About a decade ago, the discovery and identification of chlamydiae related endosymbionts in amoeba led to an awareness of the diversity of chlamydiae in the environment. More recently, these environmental chlamydiae have been found in the nasal mucosa of heathly female volunteers (Amann *et al.*1997) raising the possibility that they could have potential as emerging pathogens. Other findings of chlamydia-like bacteria (*Simkania negevensis, Waddlia chondrophila*, and *Fritschea bemisiae*) as contaminants of tissue culture (Kahane *et al*. 1995), and within an aborted bovine fetus (Rurangirwa *et al.* 1999), have broadened the range of chlamydiae in our environment. Identification of these new "Environmental" chlamydiae have caused a reorganization of Chlamydia taxonomy that introduced three new families, *Parachlamydiaceae/Neochlamydia, Simkaniaceae* and *Waddliaceae*, classifying these novel chlamydiae within the order of the *Chlamydiales* (Everett et al. 1999, Molmeret et al.2005).

It is now firmly established that members of *Parachlamydiaceae* are obligately intracellular bacteria that naturally infect free-living amoebae (FLA; Greub *et al*. 2002a).

Parachlamydiaceae (environmental chlamydia) are closely related to the *Chlamydiaceae* (pathogenic chlamydia), sharing 80 to 90% sequence homology of rRNA genes (Amann *et al*. 1997). Like chlamydiae, parachlamydiae are extremely successful intracellular organisms in part due to their unique biphasic developmental cycle consisting of two morphologically and biochemically distinct forms (Moulder 1991). The elementary body (EB) is the metabolically inactive extracellular form capable of initiating infection upon attaching to a host cell. Following internalization, a series of biochemical processes trigger the EB to differentiate into the metabolically active, non-infectious reticulate body (RB), which divides by binary fission. Following multiple rounds of division, the RB converts back into the EB leading to lysis of the host cell and release of EBs, which initiate the next round of infection. Parachlamydia, unlike pathogenic *Chlamydia*, have a third morphological stage, the crescent body stage, which is associated with prolonged incubation (Greub *et al.* 2002b).

Free living amoebae are known to be natural hosts of several diverse intracellular environmental bacteria (*Legionella pneumophila, Mycobacterium spp., Francisella tularensis*, and *Rickettisa typhi,* among others; Molmeret *et al.*2005), but the majority of endosymbionts of FLA which have been identified, belong to the order *Chlamydiale* (Horn *et al.* 2004b). To date, twelve environmental parachlamydia isolates have been discovered (Collingro *et al.* 2005). There is evidence that a few of these environmental isolates may be associated with diseases of humans. Epidemiologically, parachlamydia have been associated with an outbreak of humidifier-associated fever (Birtles *et al.* 1997) and several pneumonia cases (Birtles *et al.* 1997, Greub *et al.* 2003a). Also, a patient with adult Kawasaki syndrome was found to have a four-fold rise in antibody titer to

Parachlamydia acanthamoebae (Marrie *et al.* 2001). Further evidence supporting the idea that environmental chlamydia may be capable of infecting humans is the PCR detection of parachlamydiae 16S rDNA in sputum and bronchoalveloar lavage specimens from respiratory disease patients (Corsaro *et al.* 2001, Corsaro *et al.* 2002), and from mononuclear cells of a patient with bronchitis (Ossewarde and Meijer 1999). In a serological study of patients with community-acquired pneumonia, 2.2% were seropositive (>1/50 titre) for parachlamydiae (Marrie *et al.* 2001). From these results, it has been suggested that parachlamydiae may be pathogenic but only under special clinical conditions.

It has also been shown that *P. acanthamoeba* strain Hall's coccus, was able to enter and multiply within human macrophages (Greub *et al.* 2003). Recent co-cultivation techniques isolated a *Parachlamydia* sp. UV-7 which was able to infect Vero (monkey), NCI (human), and HeLa (human) cells; however, the infected cells eventually underwent apoptosis (Collingro *et al.* 2005). In contrast to this, some species of *P. acanthamoeba* (Bn9, Hall's coccus) were unable to grow in mammalian cell lines such as McCoy (mouse) or human cell lines, such as Vero, P388D1 macrophage-like cells, or embryonic lung fibroblasts (Maurin *et al.* 2002). In my own studies, I found that *P. acanthamoeba* UWE25 was unable to infect HeLa cells and RAW (mouse) macrophages. Since so few studies have shown an association between environmental parachlamydiae and clinical disease, at this time it is difficult to definitively state that they are pathogenic in humans. However, it is still important to have a better understanding of these symbiotic organisms, in order to devise suitable therapeutic interventions for human infections.

The recent *in silico* analyses of the complete genome sequence of the *Parachlamydia*-related symbiont UWE25 of *Acanthamoeba sp.* (isolated from a soil sample in Washington State) has provided insights into its interaction with its amoeba host. Although the UWE25 genome is twice as large as the genome of pathogenic chlamydiae (2.4Mb vs. 1-1.2Mb), like the pathogenic chlamydiae, several essential biosynthetic pathways are truncated in UWE25. Consequently, the symbiont UWE25 relies on the import of a number of metabolites such as several amino acids, nucleotides and cofactors, from its amoeba host (Horn *et al*. 2004). Pathogenic chlamydiae also exhibit reduced central metabolic and biosynthetic pathways and are auxotrophic for most amino acids and nucleotides (McClarty 1999).

All eukaryotic and prokaryotic organisms require a balanced supply of nucleotides as precursors for RNA and DNA synthesis and as energy transducers. Chlamydiae and Parachlamydiae are capable of transporting ribonucleotides (NTPs), but not deoxyribonucleotides (dNTPs) directly from the host cell (McClarty 1999, Horn *et al.* 2004). Genomic sequencing projects indicate that both organisms contain *nrdA* and *nrdB*, encoding the two subunits of ribonucleotide reductase required for the conversion of ribonucleotides to deoxyribonucleotides (Stephens *et al.* 1998, Horn *et al.* 2004). Ribonucleotide reductase accounts for the acquisition of three (dCTP, dGTP and dATP) of the four deoxyribonucleotides required for DNA biosynthesis (Roshick *et al.* 2000). The fourth nucleotide, deoxythymidylate triphosphate (dTTP), is produced by two well known processes. Exogenous thymidine can be directly salvaged by thymidine kinase (Tdk) or dTMP can be synthesized *de novo* from dUMP, a reaction catalysed by thymidylate synthase (ThyA; Carreras and Santi 1995). *Chlamydia* are capable of

incorporating exogenously added uridine into thymidine nucleotides, a result implying the existence of a thymidylate synthase and a dihydrofolate reductase (DHFR) in this organism (Fan *et al.* 1991, Tipples and McClarty 1991) Interestingly, *in silico* analyses of whole genome sequence data showed that while *Chlamydie* and *Parachlamydia* encode a DHFR homologue, there were no homologues of TdK or ThyA in either parasite (Stephens *et al.* 1998, Horn *et al.* 2004). This leads to the question if parachlamydiae do not encode for the only enzyme capable of dTMP formation, how do they obtain thymidine nucleotides?

Until recently, ThyA was thought to represent the only enzyme capable of catalyzing the *de novo* formation of dTMP (thymidylate) *in vivo*. ThyA carries out the reductive methylation of deoxyuridine 5'-monophosphate (dUMP) using methylenetetrahydrofolate (CH_2-H_4folate) as a one-carbon donor and source of reducing equivalents, generating dTMP and dihydrofolate (H_2folate) as products. Since reduced folates are essential for many physiological functions, H_2folate is rapidly reduced by NADPH to H_4folate by the action of DHFR. The subsequent regeneration of CH_2-H_4folate is carried out by serine hydroxymethyltransferase, by the addition of a one carbon unit donated from serine to H_4folate, producing CH_2-H_4folate and glycine. Together these reactions represent the thymidylate cycle (Figure 1; Carreas and Santi 1995, Myllykallio *et al.* 2002).

Recently the existence of a novel family of thymidylate-synthesizing enzymes, called thymidylate synthase complementing proteins or flavin-dependent thymidylate synthases (FDTS), encoded by *thyX,* has been described (Kuhn *et al.* 2002, Myllykallio *et al* 2002, Mathews *et al.* 2003, Leduc *et al.* 2004). All members of the family
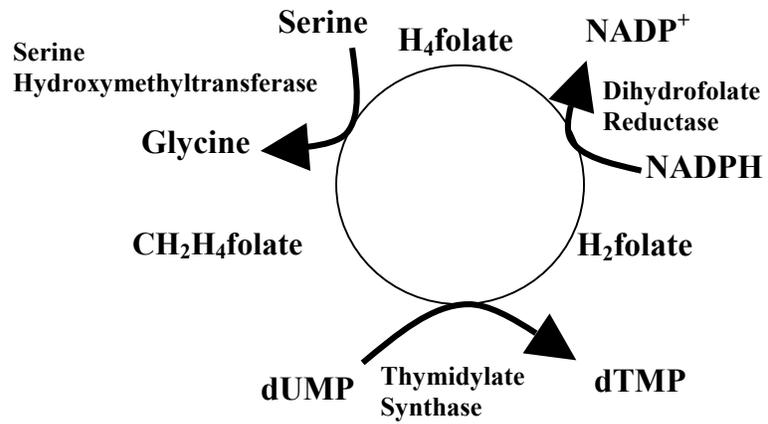
Figure 1. Schematic representation of the thymidylate cycle for ThyA as described by Carreras et al 1995.

contain a conserved ThyX motif consisting of (T/RHRX$_{7-8}$S). The first discovered member of ThyX family was a gene encoding a protein shown to complement a thymidine-requiring mutant of *Dictyostelium discoideum* (Dynes and Firtel 1989, Myllykallio *et al.* 2002). *D. discoideum* is the only known eukaryote with a ThyX gene (Leduc *et al.* 2004). Recent analyses indicate that homologues of *thyX* are present in upwards of 30% of currently sequenced microbial genomes (Myllykallio *et al.* 2002). Interestingly, many of the genomes containing *thyX* lack *folA,* encoding DHFR (Myllykallio *et al.* 2002, Myllykallio *et al.* 2003). This information coupled with the observation that NADH or NADPH are required for *Helicobacter pylori* and *C. trachomatis* ThyX dTMP synthesis activity has led to the speculation 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 (Myllykallio *et al.* 2002, Mathews *et al.* 2003, Myllykallio *et al.* 2003, Agrawal *et al.* 2004, Leduc *et al.* 2004, Griffin *et al.* 2005). This could account for the lack of a DHFR (*folA*) homologue which performs the essential reaction of reducing folates in many ThyX organisms.

Despite being twice as large as the typical ThyX proteins ($\approx$26-30 kDa), cross species *in silico* analyses have shown that *Parachlamydia* PC0155 (63.2kDa) is a distantly related homologue of ThyX (Figure 2). Interestingly PC0155 is very similar in size to *C.trachomatis* CT632 (60.9 kDa), which was recently shown to encode a novel ThyX (Griffin *et al* .2005). In this thesis, I report on the cloning and characterization of *Parachlamydia* UWE25 PC0155. My results show that PC0155 does indeed encode a thymidylate synthesizing protein.

