Carbon and Energy Metabolism In *Chlamydia trachomatis*

By

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Submitted to the Faculty of Graduate Studies

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Carbon and Energy Metabolism in *Chlamydia trachomatis*

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Emma Iliffe-Lee

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

Doctor of Philosophy

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ABSTRACT

For years, the obligate intracellular eubacteria chlamydiae, were speculated to be energy parasites, completely dependent on the host cell for high-energy intermediates. In order to gain a better understanding of chlamydial biochemistry and intracellular parasitism, carbon and energy metabolism was studied. Four *C. trachomatis* genes encoding energy-producing enzymes of the glycolytic and pentose phosphate pathways specifically, glyceraldehyde-3-phosphate dehydrogenase (GA3PDH), phosphoglycerate kinase (PGK), pyruvate kinase (PK) and glucose-6-phosphate dehydrogenase or zwichenferment (ZWF) were cloned, sequenced and expressed as recombinant proteins in *Escherichia coli*. Results indicate that the deduced amino acid sequence obtained showed high homology to other respective GA3PDH, PGK, PK, and ZWF enzymes, all four genes were expressed during the chlamydial life cycle and the recombinant proteins were active. The key, regulatory enzyme PK was further analyzed and kinetic studies showed that *C. trachomatis* PK (CTPK) requires cations for activity, can use alternative NDPs as phosphate acceptors and is allosterically inhibited by ATP, GTP and AMP, a metabolite that normally activates bacterial PKs. Surprisingly, CTPK is also allosterically activated by fructose-2,6-bisphosphate, a metabolite found only in eukaryotes. Studies on glycogen and carbon metabolism in *C. trachomatis* indicated that chlamydia was capable of limited growth in the presence of certain gluconeogenic substrates but was not able to synthesize glycogen from these substrates. In addition, unlike other bacteria, *C. trachomatis* was unable to regulate transcription of central metabolism genes in response to type or amount of carbon found in its growth environment. Information generated from these

studies can be used towards the development of new treatments for chlamydial diseases and towards the development of a cell-free growth system for chlamydiae.

INTRODUCTION

Chlamydiae

Chlamydiae are obligate intracellular eubacterial parasites that infect a wide range of eukaryotic host cells and cause a variety of diseases in human, animals and birds (Fraiz and Jones, 1988). Chlamydiae are classified in their own order, *Chlamydiales*, with one family, *Chlamydiaceae*, and a single genus, *Chlamydia*. Molecular analysis of rRNA sequences confirms that chlamydiae are eubacteria with only very distant relationships to other eubacterial orders (Weisburg *et al*., 1986). Currently, the genus *Chlamydia* consists of four species, *C. trachomatis*, *C. psittaci* (Page, 1968), *C. pneumoniae* (Grayston, 1989) and *C. pecorum* (Fukushi and Hirai, 1993). *C. trachomatis* is a human pathogen responsible for a variety of sexually transmitted diseases and the blindness, trachoma. *C. pneumoniae*, also a human pathogen, is found worldwide. Seroepidemiological studies indicate that more than 60% of adults have had some exposure to this organism in their lifetimes (Schachter, 1999). *C. pneumoniae* is an important cause of respiratory disease causing up to 10% of all cases of community-acquired pneumoniae and 5% of bronchitis and sinusitis cases (Kuo *et al*., 1995). Recently, *C. pneunomiae* has been reported to be associated with a variety of chronic or acute diseases such as atherosclerosis, asthma, sacracoidosis, otitis media, erythema nodosum and Reiter's syndrome (Kuo *et al*., 1995). *C. pecorum* affects mammals and *C. psittaci* primarily cause avian and animal disease but can also cause a pneumonia-like illness in humans (psittacosis).

C. trachomatis is divided into three biovars: trachoma, lymphogranuloma venereum (LGV), and murine based on the disease each causes (Moulder, 1988). Comparative DNA sequence analysis indicates that the trachoma and LGV biovars appear to be essentially identical (>95%) where as the murine biovar is more distantly related (Stephens, 1999a). The mouse biovar consists of a single serotype, mouse pneumonitis [MoPn]. LGV biovar consists of four serovars, L1, L2, L2a, and L3, and attacks the lymphatic and subepithelial tissues. LGV is the cause of the sexually transmitted disease lymphogranuloma venereum (Schachter, 1999). The trachoma biovar consists of at least 15 serovars with several sub-types now recognized and is believed to be limited to columnar epithelial cells at mucosal surfaces (endocervix, urethra, epididymis, endometrium, oviduct, conjunctiva, nasopharynx, and lower respiratory tract) (Schachter, 1999). Trachoma serovars A, B, Ba and C are the leading cause of the preventable form of blindness, trachoma (Fraiz and Jones, 1988). The worldwide prevalence of trachoma is often quoted as 400-600 million and an estimated 6 million cases suffer severe visual impairment (Schachter, 1999). Trachoma is endemic primarily in developing areas such as Africa, India, the Middle and Far East as well as Latin America. Trachoma serovars D-K, including Da, and Ia are the most common sexually transmitted bacterial pathogens, with an estimated 90 million new cases occurring each year worldwide (World Health Organization, 1996). Of these, about 4 million occur in the United States (Schachter, 1999). *C. trachomatis* genital infection can cause a wide variety of diseases including, conjunctivitis (via hand-eye contact), cervicitis, salpingitis, endometritis, urethritis, epididymitis, arthritis (Reiter's syndrome), sterility, ectopic pregnancy and pelvic inflammatory disease (Schachter, 1999).

The wide distribution of chlamydiae in the animal kingdom suggests that they are extraordinarily enduring and evolutionary successful pathogens. One of the reasons for chlamydiae's success as a pathogen is no doubt linked to its highly specialized biphasic growth cycle which consists of two distinct bacterial forms, the extracellular form termed the elementary body (EB) and the intracellular form, the reticulate body (RB) (Moulder, 1991). The chlamydial developmental cycle takes place within the confines of a membrane-bound vacuole, the chlamydial inclusion, which avoids fusion with the host cell lysosomes.

The EB is capable of initiating infection by attaching to and entering the host cell. The EB contains a condensed nucleoid that is mediated by histone-like proteins and is metabolically inactive. It is small (0.3 μm in diameter), and osmotically stable due to the high disulfide bond cross-linkage exhibited by the cysteine-rich outer membrane proteins found in the cell envelope. The cysteine-rich envelope proteins include: i) The major outer membrane protein or MOMP which is 40 kDa and encoded by *ompA*, ii) the outer membrane cysteine-rich protein B or OmcB which is encoded by *omcB* and posttranscriptionally processed into two proteins of 60 kDa and iii) OmcA, a lipoprotein of 12-15 kDa encoded by *omcA*. MOMP is present in the largest quantity (Moulder, 1991).

The cell envelope of chlamydiae is gram negative in that it includes an inner membrane and a lipopolysaccharide-containing outer membrane (Hatch, 1999). One important difference however, is the apparent lack of peptidoglycan (PG) (Barbour *et al.*, 1982; Fox *et al.*, 1990). Peptidoglycan forms a rigid barrier between the cell and the extracellular environment and is responsible for the osmotic integrity of the bacteria. The

inability to detect PG in chlamydiae conflicts with information from the genome sequence which indicates that most of the genes required for PG synthesis are present (Stephens *et al.*, 1998). Furthermore, chlamydiae are sensitive to drugs that inhibit PG synthesis such as penicillin G and D-cycloserine, and contain penicillin-binding proteins (Barbour *et al.*, 1982). PG is likely present in chlamydiae but only in very trace amounts and therefore has a small role in structural stability. Instead the presence of cysteine-rich outer membrane proteins which are cross-linked by disulfide bonds is thought to compensate for the lack of PG. Other proteins or structures found in the envelope include polymorphic outer membrane proteins (POMPs), heat shock protein (Hsp) 70, type III secretion apparatus, and glycoproteins (Hatch, 1999; Raulston, 1995).

Once the EB has attached and entered the host cell, it differentiates into the larger (1.0 µm in diameter), RB form. RBs have a relaxed nucleoid and are metabolically active. The transformation from EB to RB includes a reduction in the disulfide bonds in MOMP and the disappearance of OmcB and OmcA. This change in envelope character results in an increase in membrane fluidity, size and osmotic fragility. RBs divide by binary fission with a doubling time of ~2 h. The chlamydial developmental cycle is asynchronous with EBs, RBs and intermediate forms (chlamydial bodies in between the EB and RB stage), located within the same inclusion. By 16-20 h. post infection (p.i.), some of the RBs begin to differentiate back into EBs while others continue to replicate. The molecular signals involved in differentiation from EB to RB or RB to EB are poorly understood. Approximately 36-72 h p.i. depending upon the species or strain, most of the organisms are in the EB form and lysis or release from the host cell occurs. EBs can then go on to initiate a new round of infection (Moulder, 1991).

The precise mechanisms of chlamydial entry including the identification of a chlamydial ligand and a host cell receptor remain uncertain. A number of chlamydial ligands have been proposed and characterized. These include the glycosaminoglycan (GAG), heparan sulfate (HS), MOMP (Su *et al.*, 1990; Su *et al.*, 1988; Swanson and Kuo, 1991; Swanson and Kuo, 1994), hsp70, POMPs, and the thermolabile 38 kDa membrane protein (Joseph and Bose, 1991a and 1991b). Several studies suggest a strong role for HS in the EB attachment process, however it remains controversial whether the HS is a chlamydial ligand, or host ligand (Chen and Stephens, 1994; Chen and Stephens, 1997; Chen *et al.*, 1996; Davis and Wyrick, 1997; Kuo and Grayston, 1976; Rasmussen-Lathrop *et al.*, 2000; Stephens, 1994; Stephens *et al.*, 2000; Su *et al.*, 1996; Taraktchoglou *et al.*, 2001; Zhang and Stephens, 1992).

Similar to the attachment process, the molecular mechanisms of uptake/entry are unclear. Chlamydiae enter non-professional phagocytes with high efficiency strongly suggesting that they evoke the host to initiate internalization (Byrne and Moulder, 1978). Evidence for receptor-mediated endocytosis into clathrin-coated pits (Hondinka *et al.*, 1988; Hondinka and Wyrick, 1986), microfilament-dependent (phagocytic) uptake into non-clathrin-coated vesicles (Ward and Murray, 1984) as well as evidence for both mechanisms (Wyrick *et al.*, 1989) has been presented. Clearly, chlamydiae manipulate the host to allow entry however the nature of the host signaling cascades and the cellular processes activated are poorly understood. Evidence for a role of cyclic nucleotides (Ward and Salari, 1980; Ward and Salari, 1982) and tyrosine phosphorylation (Birkelund *et al.*, 1994; Fawaz *et al.*, 1997) in chlamydial entry has been presented. Recent studies on the resistance of chlamydiae-infected cells to apoptosis suggests communication

between the host cell signaling pathways and chlamydia (Fan *et al.*, 1998). In addition, the proposed type III chlamydial secretion system may also have a function in chlamydial entry as type III secretion systems in other gram-negative bacteria have been shown to inject proteins into eukaryotic host cells and disrupt cell signaling pathways (Hueck, 1998).

Once the EB has achieved entry into the host cell, the chlamydial developmental cycle can take place within the chlamydial inclusion. Intracellular survival of chlamydiae is dependent on the ability to enter and replicate inside a host cell. One of the survival mechanisms chlamydia employs is to avoid fusion with host cell lysosomes. It is well documented that the chlamydial inclusion is not lysosomal in character in that lysosomal (Friis, 1972; Lawn *et al.*, 1973) and secondary lysosomal (Wyrick and Brownridge, 1978) markers are not found in the chlamydial inclusion. Studies suggest that chlamydial avoidance of lysosomal fusion may be due to some intrinsic property of the cell wall (Eissenberg and Wyrick, 1981; Eissenberg *et al.*, 1983; Levy and Moulder, 1982) as well as through the action of chlamydial specific protein(s) (Scidmore *et al.*, 1996b). The chlamydial inclusion is also non-fusogenic with endocytic vesicles (Heinzen *et al.*, 1996; van Ooij *et al.*, 1997). Within 2 h p. i., endocytosed EBs are concentrated in the region of the Golgi apparatus. Host microtubules and microfilaments appear to play a role in trafficking chlamydia to the peri-Golgi location (Clausen *et al.*, 1997; Ridderhof and Barnes, 1989; Schramm and Wyrick, 1995). Furthermore, trafficking studies using C6-NBD-ceramide indicate that chlamydia acquire sphingomyelin from the vesicles originating from the trans-Golgi network where it is trafficked to the chlamydial inclusion and incorporated into the cell walls of the bacteria (Hackstadt *et al.*, 1996;

Hackstadt *et al.*, 1995; Scidmore *et al.*, 1996a). Collectively, these studies suggest that the chlamydial inclusion may evade lysosomal fusion by appearing to the host as a secretory vesicle which is not destined to fuse with lysosomes (Hackstadt, 1999b).

Intracellular Parasitism

Intracellular parasites have evolved diverse strategies for survival inside host cells. Essential steps include: i) The parasite must gain entrance inside the host cell, ii) the invading organisms must evade host cellular defense mechanisms, iii) the parasite must not destroy host functions which are essential to parasite replication, iv) the parasite must multiply, v) progeny parasites must be released from the host cell and continue to invade and replicate in new hosts (Finlay and Falkow, 1989; Hackstadt *et al.*, 1998; Moulder, 1974; Moulder, 1985). In order to survive, intracellular parasites must avoid host cell defenses such as lysosomal killing. Some intracellular parasites, such as *Rickettsia*, *Shigella* and *Listeria* employ mechanisms that allow them to escape the endocytic vesicle and replicate freely, within the host cell cytoplasm (Salyers, 1994). Others such as *Coxiella burnetii* and likely *Leishmania*, do not avoid lysosomal fusion and have adapted strategies that allow them to survive within the harsh, acidic environment of the lysosome (Salyers, 1994). Most intracellular parasites however, inhabit vesicles which do not fuse with the lysosome (Hackstadt, 1999b). As discussed, it has been suggested that chlamydiae avoid fusion with the host cell lysosome by manipulating its vesicle such that it appears as an exocytic vesicle to the host (Hackstadt, 1999b).

One of the advantages of intracellular parasitism is that the organisms have the potential to access all the essential building blocks for DNA, RNA, protein and lipid synthesis that other free-living organisms must synthesize for themselves (for review see McClarty, 1994; Moulder, 1985; Moulder, 1991). Exposure to such a nutrient-rich environment may account for the small chlamydial genome (1.0 x 10^6 bp) as many redundant genes may be dispensed. Indeed, the chlamydial genome sequence indicates that many genes involved in biosynthesis of metabolites such as amino acids and nucleotides are not present (Stephens *et al.*, 1998). Unlike intracytoplasmic bacterial parasites which have direct access to the nutrient-rich environment of the host cell cytoplasm (Falkow *et al.*, 1992; Moulder, 1985), chlamydiae are sequestered in a membrane-bound inclusion. Therefore, in order to acquire nutrients from the host cell cytoplasm, chlamydiae must have a specialized transport mechanism that allows nutrients to cross not only the inclusion membrane, but also the outer and cytoplamic membranes. Possible means of nutrient exchange include: i) Fusion of the vacuole with nutrient-laden vesicles of the endosomal or lysosomal pathway that are involved in fluid-phase uptake or turnover of endogenous components, ii) open channels through the parasitophorous vacuole to the cytoplasm that allow the free exchange of low-molecular weight molecules (Desai *et al.*, 1993; Schwab *et al.*, 1994) or iii) transport proteins of host or parasite origin contained in the membrane that specifically bind and deliver metabolites from the cytoplasm to the lumen of the vacuole.

Possible means of nutrient exchange in chlamydia include MOMP, which has been shown to function as a porin (Bavoil *et al.*, 1984), an ATP translocase which has been previously demonstrated to exchange ATP for ADP (Hatch *et al.*, 1982; Tjaden *et*

al., 1999) and the genome sequence indicates that chlamydiae encodes for a wide variety of transport systems for acquiring metabolites (Kalman *et al.*, 1999; Stephens *et al.*, 1998). The use of specific transporters as a means for nutrient exchange by vacuole-bound parasites however, has not yet been documented. An additional means of nutrient exchange between chlamydia and the host cell cytoplasm may be accomplished by the spike-like projections found on the surface of both EBs and RBs (Matsumoto, 1988; Nichols *et al.*, 1985). RBs have been documented to have close contact with the luminal surface of the inclusion and the spikes apparently penetrate the inclusion membrane. However, there has been no direct evidence for nutrient transfer through these structures (Hackstadt, 1999b). Passive diffusion as a means of exchange also does not seem likely because the chlamydial inclusion does not contain pores that allow passive diffusion of tracer molecules as small as 520 Da (Heinzen and Hackstadt, 1997).

Biochemistry: Energy and Carbon Metabolism in Chlamydiae

For nearly forty years, chlamydiae were speculated to be energy parasites, incapable of generating their own ATP or other high-energy intermediates. Most of the biochemical evidence which supported the energy parasite hypothesis was based on negative findings: failure to detect glycolytic enzyme activities capable of generating net ATP or electron transport chain components (cytochromes, flavoproteins) required for oxidative phosphorylation (Allen and Bovarnick, 1957; and 1962; Moulder, 1991). Early work by Moulder (1970) and Gill and Stewart (1970b) demonstrated that L-cells infected with *C. psittaci* resulted in an increase in the rate of glycolysis and concluded that the

increase was not due to chlamydial metabolic activity, but rather was a host cell response to the infection. Later Moulder demonstrated that the increase in glycolysis due to chlamydial infection of host cells was not prevented by chloramphenicol, a prokaryotic protein synthesis inhibitor, but was completely inhibited by cycloheximide, a eukaryotic protein synthesis inhibitor (Kellogg *et al.*, 1977; Moulder, 1970). Gill and Stewart (1970a), as well as Becker and Asher (1972) demonstrated that the yield of chlamydia is reduced when L-cells are treated with eucaryotic inhibitors of mitochondrial function, and suggested that chlamydiae depend on mitochondrial ATP for growth and development. Hatch (1975) used a labeling technique to demonstrate that chlamydiae draw on the host cell for NTPs for biosynthesis of its own RNA. Other studies also demonstrated that chlamydiae are auxotrophic for three of the four NTPs and draw on the host cell for these NTPs (McClarty, 1994; McClarty and Tipples, 1991; Tipples and McClarty, 1993). Finally, Tipples and McClarty (1993) showed that *C. trachomatis* grows well in a mutant cell line with a severely compromised mitochondrial function and suggested that the source of ATP, that is whether it is produced by glycolysis or respiration, is not important. All of these *in situ* findings supported the energy parasite hypothesis.

Strong positive support for the energy parasite hypothesis came from the characterization of an ATP-ADP translocase, similar to that found in mitochondria (Fiore *et al.*, 1998), chloroplasts (Mohlmann *et al.*, 1998) and *Rickettsia prowazekii* (Krause *et al.*, 1985). The ATP-ADP translocase allowed for the exchange of host cell ATP for parasite ADP resulting in the net gain of high-energy phosphate (Hatch *et al.*, 1982). The

sequence of the *C. trachomatis* serovar D genome also indicates that chlamydia contains two paralogs for the ATP/ADP translocator (*adt1* and *adt2*) (Stephens *et al.*, 1998). The *adt1* and *adt2* gene products share 41% identity and 39% identity to the ATP/ADP translocase in *R. prowazekii* (accession number S65530) respectively, which was originally cloned and characterized as an obligate ATP/ADP exchanger by Krause *et al.* (1985). Recently the chlamydial *adt*1 and *adt*2 gene products were cloned and expressed as recombinant proteins in *E. coli* (Tjaden *et al.*, 1999). The *adt*1 product was characterized as a nucleoside phosphate transporter and was shown to exchange ATP for ADP where as the *adt*2 gene product was suggested to be a nucleoside triphosphate/H^+symporter and was able to take up NTPs. Together, the above findings supported the energy parasite hypothesis.

The genome sequence of *C. trachomatis* serovar D indicates that chlamydia encode for several energy-producing enzymes (Stephens *et al.*, 1998) however to definitively demonstrate that chlamydiae can generate ATP through catabolic reactions remains complicated. Conditions for cell free growth have not been established and no gene transfer system has been developed. Host free RBs are difficult to purify and show limited metabolic activity (Hatch, 1988). Furthermore, it is difficult to study energy metabolism *in situ* since few procaryotic or eucaryotic-specific inhibitors of energy metabolism are available.

To determine whether *Chlamydia trachomatis* contains functional enzymes which can produce energy (ATP) or reducing power (NAD(P)H), three glycolytic enzymes, specifically pyruvate kinase (PK), phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and one pentose phosphate pathway enzyme,

glucose-6-phosphate dehydrogenase or zwichenferment (ZWF), were identified, cloned and characterized. PK and PGK result in the production of ATP via substrate phosphorylation, GAPDH results in the production of NADH, and ZWF produces NADPH. Later in the course of this thesis project, the genome sequencing project (Stephens *et al.*, 1998) became available and the cloned genes of the *C. trachomatis* L2 serovar were found to have > 95% homology to serovar D. The data presented in this thesis, together with the information generated by genome sequence analysis indicates that chlamydia contains complete and functional Embden-Meyerhof-Parnas (EMP) and pentose phosphate pathways (PPP) as well as portions of the Tricarboxylic Acid (TCA) Cycle (Fig. 1).

The genome sequence also indicates that chlamydia contains a total of 894 open reading frames that include several genes that encode for various chemical reactions necessary to replicate itself (Stephens *et al.*, 1998). Metabolic reactions can be categorized into assembly, polymerization, biosynthetic and fueling reactions, based on their primary function in growth (see McClarty (1999), for review). This thesis project focused mainly on the fueling reactions, which produce the key metabolic precursors (Table 1) for most biosynthetic pathways. Biosynthetic reactions/pathways produce the building blocks such as nucleotides, amino acids, fatty acids and various sugars (Neidhardt *et al.*, 1990). In addition to metabolic precursors, fueling reactions also produce reducing power (NADH, NADPH), and conserve the metabolic energy (proton motive force [PMF], ATP) needed for biosynthesis. Chlamydiae encode the enzymatic machinery, which is collectively called central metabolism to produce all 12 key metabolic precursors for most biosynthetic pathways (McClarty, 1999; Neidhardt *et al.*,

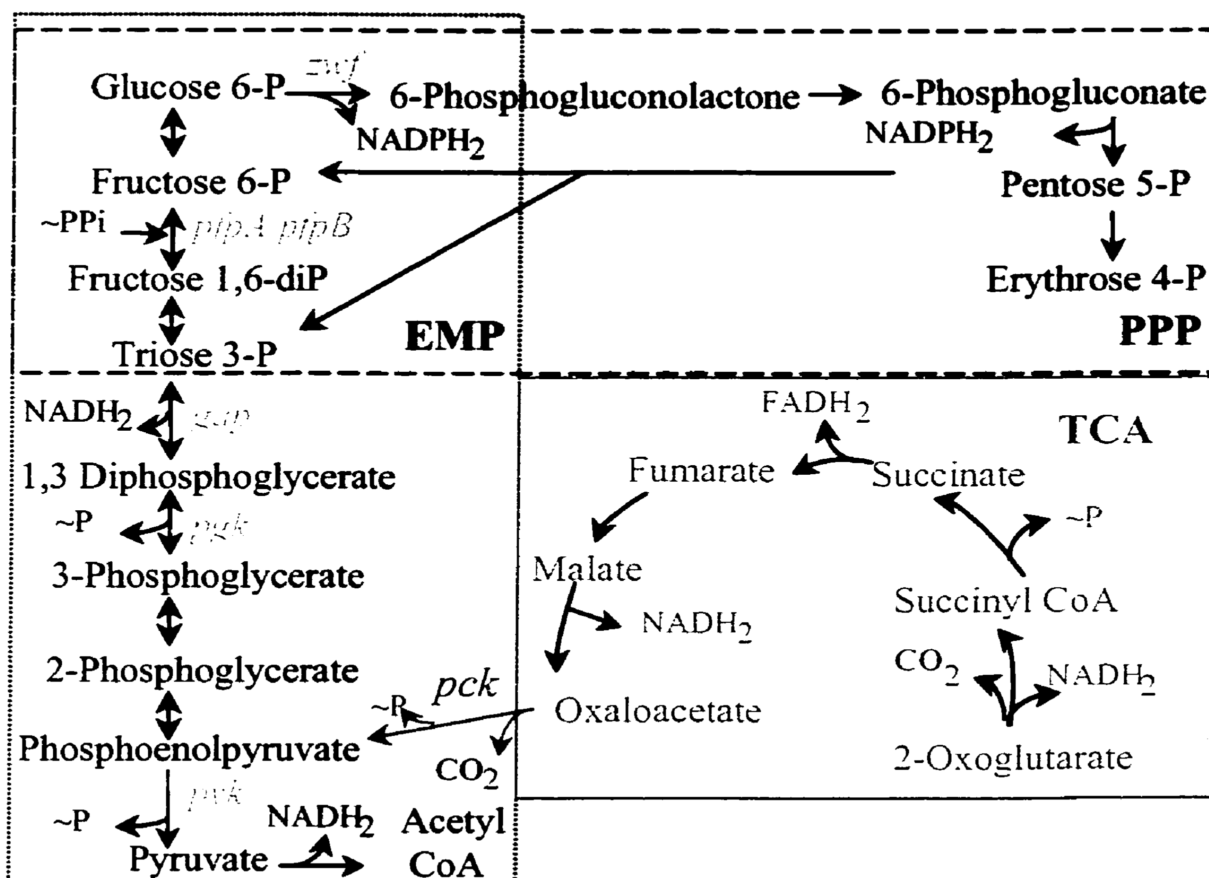


Figure 1. The Embden-Meyerhof Parnas (EMP) pathway, pentose phosphate pathway (PPP) and partial tricarboxylic acid cycle (TCA) in *C. trachomatis* as deduced from our studies and the serovar D genome sequence (Stephens *et al.*, 1998). The EMP is enclosed in a dotted line, the PPP is enclosed in a dashed outline, and the TCA is bordered by a solid line. The TCA cycle and the EMP are connected by the gluconeogenic reaction: oxaloacetate + GTP → phosphoenolpyruvate + GDP + $C0_2$ catalyzed by phosphoenolpyruvate carboxykinase (PEPCK).

1990). Central metabolism is divided into the EMP pathway, which converts glucose-6-P to pyruvate; PPP, which oxidizes glucose-6-P to CO_2; and the TCA cycle, which oxidizes acetyl-CoA to CO_2. Six of the 12 precursors come from the EMP pathway, two come from the PPP, and three more come from the TCA cycle; the last one is provided by pyruvate dehydrogenase (*pdh*), a linker reaction (McClarty, 1999; Neidhardt *et al.*, 1990).

Table 1. Precursor metabolites and their products in chlamydiae

Source pathway	Precursor metabolite	Building block(s)	Macromolecule(s) made
Glycolysis	Glucose-6-P	ADP-glucose	Glycogen
	Fructose-6-P	UDP-N-acetylglucosamine	Peptidoglycan
	Glyceraldehyde-3-P	Glycerol-3-P	Phospholipids
	3-P-glycerate		
	Phosphoenolpyruvate	Chorismate, KDO	Folates (?), LPS
	Pyruvate		
Pentose phosphate	Ribose-5-P	KDO	LPS
	Erythrose	Chorismate	Folates (?)
TCA cycle	Oxaloacetate	Phosphoenolpyruvate	Folates (?), LPS
	Succinyl-CoA		
	2-Oxoglutarate	Glutamate (?)	Protein
Linker reaction	Acetyl-CoA	Acetyl-CoA	Fatty acids

(McClarty, 1999).

EMP Pathway

The EMP or glycolytic pathway is a central metabolic pathway and is present, at least in part, in virtually all organisms. In addition, the enzymes involved are highly conserved (Fothergill-Gilmore and Michels, 1993; Fraenkel, 1996). Chlamydiae contains homologs of all enzymes in glycolysis except for hexokinase, the first enzyme in the EMP pathway which converts glucose to glucose-6-P (Stephens *et al.*, 1998)(Fig. 2). The lack of hexokinase is in agreement with an earlier study which could not detect chlamydia-specific hexokinase activity (Vender and Moulder, 1967). Chlamydiae however do contain homologs to two carbohydrate transporters namely SodiTi, a dicarboxylate transporter that likely supplies dicarboxylic acids to the TCA cycle, and UphC, a hexose-P transporter which could supply chlamydia with host glucose-6-P (Island *et al.*, 1992; Stephens *et al.*, 1998). Chlamydiae also contains homologs of two components, enzyme 1 (*pts1*) and Hpr (*ptsH*) but, lacks the homolog of the sugar-specific enzyme II (EII) of the phosphoenolpyruvate:phosphotransferase (PTS) system (Stephens *et al.*, 1998). The PTS system is the main sugar uptake system in many bacteria and primarily functions by phosphorylating and concomitantly transporting sugars (mono- and dissacharides) and other sugar derivatives. It also plays a central role in regulating gene expression, chemotaxis, and metabolism (Deutscher *et al.*, 1997; Luesink *et al.*, 1999; Saier *et al.*, 1995). It is likely that chlamydiae use the *uphC* gene product to acquire glucose-6-P from the host, which serves as the primary energy source in chlamydia and that the PTS system is involved in regulating gene expression rather than sugar transport (McClarty, 1999).

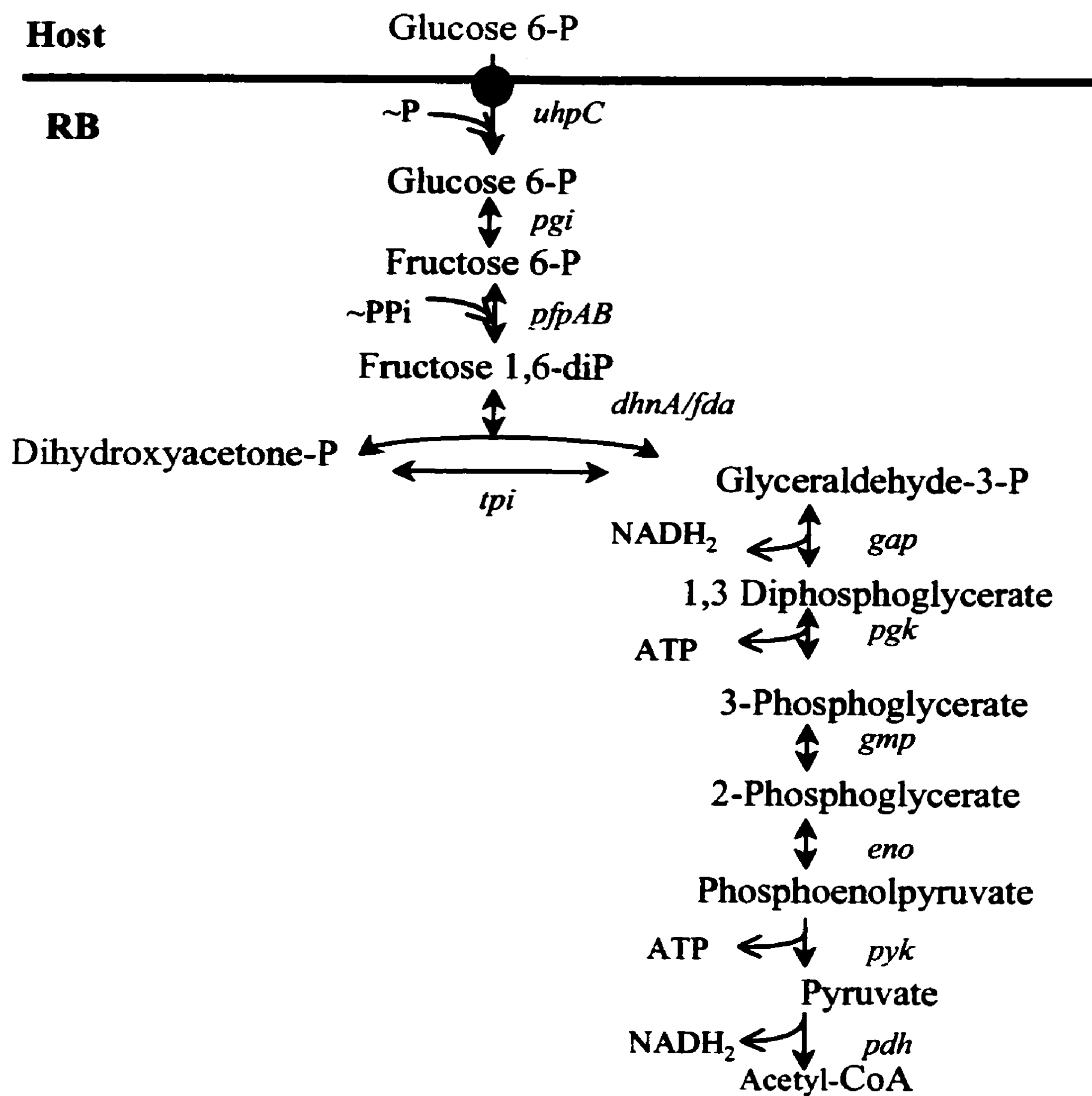


Figure 2. The Embden-Meyerhof-Parnas (EMP) pathway in *C. trachomatis* as deduced from the serovar D genome sequence (Stephens *et al.*, 1998) and from work in this thesis.

All of the chlamydial glycolytic enzymes show high homology to other respective prokaryotic and/or eukaryotic enzymes except for phosphofructose kinase (PFK) and fructose-1,6-bisphosphate aldolase (ALD). Information from the genome sequencing project indicates that *C. trachomatis* does not contain a typical bacterial *pfk*, rather it contains two genes, *pfpA* and *pfpB* which show high homology to pyrophosphate-dependent phosphofructose kinases (PPi-PFKs). PPi-PFK catalyzes the same reaction as PFK, except that uses PPi instead of ATP, and it is reversible (fructose-6-P + PPi $\leftrightarrow$ fructose-1,6-BP + Pi) (Mertens, 1991). PPi-PFK was first discovered in 1974, in the parasitic amoeba *Entamoeba histolytica* (Reeves *et al.*, 1974). Subsequently, it has been found in some bacteria, including *Borrelia burgdorferi* (Fraser *et al.*, 1997), *Treponema pallidum* (Fraser *et al.*, 1998), *Propionibacterium shermanii* (Mertens, 1991), protozoa such as *Giardia lambia* (Mertens, 1990), *Toxoplasm gondii* (Peng and Mansour, 1992), *Trichomonas vaginalis* (Mertens *et al.*, 1989), protists (Mertens, 1991) and plants (Todd *et al.*, 1995). Generally there are two different PPi-PFK classes: 1) Those found in higher plants which also contain ATP-PFKs and sometimes fructose-1,6-bisphosphatase (F16BPase), an important gluconeogenic enzyme which catalyzes the PFK reaction in the reverse direction (F16BP + ADP $\rightarrow$ F6P + ATP). Their PPi-PFK is a heterotetrameric enzyme composed of two non-identical subunits, the regulatory noncatalytic α-subunit and the catalytic β-subunit (Todd *et al.*, 1995). The enzyme is also allosterically regulated by fructose-2,6-bisphosphate (F26BP) (Alves *et al.*, 1996; Mertens, 1991). 2) All other PPi-PFK-containing organisms contain only small amounts of ATP-PFK or F16BPase, if any, and their PPi-PFKs are generally composed of two or four identical subunits and are not allosterically regulated (Alves *et al.*, 1996; Mertens, 1991).

In most organisms, ATP-PFK catalyzes the first irreversible step in glycolysis and is the main control point of this pathway. Bacterial and mammalian PFKs are composed of four identical subunits whereas the yeast PFK is an octomer composed of two nonidentical regulatory (α) and catalytic (β) subunits (Alves *et al.*, 1996; Michels *et al.*, 1997). The distinct enzyme, F16BPase is required to catalyze the PFK reaction in the reverse direction. The regulation of these two enzymes is critical in controlling the flux through the glycolytic and gluconeogenic pathways (Fothergill-Gilmore and Michels, 1993; Mertens, 1991). PPi-PFKs only need the one enzyme to perform both glycolytic and gluconeogenic reactions because the enzyme is reversible (Mertens, 1991). In theory, the use of PPi-PFK instead of ATP-PFK could improve ATP yield produced during glycolysis. PPi-PFK uses PPi, a byproduct that would normally be hydrolyzed to inorganic phosphate and thus spares an ATP to be used for another reaction (Mertens, 1991). Work on *C. trachomatis pfpA* and *pfpB* genes and gene products is presented in this thesis.

ALD catalyzes the reversible split of fructose-1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Fraenkel, 1996). ALD are classified into class I and class II based on the mechanism of the reaction (Fothergill-Gilmore and Michels, 1993). The chlamydial homolog of ALD has high homology to an *E. coli* gene product, *dnhA* which was recently distinguished as a class I aldolase (Thomson *et al.*, 1998). Both of these enzymes show low sequence identity with other known aldolases in both class I and II making these enzymes rather unusual.

Glycolysis provides a net gain in the universal energy transducer ATP via substrate level phosphorylation reactions; the formation of ATP by a reaction between

ADP and a phosphorylated intermediate of a fueling pathway. Phosphoglycerate kinase and pyruvate kinase are the two enzymes in the glycolytic pathway which generate ATP via substrate phosphorylation (Fothergill-Gilmore and Michels, 1993). Both of these enzymes were cloned and characterized during the course of this thesis project.

Pyruvate kinase (PK) (EC 2.7.1.40) is a key enzyme in the glycolytic pathway that catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, producing pyruvate and ATP (PEP + MgADP + H^+ → pyruvate + MgATP). Generally, the reaction is irreversible and is one of the major control points in glycolysis. In most organisms, PK is a typical allosteric enzyme, controlled by one or more effectors and is dependent on both monovalent and divalent cations for activity (Fothergill-Gilmore and Michels, 1993).

In mammals, four isoenzymes (M1, M2, L and R) exist which are expressed in a tissue-specific manner and reflect the different metabolic requirements of the expressing tissue (reviewed by Muirhead, (1990). The M2 isoenzyme is widely distributed in vertebrate tissue such as kidney, intestine, lung fibroblasts, testis, and stomach. The L and R isoenzymes are found in the liver and erythrocytes respectively. The M2, L and R isoenzymes all display sigmoidal kinetics with respect to PEP and all are allosterically regulated by a number of effectors, most importantly being fructose-1,6-bisphosphate (F16BP). The isoenzymes R and L are also regulated by reversible protein kinase-mediated phosphorylation. The M1 isoenzyme is present in skeletal muscle, brain and heart and is regarded as unregulated because it shows hyperbolic saturation kinetics with regard to its substrates under most metabolic conditions. In the case of the allosteric PKs, the conformation of the enzyme is converted from the inactive T-state to the active R-

state upon binding of ligands such as PEP or F16BP (Imamura and Tanaka, 1982). Interestingly, the M1 protein is thought to have evolved from the prototypic allosteric PK, locked in an active R-like conformation for energy metabolism in specialized energy-consuming tissues such as brain and heart (Valentini *et al.*, 2000).

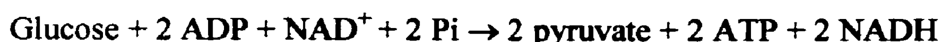
In vascular plants and green algae, PK exists as both cytosolic and plastid isoenzymes that differ in their respective physical, immunological and kinetic characteristics (Smith *et al.*, 2000; Lin *et al.*, 1989; Turner and Plaxton, 2000). Plant PK may exist as a monomer, homotetramer, heterotetramer or heterohexamer depending on the species, tissue and intracellular location (Smith *et al.*, 2000; Lin *et al.*, 1989; Turner and Plaxton, 2000; Podesta and Plaxton, 1991).

Most bacterial PKs show basal activity without effectors and are generally classified into two types, type I PK and type II PK. Type I PKs are dominant under growth conditions which favor glycolysis and are allosterically activated by F16BP and are inhibited by ATP and succinyl-CoA. The activator F16BP acts by increasing the affinity of the enzyme for PEP, and also relieves some of the inhibitory effects of ATP. Type II PKs dominate under gluconeogenic conditions and are activated by AMP, and sugar monophosphates such as ribose-5-P and glucose-6-P. *E. coli* and *S. typhimurium* contain both type I and type II isoenzymes whereas the majority of other prokaryotes contain either type I (some *Enterobacteriaceae*) or type II (*Pseudomonas citronellolis, Bacillus, Streptococcus mutans* and *Halobacterium*) where some differences within the types can occur (Fothergill-Gilmore and Michels, 1993). In contrast to animal, plant and bacterial PKs, protozoan parasitic PKs such as *Leishmania* and *Trypanosomes* have the

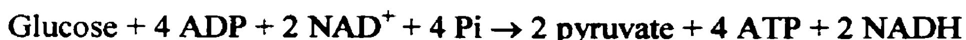
unusual property of being allosterically regulated by sub-micromolar concentrations of fructose-2,6-bisphosphate (F26BP) (Ernest *et al.*, 1998; Rigden *et al.*, 1999).

Phosphoglycerate kinase, an important enzyme in glycolysis, catalyzes the reversible transfer of a phosphoryl-group from 1,3-bisphosphoglycerate to ADP to form 3-phosphoglycerate and ATP. Typically, PGK is a monomeric enzyme with a mass of approximately 46 kDa, although dimeric and tetrameric PGKs have been found in the archaea *P. woesei* and *S. solfataricus* respectively (McHarg *et al.*, 1999). PGK is known to be activated by low concentrations of anions and is inhibited by high concentrations of non-substrate anions (McPhillips *et al.*, 1996).

In general, the sum reaction for glycolysis is:


Glucose + 2 ADP + NAD^+ + 2 Pi → 2 pyruvate + 2 ATP + 2 NADH

In contrast, the balance sheet for chlamydial glycolysis is:


Glucose + 4 ADP + 2 NAD^+ + 4 Pi → 2 pyruvate + 4 ATP + 2 NADH

Chlamydia has the potential to produce 4 ATP molecules, two from each triose arm of the EMP pathway. One ATP is saved because glucose-6-P is acquired from the host, and the other is saved because PPi is used as the phosphate donor versus ATP in the PFK reaction (McClarty, 1999).

Another important feature of glycolysis is that it produces $NADH^+$, the universal source of reducing power. Glyceraldehyde-3-P dehydrogenase (GAPDH) is an essential enzyme in the glycolytic pathway, which produces NADH. It catalyzes the oxidative

phosphorylation of glyceralde-3-phosphate into 1,3-bisphosphoglycerate (Fothergill-Gilmore and Michels, 1993). The reaction involves both an oxidation and phosphorylation of the substrate. The enzyme functions as a homotetramer consisting of four subunits with a molecular mass of about 150 kDa (Talfournier *et al.*, 1998; Yun *et al.*, 2000). Each subunit consists of two domains; the N-terminal coenzyme or NAD^+ binding domain (residues 1-148 and 311-330) and the C-terminal or catalytic domain (149-330) (*E. coli* GAPDH numbering) (Branlant and Branlant, 1985; Talfournier *et al.*, 1998). In addition to its role in glycolysis, GAPDH has been implicated in several other roles such as membrane transport, membrane fusion, microtubule assembly, nuclear RNA export, protein phosphotransferase/kinase reactions, DNA replication, DNA repair, neuronal disorders and apoptosis. (Berry and Boulton, 2000; Sirover, 1999). Whether *C. trachomatis* GAPDH also has additional roles remains to be defined. The cloning and characterization of *C. trachomatis* GAPDH enzyme is presented in this thesis.

The Pentose Phosphate Pathway

The PPP may occur by both an oxidative (*zwf* and *gnd*) and nonoxidative branch (*tkt*, *tal*, *rpi* and *rpe*). The oxidative branch generates NADPH, a major source of reducing power, which can be used for biosynthetic reactions such as fatty acid biosynthesis (Fraenkel, 1996; Neidhardt, 1990). The nonoxidative branch generates pentose phosphates and erythrose-4-P, two of the twelve precursor metabolites (Table 1). Chlamydiae contain all the homologs of the enzymes in the PPP except for 6-phosphogluconolactonase (*pgl*) (Stephens *et al.*, 1998)(Fig. 3), a gene found nonessential

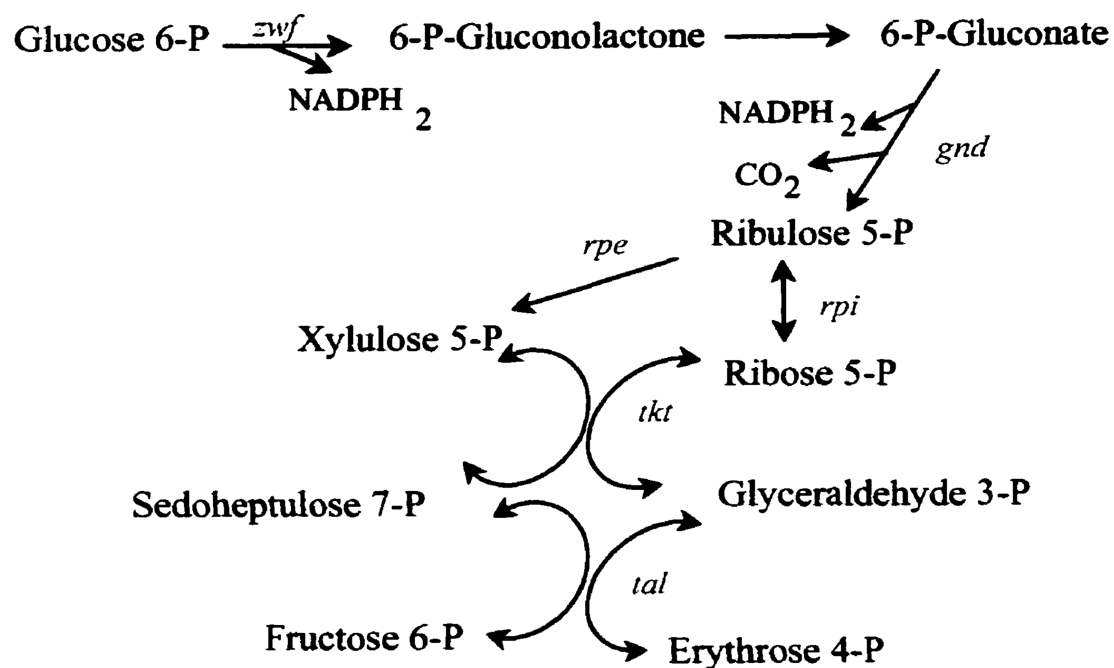


Figure 3. The PPP in chlamydiae as deduced from studies in this thesis and *C. trachomatis* serovar D genome sequence (Stephens *et al.*, 1998; McClarty, 1999).

in *E. coli* (Fraenkel, 1996). Early studies demonstrated the presence of glucose-6-P dehydrogenase (*zwf*) and 6-phosphogluconate dehydrogenase (*gnd*) activity in extracts prepared from *C. psittaci* (Moulder *et al.*, 1965). Glucose-6-P dehyrogenase (G6PDH) or zwichenferment (ZWF) catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone with the concomitant reduction of NADP to NADPH. This enzyme catalyzes the initial and rate limiting step in the PPP generating the main source of NAPDH which can be used for reducing power in most cells (Cosgrove *et al.*, 1998). G6PDH was cloned and characterized from *C. trachomatis* and is discussed in this thesis.

The Tricarboxylic Acid Cycle

The TCA cycle, or citric acid cycle is the final common pathway for the oxidation of fuel molecules such as amino acids, fatty acids and carbohydrates (Stryer, 1988). In order for the TCA cycle to operate, it requires a continuous supply of NAD^+ and FAD. One cycle of the TCA cycle results in the production of four electron carriers, (three NADH molecules and one $FADH_2$ molecule) as well as one high-energy phosphate bond (GTP). These electron carriers yield eleven molecules of ATP when they are oxidized by O_2 in the electron-transport chain (respiration) with the concomittant production of the electron acceptors (NAD^+ and FAD). These electron carriers or cofactors can also be reoxidized by fermentation, which involves the reduction of pyruvate to lactate. It is likely that chlamydiae regenerates NAD^+ and FAD molecules through the use of the electron-transport chain because chlamydiae contains homologs for the components in the electron-transport chain but lacks the lactate dehydrogenase homolog required for

fermentation (Stephens *et al.*, 1998). Another function of the TCA cycle is to provide intermediates for biosynthesis (Stryer, 1988). Three more of the precursor metabolites, oxaloacetate, 2-oxoglutarate and succinyl-CoA are produced (Table 1).

Information from the chlamydial genome sequence indicates that the TCA cycle is incomplete (Fig. 4). The first three enzymes, citrate synthase (*gltA*), aconitase (*acn*), and isocitrate dehydrogenase (*icd*) are missing (Stephens *et al.*, 1998). Interestingly, several other prokaryotic genomes that have been sequenced also contain incomplete TCA cycles where the last part of the oxidative cycle leading from succinate to oxaloacetate is the most highly conserved and the initial steps from acetyl-CoA to 2-oxoglutarate show the least conservation (Huynen *et al.*, 1999). As a result of the lack of initial steps, acetyl-CoA cannot enter the chlamydial TCA cycle. Instead, the cycle begins with 2-oxoglutarate and ends in oxaloacetate. The cycle can potentially function as long as there is an input of carbon and according to the chlamydial genome, this could be accomplished in two ways.

First, chlamydia contains a homolog for a dicarboxylate translocator (*sodiTi*) which shows highest homology (50%) to spinach (*Spinacia oleracea*) *sodiTi* in chloroplast envelopes (accession no. U13238). The spinach *sodiTi* encodes for a dicarboxylate exchanger, allowing the entry of one dicarboxylate into the chloroplast in exchange for another (Weber *et al.*, 1995). In chlamydia, the SodiTi transporter may allow for the transport of 2-oxoglutarate from the host in exchange for oxaloacetate to help fuel the partial TCA cycle. Chlamydia contains the necessary machinery to oxidize 2-oxoglutarate into oxaloacetate, which could then be returned to the host cell. Oxaloacetate could also be coverted into malate because *C. trachomatis* contains a

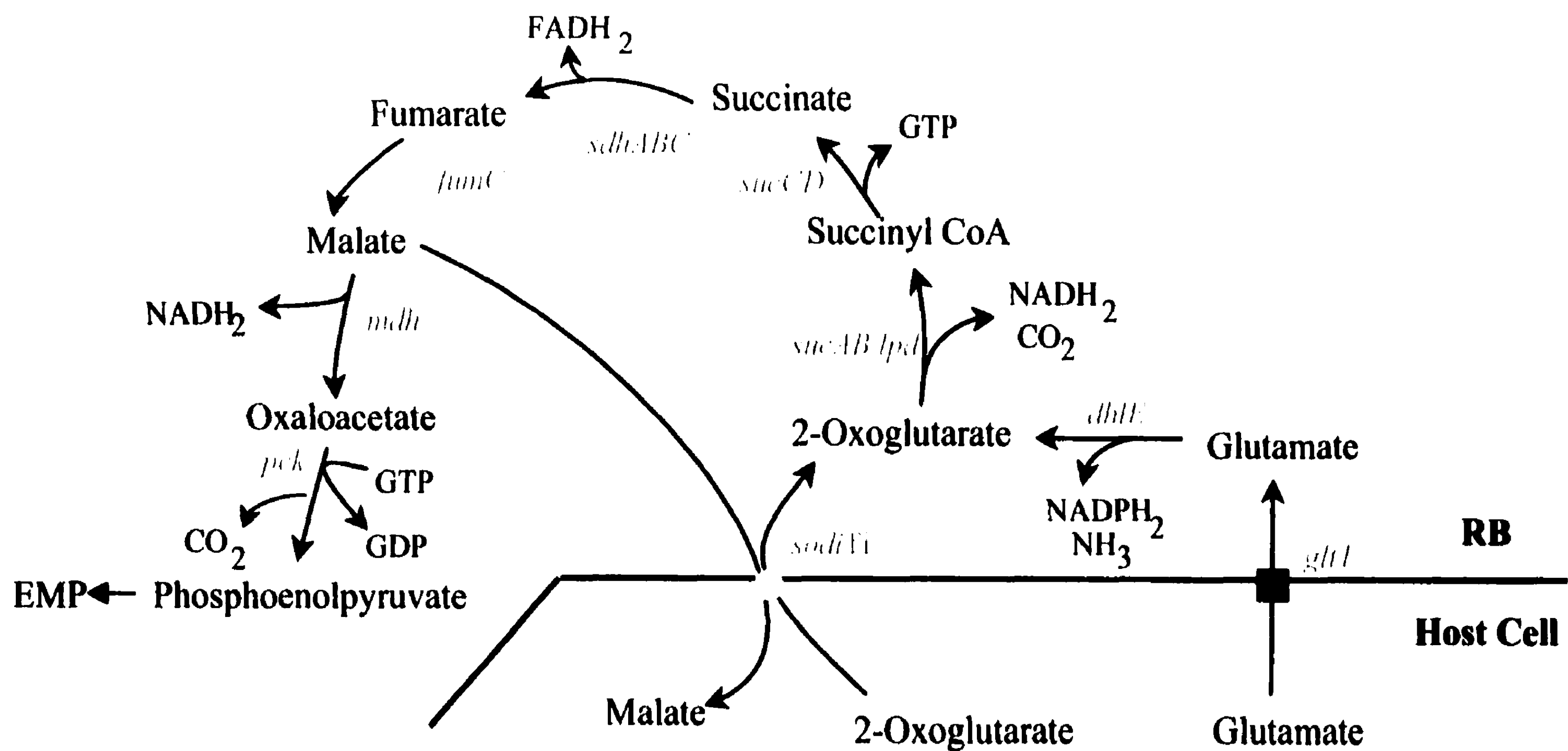


Figure 4. The tricarboxylic (TCA) cycle in chlamydiae. The TCA cycle is incomplete in chlamydiae; there is no entry of acetyl-CoA into the cycle. As a result, an alternative carbon source is required to keep the cycle functioning. Two possible senarios are depicted. One involves the SodiTi exchanger and the other involves the glutamate transporter (*gltT*) and glutamate dehydrogenase (*dhlE*) . See "Introduction" for details, (McClarty, 1999).

homolog of malate dehyrogenase (*mdh*). Malate could then be returned to the host cell by the SodiTi exchanger. Although the SodiTi exchanger would only allow for the net gain of a carbon, it would allow the cycle to function where one turn would result in the production of two NADH molecules, one $FADH_2$ molecule and one high-energy phosphate (GTP) (McClarty, 1999).

Secondly, chlamydia could obtain glutamate from the host cell through a glutamate transporter homolog (*gltT*). Early studies with *C. psittaci* extracts showed that glutamate could be deaminated to 2-oxoglutarate which could then be decarboxylated to succinate (Weiss, 1967). This sequestration of glutamate from the host cell would result in the net gain of five carbons. Chlamydiae also have a homolog to a dehydrogenase (*dhlE*) which could be used to convert glutamate into 2-oxoglutarate (glutamate + $NAD(P)^+$ + H_20 → 2-oxoglutarate + NH_3 + NAD(P)H). The 2-oxoglutarate could then enter the partial TCA cycle and get oxidized to oxaloacetate resulting in the production of NADH, FADH and GTP as described above (McClarty, 1999).

Gluconeogenesis

All cells require glucose for growth and gluconeogenesis is the synthesis of glucose from non-carbohydrate sources such as lactate, pyruvate, glycerol and most amino acids (Stryer, 1988). Several of the reactions that convert pyruvate into glucose are common to glycolysis. However, three of the glycolytic reactions are irreversible and therefore require bypass reactions, which are provided by gluconeogenesis. The irreversible reactions are hexokinase, phosphofructose kinase and pyruvate kinase. As

discussed, chlamydia does not contain a hexokinase and the phosphofructose kinase homolog is PPi-dependent and irreversible and therefore does not require a gluconeogenic bypass reaction. Chlamydiae contains a homolog for phosphoenolpyruvate carboxykinase (PEPCK) which catalyzes the reaction: oxaloacetate + GTP → phosphoenolpyruvate + CO_2 (Stephens *et al.*, 1998). This reaction bypasses the irreversible pyruvate kinase reaction and results in the formation of PEP, which can then move through the gluconeogenic pathway and allow for the formation of other glycolytic intermediates (see Fig. 1). Interestingly, PEPCK is the only direct link between the EMP pathway and the TCA cycle in chlamydiae. A further look at the chlamdial genome suggests that chlamydia could use glutamate as a carbon and energy source. As discussed previously, glutamate could be taken from the host via a glutamate transporter (*gltT*) and could be converted to 2-oxoglutarate through use of a dehydrogenase (*dhlE*). The 2-oxoglutarate could then move through the partial TCA cycle and into the EMP pathway via the PEPCK homolog (McClarty, 1999).

Respiration

The failure to detect oxygen consumption, flavoproteins and cytochrome respiratory enzymes in *C. psittaci* extracts (Allen, 1957; Allen, 1962) led to the conclusion that chlamydiae do not contain an electron transport chain. Results from the genome sequence however indicate that chlamydiae contain all the necessary components required for a functional electron transport chain (Fig. 5) (McClarty, 1999; Stephens *et al.*, 1998). These results indicate that chlamydia has at least the genetic capability to

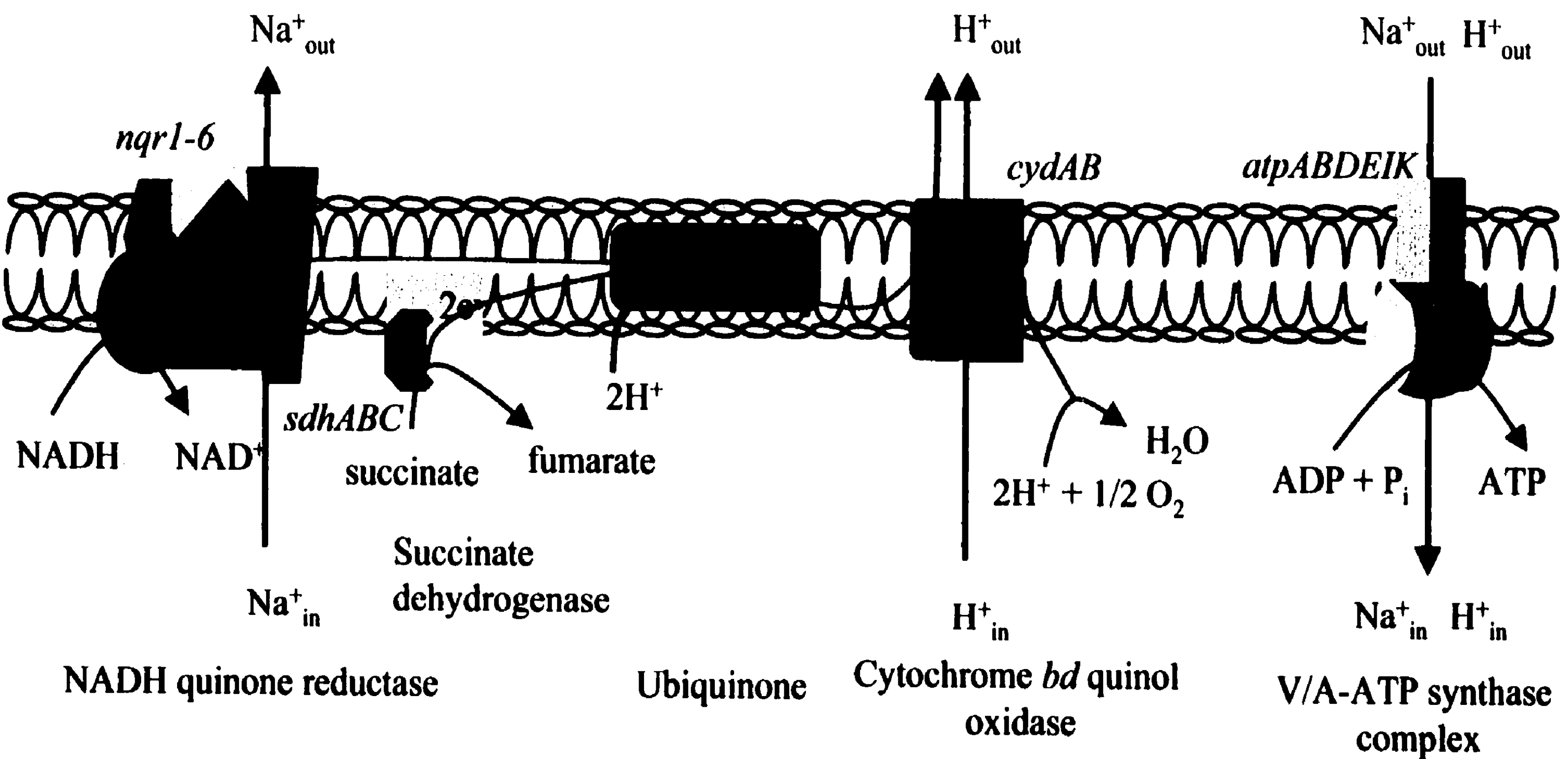


Figure 5. The electron transport chain and V/A-ATP synthase complex in chlamydiae as deduced from the *C. trachomatis* D genome (Stephens *et al.*, 1998). The proposed H^+ and/or Na^+ ion gradients for ATP synthesis in chlamydiae is depicted (McClarty, 1999).

generate ATP via respiration. Electron transport or respiratory chains are composed of membrane-bound protein complexes (dehydrogenases and reductases) and electron carriers (quinones) (Stryer, 1988). Electrons of NADH are passed to a common quinone pool (ubiquinone) via dehydrogenases or reductases (NADH-Q reductase or dehyrogenase). Ubiquinone also accepts electrons from $FADH_2$ via dehyrogenases (i.e. succinate dehydrogenase). Electrons of ubiquinone are then passed to the ultimate electron acceptor 0_2, to form H_20 via oxidases (cytochrome oxidase) (Stryer, 1988). The flow of electrons through the protein complexes leads to the pumping of protons from the inner member to the outer membrane creating a proton electrochemical potential gradient (PMF) across the cytoplasmic membrane. The PMF can be used by other membrane proteins to help transport solutes or generate ATP (McClarty, 1999; Stryer, 1988).

Chlamydia contains the six homologs that are known to comprise the Na^+-dependent NADH-quionone reductase complex, the primary electron transporter in the respiratory chain. Chlamydia also contains homologs to some of the enzymes in the ubiquinone biosynthesis pathway, although it may be that chlamydia obtains ubiquinone from the host cell. Homologs of all the subunits of succinate dehydrogenase as well as cytochrome oxidase are also present in chlamydia. Together then, it appears that chlamydiae have a redox-driven Na^+ pump via the Na^+-dependent NADH-quinone reductase and a H^+ pump generated by the electron transport chain creating both a Na^+ and electrochemical H^+ gradient. These gradients can be used to transport solutes and nutrients. Specifically, the PMF can be used to synthesize ATP. The chlamydial genome also contains homologs of a V_1V_0-type ATPase complex which may be used to generate

ATP either using Na^+ and /or H^+ gradient as the driving force (McClarty, 1999; Stephens *et al.*, 1998).

Glycogen Metabolism

Glycogen is a branched glucose containing polysaccharide, which represents a major carbon and energy reserve in many bacteria (Neidhardt *et al.*, 1990; Preiss, 1996). Its biosynthesis from glucose-1-phosphate is catalyzed by at least three enzymes: ADP-glucose pyrophosphorylase (AGP) which adds ADP onto glucose-1-phosphate, glycogen synthase (GS) adds ADP-glucose units onto polyglucosyl chains and the branching enzyme (BE) catalyzes the formation of the branched α-1,6-glucosidic linkages from the growing polyglucose chain (Preiss, 1996). Several enteric bacteria and bacilli are known to accumulate glycogen when cell growth becomes limited by nitrogen but carbon is still available (Neidhardt *et al.*, 1990; Preiss, 1996). Sequestering carbon from competing organisms and storing it as glycogen provides a readily usable energy source when growth is again possible. Glycogen synthesis in *E. coli* is highly regulated by both allosteric and genetic mechanisms which are primarily controlled by the availability of carbon and nitrogen (Preiss, 1996). Bacillus has been suggested to use glycogen as a carbon and energy source to form spores (Preiss, 1996; Slock and Stahly, 1974). Like *E. coli*, the amount of glycogen synthesized is dependent upon the availability of carbon and regulation has been suggested to involve different sporulation sigma factors (Kiel *et al.*, 1994; Takata *et al.*, 1997). Humans also use glycogen as a carbon and energy store, however unlike bacteria; humans' use UDP-glucose as the glucose donor as apposed to

ADP-glucose. The regulation of glycogen metabolism in humans is very complex and involves hormonal as well as allosteric regulation (Stryer, 1988).

The presence of glycogen in *C. trachomatis* inclusions has been observed for several decades, however its biological role and regulatory properties in chlamydia are poorly understood. Glycogen, which can be visualized with iodine staining, has only been detected in the inclusion of *C. trachomatis* and not in *C. psittaci* or *C. pneumoniae* (Moulder, 1991). The accumulation of glycogen appears in inclusions of *C. trachomatis* 20-30 h after infection peaks at 30-60h p.i. and then gradually declines (Moulder, 1991). Glycogen particles first appear in RBs but are most commonly seen in EBs (Chiappino *et al.*, 1995). The accumulation of glycogen in the inclusion is speculated to be due to the rupturing of RBs and intermediate forms (IF) which release glycogen particles into the inclusion (Chiappino *et al.*, 1995).

Several studies strongly suggest that *C. trachomatis* is metabolically capable of generating glycogen (Fan and Jenkin, 1970; Jenkin and Fan, 1971; Moulder, 1991; Weigent and Jenkin, 1978). Fan and Jenkin (1970) demonstrated that labeled glucose was incorporated into glycogen at much higher rates in *C. trachomatis*-infected HeLa cells compared to uninfected cells. Later they showed that infected lysates preferentially incorporate ADP-glucose at high rates in contrast to uninfected HeLa cells which only incorporate UDP-glucose at slower rates (Jenkin and Fan, 1971). Studies have also shown that chloramphenicol and penicillin inhibited glycogen synthesis in *C. trachomatis*-infected cells whereas cycloheximide did not (Moulder, 1991). Further support that glycogen is synthesized by chlamydial enzymes comes from the genome sequence (Stephens *et al.*, 1998). The *C. trachomatis* genome contains homologs for all the genes

required for synthesizing glycogen from glucose-6-phosphate (Read *et al.*, 2000; Stephens *et al.*, 1998) (Fig. 6). It contains homologs of *glgC*, *glgA* and *glgB*, which encode for AGP, GS and BE, respectively, as well as the homolog for the gene encoding for phosphoglucosemutase *mrsA*, which converts glucose-6-phosphate into glucose-1-phosphate. In addition, orthologs for the glycogen-degrading enzymes glycogen hydrolase (GlgX) and glycogen phosphorylase (GlgP) are also present implying that *C. trachomatis* can not only synthesize and store glycogen, but they can also break it down to obtain glucose-1-phosphate. Surprisingly, despite the fact that *C. pneumoniae* and *C. psittaci* strain GPIC are not known to accumulate glycogen, their genomes contain an identical complement of glycogen metabolizing genes (Kalman *et al.*, 1999; Read *et al.*, 2000; http://www.tigr.org/).

All cells require glucose for growth. When growing on gluconeogenic carbon sources or in a nutrient rich environment where glucose has become limiting, enzymes required for *de novo* glucose synthesis are induced. For *E. coli* there are a wide variety of substrates including various sugars, amino acids and dicarboxylic acids, which can serve as gluconeogenic carbon sources (Lin, 1996; McFall and Neuman, 1996). Detailed analysis of chlamydial genome sequence data (Kalman *et al.*, 1999; Read *et al.*, 2000; Stephens *et al.*, 1998) suggests that host derived glucose-6-phosphate is the primary carbon and energy source used to support parasite growth (McClarty, 1999). It was also noted, however, that chlamydiae contained key gluconeogenic enzymes and that it was possible that host derived glutamate or dicarboxylic acids could potentially support chlamydial growth (McClarty, 1999). The effect of various carbon sources on *C.*

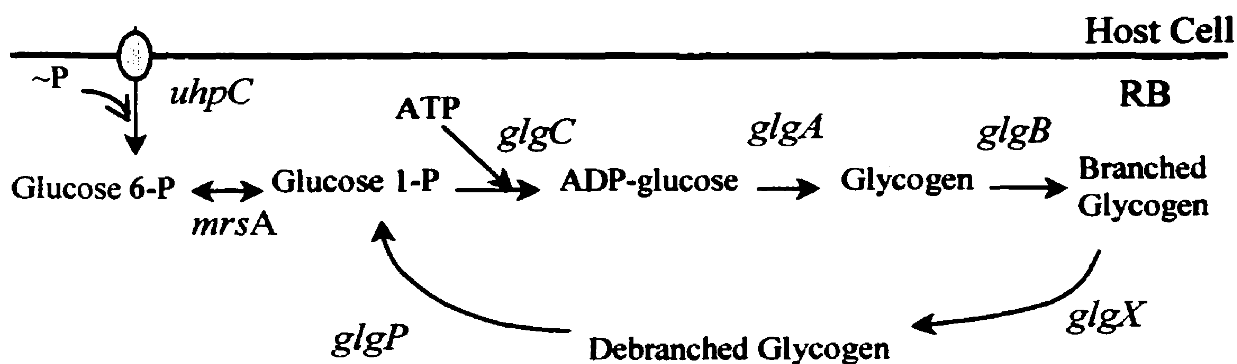


Figure 6. Glycogen metabolism in chlamydiae as depicted from *C. trachomatis* genome sequence (Stephens *et al.*, 1998). Chlamydia contains a homolog for a hexose-P transporter (*uphC*) which could be used to transport host glucose-6-P into chlamydiae. See "Introduction" for details.

trachomatis growth, central carbon gene expression and glycogen metabolism is discussed in this thesis.

The projects in this thesis were undertaken to provide more information about carbon and energy metabolism in chlamydiae. Specifically, the need to clarify the energy parasite hypothesis was approached by isolating and characterizing key energy-producing enzymes in the glycolytic and pentose phosphate pathways. Kinetic analysis of pyruvate

kinase, a major regulatory enzyme in the glycolytic pathway, revealed unique properties which may be used towards the development of novel chemotherapeutic agents to treat chlamydial disease. In addition, studies on other aspects in chlamydial carbon metabolism, such as glycogen metabolism and the response chlamydia has with its environment in terms of availability and type of carbon source found in the culture media are also presented in this thesis. Together, these findings are discussed in terms of the evolution and adaptation of chlamydiae to a stable nutrient environment inside of an eukaryotic host cell.

MATERIALS AND METHODS

1. Materials

Restriction enzymes, taq polymerase and superscript reverse transcriptase were purchased from Life Biotechnologies. All components in the enzyme assays and all chemicals were purchased from Sigma Chemical Co. The RNA isolation kit was purchased from Qiagen and the plasmid purification kit was obtained from Promega. The random primer labeling kit and the DNA cycle sequencing kit were bought from Life Biotechnologies. D-[U-^{14}C] glucose (261 mCi/mmol) and L-[U-^{14}C] glutamate (282 mCi/mmol) were obtained from New England Nuclear, Dupont Canada Inc. Cell culture medium, fetal bovine serum and cell culture grade glucose, oxaloacetate, malate, glutamate and α-ketoglutarate were obtained from Life Technologies Inc. Anthrone, glycogen and the glucose diagnostic kit were purchased from Sigma Chemical Co. Oligonucleotides were purchased from Life Biotechnologies, or synthesized on a Beckman DNA synthesizer.

2. *Chlamydia trachomatis* strains and propagation

C. trachomatis L2/434/Bu was originally obtained from C.C. Kuo, University of Washington (Seattle, WA) and has been maintained in our laboratory since that time. *C. trachomatis* L2/434/Bu was used throughout this study and was grown as previously described (Tipples and McClarty, 1991). Unless otherwise indicated, 1 μg/ml

cycloheximide was present in the post infection growth medium. HeLa cells were infected with *C. trachomatis* at a multiplicity of infection of 3-5 infection forming units per cell. Mock-infected (MI) host cell cultures were treated in the same fashion as infected cells, except that chlamydiae were not added.

3. Cell lines and culture conditions

The wild-type HeLa 229 cells were obtained from R. Brunham, University of British Columbia, Center for Disease Control and are continuously maintained in our laboratory. They are routinely cultured at 37°C on the surface of plastic tissue culture flasks (Corning Glass Works) in minimal essential medium (Life Biotechnologies) containing 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Life Biotechnologies) in an atmosphere of 5% CO_2-95% humidified air. HeLa cells were infected with *C. trachomatis* as previously described (Tipples and McClarty, 1991). For experiments where special carbon source conditions were employed, following infection the chlamydial inoculum was removed and the cell monolayer was washed three times with sterile phosphate buffered saline. The chlamydiae-infected cells were then cultured in glucose free Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 5 mM pyruvate and the indicated concentration of glucose (0, 0.1, 1 or 10 mg/ml) or 20 mM malate, glutamate, α-ketoglutarate or oxaloacetate (± cycloheximide), and 10% heat inactivated dialyzed fetal bovine serum. The wild type mouse L929 cell line was provided by K. Coombs, University of Manitoba, Winnipeg. The L929 cells were grown in D-MEM and 10% fetal bovine serum.

4. Preparation of RB extracts for enzyme assays

Suspension cultures of mouse L929 cells were used as the host for preparing RBs which were highly purified through Renografin density gradients as previously described (Caldwell *et al.*, 1981; Fan *et al.*, 1992). Purified RBs were lysed, and crude cell extract was prepared as described (Fan *et al.*, 1992). Purified sham extracts were prepared from MI mouse L929 cells by the same procedure used to purify RBs from infected mouse cells.

5. *E. coli* strains used for molecular cloning

MC1061 *(hsdR2 hsdM+ hsdS+ araD139* Δ*(ara-leu)*$_{7697}$$\Delta$*(lac)*$_{X74}$*galE15 galK16 rpsL (Str*^{r}*)mcrA mcrB1)* was obtained from Bjorne Hove-Jensen, Denmark.

DH5α (supE44Δ*lacU169 (*ϕ*80lacZ*Δ*M15) hsdR17recA1 endA1 gyrA96 thi-1 relA1*) was obtained from B. Triggs-Raine, University of Manitoba.

6. Construction of degenerate oligonucleotide primers

PK degenerate oligonucleotide primers were designed based on the consensus amino acid sequence alignments of PKs from human (M2), yeast (*Saccharmyces cerevisiae*), *Lactococcus lactis*, *Escherichia coli* and *Bacillus stearothermophilus*. PK degenerate oligonucleotide primers PKd5 (5'-

TT(A/G/T)AA(T/C)TT(T/C)TC(T/C/A)CA(T/C)GG-3') and PKd3 (5'-(A/G)GA(T/C)TC(A/C/T)CC(A/G/T)GA(T/C)AACAT-3') are derived from amino acid positions ^{33}LNFSHG^{38} and ^{315}SEGSLM^{310} respectively in *E. coli* PK (*E. coli* PK GenBank accession number S29004). PGK degenerate oligonucleotide primers were designed based on the consensus amino acid sequence alignments of PGKs from human, yeast (*Saccharmyces cerevisiae*), *Penicillum chrysogenum, Escherichia coli, Bacillus megaterium* and *Plasmodium falciparum*. PGK degenerate oligonucleotide primers PGKd5 (5'-GT(A/T)ATGGA(C/T)GC(A/T)TT(C/T)GG(T/A)AC(T/A)GC(T/A)CA-3') and PGKd3 (5'-ACCTTC(C/A/T)AC(G/A)AATTC(G/A)AG(G/A)AA(T/A)GC(GT)CC-3'), are derived from amino acid ^{137}VMDAFGTAH^{145} and ^{372}GEVFELAF^{364} respectively in *E. coli* PGK (*E. coli* PGK accession number TVECG). GAP primers consisted of a 5' primer GAP5 (3002 ^{b}-GTTGATAGAAGAGTCATTGGG-3021) and a 3' primer GAP3 (3387-CCATAACCAAACATCCATCCG-3367) which are numbered according to sequence data reported by (Gu *et al.*, 1995). In each case the chlamydial codon preference was used. ZWF was previously identified as an ORF located downstream of an operon containing CTP synthetase (Wylie *et al.*, 1996).

7. Molecular cloning of *C. trachomatis gap*, *pgk*, *pyk* and *zwf*

a) Construction of probes

Each set of primers (GAP5 & GAP3, PGKd5 & PGKd3, PKd5 & PKd3; see construction of degenerate oligonucleotide primers) were used for PCR with *C.*

trachomatis serovar L2 genomic DNA as template. The PCRs were carried out in 100 µl of solution which contained 800 ng of *C. trachomatis* L2 genomic DNA, 3 µM of degenerate primers or 0.5 µM of GAP primers, all four deoxyribonucleoside triphosphates (dNTPs) (each 0.25 mM), 10 µl of 10 x PCR buffer (Perkin Elmer), and 2.5 u of Taq polymerase (Perkin-Elmer). The reactions were conducted in 35 cycles with the following program: 1 min at 95°C, 1 min at 55°C, 2 min at 70°C. The 385 bp PCR product (GAP) from the GAP5 and GAP3 primer reaction, the 705 bp PCR product (PGK) from the PGKd5 and PGKd3 primer reaction and the 846 bp PCR product (PK) from the PKd5 and PKd3 primer reaction were of anticipated size and were isolated and purified from an 0.8% agarose gel using an electro-eluter. The purified probes were stored at –20°C and used later to screen a *C. trachomatis* L2 *Hin*dIII library via colony blot hybridization.

b) Colony blot hybridization

Forty µl of competent *E. coli* MC1061 was transformed with 20 ng of recombinant *C. trachomatis* L2 *Hin*dIII genomic library prepared previously (Tipples and McClarty, 1995), by electroporation. After 90 min of growth in SOC medium (Sambrook, 1989) the cells were plated onto large LB agar plates containing 50 µg/ml of ampicillin, at an appropriate dilution to give approximately 100-200 colonies/plate. The colonies from each plate were transferred onto individual nylon membranes which were denatured (0.5 M NaOH, 1.5 M NaCl), neutralized (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) and washed with 2 x SSC (Sambrook, 1989). The membranes were dried at 80°C for 2 h and

prehybridized with 40 ml of pre-hybridization solution (6 x SSC, 5 x Denhardts solution, 0.5% SDS and 100 ng/ml of denatured, fragmented salmon sperm DNA) at 65°C for 1 h. The prehybridization solution was discarded and the membranes were incubated with 40 ml of hybridization solution (6 x SSC, 0.5% SDS, 100ng/ml denatured salmon sperm) containing the 385 bp GAP PCR product labeled with α-^{32}PdATP using the random primer DNA labeling system from GIBCO. The membranes were dried and exposed to x-ray film for 12 h. at -80°C and positive colonies were identified. The nylon membranes were then stripped and probed with either the ^{32}P labeled 705 bp PGK PCR product or the ^{32}P labeled 846 bp PK PCR product to isolate positive PGK and PK clones respectively.

8. DNA sequencing

The BRL Life Biotechnologies double-stranded cycle sequencing kit was used for sequencing and the protocol supplied with the kit was followed. The reaction products were run on a 6% polyacrylamide gel. Following the electrophoresis, the gel was dried and then exposed to film overnight.

The sequencing data was analyzed using PC/GENE software purchased from IntelliGenetics, Inc (Mountain View, California), and also by sequence-homology searching of the data in Genbank.

Analysis of nucleotide sequence for open reading frames coding for polypeptides was done assuming that the start codon/methionine codon was ATG or GTG, and the stop codons were TAA, TAG, or TGA.

9. Reverse-transcriptase-PCR (RT-PCR)

For the time course experiments total RNA was isolated from *C. trachomatis* L2-infected HeLa cells (3.0 X 10^7 cells per 150 cm^2 flask) cultured in complete D-MEM supplemented with 10% fetal bovine serum at 2, 6, 16, 24, 36, 48 h p.i using the RNA extraction kit from Qiagen. For experiments where alternative carbon sources were used total RNA was isolated from *C. trachomatis* L2-infected HeLa cells that were cultured in glucose free D-MEM supplemented with the indicated amount and source of carbon (0, 1, 10 mg/ml glucose or 20 mM glutamate ± cycloheximide) at 24 hours p.i. RT-PCR was performed using Superscript™ Reverse Transcriptase (Life Techonologies Inc.) according to the manufacturer's instructions. cDNA resulting from reverse transcription was ethanol precipitated, resuspended in ddH_20 and stored at -20°C as template for PCR amplification. To detect any changes in the level of expression of the various genes, the PCR reaction was maintained in the linear range by using 30 cycles.

10. *E. coli* strains used for complementation and enzyme studies

E. coli BL21 (DE3) *(hsdS gal λclts857 ind1 Sam7 nin5lacUV5-T7 gene 1)* was obtained from Novagen, Inc.

E. coli DS112 *(K-12, F⁻λ⁻ΔgapA::Cm)* was obtained from Seta *et al.*, (1997) (Seta *et al.*, 1997)

**E. coli* DF264 (*garB10 fhuA22 ompF627 fadL701*(T_2^R)*relA1 zgf-210::Tn10 pgk-2 pit-10 spoT1 rrnB-2 mcrB1 creC510*) (Thomson *et al.*, 1979).

E. coli PB25 (*supE thi* Δ(*lac-proAB*)(*F' traD36 proAB lacI^q^Z*Δ*M15*)Δ*pykA::kan pykF::cat*) was obtained from Ponce *et al.*, (1995)(Ponce *et al.*, 1995).

**E. coli* DF2000 (*garB10 fhuA22 ompF627*(T_2^R) *zwf-2fadL701*(T_2^R) *relA1 pit-1spoT1 rrnB-2 pgi-2 mcr B1 creC510*) (Fraenkel, 1968).

**E. coli* DF456 (*fhuA2, lacY1, tsx-6, glnV44(AS), gal-6, λ-, gatC49, gatA50, srlC?-49, recA1, argG6, rspL104, xylA7, mtlA2, pfk300::Mu, metB1*) (Thomson *et al.*, 1979).

**E. coli* strains DF264, DF2000 and DF456 were obtained from the *E. coli* Genetic Stock Centre, Yale University, New Haven, CT.

11. *E. coli* culture media

LB broth, LB agar and SOC broth were prepared according to Sambrook *et al* (1989). Minimal media consisted of: 1 x M63 minimal salts (Sambrook, 1989), 0.1% casamino acids, 2 μg/ml thiamine, and 4 μg/ml $MgSO_4$

E. coli DS112 Permissive media consisted of minimal media, 12.5 mM glycerol, 25 mM malate and 34 μg/ml chloramphenical.

Selective media consisted of minimal media, 10 mM glucose and 34 μg/ml chloramphenical.

E. coli DF264 Permissive media consisted of minimal media, 12.5 mM glycerol

Selective media consisted of minimal media and 10 mM glucose.

E. coli PB25 Permissive media consisted of minimal media, 10 mM glucose, 30 μg/ml kanamycin and 25 μg/ml chloramphenical.

Selective media consisted of minimal media, 15 mM ribose, 30 μg/ml kanamycin and 25 μg/ml chloramphenical.

E. coli DF2000 Permissive media consisted of minimal media and 10 mM gluconate.

Selective media consisted of minimal media and 10 mM glucose.

E. coli DF456 Permissive media consisted of LB plates.

Selective media consisted of minimal media and 0.4% mannitol.

E. coli containing plasmids conferring ampicillin-resistance were selectively grown in the presence of 50 μg/ml ampicillin. Agar plates contained the described media with 2% agar.

12. Construction of expression vectors

pUC19 (Sambrook, 1989) was used as an expression vector for the cloned chlamydial *gap*, *pgk*, *pyk* and *zwf* genes. Four sets of PCR primers specifically 5SGAP and 3BGAP, 5HPGK and 3SPGK, 5SPK and 3SPK and 5SZWF and 3BZWF (Table 3) were used for PCR with *C. trachomatis* L2 genomic DNA to generate GAPDH, PGK, PK and ZWF PCR gene products respectively, which contained enzyme sites for cloning into pUC19. Each gene was inserted into pUC19 vector downstream of the lac promoter yielding pUC19-GAPDH (pCTGAPDH), pUC19-PGK (pCTPGK), pUC19-PK (pCTPK) and pUC19-ZWF (pCTZWF) respectively (Table 3). Each construct allows for expression either by a fortuitous *E. coli* RNA polymerase recognition of a chlamydial promoter or from the β–galactosidase promoter present in the plasmid. *E. coli* DH5α was transformed by electroporation with pCTGAPDH, pCTPGK, pCTPK or pCTZWF. Recombinants were selected and used for preparation of cell extract.

The pQE-80L expression plasmid was purchased from Qiagen and was used for kinetic studies on pyruvate kinase. Expression of recombinant proteins cloned into pQE expression vectors is from a phage T5 promoter, which is regulated by lac repressor protein. The pQE plasmid contains the lacI^{q} gene, allowing the use of any *E. coli* strain. Expression of recombinant proteins encoded by pQE vectors is induced by IPTG which binds to the lac repressor protein and inactivates it, permitting the host cell's RNA polymerase to transcribe sequences downstream from the promoter. The oligonucleotide PCR primers 5'-CCCCGGTACCATCGCTAGAACGAAA-3' and 5'-CCCCGTCGACCAGAAACCCCGGTGAAC-3' used for cloning chlamydial PK into pQE-80L was based on published *C. trachomatis* L2 genome sequence information

(Iliffe-Lee and McClarty, 1999). The underlined portions of the primers indicate the *Kpn*I and *Sal*I restriction sites included for cloning purposes.

13. Preparation of competent *E. coli* for electroporation

50 ml of LB or permissive media was inoculated with a single *E. coli* colony and incubated overnight at 37°C. The overnight culture was then used to inoculate 1 L of media. This culture was incubated at 37°C until an OD of 0.6 at 600 nm was reached. The culture was then chilled on ice for 10 min. Following the chilling, cells were centrifuged at 3, 000 x g for 12 min at 4°C. The cells were then resuspended in 200 ml of sterilized ice-cold water and centrifuged as before. The cells were resuspended in 100 ml of sterilized ice-cold water and again centrifuged as described. The pellet was then resuspended in 10 ml ice-cold 10% glycerol and centrifuged at 4, 500 x g for 10 min. Finally, the pellet was resuspended in 2 ml of ice-cold glycerol, aliquoted into smaller fractions, and stored at -80°C.

14. Complementation studies for CTGAPDH, CTPGK, CTPK and CTZWF

E. coli was transformed by electroporation by using the Bio-Rad Gene Pulser. Conditions were set at 2.5 kV/resistance high voltage, resistance of 200 ohms, charging voltage of 1.8 kV, field strength of 12.25 kV/cm and desired pulse length of 3-4 milliseconds.

Approximately 20 ng of plasmid DNA (pCTGAPDH, pCTPGK, pCTPK or pCTZWF) was mixed with 40 µl of competent *E. coli* cells (DS112, DF264, PB25 or DF20000 respectively) and then transferred to a cold electroporation cuvette. Following the electroporation, 1 ml of SOC was added to the suspension and then transferred to a sterile tube and incubated at 37°C for 1.5 h. The cells were centrifuged at 3, 000 x g for 10 min and resuspended into minimal media. The cells were then centrifuged as before, and again resuspended in minimal media. The cells were then plated onto the selective media containing the appropriate antibiotics and incubated at 37°C.

15. Preparation of bacterial cell extracts for enzyme assays

DS112, DF264, PB25 and DF2000 competent cells were transformed by electroporation with pCTGAPDH, pCTPGK, pCTPK or pCTZWF respectively. The competent cells were also transformed with pUC19 as a control. The transformed cells were then incubated for 90 min at 37°C in SOC. The cells were washed 2 x with M63 minimal media and plated onto permissive medium containing the appropriate antibiotics. Plates were incubated at 37°C until colonies appeared. Single colonies were picked and grown in permissive media (500 ml) containing appropriate antibiotics for 36 h. Cells were pelleted by centrifugation and resuspended in appropriate buffer: 40 mM triethanolamine-HCl pH 7.5 (for strains DS112 alone or containing pUC19 or pCTGAPDH; or strain DF264 alone or containing pUC19 or pCTPGK); 10 mM Tris-buffer pH 7.5 (for strain PB25 alone or containing pUC19 or pCTPK); or 100 mM Tris-HCl pH 7.6 (for strain DF2000 alone or containing pUC19 or pCTZWF). Lysozyme was

added to a final concentration of 350 μg/ml and cells were frozen at -70°C. All cell extracts were then thawed at 4°C and lysed by sonication (three 20-s pulses at a probe intensity of 40). The extracts were then centrifuged (150, 000 x g for 2 h) to remove particulate $NADH_2$-oxidase activity. Supernatants were aliquoted and stored at -70°C for enzyme analysis.

16. Crude GAPDH, PGK, PK and ZWF enzyme assays

All enzyme assays were carried out in a final volume of 1 ml, at 25°C (Table 6). GAPDH assay conditions were adapted from Seta *et al.* (1997) and consisted of 40 mM triethanolamine-HCl pH 7.5, 2.0 mM EDTA pH 8.0, 50 mM K_2HP0_4, 1 mM NAD and extract. The reaction was started with the addition of 1 mM G3P. PGK activity was measured in the back reaction leading from 3-phosphoglycerate (3PGA) to 1,3-diphosphoglycerate adapted from Maitra and Lobo, (1971). The reaction mixture consisted of 40 mM triethanolamine-HCl pH 7.5, 5 mM $MgCl_2$, 0.2 mM EDTA, 30 mM $(NH_4)_2SO_4$, 100 mM NaCl, 2 mM ATP, 3.45 units of glyceraldehyde-3-phosphate dehydrogenase, 0.2 mM NADH and extract. The reaction was started with the addition of 5 mM 3PGA. PK activity was measured in a coupled reaction with lactate dehydrogenase leading from PEP to lactate adapted from Malcovati and Valentini, (1982). The reaction mixture consisted of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 2 mM ADP, 0.2 mM NADH and 10 units of lactate dehydrogenase. The reaction was started with the addition of 10 mM PEP (+/- 1 mM F16BP or +/- 1 mM AMP). ZWF assay conditions were adapted from Banerjee and Frankel (1972) and consisted of 100 mM Tris-HCl pH

7.6, 10 mM $MgCl_2$, 0.2 mM NADP and extract. The reaction was started with the addition of 1 mM G6P. In all enzyme assays, the samples were first measured for background readings in a spectrophotometer at a wavelength of 340 nm for 5 min with the addition of either NAD(P) or NADH. The background readings were subtracted from the readings containing the substrate. The appearance of NAD(P)H (ZWF, GAPDH) or the oxidation of NADH (PK, PGK) was calculated using the NADH molar extinction coefficient of 6.22 x 10^3 $M^{-1}cm^{-1}$ using the Beer-Lambert relationship: $A=\varepsilon cl$ (where A=absorbance, ε=molar extinction coefficient, *c*=concentration and l=path length) (Eisenthal R., 1992).

17. Molecular cloning, sequencing and expression of *C. trachomatis pfpA* and *pfpB*

Primer sets 5PFPA: 5'-CCCC**CTGCAG**TCCGTGCAAGAATGGTG-3';
3PFPA: 5'-CCCC<u>GTCGAC</u>AGAACCCCTAGAGAAGTC-3'; and
5PFPB: 5'-CCCC**CTGCAG**CCGTTGTATCCTTACGTC-3';
3PFPB: 5'CCCC<u>GTCGAC</u>TAGCTCAGGTGGTTAGAGC-3' were constructed for molecular cloning of *C. trachomatis* L2 *pfpA* and *pfpB* respectively. The bold lettering indicates *Pst*I sites where as the underlined portions indicates *Sal*1 sites for cloning into pUC-19. Primers were designed based on genome sequence information from *C. trachomatis* D serovar. Primer sets 5PFPA and 3PFPA, and 5PFPB and 3PFPB were used for PCR with *C. trachomatis* L2 genomic DNA to generate PFPA and PFPB PCR gene products respectively. Each gene was inserted into pUC19 vector downstream of the lac promoter yielding pCTPFPA and pCTPFPB plasmids respectively. The plasmids were

sequenced by the BRL Life Biotechnologies double-stranded cycle sequencing kit and analyzed using PCGENE and by sequence-homology searching of the data in Genbank.

DF456 cells were made competent and were transformed by electroporation with pCTPFPA, pCTPFPB, or pUC19 as a control and plated on LB agar ampicillin plates. Single colonies were picked and grown in 1 L of LB media containing 100 μg/ml of ampicillin and then pelleted by centrifugation. The pellet was resuspended in buffer (100 mM Tris-HCl pH 7.5) and frozen at -70°C. The extract was then thawed, sonicated (150,000 x g for 2 h), centrifuged, aliquoted and stored at -70°C for enzyme analysis.

18. Crude ATP-PFK and PPi-PFK enzyme assays

Enzyme assays were carried out in a final volume of 1 ml, at 25°C. ATP-PFK assay conditions were adapted from (Yuan *et al.*, 1988) and consisted of 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM NH_4Cl, 1 mM F6P, 0.2 mM NADH, 0.6 units aldolase, 6 units triose-P-isomerase, 1 unit of glycerolphosphate dehydrogenase and 1 mM ATP. PPi-PFK assay conditions were identical except that 1 mM ATP was replaced with 1 mM sodium pyrophosphate (PPi). The assay was started with the addition of 1 mM F6P. The samples were first measured for background readings in a spectrophotometer at a wavelength of 340 nm for 5 min with the addition of NADH. The background readings were subtracted from the readings containing the substrate. The oxidation of NADH was calculated using the NADH molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$.

19. Expression and purification of *C. trachomatis* PK

The pQE80L vector places a 6 x His tag at the N-terminus of the recombinant protein allowing the His-tagged chlamydial PK recombinant protein to be purified by metal chelation affinity chromatography according to the manufacturer's instructions. Briefly, *E. coli* strain DH5α was transformed with the pQE80L-CTPK plasmid. The bacterial culture was grown in 500 ml of LB media containing 100 μg/ml of ampicillin at 37°C to an OD of 0.6 at 600 nm. IPTG was added to a final concentration of 1 mM and incubated for 3.5 h. Bacteria were harvested by centrifugation, resuspended in 16 ml of binding buffer (5 mM imidazole, 1 M NaCl, 20 mM Tris-HCl pH 7.9) and quickly frozen at -80°C. All subsequent procedures were carried out at 4°C. Cells were then thawed, sonicated and centrifuged at 45, 000 x g for 1 h. The supernatant was collected and filtered using a 0.45 micron membrane. The filtered supernatant was then passed through the metal chelation column, washed with binding buffer, and then washed with wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9). The protein was eluted with buffer containing 1 M imidazole, 0.5 M NaCl and 20 mM Tris-HCl pH 7.9, and concentrated using the centriprep centrifugal Amicon YM-30 filter device from Millipore. The chlamydial recombinant PK concentrated enzyme was washed 2 x with 10 mM Tris-HCl pH 7.3 and resuspended in PK storage buffer (10 mM Tris-HCl pH 7.3, 10 mM $MgCl_2$, 50 mM KCl, 1 mM mercaptoethanol, 0.2mM EDTA, 0.1 mg/ml BSA, 15% glycerol) and stored at -80°C.

20. Kinetic Analysis of *C. trachomatis* PK

PK activity was determined at 25°C by the lactate dehydrogenase coupled spectrophotometric assay as described previously (Iliffe-Lee and McClarty, 1999). The standard reaction mixture contained: 10 mM Tris-HCl pH 7.3, 10 mM $MgCl_2$, 100 mM KCl, 10 mM PEP, 2 mM ADP, 0.2 mM NADH and 10 units of lactate dehydrogenase in a final volume of 1 ml. The reaction was started by the addition of PEP. One unit of enzyme activity corresponds to the oxidation of 1 μmol of NADH or production of 1 μmol of pyruvate per minute under the above conditions.

Kinetic parameters for PEP were determined at fixed concentration of 2 mM ADP either in the absence or in the presence of an effector at a fixed concentration of either 1 or 10 mM. The various effectors tested included: 1 mM F26BP, R5P, G1P, G6P, F1P, F6P, 3PGA, 1 GMP and 1 and 10 mM F16BP. In all cases, at least 8 different PEP concentrations were used for each enzyme assay.

The kinetic parameters for ADP were determined at fixed concentration of 1 and 10 mM PEP in the presence or absence of 1 mM F26BP. In all cases, at least 8 different ADP concentrations were used.

For F26BP, PEP was fixed at 1 mM and ADP at 2 mM. For ATP, GTP or AMP, PEP was fixed at 10 mM PEP and ADP at 2 or 0.3 mM. For KCl or $MgCl_2$, PEP was fixed at 10 mM and ADP at 2 mM.

In the presence of a fixed concentration of an inhibitor, (0, 0.1, or 1 mM ATP; 0, 0.1, or 1 mM GTP; 0, or 1 mM AMP; or 0, 2, or 10 mM Pi) kinetic parameters for PEP were determined at a fixed concentration of 2 mM ADP in the both the presence or absence of 1 mM F26BP, where at least 8 different concentrations of PEP were used for each assay. Kinetic parameters for ADP in the presence of a fixed inhibitor concentration

(0, 0.5, 1.0, 2.0, or 3.0 mM ATP; 0, 0.5, 1.0 or 2.0 mM GTP; or 0, 0.5, 1.0, 2.0, or 3.0 mM AMP) was determined at 10 mM PEP.

All measurements were done in triplicate and the mean and standard error of the mean (S.E.M.) were calculated. When hyperbolic kinetics were obtained, the Michaelis-Menten equation was used; K_m = the substrate giving one half the maximal velocity (V_{max}) (Eisenthal R., 1992). These calculations were fit using nonlinear least-squares regression computer kinetics program supplied by GraphPad PRISM 3.0 software (San Diego, CA). When sigmoidal kinetics were obtained, the Hill equation was used which was modified and fit into the nonlinear least squares method as shown in

equation 1: $$v = \frac{V_{max}[S]^n}{K^n app + [S]^n}$$

Where V_{max} is the maximal velocity of each data set, [S] is the concentration of the variable substrate, n is the Hill coefficient and Kapp is a complex steady state kinetic equilibrium constant that is equivalent to K_m in Michaelis-Menten when n=1 (Nimmo and Bauermeister, 1977). n can be interpreted as a minimum estimate of the number of subunits in the enzyme. If n= 1, there is no cooperativity; if $n > 1$, there is positive cooperativity (the binding of a substrate molecule to the first site on the enzyme facilitates binding to the second); if $n < 1$, there is negative cooperativity (the binding of the substrate molecule to the first site inhibits the binding of the second) (Eisenthal R., 1992; Nimmo and Bauermeister, 1977). The program was supplied by GraphPad PRISM 3.0 software. The apparent $S_{0.5}$ (the substrate giving one-half the V_{max}) was determined by the Hill plot {($\log v/V_{max}$-v) versus log [S]}. The Hill plot is found to describe the binding of ligands to allosteric proteins in the region of 50% saturation (10 to 90%)

(Cornish-Bowden and Koshland, 1975). Inhibition constants, K_i (inhibitor concentration producing 50% inhibition of enzyme activity), were determined from Dixon plots (Dixon M., 1979).

21. Quantification of glycogen and glucose

Glycogen was quantified by the anthrone reaction (adapted from Roe and Dailey, 1966). HeLa cells (2 x 10^6 cells/5-cm dish) were infected with *C. trachomatis* as described (Tipples and McClarty, 1991) then incubated at 37°C with the indicated culture medium. At 40 h p.i. the medium was aspirated and the cell monolayer was rinsed three times with ice-cold phosphate buffered saline, then 0.5 ml of 10% KOH was added. The cell monolayer from each dish was harvested with a rubber policeman and transferred to 1.5 ml microcentrifuge tube. The tubes were boiled for 20 min at 100°C and then cooled to room temperature. Sufficient 100% tricholoroacetic acid was added to obtain a final concentration of 10%. The tubes were microfuged for 10 min at 10, 000 x g, the supernatant was transferred to a screw capped microfuge tube and 1 ml of anhydrous ethanol was added followed by centrifugation at 4,000 x g for 15 min. The supernatant was then discarded and the pellet was washed with 70% ethanol and air-dried. The precipitate was resuspended in 0.5 ml of distilled water and then 1 ml of 0.2% anthrone (0.2 g of anthrone in 100 ml of H_2SO_4, prepared fresh) was added. The tubes were boiled for 20 min at 100°C, and the color that developed was measured in a spectrophotometer at 620 nm. The concentration of glycogen was determined using a glucose standard curve

(Roe and Dailey, 1966). The concentration of glucose in the media was determined using a glucose diagnostic kit from Sigma Chemical Co., employing a glucose standard curve.

22. Incorporation of radiolabeled glucose or glutamate into glycogen of uninfected and *C. trachomatis*-infected HeLa cells

D-[U-^{14}C] glucose (2 μCi/5 cm dish) or L-[U-^{14}C] glutamate (2 μCi/5 cm dish) was added to the appropriate culture dishes immediately following infection. At 40 h. p.i. monolayers were harvested and glycogen was isolated as described above except that 1 mg/ml of bovine liver glycogen was added as carrier immediately after the addition of 10% KOH. The dried glycogen pellet was resuspended in 0.5 ml distilled water, and the radioactivity incorporated was determined by adding 100 μl to 5 ml of Universol scintillation fluid (ICN Biomedicals) and counting in a Beckman LS 5000 scintillation counter.

23. Nucleotide pool measurements

Nucleotides were extracted and quantitated as previously described (Tipples and McClarty, 1993). Briefly, uninfected and *C. trachomatis*-infected HeLa cells (1.5 X 10^7 cells per 75 cm^2 flask) were cultured in glucose free D-MEM supplemented with the indicated amount and source of carbon (0, 0.1, 1 or 10 mg/ml glucose or 20 mM glutamate, malate, α-ketoglutarate or oxaloacetate ± cycloheximide). At 30 hours p.i. the cell monolayer was harvested and resuspended in 250 μl of 10% trichloroacetic acid and

placed on ice for 30 min. The suspension was microfuged for 1 min and extracted nucleotides were neutralized with 78.1:21.9 (v/v) freon-tri-N-octylamine. Nucleotides were separated on a Whatman Partisil 5 SAX HPLC column using 0.55 M ammonium phosphate buffer (pH 3.5, 2.5% acetonitrile) as previously described (Tipples and McClarty, 1993).

24. **Infectivity titration assay**

Infectivity of *C. trachomatis* EBs was titrated by determination of inclusion forming units (IFUs) on HeLa cells as described by Tipples and McClarty (1991) except that inclusions were visualized by indirect immunofluorescence employing polyclonal antisera against formalin-killed *C. trachomatis* L2 EBs and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin serum (Zymed Laboratories, Inc).

RESULTS

A. ENERGY METABOLISM IN *C. TRACHOMATIS*

1. Identification, cloning and characterization of energy-producing genes in *C. trachomatis*

a) Enzyme assays with crude RB extracts

For years, chlamydia was defined as an 'energy parasite', completely dependent on the host cell for high-energy metabolites. To determine whether *C. trachomatis* L2 encodes for glucose metabolizing enzymes that produce energy either in the form of ATP or NAD(P)H, highly purified RB extracts were prepared and assayed for GAPDH, PGK, PK and ZWF activity. Specific activities of 220, 17, 340 and 45 nmols min^{-1} mg^{-1} were detected for GAPDH, PGK, PK and ZWF respectively. In all cases these levels of activity were 30-100 fold above background, as detected in extract prepared from sham infected host cells. As a result of our ability to assay these enzymes, a cloning strategy was designed to isolate chlamydial DNA encoding PK, PGK and GAPDH using an amino acid homology-based polymerase chain reaction (PCR) approach (see "Materials and Methods" for details). ZWF was putatively identified as an ORF downstream of an operon containing CTP synthetase (Tipples and McClarty, unpublished).

b) Cloning of *C. trachomatis gap*, *pgk* and *pyk* genes

GAPDH primers were constructed based on partial sequence information (Gu *et al.*, 1995). Degenerate oligonucleotide PGK and PK primers were designed based on the consensus amino acid sequences from a number of different prokaryotic and eukaryotic PGK and PK proteins (see "Materials and Methods"). In both cases, the chlamydial codon preference was used to design the PCR primers. The primers were used for PCR with *C. trachomatis* L2 genomic DNA as template. The PCR fragments generated were of anticipated size and were used as hybridization probes to screen a *C. trachomatis* L2 *Hin*dIII DNA library. Positive clones were identified for each and their respective inserts were sequenced.

c) Characterization of *C. trachomatis* L2 *gap*, *pgk*, *pyk* and *zwf* ORFs

The complete nucleotide sequences of *C. trachomatis* L2 *gap*, *pgk*, *pyk* and *zwf* are shown in Fig. 7A-D respectively. The detection of the protein coding regions in the chlamydial sequence was based on ATG or GTG start codons, and TAA, TGA, or TAG stop codons. All nucleotide sequences were confirmed by cycle sequencing both strands of the double-stranded DNA. The *C. trachomatis* L2 *gap*, *pgk*, *pyk*, and *zwf* sequences have been deposited in GenBank with accession numbers U83198, U83197, U83196, U83195 respectively. The restriction map and the sequencing strategy of the isolated clones pHGAP6, pHPGK3 and pHPK1 are shown in Fig. 8A-C, and the restriction map of *C. trachomatis* L2 *zwf* is shown in Fig. 9.

Figure 7. Nucleotide and deduced amino acid sequence of *C. trachomatis* L2 A) glyceraldehyde-3-phosphate dehydrogenase (CTGAPDH), B) phosphoglycerate kinase (CTPGK), C) pyruvate kinase (CTPK) and D) glucose-6-phosphate dehydrogenase (CTZWF). The agt (start/methionine codon) or gtg (start/valine codon) and stop codons (taa, tga, tag) in each respective nucleotide sequence are highlighted in bold. The first and last amino acid is also highlighted in bold in each respective amino acid sequence.