Phylogenetic tree
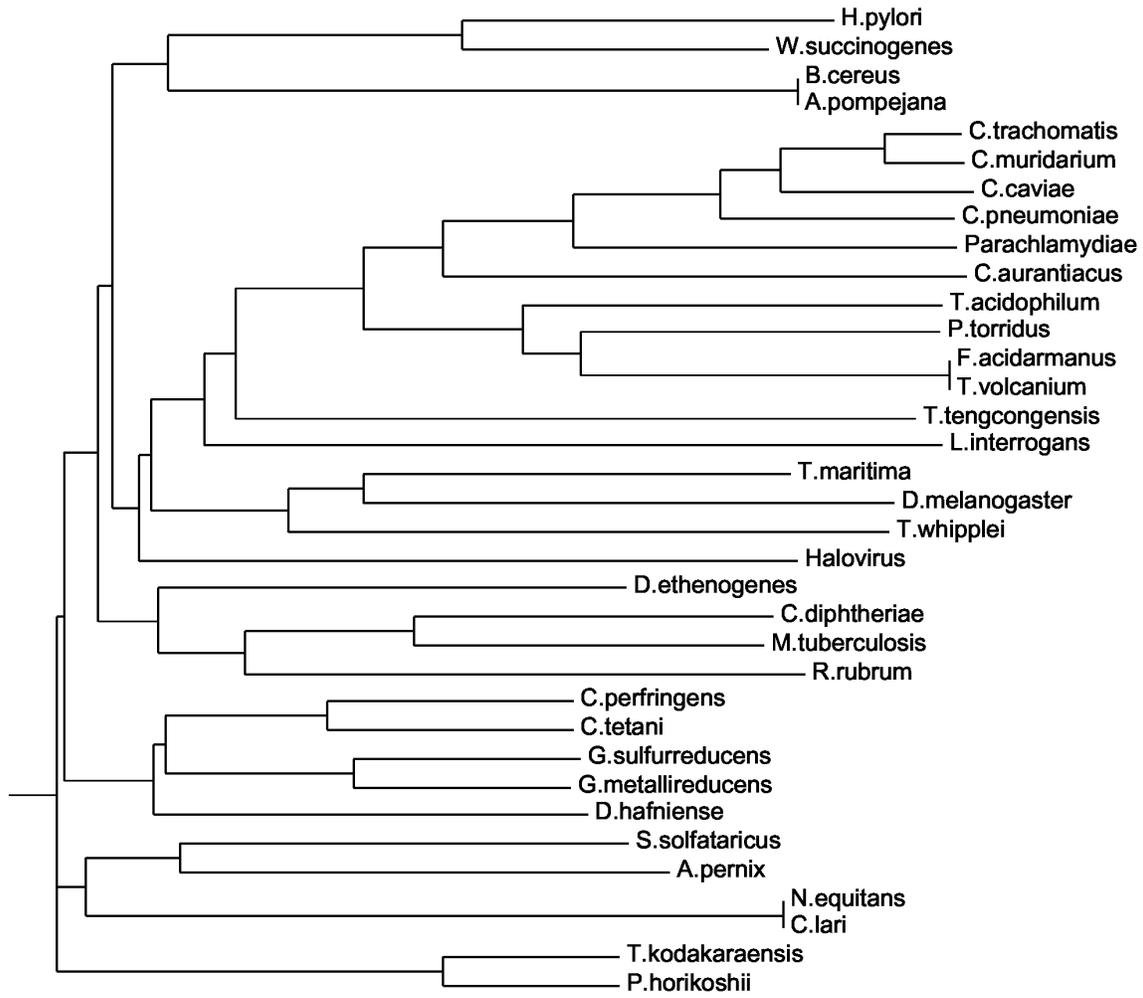
0.1

Figure 2. Phylogenic tree of ThyX homologs. ThyX homologues were aligned using ClustalW and the dendogram was constructed using Tree Draw (see appendix for organism abbreviations).

MATERIALS AND METHODS

1.   **Materials**

[5-^{3}H] dUMP(15 Ci/mmole) 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. All other chemicals were of reagent grade or better and were purchased from Sigma Chemical Co. Trizol reagent for RNA isolation, RT-PCR kit, Taq Polymerase, restriction enzymes, DNase 1, and oligonucleotide primers were purchased from Invitrogen Life Technologies.   The pQE80-L plasmid, PCR product and mini-prep purification kits were obtained from Qiagen. The Rapid DNA ligation kit and the High Pure PCR Product Purification Kits were purchase from Roche. Purified 5,10-methylenetetrahydrofolate (5, 10-CH$_2$H$_4$folate) was kindly provided by Dr. Rudolf Moser at Merck Eprova EG, Schaffhausen Switzerland.

2.   **Strains and Culture Conditions**

Acanthamoeba catellanii was purchased from ATCC, Virginia, USA. *Acanthamoeba catellanii* was grown as a monolayer in T75 cm^2 tissue culture flasks containing 20 mL PYNFH medium (ATCC catalog # 327-X), at room temperature (20-24°C). Fresh media was applied every 3-4 days. When the cultures formed a confluent monolayer, the flask was vigorously agitated, and the cells were passaged 1:5 into a fresh flask. The cells were confluent again in approximately 7-10 days.

P. acanthamoeba strain UWE25 was kindly provided by Matthias Horn, Universität Wien, Althanstr. 14, A-1090 Vienna, Austria. *Parachlamydia acanthamoeba*

strain UWE25 was grown at room temperature (20-24°C) in T75 cm^2 tissue culture flasks with 20 mL TSY medium (30.0 g trypticase soy broth, 10.0 g yeast extract, add up to 1L of distilled water, pH 7.3, autoclave). When cultures formed a monolayer, approximately every 3-4 days, the culture was passaged (1:5) by vigorous agitation. Fresh media was applied every 2 days.

3.   *E. coli* strains used for protein expression and complementation

XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacI^{q}ZΔM15 Tn10 (Tet^r)*] was obtained from Stratagene.

MH$_{2720}$ *thyA$^-$* (*thyA114(Stable)::Mu*) was obtained from the *E. coli* Genetic Stock Center (New Haven, Ct.)

4.   *E. coli* culture media

LB Broth consisted of 10 g tryptone (Fisher), 5 g yeast extract and 5 g NaCl in 1 L. The pH was adjusted to pH 7.0 and autoclaved.

TB (Terrific Broth) consisted of 12 g bacto-tryptone, 24 g yeast extract, 4 mL glycerol in 900 mL distilled water, then was autoclaved. Then 100 mL of sterile solution of 0.17 M KH$_2$PO$_4$, 0.72 M K$_2$HPO$_4$ (2.31 g, 12.54 g) was dissolved in 900 mL distilled water. After the salts were dissolved the solution was made up to 1L, then was autoclaved.

LB Agar consisted of 15 g agar added to 1 L of LB broth prior to autoclaving. When required, 100 μg/mL ampicillin and 40 μg/mL thymidine were added to the medium.

SOC consisted of 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.2 g KCl, adjusted to pH 7.0 in 1 L. Sterile $MgCl_2$ and glucose were added to the medium after it was autoclaved to a final concentration of 10 mM and 20 mM, respectively.

Minimal Medium consisted of 1 x M9 salts (200 mM Na_2HPO_4, 110 mM KH_2PO_4, 94 mM NH_4Cl, 43 mM NaCl, 135 mM $CaCl_2$), 2.0% agarose, 0.2% casamino acids, 2 mM $MgSO_4$, 2.5 mg/mL glucose, 50 µg/mL thiamine, 50 µg/mL cysteine and 200 µM serine. When required, 100 µg/mL ampicillin and 40 µg/mL thymidine were added to the medium.

5.   Cryopreservation of Strains

Harvested amoebae and amoebae containing *Parachlamydia* UWE25 (approximately 2 x 10^6/mL in growth medium) were mixed 1:1 with cell-culture-grade 20% dimethyl sulfoxide (DMSO) to give a final concentration of 10%. This mixture was frozen and subsequently stored in liquid nitrogen (Schuster 2002).

6.   Isolation of *Parachlamydia* UWE25 genomic DNA

DNA from UWE25 *Acanthamoeba* habouring *Parachlamydia* UWE25 were isolated by growing the infected amoebae in T25 culture flasks. Once a monolayer was established, the cells were centrifuged at 16 000 rpm for 20 minutes. The pellet was then resuspended in 250 uL of 0.5mM NaOH, and incubated at room temperature for 5 minutes, then neutralized by adding 300 uL 1M Tris-Cl pH 8.0. This stock was then diluted and used for PCR.

7.   Molecular cloning of *Parachlamydia* UWE25 PC0155

Parachlamydia UWE25 PC0155 was cloned into the pQE-80L expression plasmid purchased from Qiagen.   The pQE-80L-PC0155 construct generated an N-terminal penta-histidine tagged enzyme used for *in vitro* enzyme assays and kinetic studies.   The expression plasmid contains a phage T5 promoter regulated by a *lac* repressor protein.   Addition of IPTG induces the expression of the recombinant protein. Cloning of *Parachlamydia* PC0155 was performed using the following primers were designed based on published sequence of the PC0155 gene of *Parachlamydia* UWE25: forward 5'CCC CGG TAC CAT GCT AAC AGA TCA TTA TCA AG-3' and reverse 5'CCC CGT CGA CTT AAA TAA GAA GAG ATT GTT G-3'. The underlined portions of the primers indicate the Kpn1(forward) and Sal1(reverse) restriction sites. These sites were included so that the PCR fragment could be restricted and subsequently ligated into the pQE80L vector, cut with the same two enzymes, directly.   PCR was carried out in a total volume of 50 µL containing 1 x PCR buffer, 1.5 mM MgCl$_2$, 0.25 mM of all four deoxyribonucleoside triphosphates (dNTPs), 50µM 5'Kpn-pCT*ThyX* and 3'Sal-pCT*ThyX* primers, 2.5 U Taq polymerase and *Parachlamydia* UWE25 genomic DNA.   The following program was carried out for 35 cycles: 1 min at 95°C, 1 min at 60°C , 2 min at 72°C, and 1 cycle at 72°C for 8 min. The PC0155 gene product was purified from a 0.8% agarose gel using a Qiaquick gel extraction kit (Qiagen) following the manufacturer's protocol.   The purified PCR PC0155 product and as purified pQE-80L plasmid were digested overnight with *Kpn*I/*Sal1* at 37°C. Then each digested product was purified again with a PCR clean up kit (Roche) following the manufacturer's protocol. The PC0155 and pQE-80L *Kpn1/Sal1* digested products were then run on a 0.8% agarose gel

and purified using the Roche PCR clean up kit (Roche). The digested PC0155 PCR product and pQE-80L were ligated using the Rapid Ligation Kit (Roche), according to maufacturer's protocol. The resulting pQE-80L PC0155 construct was transformed into XL1-Blue calcium chloride competent cells.