A) CTGAPDH Nucleotide Sequence

1 agctaagaaa aataccttag ctgcaagaag attagctgta gggcgtctta tggtcagata

61 taatacgttg actagcaaag aggctcgcca agttaaagct ggagatttgt ctgcttataa

121 tgttgataga agagtcattg ggaagttatt tgatgtgtta gcaaccaggt tttcttcgag

181 aaatcgcggg tatacgcgca ttttgaagtt gcaaaatagg gttggtgata atgctcaaaa

241 gtgtatcata gaatttttag catagtgatg ctaatttttc gaaaacactg actacctggg

301 atttagca**at g**agaattgtg attaatggtt ttggacggat tgggcgatta gttttaagac

361 agattctgaa aaggaattct cccatagaag ttgtagctat taatgattta gtcgcaggag

421 atcttttaac atatttattt aaatatgatt ccacacacgg atctttcgct cctcaagcaa

481 cattttcgga tggatgtttg gttatgggag aaagaaagat ccgtttctta gcggaaaaag

541 acgttcaaaa gcttccttgg aaggatttgg atgttgatgt cgtcgtcgaa agtactggat

601 tgtttgtcaa tagggatgat gctgcaaagc atttggactc tggagcaaag agagtgttga

661 tcacagctcc tgcgaaaggc gatgtcccta cgtttgttat gggagttaac catcagcagt

721 ttgacccagc tgacgtcatc atttctaatg cttcctgtac taccaattgt ttagctcctt

781 tggccaaagt tctattggat aattttggta tagaagaagg gctaatgaca acagttcacg

841 ctgcaacagc tacgcagagt gtggttgatg gcccttctcg taaggattgg agaggggggta

901 gaggagcttt tcagaatatt atcccggctt cgacaggagc tgctaaagct gtagggttgt

961 gtttgcctga gcttaaagga aaattaacag gaatggcctt tagagtgcct gtagcagatg

1021 tttctgtagt agatttaact gttaagttga gctcagccac gacgtacgag gctatctgtg

1081 aagctgtgaa gcatgcagca aacacgagca tgaagaatat tatgtactac acggaagaag

1141 ctgtagtctc ttctgatttt attggctgtg agtattcatc tatattcgat gctcaagccg

1201 gggttgcttt gaacgatcga tttttcaaat tggtagcttg gtatgataat gaaataggct

1261 atgcaactcg catagtggat ttattagagt acgtacaaga aaactct**aaa** taaaggttcg

1321 ttcgtgtatt ttacaagaga tccagtcata gagactgtta ttacatctag agaaggatat

Fig. 7A

1381 aagttatcca ttcgtaattc gaaacacttg tcccaagatc cttttgtcgt tgaggctata

1441 gaggttgtcc gtttaggagg gactagtttt ttccgtaatt gtgatcatag taagccgttt

1501 ttactgccag catctgatta tgaagtgatg gaaatccggg atgctaaaat caacc

CTGAPDH Deduced Amino Acid Sequence

1 MTTWDLA**M**RI VINGFGRIGR LVLRQILKRN SPIEVVAIND LVAGDLLTYL FKYDSTHGSF

61 APQATFSDGC LVMGERKIRF LAEKDVQKLP WKDLDVDVVV ESTGLFVNRD DAAKHLDSGA

121 KRVLITAPAK GDVPTFVMGV NHQQFDPADV IISNASCTTN CLAPLAKVLL DNFGIEEGLM

181 TTVHAATATQ SVVDGPSRKD WRGGRGAFQN IIPASTGAAK AVGLCLPELK GKLTGMAFRV

241 PVADVSVVDL TVKLSSATTY EAICEAVKHA ANTSMKNIMY YTEEAVVSSD FIGCEYSSIF

301 DAQAGVALND RFFKLVAWYD NEIGYATRIV DLLEYVQENS **K**

Fig. 7A

B) CTPGK Nucleotide sequence

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   1 tttattacag ttcctgctgg agcggcgctt tctacgtatt ttttttgtta ctgcgcgcac
  61 tattttgtta agcgcaactc tctatatgca tactatatgc agactaaaaa tgttttgtct
 121 gagcgattct tcatgtggca ctgagataaa ggcttgagtt ttcctttgct taggcctata
 181 agaaaattta ggttaaggat cagataagca tggataaatt atcgataaga gacctttctc
 241 ttgaagggaa aaaggtacta gttcgtgtag attttaatgt tcctattaaa gatggaaaga
 301 ttttagatga tgtgcgtatt cgtagcgcaa tgcctacgat ccattatctt ttgaaacaag
 361 atgcagcagt cattttggtg agccatttag gacgcccaaa gggaggcgta tttgaagagg
 421 catattcatt agctcctatt gttcctgtgc tagaggggta tttagggcat catgtgcctc
 481 tttctccaga ttgtatagga gaagtcgcgc gacaggcggt cgcgcaactt tctcctggta
 541 gagttcttct tttagagaat gtacgtttcc ataaggggga agaacatcct gacgaggatc
 601 ctagttttgc tattgagctt gctgcttatg cagattttta tgtgaatgat gctttcggga
 661 catctcatcg taagcatgct tctgtatatc gggtgccaca actattccct gaccgggcag
 721 ccgcaggctt ccttatggaa aaagaattag aatttttggg ccagcatcta ttagttgagc
 781 ctaaacgtcc tttcactgct attttaggag gcgcgaaaat gtcttcgaaa ataggagtaa
 841 tcgaggcgct actttcgtgc gtggatcatc tcgtattagc tgggggtatg ggtacacct
 901 ttttaagggc tatgaatcgc caggtaggga attcattagt ggaagaatca gggatccctt
 961 tagcgaaaaa agtattagag aaagctcaag ctctgggggt gaagatccat cttccagtgg
1021 atgcgaaggt cgctaaacag tgtgactctg gagaggattg gagggagctg tctatacagg
1081 aaggaatccc tgaaggatta gcaggttttg atattggggc acagacaata gaactatttt
1141 ctaaggtgat tcaggagtcg gcaacgatat tttggaatgg tcctgtcggg gtatacgaag
1201 tccctccttt tgatcaagga tcgaaggcaa tagcacaatg tctcgcgagc cattcttctg
1261 ctgtgactgt ggttggggga ggcgatgcgg ctgctgtagt agctcttgca gggtgtactt
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Fig. 7B

1321 cacagatctc ccacgtatct acagggggag gcgcttcctt agaattctta gaaaaaggta

1381 gtcttcctgg tacggaaata ctatctccag ctcaaagc**ta a**atatctgcg tatgcattct

1441 ttgttaatga aaagccctgt ttatcggggc tttttttgt ttaaaaggag gtctgaattc

1501 gagatttaga atgattaaga tcctttgcaa aaaagataac tcttaacccc attgttttta

1561 attaattaga aactaatttt cgttcgttta aaaacagaac aattgttttt cttaaaaaga

1621 agtttttaaa atttaataaa aatagttgga attaaaagtt attgcttcgg cggaggattt

1681 atgagtattc gacctactaa tgggagtgga aatggatacc cgtctattaa tccttctaac

1741 gataatcaag acggtcttgt gcaatggacc tctgggccta attacggagg ccatacggta

1801 tcttctcgag gaggatttca agggatatgc gtacgaatag ccgatttat

CTPGK Deduced Amino Acid Sequence

1 MS**M**DKLSIRD LSLEGKKVLV RVDFNVPIKD GKILDDVRIR SAMPTIHYLL KQDAAVILVS

61 HLGRPKGGVF EEAYSLAPIV PVLEGYLGHH VPLSPDCIGE VARQAVAQLS PGRVLLLENV

121 RFHKGEEHPD EDPSFAIELA AYADFYVNDA FGTSHRKHAS VYRVPQLFPD RAAAGFLMEK

181 ELEFLGQHLL VEPKRPFTAI LGGAKMSSKI GVIEALLSCV DHLVLAGGMG YTFLRAMNRQ

241 VGNSLVEESG IPLAKKVLEK AQALGVKIHL PVDAKVAKQC DSGEDWRELS IQEGIPEGLA

301 GFDIGAQTIE LFSKVIQESA TIFWNGPVGV YEVPPFDQGS KAIAQCLASH SSAVTVVGGG

361 DAAAVVALAG CTSQISHVST GGGASLEFLE KGSLPGTEIL SPAQ**S**

Fig. 7B

C) CTPK Nucleotide Sequence

1 cactcaacga atcctttctc attttaaatt ctccacaccc attcctatcg aacgcttttt

61 taaagcgtag cattgcggtt gctaaatatt ttgtatagtt gaaggcttct ttcatttcgg

121 atattctaga agatattcta ctcactaata ccggtatccc gattt**atg**at cgctagaacg

181 aaaattattt gtacgatagg ccctgcaacc aatacccctg agatgctgga aaagcttctc

241 gatgcaggga tgaatgtagc tcgccttaat tttagccacg ggacccatga aagccatggc

301 cggaccatcg ctattcttaa agaactacga gagaagcgcc aagttccttt agctattatg

361 ctagatacaa aaggtcccga aattcgttta ggccaagtag aatctcctat aaaagtacag

421 cctggggatc gtcttactct cgttagcaaa gaaattttag gatccaaaga aagcgcgtta

481 ctctttatcc aagttgtgta ttccccttat gttagagaac gagctcctgt tctcattgat

541 gatgggtata tccaagcagt ggtggtcaat gctcaagagc atatggtgga aatagagttt

601 caaaattcag gagaaataaa atccaacaaa tctcttagca tcaaagatat cgatgttgct

661 cttcctttca tgacagagaa ggatattgca gacttaaaat ttggggtaga acaagaactc

721 gatcttatcg ctgcttcgtt cgtcagatgt aatgaagata ttgacagcat gcgtaaagtt

781 ttggaaagct ttggtcgtcc taatatgccc atcattgcca aaatagaaaa tcatttagga

841 gtacaaaatt tccaagagat cgctagagct gctgatggta tcatgattgc acgcggggat

901 cttggtattg aattgtctat tgttgaagtt cctggactac aaaaatttat ggcccgagca

961 tcgagggaaa cgggtcggtt ttgtatcact gcaacgcaaa tgctcgagtc aatgattcgc

1021 aaccccccttc ctacacgagc cgaagtctct gacgttgcca acgccattta cgatggaacc

1081 tctgcagtca tgttgtctgg agaaactgcc tcaggagccc atcctgtaca tgcagtaaaa

1141 acaatgcgtt ccattatcca agagactgag aagactttcg attaccacgc ttttttccag

1201 ctgaacgaca aaaacagcgc tctcaaagtt tctccttatc ttgaagccaa ttgggttttc

1261 tggatccaaa ttgcagaaaa agcatctgcc aaagccatta ttgtgtatac ccagacggga

Fig. 7C

1321 gggtctccga tgtttttatc caaatatcga ccttatctcc ctattattgc tgttacccct

1381 aaccgcaatg tgtactatcg tttagctgta gaatggggag tatatcctat gctaaccctg

1441 gaatcgaacc gtacagtctg gcgtcaccaa gcttgtgtat atggagtaga aaaaggaatt

1501 ctttctaact atgataaaat tcttgtcttc agccgcggag ctgggatgca agataccaac

1561 aatctcacct tgacaactgt gcatgatgcg ctatcccct ctcttgacga gatagttcca

1621 **taa**tcattga aaccatatag caggtatgtc ttctatcgtt agactttctg gtattactgt

1681 aaggaattta aaaacattac agtagagttt tgtctcgaga gatcgttttg ttcaccgggg

1741 tttctggatc gaagtcttct ctt

CTPK deduced amino acid sequence

1 MFYSLIPVSR **FM**IARTKIIC TIGPATNTPE MLEKLLDAGM NVARLNFSHG THESHGRTIA

61 ILKELREKRQ VPLAIMLDTK GPEIRLGQVE SPIKVQPGDR LTLVSKEILG SKESALLFIQ

121 VVYSPYVRER APVLIDDGYI QAVVVNAQEH MVEIEFQNSG EIKSNKSLSI KDIDVALPFM

181 TEKDIADLKF GVEQELDLIA ASFVRCNEDI DSMRKVLESF GRPNMPIIAK IENHLGVQNF

241 QEIARAADGI MIARGDLGIE LSIVEVPGLQ KFMARASRET GRFCITATQM LESMIRNPLP

301 TRAEVSDVAN AIYDGTSAVM LSGETASGAH PVHAVKTMRS IIQETEKTFD YHAFFQLNDK

361 NSALKVSPYL EANWVFWIQI AEKASAKAII VYTQTGGSPM FLSKYRPYLP IIAVTPNRNV

421 YYRLAVEWGV YPMLTLESNR TVWRHQACVY GVEKGILSNY DKILVFSRGA GMQDTNNLTL

481 TTVHDALSPS LDEIV**P**

Fig. 7C

D) CTZWF Nucleotide Sequence

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   1 taggacccct catggcaaag ccatgtttta tgagagagaa atctttttag gcacatattc
  61 gtttttttgg tattgtgggc tctctcttaa aaaattaaca ctctatacta ggttgtacct
 121 tggaagaaat taaagacttt ggccccacat taccagcctg ccctccctgt atcgtggtta
 181 tttttggtgc tacaggagac ttgacctcta ggaagctctt tcctgcttta tacaatttaa
 241 caaaggaagg acgtctatcc gaaaactttg tttgtgttgg gtttgctagg cgacctaagt
 301 ctcatgagca atttctcgaa gaaatgaagc ttgccgttca gcatttctct cactcatcgg
 361 aaatagatat tcgagtttgg gaaagtctgg aaaatagaat cttttaccac caagctaatt
 421 tttctgatgc cgaaggctac tctgctctga aagcttattt ggagcaacta gatcaacaat
 481 atggaacaca agggaatcgt ctttttttatt tatcaacacc accagattat ttccaggaaa
 541 tcatccgcaa tttaaatcgg catcagctat tctatcatga acaaggagca caacagcctt
 601 ggtctcggct aattatagaa aagccttttg gagttaattt agaaacagct cgagagcttc
 661 aacaatgcat tgatgccaat attgatgaag agtcggttta tcgaatagac cattatttag
 721 gaaaagaaac ggttcaaaac attctgacta ttcgttttgc taatactctc tttgagtctt
 781 gctggaattc tcagtacata gatcatgtgc aaatcagcgt tagcgaatca attggtatag
 841 gatctcgagg gaatttcttc gaaaagtcgg gcatgctacg agacatggta cagaatcatt
 901 tgacgcagct gctatgtcta ctgactatgg aacctccttc tgaattttct tcagaagaaa
 961 taaaaaaaga aaaaattaaa attctaaaga aaattcttcc tatccgcgaa gaagatgctg
1021 ttcgtggcca atatggtgaa gggattgtgc aagatgtttc agttctgggc tatcgggagg
1081 aagaaaatgt cgatcgaat tcttcagtag aaacctacgt tgcattaaaa ttatttatcg
1141 acaatcctcg ctggaaaggg gttccctttt acttacaagc agggaaacgt cttactaaaa
1201 gaacaacaga tatcgctgtg atctttaaaa aatccagcta caatttattc aatgcagaga
1261 attgtccttt gtgtccgtta gaaaatgatt tacttattat tcgtattcaa ccggatgaag
```

Fig. 7D

1321 gtgttgcgct acaatttaac tgcaaggttc caggaacaaa taagctcgta cgtcctgtaa

1381 aaatggactt ccgttacgac agctatttta atactgttac tcccgaagct tatgaacggt

1441 tactgtgcga ctgtatcctt ggggacagaa cgctattcac tagcaatgaa gaagtcttag

1501 catcttggga actattttct cctctattag aaaaatggtc tcaagtacac cctatattcc

1561 ctaactatat ggccggatct ttacgtcctc aagaagctga tgaactatta tctagagatg

1621 gaaaagcttg gcggccctat **taa**tttgttt tgcaagaggt tatatacatg gctaccctta

1681 ttagctaaat gatgcgaata gaatgcttat cgctgactct caagaagagt ttttacaaat

1741 cgcatgttat gattggatct ctacagcaaa taaagcgatt cacaaacgcg gtgcattcta

1801 tgtcgctc

CTZWF Deduced Amino Acid Sequence

1 MLGCTLEEIK DFGPTLPACP PCI**V**VIFGAT GDLTSRKLFP ALYNLTKEGR LSENFVCVGF

61 ARRPKSHEQF LEEMKLAVQH FSHSSEIDIR VWESLENRIF YHQANFSDAE GYSALKAYLE

121 QLDQQYGTQG NRLFYLSTPP DYFQEIIRNL NRHQLFYHEQ GAQQPWSRLI IEKPFGVNLE

181 TARELQQCID ANIDEESVYR IDHYLGKETV QNILTIRFAN TLFESCWNSQ YIDHVQISVS

241 ESIGIGSRGN FFEKSGMLRD MVQNHLTQLL CLLTMEPPSE FSSEEIKKEK IKILKKILPI

301 REEDAVRGQY GEGIVQDVSV LGYREEENVD PNSSVETYVA LKLFIDNPRW KGVPFYLQAG

361 KRLTKRTTDI AVIFKKSSYN LFNAENCPLC PLENDLLIIR IQPDEGVALQ FNCKVPGTNK

421 LVRPVKMDFR YDSYFNTVTP EAYERLLCDC ILGDRTLFTS NEEVLASWEL FSPLLEKWSQ

481 VHPIFPNYMA GSLRPQEADE LLSRDGKAWR **PY**

Fig. 7D

Figure 8. Schematic outline, restriction map and sequencing strategy of A) pHGAP6, B) pHPGK3 and C) pHPK1 clones which contain chlamydial DNA inserts. pHGAP6, pHPGK3 and pHPK1 were isolated by using a PCR-generated probe for colony hybridization screening of a partially digested *Hind*III *C. trachomatis* L2 DNA library. The *thin solid lines* represents the chlamydial DNA insert, and *the thicker solid lines* represents the predicted coding regions of *C. trachomatis gap*, *pgk* and *pyk* and contained in plasmids pHGAP, pHPGK3 and pHPK1 respectively. The *thinner dashed line* represents the pUC19 cloning vector. Selected restriction enzyme sites are marked: H, *Hind*III; E, *Eco*RI; and S, *Sal*I. Thin small arrows represent the individual regions sequenced and the direction of the sequence. Thick small arrows represent degenerate primers GAP5 and GAP3 (GAPDH), PGKd5 and PGKd3 (PGK), and PKd5 and PKd3 (PK) which were used to initiate sequencing of the putative *C. trachomatis* PK, PGK and GAPDH genes respectively. Selected areas used for RT-PCR and S. blot analysis are also indicated by an arrow (↔).

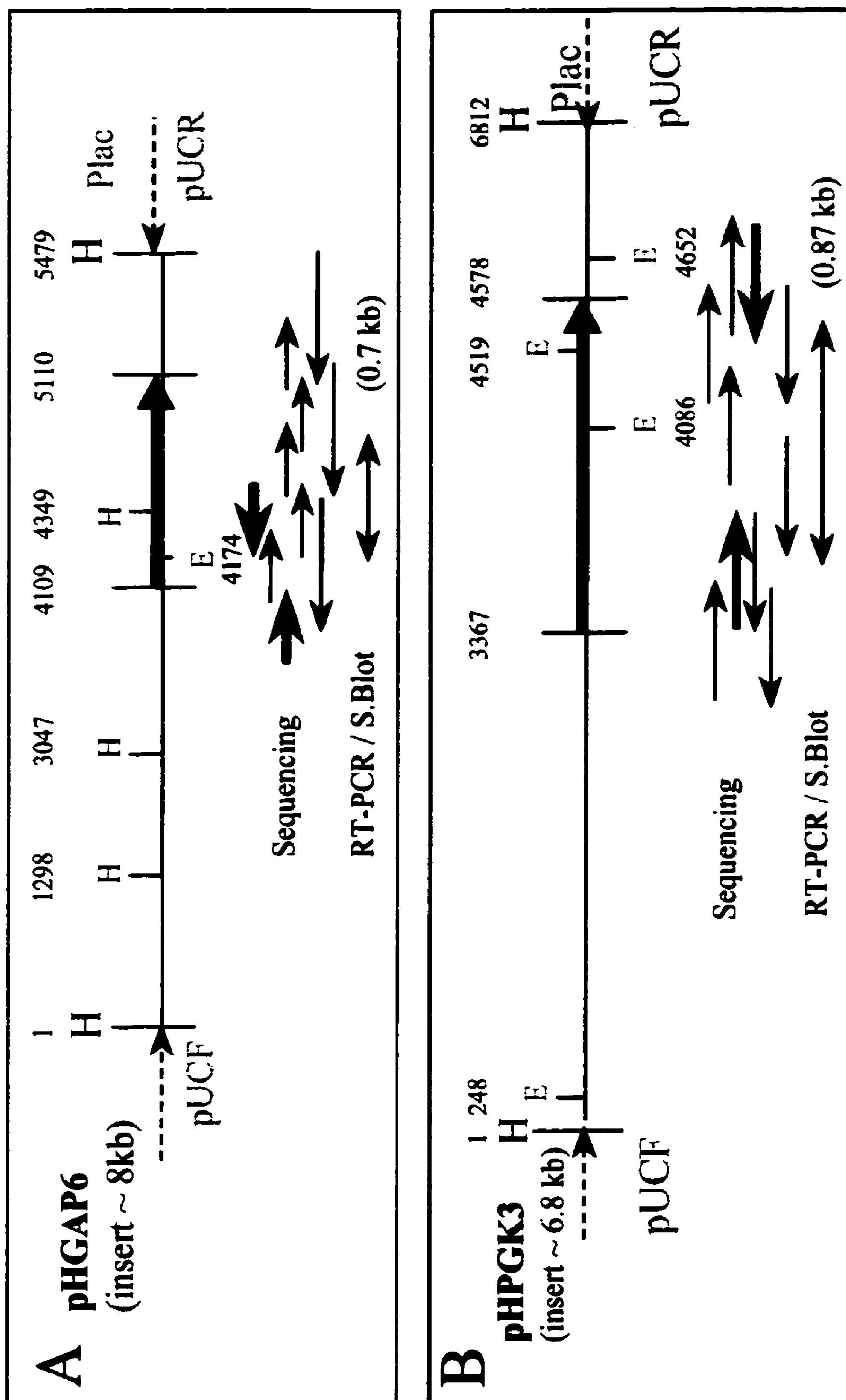


Fig. 8

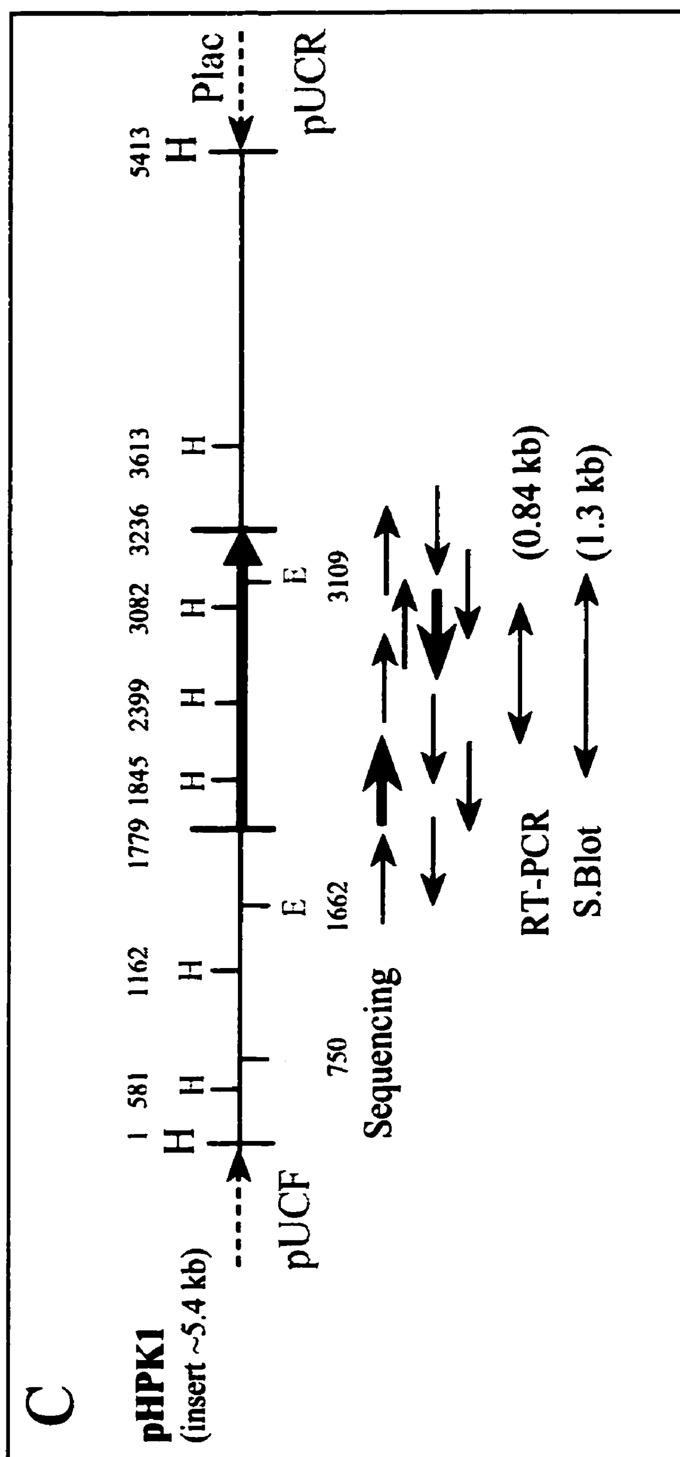


Fig. 8

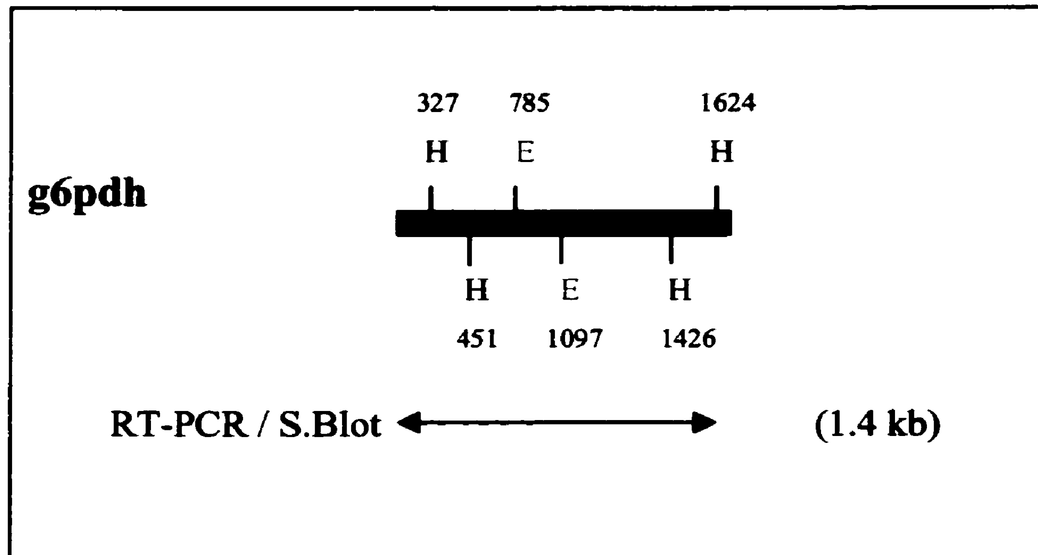


Figure 9. Restriction map of *C. trachomatis* L2 ZWF. The thick line represents the entire nucleotide sequence of ZWF. Selected restriction enzyme sites are marked: H, *Hin*dIII and E, *Eco*RI. Selected area used for RT-PCR and S.blot analysis is indicated by an arrow.

Open reading frames (ORFs) were identified for each clone using PC/Gene Software. The predicted amino acid sequence of these clones as well as pH11, which contains the putative ZWF ORF (Tipples unpublished), were then compared with the translated GenBank database and were found to show significant homology to other known *gap*, *pgk*, *pyk* and *zwf* genes. Characteristics of the cloned ORFs are summarized in Table 2. The amino acid sequences of the putative *C. trachomatis* L2 GAPDH, PGK, PK and ZWF ORFs show the highest homology to *E. coli gap*A, *Thermotoga maritima pgk*, *Bacillus stearothermophilus pyk* and *Nostoc* PCC73102 *zwf* respectively, indicating that the chlamydial energy-producing genes are homologous to a diverse field of bacterial species. While this work was in progress, results from the *C. trachomatis* serovar D genome sequencing project became available (Stephens *et al.*, 1998). Our cloned GAPDH, PGK, PK and ZWF sequences have > 95% amino acid homology to those present in serovar D.

Alignments of the deduced amino acid sequences of GAPDH, PGK, PK and ZWF enzymes from diverse species *C. trachomatis* L2, *E. coli*, *B. subtilus*, Human and *Nicotiana tabacum* (tobacco cytosolic enzymes) are shown in Figure 10A-D respectively. Crystal structures of NAD^+-bound GAPDH have been determined from a number of organisms including American lobster (Moras *et al.*, 1975), humans (Watson *et al.*, 1972), *B. stearothermophilus* (Skarzynski *et al.*, 1987), *T. maritima* (Korndorfer *et al.*, 1995), *T. aquaticus* (Tanner *et al.*, 1996), *E. coli* (Duee *et al.*, 1996), *L. mexicana* (Kim *et al.*, 1995), *T. brucei* (Vellieux *et al.*, 1993) and *T. cruzi* (Souza *et al.*, 1998). Studies from *B. stearothermophilus* indicated that a conformational change of the protein is induced

Table 2. Characteristics of the amino acid sequences deduced from the *C. trachomatis gap*, *pgk*, *pyk* and *zwf* cloned open reading frames

Name of clone	Number of amino acids	Molecular mass (kDa)	Isoeletric point	Enzyme	Identity to known protein	Organism and gene	Ref[a]
PHGAP6	334	37.0	5.48	Glyceralde-hyde-3-P-DHG	59.5%	*E. coli gap*A	P06977
PHPGK3	403	44.7	5.56	Phospho-glycerate kinase	49.4%	*T. maritima*	P36204
PHPK1	485	53.8	5.95	Pyruvate kinase	42.2%	*B. stearo-thermophilus*	Q02499
PH11	489	54.2	5.25	Glucose-6-P-DHG	38.0%	*Nostoc punc-forme*	317993

[a]Data Library Accession Numbers

Figure 10. Comparison of the deduced amino acid sequences from *C. trachomatis* L2 (CHLTR), *B. subtilis* (BACSU), *E. coli* (ECOLI), HUMAN, and *Nicotiana tabacum* (TOBAC) of A) GAPDH, B) PGK, C) PK and D) ZWF proteins. Identical residues are indicated by an asterisk (*), and similarity between amino acids is shown by dots (:.). Important residues implicated in substrate binding, effector binding and catalysis are indicated by the plus sign (+). Alignments are done by the FASTP program.

```
                            ++                     +
HUMAN       -----GKVKVGVNGFGRIGRLVTRAAFNSGK-VDIVAINDPFIDLNYMVYMFQYDSTHGK
TOBAC       ---------------GRIGRLVARVALQRDD-VELVAVNDPFISTDYMTYMFKYDSVHGQ
ECOLI       ------TIKVGINGFGRIGRIVFRAAQKRSD-IEIVAIND-LLDADYMAYMLKYDSTHGR
CHLTR       MTTWDLAMRIVINGFGRIGRLVLRQILKRNSPIEVVAIND-LVAGDLLTYLFKYDSTHGS
BACSU       ------AVKVGINGFGRIGRNVFRAALNNPE-VEVVAVND-LTDANMLAHLLQYDSVHGK
                           ***** * *   :  . :::**:** :   : :.::::***.**

HUMAN       F-HGTVKAENGKLVINGNP-ITIFQERDPSKIKWGDAGAEYVVESTGVFTTMEKAGAHLQ
TOBAC       WKHHELKVKDEKTLLFGEKSVRVFGIRNPEEIPWAEAGADFVVESTGVFTDKDKAAAHLK
ECOLI       F-DGTVEVKDGHLIVNGKK-IRVTAERDPANLKWDEVGVDVVAEATGLFLTDETARKHIT
CHLTR       F-APQATFSDGCLVMGERK-IRFLAEKDVQKLPWKDLDVDVVVESTGLFVNRDDAAKHLD
BACSU       L-DAEVSVDGNNLVVNGKT-IEVSAERDPAKLSWGKQGVEIVVESTGFFTKRADAAKHLE
                    ..   ::  .  : .   ::  :: * . ..: *.*:**.*     *  *:
                                                   +++
HUMAN       GGAKRVIISAPSAD-APMFVMGVNHEKYDNS-LKIISNASCTTNCLAPLAKVIHDNFGIV
TOBAC       GGAKKVVISAPSKD-APMFVVGVNEKEYKPE-YDIVSNASCTTNCLAPLAKVINDRFGIV
ECOLI       AGAKKVVMTGPSKDNTPMFVKGANFDKYAG--QDIVSNASCTTNCLAPLAKVINDNFGII
CHLTR       SGAKRVLITAPAKGDVPTFVMGVNHQQFDPA-DVIISNASCTTNCLAPLAKVLLDNFGIE
BACSU       AGAKKVIISAPANEEDITIVMGVNEDKYDAANHDVISNASCTTNCLAPFAKVLNDKFGIK
            .***:*:::.*:      :* *.* .::      ::************:***: *.***
                 + +                               +
HUMAN       EGLMTTVHAITATQKTVDGPSGKLWRDGRGALQNIIPASTGAAKAVGKVIPELNGKLTGM
TOBAC       EGLMTTVHSLTATQKTVDGPSMKDWRGGRATSFNIIPSSTGAAKAVGKVLPALNGKLTGM
ECOLI       EGLMTTVHATTATQKTVDGPSHKDWRGGRGASQNIIPSSTGAAKAVGKVLPELNGKLTGM
CHLTR       EGLMTTVHAATATQSVVDGPSRKDWRGGRGAFQNIIPASTGAAKAVGLCLPELKGKLTGM
BACSU       RGMMTTVHSYTNDQQILDLP-HKDYRRARAAAENIIPTSTGAAKAVSLVLPELKGKLNGG
            .*:*****: *  *. :* *  * :* .*.:  ****:********.  :* *:***.*
              +                                               +
HUMAN       AFRVPTANVSVVDLTCRLEKPAKYDDIKKVVKQASEGPLKGILGYTEHQVVSSDFNSDTH
TOBAC       AFRVPTVDVSVVDLTVRLEKEASYDDIKAAIKEESEGKLKGILGFTEDDVVSTDFVGDSR
ECOLI       AFRVPTPNVSVVDLTVRLEKAATYEQIKAAVKAAAEGEMKGVLGYTEDDVVSTDFNGEVC
CHLTR       AFRVPVADVSVVDLTVKLSSATTYEAICEAVKHAANTSMKNIMYYTEEAVVSSDFIGCEY
BACSU       AMRVPTPNVSLVDLVAELNQEVTAEEVNAALKEAAEGDLKGILGYSEEPLVSGDYNGNKN
            *:***. :**:***. .*.. .. : :  .:*  ::  :*.:: ::*. :** *: .
                                  + +   +
HUMAN       SSTFDAGAGIALNDHFVKLISWYDNEFGYSNRVVDLMAHMASKE-
TOBAC       SSIFDAKAGIALSKNFVKLVSWYDNEWGYSSRVIDLICHMASVA-
ECOLI       TSVFDAKAGIALNDNFVKLVSWYDNETGYSNKVLDLIAHISK---
CHLTR       SSIFDAQAGVALNDRFFKLVAWYDNEIGYATRIVDLLEYVQENSK
BACSU       SSTIDALSTMVMEGSMVKVISWYDNESGYSNRVVDLAAYIAKKGL
            :* :** : :.:.  :.*:::***** **:.:::**  :: .
```

Fig. 10A

B

```
TOBAC   MASATASHTLCGIPATSSSTTNKAIAPSSARFLAKTPLRRLGFAGAAADSLFTNHVATKL
BACSU   ------------------------------------------------------------
ECOLI   ------------------------------------------------------------
CHLTR   ------------------------------------------------------------
HUMAN   ------------------------------------------------------------

                                                ++          +
TOBAC   RSLKSSSKPIRGVASMAKKSVGDLTAAELKGKKVFVRADLNVPLDDNQNITDDTRIRAAV
BACSU   ---------------MNKKTLKDI---DVKGKVVFCRVDFNVPMKDGE-VTDDTRIRAAL
ECOLI   ----------------SVIKMTDL---DLAGKRVFIRADLNVPVKDGK-VTSDARIRASL
CHLTR   -------------MSMDKLSIRDL---SLEGKKVLVRVDFNVPIKDGK-ILDDVRIRSAM
HUMAN   -------------SLSNKLTLDKL---DVKGKRVVMRVDFNVPMKNNQ-ITNNQRIKAAV
                      .: .:     .: ** *. *.*:***:.:.: : .: **::::
                   +  +
TOBAC   PTIKHLMANGAK-VILSSHLGRP-KG-VTPKYSLAPLVPRLSELLGIQVVKVEDCIGPEV
BACSU   PTIKHLADQGAK-VLLASHLGRP-KGEVVEELRLTPVAARLGELLGKEVKKADEAYGDAV
ECOLI   PTIELALKQGAK-VMVTSHLGRPTEGEYNEEFSLLPVVNYLKDKLSNPVRLVKDYLDG--
CHLTR   PTIHYLLKQDAA-VILVSHLGRPKGGVFEEAYSLAPIVPVLEGYLGHHVPLSPDCIGEVA
HUMAN   PSIKFCLDNGAKSVVLMSHLGRPDGVPMPDKYSLEPVAVELKSLLGKDVLFLKDCVGPEV
        *:*.    :.*  *:: ******          * *:.  *    *.  *    :  .
                   +
TOBAC   EKLVASLPEGGVLLLENVRFYKEEEK--NEPE-------------FAKKLASLADLYVND
BACSU   KAQISEMKDGDVLVLENVRFYPGEEK--NDPE-------------LAKAFAELADVYVND
ECOLI   ---VDVA-EGELVVLENVRFNKGEKK--DDET-------------LSKKYAALCDVFVMD
CHLTR   RQAVAQLSPGRVLLLENVRFHKGEEHPDEDPS-------------FAIELAAYADFYVND
HUMAN   EKACANPAAGSVILLENLRFHVEEEGKGKDASGNKVKAEPAKIEAFRASLSKLGDVYVND
             * :::***:**   *:    .:                  :    :    *.:* *
  +   +                                                +     +
TOBAC   AFGTAHRAHASTEGVTKFLK-PSVAGFLLQKELDYLVGAVSN-PKRPFAAIVGGSKVSSK
BACSU   AFGAAHRAHASTAGIAEHL--PAVAGFLMEKELDVLGKAVSN-PDRPFTAIIGGAKVKDK
ECOLI   AFGTAHRAQASTHGIGKFAD-VACAGPLLAAELDALGKALKE-PARPMVAIVGGSKVSTK
CHLTR   AFGTSHRKHASVYRVPQLFPDRAAAGFLMEKELEFLGQHLLVEPKRPFTAILGGAKMSSK
HUMAN   AFGTAHRAHSSMVGVNLPQK---AGGFLMKKELNYFAKALES-PERPFLAILGGAKVADK
        ***::** ::*   :         .* *:  **: :   :    * **: **:**:*:  *

TOBAC   IGVIESLLEKCDILLLGGGMIFTFYKAQG-LSVGSSLVEEDKLELATSLLEKAKAKGVSL
BACSU   IGVIESLLDKVDNLIIGGGLAYTFVKALG-YEVGKSLLEEDKIELAKSFMDRAKEKGVNF
ECOLI   LTVLDSLSKIADQLIVGGGIANTFIAAQG-HDVGKSLYEADLVDEAKRLLTTCN-----I
CHLTR   IGVIEALLSCVDHLVLAGGMGYTFLRAMN-RQVGNSLVEESGIPLAKKVLEKAQALGVKI
HUMAN   IQLINNMLDKVNEMIIGGGMAFTFLKVLNNMEIGTSLFDEEGAKIVKDLMSKAEKNGVKI
        : ::: :  .  : :::.**:  **   .  .  .:*.** : .    .. .:  .:    :
                                                                +
TOBAC   LLPSDVVIADKFAPDANSKIVPASA-IPDGWMGLDIGPDSVKTFNDALDTTKTVIWNGPM
BACSU   YMPEDVLVADDFSNDANVKIVPISE-IPSDLEAIDIGTKTRETYADVIKNSKLVVWNGPM
ECOLI   PVPSDVRVATEFSETAPATLKSVND-VKADEQILDIGDASAQELAEILKNAKTILWNGPV
CHLTR   HLPVDAKVAKQCDSGEDWRELSIQEGIPEGLAGFDIGAQTIELFSKVIQESATIFWNGPV
HUMAN   TLPVDFVTADKFDENAKTGQATVASGIPAGWMGLDCGPESSKKYAEAVTRAKQIVWNGPV
         :* *   * .         .    :  .   :* *  : :   . :  :  :.****:
   +                                      ++                +++
TOBAC   GVFEFDKFAVGTEAIAKKLADLSGKGVTTIIGGGDSVAAVEKVGVASVMSHISTGGGASL
BACSU   GVFEIDLFAQGTKAVAEALAEA--KDTYSVIGGGDSAAAVEKFGLADKMSHISTGGGASL
ECOLI   GVFEFPNFRKGTEIVANAIADS---EAFSIAGGGDTLAAIDLFGIADKISYISTGGGAFL
CHLTR   GVYEVPPFDQGSKAIAQCLASHS--SAVTVVGGGDAAAVVALAGCTSQISHVSTGGGASL
HUMAN   GVFEWEAFARGTKALMDEVVKATSRGCITIIGGGDTATCCAKWNTEDKVSHVSTGGGASL
        **:*    *  *:: : . :..            :: ****: :      .  . :*::****** *

TOBAC   ELLEGKVLPGVIALDEADAPVAV
BACSU   EFMEGKELPGVAALNDK------
ECOLI   EFVEGKVLPAVAMLEERAKK---
CHLTR   EFLEKGSLPGTEILSPAQS----
HUMAN   ELLEGKVLPGVDALSNIL-----
        *::*    **..  *.
```

Fig. 10B

```
ECOLI    -----------------------------------------MKKTKIVCTIGPKTESEEML
BACSU    -----------------------------------------MRKTKIVCTIGPASESIEML
HUMAN    SKPHSEAGTAFIQTQQLHAAMADTFLEHMCRLDIDSPPITARNTGIICTIGPASRSVETL
TOBAC    ----------------MAIENNNNGVNFCTVKR--P-----KTKIVCTLGPASRSVPMI
CHLTR    ---------------------------------------MIARTKIICTIGPATNTPEML
                                                  .* *:**:** :.:   :

                 + ++                                             +
ECOLI    AKMLDAGMNVMRLNFSHGDYAEHGQRIQNLRNVMSKTG------KTAAILLDTKGPEIRT
BACSU    TKLMESGMNVARLNFSHGDFEEHGARIKNIREASKKLG------KNVGILLDTKGPEIRT
HUMAN    KEMIKSGMNVARLNFSHGTHEYHAETIKNVRTATESFASDPILYRPVAVALDTKGPEIRT
TOBAC    EKLLRAGMNVARFNFSHGSHDYHQETIDNLRQAMESTG---ILC---AVMLDTKGPEIRT
CHLTR    EKLLDAGMNVARLNFSHGTHESHGRTIAILKELREKRQ------VPLAIMLDTKGPEIRL
          ::: :**** *:*****  .   *    *   ::     ..         .: *********

ECOLI    MKLE--GGNDVSLKAGQTFTFTTDKSVIGNSE--MVAVTYEGFTTDLSVGNTVLVDDGLI
BACSU    HTME--NGG-IELETGKELIISMD-EVVGTTD--KISVTYEGLVHDVEQGSTILLDDGLI
HUMAN    GLIKGSGTAEVELKKGATLKITLDNAYMEKCDENILWLDYKNICKVVEVGSKIYVDDGLI
TOBAC    GFLK--DAKPVQLKQGQ--EITISTDYSIKGDESMICMSYKKLAEDVKPQSVILCADGQI
CHLTR    GQVE----SPIKVQPGDR-LTLVSKEILGSKE-SALLFIQVVYSPYVRERAPVLIDDGYI
          ::       :.:: *        .        . :   :  .        :      :    ** *

ECOLI    GMEVTAIEGNK--VICKVLNNGDLGENKGVNLPGVSIALPALAEKDKQD-LIFGCEQGVD
BACSU    GLEVLDVDAAKREIKTKVLNNGTLKNKKGVNVPGVSVNLPGITEKDARD-IVFGIEQGVD
HUMAN    SLQVK--QKGADFLVTEVENGGSLGSKKGVNLPGAAVDLPAVSEKDIQD-LKFGVEQDVD
TOBAC    TFTVLSCDKENGLDRCRCENTAVLGERKNVNLPGVIVDLPTLTDKDKDDILNWGVPNHID
CHLTR    QAVVVN--AQEHMVEIEFQNSGEIKSNKSLSIKDIDVALPFMTEKDIAD-LKFGVEQELD
           *             .  *  .  :  ..*.:.:  .   :  ** :::**  *  :  :*   : :*
                                                    + +                   +
ECOLI    FVAASFIRKRSDVIEIREHLKAHGGENIHIISKIENQEGLNNFDEILEASDGIMVARGDL
BACSU    FIAPSFIRRSTDVLEIRELLEEHNAQDIQIIPKIENQEGVDNIDAILEVSDGLMVARGDL
HUMAN    MVFASFIRKASDVHEVRKVLGEK-GKNIKIISKIENHEGVRRFDEILEASDGIMVARGDL
TOBAC    MIALSFVRKGSDLVEVRKLLGEH-AKNILLMSKVENQEGVANFDDILLNSDAFMVARGDL
CHLTR    LIAASFVRCNEDIDSMRKVLESFGRPNMPIIAKIENHLGVQNFQEIARAADGIMIARGDL
         ::  **:*   *:  .:*: *        ::  ::.*:**: *:  .:: *       :*.:*:*****
                                                   +
ECOLI    GVEIPVEEVIFAQKMMIEKCIRARKVVITATQMLDSMIKNPRPTRAEAGDVANAILDGTD
BACSU    GVEIPAEEVPLVQKELIKKCNALGKPVITATQMLDSMQRNPRPTRAEASDVANAIFDGTD
HUMAN    GIEIPAEKVFLAQKMMIGRCNRAGKPVICATQMLESMIKKPRPTRAEGSDVANAVLDGAD
TOBAC    GMEIPIEKIFLAQKVMIYKCNIQGKPVVTATQMLESMIKSPRPTRAEATDVANAVLDGTD
CHLTR    GIELSIVEVPGLQKFMARASRETGRFCITATQMLESMIRNPLPTRAEVSDVANAIYDGTS
         *:*:.   ::       **  :      .       :   :  *****:**  :.*  *****   *****:  **:.

ECOLI    AVMLSGESAKGKYPLEAVSIMATICERTDRVMNSRLEFNN-----DNRKLRITEAVCRGA
BACSU    AIMLSGETAAGSYPVEAVQTMHNIASRSEEALNYKEILSKR-R--DQVGMTITDAIGQSV
HUMAN    CIMLSGETAKGDYPLEAVRMQHLIAREAEAAIYHLQLFEEL-RRLAPITSDPTEATAVGA
TOBAC    CVMLSGETAAGAYPDLAVGTMAKICIEAESTIDYPDVFKRI-MSNAPVPMSPLESLASSA
CHLTR    AVMLSGETASGAHPVHAVKTMRSIIQETEKTFDYHAFFQLNDKNSALKVSPYLEANWVFW
         .:*****:*  *  :*   **       *    .:: .:        :.                :  :
                   +  +  +
ECOLI    VETAEKLDAPLIVVATQGGKSARAVRKYFPDATILALTT-------------NEKTAHQL
BACSU    AHTAINLNAAAIVTPTESGHTARMIAKYRPQAPIVAVTV-------------NDSISRKL
HUMAN    VEASFKCCSGAIIVLTKSGRSAHQVARYRPRAPIIAVTR-------------NPQTARQA
TOBAC    VRTANSAKAALILVLTRGGSTAKLVAKYRPGMPILSVVVPEIKTDSFDWTCSDESPARHS
CHLTR    IQIAEKASAKAIIVYTQTGGSPMFLSKYRPYLPIIAVTP-------------NRNVYYRL
          .  :  .    :    *:.   *.  *  :.     :  :*  *    .*:::.              :   .     :

ECOLI    VLSKGVVPQLVKE------ITSTDDFYRLGKELALQSGLAHKGDVVVMVSGALVPSGTTN
BACSU    ALVSGVFAESGQN------ASSTDEMLEDAVQKSLNSGIVKHGDLIVITAGTVGESGTTN
HUMAN    HLYRGIFPVLCKDPVQEAWAEDVDLRVNFAMNVGKARGFFKKGDVVIVLTGWRPGSGFTN
TOBAC    LIFRGLVPVLHAGSARASHEESTEEALDFALQHAKTKGLCKQGDSVVALH--R--VGTAS
CHLTR    AVEWGVYPMLTLE-------SNRTVWRHQACVYGVEKGILSNYDKILVFSRGAG-MQDTN
          :   *: .                  .          .       .       *:    :  *  ::                :.

ECOLI    TASVHVL-----------------------------------------------------
BACSU    LMKVHTVGDIIAKGQGIGRKSAYGPVVVAQNAKEAEQKMTDGAVLVTKSTDRDMIASLEK
HUMAN    TMRVVPVP----------------------------------------------------
TOBAC    VIKIVTVK----------------------------------------------------
CHLTR    NLTLTTVHDALSPSLDEIVP----------------------------------------
            :   :

ECOLI    ------------------------------------------------------------
BACSU    ASALITEEGGLTSHAAVVGLSLGIPVIVGLENATSILTDGQDITVDASRGAVYQGRASVL
HUMAN    ------------------------------------------------------------
TOBAC    ------------------------------------------------------------
CHLTR    ------------------------------------------------------------
```

Fig. 10C

```
CHLTR    ------------------------------------------------------------
BACSU    ------------------------------------------------------------
TOBAC    MVTLYSSPSTHSSGPVASYSNSSIGLYNYHHNKQIAVSSILSRKFGSLQINQKPFWNAVR
HUMAN    ------------------------------------------------------------
ECOLI    ------------------------------------------------------------

                                                                +
CHLTR    -------------------------MLG----CTLEEIKDFGPTLPACPPCIVVIFGATG
BACSU    -----------------------------------------MKTN--QQPKAVIVIFGATG
TOBAC    MQDGAVATPPSKIENETPLKKLKNGILPVAPPKEQKDTIDFDSN-KAKSTVSITVVGASG
HUMAN    ------------AEQVALSRTHVCGIL------R-EELFQGDAF-HQSDTHIFIIMGASG
ECOLI    -----------------------------------------MAVT-QTAQACDLVIFGAKG
                                                          . :.**.*
  +                            +
CHLTR    DLTSRKLFPALYNLTKEGRLSENFVCVGFARRPKSHEQFLEEMKLAVQHFSHSSEIDIR-
BACSU    DLAKRKLYPSIHRLYQNGQIGEEFAVVGVGRRPWSNEDLRQTVKTSIS-----SSADKH-
TOBAC    DLAKKKIFPALFALYYEGCLPEHFTIFGYARSKMTDAELRNMVSKTLT-----CRIDKRE
HUMAN    DLAKKKIYPTIWWLFRDGLLPENTFIVGYARSRLTVADIRKQSEPFFK-----ATPEEK-
ECOLI    DLARRKLLPSLYQLEKAGQLNPDTRIIGVGRADWDKAAYTKVVREALETFMK-ETIDEG-
         **: :*: *::   *    ● :   .    .* .*          :     .        :
                                                              +
CHLTR    ----VWESLENRIFYHQANFSDAEGYSALKAYLEQLDQQYGTQGNRLFYLSTPPDYFQEI
BACSU    ----IDD-FTSHFYYHPFDVTNPGSYQELNVLLNQLEDTYQIPNNRMFYLAMAPEFFGTI
TOBAC    NCGEKMEQFLERCFYHSGQYDSLENFAELDKKLKEHE--AGRFSNRLFYLSIPPNIFINA
HUMAN    ---LKLEDFFARNSYVAGQYDDAASYQRLNSHMNALH--LGSQANRLFYLALPPTVYEAV
ECOLI    ----LWDTLSARLDFCNLDVNDTAAFSRLGAMLDQKN--R-ITIN---YFAMPPSTFGAI
              : :  :  :   :  .   :  *   :.  .         *    *:: .*  :
                                                              ++ +
CHLTR    IRNLNRHQLFYHEQGAQQPWSRLIIEKPFGVNLETARELQQCIDANIDEESVYRIDHYLG
BACSU    AKTLKSEGVT-----ATTGWSRLVIEKPFGHDLPSAQALNKEIREAFTEDQIYRIDHYLG
TOBAC    VRCASLSASS------AHGWTRVIVEKPFGRDSESSAALTRSLKQYLNEDQIFRIDHYLG
HUMAN    TKNIHESCMS------QIGWNRIIVEKPFGRDLQSSDRLSNHISSLFREDQIYRIDHYLG
ECOLI    CKGLGEAKLN-------AKPARVVMEKPLGTSLATSQEINDQVGEYFEECQVYRIDHYLG
          :                         *:::***:*  .  ::  :   :   : ● .::*******
 +
CHLTR    KETVQNILTIRFANTLFESCWNSQYIDHVQISVSESIGIGSRGNFFEKSGMLRDMVQNHL
BACSU    KQMVQNIEVIRFANAIFEPLWTNRYISNIQITSSESLGVEDRARYYEKSGALRDMVQNHI
TOBAC    KELVENLSVLRFSNLIFEPLWSRQCIRNVQFIFSEDFGTEGRGGYFDHYGIIRDIMQNHL
HUMAN    KEMVQNLMVLRFANRIFGPIWNRDNIACVILTFKEPFGTEGRGGYFDEFGIIRDVMQNHL
ECOLI    KETVLNLLALRFANSLFVNNWDNRTIDHVEITVAEEVGIEGRWGYFDKAGQMRDMIQNHL
         *: * *: .:**:* :*    *     *    : :    * .*   .*   :::. * :**::***:

CHLTR    TQLLCLLTMEPPSEFSSEEIKKEKIKILKKILPIR----EEDAVRGQYGEG-IVQDVSVL
BACSU    MQMVALLAMEPPIKLNTEEIRSEKVKVLRALRPIAKDEVDEYFVRGQYHAG-EIDGVPVP
TOBAC    LQILALFAMETPVSLDAEDIRNEKVKVLRSMRPLQ----LDDVIIGQYKCHTKGD-VTYP
HUMAN    LQMLCLVAMEKPASTNSDDVRDEKVKVLKCISEVQ----ANNVVLGQYVGNPDGEGEATK
ECOLI    LQILCMIAMSPPSDLSADSIRDEKVKVLKSLRRIDRSNVREKTVRGQYTAG-FAQGKKVP
          *::.:.:*.  *  .  .::.::.**:*:*: :   :         :   : ***        :

CHLTR    GYREEENVDPNSSVETYVALKLFIDNPRWKGVPFYLQAGKRLTKRTTDIAVIFKKSSYNL
BACSU    AYTDEDNVAPDSNTETFVAGKLLIDNFRWAGVPFYIRTGKRMREKSTKIVVQFKDIPMNL
TOBAC    GYTDDKTVPKDSLTPTFAAAALFIDNARWDGVPFLMKAGKALHTRSAEIRVQFRHVPGNL
HUMAN    GYLDDPTVPRGSTTATFAAVVLYVENERWDGVPFILRCGKALNERKAEVRLQFHDVAGDI
ECOLI    GYLEEEGANKSSNTETFVAIRVDIDNWRWAGVPFYLRTGKRLPTKCSEVVVYFKTPELNL
         .* ::   .   .* . *:.*   : ::* ** **** :: **  :   : :.: : *:    ::

CHLTR    FNAENC-PLCPLENDLLIIRIQPDEGVALQFNCKVPGTN--KLVRPVKMDFRYDSYFN-T
BACSU    YYGNEN-NMNP--N-LLVIHIQPDEGITLYLNAKKLGGA--AHAQPIKLDYCSNCNDE-L
TOBAC    YNKNFGSDLDQATN-ELVIRVQPNEAIYLKINNKVPGLG--MRLDRSNLNLLYSARYS-K
HUMAN    FHQQCK-----R-N-ELVIRVQPNEAVYTKMMTKKPGMF--FNPEESELDLTYGNRYKNV
ECOLI    FKESWQ-DLPQ--N-KLTIRLQPDEGVDIQVLNKVPGLDHKHNLQITKLDLSYSETFNQT
                     *   *  *::**:*.:   .   *   *          :::    .    .