8. Transformation of *E.coli*

Competent *E. coli* strains were prepared as follows. The desired strain of *E. coli* was inoculated into 5 mL of LB broth and incubated overnight at 37^oC, with vigorous shaking (Forma Scientific Orbital Shaker). The overnight culture was then used to inoculate 100 mL LB broth, which was incubated at 37^oC with vigorous shaking until it reached an O.D of 0.6-0.8 at 595 nm (Pharmacia Biotech Ultrospec 3000).This usually took 2-4 hours. The culture was chilled on ice for 10 min prior to being centrifuged (Beckman J2-MC centrifuge with JA-10 rotor) at 7 500 rpm for 12 min at 4^oC. The pellet was resuspended in half of the original culture volume (50 mL) of an ice-cold, sterile solution of 0.1M $CaCl_2$ (14.7g/L) and centrifuged as described above. The cell suspension was placed in an ice bath for 15 minutes and then centrifuged at 5000 rpm (Beckman J2-MC centrifuge with JA-10 rotor) for 5 minutes at 4°C. The cells were resuspended in $^1/_{15}$ of the original volume of an ice-cold, sterile solution of 0.1M $CaCl_2$ (~3.3 mL). Then 15% sterile glycerol (15 mL/100 mL distilled water, autoclave) was added to the cell suspension and stored at -80ºC in 200 μL aliquots.

Competent *E. coli* cells were transformed by adding plasmid DNA [pQE-80L-PC0155, pQE-80L-*E.coli-ThyA* (previous study, positive control, Griffin *et al*.2005), pQE-80L (no insert, vector control)] mixed with 200 μL of ice thawed $CaCl_2$ competent

E. coli cells (XL1-Blue or MH$_{2720}$) and then incubated on ice for 30 minutes. The cells were heat shocked in a preheated 42ºC water bath for 45 seconds, then placed on ice for 2 minutes. 800 μL of preheated (37ºC) SOC media was added to the cell suspension and incubated at 37ºC, shaking (Fisher Scientific, Ocelot 260300F) for 1.5 hours. The cells were plated onto selective media containing 100 μg/mL ampicillin and incubated overnight at 37ºC. For MH$_{2720}$ cells 40 μg/mL of thymidine was added to the media.

9.   DNA sequencing

DNA sequencing was carried out by the DNA Core Facility, National Microbiology Laboratory, Winnipeg, Manitoba.

10.   RNA Isolation

Total RNA was isolated from *Acanthamoeba catellanii* grown at (2.0 X 10^7 cells per T75 cm^2 flask) containing 20 mL PYNFH medium (ATCC catalog # 327-X), at 20-24°C. *P. acanthamoeba* strain UWE25 was grown at 20-24°C in T75 cm^2 tissue culture flasks with 20 mL TSY medium. Prior to RNA isolation, the infected cell monolayers were washed with 10 mL Hanks Balanced Salt Solution (Invitrogen). A total volume of 7.5 mL of Trizol was added to each T75cm^2 flask and incubated at room temperature for 5 min to allow for cell lysis. Cell lysates were transferred to diethyl pyrocarbonate (DEPC) treated tubes (1 mL DEPC/4L distilled water, soak tubes overnight in DEPC water, in fumehood, then autoclave) and stored at -80ºC in the Trizol reagent overnight to a maximum of 30 days. The cell lysate was then thawed in a 37ºC waterbath, and 1.5 ml chloroform was added to each tube (0.2 mL chloroform/ 1 mL of Trizol used) and the

samples shaken vigorously and incubated at room temperature for 3 min. The samples were centrifuged (Beckman J2-MC centrifuge with JA-20 rotor) at 12,500 rpm for 15 min at 4°C. The aqueous phase was then transferred to a new DEPC treated polypropylene tube. The RNA was precipitated by adding 3.75 mL isopropyl alcohol (0.5 mL isopropyl alcohol per 1 mL of Trizol used) and incubated at room temperature for 10 min prior to centrifugation at 12 500 rpm for 10 min at 4°C. The RNA pellet was washed with 1 mL 75% ethanol (prepared with DEPC-treated water; Invitrogen) per 1 mL Trizol used and centrifuged at 9 800 rpm for 5 min, at 4°C. The RNA pellet was then allowed to dry for 10 min at room temperature before being dissolved in 200 µL of DNase/RNase-free water (Invitrogen) and incubated at 55°C, for 10 min. The RNA samples were stored at -80°C. RNA was DNase I treated according to manufacturer's instructions for preparation for Reverse-transcriptase PCR (RT-PCR).

11.   Reverse-transcriptase PCR (RT-PCR)

RT-PCR was carried out using the ThermoScript™ RT-PCR system according to the manufacturer's instructions. The cDNA created from the reverse transcription reaction was stored at -80°C and then used as template for PCR amplification. For semi-quantitative PCR, the thermal cycling conditions consisted of 25 cycles of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C (Gene Amp PCR System 9700, Applied Biosystems). The PCR products of pCTThyX, and 16S rRNA and were analyzed on 0.8% agarose gels. Primer sequences are listed in Table 1.

12.   Complementation studies

MH$_{2720}$ cells transformed with pQE80L-pCTThyX, pQE80L-*E.coli*-TS, 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 6 000 rpm for 5 min (IEC Micromax microcentrifuge), 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 media plates containing IPTG (200 μL of 100 mM IPTG added to the surface and allowed to dry) with or without 40 μg/mL thymidine. The plates were incubated at room temperature (20-24°C) for approximately 4 days and then photographed (BioRad Gel Doc 1000).

13.   Determination of enzyme-bound flavin

Absorption spectroscopy was used to determine the presence of flavin bound to pCTThyX. Flavin was extracted from purified recombinant protein by incubation at 95°C for 10 min. The precipitated protein was pelleted by centrifugation at 10 000 rpm (IEC Micromax 120 microcentrifuge), for 10 min.   The absorption spectra of the released flavin present in the supernatant was determined spectrophotometrically (Beckman Du62 Spectrophotometer) at varying wavelengths from 250 nm to 750 nm in a 1 cm quartz cuvette (Myllykallio, Lipowski et al. 2002). The identity of the flavin was determined by comparsison of the absorption spectrum of an FAD standard as well as, FAD that was extracted from *C. trachomatis* ThyX (Griffin *et al.* 2005).

Table 1. Primers used for RT-PCR, Cloning, and Sequencing of pCTThyX

Name of primer	Sequence	*Parachlamydia* UWE25 gene
5'pCT*thyX*RT	5'CCCCGGTACCATGCTAACAGATCATTAT CAAG-3'[a,b]	PC0155
3'pCT*thyX*RT	5'CCCCGTCGACTTAAATAAGAAGAGATT GTTG-3'[a,b]	PC0155
5'16SRNA-RT	5'-ATTGAATGCTGACAGCGT-3'[a,b]	16S rRNA
3'16SRNA-RT	5'-ATTGAATGCTGACAGCGT-3'[a,b]	16S rRNA
5'*Kpn*-pCT*thyX*	5'CCCCGGTACCATGCTAACAGATCATTAT CAAG-3'[a,c]	PC0155
3'*Sal*-pCT*thyX*	5'-CCCCGTCGACTTAAATAAGAAGAGATT GTTG-3'[a,c]	PC0155
5'pCT*thyX*-seq1	5'-TAGCGATTGAAAATGTTTC-3'[a,d]	PC0155
5'pCT*thyX*-seq2	5'-CAGAATTACAAGATATCG-3'[a,d]	PC0155
5'pCT*thyX*-seq3	5'-TGTCCGATGAAGAGCTGCCGC-3'[a,d]	PC0155

[a] Primers designed based on the genome sequence of *Parachlamydia acanthamoeba* strain UWE25 (http://mips.gsf.de/services/genomes/uwe25).
[b]Primers designed for RT-PCR analysis
[c]Primers designed for cloning
[d]Primers designed for sequence analysis

14.   Expression and purification of pCTThyX for *in vitro* enzyme assays.

E. coli strain MH$_{2720}$ transformed with the pQE80L-pCT*thyX* plasmid construct was grown in 50 mL of LB media containing 100 μg/ml of ampicillin and 40 μg/ml thymidine, and grown overnight at 37°C with vigorous shaking (Forma Scientific Orbital Shaker). The overnight 50 mL culture was then inoculated into 600 mL of TB medium in a 6 L flask containing 100 μg/mL of ampicillin and 40 μg/mL thymidine at room temperature (22-24°C) to an O.D of 1.0 at 595 nm. At this time the incubator temperature was reduced to 15°C, and when the OD reached approximately 1.5, 1 mL of culture was taken for uninduced protein sample control, then pCTThyX expression was induced with IPTG at a final concentration of 150 μM. The culture was incubated for a further 15-20 h at 15°C, then 1 mL of culture was taken for induced protein sample. All subsequent procedures were carried out at 4°C. Bacteria were harvested by centrifugation at 7 000 rpm (Beckman J2-MC centrifuge with JA-10 rotor) for 10 min, at 4°C. The supernatant was removed 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 (Sonics & Materials Inc., Vibra cell), for 4 x 20 second bursts, in the presence of lysozyme, at a final concentration of 350 μg/mL. Cell lysates were clarified by ultracentrifugation at 45 000 rpm (Beckman Optima L-70K with a Ti70 rotor) for 1.5 hrs, at 4°C. Recombinant His-tagged pCTThyX was purified from the supernatant by metal chelation chromatography. Clarified lysates were passed through a 3 mL nickel-affinity charged column; the column was then washed with 15 mL of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9) followed by 20 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 pCTThyX was dialyzed overnight at 4°C in the dark against 50 mM Tris-HCl pH 7.9 containing 5% glycerol. Samples were then aliquoted and stored at -80°C.

15.   Determination of protein concentration

Protein concentrations were determined using the Bio-Rad protein assay based on the dye-binding procedure of Bradford using bovine serum albumin as the standard (Bradford 1976).

16. 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* ThyX activity (Leduc *et al*. 2004). All reactions were performed at 37°C in a nitrogen gas environment to limit decomposition of reduced folate. The optimized standard reaction mixture contained, in a total volume of 100 μL, 50 mM Tris-HCl pH 7.6, 200 μM 5, 10-CH$_2$H$_4$folate, 2 mM NADPH, 200 μM [5-^{3}H] dUMP (15 μCi/mL), 5% glycerol. Purified 5, 10-CH$_2$H$_4$folate was used in these assays. The reaction was initiated by the addition of 10 μg of purified pCTThyX and terminated by adding 300 μL of a 100 mg/mL activated charcoal suspension containing 2% TCA, to remove all nucleotides including the unused radiolabelled substrate [5-^{3}H]dUMP. The samples were mixed at room temperature for 1 hr and then centrifuged at 14 000 rpm (Eppendorf centrifuge 5417C) for 5 min to pellet

the charcoal. Radioactive H_2O was quantitated in a Beckman LS 5000 liquid scintillation counter after the addition of 5 mL of liquid scintillation fluid (Universol, ICN Biomedical). One unit of enzyme activity corresponds to the production of 1 ηmoL of dTMP synthesized per min under optimum assay conditions as described above.

RESULTS AND DISCUSSION

1.   Identification, cloning and characterization of *Parachlamydia* UWE25 PC0155

a) Identification and cloning of a *Parachlamydia* UWE25 ThyX homologue.

In silico analyses of the *Parachlamydia* UWE25 complete genome sequence indicated that open reading frame PC0155 encodes for a protein with sequence homology to ThyX proteins from a variety of bacteria (Figure 3). In addition, PC0155 is highly homologous to *C. trachomatis* CT632 an open reading frame which was recently shown to encode a protein with thymidylate synthesizing activity (Griffin *et al.* 2005). Genomic DNA was prepared from *Parachlamydia* UWE25 and PC0155 was cloned, using a PCR based strategy, into pQE80L, an *E. coli* protein expression vector which places a histidine tag on the amino terminal end of the recombinant protein (See "Materials and Methods" for cloning details).

b) Functional Complementation in *E. coli*

Functional complementation was used to test if *Parachlamydia* UWE25 cloned PC0155 could rescue the thymidine auxotrophy of *E. coli* $MH_{2720,}$ a *thyA* mutant strain. Figure 4 demonstrates that ORF PC0155 from *Parachlamydia* UWE25 was able rescue the growth of the *thyA⁻* strain of *E. coli*. The negative-control strain, containing the pQE-80-L plasmid without insert, was unable to grow on medium lacking thymidine. The positive-control strain, expressing *E.coli* ThyA, was also able to complement the growth defect of the *thyA⁻* mutant strain. The insert present in the recombinant plasmid was sequenced to verify that it was PC0155.

Figure 3. Sequence alignment of a diverse set of ThyX homologs from several bacteria. The alignment was constructed with a ClustalW program. *Parachlamydia C. trachomatis, Thermoplasma acidophilum,* and *Thermoanaerobacter tengcongensis* (Accession numbers NP220149, YP007154, NP623772, CAC12549, respectively) were used for the alignment. The conserved residues making up the ThyX motif are in bold.

```
Parachlamydia     MLTDDYEEFTESQIQTLKRYVTNTSSHVFALRNLPEVIKGALFSRYSRSS-LGLRSLLLK 59
C.trachomatis     MLSKE-GGFSEEQRARLSHFVTNLDSPIFALKNLPEVVKGALFSKYSRST-LGLRALLLK 58
T.acidophilum     ------------------MSFSNEDRDVFIIKTDRMIDRGALMSRYSRASDPDIRSVFHR 42
T.tengcongensis   ---MQMRKLTPEEEKLLQNFVTDVYGPVYFIHSLPEFIIPPINSKVSRKD-TSWRLNILE 56
                           .::     ::  ::.   .     .:  *:  **   . *   : .

Parachlamydia     EFISNNDETAFEDIVGVAFKEEEKQQTHQSEAIRKAQAFYDRILDGYGDDSIGELGGAHL 119
C.trachomatis     EFLDGE---------GGNFLDDDQQ--DCELGIQKAADFYRRVLDNFGDDSVGELGGAHL 107
T.acidophilum     EFEGNQ------------------------KRSEDFYRRIFLEYGDESIAELVTAQV 75
T.tengcongensis   SLTDGD---------LDITEYLPKHSIPMERAIEKAREFHEKWVEKFGHSSIAEQHLMHL 107
                  .: ..:                      .:: *: : . :*..*:.*    ::

Parachlamydia     AIENVSMMAAKLIE**DHR**IGGSPLEK**S**TRYIYFDQKVNGEYLFYR-EPILMTSAYRDSYVQ 178
C.trachomatis     ALEQVSMLAAKILE**DAR**IGGSPLEK**S**SRYVYFDQKVNGEYLYYR-DPILMTSAFKDVFLD 166
T.acidophilum     GIQNVSNVISKVIE**EIR**IGLSYLEK**S**TRYVAYDRKVDGHYLFMQAEKIGLSGEAAREYTD 135
T.tengcongensis   CVEDVSRLLSGDIELMNRRPAFIEW**S**QRY----QKPTREKVVIP-PELEKEPVLKEKFLK 162
                   :::**  : :   :*   .   : :* * **    :*  .:    :         : .

Parachlamydia     TCNMLFDTYSRLIPPMTAIIEQRFPKD--------------------PSISKTAYTAALR 218
C.trachomatis     TCDFLFNTYSDLIPQVRSHFEKLYPKD--------------------PEVSQSAYTVSLR 206
T.acidophilum     LCNRLFDLYSSTLPRIEEEISRQWPIESFDFNIDGNPRNYKELDENGRKLAQKSYRSSVR 195
T.tengcongensis   VWNATYDAYEKLVKVLTEYLRRAEEKK--------------------EGETEEKFLNRIE 202
                   :  ::  *.  :  :   : :  .                      ::  :   :.

Parachlamydia     AKVLDCLRGLLPAGTLTNMGIYGNGRFFEHLIHRMNCQNLAELQDIAKRSHQELSKIIPS 278
C.trachomatis     AKVLDCLRGLLPAATLTNLGFFGNGRFWQNLLHRLQDNSLVEVRNIGEQSLTELMKIIPS 266
T.acidophilum     SRALDDARFILPASTLTNMGVSGNGRSFIHLIQKLMEYGVPESERLAHDLYEELKGEFPQ 255
T.tengcongensis   KIAFEDARYALILATRTSFAVALNALDLQDIVRKLFAHPTEEAKVLAKRIVEQGEKIAPS 262
                   .::   *   *   .* *.:.. *.    .:::::     *  . :..   :     *.

Parachlamydia     FVRRSDPTHRTHQSYAQFLETMQSELRLIAEQYLGKCDRSLQPGVRLVSSDPEAVTKVAA 338
C.trachomatis     FVSRAESHHYHHQAMVDYRRALKEQLKSFAHRYGEEREISKEAGVKLVYGDPDGLYKIAA 326
T.acidophilum     IIDDALSQHG--QDIINYKRSLASLFPYTDGGRFEKVRLIKYS------NEREEMQKVLA 307
T.tengcongensis   TLRHLTPTPYQMKVRERLKDISKKILGDKRAISSDDVVLLDYTGKG---TDFSPVDTVVA 319
                   :     .        .:    .:     .       . .      : . : .: *

Parachlamydia     ALLFSLGDKGFADIWNQCKRLSDEELARILDAGCNARENRRQKSPRALEHAEFTFEIISD 398
C.trachomatis     AYMFPYSEHTYAELLDICRNIPNEDLMRILESGASFRENRRHKSPRGLECAEFAFDITAD 386
T.acidophilum     LLMYPFAED-ASGIISRIKAMELSEASAILERIRDLRKNRRMKVGRPFEAVNYVFEVTTN 366
T.tengcongensis   HILFTYSGKEFVEVKEKVKAMKEEEKEKVIKTAVEEIGEFDHLID-AFKSVRFKFQLKIS 378
                   ::.  .     :   : :  .:   ::.  .  :         :: ..: *:: .

Parachlamydia     FGAYRDLH**RHR**ILTQERQLLCCDYGFYIPPEIQNTP-YEEAYCHALNQAKEVFDLISSEL 457
C.trachomatis     FGAYRDLQ**RHR**ILTQERQLLTTKLGYTMPSQLIDTP-MEAPFREAMEKADQAYRLIAEEF 445
T.acidophilum     YGAFRDLQ**RHR**FLSIVRKPLTVSYGFDVPPIIAKMPDLSEEYAEAMKDAERVYRIIKERY 426
T.tengcongensis   EANWHQLL**RHR**TIIFDWTEPTVENGFVIPPNVVKAG-AEDILIGALKTSEELFYELKEKM 437
                  . .:::*  *** :           . *: :*. :.   .    *:: :.. :  : ..

Parachlamydia     -PEEAQYIVPMAYNIRWYFHVNLRALQWLCEL**R**SAPAGHPSYRHIAQTMARLTCEAFPAF 516
C.trachomatis     -PEEAQYVVPLAYNIRWLFHINARGLQWLCEL**R**SQPQGHESYRKIAIDMAREVIQFHPAY 504
T.acidophilum     GAWIAQYAVPFAYRYPVVFTTNLAEATYFIEL**R**STPQAHFDLRDIAIRMYNEIKSVHPSL 486
T.tengcongensis   -PEVSPYVVTNAHKRLVVMNADLWAFDHFANL**R**CTKEAQWDIRETSFKMLGLIREVAPQL 496
                   . : * *. *:.    :  :     : :**.  .:. *.: *   . *

Parachlamydia     ERFFKFVDYEGYELGRLDQEQRKIDKQQSLLI 548
C.trachomatis     ELFLKFVDYSETDLGRLQQESRKKS------- 529
T.acidophilum     AGIIKFVDTGDYPLGRLSAEVRKNVKAGGI-- 516
T.tengcongensis   AFFMARRKRA---------------------- 506
                   ::    .
```

Results of the sequence analysis indicated that the encoded open reading frame did correspond to *Parachlamydia* PC0155. Figure 5 shows the complete nucleotide sequence of the PC0155 gene and the amino acid sequence of the translated open reading frame. PC0155 shows low sequence similarity with the newly identified ThyX family of thymidylate synthase complementing proteins (Figure 3; Myllykallio *et al*. 2002, Leduc *et al*. 2004). However, PC0155 does contain several of the highly conserved residues that are thought to be essential for the ThyX activity (Kuhn *et al*. 2002, Myllykallio, *et al*. 2002, Leduc *et al*. 2004). There is a common motif termed the ThyX motif $(T/RHRX_{7-8}S)$ which has been identified and shown to be present among all the members of the ThyX family of proteins (Myllykallio *et al*. 2002, Mathews *et al*. 2003, Leduc, Graziani et al. 2004, Liu and Yang 2004). Surprisingly, *Parachlamydia* UWE25 PC0155 does not contain a fully conserved ThyX motif, as indicated in the amino acid sequence alignment. There are two regions, one in the N-terminal region and one in the C-terminal region that have a partial ThyX motif, which may complement each other in the tertiary structure to form the complete motif. The N-terminal region contains $DHRX_8S$, while the C-terminal region contains $RHRX_7L$. Of particular significance, PC0155 is similar in primary sequence to *C. trachomatis* CT632 (Figure 3) which was recently shown to encode a protein with thymidylate synthesizing activity (Griffin *et al*. 2005).

strain   MH2720
mutation   *thyA*⁻
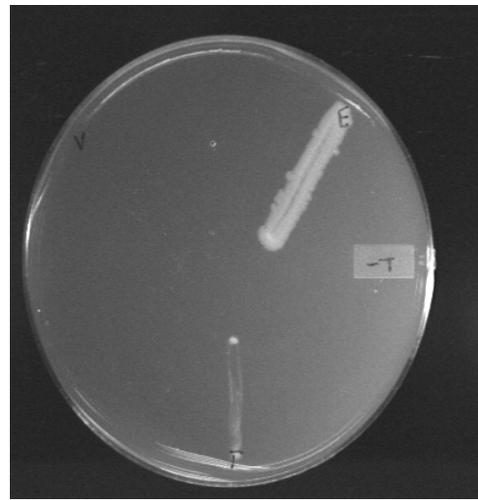expressed   pCT *thyX*
gene   or EC *thyA*

strain   MH2720
mutation   *thyA*⁻
expressed   pCT *thyX*
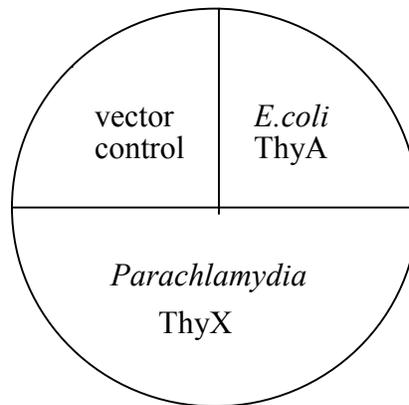gene   or EC *thyA*