CHLTR    VTPEAYERLLCDCILGDRTLFTSNEEVLASWELFSPLLEKWSQVHPIFPNYMAGSLRPQE
BACSU    NTPEAYEKLIHDCLLGDATNFAHWDEVALSWSFVDSISETWAANKTLSPNYESGSMGPKE
TOBAC    EIPDAYERLLLDAIEGERRLFIRSDELDAAWSLFTPVLKELEDKKIVPEYYPYGSRGPIG
HUMAN    KLPDAYERLILDVFCGSQMHFVRSDELREAWRIFTPLLHQIELEKPKPIPYIYGSRGPTE
ECOLI    HLADAYERLLLETMRGIQALFVRRDEVEEAWKWVDSITEAWAMDNDAPKPYQAGTWGPVA
          .:***:*:  : :  *      *    :*:   :*   . .: .        :        *   *:   *

CHLTR    ADELLSR-----DG-KAWRPY---
BACSU    SDDLLVK-----DG-LHWWNI---
TOBAC    AHYLAAR----YK--VRWGDLV--
HUMAN    ADELMKRVGFQYEGTYKWVNPHKL
ECOLI    SVAMITR-----DG-RSWNEFE--
         :  :  :       .    ●
```

Fig. 10D

upon Asp338 and Glu345 (WNGPMGVFE); Gly376, Asp377, Ser378 (GGGDS)NAD$^+$ binding (Yun *et al.*, 2000). Important residues believed to be involved in NAD$^+$ binding include (*C. glutamicum* numbering) (Eikmanns, 1992), Arg12 and Ile13 (INGFGRIGR); Asp36 (INDL); Asn315 and Tyr319 (WYDNEIGY) (Branlant and Branlant, 1985; Souza *et al.*, 1998). Amino acids postulated to be involved in the catalytic mechanism include Ser152, Cys153 and Thr154 (ASCTTN); and His180 (TTVH) (*C. glutamicum* numbering) (Eikmanns, 1992). The amino acid sequence of *C. trachomatis* L2 GAPDH contains all of these conserved residues.

The crystallographic structures of PGK from horse muscle (Banks *et al.*, 1979), yeast (Watson *et al.*, 1972), pig muscle (Harlos *et al.*, 1992), *B stearothermophilus* (Chandra *et al.*, 1998; Davies, 1994), *T. brucei* (Bernstein *et al.*, 1997) and *T. maritima* (Auerbach *et al.*, 1997) are remarkably similar (McHarg *et al.*, 1999). These crystallographic studies indicate that PGK consists of two relatively equal sized domains, the N- and C- terminal domains, connected by a well-conserved hinge region. 3PGA is found to bind to a cluster of basic amino acids in the N-terminal domain as illustrated by the pig muscle (Harlos *et al.*, 1992) and *T. brucei* PGK (Bernstein *et al.*, 1997). Specifically, studies from *T. brucei* PGK indicate that regions involved in 3PGA binding include Asp24 and Asn26 (DFNVPL); Arg39 (RIR); His62 and Arg65 (SHLGRP); Arg135 (LENVRF); Gly168 and Arg172 (DAFGTAHR); and Gly398, Gly399 and Ala400 (STGGGA) (*T. brucei* numbering) (Bernstein *et al.*, 1997). These residues involved in 3PGA binding are conserved in *C. trachomatis* PGK. MgADP has been documented to bind to the C-terminal domain (Davies *et al.*, 1993). Studies from *T. brucei* PGK indicate that MgADP binds to Ala218 and Lys223 (RPLVAIVGGAK); (*T.*

brucei numbering) (Bernstein *et al.*, 1997). These residues involved in MgADP binding are also conserved in *C. trachomatis* PGK. In most of the reported PGK structures, the substrates are bound too far apart to allow for transfer of the phosphoryl group between them, hence a hinge-bending mechanism of catalysis has been proposed (Pappu *et al.*, 1997). Basically, upon binding of the substrates, the PGK enzyme undergoes a conformational change in which the hinge region bends to permit interaction of substrates. Further studies on *C. trachomatis* PGK will have to be done to determine the substrate binding sites and mechanism of catalysis.

PK has been structurally characterized from a number of prokaryotes and eukaryotes and in most cases has been found to exist as a tetramer of identical subunits with a subunit molecular mass of about 55 kDa (Fothergill-Gilmore and Michels, 1993). The structures of unregulated M1 isoenzymes from cat (Muirhead *et al.*, 1986) and rabbit (Larsen *et al.*, 1994), as well as allosterically regulated enzymes from *E. coli* (Mattevi *et al.*, 1995), *S. cerevisiae* (Jurica *et al.*, 1998) and *L. mexicana* (Rigden *et al.*, 1999) have provided excellent models of the enzyme structure including the active site and F16BP binding site. *C. trachomatis* PK amino acid sequence contains important residues (rabbit muscle numbering) in pyruvate binding T327 (ATQM), K269 (SKIEN); K^+ binding N74, S76 (NFSHG) and Mn^{2+} (Mg^{2+}) binding E271 (SKIEN), D295 (MVARGDLG) (Larsen *et al.*, 1994). Sites suggested to be involved in ADP binding include N74 and H77 (NFSHG), and 119R (PEIRT) (rabbit muscle numbering) (Jurica *et al.*, 1998) are also conserved in the *C. trachomatis* PK sequence. The crystal structure of yeast PK in the presence of F16BP indicates that the 6-phosphate interacts with S402, S404 and T407 (Jurica *et al.*, 1998). The sequence S402-TSGTT407 has been described to constitute a

well defined phosphate binding pocket and is likely conserved in many allosterically regulated PKs (Fothergill-Gillmore *et al.*, 2000). This also holds true for the CTPK enzyme as these residues correspond to the very similar amino acids of T382, T384 and S387 respectively (*C. trachomatis* numbering). Interestingly, these CTPK residues are identical to those found in the trypanosome PK, and both trypanosome PK (Ernest *et al.*, 1998) and CTPK (this work; see "Results: Kinetic analysis of CTPK") have been shown to be allosterically activated by F26BP. The 1'-phosphate group of F16BP has been found to interact with R459 in Yeast PK which is conserved among many PK enzymes that are allosterically regulated by F16BP (Jurica *et al.*, 1998). CTPK contains a Y439 in this position. Whether this is important in phosphate binding remains to be determined. Definitive sites involved in F26BP binding have yet to be resolved (Fothergill-Gillmore *et al.*, 2000).

The crystal structure of *L. mesenteroides* G6PDH (Rowland *et al.*, 1994) and human G6PDH (Au *et al.*, 1999) has been solved and are similar suggesting that the 3-D structure of G6PDH is essentially conserved. Studies on the crystal structure of *L. mesenteroids* G6PDH indicate that the enzyme is a dimeric molecule and each subunit consists of two domains; the smaller domain contains the coenzyme binding site, whereas the other domain contains a $\beta + \alpha$ fold which is predicted to be involved in catalytic activity (Levy and Moulder, 1982; Scopes *et al.*, 1998). Residues 12-18 (*L. mesenteroids* numbering) form the dinucleotide binding fingerprint or coenzyme-binding motif of Gly-X-X-Gly-X-X-Ala/Gly (Persson *et al.*, 1991; Vought *et al.*, 2000). Arg46 (GTA<u>R</u>), has been shown to interact with $NADP^+$ and Thr14 (FGG<u>T</u>GDL) has also been implicated in coenzyme binding (Vought *et al.*, 2000). Residues Asp177 (F<u>R</u>IDH) and His 240

(QNHTMQ) have been described to be involved in catalytic site. His178, Tyr179, Lys182 (RIDHYLGKE); and Lys343 (GKRLAAK) interact with G6P (Vought *et al.*, 2000). *C. trachomatis* G6PDH amino acid sequence contains all of these residues except for Lys343, which is replaced with an arginine.

2. Southern hybridization

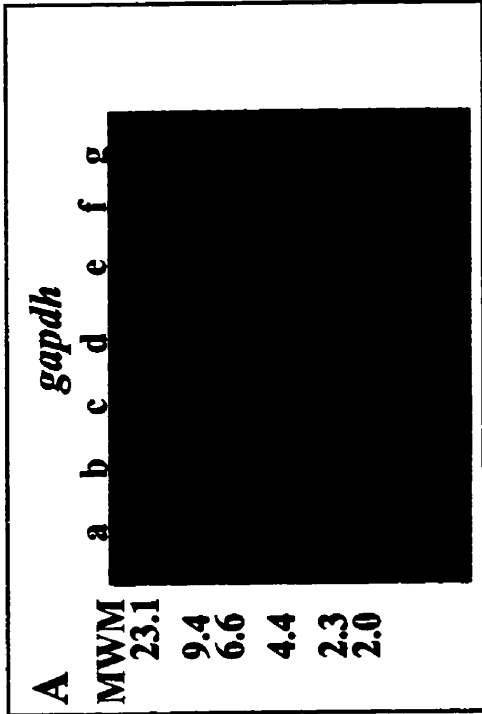
To demonstrate that the cloned *gap*, *pgk*, *pyk* and *zwf* genes were *C. trachomatis* specific; a southern hybridization was done. The primer pairs G5B and G3C, FA and R1B, F1B and RcF4, and R4 and 11B (Table 3) were used for PCR with *C. trachomatis* L2 genomic DNA as template to generate PCR products corresponding to GAPDH, PGK, PK and ZWF ORFs respectively. The PCR product were random primer α-^{32}PATP-labeled and were used individually to probe southern blots of genomic DNA from several sources completely digested with a number of restriction enzymes. Hybridizations were done overnight at 65°C and then subjected to high stringency washing with the last washing steps at 0.1 x SSC, 0.1% SDS at 65°C.

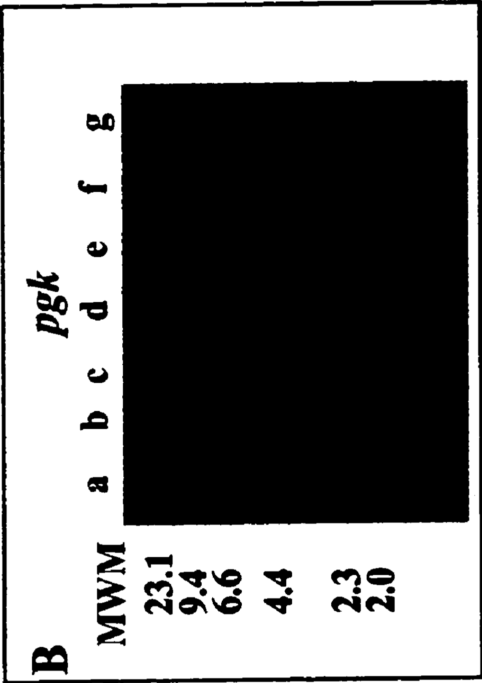
Figure 11 shows the resulting autoradiograms. Southern hybridizations of *Sal*1 digested genomic DNA from *E. coli* XL1-blue, *Acholeplasma laidlawii* (mycoplasma), *C. psittaci* Cal10, *C. psittaci* 6BC (Fig. 11A-D, lanes a-d respectively), and *Sal*1, *Hind*III and *Eco*RI digested genomic DNA from *C. trachomatis L2* (Fig. 11A-D, lanes e-g respectively) were probed with either chlamydial *gap* (Fig. 11A), chlamydial *pgk* (Fig. 11B), chlamydial *pyk* (Fig. 11C), or chlamydial *zwf* (Fig. 11D). Genomic DNA from *E. coli* XL1-blue, *A. laidlawii*, *C. psittaci* Cal10, *C. psittaci* 6BC probed with chlamydial

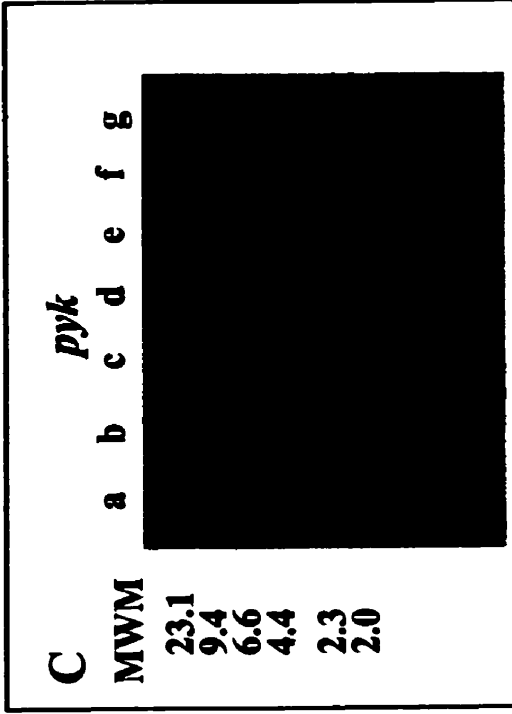
Table 3. Primers used for RT-PCR, southern blot analysis and expression of *C. trachomatis gap, pgk, pyk* and *zwf*

Name of primer	Sequence	Purpose/ *C. trachomatis* gene specificity
5'F1B	222-ATGCTGGAAAAGCTTCTCGATGC-245 [a]	s.b. [z] / *pyk*
3'RcF4	1484-CCATATACACAAGCTTGGTGACG-1462 [b]	s.b.; rt [aa] / *pyk*
5'FcR1	650-TCGATGTTGCTCTTCCTTTCA-670 [c]	rt / *pyk*
5'G5B	378-CTCCCATAGAAGTTGTAGC-397 [d]	s.b.; rt / *gap*
3'G3C	1079-ACAGATAGCCTCGTACG-1063 [e]	s.b.; rt / *gap*
5'FA	433-CTCCTATTGTTCCTGTGC-450 [f]	s.b.; rt / *pgk*
3'R1B	1318-GTACACCCTGCAAGAGCT-1301 [g]	s.b.; rt / *pgk*
5'R4	206-CTCTAGGAAGCTCTTTCCTGC-226 [h]	s.b.; rt / *zwf*
3'11B	1637-GGGCCGCCAAGCTTTTCC-1620 [i]	s.b.; rt / *zwf*
23S5'	221-GGGTTGTAGGATTGAGGA-238 [j]	rt / 23S rRNA
23S3'	1376-GTTTTAGGTGGTGCAGGA-1394 [k]	rt / 23S rRNA
5'EUO	CAACAAGATACAGGGGTC [l]	rt / *euo*
3'EUO	ATTTTCTGCGTCTGCCA [m]	rt / *euo*
5'MOMP	AGTTCTGCTTCCTCCTTG [n]	rt / *ompA*
3'MOMP	GTCTCAACTGTAACTGCG [o]	rt / *ompA*
5'OMP	GCGAGTTTATTTGCTAGCG [p]	rt / *omcB*
3'OMP	AAGTACCACAGTCAGAGC [q]	rt / *omcB*
5SPK	CCCC**GTCGAC**TTCTCCACACCCATTCC [r]	expr. [bb] / *pyk*
3SPK	CCCC**GTCGAC**CAGAAACCCCGGTGAAC [s]	expr. / *pyk*
5SGAP	CCCC**GTCGAC**GATAGAAGAGTCATTGGG [t]	expr. / *gap*
3BGAP	CCCC**GGATCC**AAGGATCTTGGGACAAG [u]	expr. / *gap*
5HPGK	CCCC**AAGCTT**TTTGTTACTGCGCGCAC [v]	expr. / *pgk*
3SPGK	CCCC**GTCGAC**TATCCATTTCCACTCCC [w]	expr. / *pgk*
5SZWF	CCCC**GTCGAC**GGTATTGTGGGCTCTCTC [x]	expr. / *zwf*
3BZWF	CCCC**GGATCC**TCTTCTTGAGAGTCAGCG [y]	expr. / *zwf*

[a-i]Primers are numbered according to the sequence data deposited in GenBank (see experimental procedures).
[j,k]Primers numbered according to sequence data in GenBank (accession # AE001345)
[l-q]Primers were designed according to the *C. trachomatis D* genome project (Stephens *et al*., 1998)(http://chlamydia-www.berkeley.edu:4321/)
[r,s,t,u,v,w,x,y]**Bold** includes *Sal*I [r,s,t,w,x] sites, *Bam*HI[u,y] sites and a *Hind*III[t] site for cloning into pUC-19 and underlined portions correspond to nucleotides 29-45[r] and 1746-1730[s] in GenBank (U83196), 125-142[t] and 1422-1407[u] in GenBank (U83198), 44-60[v] and 1719-1702[w] in Genbank (U83197), and 69-86[x] and 1728-1711[y] in GenBank (U83195).
[z]s.b., southern blot analysis
[aa]rt, RT-PCR
[bb]expr., expression


A
MWM
23.1
9.4
6.6
4.4
2.3
2.0
a b c d e f g
gapdh


B
MWM
23.1
9.4
6.6
4.4
2.3
2.0
a b c d e f g
pgk


C
MWM
23.1
9.4
6.6
4.4
2.3
2.0
a b c d e f g
pyk

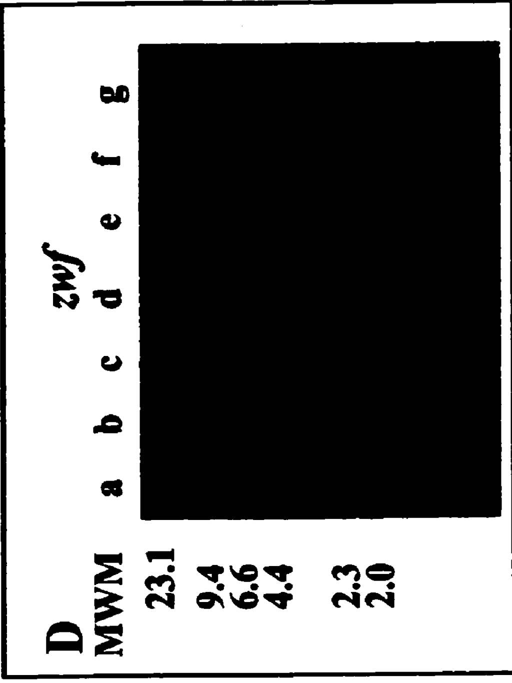

D
MWM
23.1
9.4
6.6
4.4
2.3
2.0
a b c d e f g
zwf

Figure 11. Southern hybridizations using *C. trachomatis* A) *gap*, B) *pgk*, C) *pyk* and D) *zwf* gene sequence as a probe. Two oligonucleotide primers which flanked the chlamydial *gap*, *pgk*, *pyk* or *zwf* genes were used to generate a 701 bp, 885 bp, 1263 bp or 1431 bp DNA fragment by PCR amplification respectively. Each DNA fragment was random primer labeled with α-^{32}P-ATP and used as a probe for the Southern blots. Equivalent amounts of restriction enzyme digested genomic DNA was present in each lane. Hybridization was carried out overnight at 65°C and then subjected to high stringency washing. MWM, molecular weight markers; Lane a, *E. coli* XL1-blue DNA digested with *Sal*1; Lane b, *Acholeplasma laidlawii* DNA digested with *Sal*1; Lane c, *C. psittaci* Cal10 digested with *Sal*1; Lane d, *C. psittaci* 6BC digested with *Sal*1; Lanes e-g, *C. trachomatis* L2 DNA digested with *Sal*1 (e), *Hind*III (f) and *EcoR*I (g).

gap (Fig. 11A, lanes a-d), chlamydial *pgk* (Fig. 11B, lanes a-d), chlamydial *pyk* (Fig. 11C, lanes a-d) and chlamydial *zwf* (Fig. 11D, lanes a-d) showed no binding.

C. trachomatis L2 genomic DNA digested with *Sal1*, *HindIII* or *EcoRI* probed with chlamydial *gap* showed a single band at about 9.3 kb (Fig. 11A, lane e); a double band at about 1.6 kb and 1.3 kb (Fig. 11A, lane f); and a single band at 6.4 kb (Fig. 11A, lane g) respectively upon hybridization. These results correspond to the restriction map determined for pHGAP6 (Fig. 8A).

Figure 11B shows a band at about 10.5 kb (lane e); a band at 6.8 kb (lane f); and a double band at 3.8 kb and 0.44 kb (lane g) when *C. trachomatis* L2 *Sal1*, *HindIII* or *EcoRI* digested DNA is hybridized with the chlamydial *pgk* gene respectively. These results also correspond to the restriction map analysis of pHPGK3 as shown in Fig. 8B.

Hybridization of *C. trachomatis* L2 *Sal1*, *HindIII* or *EcoRI* digested DNA with chlamydial *pyk* is shown in Fig. 11C and results in a single band of 9.1 kb (lane e); three bands at 0.5 kb, 0.6 kb and 0.7 kb and a single band at 1.8 kb (lane e); and a double band at 1.6 kb and at 5.0 kb (lane g) respectively. Like *gap* and *pgk* hybridizations, these results correspond to the restriction map analysis of pHPK1 shown in Fig. 8C.

Finally Fig. 11D, lanes e-g show the results from probing *C. trachomatis* L2 *Sal1*, *HindIII* or *EcoRI* digested DNA with the chlamydial *zwf* which show single bands and correspond to the restriction sites determined from the ZWF nucleotide sequence (Fig. 9). Together, these results indicate that *C.trachomatis* L2 *gap*, *pgk*, *pyk* and *zwf* genes are *C. trachomatis* specific and are present as single copy genes. Furthermore, there was no cross-hybridization with *E. coli*, *Acholeplasma laidlawii*, or *Chlamydia psittaci* DNA.

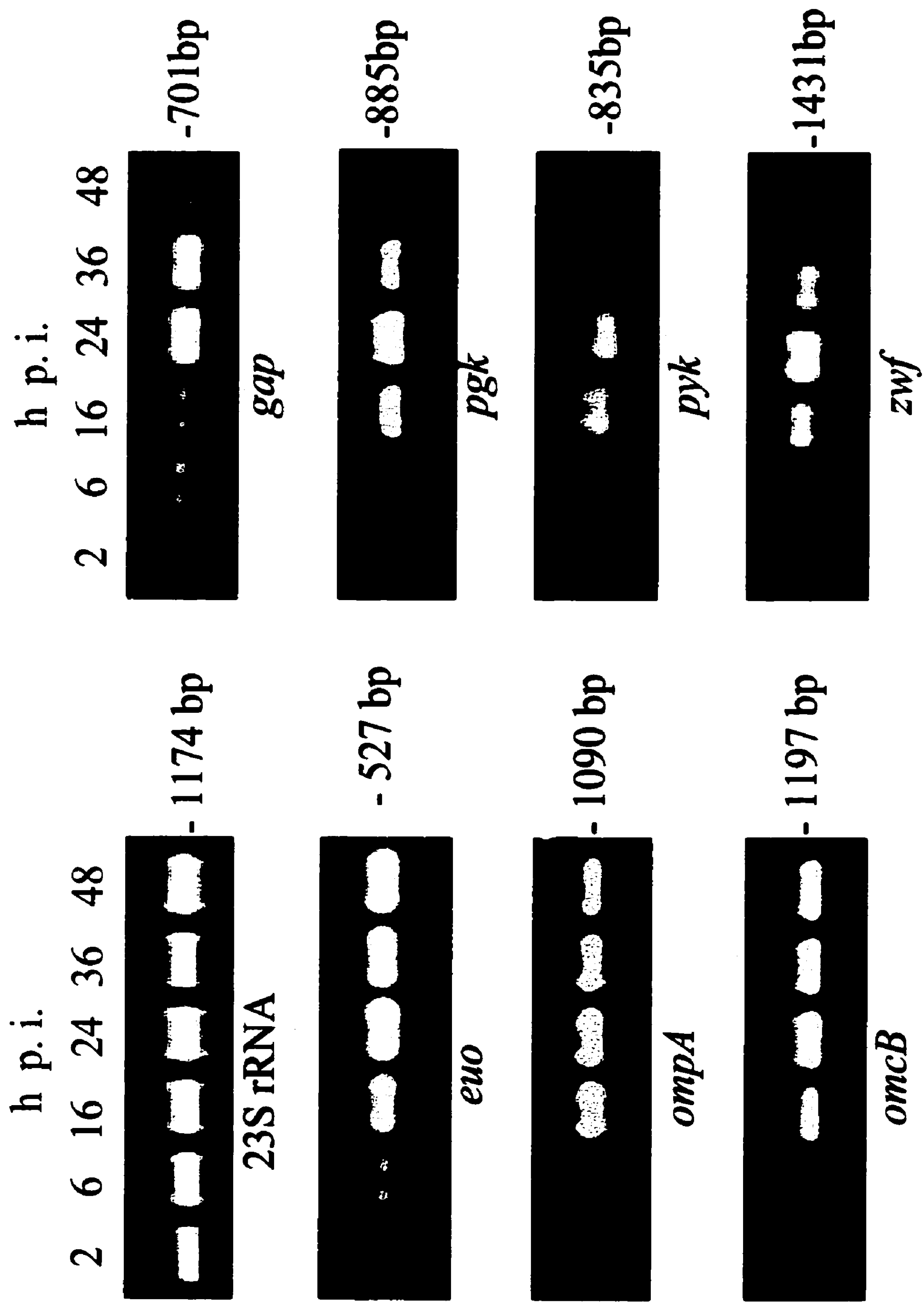
3. Stage-specific expression of *C. trachomatis* L2 *gap*, *pgk*, *pyk* and *zwf* using RT-PCR

To determine whether *C. trachomatis* L2 *gap*, *pgk*, *pyk* and *zwf* transcripts are differentially expressed throughout the chlamydial life cycle, RT-PCR was used (Fig. 12). Total RNA from L2-infected L929 cells was isolated at 2, 6, 16, 24, 36 and 48 h p.i. The RNA was synthesized into total cDNA using reverse transcriptase and random hexamer primers. The cDNA was precipitated and stored as template for PCR reactions. Primers specific and within the coding region of each gene (Table 3) were used for RT-PCR analysis. The PCR reaction was maintained in the linear range of 30 cycles in order to detect any changes in the level of gene expression at the different time points post infection.

Primers specific to chlamydial 23S rRNA were used to detect the presence of chlamydial RNA. The amount of cDNA used as template at each time point was adjusted so that the 23S rRNA PCR products were of similar intensity when run on an agarose gel (Fig. 12). This amount of cDNA was kept constant for subsequent reactions. In addition, RNA samples from each time point were subjected to PCR minus the reverse transcription step to ensure that contaminating genomic DNA was not being amplified in the PCR reaction (data not shown).

EUO, a protein known to be expressed early in the chlamydial life cycle (Wichlan and Hatch, 1993), was detected as early as 2 h p.i. Transcript for *ompA*, a constitutively expressed gene which encodes for MOMP (Stephens, 1988), was first detected at 6 h p.i., reached a peak at approximately 16-24 h p.i., and declined thereafter. The pattern of

MOMP expression is not unexpected, especially at the peak region, given that this is the time when the majority of the organisms are in the metabolically active RB stage. As previously reported, the expression of *omcB* which encodes for a 60 kDa cysteine-rich outer membrane protein (CRP) present only in EBs (Hackstadt *et al.*, 1985), is detected at 16 h p.i. and later (Hatch *et al.*, 1986). Results shown in Figure 12 indicate that transcription of *gap*, *pgk*, *pyk* and *zwf* is delayed relative to EUO, and like MOMP, peak at approximately 24 h p.i., and decline thereafter when fewer RBs are undergoing division and are beginning to differentiate back into EBs.



h p.i.
2 6 16 24 36 48
gap
-701bp
pgk
-885bp
pyk
-835bp
zwf
-1431bp
h p.i.
2 6 16 24 36 48
23S rRNA
- 1174 bp
euo
- 527 bp
ompA
- 1090 bp
omcB
- 1197 bp

Figure 12. RT-PCR analysis of total RNA extracted from chlamydiae infected cells at different time points in the chlamydial life cycle. Each lane contains RNA samples subjected to RT-PCR analysis. Time points indicate the number of hours post-infection at which the RNA sample was isolated. Primers employed are shown in Table 3. RT-PCR using 23S rRNA primers, *euo* primers, *ompA* primers, *omcB* primers, *gap* primers, *pgk* primers, *pyk* primers and *zwf* primers are shown. The size of each band is shown in base pairs (bp).

4. Complementation Studies

To determine if the putative *gap*, *pgk*, *pyk* and *zwf* genes encode active enzymes; genetic complementation experiments were performed. Genotypes of the *E. coli* strains as well as plasmids used for the complementation and enzyme studies are shown in Table 4. Each strain was made competent as described in "Materials and Methods". *E. coli* contains two genes encoding for GAPDH, *gap*A and *gap*B. It has previously been shown that *E. coli* strain DS112, which contains a chloramphenicol resistance cassette inserted into *gap*A, has little GAPDH activity, suggesting that *gap*A is responsible for the majority of GAPDH activity detected in wild type *E. coli* (Seta *et al.*, 1997). In addition, strain DS112 has been shown to grow on glycerol supplemented with succinate or malate but is unable to grow on glucose or glycerol as the only carbon source (Seta *et al.*, 1997). DS112 was transformed with pCTGAPDH and was plated on glucose. Results demonstrate that the chlamydial *gap* gene is capable of complementing the mutation in strain DS112 (Table 5).

E. coli strain DF264 was used for PGK complementation studies. DF264 contains a point mutation in the only copy of the *pgk* gene rendering it inactive (Thomson *et al.*, 1979). Thomson *et al.* (1979) have previously demonstrated that DF264 does not grow on glucose but grows on minimal medium supplemented with glycerol and malate. Growth complementation experiments indicated that the chlamydial pgk gene (Table 5) complements the mutation in strain DF264.

Similar to *gap* genes, *E. coli* contains two genes encoding for PK, *pyk*F (*pyk*I) and *pyk*A (*pyk*II). *E. coli* strain PB25 contains a kanamycin resistant cassette inserted into

Table 4. Bacterial strains and plasmids used for *C. trachomatis gap, pgk, pyk* and *zwf* complementation and enzyme studies.

Strain or plasmid	Relevent genotype/comment	Source/reference
C. trachomatis L2	434/Bu	(Tipples and McClarty, 1995)
E. coli		
MC1061	*hsdR2 hsdM+ hsdS+ araD139* Δ*(ara-leu)*$_{7697}$$\Delta$*(lac)*$_{X74}$*galE15 galK16 rpsL (Str^r) mcrA mcrB1*	R. Brunham
DH5α	supE44Δ*lacU169 (ϕ80lacZ*Δ*M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*)	(Wylie *et al.*, 1996)
BL21	(DE3) *(hsdS gal λclts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)*	(Wylie *et al.*, 1996)
DS112	*(K-12, F$^-$λ$^-$*Δ*gapA::Cm)*	(Seta *et al.*, 1997)
DF264	*garB10 fhuA22 ompF627 fadL701(*T_2^R*) relA1 zgf-210::Tn10 pgk-2 pit-10 spoT1 rrnB-2 mcrB1 creC510)*	(Thomson *et al.*,1979)
PB25	*supE thi* Δ*(lac-proAB) (F' traD36 proAB lacI^{q}Z*Δ*M15)*Δ*pykA::kan pykF::cat*	(Ponce *et al.*, 1995)
DF2000	*garB10 fhuA22 ompF627(*T_2^R*) zwf-2fadL 701(*T_2^R*) relA1 pit-1spoT1 rrnB-2 pgi-2* mcr B1 creC510	(Fraenkel, 1968)
Plasmids		
Molecular cloning		
pHGAP6	Contains the *C. trachomatis* L2 *gap* gene fragment in pUC19 (~8.0 kb)	This work
pHPGK3	Contains the *C. trachomatis* L2 *pgk* gene fragment in pUC19 (~6.8 kb)	This work
pHPK1	Contains the *C. trachomatis* L2 *pyk* gene fragment in pUC19 (~5.4 kb)	This work
pH11	Contains the *C. trachomatis* L2 *zwf* gene in pUC19 (~2.0 kb)	(Tipples and McClarty, unpublished)
Enzyme Analysis		
pCTGAPDH	Contains the *C. trachomatis* L2 *gap* gene in pUC-19 (~1.6 kb)	This work
pCTPGK	Contains the *C. trachomatis* L2 *pgk* gene in pUC-19 (~1.9 kb)	This work
pCTPK	Contains the *C. trachomatis* L2 *pyk* gene in pUC-19 (~1.8 kb)	This work
pCTZWF	Contains the *C. trachomatis* L2 *zwf* gene in pUC-19 (~2 kb)	This work

Table 5: Complementation by mutant *E. coli* strains[a] by *C. trachomatis* L2 DNA inserts

E. coli Strain	Growth on carbon source			
	Glucose	Ribose	Gluconate	Malate and Glycerol
DS112 (Δ*gap*A)	no	ND	ND	yes
DS112-pUC19	no	ND	ND	yes
DS112-pCTGAPDH	**yes**	ND	ND	yes
DF264 (*pgk*-)	no	ND	ND	yes
DF264-pUC19	no	ND	ND	yes
DF264-pCTPGK	**yes**	ND	ND	yes
PB25 (Δ*pyk*A Δ*pyk*F)	yes	no	yes	ND[b]
PB25-pUC19	yes	no	yes	ND
PB25-pCTPK	yes	**yes**	yes	ND
DF2000 (*zwf*-)	no	ND	yes	ND
DF2000-pUC19	no	ND	yes	ND
DF2000-pCTZWF	**yes**	ND	yes	ND

[a]*E. coli* strains carrying the indicated plasmids were grown in M63 minimal medium containing the appropriate antibiotics supplemented with either 10 mM glucose, 10 mM gluconate, 15 mM ribose, or 12.5 mM glycerol and 25 mM malate.
[b]ND, not done.

*pyk*A and a chloramphenicol resistant cassette inserted into *pyk*F (Ponce *et al*., 1995). It has been previously demonstrated that strain PB25 lacks PK activity and is unable to grow on ribose as the sole carbon source but can grow on medium containing glucose or glycerol (Ponce *et al*., 1995). Complementation experiments indicated that the chlamydial *pyk* gene is capable of complementing the mutations in strain PB25 (Table 5).

E. coli also contains a single gene, *zwf*, encoding for glucose-6-phosphate dehydrogenase. *E. coli* strain DF2000 contains a point mutation in the *zwf* gene and in the

pgi (phosphoglucose isomerase) gene, making it incapable of growing on glucose. *E. coli* DF2000 transformed with the chlamydial *zwf* gene (pCTZWF) is capable of growing on glucose selective medium (Table 5). The results from the complementation studies indicate that *C. trachomatis* GAPDH, PGK, PK and ZWF encoding genes are able to complement the respective mutant *E. coli* strains suggesting these genes encode for active enzymes.

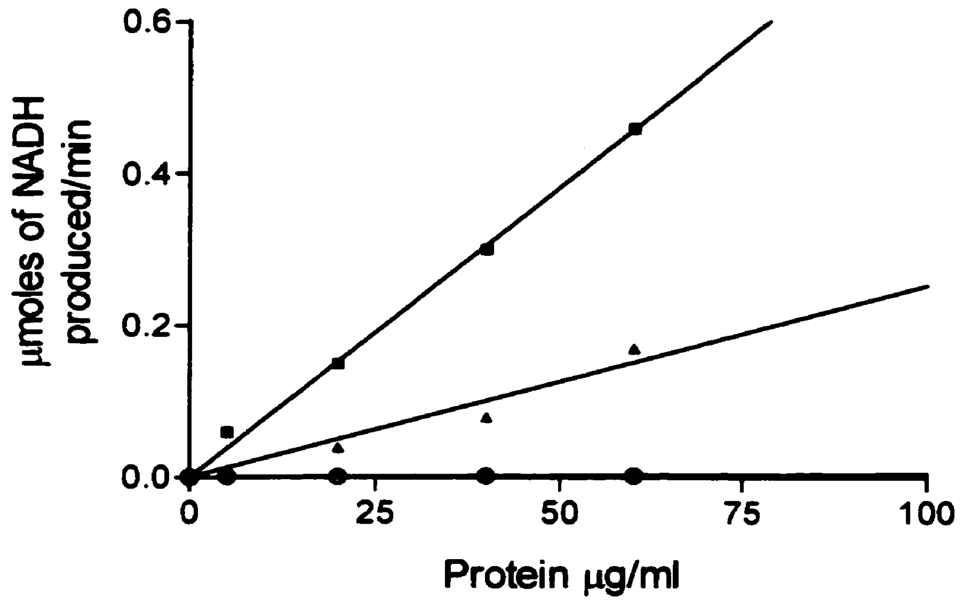
5. *In vitro* enzyme analysis of *C. trachomatis* GAPDH, PGK, PK and ZWF recombinant enzymes

To further demonstrate that *C. trachomatis* L2 encodes for functional GAPDH, PGK, PK and ZWF proteins, *in vitro* enzyme assays were performed. Assay conditions and cellular extract prepared for *in vitro* GAPDH, PGK, PK and ZWF enzyme assays are described in "Materials and Methods". These enzymes have been well studied in *E. coli* and therefore extract prepared from *E. coli* BL21 was used as a positive control where as extracts prepared from DS211 ± pUC19, DF264 ± pUC19, PB25± pUC19 or DF2000 ± pUC19 were used as negative controls.

For each assay, the effect of protein concentration and incubation time on enzyme activity was done in order to optimize the conditions for the various assays (Figs. 13-16). Results presented in Fig. 13A indicate that GAPDH assays are linear with respect to increasing protein concentrations from crude extract prepared from DS112 containing pCTGAP or from BL21. Similarly, Fig. 13B shows that GAPDH activity was linear and then levels out with respect to incubation time from assays containing DS112-pCTGAP

or BL21 extracts. As expected no activity was observed regardless of the amount of protein added or length of incubation from assays containing the DS112 or DS112-pUC19 extract (Fig. 13). Like GAPDH, PGK (Fig. 14), PK (Fig. 15) and ZWF assays (Fig. 16) containing crude extracts from either DF264-pCTPGK, PB25-pCTPK or DF2000-pCTZWF respectively or from BL21 were also linear with respect to protein concentration and length of incubation. Similarly, PGK, PK or ZWF assays which contained the negative control extracts DF264 and DF264-pUC19; PB25 and PB25-pUC19; or DF2000 and DF2000-pUC19 lacked either PGK, PK or ZWF activity respectively. These preliminary experiments allowed for an estimation of optimal GAPDH, activity to occur at extract containing 20 μg protein and an incubation time of 3 minutes. For PGK, PK and ZWF assays, optimal activity was estimated to occur at extract containing 20 μg protein and at an incubation time of 5 minutes. All subsequent assays were performed in triplicate using these optimal conditions.

A)



B)

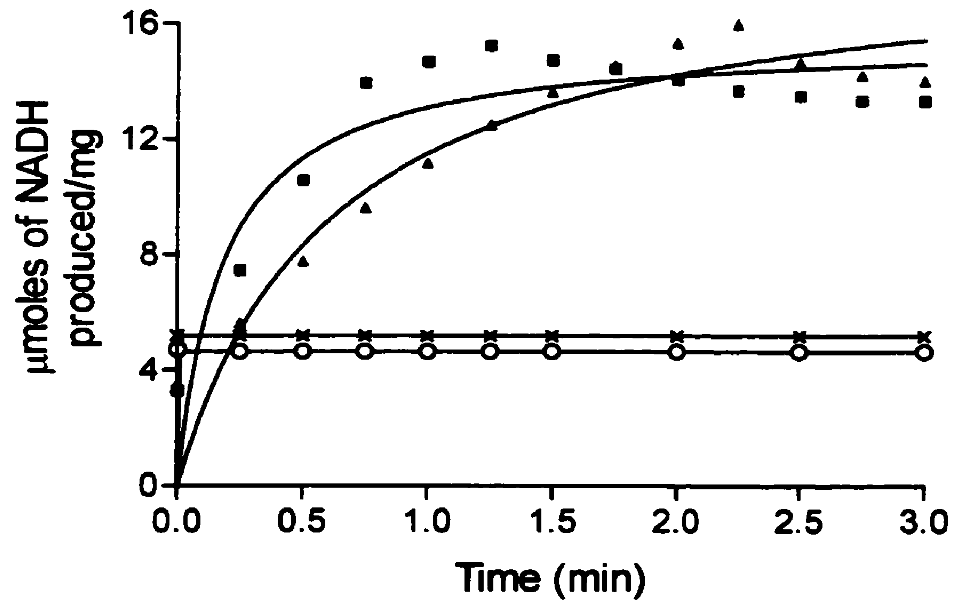
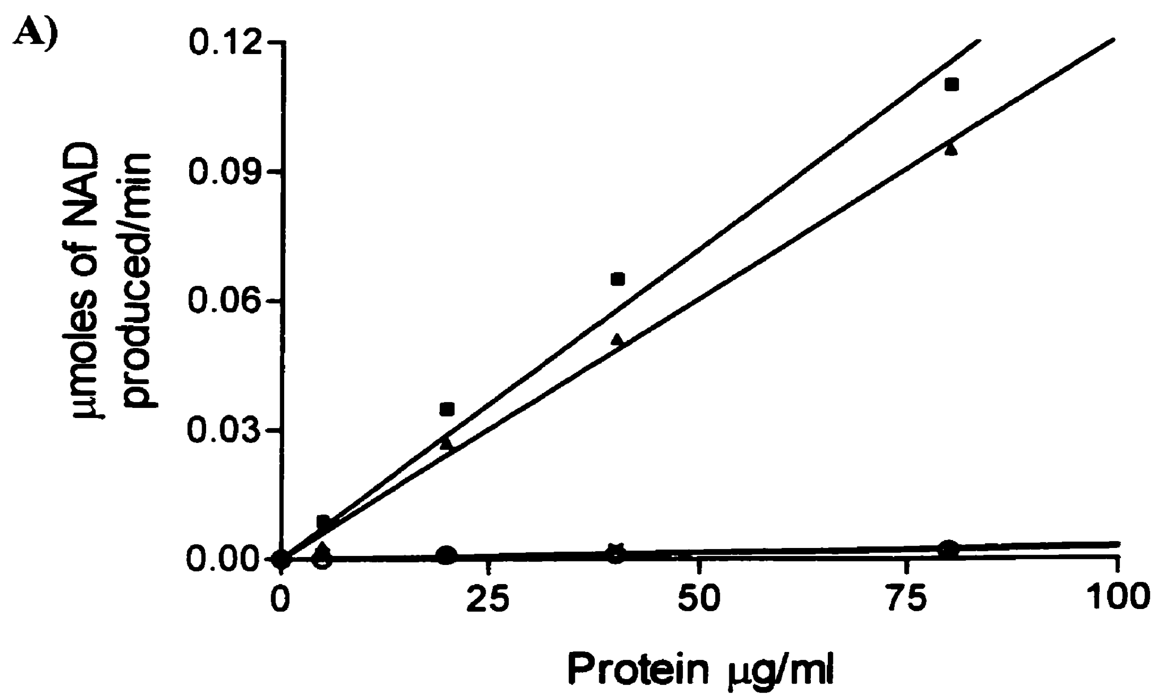


Figure 13. Optimization of *in vitro* GAPDH assay: NADH formation in the presence of A) increasing protein concentrations and B) increasing incubation time. The pre-reaction mixture contained: 40 mM triethanolamine-HCl pH 7.5, 2.0 mM EDTA pH 8.0, 50 mM K_2HPO_4, 1 mM NAD for a final volume of 1 ml. A) The indicated amount of protein extract [(■) DS112-pCTGAP; (▲) BL21; (✕) DS112; (O) DS112-pUC19] was added and the reaction was started with the addition of 1 mM glyceraldehyde-3-phosphate (G3P). B) 20 µg of extract [(■) DS112-pCTGAP; (▲) BL21; (✕) DS112; (O) DS112-pUC19] was added and the reaction was followed until the time indicated. Samples were measured in a spectrophotometer at a wavelength of 340 nm for 3 minutes. Each assay was done in duplicate with results varying less than 10%.


A)
0.12
0.09
0.06
0.03
0.00
µmoles of NAD produced/min
0
25
50
75
100
Protein µg/ml

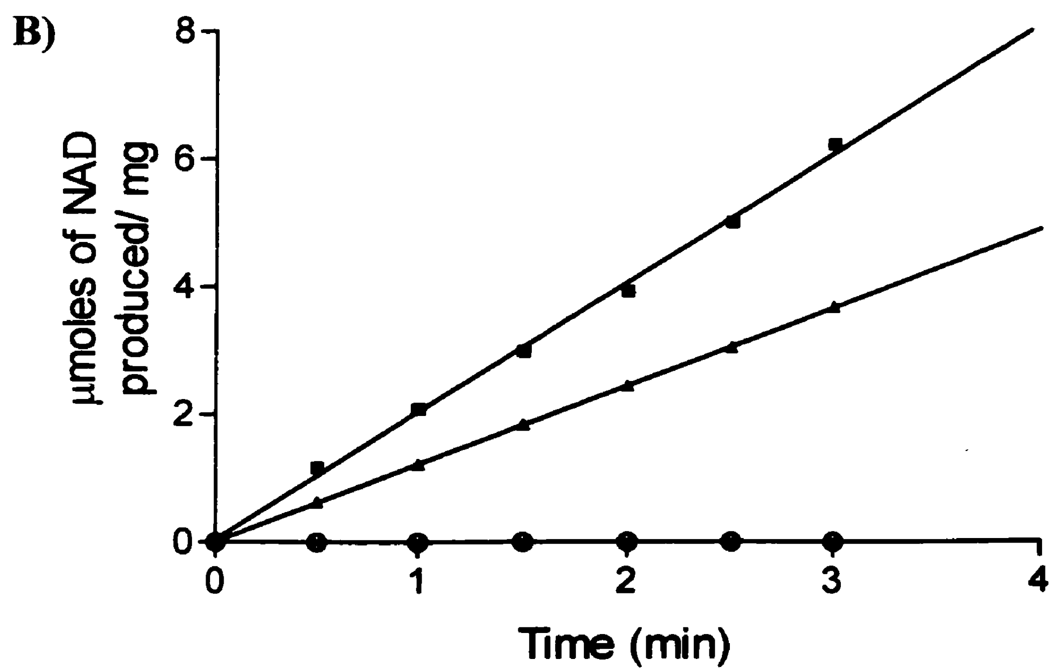
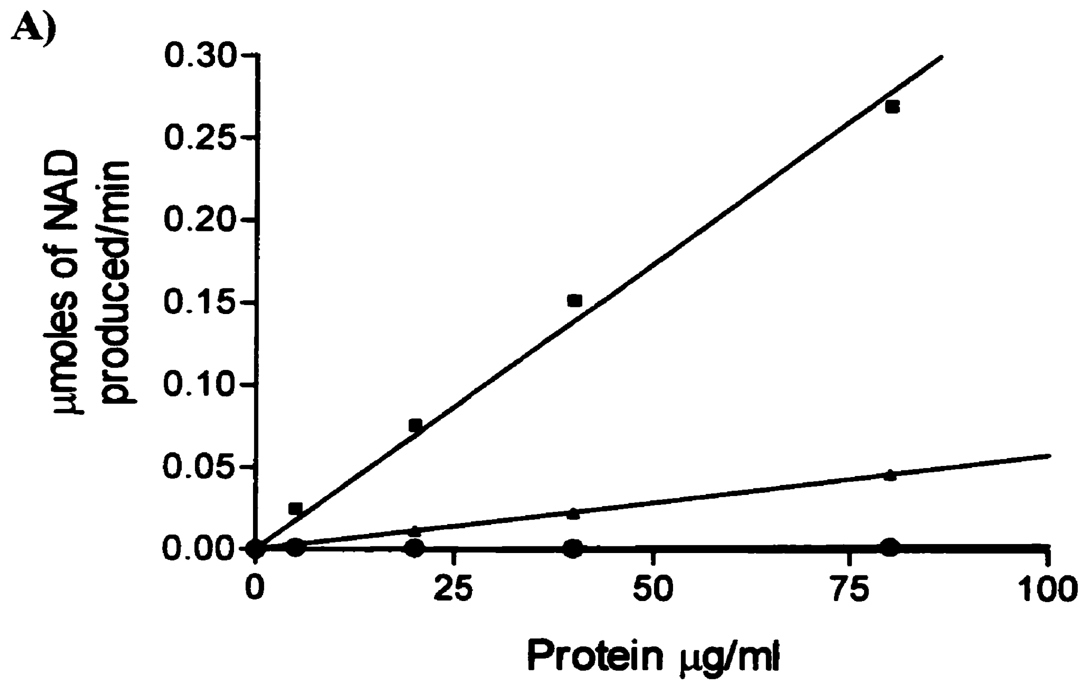

B)
8
6
4
2
0
µmoles of NAD produced/ mg
0
1
2
3
4
Time (min)

Figure 14. Optimization of *in vitro* PGK assay: NAD formation in the presence of A) increasing protein concentrations and B) increasing incubation time. The pre-reaction mixture contained: 40 mM triethanolamine-HCl pH 7.5, 5.0 mM $MgCl_2$, 0.2 mM EDTA pH 8.0, 30 mM $(NH_4)_2SO_2$, 100 mM NaCl, 2 mM ATP, 3.45 units of glyceraldehyde-3-phosphate dehydrogenase, 0.2 mM NADH for a final volume of 1 ml. A) The indicated amount of protein extract [(■) DF264-pCTPGK; (▲) BL21; (✕) DF264; (O) DF264-pUC19] was added and the reaction was started with the addition of 5 mM 3-phosphoglyceraldehyde (3PGA) and measured by a spectrophotometer at a wavelength of 340 nm for 5 minutes. B) 20 µg of extract [(■) DF264-pCTPGK; (▲) BL21; (✕) DF264; (O) DF264-pUC19] was added and the reaction was followed until the time indicated. Each assay was done in duplicate with results varying less than 10%.


A)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
μmoles of NAD produced/min
0
25
50
75
100
Protein μg/ml

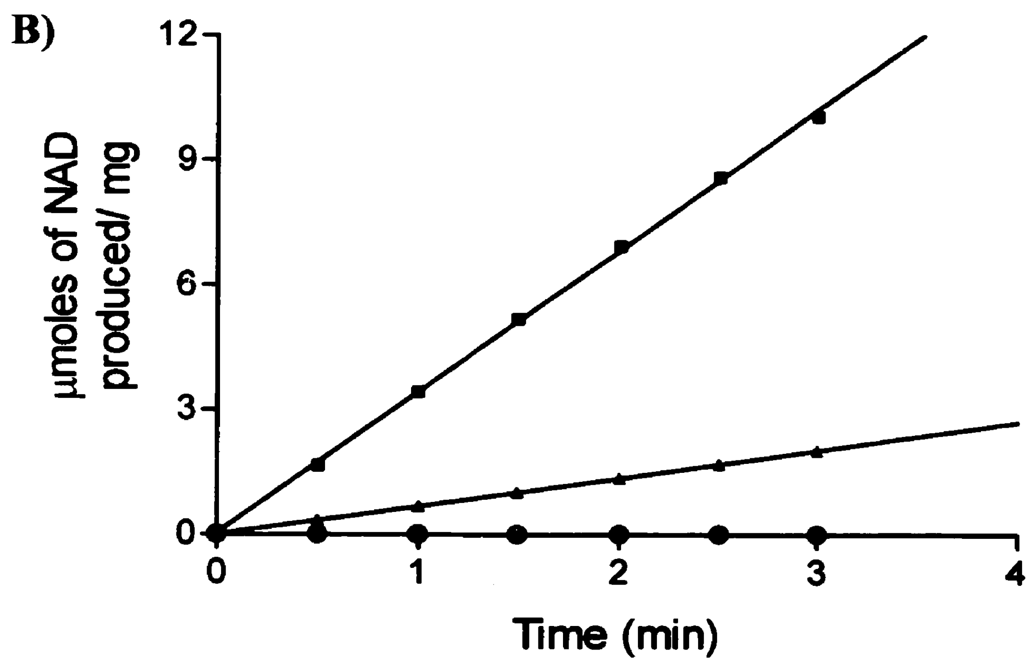
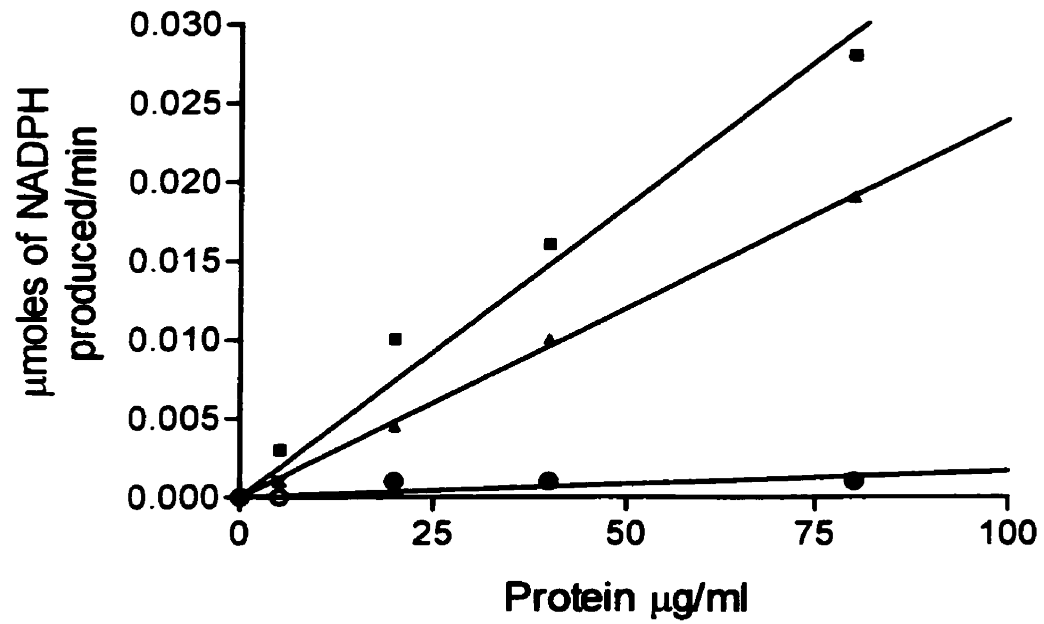

B)
12
9
6
3
0
μmoles of NAD produced/ mg
0
1
2
3
4
Time (min)

Figure 15. Optimization of *in vitro* PK assay: NAD formation in the presence of A) increasing protein concentrations and B) increasing incubation time. The pre-reaction mixture contained: 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 2 mM ADP, 0.2 mM NADH and 10 units of lactate dehydrogenase for a final volume of 1 ml. A) The indicated amount of protein extract [(■) PB25-pCTPK; (▲) BL21; (✕) PB25; (O) PB25-pUC19] was added and the reaction was started with the addition of 10 mM phosphoenolpyruvate (PEP). B) 20 μg of extract [(■) PB25-pCTPK; (▲) BL21; (✕) PB25; (O) PB25-pUC19] was added and the reaction was followed until the time indicated. Samples were measured in a spectrophotometer at a wavelength of 340 nm for 3 minutes. Each assay was done in duplicate with results varying less than 10%.

A)



B)

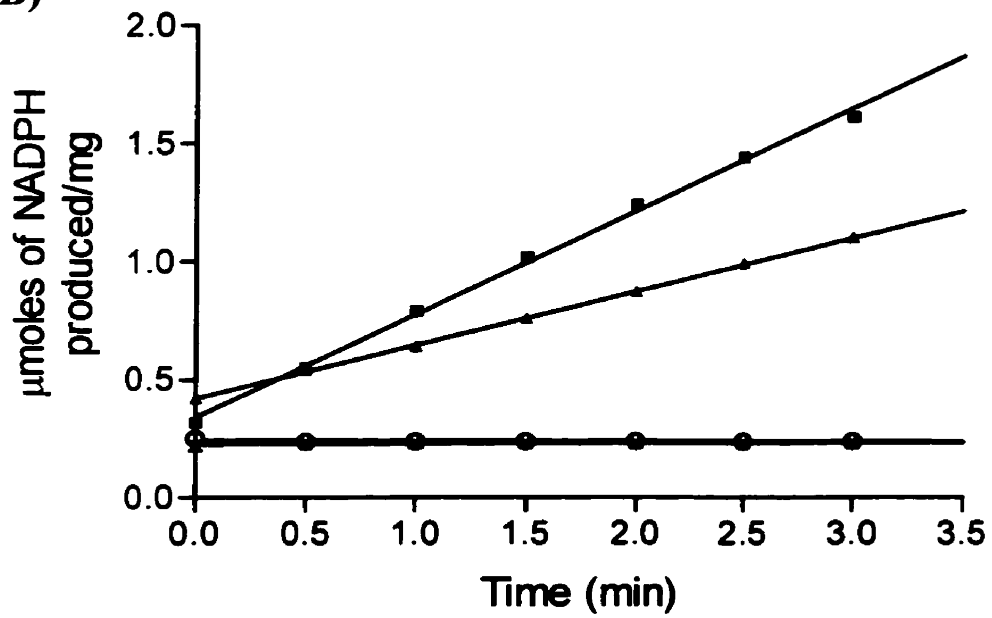


Figure 16. Optimization of *in vitro* ZWF assay: A) NADPH formation in the presence of A) increasing protein concentrations and B) increasing incubation time. The pre-reaction mixture contained: 100 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 0.2 mM NADP and 10 units of lactate dehydrogenase for a final volume of 1 ml. A) The indicated amount of protein extract [(■) DF2000-pCTZWF; (▲) BL21; (✕) DF2000; (O) DF2000-pUC19] was added and the reaction was started with the addition of 1 mM glucose-6-phosphate. The reaction was followed spectrophotometrically for 5 minutes at a wavelength of 340 nm. B) 20 μg of extract [(■) DF2000-pCTZWF; (▲) BL21; (✕) DF2000; (O) DF2000-pUC19] was added and the reaction was followed until the time indicated. Each assay was done in duplicate with results varying less than 10%.

The results from the *in vitro* GAPDH, PGK, PK and ZWF enzyme assays are shown in Table 6. Extract prepared from *E. coli* BL21 was found to have a level of GAPDH activity comparable to that reported by others (Irani and Maitra, 1977; Maitra and Lobo, 1971; Seta *et al.*, 1997). Extract prepared from the GAPDH mutant strain DS112 or strain DS112 containing pUC19 showed a very low level of activity which is in agreement with others (Seta *et al.*, 1997). Extract prepared from strain DS112 containing pCTGAPDH showed a high level of activity demonstrating that the recombinant chlamydial GAPDH is active. Similarly, extract prepared from *E. coli* BL21 showed PGK activity, whereas no PGK activity was detected in DF264 or DF264-pUC19 extracts. Extract prepared from *E. coli* strain DF264 containing pCTPGK showed PGK activity.

For PK enzyme assays, cellular extract prepared from the positive control *E. coli* BL21 was found to have a level of PK activity comparable to other findings (Pertierra and Cooper, 1977; Somani *et al.*, 1977; Valentini *et al.*, 1979). Extracts prepared from the PK mutant *E. coli* strain PB25 and strain PB25 containing pUC19, showed no detectable PK activity which is in agreement with Ponce *et. al.* (1995). In contrast, extract prepared from PB25 containing pCTPK, expressing recombinant chlamydial PK, showed a high level of PK activity.

E. coli PKF is known to be allosterically activated by fructose 1,6 bisphosphate (F16BP) (Malcovati and Valentini, 1982; Waygood *et al.*, 1976). In keeping with this, we found that the addition of F16BP increases *E. coli* PK activity. In contrast, addition of F16BP did not result in an increase of recombinant chlamydial PK activity (Table 6).

Table 6. GAPDH, PGK, PK and ZWF activity in crude extracts prepared from *E. coli* strains[a]

E. coli Strain[b]	GAPDH[c]	PGK[d]	PK[e]	PK + AMP[f]	PK +FBP[g]	ZWF[h]
BL21	3.60 ± 0.85	1.23 ± 0.65	0.54 ± 0.09	0.66 ± 0.04	1.26 ± 0.29	0.24 ± 0.04
DS112 (Δ*gapA*)	<0.01	1.78 ± 0.73	ND	ND	ND	ND
DS112-pUC19	<0.01	1.21 ± 0.48	ND	ND	ND	ND
DS112-pCTGAP	13.40 ± 0.83	ND	ND	ND	ND	ND
DF264 (*pgk-*)	6.30 ± 0.58	<0.01	ND	ND	ND	ND
DF264-pUC19	5.67 ± 0.73	<0.01	ND	ND	ND	ND
DF264-pCTPGK	3.64 ± 0.82	2.20 ± 0.57	ND	ND	ND	ND
PB25(Δ*pykA*Δ*pykF*)	4.80 ± 0.37	4.53 ± 0.43	<0.01	<0.01	<0.01	<0.01
PB25-pUC19	5.70 ± 0.45	ND	<0.01	<0.01	<0.01	<0.01
PB25-pCTPK	ND	ND	3.47 ± 0.61	3.09 ± 0.31	2.02 ± 0.55	0.47 ± 0.13
DF2000 (*zwf-*)	5.88 ± 0.63	1.70 ± 0.13	ND	ND	ND	ND
DF2000-pUC19	6.35 ± 0.48	2.30 ± 0.31	ND	ND	ND	ND
DF2000-pCTZWF	ND	ND	ND	ND	ND	ND

[a]Specific activity values are presented as the average of three independent measurements expressed in micromole per mg per minute ± S.D. Cultures are grown aerobically for 36 h in M63 medium supplemented with either malate and glycerol (PGK & GAP), glucose (PK), or gluconate (ZWF) as carbon source.

[b]The *E. coli* strains carrying the indicated plasmids were used for extract preparation.

[c]GAPDH activity was determined at 25°C in 40 mM triethanolamine-HCl pH 7.5 buffer containing 2.0 mM EDTA pH 8.0, 1 mM NAD and 50 mM K_2HPO_4. The reaction was started by the addition of 1 mM G-3-P.

[d]PGK activity was determined at 25°C in 30mM triethanolamine-HCl pH 7.5 buffer containing 5mM $MgCl_2$, 0.2mM EDTA, 30mM $(NH_4)_2SO_4$, 100 mM NaCl, 2 mM ATP, and 0.2 mM NADH. The reaction was starting by the addition of 5 mM 3-PGA.

[e]PK activity was determined at 25°C in 10 mM Tris pH 7.5 buffer containing 10 mM $MgCl_2$, 50 mM KCl, 2 mM ADP, 0.2 mM NAD and 10 units of lactate dehydrogenase. The reaction was started with the addition of 10 mM PEP.

[f]PK activity was determined as in c, with the addition of 1 mM FBP along with PEP to start the reaction.

[g]PK activity was determined as in c, with the addition of 1 mM AMP along with PEP to start the reaction.

[h]ZWF activity was determined at 25°C in 100 mM Tris-HCl pH 7.6 buffer containing 10 mM $MgCl_2$ and 0.2 mM NADP. The reaction was started with the addition of 1 mM G-6-P.

[i]The sensitivity of the assays are 0.01 $\mu mol\ min^{-1}\ mg^{-1}$.

[j]ND, not done.

E. coli PKA is known to be activated by AMP and some sugar monophosphates such as ribose-5-phosphate (Malcovati and Valentini, 1982; Waygood *et al.*, 1975). We found that AMP had no significant effect on chlamydial PK activity prepared from crude extract.

For the ZWF assay, extract prepared from strain DF2000 or from strain DF2000 containing pUC19 showed no detectable activity under our assay conditions, however, extract prepared from strain DF2000 containing pCTZWF showed ZWF activity. As an additional control, the negative control extracts were also assayed for an additional enzyme to ensure the prepared extracts were functional (Table 6). Together these results indicate that *C. trachomatis* encodes for functional GAPDH, PGK, PK and ZWF enzymes.

6. Cloning and characterization of *C. trachomatis* L2 *pfpA* and *pfpB*

a) Cloning and sequence analysis of *C. trachomatis* L2 *pfpA* and *pfpB*

Information from the genome sequencing project indicates that *C. trachomatis* does not contain a typical eubacterial *pfk*, rather it contains two genes, *pfpA* and *pfpB* which show high homology to PPi-PFKs. As mentioned, PPi-PFK catalyzes the same reaction as PFK, except that it uses PPi instead of ATP, and it is reversible. We used the information from the genome sequencing project to generate primers in order to clone *C. trachomatis* L2 *pfpA* and *pfpB* (see "Materials and Methods" for details).

The complete nucleotide and deduced amino acid sequences of *C. trachomatis* L2 *pfpA* and *pfpB* are shown in Fig. 17. The nucleotide sequences were confirmed by cycle sequencing both strands of the double-stranded DNA. Open reading frames (ORFs) were then compared with the translated GenBank database and were found to show >95% identity to the *C. trachomatis* D *pfpA* and *pfpB* genes respectively. Characteristics of the cloned ORFs are summarized in Table 7. The amino acid sequences of the putative *C. trachomatis* L2 PFPA and PFPB ORFs, show the highest homology (excluding *C. trachomatis* D), to *Spirochaeta thermophilia* PFP (~40%). *C. trachomatis pfpA* and *pfpB* amino acid sequences show about 35% identity to each another. Alignments of the deduced amino acid sequences of *C. trachomatis* L2 PFPA and PFPB, *Treponema pallidum* PFP, *Entamoeba histolytica* PFP, *Ricinus communis* PFPA and PFPB, *E. coli* PFKA and Human PFKL enzymes are shown in Figure 18. Interestingly, *C. trachomatis* PFPA and PFPB amino acid sequences show higher homology to *R. communis* PFPB (the catalytic subunit) than *R. communis* PFPA (the regulatory subunit) suggesting that both genes may encode for catalytic enzymes (Table 7).

Although sequence analysis indicates that the PPi-PFK family differs substantially from the ATP-PFK family, most of the active sites in the amino-terminal half are conserved between these two families (Ding *et al.*, 2000). Several PFK crystallographic, site-directed mutagenic and other studies have identified a number of catalytically important residues (Auzat *et al.*, 1994; Evans and Hudson, 1979; Poorman *et al.*, 1984; Rypniewski and Evans, 1989; Shirakihara and Evans, 1988). The MGR motif (residues 169-171, *E. coli* PFK numbering) is found in all PPi-PFK and in most ATP-PFK sequences and has been implicated in F6P binding (Shirakihara and Evans, 1988). The R

Figure 17. Nucleotide and deduced amino acid sequence of *C. trachomatis* L2 A) pyrophosphate dependent phosphofructose kinase gene A (*pfpA*) and B) pyrophosphate dependent phosphofructose kinase gene B (*pfpB*). The start (atg/methionine {M}) and stop codons (taa, tga, tag) in each nucleotide sequence is highlighted. The first and last residues in the amino acid sequences are marked in bold.

A) CTPFPA nucleotide sequence

1 **atg**tcgtcga ataaacatgc ttctctttgt caaaagacgc cttctttgtg tcgggagctt

61 caaaaagctc ctgctcttct attaacagaa gacataaggt ttaaagctct tcttaatgaa

121 cgcattgact ctgttgcaga actatttcca tgcacttata actctcccta ctacaaattt

181 atttcgaagt ccgatctttc cgctgagacc tttcccctta aagtgggcgt tatgctttct

241 ggaggcccag ctcctggtgg gcacaatgtc atcttaggat tgctacacag tattaaaaag

301 ctccatccga atagtcagct tttaggattt attcgcaatg gagaaggact tctcaataat

361 aatactgtag aaatcacaga tgaattcatt gaagagtttc gtaactctgg aggctttaat

421 tgcataggaa caggtcgcac taatatcata accgaagaaa acaaagcgcg ctgtttacaa

481 acagcaaatg aactcgattt agatggatta gtgattattg gaggcgatgg ttcgaataca

541 gccacggcga ttcttgctga atattttgct aagcatcaag caaaaacggt attagttggt

601 gttcctaaaa ctattgatgg agatttgcag cacctatttt tagacctcac atttgggttt

661 gatactgcta ctaaatttta ttcatccatc atcagcaaca tttctagaga cgcattatcg

721 tgtaaaggcc actatcattt tattaaacta atgggccggt cttcttctca tatcacgcta

781 gaatgcgcac tacagactca cccaaatatt gctcttatag gcgaagagat tgcagaaaaa

841 agcatctcct tagaaacatt aatccatgat atttgtgaaa caatagcaga tcgagctgct

901 atgggggaaat accatggcgt tattctcatc cctgaaggag tcattgagtt tattcctgaa

961 atacagtctc tggttaaaga aattgaatcc attccagagc aggagaatct ttaccaagct

1021 ttatccttat cttctcagca acttttatgc caatttccgg aagatatttg ccatcagctc

1081 ttgtataata gagatgctca tggcaacgtc tatgtatcaa aaattagtgt tgataaactt

1141 ctgattcatc tagttcgtca acatttagaa acacatttta gacaagttcc cttcaatgca

1201 atctcccatt ttttaggtta tgaagggcgt tcaggaactc ctacacattt tgataatgtg

1261 tatagctata acttaggata tggtgctggg gttctcgttt ttaaccgctg taatgggtat

1321 ttatccacga tcgaaggtct aactagccct attgaaaaat ggcgattgcg cgctttaccc

1381 attgttcgaa tgttgacgac caagcagggg aaagacagta aacattatcc tctgataaaa

1441 aaaagattgg tagatattgc tagtcctgtt tttaataagt tctcactgta tcggaaaatc

1501 tgggctttag aagactccta tcgctttgta gggccattac aaatacattc tccggaggat

1561 gctcattctg atgattttcc tcctttaatt ttgtttttga atcataatga atggcaaaaa

1621 cgctgttcta tttgtttaga aatccccgat caggattat**t aa**

Fig. 17A

CTPFPA Deduced Amino Acid Sequence

1 **M**SSNKHASLC QKTPSLCREL QKAPALLLTE DIRFKALLNE RIDSVAELFP CTYNSPYYKF

61 ISKSDLSAET FPLKVGVMLS GGPAPGGHNV ILGLLHSIKK LHPNSQLLGF IRNGEGLLNN

121 NTVEITDEFI EEFRNSGGFN CIGTGRTNII TEENKARCLQ TANELDLDGL VIIGGDGSNT

181 ATAILAEYFA KHQAKTVLVG VPKTIDGDLQ HLFLDLTFGF DTATKFYSSI ISNISRDALS

241 CKGHYHFIKL MGRSSSHITL ECALQTHPNI ALIGEEIAEK SISLETLIHD ICETIADRAA

301 MGKYHGVILI PEGVIEFIPE IQSLVKEIES IPEQENLYQA LSLSSQQLLC QFPEDICHQL

361 LYNRDAHGNV YVSKISVDKL LIHLVRQHLE THFRQVPFNA ISHFLGYEGR SGTPTHFDNV

421 YSYNLGYGAG VLVFNRCNGY LSTIEGLTSP IEKWRLRALP IVRMLTTKQG KDSKHYPLIK

481 KRLVDIASPV FNKFSLYRKI WALEDSYRFV GPLQIHSPED AHSDDFPPLI LFLNHNEWQK

541 RCSICLEIPD QD**Y**

B) CTPFPB Nucleotide Sequence

1 **atg**gagctac tctctgtaaa taagagctac tttgaactac aaagactaca ctatcgtcca

61 gatactctga gtctattgaa tagcttgtgt tcgatgcata ttcaggaaaa gccttcttcc

121 gaaccggctt cagatttgct agctaagcat attcctcacc tatgtgctct cccagacctc

181 actcttcaaa aagatgctcc ttcttcttct gagcctttac gtatcggagt tttactgtca

241 ggaggacagg ctcctggcgg tcataacgta gtcatcggat tatttgaagg attacgcgcc

301 tttaataaag aaacaaagct cttcggtttt attaaaggcc ctcttggact tattcgagga

361 ttatataagg atctagatat ctctgttatc tatgattatt acaatgctgg agggtttgat

421 atgctctctt ctagcagaga aaaaatcaaa acaaaagaac agaagagcgc tattcttgct

481 acagtaaaaa aaatgaaact ccacggtctg ctcattgtag gaggagataa ctccaataca

541 gacactgcaa tgctagcaga atattttatc gagcataatt gccctacagc agttattggt

601 gtccctaaga ctatcgatgg ggatttaaaa aacgcttgga tagaaactcc tttaggattt

661 catacatctt gccgcactta ttctgaaatg atcggaaatt tggaaaaaga tgttctttct

721 actcgcaaat accatcattt tgtcaaattg atgggtgaac aagcttctca cagtacgttg

781 gaatgcggtc ttcagacact gcctaatatt accctaatag gagaagaagt tgctgttcaa

Fig. 17A and B

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 841 catgcctctt tacaaagctt aagtctcagt attgctcaag ggttgatcga gcgctttcat
 901 agagggaaag actacagtac tattctcatt cctgaaggct taatcaaaca aatccctgat
 961 acaaaacgat taatccaaga attgaacact ttaattgctg aagaacaatt ttctgttcat
1021 aatttggacc aacaattatc cccaatggct atcgaaactt tctcttctct tccagaaaat
1081 attcgagacc aattacttct agatcgagat tcttatggga atattcgggt atctaaaatt
1141 gccattgaag agcttttagc ttctttagtt agcaaagaaa tctctaagct tgaacctaca
1201 atgtcctttt ctcctgtaac acattttcta ggatacgaat ctcgagcaag tttcccttct
1261 aattttgatt ccaattatgg tttagcatta ggaatagctg cttctctctt cttagtaaga
1321 gggaaaacag ggtatatggt cacgataggc aatctagcag aaacttatac cgaatggacc
1381 atcgcagcga ctcctttata caaaatgatg cacttagaaa aacggttcaa tcaagagact
1441 ccagtaatca aaacagattc tgtatctcca gatgccccta tggcaaaata tttacataaa
1501 atgaaagaga tctgtttatt agaagattcg taccgattcc ccggaccgtt acaatatttc
1561 gaagaacaag ctcttgttga tcagcgtcca ctaacattac tttgggaaaa aggaaaatta
1621 tcggagaata acgcgacaaa attctaa
```