A
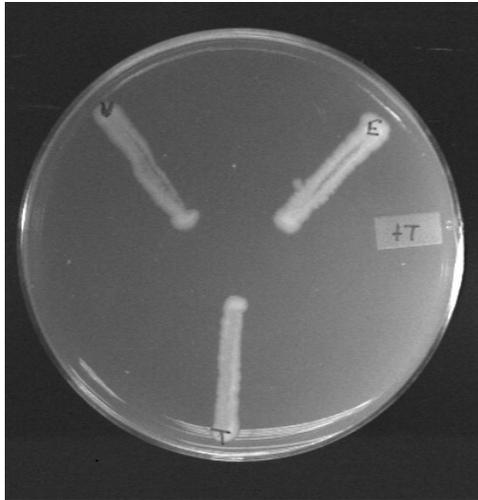
B

C

Figure 4. Complementation analysis of *Parachlamydia* UWE25 PC0155 in a *thyA*-strain of *E.coli* (MH2720). The gene encoding ORF PC0155 was cloned into the pQE-80L expression vector. Vector without insert and *E.coli* ThyA were used as contols. Constructs were transformed into *E.coli* MH2720 (*thyA*⁻) and growth was observed on M9 plates plus IPTG. (A) containing thymidine or B) lacking thymidine. C) Location of the source of the gene that has been transformed into the *thyA*⁻ strain of *E. coli*. Vector control, *E.coli thyA* contol, and *parachlamydia* UWE25 *thyX* .

```
   1  atgctaacagatgattatgaagagttcactgaatcgcaaattcaa
      M  L  T  D  D  Y  E  E  F  T  E  S  Q  I  Q
  46  acattaaagcgatatgttaccaatacatctagtcatgttttcgca
      T  L  K  R  Y  V  T  N  T  S  S  H  V  F  A
  91  ttgagaaatttacccgaagtcattaaaggcgcgctttttttcgcgt
      L  R  N  L  P  E  V  I  K  G  A  L  F  S  R
 136  tattcgcgatctagtctgggattaagaagtttattacttaaagaa
      Y  S  R  S  S  L  G  L  R  S  L  L  L  K  E
 181  ttcatttctaacaatgatgaaacggcatttgaagatattgttgga
      F  I  S  N  N  D  E  T  A  F  E  D  I  V  G
 226  gtagctttcaaagaagaagagaaacaacaaactcatcaaagtgag
      V  A  F  K  E  E  E  K  Q  Q  T  H  Q  S  E
 271  gccattagaaaagcgcaagctttttacgatcgaattttagatggt
      A  I  R  K  A  Q  A  F  Y  D  R  I  L  D  G
 316  tatggagatgactcaattggcgaattaggaggagctcatctagcg
      Y  G  D  D  S  I  G  E  L  G  G  A  H  L  A
 361  attgaaaatgtttctatgatggccgctaaactgattgaagaccat
      I  E  N  V  S  M  M  A  A  K  L  I  E  **D** **H**
 406  cgtattggaggatctcctcttgaaaaatcgactcgctatatttat
      **R**  I  G  G  S  P  L  E  K  **S**  T  R  Y  I  Y
 451  tttgatcaaaaagtgaatggagaataccttttctatcgagaacca
      F  D  Q  K  V  N  G  E  Y  L  F  Y  R  E  P
 496  attttaatgacttcagcttatcgcgatagttatgtgcaaacctgt
      I  L  M  T  S  A  Y  R  D  S  Y  V  Q  T  C
 541  aatatgttattcgatacgtatagtcgtttaattcctccaatgaca
      N  M  L  F  D  T  Y  S  R  L  I  P  P  M  T
 586  gctattattgaacaacgattcccccaaagatccctctatttctaaa
      A  I  I  E  Q  R  F  P  K  D  P  S  I  S  K
 631  accgcttatacagccgcgttgcgagctaaagttttagactgttta
      T  A  Y  T  A  A  L  R  A  K  V  L  D  C  L
 676  cgaggcttactacctgccggaacccttaccaatatgggaatttac
      R  G  L  L  P  A  G  T  L  T  N  M  G  I  Y
 721  ggaaatggtcggttttttgaacatcttattcacagaatgaactgt
      G  N  G  R  F  F  E  H  L  I  H  R  M  N  C
 766  caaaatttggcagaattacaagatatcgctaaaagaagtcatcaa
      Q  N  L  A  E  L  Q  D  I  A  K  R  S  H  Q
 811  gagctttctaaaatcattccttcgtttgtacgccgctcagatcca
      E  L  S  K  I  I  P  S  F  V  R  R  S  D  P
 856  acccatcgtacgcatcaaagttacgcccagttttttagaaacaatg
      T  H  R  T  H  Q  S  Y  A  Q  F  L  E  T  M
 901  caatcggaacttagactaattgccgagcaatatcttggtaaatgt
      Q  S  E  L  R  L  I  A  E  Q  Y  L  G  K  C
 946  gatcgatcacttcaacccggagttcgtctggtttcttccgatcca
      D  R  S  L  Q  P  G  V  R  L  V  S  S  D  P
 991  gaagctgtcactaaggtcgccgctgctcttctgtttttcactaggg
      E  A  V  T  K  V  A  A  A  L  L  F  S  L  G
1036  gacaaaggatttgcagacatttggaatcaatgtaagcggttgtcc
      D  K  G  F  A  D  I  W  N  Q  C  K  R  L  S
1081  gatgaagagctcgcgcgaattttagatgccggttgtaatgcacgt
      D  E  E  L  A  R  I  L  D  A  G  C  N  A  R
1126  gaaaataggcgacaaaaatctccaagagctctcgaacatgctgaa
      E  N  R  R  Q  K  S  P  R  A  L  E  H  A  E
```

```
1171  tttactttcgaaatcatctcagattttggcgcttatcgagattta
      F   T   F   E   I   I   S   D   F   G   A   Y   R   D   L
1216  catagacatcgcattttaacgcaagaacgacaacttctttgttgc
      H   R   H   R   I   L   T   Q   E   R   Q   L   L   C   C
1261  gactacggattttatattcctccagaaattcaaaacactccttac
      D   Y   G   F   Y   I   P   P   E   I   Q   N   T   P   Y
1306  gaagaagcatattgtcatgcactcaaccaagctaaagaagttttt
      E   E   A   Y   C   H   A   L   N   Q   A   K   E   V   F
1351  gatttgatttcaagcgaacttcctgaagaagctcaatatatcgtt
      D   L   I   S   S   E   L   P   E   E   A   Q   Y   I   V
1396  ccaatggcgtataatattcgttggtattttcatgtgaatttgcgt
      P   M   A   Y   N   I   R   W   Y   F   H   V   N   L   R
1441  gcacttcagtggctctgcgaattacgttctgctccagcaggtcat
      A   L   Q   W   L   C   E   L   R   S   A   P   A   G   H
1486  ccaagttatcgccatatagctcaaacaatggctagactaacctgt
      P   S   Y   R   H   I   A   Q   T   M   A   R   L   T   C
1531  gaagcctttcctgctttcgaaagatttttttaaattgtcgattac
      E   A   F   P   A   F   E   R   F   F   K   F   V   D   Y
1576  gaaggatacgaattaggaagactcgatcaagaacaaagaaaaatt
      E   G   Y   E   L   G   R   L   D   Q   E   Q   R   K   I
1621  gataaacaacaatctcttcttatt**taa** 1647
      D   K   Q   Q   S   L   L   I   *
```

Figure 5. **Nucleotide and amino acid sequence of *Parachlamydia* UWE25 thymidylate synthase complementing protein (ORF PC0155).** The start (atg/methionine [M]) and stop codon taa, in the nucleotide sequence are highlighted in bold type. The residues representing putative N and C terminal ThyX motifs are indicated in bold underlined type.

c) Identification and purification of *Parachlamydia* UWE25 PC0155

In order to determine if recombinant PC0155 had thymidylate synthesizing

activity, an *in vitro* enzyme assay was developed. Recombinant PC0155 was expressed

in *E. coli* MH$_{2720}$ (*thyA*$^-$), using the pQE80-L expression system. *E. coli* MH$_{2720}$ was used

as a host because it lacks thymidylate synthesizing activity, due to an inactivating

mutation in its ThyA encoding gene. As such any thymidylate synthesizing activity

detected with recombinant PC0155 would have to result from the parachlamydia

recombinant protein. Purification of recombinant PC0155 by nickel-affinity

chromatography typically yielded 2-3 mg/L of purified protein, using standard published

protocols for culture growth and recombinant protein induction. In an attempt to improve

protein yield, so that enough PC0155 would be produced for subsequent assays, a

modified procedure involving culturing at lower incubation temperatures, as described in

"Materials and Methods", was tried. This modified protocol increased recombinant

protein yield by approximately two fold, to about 5mg/L.

On SDS-polyacrylamide gel electrophoresis recombinant PC0155 showed one

predominant band with an apparent molecular mass of ≈ 63.2 kDa (Figure 6). The

purified protein was a pale yellow in color, suggesting the presence of an enzyme bound

flavin molecule, similar to that reported for *H. pylori* and *C.trachomatis* ThyX

(Myllykallio *et al*. 2002, Griffin *et al*.2005).
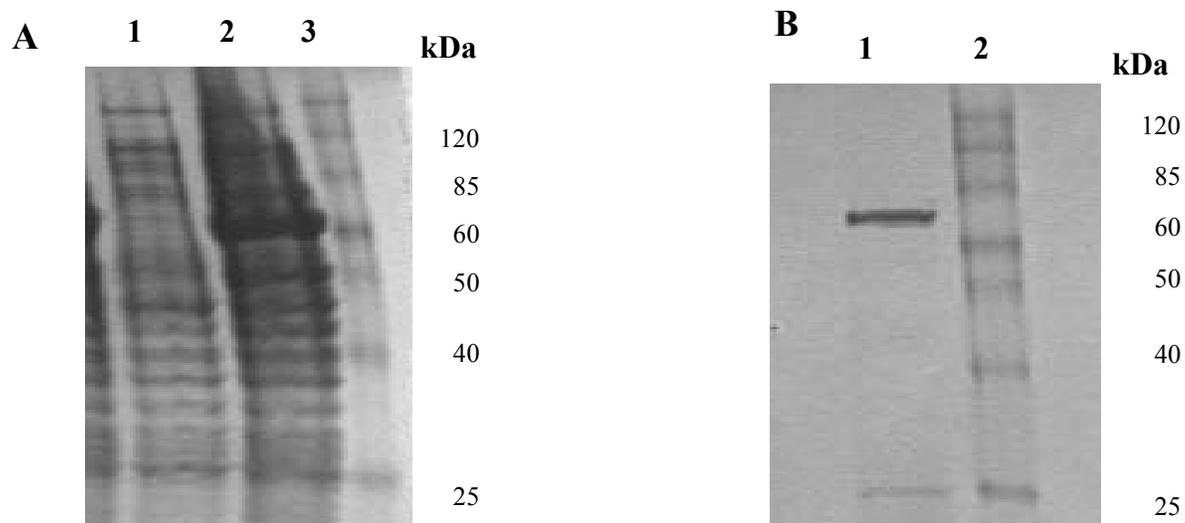
Figure 6. SDS-Polyacrylamide gel electrophoresis of recombinant Parachlamydia ThyX. A) Samples of crude cellular extracts were run on a 12% SDS-Polyacrylamide gel along with the molecular weight markers. The gel was then stained in a coomassie brilliant blue staining dye solution. Lane 1 uninduced crude recombinant pCTThyX and Lane 2, induced crude recombinant pCTThyX and Lane 3, molecular weight markers. B) A purified sample of 2µg recombinant pCTThyX was run on a gel. Lane 1, purified recombinant pCTThyX and Lane 2, molecular weight markers.