CTPFPB Deduced Amino Acid Sequence

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  1 MELLSVNKSY FELQRLHYRP DTLSLLNSLC SMHIQEKPSS EPASDLLAKH IPHLCALPDL
 61 TLQKDAPSSS EPLRIGVLLS GGQAPGGHNV VIGLFEGLRA FNKETKLFGF IKGPLGLIRG
121 LYKDLDISVI YDYYNAGGFD MLSSSREKIK TKEQKSAILA TVKKMKLHGL LIVGGDNSNT
181 DTAMLAEYFI EHNCPTAVIG VPKTIDGDLK NAWIETPLGF HTSCRTYSEM IGNLEKDVLS
241 TRKYHHFVKL MGEQASHSTL ECGLQTLPNI TLIGEEVAVQ HASLQSLSLS IAQGLIERFH
301 RGKDYSTILI PEGLIKQIPD TKRLIQELNT LIAEEQFSVH NLDQQLSPMA IETFSSLPEN
361 IRDQLLLDRD SYGNIRVSKI AIEELLASLV SKEISKLEPT MSFSPVTHFL GYESRASFPS
421 NFDSNYGLAL GIAASLFLVR GKTGYMVTIG NLAETYTEWT IAATPLYKMM HLEKRFNQET
481 PVIKTDSVSP DAPMAKYLHK MKEICLLEDS YRFPGPLQYF EEQALVDQRP LTLLWEKGKL
541 SENNATKF
```

Fig. 17B

Table 7: Characteristics of the amino acid sequences deduced from *C. trachomatis pfpA* and *pfpB* cloned genes

Name of clone	Number of amino acids	Molecular mass (kDa)	Enzyme	Identity to known protein	Organism and gene	Reference[a]
pCTL2PFPA	553	62.85	Pyrophosphate-dependent phosphofructose kinase (PPi-PFK)	40.00%	*S. thermophilia pfp*	AF307859
				37.48%	*R. communis pfpB*	Z32850
				37.48%	*R. communis pfpA*	Z32849
				25.00%	*E. coli pfkA*	X02519
pCTL2PFPB	548	62.26	Pyrophosphate-dependent phosphofructose kinase (PPi-PFK)	42.00%	*S. thermophilia pfp*	AF307859
				40.46%	*R. communis pfpB*	Z32850
				33.06%	*R. communis pfpA*	Z32849
				28.00%	*E. coli pfkA*	X02519

[a]GenBank Accession numbers

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T.pall.pfp          ----------MSISLLQQERHRYLPKVPDLLRGDFRRVCARRG-LSTTAVADYDALRSLF 49
E.hist.pfp          ----------MSLSALHKYRLQYKPVLPKHIA-DIDNITIEEG-AKTQSAVNQKELSELF 48
R.communis.pfpB     MATPNSGRAASVYSEVQSSRIEHVLPLPSVLN---HPFKIVQG-PPSSAAGNPDEIAKLF 56
C.trach.pfpB        -----MELLSVNKSYFELQRLHYRPDTLSLLN-SLCSMHIQEK-PSSEPAS--DLLAKHI 51
R.communis.pfpA     --MDSDFGIPRELSDLQKLRSLYKPELPPCLQGTTVRVELGDG-TTACSEAGAHTISRSF 57
C.trach.pfpA        -----------MSSNKHASLCQKTPSLCRELQKAPALLLTEDIRFKALLNERIDSVAELF 49
E.coli.pfkA         ------------------------------------------------------------
Human.pfkL          ------------------------------------------------------------

                                                            +             +
T.pall.pfp          ARTYGQPLVNFVNASEKNEDSPMETAPEPRGLRVAIVLSGGQAPGGHNVIAGLFDGLKRW 109
E.hist.pfp          KHTYGLPICNIVAG--KN-------ADIHRVIRCGFILSGGPAAGGHNVVAGLFDGLMKG 99
R.communis.pfpB     PNLFGQPSAMLVPDVADS-------LDSNQQLKIGLVLSGGQAPGGHNVISGIFDYLQDR 109
C.trach.pfpB        PHLCALPDLTLQKDAPSS----------SEPLRIGVLLSGGQAPGGHNVVIGLFEGLRAF 101
R.communis.pfpA     PHTYGQPLAHFLRATAKVAD--AHIISEHPAMRVGVVFCGRQSPGGHNVVWGLHNALKIH 115
C.trach.pfpA        PCTYNSPYYKFISKSDLS--------AETFPLKVGVMLSGGPAPGGHNVILGLLHSIKKL 101
E.coli.pfkA         ------------------------------MIKKIGVLTSGGDAPGMNAAIRGVVR--SAL 29
Human.pfkL          -----MAAVDLEKLRASG-----------AGKAIGVLTSGGDRQGMNAAVRAVTR--MGI 42
                                                      ..: .*    * : .: .:
                                +                                ++ + +
T.pall.pfp          HADSVLIGFLGGPAGVLSGD--HIEICADRVDAYRNTGGFDLIGSGRTKIESESQFAAAA 167
E.hist.pfp          NKENKLYGFRCGAGGILSND--YIEITAELVDKHRNTGGFDLVGSGRTKIETEEQFATAF 157
R.communis.pfpB     AKGSILYGFRGGPAGIMKCN--YVQLTADYIHPYRNQGGFDMICSGRDKIETPEQFKQAE 167
C.trach.pfpB        NKETKLFGFIKGPLGLIRGL--YKDLDISVIYDYYNAGGFDMLSSSREKIKTKEQKSAIL 159
R.communis.pfpA     NPNSTLLGFLGGSEGLFAQK--TLEVTDDILSTYKNQGGYDLLGRTKDQIRTTEQVHAAL 173
C.trach.pfpA        HPNSQLLGFIRNGEGLLNNN--TVEITDEFIEEFRNSGGFNCIGTGRTNIITEENKARCL 159
E.coli.pfkA         TEGLEVMGIYDGYLGLYEDR--MVQLDRYSVSDMINRGGTFLGSARCPEFRDENIRAVAI 87
Human.pfkL          YVGAKVFLIYEGYEGLVEGGENIKQANWLSVSNIIQLGATIIGTARSKAFTTREGRRAAA 102
                         :  :  .  *:        :     :    : *.          :   .
                                  +++++ ++
T.pall.pfp          QTVTRMALDALVVVGGDDSNTNAALLAEHFVN-----------------SGISTKVIGVP 210
E.hist.pfp          KHITALKLNAMVVVGGDDSNTNAALLAEYFAA-----------------HGSDCVFVGVP 200
R.communis.pfpB     ETAGKLDLNGLVVIGGDDSNTNACLLAENFRS-----------------KNLKTRVIGCP 210
C.trach.pfpB        ATVKKMKLHGLLIVGGDNSNTDTAMLAEYFIE-----------------HNCPTAVIGVP 202
R.communis.pfpA     TTCKNLKLDGLVIIGGVTSNTDAAQLAETFAE-----------------AKCPTKVVGVP 216
C.trach.pfpA        QTANELDLDGLVIIGGDGSNTATAILAEYFAK-----------------HQAKTVLVGVP 202
E.coli.pfkA         ENLKKRGIDALVVIGDGGSYMGA-MRLTEMG-----------------------FPCIGLP 124
Human.pfkL          NNLVQHGITNLCVIGGDGSLTGANIFRSEWGSLLEELVAEGKISETTARTYSHLNIAGLV 162
                           :  : ::*.   *   :                                  ●
                    +++++                           +      + +      +++
T.pall.pfp          KTIDGDLKNEAIETSFGFDTATKTYSELIGNIARDACSARKYWHFIKLMGRSASHIALEC 270
E.hist.pfp          KTIDGDLKNQYIETSFGFDTACKTYSELIGNIQRDAISSRKYWHFIKVMGRSASHIALEA 260
R.communis.pfpB     KTIDGDLKCKEVPTSFGFDTACKIYSEMIGNVMIDARSTGKYYHFVRLMGRAASHITLEC 270
C.trach.pfpB        KTIDGDLKNAWIETPLGFHTSCRTYSEMIGNLEKDVLSTRKYHHFVKLMGEQASHSTLEC 262
R.communis.pfpA     VTLNGDLKNQFVETNVGFDTICKVNSQLISNVCTDALSAEKYYYFIRLMGRKASHVALEC 276
C.trach.pfpA        KTIDGDLQHLFLDLTFGFDTATKFYSSIISNISRDALSCKGHYHFIKLMGRSSSHITLEC 262
E.coli.pfkA         GTIDNDIKG--TDYTIGFFTALSTVVEAIDRLRDTSSS-HQPISVVEVMGRYCGDLTLAA 181
Human.pfkL          GSIDNDFCG--TDMTIGTDSALHRIMEVIDAITTTAQS-HQRTFVLEVMGRHCGYLALVS 219
                    :::.*:         .*  :      . *. :     *    .:.:**. ..      :* .
                          +                                          +
T.pall.pfp          ALKTQPNVCLISEEVAAQSLTLAQIVQSLCDTIATRAQHGEHFGIVLVPEGLIEFIP--- 327
E.hist.pfp          ALETQPTYCIISEEVEDKKMTVSQIASEIADIVIERHKKGLNFGVVLIPEGLVEFIP--- 317
R.communis.pfpB     ALQTHPNITIIGEEVAAKKLALKDVTDYIVDVICKRADLGYNYGVILIPEGLIDFIP--- 327
C.trach.pfpB        GLQTLPNITLIGEEVAVQHASLQSLSLSIAQGLIERFHRGKDYSTILIPEGLIKQIP--- 319
R.communis.pfpA     TLQSHPNMVILGEEVAASKLTLFDLTKQVCDAVQARAEQDKYHGVILLPEGLIESIP--- 333
C.trach.pfpA        ALQTHPNIALIGEEIAEKSISLETLIHDICETIADRAAMGKYHGVILIPEGVIEFIP--- 319
E.coli.pfkA         AIAGGCEFVVVPEV---EFSRED-----LVNEIKAGIAKGKKHAIVAITEHMCDVD---- 229
Human.pfkL          ALASGADWLFIPEAPP-EAPPEDGWENFMCERLGETRSRGSRLNIIIIAEGAIDRNGKPI 278
                     :       .: *    .          : : :      .     : :.*   .
                                     +              +  +
T.pall.pfp          ---EMKALITELNEVMARRAQEFEALDTPDAQRVWIEQALSASARAVFNALPAEISTQLL 384
E.hist.pfp          ---EVIALIKELNNLLAHKKEEYSKITEFSAQKAFVCENISESCAATFKNLPDNIRKQLL 374
R.communis.pfpB     ---EVQNLIAELNEILAHDVVD---------EGGLWKKKLTSQSLQLFEFLPVAIQEQLM 375
C.trach.pfpB        ---DTKRLIQELNTLIAEEQFS----------VHNLDQQLSPMAIETFSSLPENIRDQLL 366
R.communis.pfpA     ---EVYALLKEIHGLLRQGVSP-----------NNISSQLSPWASALFEFLPPFIKKQLL 379
C.trach.pfpA        ---EIQSLVKEIESIPEQ---------------ENLYQALSLSSQQLLCQFPEDICHQLL 361
E.coli.pfkA         ---ELAHFIEKETGRETRATVLG---------HIQRGGSPVPYDRILASRMGAYAIDLLL 277
Human.pfkL          SSSYVKDLVVQRLGFDTRVTVLG---------HVQRGGTPSAFDRILSSKMGMEAVMALL 329
                          :: :   .                                 :       ●:
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                        +                                                 +
T.pall.pfp           ADRDPHGNVQVSRIDTERLLILQVTERLAQMKQEGTYTGV-FSSIAHFFGYEGRC----- 438
E.hist.pfp           LDRDPHGNVNVSAIETESFVSGIVKAEIVKRGIKVPFT-----PVHHFFGYEGRC----- 424
R.communis.pfpB      LERDPHGNVQVAKIETEKMLIQMVETELEKRKQQGTYKAH-FKGQSHFFGYEGRC----- 429
C.trach.pfpB         LDRDSYGNIRVSKIAIEELLASLVSKEISKLEPTMSFS-----PVTHFLGYESRA----- 416
R.communis.pfpA      LYPESDDSAQLSQIETEKLLAHLVEAEMNKRLKEGTYKGKKFNAICHFFGYQARG----- 434
C.trach.pfpA         YNRDAHGNVYVSKISVDKLLIHLVRQHLETHFRQVPFN-----AISHFLGYEGRS----- 411
E.coli.pfkA          AGYGGR------CVGIQNEQLVHHDIIDAIENMKRPFKGDWLDCAEKMY----------- 320
Human.pfkL           EATPDTPACVVTLSGNQSVRLPLMECVQMTKEVQKAMDDKRFDEATQLRGGSFENNWNIY 389
                                     :                  .          ::

T.pall.pfp           -----AFPSNFDADYCYTLG---------LTACLLAVHRFTGYVASVRNLTSSVAEWAVG 484
E.hist.pfp           -----AFPSNFDSTYCYALG---------YTAFILLALKKTGQICCISGLQKPAEEWICG 470
R.communis.pfpB      -----GLPTNFDSTYCYALG---------YAAGALLHSGKTGLISSVGNLGAPVAEWTVG 475
C.trach.pfpB         -----SFPSNFDSNYGLALG---------IAASLFLVRGKTGYMVTIGNLAETYTEWTIA 462
R.communis.pfpA      -----SLPSKFDCDYAYVLG---------HICYHVLAAGLNGYMATATNLKNPVNKWRCG 480
C.trach.pfpA         -----GTPTHFDNVYSYNLG---------YGAGVLVFNRCNGYLSTIEGLTSPIEKWRLR 457
E.coli.pfkA          ------------------------------------------------------------
Human.pfkL           RLLTHQKPPKEKSNFSLAILNVGAPAAGMNAAVRSAVRTGISHGHTVYVVHDGFEGLAKG 449

T.pall.pfp           GVPLTMLMNMERRHGSQ------KPVIKKALVDLEGMPFRVFSRRRASWALKTSYVYPGA 538
E.hist.pfp           GVPLTIMMNMEQRNGEM------KPVIKKALVEIEGKPFKFYQSKRAQWASAEDFVFPGA 524
R.communis.pfpB      GTALTSLMDVERRHGKF------KPVIKKAMVELEGAPFKKFASLREEWALKNRYVSPGP 529
C.trach.pfpB         ATPLYKMMHLEKRFNQE------TPVIKTDSVSPDAPMAKYLHKMKEICLLEDSYRFPGP 516
R.communis.pfpA      AAPIAAMMTVKRWAQNPGATSIGKPAIHPAAVDLKGKAYELLRLNAVKFLMDDLYRNPGP 540
C.trach.pfpA         ALPIVRMLTTKQGKDSK-----HYPLIKKRLVDIASPVFNKFSLYRKIWALEDSYRFVGP 512
E.coli.pfkA          ------------------------------------------------------------
Human.pfkL           QVQEVGWHDVAGWLGRGGSMLGTKRTLPKGQLESIVENIRIYG-IHALLVVGGFEAYEGV 508

T.pall.pfp           VQYYGPPAV--CDEPSVTIRLE-------------------------------RPAPAAN 565
E.hist.pfp           IQYFGPSEV--CDQPTKTLLLE-------------------------------QN----- 546
R.communis.pfpB      IQFMGPG----SDAASHTLLLE-------------------------------LGSVA-- 552
C.trach.pfpB         LQYFEEQAL--VDQRPLTLLWE-------------------------------KGKLSEN 543
R.communis.pfpA      LQFEGPG----ADAKPITLCVEDQDYMGRIKKLQEYLDKVRTIVKPGCSQEVLKAALSVM 596
C.trach.pfpA         LQIHSPEDAHSDDFPPLILFLN-------------------------------HNEWQKR 541
E.coli.pfkA          ------------------------------------------------------------
Human.pfkL           LQLVEARGR--YEELCIVMCVIPATISNNVPGTDFSLGSDTAVNAAMESCDRIKQSASGT 566

T.pall.pfp           SSFGHRSS---------------------------------------------------- 573
E.hist.pfp           ------------------------------------------------------------
R.communis.pfpB      ------------------------------------------------------------
C.trach.pfpB         NATKF------------------------------------------------------- 548
R.communis.pfpA      ASVTDVLLTMSSTSLDGQKPL--------------------------------------- 617
C.trach.pfpA         CSICLEIPDQDY------------------------------------------------ 553
E.coli.pfkA          ------------------------------------------------------------
Human.pfkL           KRRVFIVETMGGYCGYLATVTGIAVGADAAYVFEDPFNIHDLKVNVEHMTEKMKTDIQRG 626

Human.pfkL           LVLRNEKCHDYYTTEFLYNLYSSEGKGVFDCRTNVLGHLQQGGAPTPFDRNYGTKLGVKA 686
Human.pfkL           MLWLSEKLREVYRKGRVFANAPDSACVIGLKKKAVAFSPVTELKKDTDFEHRMPREQWWL 746
Human.pfkL           SLRLMLKMLAQYRISMAAYVSGELEHVTRRTLSMDKGF 784
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Figure 18. Comparison of the deduced amino acid sequences from PPi-PFKs from *C. trachomatis* (C.trach), *T. pallidum* (T.pall), *E. histolytica* (E.hist), *R. communis* and ATP-PFKs from *E. coli* and Human. Identical residues are indicated by an asterisk (*) and similarity between amino acids is shown by dots (:.). Important residues implicated in substrate binding, effector binding, or catalysis are indicated by the plus sign (+). Alignments are done using the ClustalW version 1.8.

of this sequence has been shown to be important for the activity of *N. fowleri* PPi-PFK (Hinds *et al.*, 1998) and the M has been shown to be important in fructose-6-phosphate binding in the *E. histolytica* PPi-PFK enzyme (Wang *et al.*, 1998). This MGR motif is conserved in *C. trachomatis pfpA* sequence, however, the R is replaced with an E in the *C. trachomatis pfpB* sequence.

The TIDXD (residues 125-129 in *E. coli* PFK) is conserved in all PFKs. The two Ds in this sequence have been shown to be catalytically important in *E. coli* PFK (Berger and Evans, 1992; Hellinga and Evans, 1987; Laine *et al.*, 1992) and in *P. freudenreichii* PPi-PFK (Green *et al.*, 1993). The T and the two Ds have also been implicated in F6P binding (Shirakihara and Evans, 1988). This TIDXD motif is conserved in *C. trachomatis pfpA* and *pfpB* sequences and is identical that of the PFP sequences shown in Fig. 18 except for *R. communis pfpA* which replaces the first D with N.

The GGDD sequence or its variation GGED is found in most PPi-PFKs whereas GGDG or GDGG (residues 102-105 *E. coli* PFK numbering) is found in most ATP-PFKs. The GGDD motif is conserved in *C. trachomatis* L2 *pfpA* however, the last D is replaced by an N in the C. *trachomatis* L2 *pfpB* sequence. The D104, G105 as well as S106 have been implicated in ATP binding in *E. coli* PFK (Shirakihara and Evans, 1988). Other residues implicated in ATP binding include G22 (SGGDA); Y42 (DGYLGL), R73 ,C74, R78 (SARCPEFR); M108, G109 (MGA) (*E. coli* numbering; (Shirakihara and Evans, 1988)) most of which are not conserved in either *C. trachomatis pfpA* or *pfpB* sequences, nor in the other *pfp* genes shown in Fig. 18. A site-directed mutagenic study also demonstrated that R423 and Y420 were important in F6P binding and R377 was important in catalysis in *E. histolytica* PPi-PFK (Deng *et al.*, 2000). All of these residues

are conserved in the *C. trachomatis pfpA* and *pfpB* sequences. Together this information indicates that *C. trachomatis pfpA* and *pfpB* genes contain important catalytic and substrate binding sites found in both ATP-PFK and PPi-PFKs, but are more similar to the PPi-PFKs.

b) *In vitro* enzyme analysis of *C. trachomatis* PFPA and PFPB recombinant enzymes

To determine whether *C. trachomatis* L2 *pfpA* and *pfpB* encode for two independent ATP-PFK or PPi-PFK enzymes or two subunits of one ATP-PFK or PPi-PFK enzyme, *in vitro* enzyme analysis was performed. As mentioned, both *pfpA* and *pfpB* show highest homology to PPi-PFK and of particular interest, both genes show higher homology to the catalytic β-subunit of the rice plant PPi-PFK rather than the regulatory α-subunit suggesting that both proteins may be functional. ATP-PFK and PPi-PFK enzyme assay conditions as well as cellular extract preparations are described in "Materials and Methods".

E. coli contains two genes encoding for ATP-PFK namely, *pfkA* and *pfkB* (Fraenkel, 1996). *pfkA* encodes for the major form of PFK, PFK-1, an allosteric enzyme activated by nucleoside diphosphates and inhibited by PEP. PFK-1 accounts for approximately 90% of the enzyme activity found in crude extracts (Fraenkel, 1996; Torres and Babul, 1991). PFK-2, which is encoded by *pfkB*, accounts for the remaining 10% of the overall PFK activity and is non-allosteric and structurally unrelated to PFK-1 (Fraenkel, 1996; Torres and Babul, 1991). *E. coli* strain DF456 is deficient in PFK-1 activity and is incapable of growth on minimal media supplemented with mannitol (Thomson *et al.*, 1979) which was

confirmed in our laboratory. DF456 was subsequently transformed with either a plasmid containing the *C. trachomatis pfpA* gene (pCTPFPA), a plasmid containing the *C. trachomatis pfpB* gene (pCTPFPB), or pUC-19 as a negative control. ATP-PFK has been well studied in *E. coli* therefore extract prepared from *E. coli* BL21 was used as a positive control.

The results from the *in vitro* PFK enzyme assays are shown in Table 8. Extract prepared from *E. coli* BL21 was found to have a level of ATP-PFK activity comparable to others (Thomson *et al.*, 1979). As expected, BL21 extract did not contain PFK activity when PPi replaced ATP. Extract prepared from the PFK-1 mutant strain DF456 or strain DF456 containing pUC19 did not contain PPi-PFK activity and showed a very low level of ATP-PFK activity which is in agreement with other findings (Thomson *et al.*, 1979). Extract prepared from strain DF456 containing pCTPFPA showed ATP-PFK activity and when PPi replaced ATP, the PFK activity rose 2 fold. These results show that recombinant chlamydial PFPA is active and is able to use both ATP and PPi as substrates however it shows greatest PFK activity with PPi.

Extract prepared from strain DF456 containing pCTPFKB also showed both ATP-PFK and PPi-PFK activity, however ATP-PFK activity was two fold higher than PPi-PFK activity. These results indicate that recombinant chlamydial PFPB contains both ATP-PFK and PPi-PFK activity but shows greater PFK activity with ATP as the phosphate donor.

When extract prepared from DF456-pCTPFPA was combined with extract prepared from DF456-pCTPFPB and assayed for PFK activity, both ATP-PFK and PPi-PFK activity was doubled. These results suggest that activity from each chlamydial PFP

Table 8. ATP-PFK and PPi-PFK activity in crude extracts prepared from *E. coli* strains[a]

E. coli Strain[b]	No ATP or PPi	ATP	PPi
BL21	0.168	0.934	0.180
DF456 (*pfkA*-)	0.112	0.201	0.121
DF456-pUC19	0.105	0.095	0.055
DF456-pCTPFPA	0.117	0.348	0.657
DF456-pCTPFPB	0.101	0.753	0.354
DF456-pCTPFPA + DF456-pCTPFPB	0.136	0.910	0.513

[a]Specific activity results are presented as the mean of two independent determinations expressed in micromole per minute per mg. ATP-PFK assays were determined at 25°C and consisted of 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2 mM NH_4Cl, 1 mM F6P, 0.2 mM NADH, 0.6 units aldolase, 6 units triose-P-isomerase, 1 unit of glycerolphosphate dehydrogenase, 1 mM ATP. PPi-PFK assay conditions were identical except that 1 mM ATP was replaced with 1 mM PPi. Enzyme assays were carried out in a final volume of 1 ml started with the addition of 1 mM F6P. The sensitivity of the assays are 0.01 μmol min^{-1} mg^{-1}.

[b]The *E. coli* strains carrying the indicated plamids were used for extract preparation

protein (PFPA and PFPB) was additive and that chlamydial PFPA and PFPB proteins are not subunits of one PFK enzyme but likely function individually as two separate enzymes. Furthermore, *C. trachomatis* PFPA likely functions as a PPi-PFK and PFPB probably functions as an ATP-PFK. Further work on highly purified *C. trachomatis* PFPA and PFPB enzymes will have to be done to clarify whether they are truly independent ATP-PFK and PPi-PFK enzymes and whether they are allosterically regulated.

7. RT-PCR analysis of *C. trachomatis* metabolic genes

The completed genome sequence project indicates that the 1,042,519 bp chromosome and 7,493 base pair plasmid of *C. trachomatis* D contains about 894 protein encoding genes, 604 with an inferred functional assignment, 35 which were similar to other hypothetical proteins deposited for other bacteria and 255 which share no homology to any known protein deposited in GenBank and have no known function (Stephens *et al.*, 1998)(http://chlamydia-www.berkeley.edu:4321/). Information from the genome sequencing project was used to construct primers for RT-PCR analysis to determine whether other metabolic genes in *C. trachomatis* L2, particularly those designated to be involved in carbon and energy metabolism, had similar expression patterns to the glucose metabolizing genes.

Twelve genes were selected and are defined, along with the primers used, in Table 9. These genes included two more glycolytic genes namely *pfpA* and *pfpB* as well as an additional PPP gene, *zwfB*. Interestingly, *zwfB* shares little homolgy to the other *zwf* gene in its genome (≈ 10 % identity) but shares highest homology (≈ 54% identity) to the *devB* gene in *Anabaena sp.* PCC7120 (accession no. P46016). *devB* is a developmentally regulated gene in heterocyst development, which functions as a glucose-6-phosphate dehydrogenase. *odpB*, a gene which shares highest homology (59% identity) to the pig heart beta subunit of pyruvate dehydrogenase (PDH) (accession no. 448581) was also analyzed. PDH is a multi-complex enzyme responsible for the conversion of pyruvate

Table 9: Primers[a] and *C. trachomatis* L2 metabolic genes analyzed in RT-PCR

C. trachomatis L2 gene analyzed	Gene description	Location/Source	Primers used for RT-PCR	
			Name of primer	Sequence
pfpA	Pyrophosphate-fructose 6-phosphate 1-transferase	Embden Meyerhof Parnas pathway	5bPFKA 3cPFKA	5'-GCGTTATGCTTTCTGGAG-3' 5'-CATAGACGTTGCCATGAG-3'
pfpB	Pyrophosphate-fructose 6-phosphate 1-transferase	Embden Meyerhof Parnas pathway	5bPFKB 3cPFKB	5'-TCCTCACCTATGTGCTCT-3' 5'-CTATGAAAGCGCTCGATC-3'
zwfB	Glucose 6-phosphate dehydrogenase	Pentose phosphate pathway	5ZWFB 3ZWFB	5'-TGCTTATCGCTGACTCTC-3' 5'CAGAAGATAGTACCCAC-3'
odpB	Pyruvate dehyrogenase beta subsunit	Central metabolism	5ODPB 3ODPB	5'-TCGAAATCCGAGAGGCTA-3' 5'-TTGACAGACTCGTAGAGG-3'
nqr5	NADH-ubiquinone oxidoreductase subunit 5	Electron transport chain	5NRQ5 3NRQ5	5'-GTGTAGCTATTTGGCCTG-3' 5'TGGTTGTGGGCTATCAGT-3'
cydA	Cytochrome oxidase subunit I	Electron transport chain	5CYDA 3CYDA	5'-CGAGAGTACAGTTTGCGT-3' 5'-TGCGTTCACGATAGGAGA-3'
nrdB	Ribonucleoside-diphosphate reductase small chain	Nucleotide metabolism	5NRDB 3NRDB	5'-TGGCTGCGCAAATAACTG-3' 5'-ATCGAAGCTCTCAATCCC-3'
sucC	Succinyl-CoA synthetase beta chain	Tricarboxylic acid cycle	5SUC 3SUC	5'-CGAACAATGGAAAGCTGG-3' 5'-ATTGCACAGCGAGTTCAG-3'
adt1	ADP/ATP translocase	Energy metabolism/ ATP transport	5ADT1 3ADT1	5'-CGATACACATGCATGAGC-3' 5'-GATGCAGCCAAGAACAGT-3'
adt2	ADP/ATP translocase	Energy metabolism/ ATP transport	5ADT2 3ADT2	5'-AAGACCACGAAAGACTCC-3' 5'-TCGAGACTCAGCATCTAC-3'
sodiTi	Dicarboxylate [2-oxoglutarate/malate] transporter	Transport protein	5SODITI 3SODITI	5'-CCATAGCAAAAGGCGTGA-3' 5'-ATCCGTAGTGTGTGAGTC-3'
aspC	Aspartate aminotransferase	Amino acid biosynthesis	5ASPC 3ASPC	5'-AATGCTAGTGTCGCTGAG-3' 5'-TGTTCGCTTGAGTGGGTT-3'

[a]Primers were designed according to the *C. trachomatis D* genome project (Stephens *et al.*, 1998)(http://chlamydia-www.berkeley.edu:4321/)

into acetyl-CoA, which can either enter the TCA cycle, or be used in fatty acid biosynthesis. *sucC*, a gene which shows highest homology (39% identity) to the succinyl-CoA synthetase *B*-subunit of *Rickettsia prowazekki* (accession no. Y11777) was also monitored. Succinyl-CoA synthetase is involved in the TCA cycle and catalyzes the reversible reaction succinate + CoA + ATP ↔ succinyl-CoA + ADP + orthophosphate. Two genes involved in the respiration chain *cydA*, which shows highest homology (43% identity) to cyanide insensitive terminal oxidase from *Pseudomonas aeruginosa* (accession no. Y10528) and *nqr5*, which shows 61% homology to Na^+-translocating NADH-quinone reductase in *Vibrio alginolyticus* (accession no. S65530) were also analyzed. The two genes involved in nucleoside phosphate transport, *adt1* and *adt2* as well as the *sodiTi* gene which encodes for a dicarboxylate transporter, were also included in the analysis. *aspC*, was also analyzed and shows 33% identity to the aspartate aminotransferase in *Synechocystis sp.* (accession no. D64000). Aspartate aminotransferase catalyzes the transamination reaction glutamate + oxaloacetate ↔ 2-oxoglutarate + aspartate involved in amino acid biosynthesis. Finally, *nrdB*, a gene which shows homology (35% identity) to the ribonucleotide-diphosphate reductase small subunit (R2 subunit) of *Plamodium falciparum* (accession no. U01322) which is part of the ribonucleotide reductase (RNR) enzyme complex was included. RNR synthesizes deoxyribonucleotides from ribonucleotides.

To analyze expression of the 12 selected metabolic genes throughout the chlamydial developmental cycle, total RNA was isolated from *C. trachomatis*-infected HeLa cells at 2, 6, 24, 36, 48 h p.i. and used as template for cDNA synthesis. The amount of cDNA used as template for each time point was then roughly equalized using primers

specific to chlamydial 23S rRNA so that the 23S rRNA PCR products were of similar intensity when run on an agarose gel. This amount of cDNA was kept constant for subsequent reactions and the primer sets for the 12 selected genes employed were within the coding region of each gene respectively (Table 9). We also used primers specific to *euo* as a control for early gene expression, primers specific to *ompA* as a control for mid-late gene expression, and primers specific to *omcB* for late gene expression as previously described (Iliffe-Lee and McClarty, 1999). Primers for 23S rRNA, *euo*, *ompA* and *omcB* are shown in Table 3. Results from Fig. 19 indicate that similar to the glucose metabolizing enzymes (*gap*, *pgk*, *pk*, *zwf*) all 12 genes (*pfpA*, *pfpB*, *zwfB*, *odpB*, *nqr5*, *cydA*, *nrdB*, *adt1*, *adt2*, *sodiTi*, *aspC*) are first detected at 2-6 h p.i., reach a maximum 16-24 h p.i, and then slowly decline thereafter except for *nrdB*, and *aspC* which remain essentially constant throughout the remainder of the life cycle.

Figure 19. RT-PCR analysis of total RNA extracted from chlamydiae-infected cells at different time points in the chlamydial life cycle. Each lane contains RNA samples subjected to RT-PCR analysis. Time points indicate the number of hours post-infection at which the RNA sample was isolated. Primers employed are shown in Table 9. RT-PCR using 23S rRNA primers, *euo* primers, *ompA* primers, *omcB* primers, *pfpA* primers, *pfpB* primers, *zwfB* primers, *odpB* primers, *nqr5* primers, *cydA* primers, *nrdB* primers, *sucC* primers, *adt1* primers, *adt2* primers, *sodiTi* primers and *aspC* primers are shown. The size of each band is shown in base pairs (bp).

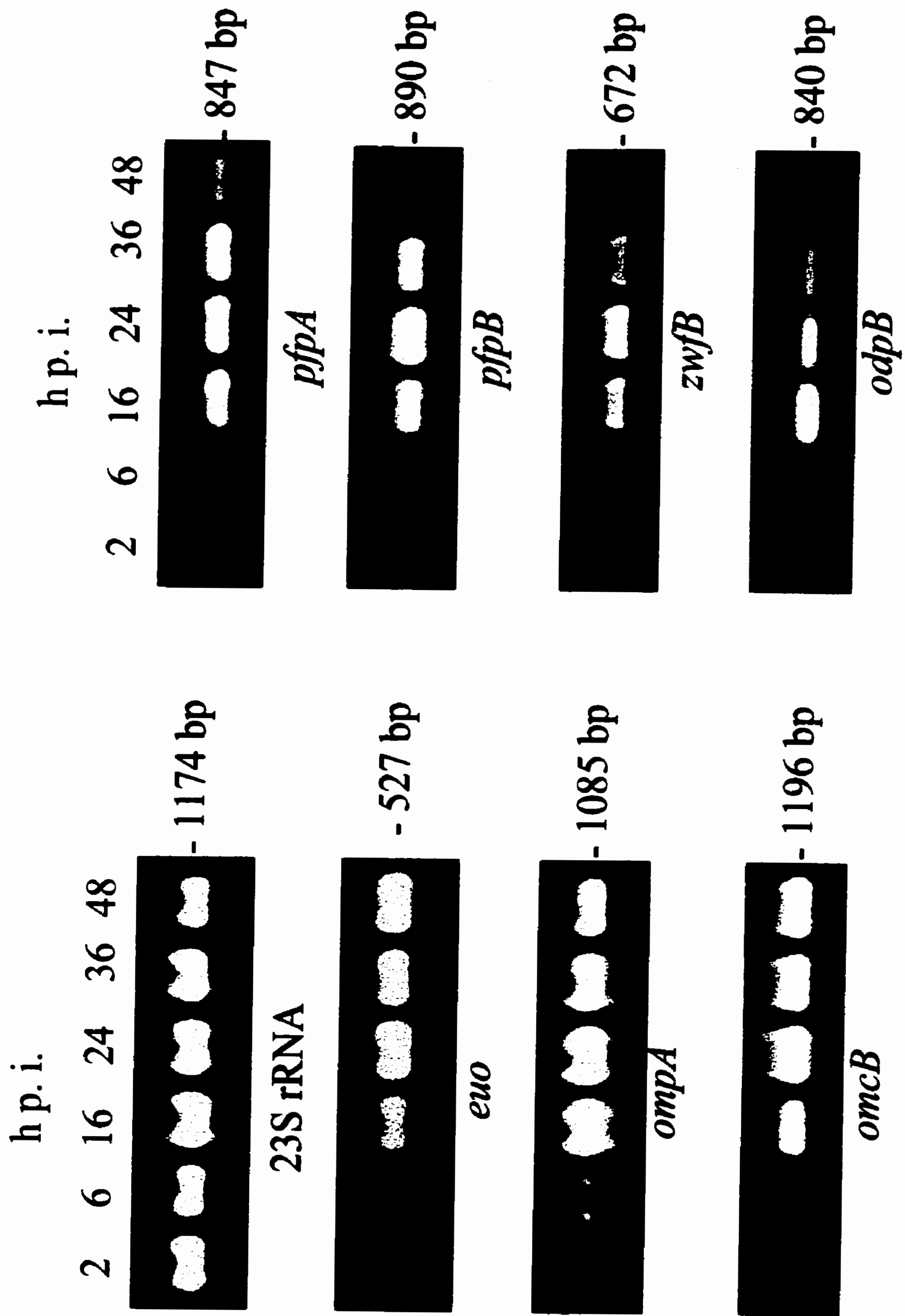


Fig. 19

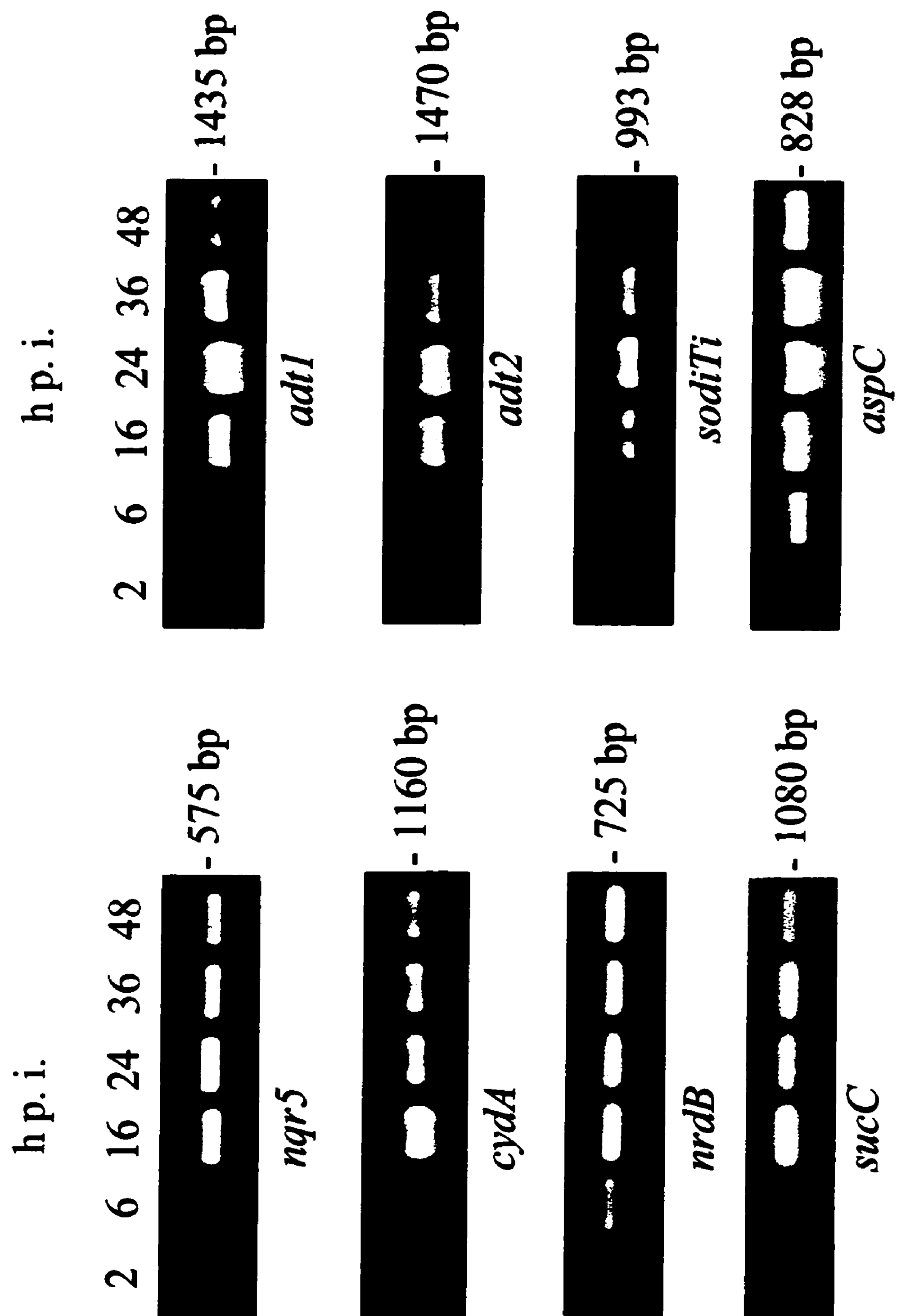


Fig. 19

B. ENZYME STUDIES ON *C. TRACHOMATIS* L2 PYRUVATE KINASE

1. Expression and purification of CTPK

Pyruvate kinase is of considerable interest because it is the final regulatory point in the catabolic Embden-Meyerhof-Parnas pathway, which controls the carbon flux of glycolytic intermediates and regulates the level of ATP in the cell. In the previous study, pyruvate kinase from *Chlamydia trachomatis* L2 was identified, cloned, sequenced, and demonstrated to be active in crude extract. In order to gain a better understanding of the regulatory properties of pyruvate kinase in chlamydia, *C. trachomatis* L2 pyruvate kinase (CTPK) was expressed and purified from *E. coli*. The kinetic properties of the enzyme were then characterized and compared to other established PK enzymes.

Recombinant full length CTPK (485 amino acids) was over expressed in *E. coli* using the pQE80L expression system. Maximal production of soluble, active enzyme was obtained by continued growth after induction with 1 mM IPTG for 3.5 h at 37°C. The yield of total protein obtained from the crude soluble extract from a 500 ml culture was about 180 mg and was assayed to give a specific activity of 1.7 units/mg. The recombinant protein was subsequently filtered and purified 32.35 fold on a metal chelation affinity column resulting in a yield of 3 mg of pure recombinant CTPK protein with a specific activity of about 55 units/mg. The purified protein was subjected to SDS/polyacrylamide gel electrophoresis along with molecular weight standards and the recombinant protein ran with a mobility close to the molecular mass (53.5 kDa) deduced from the cloned pyk gene (Iliffe-Lee and McClarty, 1999) (Fig. 20).

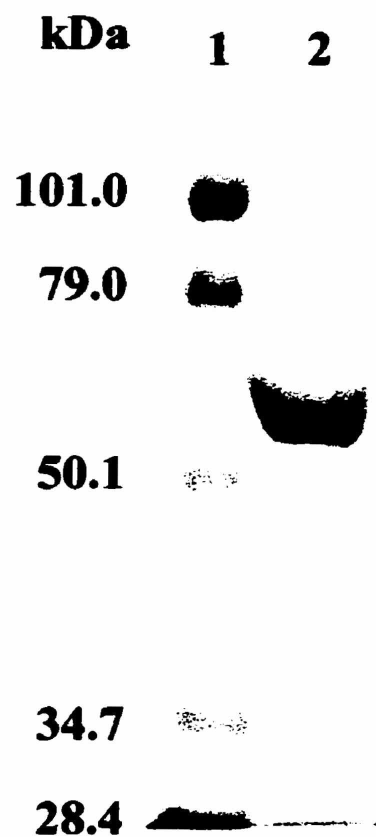


Figure 20. SDS/polyacrylamide gel electrophoresis of recombinant *C. trachomatis* L2 PK protein. A purified sample of 10 µg *C. trachomatis* recombinant PK protein was run on a 10% SDS/polyacrylamide gel along with the molecular weight markers. Sizes are indicated in kilodaltons (kDa).

2. Kinetic Analysis

a) Activity as a function of enzyme concentration

In order to optimize the amount of CTPK protein to use for the PK assay, the effect of increasing protein concentrations on CTPK activity was assessed. The results presented in Fig. 21 indicate that the amount of protein added is linear with respect to CTPK activity. Optimal activity was estimated from these preliminary assays to occur at 1 μg/ml protein. All subsequent assays were performed in triplicate using this optimal protein concentration.

b) pH optima

The activity of the purified recombinant CTPK was measured at a pH range of 5.0 to 9.0 under standard saturating conditions as described in "Materials and Methods" and is shown in Fig. 22. Under these conditions, the enzyme exhibited a broad pH/activity profile and showed a pH optimum around 7.3 which is similar to other PK enzymes (Abbe and Yamada, 1982; Lin *et al.*, 1989; Sakai *et al.*, 1986). All subsequent CTPK kinetic studies were performed at pH 7.3.

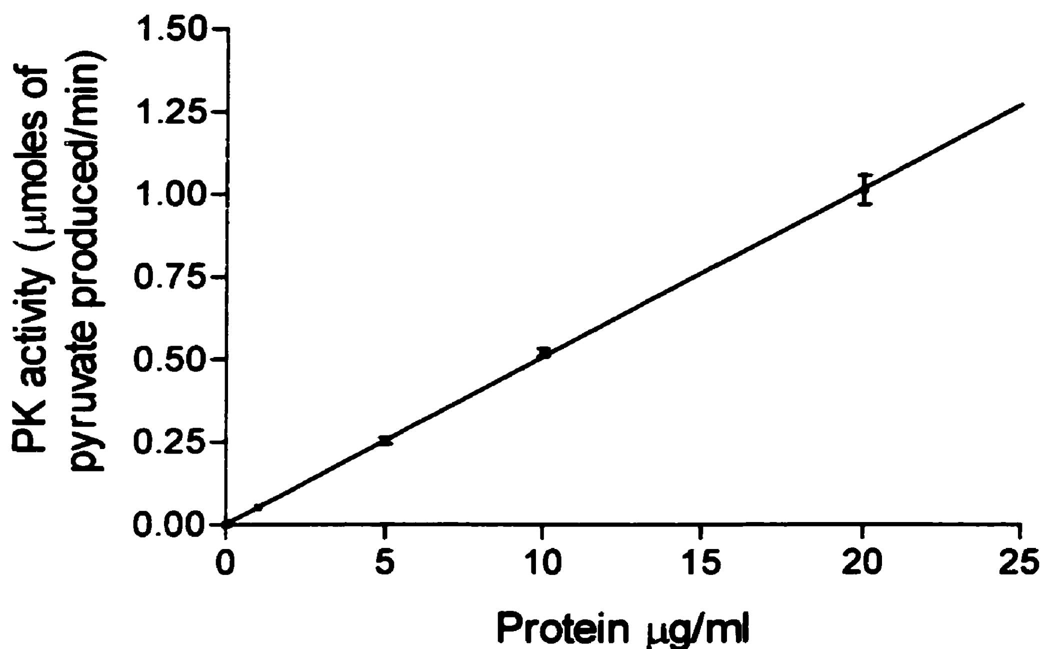


Figure 21. Optimization of *in vitro* PK assay using increasing concentrations of purified recombinant *C. trachomatis* L2 PK. The pre-reaction mixture contained: 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM KCl, 2 mM ADP, 0.2 mM NADH and 10 units of lactate dehydrogenase for a final volume of 1ml. The indicated amount of protein extract was added and the reaction was started with the addition of 10 mM phosphoenolpyruvate (PEP). The reaction was measured in a spectrophotometer at a wavelength of 340 nm for 5 minutes. Each assay was run in triplicate and the results shown are the mean ± S.E.M.

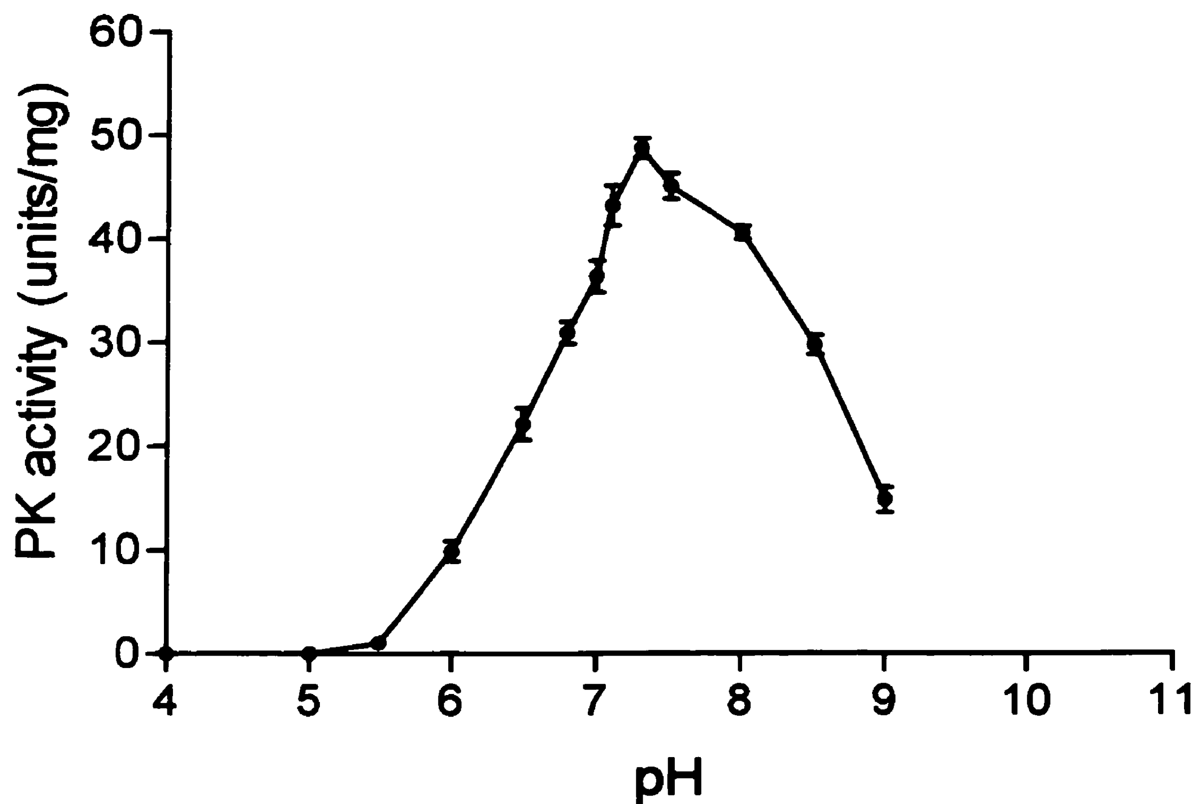


Figure 22. pH profile of purified *C. trachomatis* pyruvate kinase. Activity was determined at various pH values under standard saturation conditions as discussed in "Materials and Methods". Each point is the mean of triplicate values ± S.E.M.

c) Cofactor requirements