d) Spectroscopic characterization of PC0155 bound flavin

The pale yellow color of purified recombinant PC0155 suggested that it is a flavin-containing enzyme. To determine the presence of an enzyme bound flavin, the presumed flavin was isolated from purified PC0155 by heat denaturation, and then subjected to spectroscopic analysis. 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 7). These results are analogous to those obtained for the *H. pylori, T. maritima, C. jejuni,* and *C.trachomatis* ThyX where an oxidized flavin is bound to the ThyX protein (Myllykallio *et al*. 2002; Agrawal *et al.* 2004, Gattis and Palfey 2005,Griffin *et al* 2005).

e) PC0155 gene expression in *Parachlamydia* UWE25

The RT-PCR was used to determine whether PC0155 is expressed in *Parachlamydia* UWE25. Total RNA was isolated from *Parachlamydia* UWE25 infected *Acanthamoeba*. The RNA was synthesized into cDNA using reverse transcriptase with random hexamer primers. The cDNA was used as template for PCR reactions. Primers used for PCR were specific for the coding region of each gene indicated (Table 1).

Primers specific to parachlamydial 16S rRNA were used to detect the presence of RNA and to ensure that the amount of cDNA used was constant (Figure 8). Samples were ensured to be free of contaminating DNA by subjecting each time point to PCR minus the reverse transcription step (negative control).RNA isolated from uninfected *A. catellanii* was used as a negative control. Results shown in Figure 8 indicate that PC0155 is specifically expressed in *Acanthamoeba* infected with *Parachlamydia* UWE25.
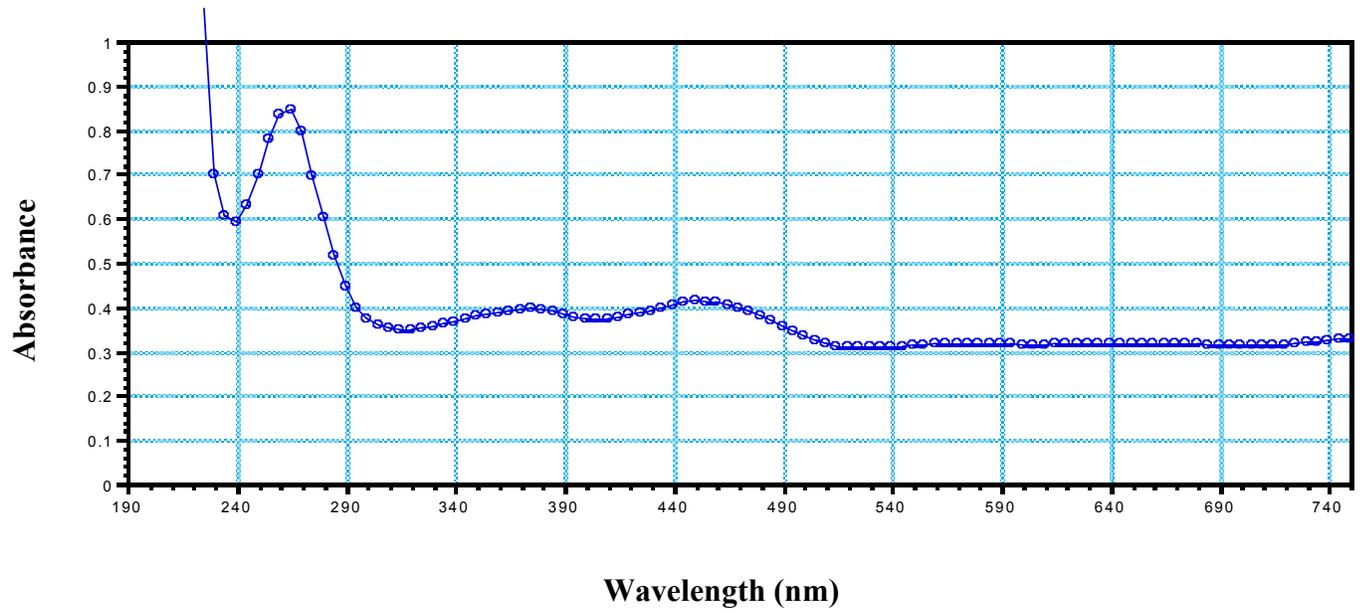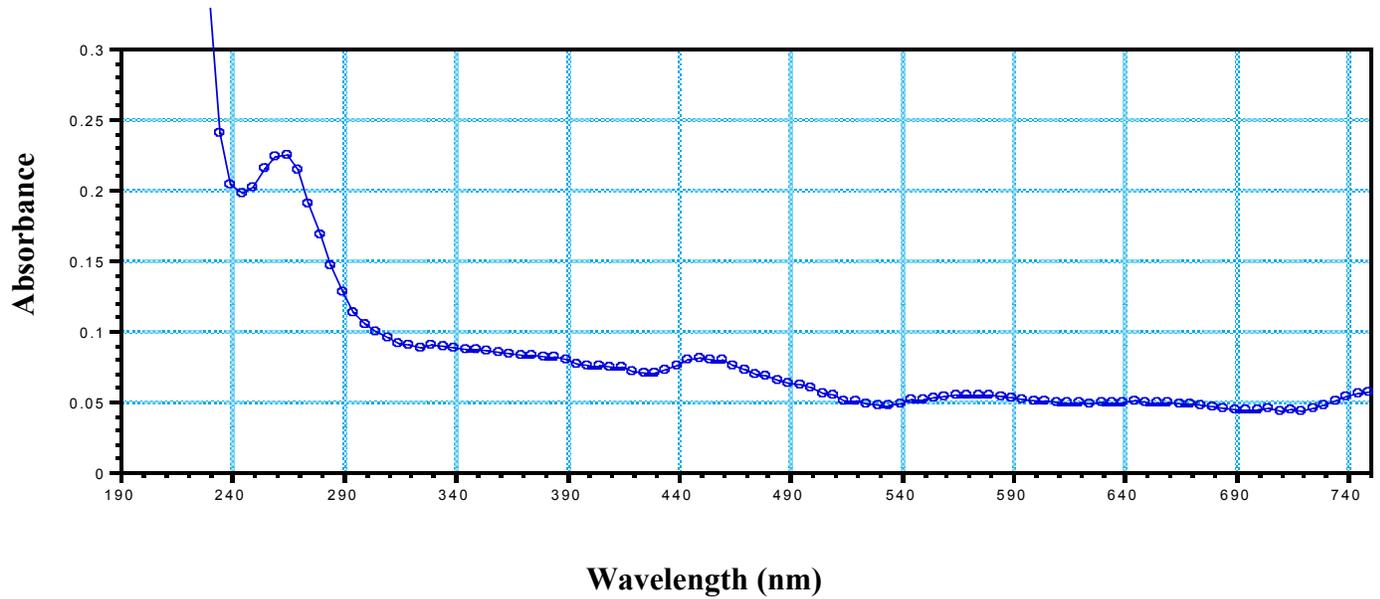
Figure 7. Spectroscopic analysis of flavin isolated from PC0155. A) Standard FAD and B) Flavin isolated from pCThyX. Flavin was isolated from pCThyX by heat denaturation of purified recombinant protein. The characteristic peaks at 260 nm, 375 nm and 450nm are indicative of oxidized FAD.
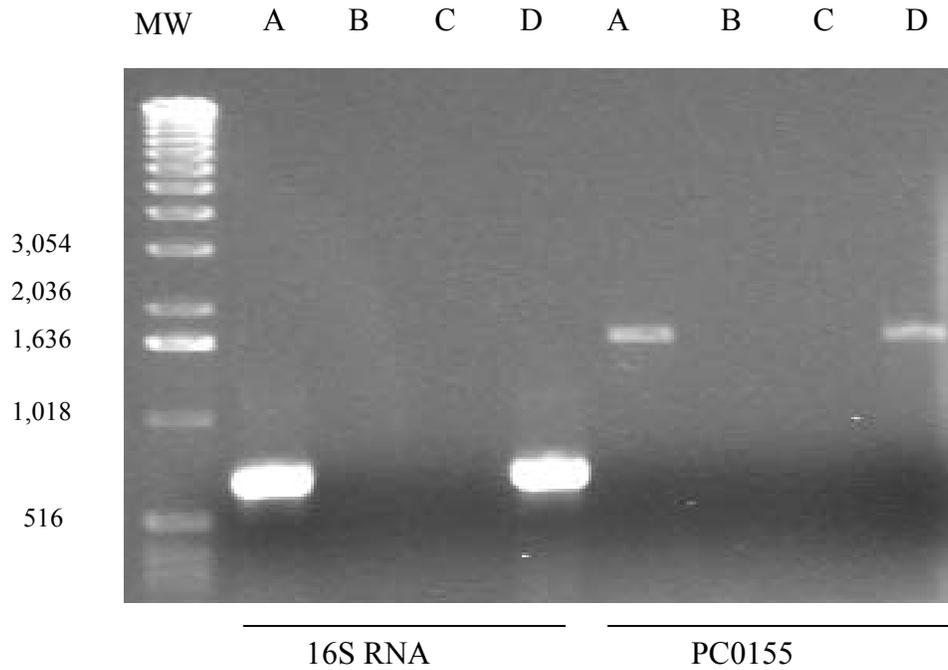
Figure 8. RT-PCR analysis of total RNA extracted from
***Parachlamydiae* UWE25 infected *Acanthamoeba*.** Each lane contains
RNA samples subjected to RT-PCR analysis using specified primers (16s
RNA and PC0155) A) *Acathamoeba* infected with *Parachlamydia*
UWE25 B) uninfected *Acathamoeba castellanii* C) negative-control D)
positive-control

2. Thymidylate Synthesizing Activity of *Parachlamydia* PC0155

Optimization of *in vitro* assay conditions

a) Cofactor requirements

Enzymatic reactions involving the highly characterized ThyA enzymes use CH_2H_4folate as both a one-carbon donor and as the source of reducing equivalents, which makes ThyA enzymes unique (Carreras and Santi 1995, Myllykallio *et al.* 2002). In contrast to ThyA, studies on *T. maritima, H. pylori* and *C. trachomatis* ThyX indicate that dTMP synthesizing activity is dependent on CH_2H_4folate, reduced flavin nucleotides, and reduced pyridine nucleotides (NADH or NADPH; Myllykallio *et al.* 2002, Griffin *et al.* 2005). To conclusively demonstrate that purified recombinant PC0155 was capable of catalyzing the formation of dTMP from dUMP an *in vitro* assay was developed and optimized. Initial results indicated that PC0155 was indeed capable of *in vitro* dTMP synthesizing activity under standard assay conditions (see "Materials and Methods" for details) with dUMP, CH_2H_4folate, and a reduced pyridine nucleotide (NADPH) present as substrates. These results indicate that PC0155 (pCTThyX) does indeed encode the parachlamydia ThyX homologue. Reactions involving *H. pylori*, *T. maritima* and *C. trachomatis* ThyX have been shown to require NADH or NADPH, with no enzymatic activity taking place in the absence of a reduced pyridine nucleotide (Myllykallio, Lipowski *et al.* 2002, Agrawal *et al.* 2004, Leduc *et al.* 2004, Griffin *et al.*2005). We wanted to determine whether pCTThyX requires an external source of reducing power for enzymatic activity to occur. pCTThyX was assayed under standard conditions in the presence of various cofactors; 2 mM of NADPH, NADH, FADH and FMN. The results

in Table 2 indicate that there is no enzyme activity in the absence of external reducing agent. In addition, there is no activity with NADH, FADH or FMN as cofactor. In contrast, dTMP-synthesizing activity is detected with the addition of NADPH. These results indicate that pCTThyX does require an external source of reducing power and that NADPH is the only reduced pyridine cofactor capable of supporting enzyme activity.

b) Nucleotide specificity

Thymidylate synthase specifically uses dUMP for the formation of dTMP; however, it has been observed under certain circumstances that ThyA is able to methylate alternative nucleotides such as dCMP (Liu and Santi 1992, Liu and Santi 1993). To determine the nucleotide specificity of pCTThyX, various nucleotides were added into the assay to compete with the natural nucleotide dUMP. Competition by other substrates suggests the ability of alternative nucleotide to enter the active site of pCTThyX. The results shown in Table 3 demonstrate the ability of various nucleotides to compete with dUMP. The competing nucleotide was added at 5-fold excess over dUMP (500 μM vs 100 μM). As expected dTMP, the product of the reaction, showed inhibition of activity since it can freely enter the active site of the enzyme, thereby competing with dUMP. Previous kinetic work on ThyA determined that dTMP is a competitive inhibitor with respect to dUMP (Daron and Aull 1978). To determine the preferred form of the ribose sugar competition with UMP was carried out. UMP does not significantly inhibit pCTThyX, indicating that, similar to *E. coli* ThyA, thymidylate synthesizing activity is specific for the deoxy- form of the ribose sugar.

Table 2. Cofactors requirement for pCTThyX dTMP synthesizing activity

Cofactor	Activity[a] (ηmoles dTMP formed/min/mg)
None	< 0.01
NADPH	1.14
NADH	< 0.01
FADH	< 0.01
FMN	< 0.01

[a] Results are the average of two determinations.
Enzyme assays were conducted at 37°C, with 200 μM CH_2H_4folate
and 200μM dUMP under standard assay conditions as described
in the "Materials and Methods".

Table 3. Nucleotide Specificity of pCTThyX

Nucleotides[a]	Activity[b] (ηmoles dTMP produced/min/mg)	Relative Activity (%)
	pCThyX[c]	pCThyX
dUMP	10.60 ±1.71	100
dUMP + TMP	6.70± 0.850	63.20
dUMP + UMP	6.56 ± 0.580	61.88
dUMP + dCMP	< 0.01	0.00

[a] Concentration of dUMP was 100 μM and 500 μM for all other nucleotides listed.
[b] Results are the mean ± standard deviations of two determinations.
[c] Enzyme assay conditions were conducted at 37°C as described in "Materials and Methods".

To demonstrate pyrimidine-base specificity, dCMP was added to the assay to compete with dUMP. Surprisingly, dCMP strongly inhibited pCTThyX activity suggesting that it competes with dUMP for the enzyme active site. It is possible that pCTThyX could have dCMP methylating activity or it may be that dCMP is an allosteric effector of the enzyme. Further kinetic studies are needed to distinguish these two possibilities.

c) Folate specificity

There are various folates that can act as one-carbon carriers and could be potentially used in the formation of dTMP. We wanted to determine if pCTThyX uses $CH_2H_4folate$ specifically or is able to use other reduced folates, such as $CHOH_4folate$ and $CH_3H_4folate$ as a methyl donor. The results presented in Table 4 indicate that $CH_2H_4folate$ was absolutely required for pCTThyX activity. There was little or no activity when other methyl-folates were added. These results indicate that pCTThyX is specific for $CH_2H_4folate$ as a one-carbon donor.

d) pH optima

Depending on the organism, ThyA enzymes demonstrate maximal activity at pH ranges from 6.5 to 8.0 (Carreras and Santi 1995). The optimal pH for pCTThyX was determined. The activity of purified pCTThyX was measured over a pH range of 6.0 to 9.0. As shown in Figure 8, pCTThyX showed maximal activity at pH 7.6, which is close to pH optimum of 7.2 for *C. trachomatis* ThyX (Griffin *et al.* 2005).

Table 4. Use of alternative folates by pCTThyX .

Cofactor[a]	Activity[b] (ηmoles dTMP produced/min/mg) ThyX[c]	Relative Activity (%) ThyX
CH_2H_4folate	8.50 ± 0.98	100
$CHOH_4$folate	0.22 ± 0.02	2.59
CH_3H_4folate	0.27 ± 0.03	3.18
None	<0.01	<1

[a] Cofactor concentrations were 500 μM for the folates, and the cofactor was replaced with H_2O for the reaction containing none.
[b] Results are the mean ± standard deviations of two determinations.
[c] Enzyme assay conditions were conducted at $37^{\circ}C$ with 100 μM dUMP and 1 mM NADPH under standard assay conditions as described in "Materials and Methods".
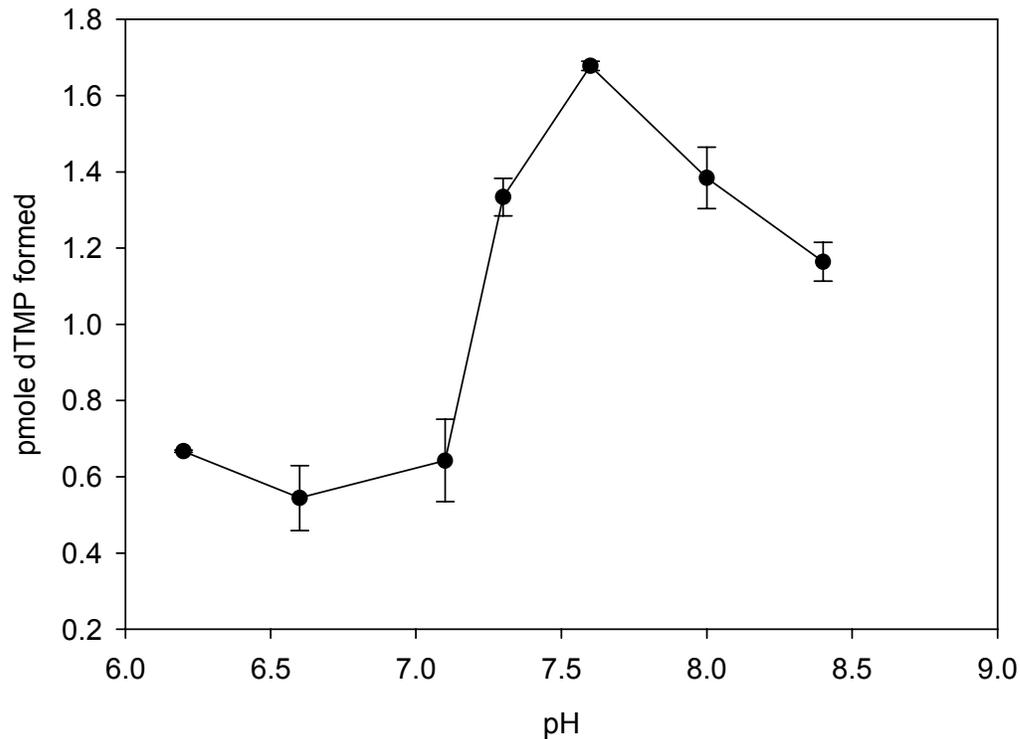
Figure 9. pH profile of purified recombinant pCTThyX activity. The pre-reaction mixture contained: 5% glycerol, 2 mM NADPH, 1 mM CH_2H_4folate, 200 μM [5-^{3}H] dUMP (15 μCi/ml) and 50 mM Tris-HCl with the indicated pH in a final volume of 100 μl. The reaction was initiated with the addition of 10 μg of purified pCTThyX. Each assay was run in triplicate and the results shown are the mean ±S.E.M.

e) dTMP synthesizing activity as a function of pCTThyX concentration

In order to determine if pCTThyX activity was linear with enzyme concentration, increasing amounts of recombinant protein were added to dTMP synthesizing assays. The results demonstrated that there is a linear relationship between the amount of enzyme added and pCTThyX activity (Figure 10).