The divalent cation Mg^{2+} and monovalent cation K^+ were absolutely required for CTPK activity (Table 10). Figure 23 shows that CTPK displayed hyperbolic kinetics with respect to KCl under saturating conditions. CTPK displayed hyperbolic kinetics with respect to $MgCl_2$ under saturating conditions for concentrations of $MgCl_2$ up to 20 mM (Fig. 24). $MgCl_2$ concentrations greater than 20 mM inhibited CTPK activity (data not shown). Substrate inhibition by high concentrations of $MgCl_2$ is found to occur with other PK enzymes (Garcia-Olalla and Garrido-Pertierra, 1987; Sakai *et al.*, 1986). The K_m determined for Mg^{2+} refers only to the assay conditions described and is expressed in terms of total Mg^{2+} concentration.

Table 10. Kinetic parameters of CTPK with KCl and $MgCl_2$[a]

Variable Substrate	Fixed Substrate	V_{max} (units/mg)	K_m (mM)	n[b]
$MgCl_2$ (0-20mM)	2 mM ADP and 10 mM PEP	56.26 ± 1.49	2.82 ± 0.02[c]	1.43 ± 0.04
KCl	2 mM ADP and 10 mM PEP	69.17 ± 1.56	36.03 ± 0.65	1.09 ± 0.02

[a] Results are means ± standard errors for three determinations. Values for V_{max} and K_m were determined by fitting data into the Michaelis-Menten equation. Enzyme assays were conducted at 25°C, pH 7.3 at saturating substrate conditions as described in "Materials and Methods".

[b] The n was determined by fitting data into the hill equation as described in "Materials and Methods".

[c] The K_m or in this case the $S_{0.5}$ was determined by Hill plot.

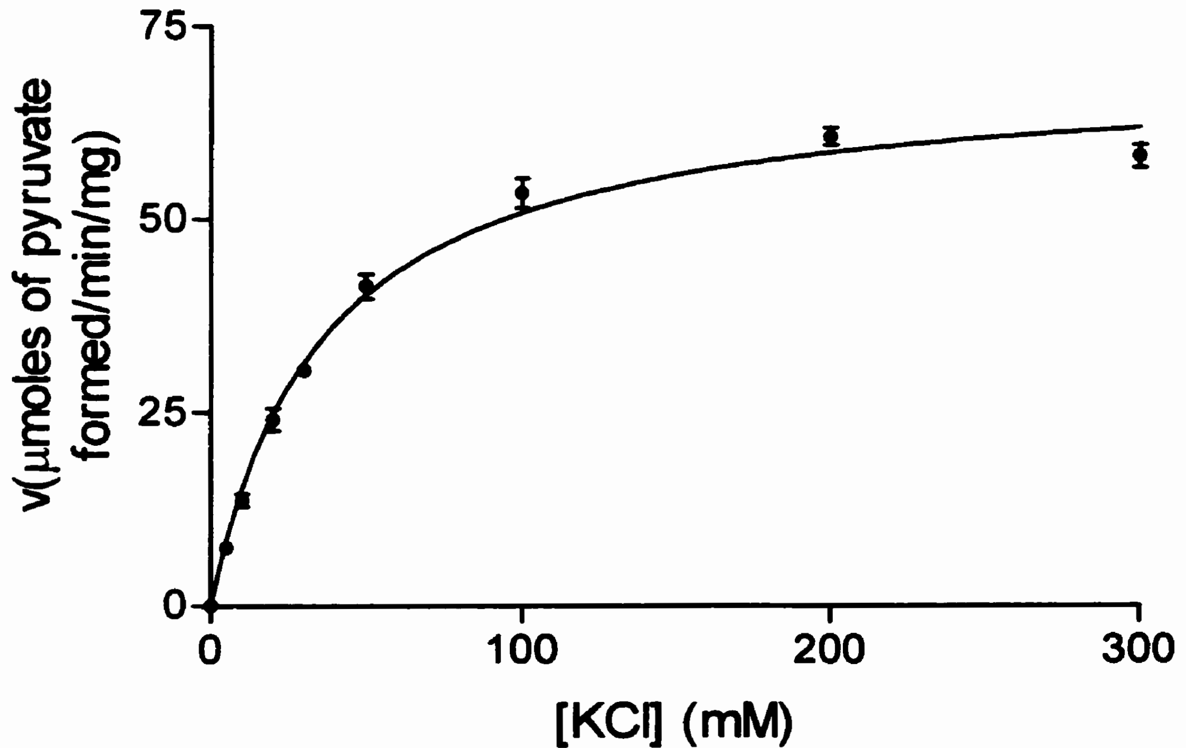


Figure 23. KCl saturation kinetics for *C. trachomatis* PK. Enzyme activity was assayed at 10 mM $MgCl_2$, 25°C and pH 7.3 as described in "Materials and Methods". The assays were performed at saturating ADP (2.0 mM) and PEP (10.0 mM) concentrations. Data was fitted into the Michaelis-Menten equation and the resulting fitted curves are shown. The assay was run in triplicate and the results shown are the mean ± S.E.M.

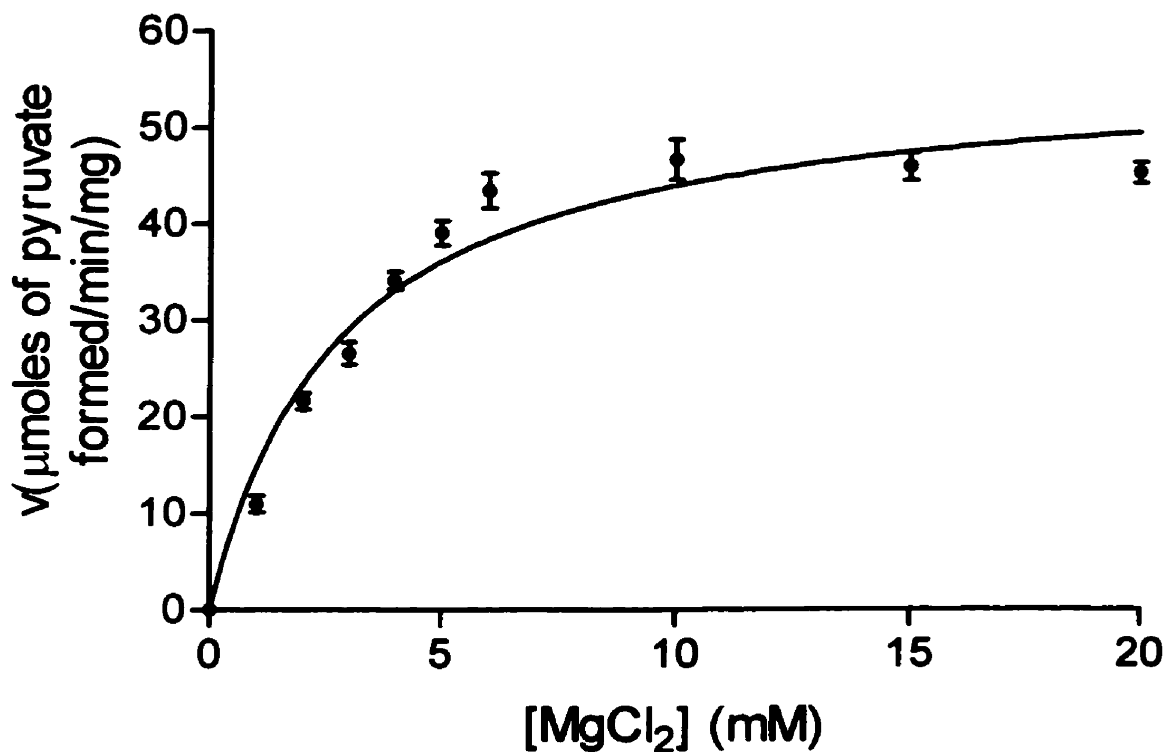


Figure 24. Kinetic properties of *C. trachomatis* PK with respect to $MgCl_2$. Enzyme activity was assayed at 25°C at pH 7.3 as described in "Materials and Methods" at non-inhibitory $MgCl_2$ concentrations. The assays were performed at saturating ADP (2.0 mM) and PEP (10.0 mM) concentrations. Data was fit into the Hill equation. Each assay was run in triplicate and the results shown are the mean ± S.E.M.

To determine whether CTPK was able to use NADPH as an alternate cofactor to NADH, CTPK was assayed under saturating ADP (2 mM) and PEP (10 mM) concentrations in the presence of 0.2 mM NADPH under standard assay conditions as described in "Materials and Methods". Results indicate that CTPK is able to use NADPH in replace of NADH but to a much lesser extent (data not shown). CTPK only reached about half its maximal velocity (V_{max} = 28.08) when using NADPH compared to NADH (V_{max} = 51.05) under identical assay conditions. These results indicate that NADH is the preferred substrate compared to NADPH and was used in all subsequent CTPK enzyme assays.

d) Nucleotide specificity

Results in Table 11 indicate that PK from *C. trachomatis* has broad specificity for nucleoside diphosphates. CTPK exhibited Michaelis-Menten kinetics with respect to each NDP tested under saturating PEP conditions at 10 mM $MgCl_2$ as described in "Materials and Methods" (Fig. 25A). The Lineweaver-Burk plot of the NDPs show a set of lines intersecting at a point on the y-axis, a characteristic described for a competitive inhibitor (Dixon M., 1979; Eisenthal R., 1992), suggesting that the NDPs compete for the same binding site on CTPK (Fig. 25B). In contrast to NDPs, CTPK was unable to use Pi as an alternate substrate to ADP as indicated by its complete absence of activity (data not shown). Taken together, these results indicate that ADP is by far the best phosphate acceptor as indicated by its lowest K_m value and highest V_{max} value.

Table 11: Use of alternative nucleoside diphosphates by *C. trachomatis* PK[a]

Nucleotide	K_m (mM)	V_{max} (units/mg)
ADP	0.63 ± 0.02	57.86 ± 1.07
GDP	2.90 ± 0.14	14.70 ± 0.30
UDP	4.92 ± 0.04	15.80 ± 0.19
IDP	5.09 ± 0.05	7.87 ± 0.36
CDP	6.26 ± 0.05	10.36 ± 0.39

[a]Results are means ± standard errors for three determinations. Kinetic parameters were obtained by fitting into Michaelis-Menten equation. Enzyme assay conditions were at 10 mM PEP, 10 mM $MgCl_2$ under standard buffer conditions as described in "Materials and Methods".

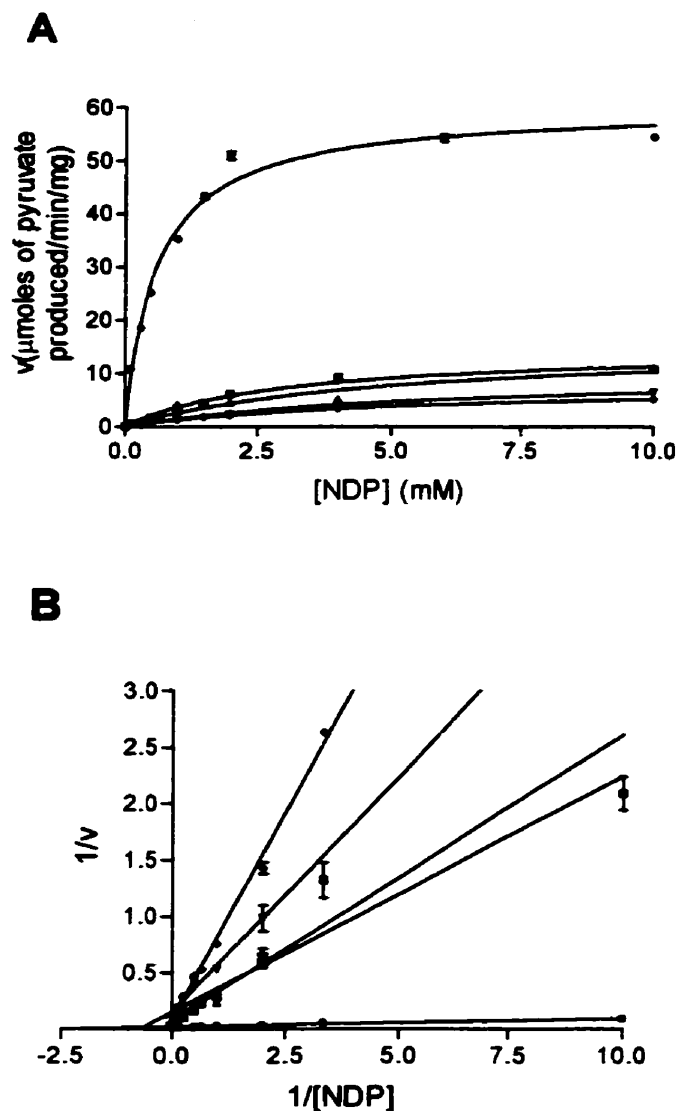


Figure 25. Effect of different nucleoside diphosphates on CTPK activity. Enzyme activity was assayed at 25°C, 10 mM $MgCl_2$ and pH 7.3 as described in "Materials and Methods". A) Michaelis-Menten and B) Lineweaver-Burk plots of CTPK activity with ADP (●), GDP (■), CDP (▼), IDP (◆) or UDP (▲) as the variable substrate under saturating PEP conditions (10 mM). Each assay was run in triplicate and the results shown are the mean ± S.E.M.

e) PEP kinetics in the absence and presence of activators

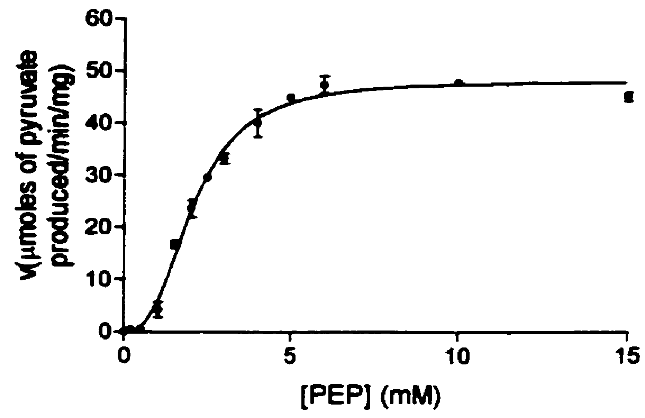
A variety of compounds were tested as possible effectors of purified CTPK including F26BP, an effector known to activate other PKs from some eukaryotic parasites (Fothergill-Gillmore *et al.*, 2000). A summary of the CTPK kinetic parameters with respect to PEP is shown in Table 12. In the absence of any effector, CTPK displayed sigmoidal kinetics with respect to PEP under saturating ADP conditions (Fig. 26A). The Hill coefficient (n) value (2.67 ± 0.24) (Table 12) indicates that CTPK showed positive cooperativity towards PEP (Cornish-Bowden and Koshland, 1975). The Lineweaver-Burk plot is concave and upward which further demonstrates the positive cooperativity of CTPK for PEP (Fig. 26C). The $S_{0.5}$ value for PEP (3.05 ± 0.05) was calculated from Hill plot (Fig. 26B) and is comparable to other PK sources (Collins *et al.*, 1995; Sakai *et al.*, 1986; Schramm *et al.*, 2000; Waygood and Sanwal, 1974).

Table 12. The effect of various metabolites on the activity of *C. trachomatis* PK with respect to PEP[a]

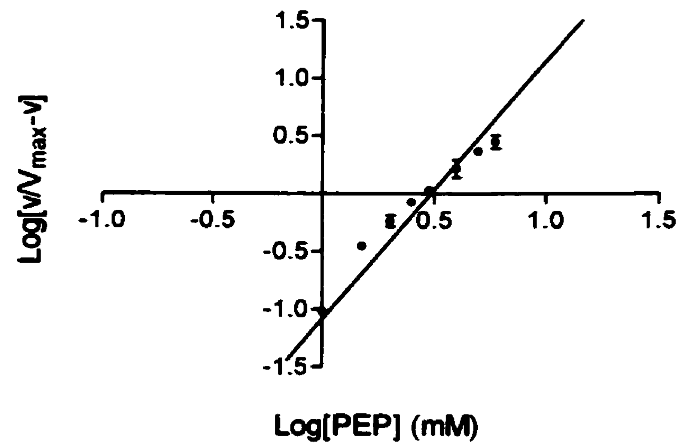
	PEP		
Effector	V_{max} (units/mg)	$S_{0.5}$ (mM)	n
None	48.15 ± 1.15	3.05 ± 0.05	2.67 ± 0.24
1 mM Ribose-5-P	53.08 ± 1.89	2.37 ± 0.17	1.68 ± 0.21
1 mM Glucose-6-P	45.90 ± 1.58	2.50 ± 0.11	1.94 ± 0.25
1 mM Glucose-1-P	47.11 ± 0.87	2.75 ± 0.08	2.03 ± 0.06
1 mM Fructose-6-P	44.95 ± 0.48	2.42 ± 0.13	2.42 ± 0.12
1 mM Fructose-1-P	46.48 ± 0.50	2.43 ± 0.13	2.25 ± 0.11
1 mM GMP	40.04 ± 1.19	3.26 ± 0.11	2.49 ± 0.31
1 mM 3PGA	41.37 ± 1.24	3.16 ± 0.04	2.77 ± 0.37
1 mM Fructose-1,6-BP	47.92 ± 1.07	3.02 ± 0.04	2.59 ± 0.23
10 mM Fructose-1,6-BP	46.31 ± 0.77	3.19 ± 0.05	2.98 ± 0.21
1 mM Fructose-2,6-BP	67.11 ± 1.32	0.17 ± 0.005	0.94 ± 0.21

[a]Results are means ± standard errors for three determinations. Kinetic parameters for V_{max} and n were obtained by fitting data into the Hill equation and $S_{0.5}$ was determined from Hill plot as described in "Materials and Methods". Enzyme assays were conducted under standard conditions at a fixed concentration of 2 mM ADP, 0 or 1 mM effector, 10 mM $MgCl_2$ and at varying concentrations (0-10 mM) of PEP.

A)



B)



C)

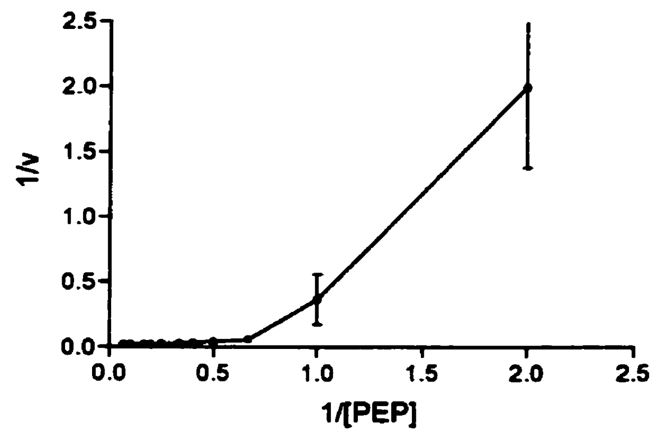
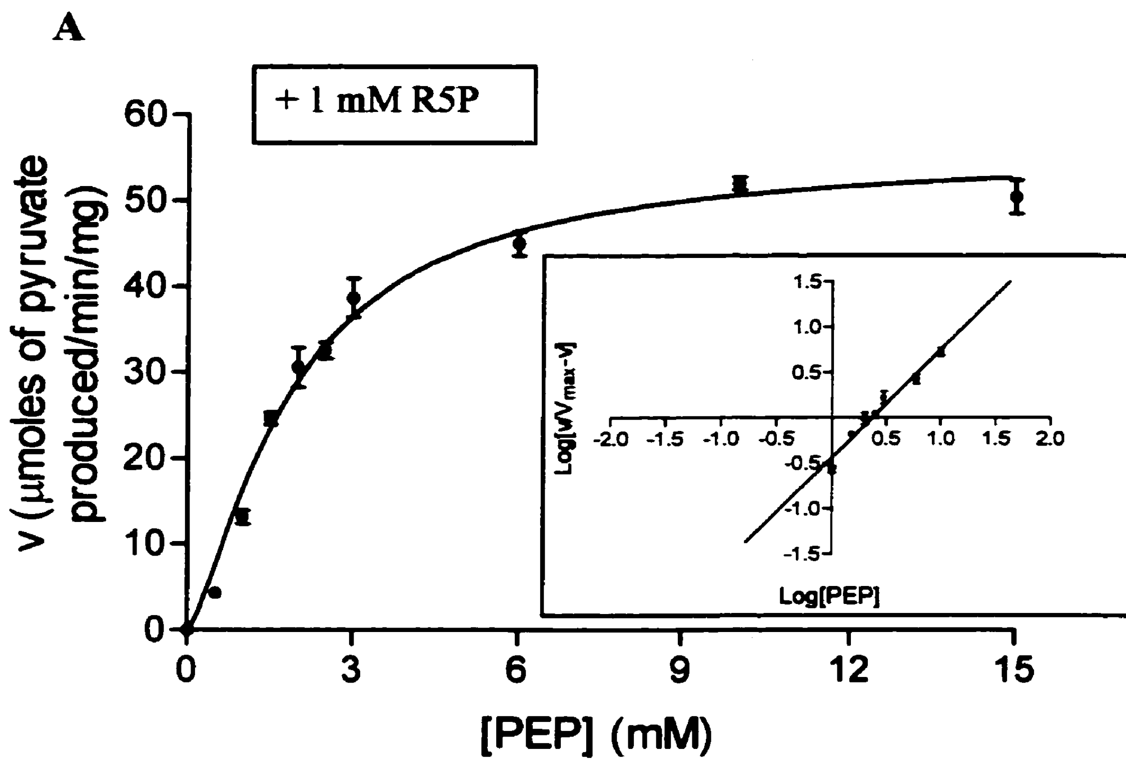


Figure 26. Saturation kinetics of CTPK with respect to PEP. Enzyme activity was determined at various concentrations of PEP at 25°C, pH 7.3 under saturating conditions of ADP as described in "Materials and Methods". A) PEP saturation curves for CTPK. Data were fitted into the Hill equation and the resulting fitted curves are shown. B) Hill plot of PEP saturation curves and C) Lineweaver-Burk plots of CTPK activity. The assay was run in triplicate and the mean ± S.E.M. are shown.

In the presence of 1 mM ribose-5-P (Fig. 27A), glucose-6-P (Fig. 27B), glucose-1-P (Fig. 27C), fructose-1-P (Fig. 27D), or fructose-6-P (Fig. 27E) CTPK displayed sigmoidal kinetics with respect to PEP, although to a lesser extent. The apparent $S_{0.5}$ for PEP was slightly reduced by about 1.2 fold and the n was reduced to a varying extent depending on the activator present. The presence of 1 mM GMP, 1 mM 3PGA, 1 or 10 mM F16BP (Figs. 27F-I respectively) had no effect on CTPK activity. The apparent $S_{0.5}$ for PEP as well as the n remained essentially the same as that found in the absence of either 3PGA, GMP or F16BP. In contrast, the presence of 1 mM F26BP had a dramatic effect on CTPK activity. The apparent $S_{0.5}$ for PEP was greatly reduced by about 17 fold and the kinetic behavior shifted from sigmoidal toward Michaelis-Menten (Fig. 27J) with an n value reduced to about 1.0. In addition, the presence of 1 mM F26BP also increased the V_{max} of the enzyme. These results suggest that F26BP is by far the most effective activator of CTPK decreasing the apparent $S_{0.5}$ for PEP to the greatest extent.

Figure 27. Effect of various metabolites on the kinetics of CTPK with respect to PEP. Assays were carried out at 25°C, pH 7.3 under saturating conditions of ADP with PEP as the variable substrate as described in "Materials and Methods". PEP saturation curves in the presence of A) 1 mM R5P, B) 1 mM G6P, C) 1 mM G1P, D) 1 mM F1P, E) 1 mM F6P, F) 1 mM GMP, G) 1 mM 3PGA, H) 1mM F16BP, I) 10 mM F16BP, J) 1 mM F26BP. Data were fitted into the Hill equation and the resulting fitted curves are shown. The hill plot of the data is shown in each inset. Each assay was run in triplicate and the mean ± S.E.M. are shown.



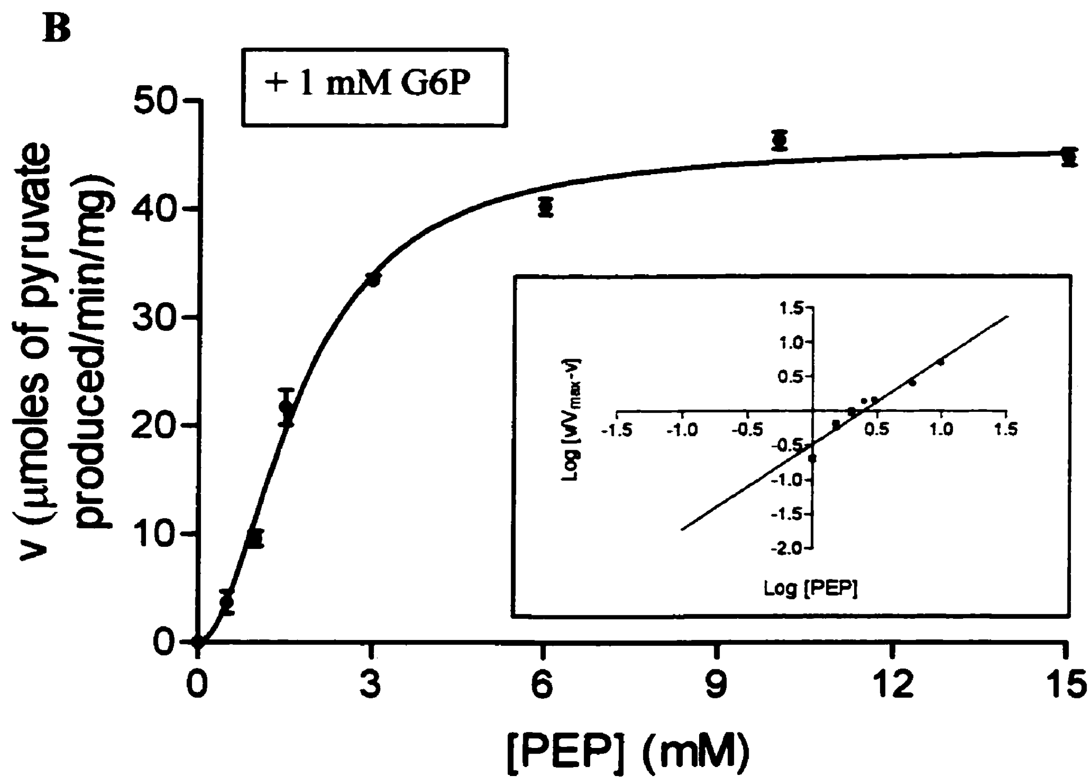
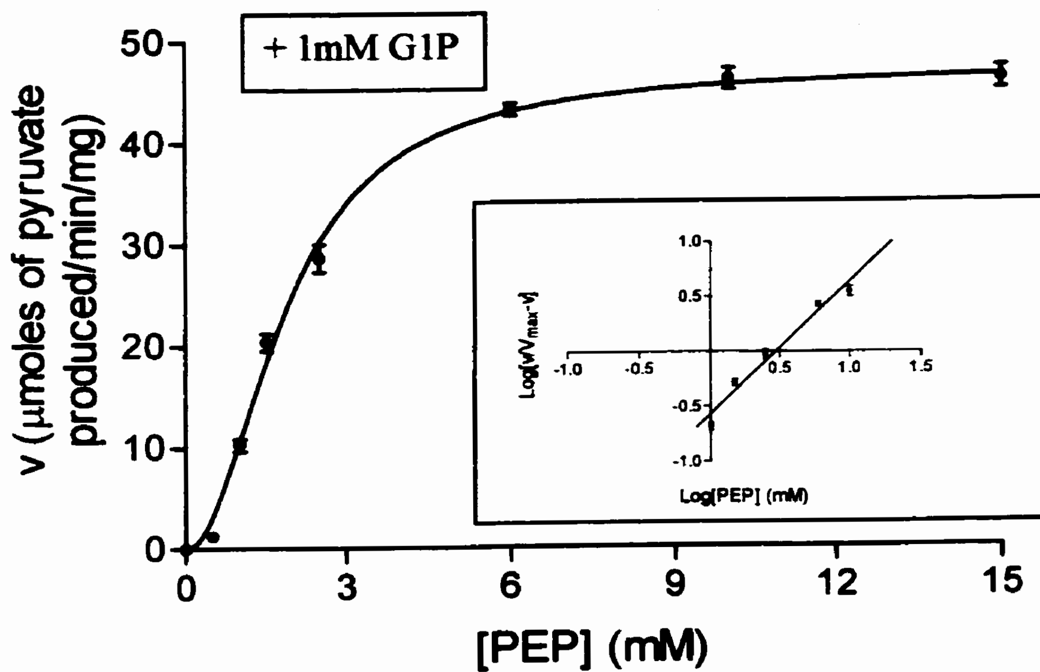


Fig. 27A and B

C



D

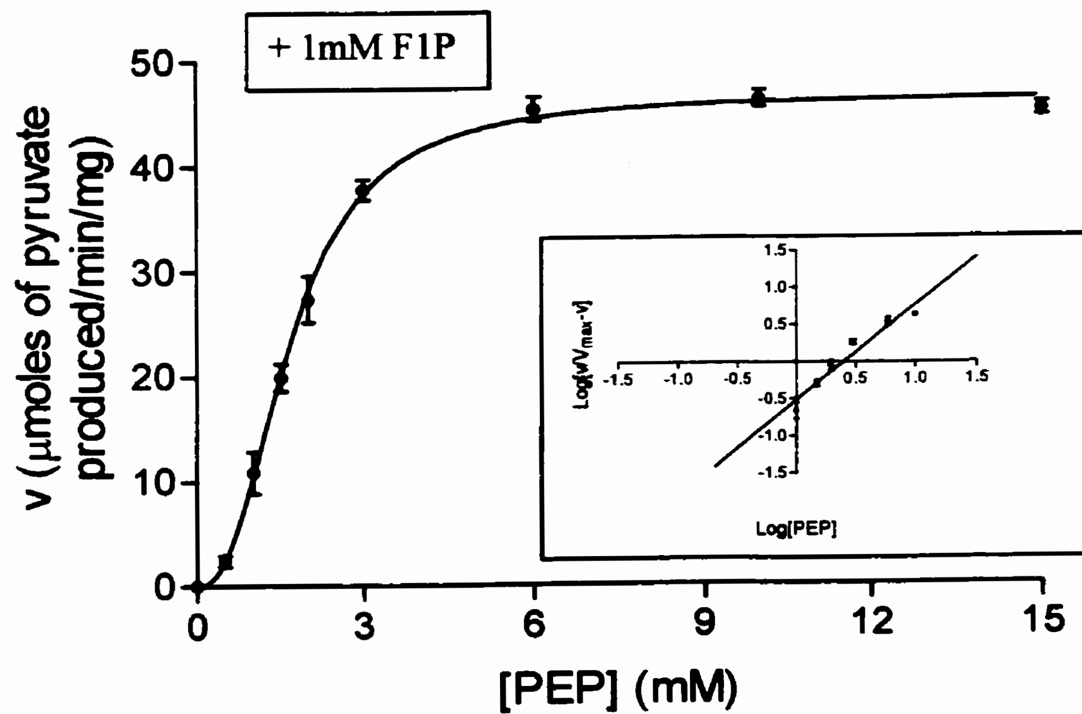
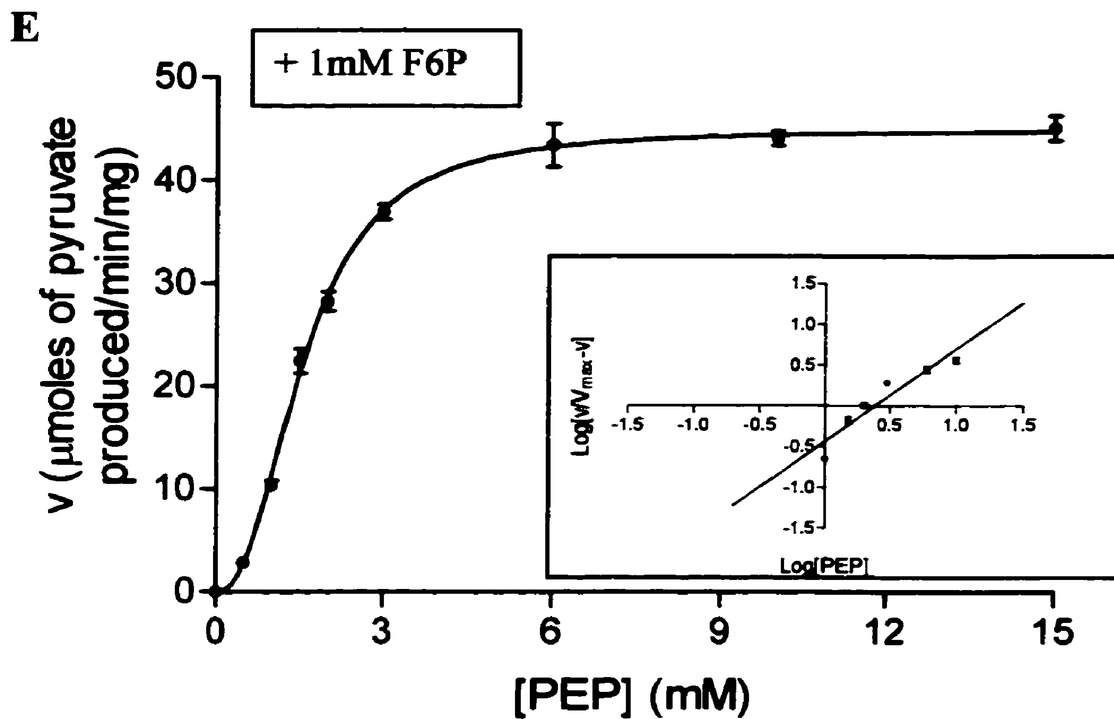


Fig. 27C and D

E



F

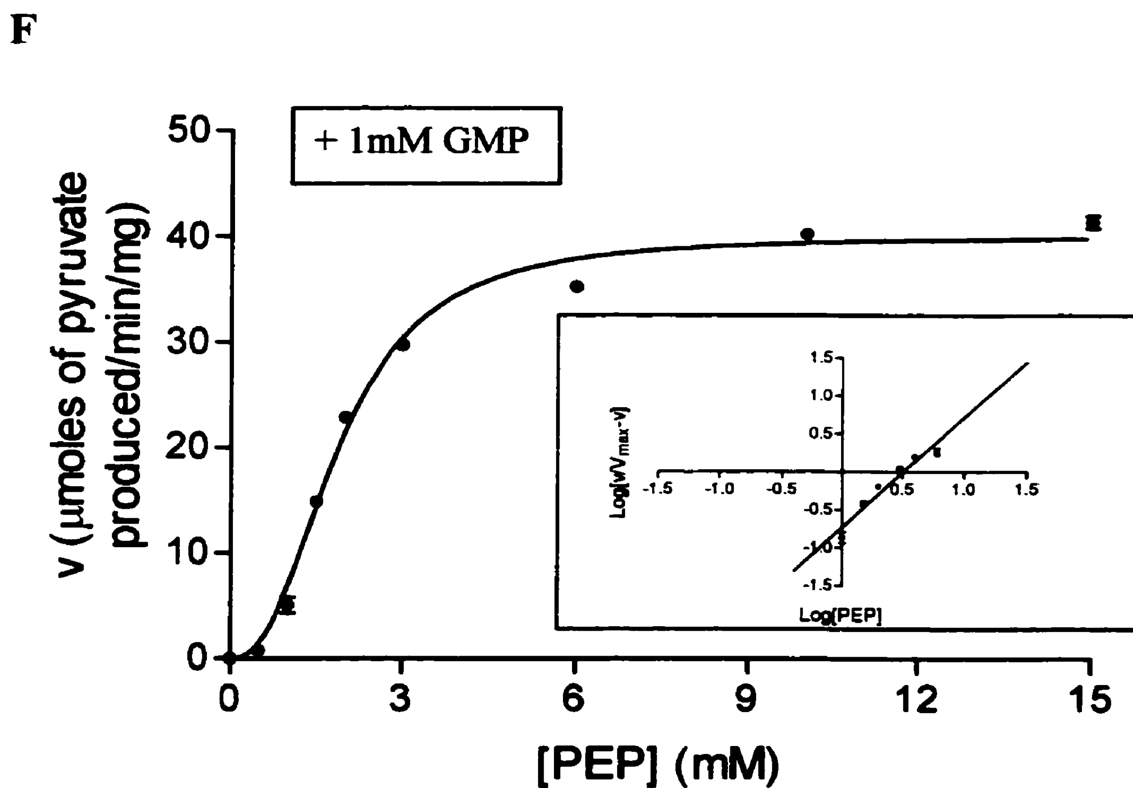
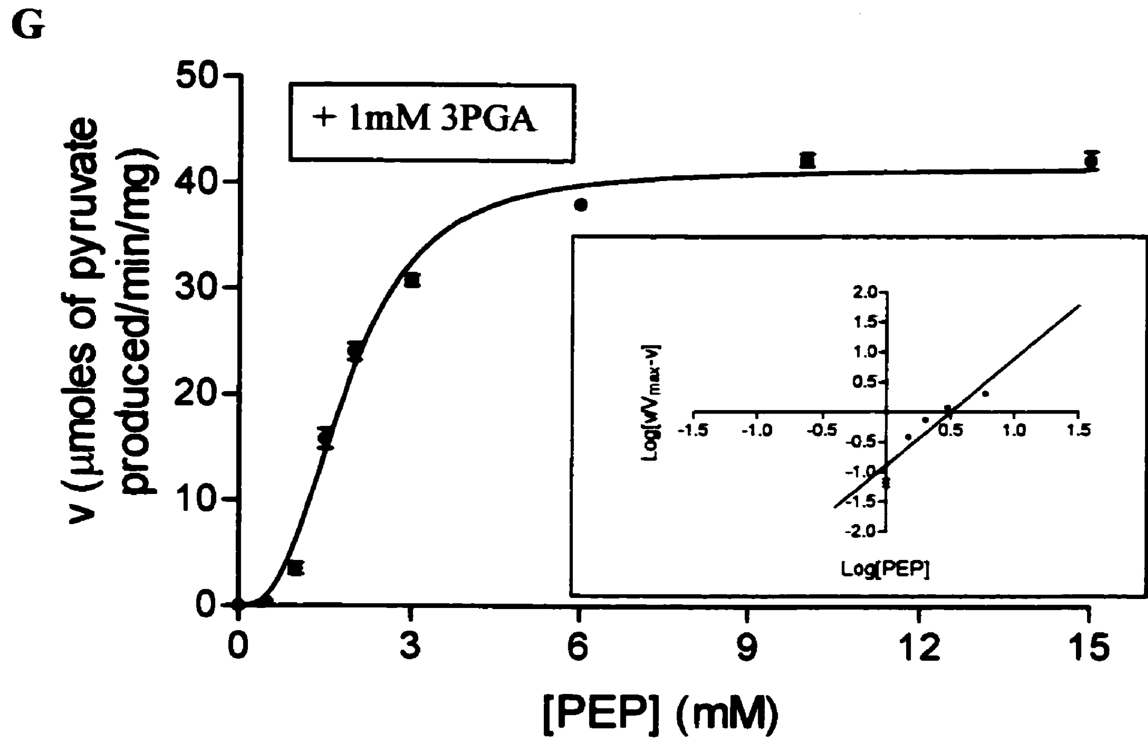


Fig. 27E and F



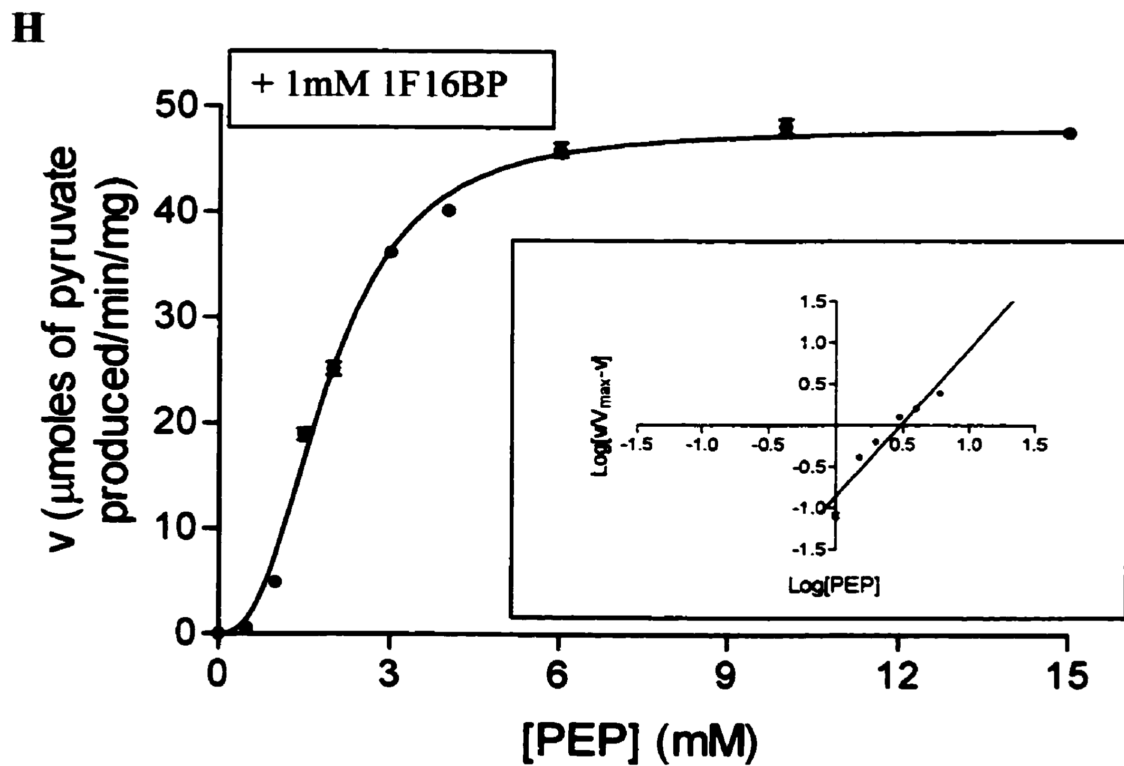
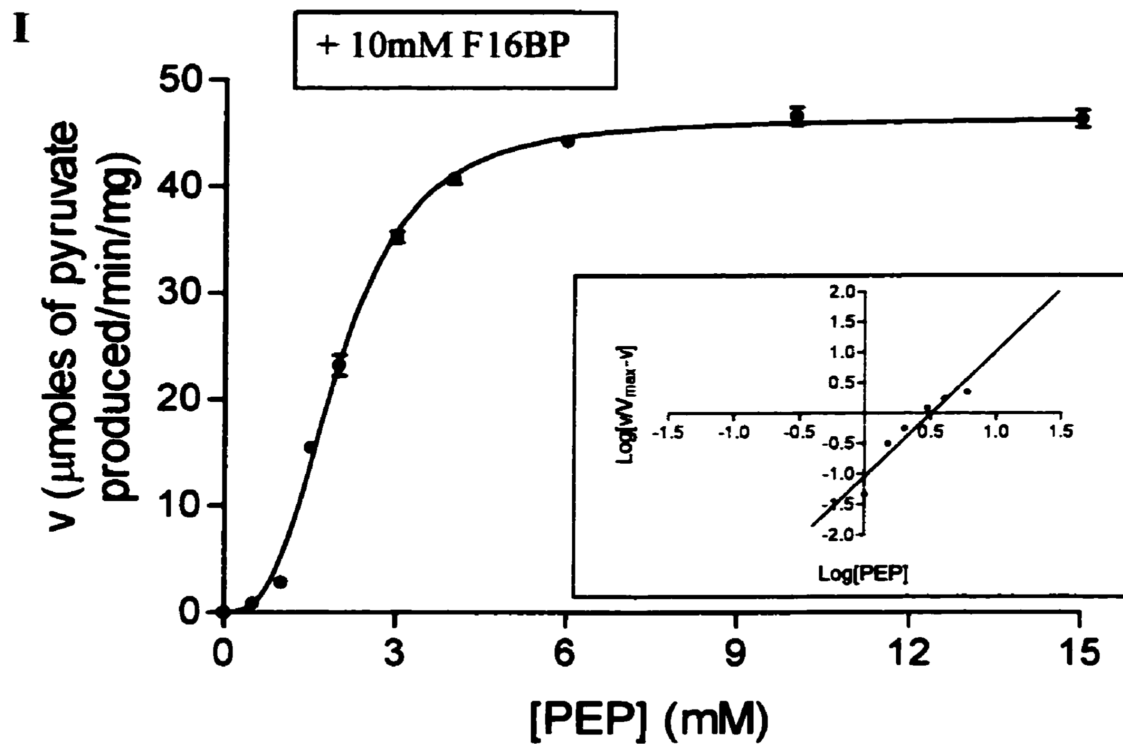


Fig. 27G and H



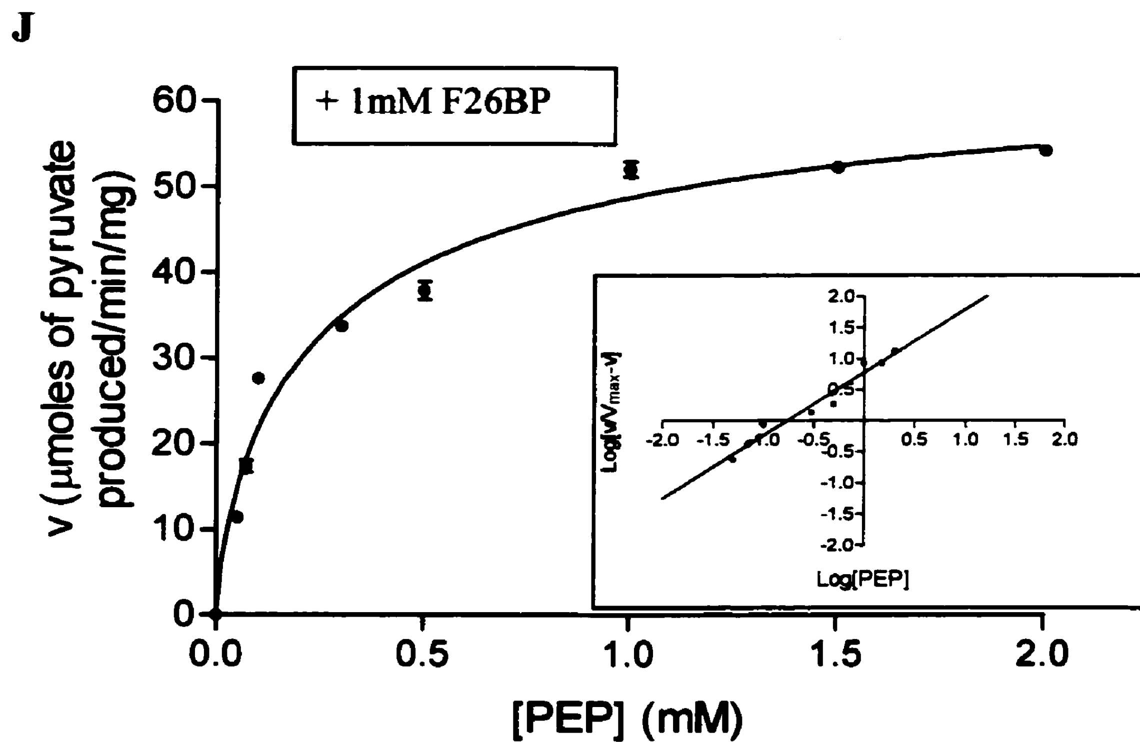


Fig. 27I and J

f) ADP kinetics in the absence and presence of F26BP

To determine CTPK kinetics with respect to ADP, only F26BP was used from the list of metabolites in Table 12, as it is the best activator of CTPK activity. CTPK was assayed under both saturating (10 mM) and subsaturating (1 mM) PEP conditions in the presence and absence of 1 mM F26BP. Figure 28A indicates that CTPK showed hyperbolic kinetics with respect to varying concentrations of ADP under saturating conditions of PEP. The addition of 1 mM F26BP slightly lowered the enzyme's apparent K_m for ADP but had no effect on the V_{max} or n (Table 13). When subsaturating PEP conditions were employed (Fig. 28B), the n was increased 1.5 fold and the enzyme only reached about half its maximal velocity. This decrease in activity is probably due to insufficient PEP concentrations. When 1 mM F26BP was added, the apparent K_m value for ADP was again slightly decreased, n was lowered to about 1.0 and maximal CTPK activity was restored (Table 13). These results indicate that the activator F26BP acts by facilitating the binding of PEP to the enzyme but has little effect on ADP.

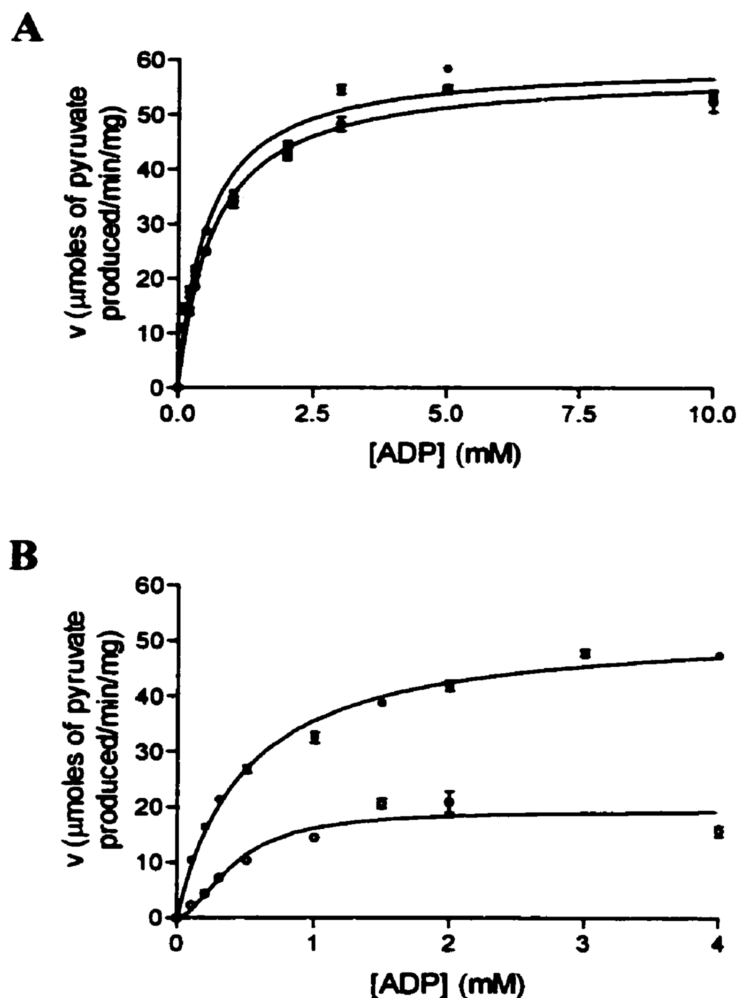


Figure 28. ADP kinetics for *C. trachomatis* PK in the absence and presence of F26BP. Enzyme activity was assayed at 25°C, pH 7.3 as described in "Materials and Methods". The assays were performed at A) saturating PEP (10 mM) conditions in the absence (o) or in the presence (•) of 1 mM F26BP, or B) subsaturating PEP (1 mM) conditions in the absence (o) or presence (•) of 1 mM F26BP. All data was fit into Michaelis-Menten equation except for data obtained under subsaturating PEP conditions in the absence of F26BP which was fit into the Hill equation. The resulting fitted curves are shown. Each assay was run in triplicate and the mean ± S.E.M. are presented.

Table 13: Kinetic parameters of *C. trachomatis* PK with ADP and F26BP[a]

Variable Substrate	Fixed Substrate	V_{max} (units/mg)	K_m or $S_{0.5}$ (mM)	n
ADP	1 mM PEP[b]	19.38 ± 2.14	0.51 ± 0.05	1.82 ± 0.68
	1 mM PEP + 1 mM F26BP	52.62 ± 4.19	0.48 ± 0.01	1.00 ± 0.02
	10 mM PEP	57.86 ± 1.07	0.63 ± 0.01	1.05 ± 0.03
	10 mM PEP + 1 mM F26BP	59.70 ± 0.81	0.53 ± 0.01	0.95 ± 0.06
F26BP[c]	2 mM ADP + 1 mM PEP	34.97 ± 0.49	0.009 ± 0.001	0.82 ± 0.05

[a]Results are means ± standard errors for three determinations. Values for V_{max} and K_m were determined by fitting data into the Michaelis-Menten equation and n was determined by Hill plot. Enzyme assays were conducted at 25°C at pH 7.3 as described in "Materials and Methods".
[b and c]Values for V_{max} and n was determined by fitting data into the Hill equation and $S_{0.5}$ values were determined by Hill plot.

g) F26BP Kinetics

Results in Table 12 indicate that of the metabolites tested, F26BP is the best activator of CTPK. To determine the kinetics of CTPK with respect to F26BP, CTPK was assayed at a fixed concentration of 2 mM ADP and 1 mM PEP at variable F26BP concentrations (Table 13). The enzyme showed slight negative cooperativity towards varying concentrations of F26BP (Fig. 29) as indicated by the n which is below 1.0 (Table 13). This negative cooperativity characteristic has also been found in yeast PK with respect to its activator, F16BP (Jurica *et al.*, 1998). The apparent $S_{0.5}$ for F26BP was found to be in the micromolar range value as calculated from Hill plots (Fig 29, inset) which is similar to *Leishmania* (Rigden *et al.*, 1999) and *Trypanosome* (Ernest *et al.*, 1998) PKs.

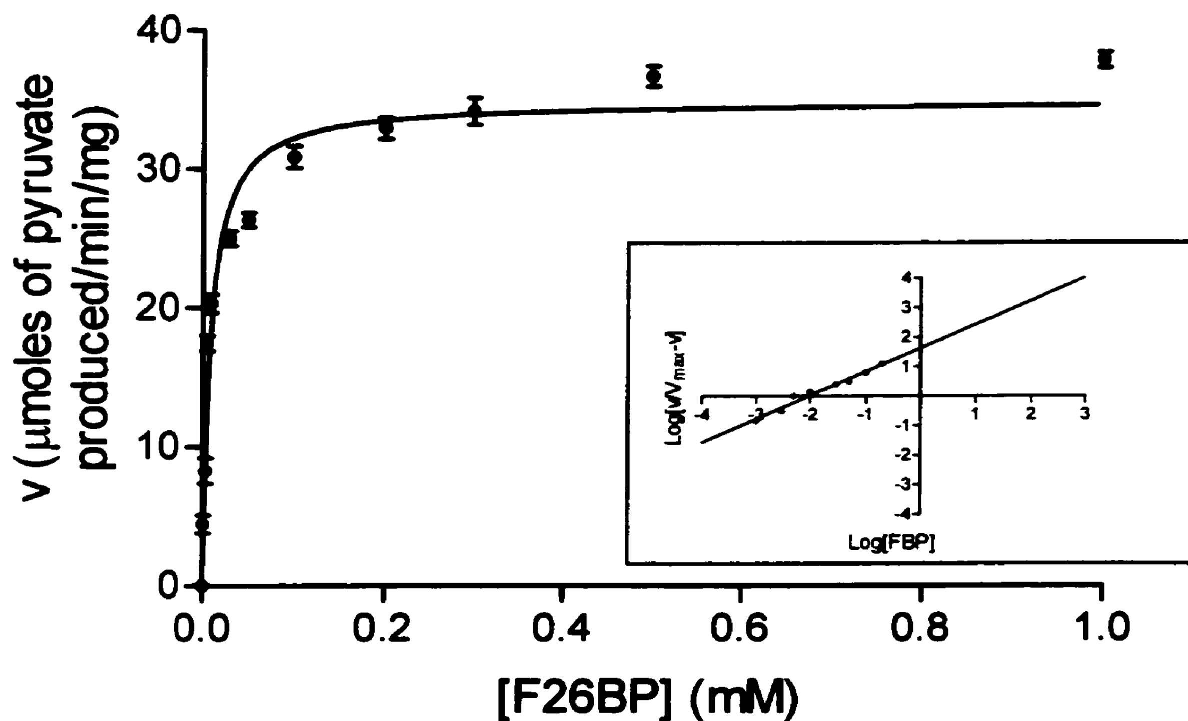


Figure 29. Kinetic parameters of *C. trachomatis* PK with respect to F26BP. Enzyme activity was assayed at 25°C, pH 7.3 as described in "Materials and Methods". The assays were performed at variable F26BP concentrations, at saturating ADP (2.0 mM) and subsaturating PEP (1.0 mM) conditions. Data were fitted into the Hill equation and the resulting fitted curves are shown. The hill plot of the data is shown in the inset. The assay was run in triplicate and the mean ± S.E.M. are shown.

h) Inhibitors

Nucleoside triphosphates have been shown to inhibit pyruvate kinases from various sources (Chuang and Utter, 1979; Lin *et al.*, 1989; Smith *et al.*, 2000; Tanaka *et al.*, 1995; Turner and Plaxton, 2000). This also holds true for the *C. trachomatis* enzyme. Under saturating ADP conditions, the presence of ATP increased the apparent $S_{0.5}$ for PEP and decreased the apparent V_{max} in a dose dependent manner (Table 14). The n values however, remained relatively consistent, regardless of the presence of ATP. Figure 30 indicates that CTPK maintained sigmoidal kinetics with respect to PEP in the presence of ATP which is further demonstrated by Lineweaver-Burk plots which are concave and upward (data not shown).

Table 14: Effect of various inhibitors on the kinetic response of *C. trachomatis* PK with respect to PEP in the absence[a] and presence[b] of F26BP

	PEP		
Inhibitor	V_{max} (units/mg)	$S_{0.5}$ or K_m (mM)	n
none	43.99 ± 1.14	2.98 ± 0.09	2.72 ± 0.29
0.1 mM ATP	35.35 ± 1.75	5.54 ± 0.05	2.79 ± 0.49
1.0 mM ATP	26.57 ± 0.33	11.00 ± 0.14	2.33 ± 0.07
0.1 mM GTP	42.23 ± 1.22	4.46 ± 0.05	2.88 ± 0.31
1.0 mM GTP	31.82 ± 1.46	7.12 ± 0.05	4.18 ± 0.85
1.0 mM AMP	33.08 ± 1.05	6.41 ± 0.17	2.54 ± 0.25
0.1 mM ATP + 1.0 mM F26BP	56.79 ± 0.72	0.29 ± 0.01	1.07 ± 0.02
1.0 mM ATP + 1.0 mM F26BP	61.02 ± 0.51	0.74 ± 0.02	0.97 ± 0.03
1.0 mM GTP + 1.0 mM F26BP	53.81 ± 0.42	0.21 ± 0.01	0.96 ± 0.05
1.0 mM AMP + 1.0 mMF26BP	63.43 ± 0.28	0.23 ± 0.02	1.04 ± 0.02

Results are means ± standard errors for three determinations. Enzyme assays were conducted at standard, saturating conditions as described in "Materials and Methods".
[a]Values for V_{max} and n were obtained by fitting data into the Hill equation and $S_{0.5}$ by Hill plot.
[b]Values for V_{max} and K_m were obtained by fitting data Michaelis-Menten equation and n was determined by Hill plot.

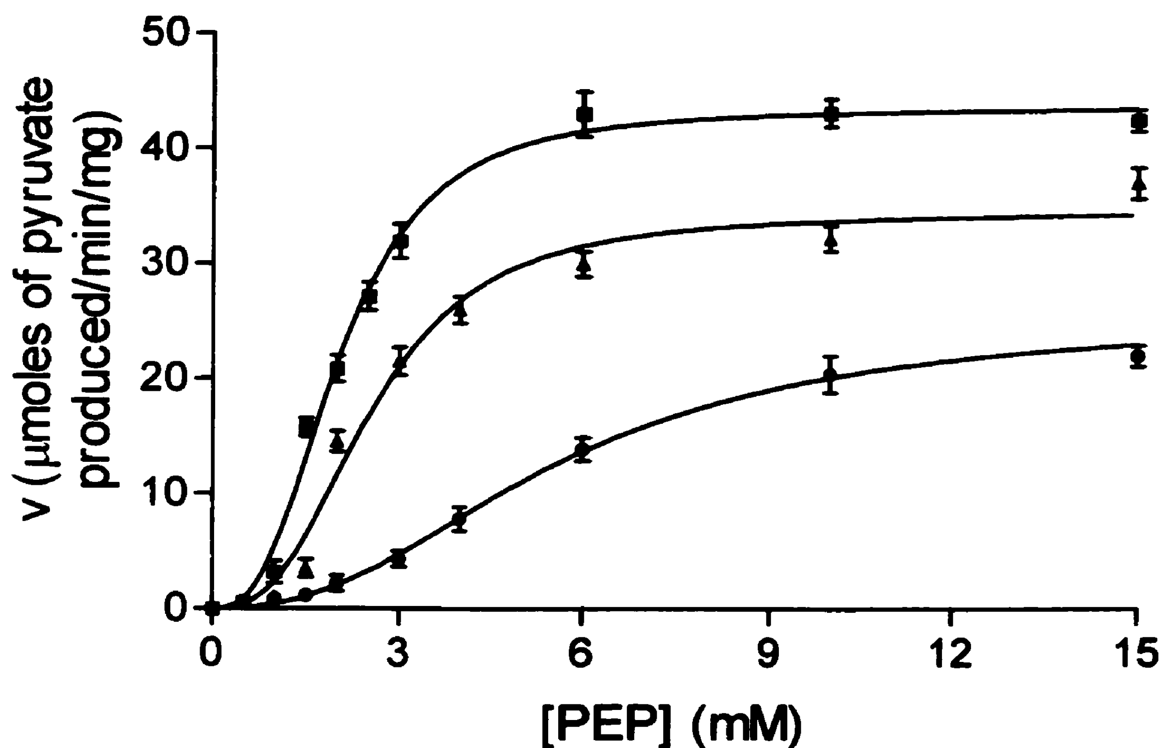


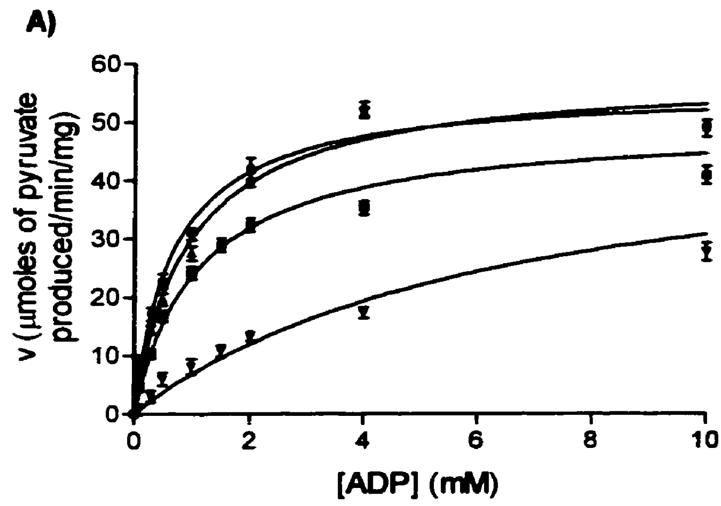
Figure 30. Effect of ATP on the kinetic response of *C. trachomatis* PK with respect to PEP. Enzyme activity was assayed at 25°C, pH 7.3 and at 10 mM $MgCl_2$ as described in "Materials and Methods". Assays were conducted at variable PEP concentrations, saturating ADP conditions in the presence of 0 mM ATP (■), 0.1 mM ATP (▲) or 1 mM ATP (●). Data were fitted into the Hill equation and the resulting fitted curves are shown. Each assay was run in triplicate and the mean ± S.E.M. are presented.

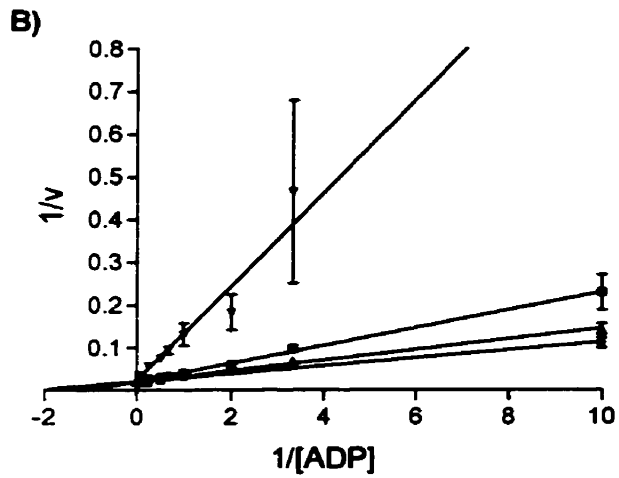
When ADP was the variable substrate, hyperbolic saturation curves were obtained when CTPK was assayed in the presence of 0, 0.5, 1 or 3 mM ATP (Fig. 31A). Increasing the concentration of ATP increased the apparent K_m for ADP but had no effect on V_{max} (Table 15). Lineweaver-Burk plots of the saturation data for the different ATP concentrations gave a series of straight lines, which intersected at a point on the y axis (Fig 31B). Using Dixon plot analysis, the K_i for ATP was determined to be 0.75 mM (Fig. 31C). These results indicate that ATP is a competitive inhibitor with respect to ADP.

Table 15: Effect of various inhibitors on the kinetic response of *C. trachomatis* PK with respect to ADP[a]

	ADP	
Inhibitor	V_{max} (units/mg)	K_m
None	57.02 ± 0.26	0.64 ± 0.01
0.5 mM ATP	56.09 ± 1.69	0.85 ± 0.01
1.0 mM ATP	50.55 ± 0.74	1.05 ± 0.03
3.0 mM ATP	50.57 ± 0.48	5.77 ± 0.01
0.5 mM GTP	51.16 ± 0.30	0.79 ± 0.01
1.0 mM GTP	49.76 ± 0.40	0.83 ± 0.01
2.0 mM GTP	51.23 ± 0.21	1.18 ± 0.04
0.5 mM AMP	54.69 ± 0.16	0.73 ± 0.01
1.0 mM AMP	55.08 ± 0.39	0.78 ± 0.01
2.0 mM AMP	56.43 ± 0.10	1.23 ± 0.04
3.0 mM AMP	47.49 ± 1.10	1.55 ± 0.06

[a]Results are means ± standard errors for three determinations. Values for V_{max} and K_m were obtained by fitting data into Michaelis-Menten equation. Enzyme assays were conducted under standard, saturating conditions at 10 mM $MgCl_2$ as described in "Materials and Methods".


A)
v (μmoles of pyruvate produced/min/mg)
60
50
40
30
20
10
0
0
2
4
6
8
10
[ADP] (mM)


B)
1/v
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
-2
0
2
4
6
8
10
1/[ADP]

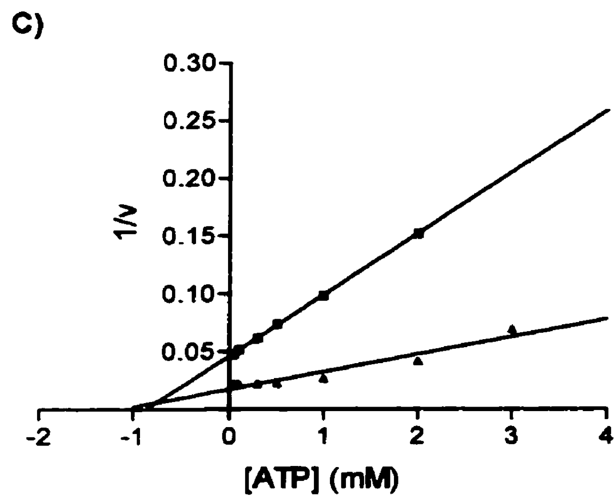

C)
1/v
0.30
0.25
0.20
0.15
0.10
0.05
-2
-1
0
1
2
3
4
[ATP] (mM)

Figure 31. Effect of ATP on the kinetic response of *C. trachomatis* PK with respect to ADP. Enzyme activity was assayed under saturating conditions of PEP (10 mM) at 10 mM $MgCl_2$, 25°C; pH7.3 as described "Materials and Methods". The assays were performed at either variable ADP concentrations in the presence of various ATP concentrations (0, 0.5, 1 or 3 mM), or at variable ATP concentrations in the presence of either 0.3 or 2 mM ADP. A) ADP saturation curves in the presence of 0 mM ATP (●), 0.5 mM ATP (▲), 1 mM ATP (■) or 3 mM ATP (▼). Data was fit into the Michaelis-Menten equation and the resulting fitted curves are shown. Each assay was run in triplicate and the mean ± S.E.M. are presented. B) Double-reciprocal plots of the inhibition of CTPK by ATP at 0 mM ATP (●), 0.5 mM ATP (▲), 1 mM ATP (■) or 3 mM ATP (▼). Each assay was run in triplicate and the mean ± S.E.M. are shown. C) Dixon plot of 1/v as a function of 1/[ATP] at 10 mM PEP and 0.3 mM ADP (■), and at 10 mM PEP and 2.0 mM ADP (▲). Results shown are the mean of two separate experiments.

Similar to ATP, GTP also increased the apparent $S_{0.5}$ for PEP and decreased the apparent V_{max} in a dose-dependent manner (Table 14). However, unlike ATP, increasing GTP concentrations also increased the apparent n value with respect to PEP saturation kinetics (Table 14). The presence of GTP also increased the K_m for ADP (Table 15) in a dose-dependent manner and shifted PEP (Fig. 32) and ADP saturation curves (Fig. 33A) to the right. Lineweaver-Burk (Fig. 33B) and Dixon plots (Fig. 33C) indicate that GTP is also a competitive inhibitor with respect to ADP and has a K_i of 0.85 mM.

In contrast to most PK enzymes, CTPK was also inhibited by AMP (Table 14 and 15), an effector that usually activates bacterial PKs (Garcia-Olalla and Garrido-Pertierra, 1987; Sakai *et al.*, 1986; Tanaka *et al.*, 1995; Waygood *et al.*, 1975). Similar to ATP and GTP, the presence of increasing concentrations of AMP corresponded with an increase in the apparent $S_{0.5}$ for PEP (Table 14) and K_m for ADP (Table 15) which is reflected in the rightward shift in both PEP (Fig. 34) and ADP (Fig. 35A) saturation curves. Similar to ATP, the presence of AMP had little effect on n values with respect to PEP saturation kinetics (Table 14). Lineweaver-Burk plots of the various AMP concentrations (Fig. 35B) with respect to ADP gave the same pattern of lines as found in the presence of GTP (Fig. 33B) and ATP (Fig. 31B) indicating that AMP is also a competitive inhibitor of ADP. The K_i of AMP was deduced from a Dixon plot to be 0.90 mM (Fig. 35C). Concentrations up to 10 mM Pi were also tested, but were not found to inhibit CTPK activity (data not shown). Together these results indicate that ATP is a slightly better inhibitor of CTPK compared to GTP and AMP as it increased the apparent $S_{0.5}$ for both ADP and PEP to a greater extent.

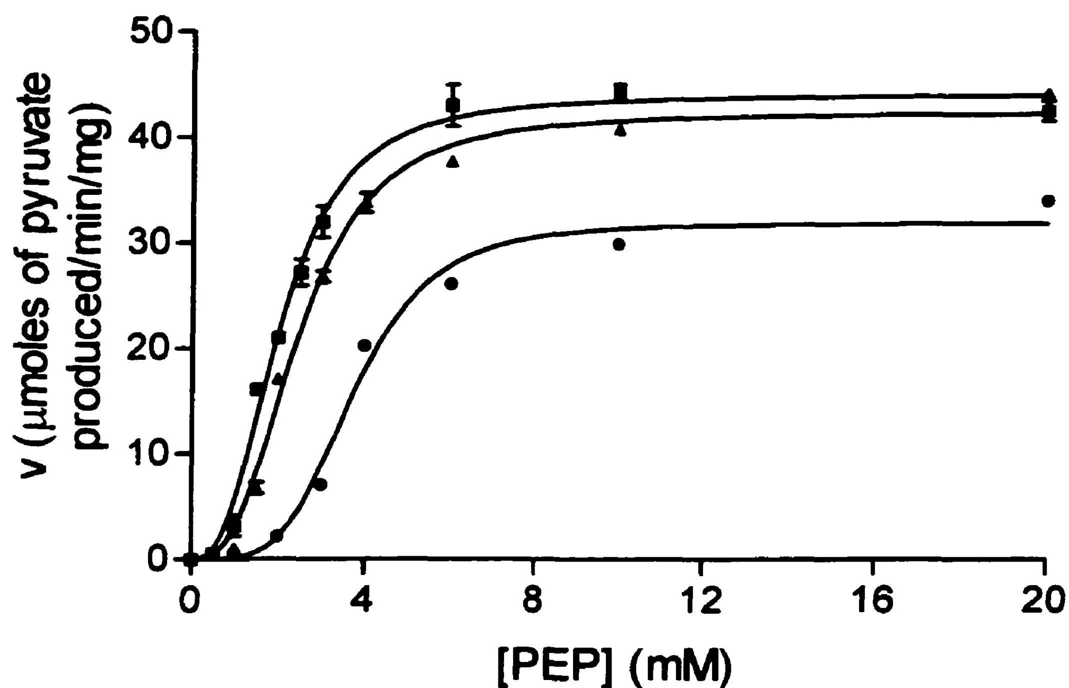
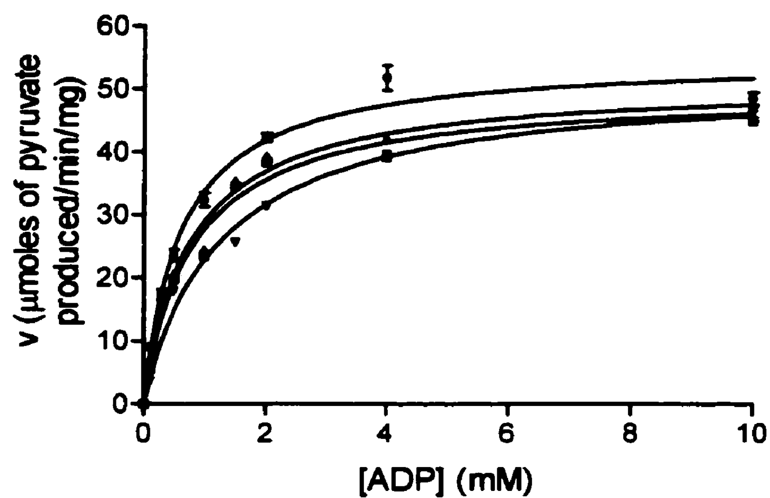
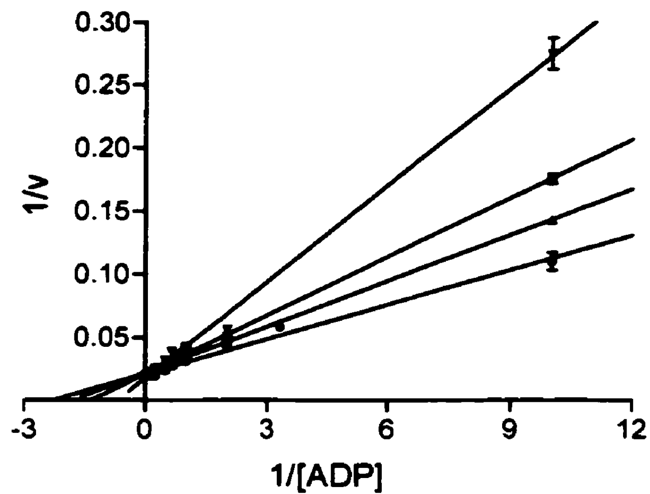


Figure 32. Effect of GTP on the kinetic response of *C. trachomatis* PK with respect to PEP Enzyme activity was assayed at 25°C, pH 7.3 and 10 mM $MgCl_2$ as described in "Materials and Methods". Assays were conducted at variable PEP concentrations, saturating ADP conditions in the presence of 0 mM GTP (■), 0.1 mM GTP (▲) or 1 mM GTP (●). Data were fitted into the Hill equation and the resulting fitted curves are shown. Each assay was run in triplicate and the mean ± S.E.M. are presented.

A



B



C

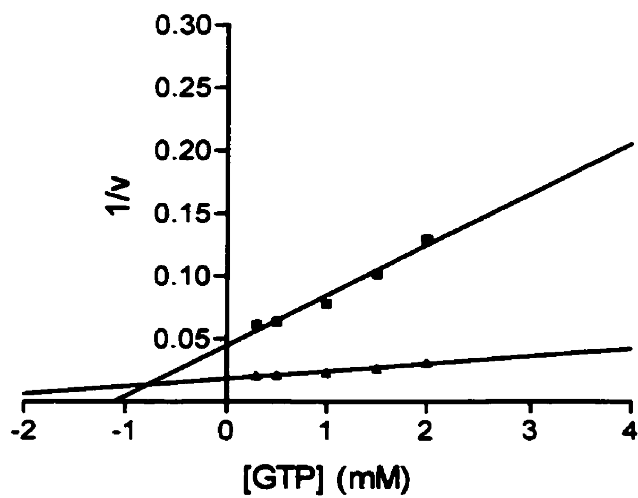


Figure 33. Effect of GTP on the kinetic response of *C. trachomatis* PK with respect to ADP. Enzyme activity was assayed under saturating conditions of PEP (10 mM) at 10 mM $MgCl_2$, 25°C; pH7.3 as described "Materials and Methods". The assays were performed at either variable ADP concentrations in the presence of various GTP concentrations (0, 0.5, 1 or 3 mM), or at variable GTP concentrations in the presence of either 0.3 or 2 mM ADP. A) ADP saturation curves in the presence of 0 mM GTP (●), 0.5 mM GTP (▲), 1 mM GTP (■) or 3 mM GTP (▼). Data was fit into the Michaelis-Menten equation and the resulting fitted curves are shown. Each assay was run in triplicate and the mean ± S.E.M. are presented. B) Double-reciprocal plots of the inhibition of CTPK by GTP at 0 mM GTP (●), 0.5 mM GTP (▲), 1 mM GTP (■) or 3 mM GTP (▼). Each assay was run in triplicate and the mean ± S.E.M. are shown. C) Dixon plot of 1/v as a function of 1/[GTP] at 10 mM PEP and 0.3 mM ADP (■), and at 10 mM PEP and 2.0 mM ADP (▲). Results shown are the mean of two separate experiments.

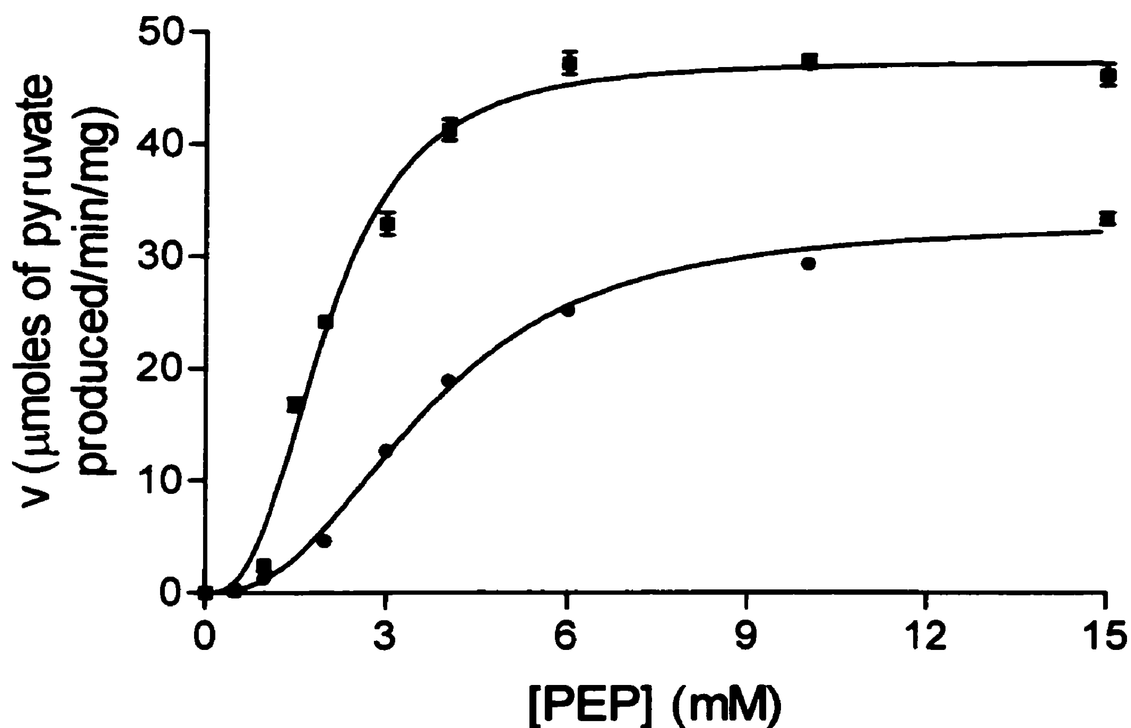
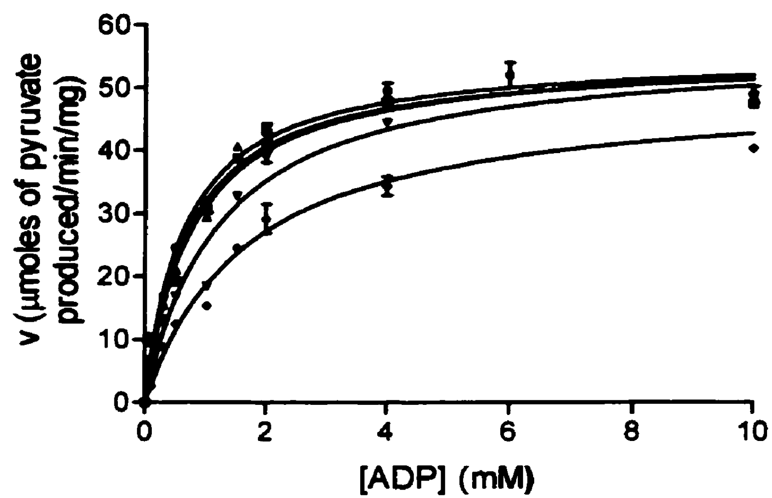
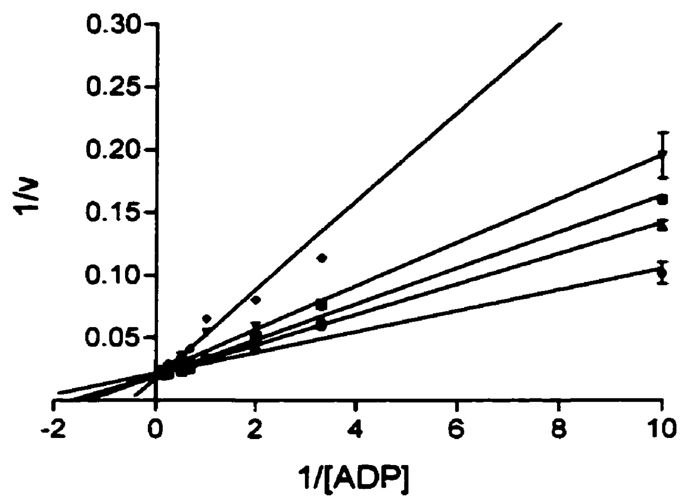


Figure 34. Effect of AMP on the kinetic response of *C. trachomatis* PK with respect to PEP. Enzyme activity was assayed at 25°C, pH 7.3 and at 10 mM $MgCl_2$ as described in "Materials and Methods". Assays were conducted at variable PEP concentrations, saturating ADP conditions in the presence of 0 mM AMP (■) or 1 mM AMP (●). Data were fitted into the Hill equation and the resulting fitted curves are shown. Each assay was run in triplicate and the mean ± S.E.M. are presented.

A



B



C

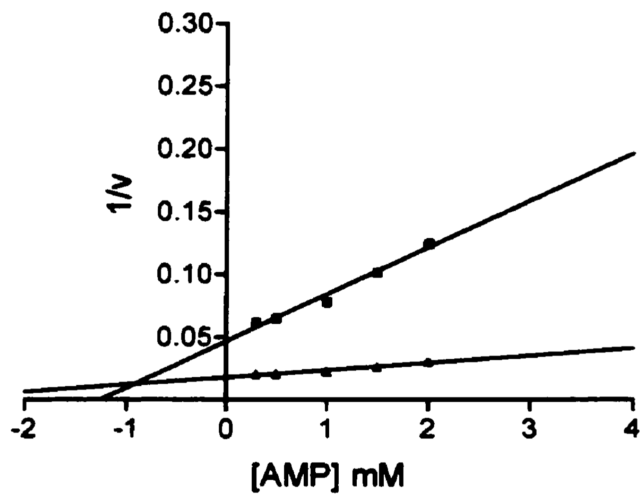
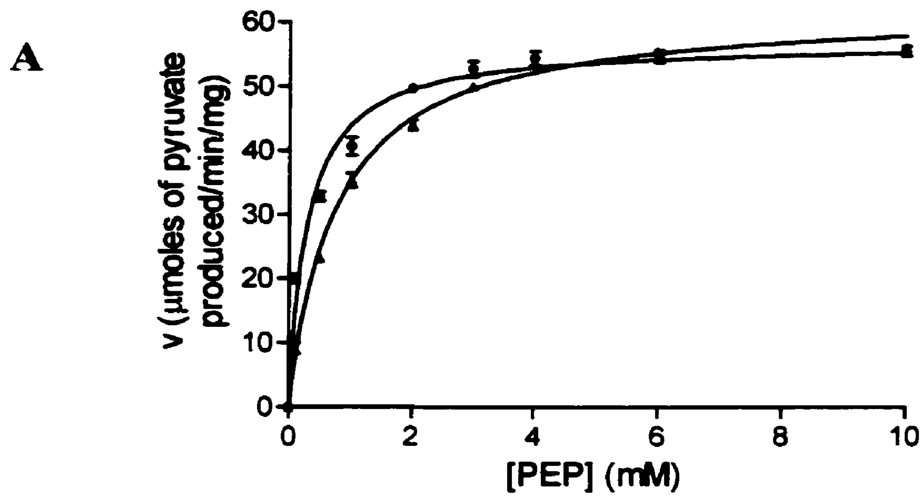


Figure 35. Effect of AMP on the kinetic response of *C. trachomatis* PK with respect to ADP. Enzyme activity was assayed under saturating conditions of PEP (10 mM) at 10 mM $MgCl_2$, 25°C; pH7.3 as described "Materials and Methods". The assays were performed at either variable ADP concentrations in the presence of various AMP concentrations (0, 0.5, 1, 2 or 3 mM), or at variable AMP concentrations in the presence of either 0.3 or 2 mM ADP. A) ADP saturation curves in the presence of 0 mM AMP (●), 0.5 mM AMP (▲), 1 mM AMP (■), 2 mM AMP (▼) or 3 mM AMP (♦). Data was fit into the Michaelis-Menten equation and the resulting fitted curves are shown. Each assay was run in triplicate and the mean ± S.E.M. are presented. B) Double-reciprocal plots of the inhibition of CTPK by AMP at 0 mM AMP (●), 0.5 mM AMP (▲), 1 mM AMP (■), 2 mM AMP (▼) or 3 mM AMP (♦). Each assay was run in triplicate and the mean ± S.E.M. are shown. C) Dixon plot of 1/v as a function of 1/[AMP] at 10 mM PEP and 0.3 mM ADP (■), and at 10 mM PEP and 2.0 mM ADP (▲). Results shown are the mean of two separate experiments.

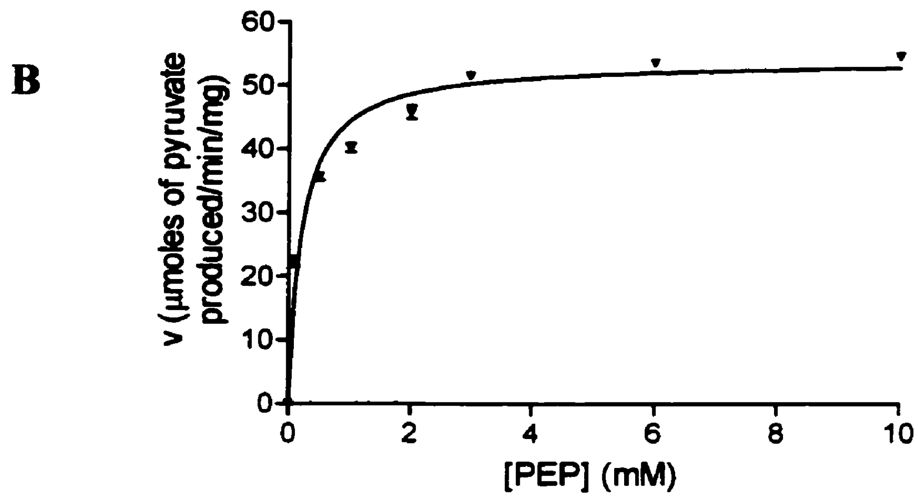
i) Effect of F26BP and various inhibitors on C. trachomatis PK activity

To determine whether the activator F26BP could relieve the inhibitory effects of ATP, GTP or AMP, CTPK was assayed at 2 mM ADP, 1 mM F26BP with PEP as the variable substrate in the presence of the different inhibitors (Table 14). In the presence of 0.1 mM ATP, F26BP almost completely reversed the inhibitory effects of ATP. The apparent $S_{0.5}$ for PEP was dramatically lowered and the sigmoidal curve was transformed into a hyperbolic one (Fig. 36A), converting the n to about 1.0. Similarly, F26BP was also very effective in relieving the inhibition exerted by the presence of 1.0 mM ATP, GTP or AMP (Table 14). In each case, the PEP saturation curves were shifted towards the left converting the sigmoidal curve into a hyperbolic one (Fig. 36A-C respectively). These results indicate that F26BP is not only able to facilitate the binding of PEP to CTPK, but is also able to relieve the inhibitory effects of ATP, GTP or AMP.

A



B



C

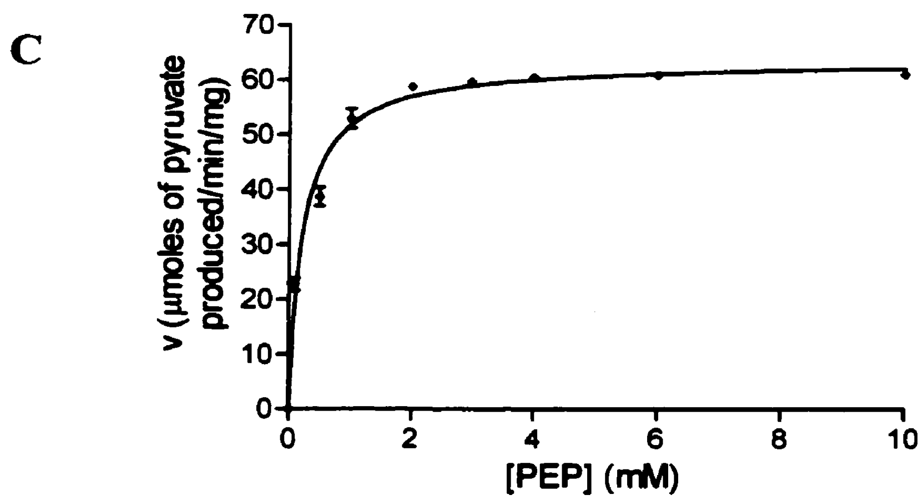


Figure 36. Effect of F26BP on the kinetic response of CTPK with respect to PEP in the presence of various inhibitors. Enzyme activity was measured under standard, saturating conditions at 25°C, pH 7.3, 10 mM $MgCl_2$ as described in "Materials and Methods". Assays were conducted at 2 mM ADP, 1 mM F26BP, variable PEP concentrations in the presence of A) 0.1 mM ATP (●) or 1 mM ATP (▲); B) 1 mM GTP or C) 1 mM AMP. All data was fit into Michaelis-Menten equation and the resulting fitted curves are shown. Each assay was run in triplicate and the mean ± S.E.M. are presented.

C. CARBON METABOLISM IN *C. TRACHOMATIS*

1. Metabolic pathways in *C. trachomatis* as inferred from the genome sequence

A carbon source such as glucose is an absolute necessity for cell growth. When growing on gluconeogenic carbon sources or in a nutrient rich environment where glucose has become limiting, enzymes required for *de novo* glucose synthesis are induced. For *E. coli* there are a wide variety of substrates including various sugars, amino acids and dicarboxylic acids, which can serve as gluconeogenic carbon sources (Lin, 1996; McFall, 1996). Detailed analysis of chlamydial genome sequence data (Kalman *et al.*, 1999; Read *et al.*, 2000; Stephens *et al.*, 1998) suggests that host derived glucose-6-phosphate is the primary carbon and energy source used to support parasite growth (McClarty, 1999). It was also noted, however, that chlamydiae contained key gluconeogenic enzymes and that it was possible that host derived glutamate or dicarboxylic acids could potentially support chlamydial growth (McClarty, 1999). A summary of potential routes of carbon metabolism in chlamydiae, as deduced from the available genome sequencing projects (Kalman *et al.*, 1999; Read *et al.*, 2000; Stephens *et al.*, 1998), is presented in Figure 37.

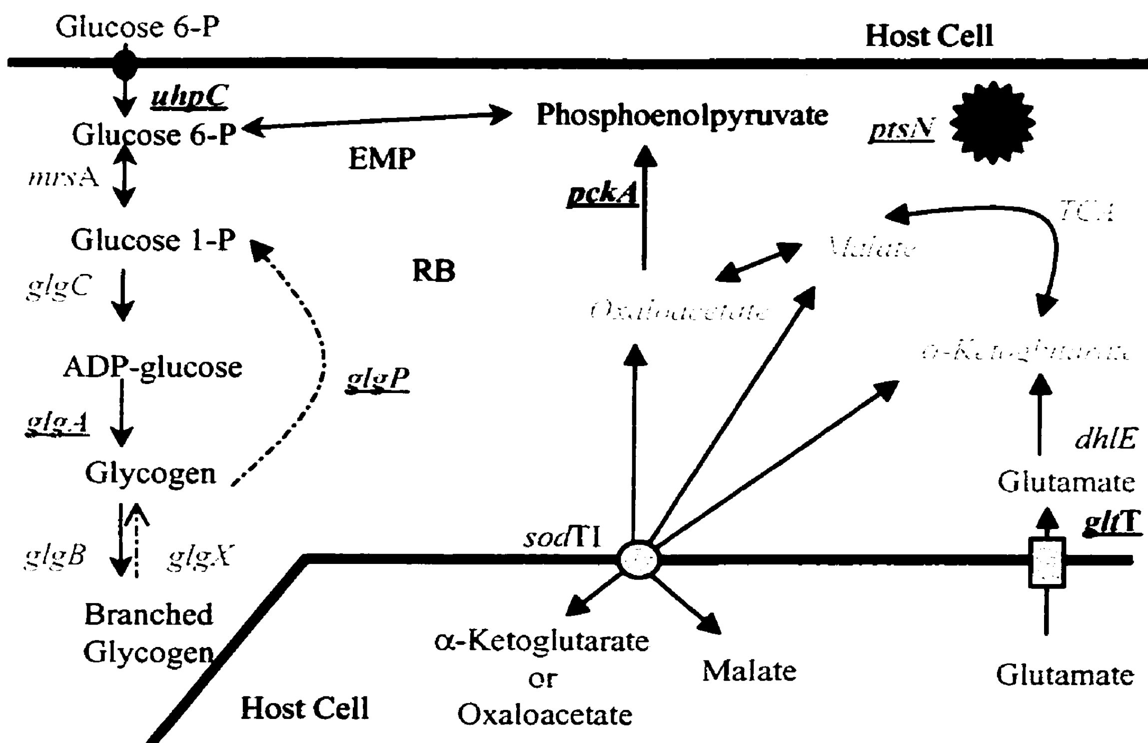


Figure 37. Pathways involved in carbon metabolism in *C. trachomatis* as deduced from the genome sequence (Stephens *et al.*, 1998; Kalman *et al.*, 1999; Read *et al.*, 2000). Gene homologues are designated according to the genome project (Stephens *et al.*, 1998). See Table 19 for details. EMP, Embden-Meyerhof-Parnas (pathway); TCA tricarboxylic acid (cycle). Genes analyzed in this study are underlined and in bold.

Several different carbon options were examined but glutamate, oxaloacetate, malate and 2-oxoglutarate were selected because information from the genome sequence indicates that chlamydia contains the necessary genes to metabolize these carbon sources (Fig. 37) into the the 12 precursor metabolites (Table 1) that ultimately construct macromolecules such as DNA, RNA, lipids, and LPS that make up the cell.

Glutamate may be acquired from the host cell cytoplasm through a glutamate transporter homolog, *gltT* (Fig. 37). Glutamate could then be converted into 2-oxoglutarate by the homolog of a gene (*dhlE*) that encodes for a dehydrogenase. The 2-oxoglutarate formed could then enter the partial TCA cycle and get oxidized to oxaloacetate, which would provide three precursors, oxaloacetate, succinyl-CoA and 2-oxoglutarate. The ortholog for phosphoenolpyruvate carboxykinase (*pck*A), which connects the tricarboxylic cyclic (TCA) cycle with the Embden-Meyerhoff-Parnas (EMP) pathway, could convert oxaloacetate into phosphoenolpyruvate (PEP). PEP could then go on to form glucose-6-P through gluconeogenesis. These gluconeogenic reactions would provide five more precursors, PEP, 3-P glycerate, glyceraldehyde 3-P, fructose-6-P and glucose-6-P. Glucose-6-P can either enter the glycogen synthetic pathway, the EMP pathway which would provide an additional 2 precursors pyruvate and acetyl-CoA, or the pentose phosphate pathway (PPP) and provide the last 2 precursors, ribose-5-P and erythrose-4-P (McClarty, 1999).

Malate or 2-oxoglutarate could be obtained from the host through a dicarboxylate translocator (SodiTi) which would allow for the transport of 2-oxoglutarate from the host in return for malate or vice versa. Malate or 2-oxoglutarate could then move through the TCA cycle, EMP pathway and PPP providing the necessary intermediates required for

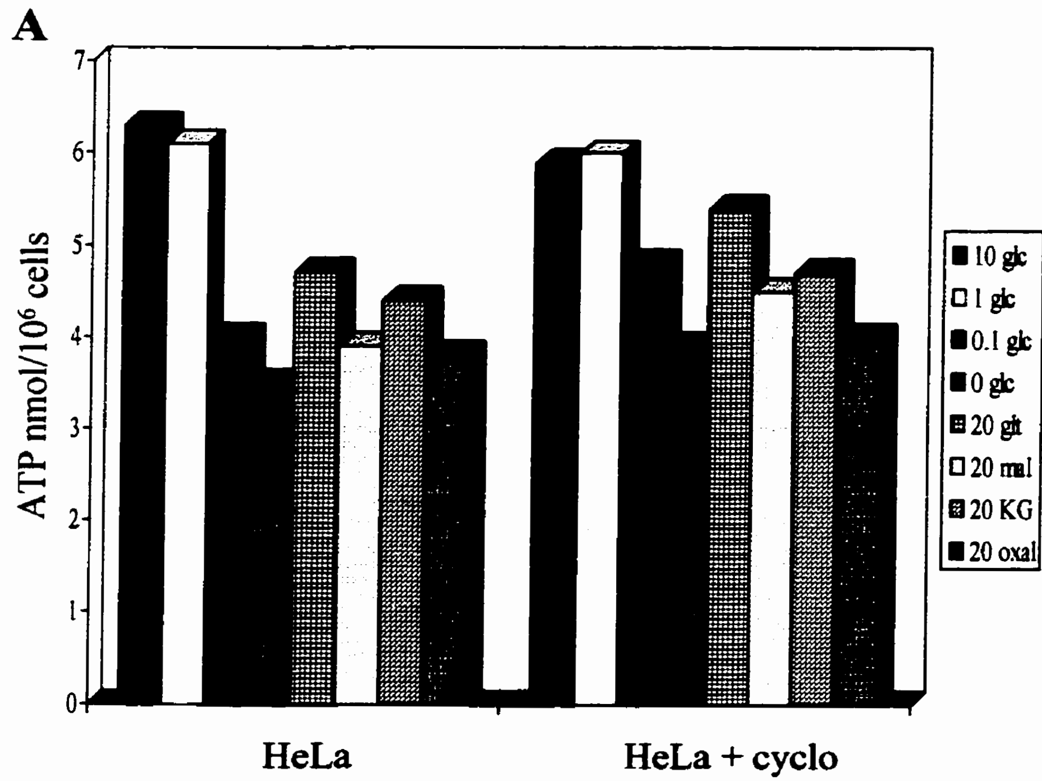
survival and glycogen synthesis as described above. Finally, oxaloacetate may also be transported from the host through the dicarboxylate exchanger (SodiTi) in exchange for malate and then enter the TCA cycle via a transamination reaction encoded by *aspC* gene product which would convert oxaloacetate + glutamate → aspartate + 2-oxoglutarate.

With free-living bacteria it is straightforward to control the source of carbon and nitrogen in the culture medium and monitor the effect on growth and gene expression. The situation is much more complicated when working with chlamydiae because of their obligate intracellular growth requirement. It is difficult to regulate the availability of nitrogen because according to the genome sequencing projects, chlamydiae have few genes for nitrogen metabolism and are auxotrophic for most amino acids (Kalman *et al.*, 1999; Read *et al.*, 2000; Stephens *et al.*, 1998). Therefore chlamydiae must obtain most amino acids directly from the host. In contrast, the chlamydial genome sequence indicates that in addition to glucose, several gluconeogenic substrates could potentially serve as carbon sources. Based on this information the effect of different carbon sources on the production of infectious EB progeny, the accumulation of glycogen, and the expression of various genes required for the utilization of the substrate were monitored.

2. Effect of culture conditions containing various carbon substrates and concentrations on the NTP pool size

As a first experiment the effect of the various culture conditions had on the overall health of the host HeLa cells was examined. This is an important parameter given the obligate intracellular growth requirement of chlamydiae. As a gauge we chose to

measure the size of the host cell ATP pool because numerous studies have shown that chlamydiae depend on the host cell as their source of nucleotides (Hatch, 1988; McClarty and Tipples, 1991; Tipples and McClarty, 1993). Nucleotides were extracted from uninfected and *C. trachomatis*-infected HeLa cells after 30 hr. and the ATP pool was quantitated following separation by HPLC as described in "Materials and Methods". Results presented in Figure 38 indicate that while there was fluctuation in the size of the ATP pool, it was never depleted under any of the culture conditions. The size of the pools of the three other ribonucleoside triphosphates, GTP, CTP and UTP fluctuated in a similar fashion as ATP (data not shown).


A
ATP nmol/10⁶ cells
0
1
2
3
4
5
6
7
10 glc
1 glc
0.1 glc
0 glc
20 glt
20 mal
20 KG
20 oxal
HeLa
HeLa + cyclo

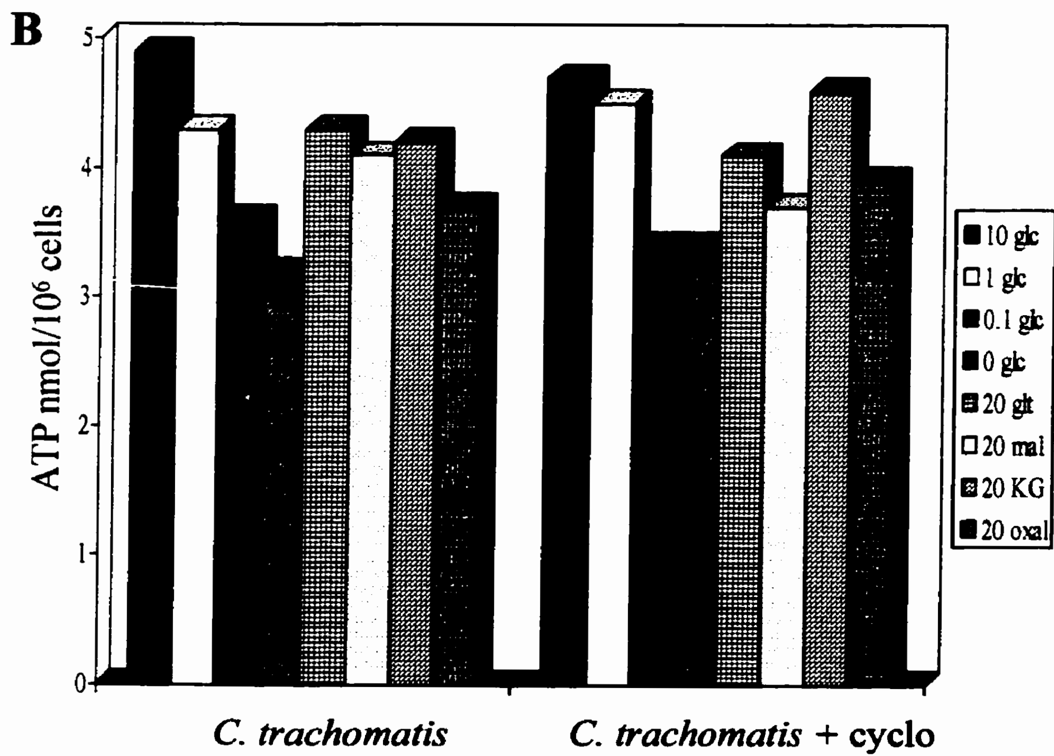

B
ATP nmol/10⁶ cells
0
1
2
3
4
5
10 glc
1 glc
0.1 glc
0 glc
20 glt
20 mal
20 KG
20 oxal
C. trachomatis
C. trachomatis + cyclo

Figure 38. Effect of various glucose concentrations and gluconeogenic substrates on ATP pool size in A) uninfected and B) *C. trachomatis*-infected HeLa cells. *C. trachomatis*-infected and uninfected HeLa cells were cultured in media containing different glucose concentrations (10, 1, 0.1 or 0 mg/ml) or gluconeogenic substrates (20 mM glutamate, 20 mM malate, 20 mM α-ketoglutarate or 20 mM oxaloacetate) in the presence or absence of cycloheximide. ATP pools were extracted 30 h p.i. as described in "Materials and Methods". HeLa, uninfected HeLa cells; HeLa + cyclo, uninfected HeLa cells + cycloheximide; L2, *C. trachomatis* L2-infected HeLa cells; L2 + cyclo, *C. trachomatis* L2-infected HeLa cells + cyclohexmide. Results represent the average of two determinations.

3. Effect of various culture conditions on the yield of infectious chlamydial EBs

The ability of the host HeLa cells cultured under the various conditions to support chlamydial growth was assessed by enumerating the number of infectious EB progeny present at 48 h p.i. by infectivity titration. As shown in Table 16 the number of infection forming units (IFUs) recovered from the cells cultured under the various conditions varied dramatically. The maximum yield of EBs was obtained from cultures incubated in the presence of 10 mg/ml glucose. With lower amounts of glucose the EB yield was reduced by several logs. This was especially true for cultures incubated in the absence of the cycloheximide, an eukaryotic protein synthesis inhibitor which results in reduced competition for nutrients by the host cell (Moulder, 1991). Interestingly, cycloheximide did not significantly increase the number of EBs recovered from cultures incubated with 10 mg/ml glucose. In the absence of glucose supplementation no infectious progeny were recovered even in the presence of cycloheximide. When glucose was replaced with the gluconeogenic substrates; glutamate, malate, α-ketoglutarate, or oxaloacetate infectious EBs were isolated indicating that chlamydia could complete its developmental cycle, however, the yield of EBs was reduced by several logs (4-5) compared to growth in the presence of non-limiting concentrations of glucose. Addition of cycloheximide led to a substantial increase (2-10 fold) in the yield of infectious progeny from cultures incubated with gluconeogenic substrates.

Table 16. Effect of glucose concentrations or various carbon sources on the production of infectious chlamydial EBs[a]

Substrate	Concentration (mg/ml)	Cycloheximide (1 μg/ml)	IFUs[b] recovered		Substrate	Concentration (mM)	Cycloheximide (1 μg/ml)	IFUs recovered
Glucose	0	-	0		Glutamate	20	-	4.3×10^4
	0	+	0				+	4.9×10^5
	0.1	-	1.4×10^5		Malate	20	-	1.3×10^4
	0.1	+	1.9×10^6				+	5.4×10^4
	1	-	5.0×10^6		α-Ketoglutarate	20	-	1.6×10^4
	1	+	5.1×10^8				+	4.9×10^4
	10	-	2.4×10^9		Oxaloacetate	20	-	1.1×10^4
	10	+	3.2×10^9				+	1.9×10^4

[a]*C. trachomatis*-infected HeLa cells were grown in media containing the indicated substrate (+/- cycloheximide). The effect of the described carbon conditions on the production of infectious EBs was assessed by infectivity titration 48 h p.i. as described in "Materials and Methods". The data represents the average of three determinations.

[b]IFU; infectious forming unit

4. Effect of various carbon conditions on glycogen stores of HeLa and *C. trachomatis*-infected HeLa cells

Results presented in Table 16 indicate that of the gluconeogenic substrates tested, glutamate was the best at supporting chlamydiae growth therefore it was used as an alternative carbon source in subsequent experiments. The question as to whether chlamydiae would accumulate glycogen when cultured on a gluconeogenic substrate was addressed. In addition, the amount of glycogen stored by *C. trachomatis* was also investigated to see if glycogen accumulation would change in response to different glucose concentrations in the culture medium as found in most eukaryotes and prokaryotes that can synthesize glycogen (Neidhardt, 1990; Preiss, 1996; Slock and Stahly, 1974; Voet D. and Voet J., 1990). Uninfected and *C. trachomatis*-infected HeLa cells were cultured in medium containing either 1 mg/ml glucose, 10 mg/ml glucose or 20 mM glutamate in the presence or absence of cycloheximide. At 40 h p.i. the amount of glucose that remained in the media as well as the amount of intracellular glycogen accumulated was determined (Table 17).

When uninfected HeLa cells were cultured in medium containing 1 mg/ml glucose essentially all of the glucose in the media was utilized. In contrast, the majority of glucose remained in the medium when the initial concentration was 10 mg/ml and a substantial amount of glycogen was stored. Not surprisingly under both conditions, addition of cycloheximide to the culture medium reduced the amount of glucose consumed. Interestingly, in the presence of 1 mg/ml, but not 10 mg/ml glucose, glycogen stores were higher in cultures incubated in the presence of cycloheximide. This result

Table 17. Effect of various culture conditions on glycogen stores in MI and *C. trachomatis* infected HeLa cells[a].

Media supplement	Glucose in media[b] (mg/ml)		Glycogen (μg/ml)[c]	
	MI-HeLa cells[d]	L2-HeLa cells[e]	MI-HeLa cells	L2-HeLa cells
1 mg/ml glucose	<0.01	<0.01	5.95	26.01
1 mg/ml glucose + cycloheximide	0.44	0.26	42.20	59.68
10 mg/ml glucose	7.21	8.43	87.41	101.13
10 mg/ml glucose + cycloheximide	8.93	8.71	35.90	51.78
20 mM glutamate	<0.01	<0.01	5.56	6.34
20 mM glutamate + cycloheximide	<0.01	<0.01	3.71	2.59

[a]Cells were either MI or *C. trachomatis*-infected confluent monolayers (2 x 10^6) grown in media with the supplement indicated in the presence or absence of cycloheximide (1 μg/ml) as described in "Materials and Methods". The data represents the average of two determinations.

[b]The amount of glucose remaining in the media was analyzed 40 h p.i. as described in "Materials and Methods". The sensitivity of the assay was 0.01 mg/ml.

[c]Glycogen was isolated from cells 40 h p.i. and was determined by the anthrone method.

[d]MI-HeLa cells, mock-infected HeLa cells.

[e]L2-HeLa cells, *C. trachomatis* L2-infected HeLa cells

suggests that when glucose becomes limiting growing HeLa cells draw upon their glycogen stores as a source of carbon That is, after 40 h., the 1 mg/ml of glucose in the media is depleted by the growing HeLa cells and the cells revert to degrading their glycogen stores to obtain glucose. Furthermore, in the presence of excess glucose (ie: when HeLa cells were cultured in 1 mg/ml glucose + cylcoheximide and 10 mg/ml glucose ± cycloheximide), glucose remains in the media after 40 h and growing HeLa cells will increase the amount of glycogen they accumulate. As expected, when glutamate (+/- cycloheximide) was the primary carbon substrate no glucose was found in the medium and the amount of intracellular glycogen detected was similar to that seen under limiting glucose conditions.

In general, glucose utilization trends were similar with *C. trachomatis*-infected HeLa cells as they were with uninfected controls (Table 17). In keeping with earlier observations with *C. trachomatis* (Matsumoto *et al.*, 1998; Moulder, 1991; Weigent and Jenkin, 1978), chlamydiae-infected cultures that were incubated in the presence of glucose always contained more glycogen than did the uninfected control cultures. Interestingly, in contrast to uninfected HeLa cells which contained dramatically different amounts of glycogen depending on the culture conditions (i.e. glucose concentration or +/- cycloheximide), the proportion of glycogen that could be attributed to *C. trachomatis* infection (i.e. infected – uninfected) remained relatively constant (15-20 μg/ml) irrespective of the culture conditions. When infected cells were cultured in the presence of the gluconeogenic substrate glutamate (± cycloheximide), *C. trachomatis* did not increase glycogen stores over the host cell background.

5. Incorporation of D-[U-^{14}C] glucose or L-[U-^{14}C] glutamate into glycogen in uninfected and *C. trachomatis*-infected HeLa cells

The results presented above suggest that *C. trachomatis* does not increase the amount of glycogen stored in response to excess glucose and does not accumulate glycogen when glutamate is the primary carbon source. As a more sensitive assay for glycogen accumulation, uninfected and *C. trachomatis*-infected HeLa cells were cultured in the presence of radiolabeled glucose or glutamate for 40 hours and then the amount of radioactivity associated with intracellular glycogen was determined. When uninfected HeLa cells were cultured in 1 mg/ml [U-^{14}C] glucose little radioactivity was incorporated into glycogen, again suggesting that glucose was limiting under these growth conditions (Table 18). In contrast, uninfected HeLa cells cultured in medium containing 10 mg/ml [U-^{14}C] glucose incorporated substantial amounts of radioactivity into glycogen. As expected, regardless of the amount of glucose in the medium, in the presence of cycloheximide little glycogen synthesis occurred as indicated by low amounts of radioactivity associated with intracellular glycogen. When uninfected HeLa cells were incubated in the presence of [U-^{14}C] glutamate there was essentially no radioactivity incorporated into intracellular glycogen.

In comparison to uninfected HeLa cells, *C. trachomatis*-infected cells cultured in the presence of 1 mg/ml [U-^{14}C] glucose (+/- cycloheximide) resulted in a 10-20 fold elevation in [U-^{14}C] glucose incorporation into glycogen (Table 18) which is consistent with data in Table 17 and with earlier findings (Fan and Jenkin, 1970; Weigent and Jenkin, 1978). When the medium was supplemented with 10 mg/ml [U-^{14}C] glucose in

the absence of cycloheximide, only a slightly larger amount of radioactivity was associated with intracellular glycogen compared to the uninfected control. When *C. trachomatis*-infected cells were cultured in the presence of cycloheximide and 10 mg/ml [U-^{14}C] glucose, the amount of radioactivity incorporated into glycogen was increased compared to infected-cells in the absence of cycloheximide but was essentially the same as infected cultures incubated in the presence of 1mg/ml glucose and cycloheximide. Consistent with data in Table 17, these results suggest that unlike HeLa cells, *C. trachomatis* does not dramatically increase the amount of glycogen synthesized in response to excess glucose. As with uninfected cells, radiolabeled glutamate was poorly incorporated into glycogen in *C. trachomatis*-infected cells suggesting that there is limited glycogen accumulated from the gluconeogenic substrate glutamate.

Table 18. Incorporation of D-[U-^{14}C] glucose or L-[U-^{14}C] glutamate into glycogen of MI and *C. trachomatis*-infected HeLa cells[a]

Media supplement(s)	Radiolabeled precursor	Glycogen (dpm/10^6 cells)	
		MI-HeLa cells	L2-HeLa cells
1 mg/ml glucose	[U-^{14}C] glucose	584.45	12704.06
1 mg/ml glucose + cycloheximide	[U-^{14}C] glucose	2157.31	21344.00
10 mg/ml glucose	[U-^{14}C] glucose	34377.55	36729.05
10 mg/ml glucose + cycloheximide	[U-^{14}C] glucose	5872.90	21884.20
20 mM glutamate	L-[U-^{14}C] glutamate	37.98	43.32
20 mM glutamate + cycloheximide	L-[U-^{14}C] glutamate	28.89	32.63

[a][U-^{14}C] glucose or [U-^{14}C] glutamate incorporation into glycogen isolated from MI-HeLa cells (mock-infected HeLa cells) or L2-HeLa cells (*C. trachomatis* L2-infected HeLa cells) was determined after a 40 h. labeling period as described in "Materials and Methods". Results represent the average of two determinations.

6. Evaluation of the expression of *C. trachomatis* L2 genes involved in carbon metabolism using RT-PCR.

To determine whether *C. trachomatis* could regulate central metabolism gene expression in response to the type or amount of carbon available as found in other bacteria (Kiel *et al.*, 1994; Preiss, 1996; Takata *et al.*, 1997), semi-quantitative RT-PCR was employed. Based on information provided by the chlamydiae genome sequencing projects as summarized in Figure 37, the expression of several genes required for carbon metabolism was monitored. The set of genes includes substrate transporters, genes encoding key enzymes of gluconeogenesis, genes required for glycogen synthesis and degradation and *ptsN,* a gene that has been proposed to encode a potential regulator of glycolytic/gluconeogenic flux (Table 19) (Stephens, 1999b).

As a first experiment we monitored the expression of the various genes during the course of a chlamydial developmental cycle by RT-PCR as previously described (Iliffe-Lee and McClarty, 1999). Total RNA was isolated from *C. trachomatis*-infected HeLa cells cultured in medium containing 1 mg/ml glucose + cycloheximide at 2, 6, 16, 24, 36, 48 h p.i. and used as template for cDNA synthesis. The amount of cDNA used as template for each time point was then roughly equalized using primers specific to chlamydial 23S rRNA so that the 23S rRNA PCR products were of similar intensity when run on an agarose gel (Fig. 39A). This amount of cDNA was kept constant for subsequent reactions and primers employed were specific and within the coding region of each gene (Table 19). The expression profile of chlamydial 23S rRNA, *euo, ompA* and *omcB* is consistent with our previous results (Fig. 12) (Iliffe-Lee and McClarty, 1999).

Table 19. Primers used in RT-PCR and *C. trachomatis* L2 genes analyzed in carbon metabolism

C. trachomatis L2 Genes Analyzed	Gene Description[a]	Primers used for RT-PCR[b]	
		Name of Primer	**Sequence**
23S rRNA	Ribosomal subunit	5 23S rRNA 3 23S rRNA	5'-GGGTTGTAGGATTGAGGA-3' 5'-GTTTTAGGTGGTGCAGGA-3'
euo	Early upstream open reading frame	5 EUO 3 EUO	5'-CAACAAGATACAGGGGTC-3' 5'-ATTTTCTGCGTCTGCCA-3'
ompA	Major outer membrane protein	5 MOMP 3 MOMP	5'-AGTTCTGCTTCCTCCTTG-3' 5'-GTCTCAACTGTAACTGCG-3'
omcB	60 kDA cysteine-rich protein	5 60 kDa CRP 3 60 kDa CRP	5'-GCGAGTTTATTTGCTAGCG-3' 5'-AAGTACCACAGTCAGAGC-3'
glgA	Glycogen synthase	5 GS 3 GS	5'-ATCACACAACGGAAGTGG-3' 5'-TAGGTTGTCACTGCTTCC-3'
glgP	Glycogen phosphorylase	5 GP 3 GP	5'-GACGTTGGTTGGCTCTTT-3' 5'-CAGATGCCTTGAGGATAG-3'
pckA	Phosphoenolpyruvate carboxykinase	5 PCK 3 PCK	5'-GGTTATGCGATGGTTCAG-3' 5'-TCGTGAATAGTGAGTCCG-3'
gltT	Proton/sodium glutamate symport protein	5 GLT 3 GLT	5'-GTTTCATCCCGTGAGGAC-3' 5'-AGTGGATCTTGCTTCGTC-3'
uphC	Hexosephosphate transport protein	5 UPH 3 UPH	5'-CGATTGTTAACTCACTGG-3' 5'-CAAACAAAGATACGCAGAG-3'
ptsN	Nitrogen regulatory IIA protein containing HTH domain	5 PTSN 3 PTSN	5'-CGATTAACGATGAGTTGC-3' 5'-ATAGACATGCCTAAGTGC-3'

[a and b]Genes are designated and primers were designed according to the *C. trachomatis* D genome project (Stephens *et al.*, 1998) (Stephens *et al.*, http://chlamydia-www.berkeley.edu:4321/).

Figure 39. RT-PCR analysis of total RNA extracted from *C. trachomatis* L2-infected HeLa cells. A) Chlamydiae-infected cells were cultured in medium containing 1 mg ml^{-1} glucose + cycloheximide, and RNA was isolated at different time points in the chlamydial developmental cycle. Each lane contains RNA samples subjected to RT-PCR analysis, and each time point describes the number of hours after infection at which the RNA sample was extracted.

B) Chlamydiae-infected cells were grown in media containing various carbon conditions, and RNA was isolated 24 h after infection. The lanes labeled A, B, C, D and E contain RNA samples isolated from chlamydiae-infected cells grown in 0, 1, 10 mg ml^{-1} glucose, 20 mM glutamate or 20 mM glutamate + cycloheximide respectively. Primers used are shown in Table 19. RT-PCR using 23S rRNA primers, *euo* primers, *ompA* primers, *omcB* primers, *glgA* primers, *glgP* primers, *pckA* primers, *gltT* primers, *uphC* primers and *ptsN* primers are shown. The size of each band is shown in basepairs (bp).

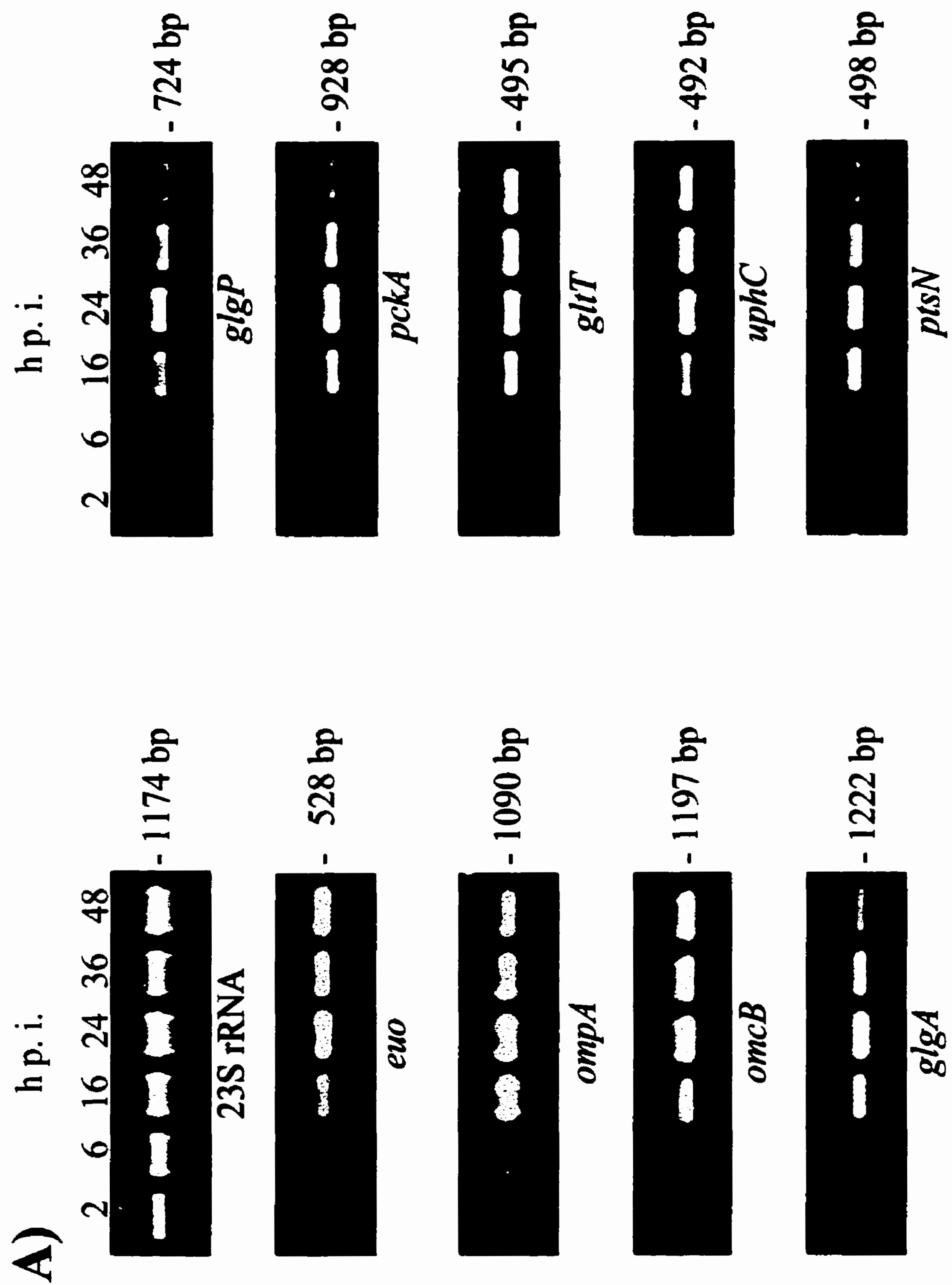


Fig. 39A

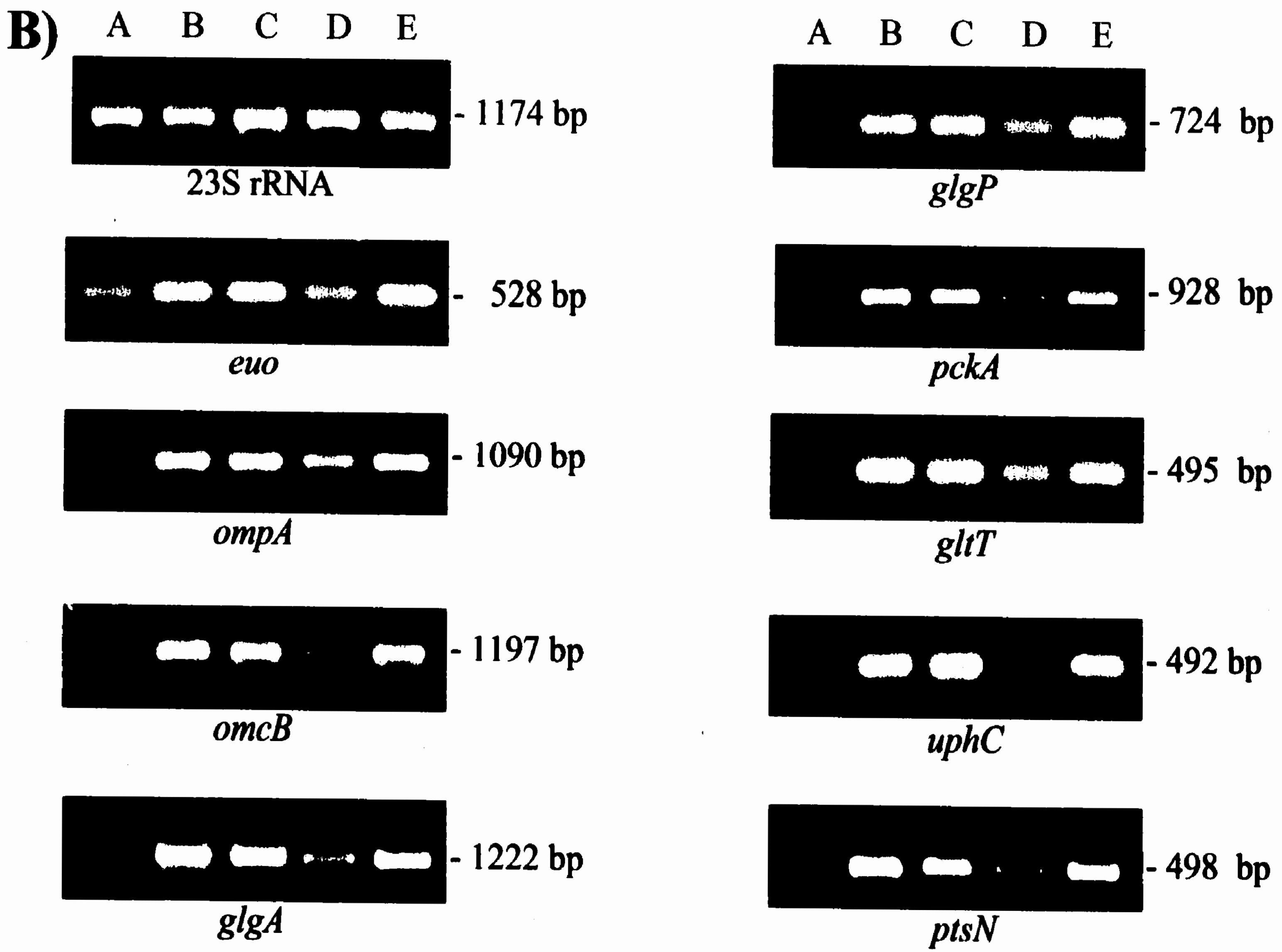


Fig. 39B

Euo is known to be expressed early in the developmental cycle (Wichlan and Hatch, 1993) and is detected at our earliest time point of 2 h p.i. As expected expression of *ompA*, (encodes for MOMP) first appears at 6 h p.i., and is present thereafter. Consistent with the fact that *omcB* is known to be expressed later in the developmental cycle when infectious EBs begin appearing (Hatch *et al.*, 1986), message is first detected at 16 h p.i. and remains high until the end. The results presented in Fig. 39A indicate that, similar to what we found with *C. trachomatis* glycolytic genes (Fig. 12) (Iliffe-Lee and McClarty, 1999), transcripts for all the carbon metabolism genes monitored except for *glgA,* first appear around 2-6 h p.i. , peak at mid-growth cycle and then decline at late time points except for *uphC* and *gltT* which remain strongly expressed at 48 h p.i. Transcript for *glgA* does not appear until about 16 h. p.i., however at a higher number of cycles it does appear at 8 h. p. i. (data not shown).

To monitor carbon metabolism gene expression in response to various culture conditions, total RNA was isolated from *C. trachomatis*-infected HeLa cells cultured in medium containing different amounts of glucose or the gluconeogenic substrates at 24 h p.i. The RNA was used as template and the 23S rRNA primers were used as a standard for RT-PCR as described above (Fig. 39B). When infected HeLa cells were cultured in the absence of glucose (Fig. 39B, panel A) *euo* was expressed implying that EB to RB early differentiation events had taken place. However, little or no transcript was detected for all other genes suggesting that RB to EB differentiation was blocked. Under all other culture conditions tested (Fig. 39B panels B-E), all studied genes were expressed indicating that chlamydia was capable of completing its developmental cycle. When infected cells were cultured in glutamate in the absence of cycloheximide (Fig. 39B,

panel D), there was a noticeable decrease in the level of expression of all genes examined however, there was no obvious change in the pattern of expression. For example, expression of gluconeogenic genes or the putative regulator of gluconeogenic/glycolytic flux *ptsN* were not significantly altered in response to glutamate, nor did the expression of the glycogen synthesis/degradation genes obviously change in response to excess/limiting glucose.

DISCUSSION

1. Energy and Glucose Metabolism in *C. trachomatis*

One of the problems encountered when studying chlamydial metabolism is that no cell-free growth system or gene transfer system has been developed. Growth and propagation of chlamydia is labor-intensive and slow. Isolation and purification of metabolically active RBs is difficult to achieve. In addition, metabolic studies on "purified" RBs is complicated by the risk of host cell contamination. Thus detection of metabolic activity on RB preparations is not definitive proof of the presence of the enzyme in question. Moreover, the lack of activity does not prove the absence of the enzyme as RB preparations or assay conditions may be inadequate. Furthermore, it is difficult to study chlamydial metabolism *in situ*, as few procaryotic- and eukaryotic-specific inhibitors of metabolism such as energy metabolism are available. Consequently, one of the methods used to demonstrate the presence and activity of a gene product in chlamydia requires biochemical characterization of the recombinant protein.

The energy parasite hypothesis was established by Moulder about 40 years ago and simply put states that chlamydia is an energy parasite completely dependent on the host cell for high-energy intermediates (Moulder, 1962). In this thesis, I have presented evidence using a number of different methods to demonstrate that *C. trachomatis* L2 does indeed contain functional energy-producing enzymes and is capable of producing its own energy. Specifically, *C. trachomatis* contains the glucose-metabolizing enzymes GADPH, PGK, PK and ZWF. The existence of these enzymes was confirmed by a

number of experiments. i) The deduced amino acid sequence of *C. trachomatis*–specific DNA fragment cloned into pUC19 (pHGAP6, pHPGK3, pHPK1, pH11) show significant homology to other known GAPDH, PGK, PK and ZWF genes (Table 2). ii) Southern hybridizations with genomic DNA indicated that GAPDH, PGK, PK and ZWF genes were *C. trachomatis*-specific and single copy (Fig. 11). iii) Total RNA extracted from chlamydiae-infected cells at different time points p.i. subjected to RT-PCR analysis indicates that GAPDH, PGK, PK and ZWF are expressed during the chlamydial developmental cycle (Fig. 12). iv) pCTGAPDH, pCTPGK, pCTPK, pCTZWF were capable of complementing *E. coli* mutant strains DS112, DF264, PB25 and DF2000 respectively (Table 5). v) Finally, *in vitro* GAPDH, PGK, PK and ZWF activity was detected in extracts prepared from the appropriate mutant *E. coli* strains (Table 6). In addition, the recent sequencing of the entire *C. trachomatis* serovar D genome confirms the observations that *C. trachomatis* encodes proteins with homology to GAPDH, PGK, PK and ZWF proteins (Stephens *et al.*, 1998). These genes have also been identified in the genome sequence of *C. pneumoniae* (Kalman *et al.*, 1999) and *C. trachomatis* mouse pneumonitis (Read *et al.*, 2000).

In most bacterial systems, glycolytic enzymes are arranged in operons and are found clustered together in the genome. PGK and GAPDH bacterial genes are frequently found as an operon such as in *S. solfataricus* (Jones *et al.*, 1995), *X. flavus* (Meijer *et al.*, 1996) and *E. coli* (Alefounder and Perham, 1989). In contrast, the enzymes involved in glycolysis in chlamydia are dispersed throughout the genome (Stephens *et al.*, 1998). The sequence information from serovar D suggests that PK, PGK and ZWF are monocistronic while GAPDH appears to be in an operon with the ribosomal protein L17. Northern blot

analysis and/or RT-PCR, using primers within *zwf*, *pyk*, *pgk* and *gap* and within adjacent genes, will have to be done to definitively determine whether the genes are mono- or polycistronic.

RT-PCR analysis indicates that the expression of *C. trachomatis* glycolytic and pentose phosphate pathway enzymes (GAPDH, PGK, PK, ZWF) are growth related and are therefore most abundant when the majority of the chlamydial population present is in the metabolically active RB form. Transcript is detected early (~6 h p.i.) and late (~36-48 h p.i.) in the chlamydial developmental cycle when a higher number of PCR cycles (40) is used (data not shown). This pattern of expression suggests that chlamydia may be capable of generating its own ATP by substrate-level phosphorylation throughout the majority of the life cycle. Interestingly, CTP synthetase, another metabolic enzyme which synthesizes CTP, displays a RT-PCR expression pattern which is similar to the glucose-metabolizing enzymes. In addition, Western blot analysis indicated that CTP synthetase was present in chlamydial EBs (Wylie *et al.*, 1996). Furthermore, a recent study by Vandahl *et al.*, (2001) demonstrated using proteome analysis that GAPDH, PGK, PK and ZWF proteins are present in *C. pneumoniae* EBs. These results suggest that chlamydia may store some metabolic enzymes in the EB form in order to carry out initial reactions required for the early differentiation process (EB to RB differentiation) in the chlamydial developmental cycle.

The glycolytic enzyme GAPDH results in the production of NADH, PGK and PK glycolytic enzymes result in the production of ATP via substrate phosphorylation, and ZWF, an enzyme in the pentose phosphate pathway, results in the production of NADPH. GAPDH residues postulated to be involved in catalytic activity and NAD binding

(Branlant and Branlant, 1985; Eikmanns, 1992; Souza *et al.*, 1998) are conserved in the amino acid sequence of *C. trachomatis* GAPDH. Likewise, important PGK residues involved in nucleotide substrate (MgADP) and triose-sugar (3-PGA) binding (Bernstein *et al.*, 1997) are conserved in the amino acid sequence of *C. trachomatis* PGK. PK is a key regulatory enzyme in the glycolytic pathway in many organisms and is discussed in further detail below. The proposed ZWF catalytic and NADP binding residues (Jeffery *et al.*, 1993; Scopes *et al.*, 1998; Shahabuddin *et al.*, 1994) are also conserved in the amino acid sequence of *C. trachomatis* ZWF. In addition, the *in vitro C. trachomatis* GAPDH, PGK and ZWF assays performed with crude cell extracts demonstrated that chlamydial recombinant enzymes are active (Table 6). Further characterization of the kinetics of chlamydial GAPDH, PGK and ZWF enzymes will require purified recombinant enzymes.

Pyruvate kinase is an important enzyme in glycolysis which results in the production of ATP, the universal energy transducer, and pyruvate, a key carbon intermediate in catabolic and biosynthetic reactions (Boles *et al.*, 1997; Ponce *et al.*, 1995). In most organisms, PK is an allosteric enzyme, controlled by one or more effectors (Fothergill-Gilmore and Michels, 1993). Important PK amino acid residues that have been shown to interact with pyruvate, K^+, Mn^{2+} and ADP (Jurica *et al.*, 1998; Larsen *et al.*, 1994), are conserved in the *C. trachomatis* PK sequence.

PK has been studied extensively in *E. coli* and is known to be allosterically regulated (Kotlarz *et al.*, 1975; Malcovati and Valentini, 1982; Ponce *et al.*, 1995; Somani *et al.*, 1977; Waygood *et al.*, 1976; Waygood *et al.*, 1975). Two isoenzymes of PK have been identified in *E. coli*, specifically *pyk*A (type II) and *pyk*F (type I). *E. coli* PKA or type II PK is activated by AMP and responds to the energy needs of the cell

(Kotlarz *et al*., 1975). It becomes more active when grown on gluconeogenic substrates such as pyruvate or acetate (Waygood *et al*., 1975). *E. coli* PKA, as well as several other procaryotic PKs such as *Halobacterium cutirubrum*, *Pseudomonas citronellolis*, *Bacillus stearothermophilus* and *Thermus thermophilus* have been found to be activated by AMP or other intermediates of the hexose phosphate pathway such as sugar phosphate or nucleoside monophosphates (Fothergill-Gilmore and Michels, 1993). In contrast to these procaryotes, crude preparations of *C. trachomatis* PK were not significantly effected by the presence of AMP.

E. coli PKF has been reported to be activated by F16BP (Malcovati and Valentini, 1982) and is predominant under growth conditions for glycolysis when the intracellular concentration of F16BP is high (Waygood *et al*., 1976). It has been suggested that by activating PK, F16BP prevents the accumulation of glycolytic intermediates between F16BP and PEP (Mertens *et al*., 1992). *C. trachomatis* PK assays performed with crude cell extracts indicate that unlike *E. coli* PKF, *C. trachomatis* PK is not activated by F16BP (Table 6). Interestingly, the PK activity of the protist *Trichomonas vaginalis* has also been shown to be unaffected by F16BP (Mertens *et al*., 1992). The insensitivity of *T. vaginalis's* PK to F16BP is speculated to be associated with the replacement of ATP-dependent phosphofructose kinase (PFK) by pyrophosphate-dependent PFK (PPi-PFK) (Mertens *et al*., 1992). In addition to *T. vaginalis*, PPi-PFK have been found in some bacteria (*P. shermanii, B. burgdorferi* (Mertens, 1991), *A. methanolica* (Alves *et al*., 1996) plants (castor bean, potato (Todd *et al*., 1995) and protozoa (*G. lambia, T. gondii, T. foetus* (Mertens, 1991).