f) dTMP synthesizing activity as a function of reaction time

The effect of incubation time on pCTThyX dTMP synthesizing activity was carried out in order to determine if the activity was linear with respect to time. The results indicated that the activity remains linear for up to 10 min at 37˚C (Figure 11).
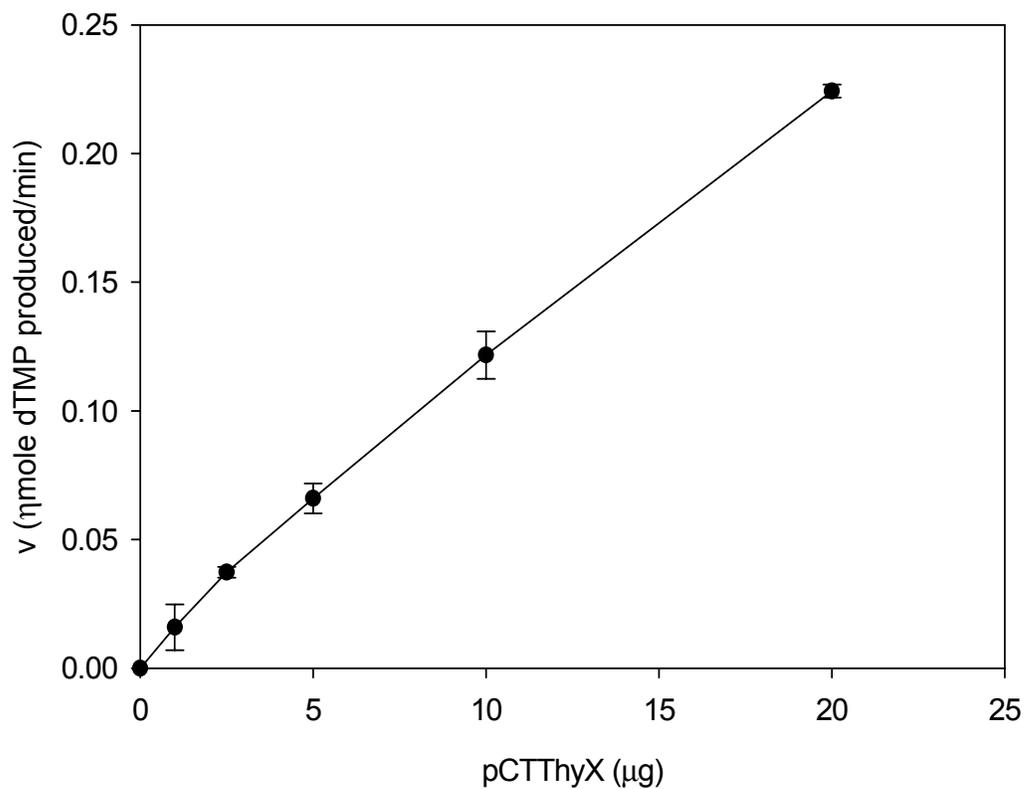
Figure 10. Linearity of pCTThyX activity with protein concentration. The pre-reaction mixture contained: 50 mM Tris-HCl pH 7.6, 5% glycerol, 2 mM NADPH, 1 mM CH$_2$H$_4$folate, and 200 μM [5-^{3}H] dUMP (15 μCi/ml) in a final volume of 100 μl. The reaction was initiated by the addition of the indicated amount of purified pCTThyX and incubated at 37°C for 20 min. Each assay was run in triplicate and the results shown are the mean ±S.E.M.
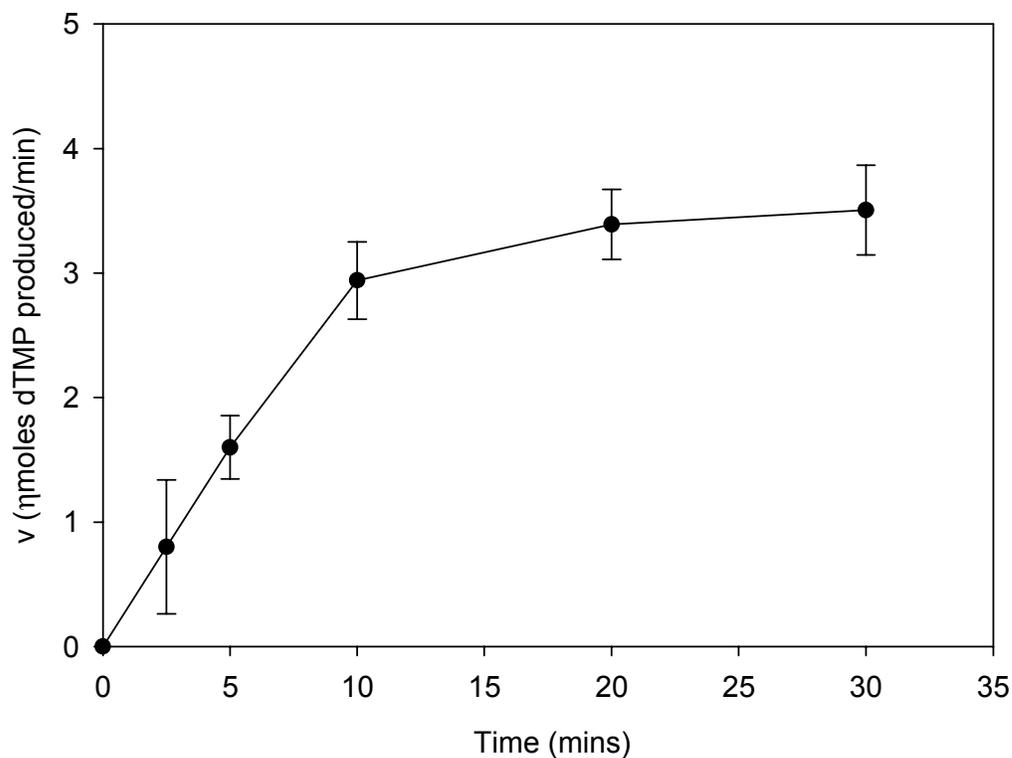
Figure 11. **Linearity of pCTThyX activity with reaction time**. The pre-reaction mixture contained: 50 mM Tris-HCl pH 7.6, 5% glycerol, 2 mM NADPH, 1 mM CH$_2$H$_4$folate and 200 μM [5-^{3}H] dUMP (15 μCi/ml) in a final volume of 100 μl. The reaction was initiated by the addition of 10 μg of purified pCTThyX and incubated at 37°C for the indicated length of time. Each assay was run in triplicate and the results shown are the mean ±S.E.M.

SUMMARY

The results presented indicate that *Parachlamydia* UWE25 open reading frame PC0155 encodes an active thymidylate synthesizing enzyme that has been grouped into a novel family of proteins termed ThyX (Kuhn *et al.* 2002, Myllykallio *et al.* 2002, Mathews *et al.* 2003, Myllykallio *et al.* 2003, Leduc et al. 2004, Griffin *et al.* 2005). Similar to pathogenic chlamydiae, *Parachlamydia* UWE25 ThyX employs CH_2H_4folate solely as a one-carbon donor, dUMP as methyl accepting substrate and uses an enzyme bound FAD molecule and external NADPH as the reducing agents. Based on the data presented in this thesis and *Parachlamydia* UWE25 genome sequence data, a schematic representation of the thymidylate synthesis cycle for parachlamydiae is presented (Figure 12).

These data emphasizes the importance of functional genomics for uncovering unique targets for rational drug design. This is especially true considering a large number of important pathogenic bacteria posses the *thyX* gene, including *H. pylori, Campylobacter jejuni, Rickettsia prowazekii, Mycobacterium turberculosis, C.trachomatis* (Myllykallio *et al*. 2002, Griffin *et al*. 2005) and parachlamydia ,and their potential pathogenic counter parts which infect free-living amoeba.

H_4folate   dTMP

Serine

Serine
Hydroxymethyltransferase   pCTThyX
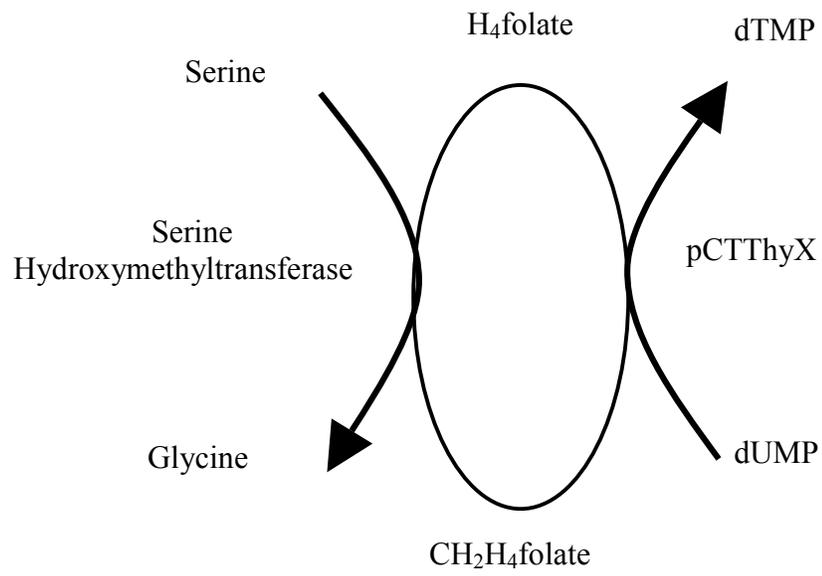
Glycine   dUMP

CH_2H_4folate

Figure 12. Schematic representation of proposed *Parachlamydia* thymidylate synthesis cycle.

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APPENDIX

Abbreviations

EB	elementary body
RB	reticulate body
RT-PCR	reverse transcriptase-polymerase chain reaction
FLA	free living amoeba
bp	base pairs
kDa	kilodalton
NTP	ribonucleoside triphosphate
dNTP	deoxyribonucleoside triphosphate
TS	thymidylate synthase
TK	thymidine kinase
DHFR	dihydrofolate reductase
$CH_2H_4folate$	methylene tetrahydrofolate
$H_2folate$	dihydrofolate
$H_4folate$	tetrahydrofolate
$CHOH_4folate$	formyl tetrahydrofolate
$CH_3H_4folate$	methyl tetrahydrofolate
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate) [oxidized form]
$NAD(P)H$	nicotinamide adenine dinucleotide (phosphate) [reduced form]
FAD	flavin adenine dinucleotide [oxidized form]
$FADH_2$	flavin adenine dinucleotide [reduced form]

FMN	flavin mononucleotide
ThyX	thymidylate synthase X
pCT*ThyX*	parachlamydia ThyX
EC*ThyA*	*E. coli* ThyA
TSCP	thymidylate synthase complementing protein
FDTS	flavin-dependent thymidylate synthase
LB	*Luria-Bertani* Medium
TCA	trichloroacetic acid
D	apargine
R	arginine
S	serine
L	leucine
H	histidine
C .caviae	*Chlamydophila caviae*
C. muridarium	*Chlamydia muridarum*
C. trachomatis	*Chlamydia trachomatis*
C .pneumoniae	*Chlamydophila pneumoniae*
C. aurantiacus	*Chloroflexus aurantiacus*
T. acidophilum	*Thermoplasma acidophilum*
P. torridus	*Picrophilus torridus*
F. acidarmanus	*Ferroplasma acidarmanus*
T. volcanium	*Thermoplasma volcanium*
T. tengcongensis	*Thermoanaerobacter tengcongensis*

C. perfringens	*Clostridium perfringens*
D. ethenogenes	*Dehalococcoides ethenogenes*
T. maritima	*Thermotoga maritima*
C. tetani	*Clostridium tetani*
G. sulfurreducens	*Geobacter sulfurreducens*
G. metallireducens	*Geobacter metallireducens*
S. solfataricus	*Sulfolobus solfataricus*
C. diphtheriae	*Corynebacterium diphtheriae*
T. kodakaraensis	*Thermococcus kodakaraensis*
D. hafniense	*Desulfitobacterium hafniense*
P. horikoshii	*Pyrococcus horikoshii*
A. pernix	*Aeropyrum pernix*
M. tuberculosis	*Mycobacterium tuberculosis*
R. rubrum	*Rhodospirillum rubrum*
B. cereus	*Bacillus cereus*
A. pompejana	*Alvinella pompejana*
N. equitans	*Nanoarchaeum equitans*
C. *lari*	*Campylobacter lari*
W. succinogenes	*Wolinella succinogenes*
H. pylori	*Helicobacter pylori*
L. interrogans	*Leptospira interrogans*
D. melanogaster	Wolbachia endosymbiont of Drosophila melanogaster
T. whipplei	Tropheryma whipplei