In most organisms, PFK plays a major role in the regulation of the glycolytic flux and catalyzes the irreversible reaction: fructose-6-phosphate + ATP $\rightarrow$ fructose 1,6-bisphosphate + ADP. ATP-dependent PFKs generally fall into three categories: the homotetrameric bacterial enzyme, the homotetrameric animal enzyme and the octameric yeast enzyme which is composed of two nonidentical subunits (Alves *et al.*, 1996). The bacterial PFK is allosterically regulated by phosphoenolpyruvate and ADP, whereas the yeast and mammalian enzymes are regulated by citrate, ATP, and fructose-2,6-bisphosphate (Alves *et al.*, 1996). PPi-PFK catalyzes the reversible reaction: fructose-6-phosphate + PPi $\leftrightarrow$ fructose 1,6-bisphosphate + Pi, using PPi as phosphate donor instead of ATP. The PPi-PFK enzyme can balance an increase in F16BP by operating in the reverse direction, whereas ATP-PFK must rely on the activation of PK by F16BP in order to balance out increases in F16BP (Mertens *et al.*, 1992). The sequence information from *C. trachomatis* serovar D indicates that chlamydia encodes two genes, *pfpA* and *pfpB*, with homology to PPi-PFK (Stephens *et al.*, 1998). Blast search results indicate that they show high homology (~ 40%) to rice plant PPi-PFK particularly to the β-subunit (Genbank accession # Z32850, see Table 7). The rice PPi-PFK is composed of two subunits where the α-subunit is speculated to be the regulatory portion of the enzyme and the β-subunit is believed to contain the catalytic activity (Mertens, 1991; Todd *et al.*, 1995). Therefore, it may be that organisms such as *C. trachomatis* and *T. vaginalis* which have PKs that are not activated by F16BP, balance their glycolytic intermediates through PPi-dependent PFKs.

In order to provide information about *C. trachomatis* L2 *pfpA* and *pfpB* genes, they were cloned into pUC-19 based on information from *C. trachomatis* serovar D

genome sequence (Stephens *et al.*, 1998) and were subsequently sequenced. The deduced amino acid sequence of the *C. trachomatis*-specific DNA fragments, (pCTPFKA, and pCTPFKB) showed high homology to other PPi-PFK enzymes (Table 7). *In vitro* enzyme analysis indicates that the recombinant chlamydial PFPA and PFPB enzymes expressed in *E. coli* mutant strain DF456, which is deficient in PFK activity, were active (Table 8). Specifically, crude extract prepared from *E. coli* mutant DF456 containing chlamydial PFPA showed both ATP-PFK and PPi-PFK activity however, PPi was the preferred substrate. In contrast, extract prepared from *E. coli* mutant DF456 containing chlamydial PFPB also showed both ATP- and PPi- dependent PFK activity, however, ATP was the preferred substrate. These results suggest that *C. trachomatis pfpA* likely encodes for a PPi-PFK, whereas as *pfpB* probably encodes for an ATP-PFK. As mentioned previously, many organisms which contain PPi-PFKs also contain ATP-PFKs (Alves *et al.*, 1996; Mertens, 1991). Higher plants contain ATP-PFKs, PPi-PFKs and sometimes a fructose-1,6- bisphosphatase. The PPi-PFKs is generally composed of two subunits, (α, regulatory and β, catalytic) and is allosterically regulated. All other PPi-PFK containing organisms contain small amounts of ATP-PFK, their PPi-PFKs are composed of two or four identical subunits and they are non-allosterically regulated. Further characterization on highly purified recombinant *C. trachomatis* PFPA and PFPB enzymes will have to be done to determine whether the enzymes catalyze reversible reactions and whether they are allosterically regulated. It will also be interesting to determine whether a regulatory relationship exists between *C. trachomatis* PFPA, PFPB and PK.

The results presented indicate that *C. trachomatis* contains functional enzymes in the glycolytic (GAPDH, PGK, PK) and pentose phosphate pathways (ZWF) which

produce energy and reducing power. This work demonstrates for the first time that chlamydia does indeed contain the biochemical machinery to generate its own energy. Previous attempts to prove that chlamydia contained enzymes capable of generating net ATP had failed. Consequently, it was speculated that many biosynthetic genes were dispensable because the nutrient-rich environment of the host cell allowed chlamydia access to metabolites that other free-living bacteria must synthesize for themselves. Chlamydiae have not only retained the genes once speculated to be lost, but the proteins encoded by these genes are biologically active. Results also indicate that *C. trachomatis* encodes for ATP- and PPi-PFK enzymes which are also found in the glycolytic pathway. Futhermore, RT-PCR analysis indicates that the expression of several of the genes involved in respiration, transport, energy or carbon metabolism (*pfpA, pfpB, zwfB, odpB, nqr5, cydA, nrdB, adt1, adt2, aspC, sodiTi*, see Table 19) as deduced from the genome sequence are similar to the expression patterns found in the glucose-metabolizing genes (*gap, pgk, pyk* and *zwf*). The genes are weakly expressed at the start of the chlamydial developmental cycle, peak in the middle (16-24 h p.i.) and gradually decline thereafter. These results suggest that like the glucose-metabolizing genes, these genes are also growth-related and are generally expressed throughout the chlamydial developmental cycle. Thus chlamydia has the enzymatic machinery to generate ATP via substrate phosphorylation and the genetic capacity to produce ATP via oxidative phosphorylation throughout the majority of its developmental life cycle.

Initial glucose metabolism (~0-6 h p.i.) required for early EB to RB differentiation might be carried out by pre-existing glycolytic enzymes already present in EBs. As mentioned, another metabolic enzyme, CTP-synthetase has been demonstrated

to be present in EBs (Wylie *et al.*, 1996). Furthermore, EBs have been shown to contain a large pool of stored ATP (Tipples and McClarty, 1993). Hatch *et. al.*, (1982) have demonstrated that RBs, but not EBs, can obtain ATP from the host cell via the ATP/ADP translocase. In addition, the two chlamydial homologs for ATP/ADP translocase, *adt1* and *adt2* have been characterized as recombinant proteins in *E. coli* and shown to exchange ATP for ADP and take up NTPs respectively (Tjaden *et al.*, 1999). In addition, RT-PCR studies suggest that expression of the ATP/ADP translocase follows the same pattern as the glucose metabolizing enzymes (Fig. 12).

In total, the data suggest that early EB differentiation may be fueled by stored ATP pools and by ATP generated from glucose metabolism carried out by pre-existing enzymes already present in EBs. As the need for ATP increases with the onset of RB multiplication, chlamydiae-generated ATP could be supplemented with ATP obtained directly from the host through use of the ATP/ADP translocator. To address the question as to whether chlamydial growth depends absolutely on an exchange of host cell ATP for parasite ADP, i.e. is an obligate energy parasite, requires the development of a genetic system in chlamydia or by drug development. Once established, this question could be addressed directly through ATP/ADP translocase gene inactivation or by drug inhibition.

2. Kinetics of *C. trachomatis* Pyruvate Kinase

Pyruvate kinase is of considerable interest because it is a major regulatory enzyme of the glycolytic pathway, controlling the flux from fructose-1,6-bisphosphate to pyruvate. In order to gain a better understanding of the regulatory properties of the key

glycolytic enzyme PK in chlamydia, *C. trachomatis* L2 PK (CTPK) was expressed and purified from *E. coli*. Results presented indicate that CTPK contains both typical and unique bacterial PK properties. A summary of the properties found in PK enzymes from eukaryotes, and prokaryotes, including CTPK is shown in Table 20.

In common with most PK enzymes, CTPK is absolutely dependent on the presence of monovalent (K^+) and divalent cations (Mg^{2+}) for enzyme activity (Fothergill-Gilmore and Michels, 1993). It is therefore not surprising that important residues in K^+ binding and Mn^{2+} (Mg^{2+}) binding (Jurica *et al.*, 1998; Larsen *et al.*, 1994) are conserved in the *C. trachomatis* L2 PK sequence (Iliffe-Lee and McClarty, 1999)(Fig. 10C). As mentioned, the CTPK amino acid sequence (Iliffe-Lee and McClarty, 1999) also contains key residues involved in PEP and ADP binding (Jurica *et al.*, 1998; Larsen *et al.*, 1994), whereas definitive sites involved in F26BP binding have yet to be resolved (Rigden *et al.*, 1999).

Several prokaryotic and eukaryotic PKs can utilize various nucleoside diphosphates as phosphate acceptors (Abbe *et al.*, 1983; Abbe and Yamada, 1982; Chuang and Utter, 1979; Kapoor and Venkitasubramanian, 1983; Lin *et al.*, 1989; Podesta and Plaxton, 1991; Sakai *et al.*, 1986; Smith *et al.*, 2000; Waygood *et al.*, 1975; Waygood and Sanwal, 1974). Likewise, CTPK is capable of using ADP, CDP, GDP, UDP and IDP as alternative phosphate acceptors with ADP serving as the best substrate. Interestingly, the broad specificity exhibited by many PK enzymes for NDPs as well as structural studies on rabbit muscle PK enzyme with bound MgATP (Rosevear *et al.*, 1987) suggests that the nucleotide portion of the NDP may exhibit a high degree of mobility (Jurica *et al.*, 1998).

Table 20. Comparison of PK characteristics from a variety of sources

PK Source	Substrate saturation curve and kinetics for PEP (mM)	Substrate saturation curve and kinetics for ADP (mM)	Activator	Inhibitor	Cation[a]	pH optima	Use of alternative NDPs	Reference
E. coli PKI	Sigmoidal $K_{0.5PEP}$ 4.0	Hyperbolic K_{mADP} 0.24	F16BP	ATP, GTP	Yes	7.0	Yes	(Waygood and Sanwal, 1974)
E. coli PKII	Hyperbolic[b] $K_{0.5PEP}$ 0.1	Hyperbolic K_{mADP} 0.08	R5P, AMP	NTPs	Yes	6.3	Yes	(Waygood *et al.*, 1975)
B. stearothermophilus PK	Sigmoidal $K_{0.5PEP}$ 2.0	Hyperbolic K_{mADP} 1.0	AMP	ATP, Pi, F16BP	Yes	7.2	Yes	(Sakai *et al.*, 1986)
Germinating Castor oil seeds cytosolic PK	Hyperbolic $K_{0.5PEP}$ 0.05	Hyperbolic K_{mADP} 0.07	?[d]	ATP, AMP, carbon intermediates	Yes	7.2	Yes	(Podesta and Plaxton, 1991)
Rat M1 PK	Hyperbolic $K_{0.5PEP}$ 0.06	Hyperbolic K_{mADP} 0.56	?	ATP, phenyl-alanine	Yes	7.5	nd	(Ikeda *et al.*, 2000)
Rat M2 PK	Sigmoidal $K_{0.5PEP}$ 0.2	Hyperbolic K_{mADP} 0.32	?	ATP, phenyl-alanine	Yes	7.5	nd	(Ikeda and Noguchi, 1998)
L. mexicana PKI	Hyperbolic $K_{0.5PEP}$ 0.22	Hyperbolic K_{mADP} 0.07	F16BP, F26BP	ATP	Yes	7.2	Yes	(Ponte-Sucre *et al.*, 1993)
L. mexicana PKII	Sigmoidal $K_{0.5PEP}$ 0.8	Hyperbolic K_{mADP} 0.07	F26BP	ATP	Yes	7.2	Yes	
T. brucei PK	Sigmoidal $K_{0.5PEP}$ 1.3	Hyperbolic K_{mADP} 0.07	F26BP	?	Yes	7.2	nd	(Ernest *et al.*, 1998)
C. trachomatis L2 PK	Sigmoidal $K_{0.5PEP}$ 3.0	Hyperbolic K_{mADP} 0.63	F26BP, R5P, Sugar-Ps	ATP, GTP, AMP	Yes	7.3	Yes	This work

[a]Enzyme activity is dependent on the presence of K^+ or Mg^{2+} or both.

[b]Data is taken from assays done under saturating conditions except for *E. coli* PKI which is done under subsaturating conditions.

[c]nd, not done.

[d]?; unknown

The $S_{0.5}$ and K_m values for PEP and ADP respectively, from CTPK were also found to be similar to some other PK enzymes. Specifically, in the absence of any effector, the CTPK $S_{0.5}$ for PEP (3.05 ± 0.05 mM) was found to be similar to that of *E. coli* (Waygood and Sanwal, 1974), *B. stearothermophilus* (Sakai *et al.*, 1986), yeast (Collins *et al.*, 1995) and *T. tenax* (Schramm *et al.*, 2000). The value of the apparent $S_{0.5}$ for PEP (0.17 ± 0.01 mM) in the presence of F26BP was found to be most similar to AMP-activated *B. stearothermophilus* PK (Sakai *et al.*, 1986). The CTPK K_m for ADP (0.63 ± 0.01 mM) was found to be similar to that of Rat PK-M1 (Ikeda *et al.*, 2000), *T. tenax* PK (Schramm *et al.*, 2000) and *B. stearothermophilus* PK (Sakai *et al.*, 1986). Interestingly, the deduced amino acid sequence of CTPK (Iliffe-Lee and McClarty, 1999) shares the highest homology to *B. stearothermophilus* PK (Accession no. S27330) and as mentioned the $S_{0.5}$ for PEP and K_m for ADP of CTPK were also found to be similar to that of *B. stearothermophilus* PK. Other CTPK kinetic properties that were related to other PK enzymes included positive cooperativity with respect to PEP (Chuang and Utter, 1979; Collins *et al.*, 1995; Ernest *et al.*, 1998; Garcia-Olalla and Garrido-Pertierra, 1987; Ikeda and Noguchi, 1998; Kapoor and Venkitasubramanian, 1983; Ponte-Sucre *et al.*, 1993; Sakai *et al.*, 1986; Waygood and Sanwal, 1974), as well as inhibition by NTPs (Chuang and Utter, 1979; Garcia-Olalla and Garrido-Pertierra, 1987; Ikeda and Noguchi, 1998; Ikeda *et al.*, 2000; Kapoor and Venkitasubramanian, 1983; Podesta and Plaxton, 1991; Sakai *et al.*, 1986; Waygood *et al.*, 1975; Waygood and Sanwal, 1974).

One of the unique properties that CTPK displayed, which differs from all other known bacterial PK enzymes, was that it was allosterically activated by F26BP. The only other recognized PK enzymes that are allosterically regulated by F26BP are the protists

belonging to the Kinetoplastida order, namely *Leishmania*, and *Trypanosoma*. These trypanosomes are unique in that the first seven enzymes in the glycolytic pathway including phosphofructose kinase (PFK), the other key glycolytic regulatory enzyme which generates F16BP, are sequestered in a peroxisome-like organelle called the glycosome (Fothergill-Gillmore *et al.*, 2000). The last three enzymes of the glycolytic pathway, including pyruvate kinase are found in the cytosol. Consequently, the feedforward activation by F16BP as found in other PK enzymes, cannot play a role in the trypanosomes because the enzymes PFK and PK are in different compartments. In the presence of F26BP, CTPK's affinity for PEP was greatly increased by 17 fold and the velocity was also slightly increased. These results are similar to *T. brucei* PK (Ernest *et al.*, 1998) and *L. mexicana* PKII enzymes (Ponte-Sucre *et al.*, 1993). Furthermore, like the trypanosomes, only micromolar concentrations of F26BP were needed to activate CTPK.

The question that remains is why chlamydia would use F26BP as an activator. To date, F26BP has only been detected in eukaryotes where it has been found in virtually all tissues (Okar and Lange, 1999). In animals, F26BP is an important regulatory metabolite that has a major role in directing carbohydrate flux. F26BP is synthesized from F6P and ATP by the enzyme 6-phosphofructo-2-kinase (6-PF-2-K) and is degraded by the enzyme fructose-2-6-bisphosphatase (F26BPase). In animals, a single polypeptide contains both the kinase and bisphosphatase activities, whereas yeast expresses a separate protein for the kinase activity and another for the bisphosphatase activity. Plants on the other hand express both bifunctional and monofunctional enzymes. The mammalian 6-PF-2-K/F26Pase is regulated at the level of gene expression via hormones and extracellular

signals, and post-translationally by phosphorylation/dephosphorylation. The F26BP formed by 6-PF-2-K allosterically activates PFK and inhibits fructose-1,6-bisphosphatase (F16BPase), an important gluconeogenic enzyme that catalyzes the PFK reaction in the opposite direction (F16BP + ADP→F6P + ATP). F26BP therefore has a major role in coordinating the two opposing pathways of glycolysis and gluconeogenesis in mammals. In the presence of high levels of glucose, the bifunctional enzyme is dephosphorylated which results in an increase in the kinase activity and thereby increases the level of F26BP. PFK is then activated and FBPase is inhibited consequently increasing glycolysis and decreasing gluconeogenesis. In the presence of glucagon, the reverse is true (Claus *et al.*, 1984; Okar and Lange, 1999).

In contrast to animals, chlamydia does not contain homologs of enzymes known to metabolize F26BP (Stephens *et al.*, 1998). However, interestingly, the *C. trachomatis* genome sequence does encode for the genes, *pfpA* and *pfpB*, which show homology to pyrophosphate dependent PFK (PPi-PFK) (Stephens *et al.*, 1998). As mentioned, PPi-PFK catalyzes the same reaction as the protein ATP-PFK except it uses PPi as the phospho donor instead of ATP and is reversible. Interestingly, plant PPi-PFKs are extremely sensitive to F26BP and are nearly inactive in its absence (Mertens, 1991). BLAST search results indicate that C. trachomatis *pfpA* and *pfpB* show high homology (~40% identity) to the rice plant PPi-PFK (Table 7). It will be interesting to determine whether the *C. trachomatis* enzymes encoded by *pfpA* and *pfpB* are also regulated by F26BP.

Information from the genome sequence also indicates that chlamydia contains a substantial number of genes for transporters that exhibit broad substrate specificity and

few genes for the biosynthesis of metabolites such as amino acids and nucleotides. This suggests that chlamydia have transport systems for acquiring many metabolites from the host cell (Kalman *et al.*, 1999; Stephens *et al.*, 1998). Therefore, chlamydia may take F26BP from the host. In keeping with our current understanding of the regulatory enzymes in carbon metabolism in mammalian cells, a decrease in host F26BP levels would ultimately result in an increase in cytoplasmic glucose levels (Claus *et al.*, 1984). This would benefit chlamydia as it is speculated that they obtain glucose in the form of glucose-6-P from the host via the UphC transporter (McClarty, 1999). It has been shown that *C. psittaci* infection increases host cell surface expression of the glucose transporter, Glt1, presumably to meet the increased demand for glucose placed on the host cell by the infection (Ojcius *et al.*, 1998). The sequestration of F26BP from the host may be another means that chlamydia uses to increase the availability of glucose in the host.

The apparent $S_{0.5}$ of chlamydial PK for F26BP was very low ($\approx$ 9 μM) indicating that only very small amounts would have to be obtained from the host in order to have a large impact on chlamydial metabolism. Furthermore, CTPK showed negative cooperativity with respect to F26BP, which states that the binding of the substrate to the first site on an enzyme inhibits the binding of the second (Eisenthal R., 1992). Thus in theory, CTPK would use the available F26BP very effeciently because only one molecule of F26BP would bind per CTPK, freeing additional F26BP molecules to bind and regulate other CTPK enzymes. Since F26BP dramatically lowers the apparent $S_{0.5}$ of chlamydial PK for PEP, the enzyme would continue to function even at very low levels of PEP. This property may be important in regulating carbon metabolism in chlamydia. Chlamydia genome sequence annotation indicates that while the Embden-Meyerhoff-

Parnas (EMP) pathway is complete, the tricarboxylic acid (TCA) cycle is incomplete. The first three enzymes citrate synthase, aconitase and isocitrate dehydrogenase are missing (Stephens *et al.*, 1998). As a result the only direct link between the EMP pathway and the TCA cycle in chlamydia is through the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK). Interestingly, in many organisms PEPCK is inhibited by PEP (Fraenkel, 1996; Jabalquinto and Cardemil, 1993; Jomain-Baum and Schramm, 1978). Furthermore, it is possible that chlamydial PFPA and PFPB are also regulated by F26BP. Taken together this suggests that carbon flux in chlamydia may ultimately be controlled by host derived F26BP. In this sense F26BP may be viewed as a signaling molecule that coordinates host and parasite carbon metabolism.

In addition to having a major role in regulating carbon flux, F26BP also plays a role in several adaptive survival strategies such as metabolic rate depression which involves a reduction of the overall metabolic rates, including ATP generating and ATP utilizing pathways in many animals. Some examples of adaptive survival strategies include hibernation (ie; bears), freeze-tolerance (ie; frogs) or hypoxia (ie; mussels) (Okar and Lange, 1999). Metabolic rate depression could occur in chlamydiae when they transform from the metabolically active RB to the inert, spore-like EB. In fact, F26BP has also been shown to be involved in mediating the biological processes of sporulation and germination in plants (Okar and Lange, 1999). Further studies on F26BP are warranted to address this fascinating aspect of chlamydial biochemistry.

Besides F26BP, other sugar phosphates such as G6P, G1P, F1P, F6P and R5P were also found to activate CTPK although not to the same extent. The activation of CTPK by both F26BP and by sugar monophosphates suggests that CTPK has properties

of both type I and type II PKs. Phylogenetic analysis of PK enzymes from prokaryotes, eukaryotes and archea also suggest that chlamydial PK is associated with both type I and type II PK isoenzymes (Schramm *et al.*, 2000).

ATP, the product of the PK reaction, was found to be an effective inhibitor of CTPK which is similar to many other PK enzymes from various sources (Garcia-Olalla and Garrido-Pertierra, 1987; Kapoor and Venkitasubramanian, 1983; Lin *et al.*, 1989; Podesta and Plaxton, 1991; Sakai *et al.*, 1986; Smith *et al.*, 2000; Srivastava and Baquer, 1985; Turner and Plaxton, 2000; Waygood and Sanwal, 1974). ATP increased CTPK's apparent $S_{0.5}$ and K_m for both PEP and ADP respectively, and was found to compete for the ADP binding site. ATP feedback inhibition is believed to play an important role in regulating PK activity *in vivo*, particularly in prokaryotes (Garcia-Olalla and Garrido-Pertierra, 1987; Sakai *et al.*, 1986; Waygood and Sanwal, 1974). High levels of ATP in the cell indicate that the energy charge is high, and the cell acts by inhibiting ATP generating reactions such as PK. Conversely, when the energy charge is low, ATP generating reactions are stimulated. Similar to ATP, GTP was also found to inhibit CTPK activity, a situation also found in other bacterial PKs (Chuang and Utter, 1979; Waygood *et al.*, 1975; Waygood and Sanwal, 1974).

AMP is also recognized as an important regulator in prokaryotes and has been found to activate several bacterial PKs (Garcia-Olalla and Garrido-Pertierra, 1987; Sakai *et al.*, 1986; Tanaka *et al.*, 1995; Waygood *et al.*, 1975). In contrast to these bacteria, AMP was found to inhibit CTPK activity when the CTPK recombinant protein was in the purified form. This property has also been observed in the plant PK of germinating castor oil seeds (Podesta and Plaxton, 1991) and in *Trypanosoma brucei* PK (Callens *et al.*,

1991). It may be that CTPK has lost its ability to respond to AMP as an activator and instead, under high AMP concentrations, the inhibitor competes for the ADP binding site. The inability of AMP to activate chlamydial PK may in part explain the unusually low energy charge that has been reported for chlamydial RBs (Tipples and McClarty, 1993).

Together, the results presented indicate that CTPK is an allosteric enzyme that differs from all other known prokaryotic PK enzymes in that it is regulated by F26BP. The data presented suggest that CTPK is probably regulated by the combined activity of host derived F26BP and ATP however, further studies on the availability of F26BP in chlamydia will have to be done in order to fully define the regulatory properties of CTPK as they exist *in vivo*. These studies will provide additional insight as to why chlamydia is such a successful pathogen and help to further define its intimate association with the host cell.

3. Glycogen and Carbon Metabolism

Based on genome sequence information, experiments were designed to determine whether chlamydiae could grow on selected gluconeogneic substrates. In addition, the ability of chlamydiae to regulate glycogen synthesis in response to changes in environmental nutrient conditions was assessed. Results presented demonstrate that *C. trachomatis* can survive in media supplemented with carbon sources other than glucose. Of the gluconeogenic substrates tested, chlamydial growth was optimal in the presence of glutamate. In addition, *C. trachomatis* infection increases the rate of glycogen synthesis and provided glucose is not limiting, the final yield of glycogen does not vary

substantially with changing glucose concentrations. In contrast, host HeLa cells substantially increased their glycogen stores in response to glucose excess. No glycogen synthesis or storage was observed when chlamydiae-infected cells were cultured in medium containing glutamate as primary carbon source. Finally RT-PCR analyses demonstrated that unlike most other organisms which tightly regulate central metabolism gene expression (Preiss, 1996; Saier *et al.*, 1996), expression of *C. trachomatis* glycogen metabolizing, hexose/dicarboxylate transporters and gluconeogenic genes remain relatively constant regardless of carbon source or amount of carbon available.

All cells require glucose for growth, however, many free-living bacteria are capable of growth on a variety of gluconeogenic substrates (Cooney and Freese, 1976; Hempfling and Mainzer, 1975; Kiel *et al.*, 1994; Preiss, 1996). The ability of any one organism to utilize a particular gluconeogenic substrate as a carbon and energy source depends on the genetic makeup of that organism i.e. the ability of the organism to transport and convert the substrate into glucose. If provided with a mixture of nutrients, bacteria are able to preferentially utilize the one that allows fastest growth. To achieve this, only enzymes necessary for utilizing the preferred nutrient are synthesized. With *E. coli* and other enteric bacteria glucose is the preferred carbon and energy source (Neidhardt *et al.*, 1990; Saier *et al.*, 1996). The ability to utilize an alternate substrate is controlled by catabolite repression, a process whereby the presence of glucose inhibits the expression of transporters and enzymes required for the metabolism of the alternate carbon source (Saier *et al.*, 1996). Catabolite repression is effected through the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) and cyclic AMP (cAMP) acting together with the cAMP receptor protein (CRP) (Saier *et al.*,

1996). cAMP and CRP also play a role in the regulation of the expression of genes involved in glycogen metabolism (Preiss, 1996). In several bacteria, carbon metabolism is also directly effected by the availabiltiy of nitrogen. As mentioned, both *E. coli* and *Bacillus* synthesize glycogen when nitrogen is limiting but carbon is still available in the growth environment.

Manipulating the availability of nitrogen or type and/or amount of carbon found in the growth environment of free-living bacteria is straightforward. In contrast, these simple experiments become very complicated when working with chlamydiae because they must be grown in the nutrient rich environment of a host cell cytoplasm. A recent study by Harper *et al.*, (2000a) demonstrated that chlamydiae transformed into aberrant forms when the amino acid content in the medium is reduced (Harper *et al.*, 2000a). Later Harper *et al.*, (2000b) showed that *C. trachomatis* infection increased the rate of amino acid transport into McCoy cells (Harper *et al.*, 2000b). These studies suggest that chlamydia is dependent on the host cell for amino acids. Furthermore, the genome sequence indicates that chlamydiae are auxotrophic for most amino acids and has several deaminases/transaminases (Stephens *et al.*, 1998). Thus, changing the carbon and energy source in the culture medium has an impact on the host cell, which may or may not have a direct or indirect effect on chlamydial growth. To help minimize the effects of the alternative carbon source on the energy status of the host cell, the medium was supplemented with pyruvate, a carbon source that could be oxidatively metabolized by the host (Voet D. and Voet J., 1990) and previously shown not to be utilized by chlamydiae (Weiss, 1967). Our results indicate that *C. trachomatis* can survive and undergo limited growth with gluconeogenic substrates as the sole carbon source,

particularly with glutamate. Interestingly, a recent study monitoring the effects of *C. psittaci* infection on host cell energy metabolism found that infection increases glutamate synthesis (Ojcius *et al.*, 1998). Our results also showed that in the presence of the gluconeogenic substrates, ATP pool size was maintained at 60-80% of values found with cells cultured in the presence of excess glucose. This suggests that the poor growth may be attributed to the actual carbon source available.

Since the chlamydia genome sequence analysis suggests that the enzymes necessary to carry out gluconeogenesis are present for the various carbon sources tested, and our RT-PCR results indicate that the genes are transcriptionally active it may be that the growth limitation is at the level of substrate transport. Chlamydiae have transporter homologues of *uhpC* and *gltT* encoding glucose phosphate and glutamate transporters respectively (Figure 37), the two carbon sources which best support chlamydial growth. The gluconeogenic substrates that are not as good at supporting growth are likely transported by the SodiTi homologue (Figure 37). It has been suggested that *sodiTi* encodes a dicarboxylate translocator that takes up oxaloacetate or α-ketoglutarate in return for malate. This exchange only results in a net gain of 1 carbon. The SodiTi homolog is therefore limited in its effectiveness as sole carbon source transporter as compared to the UphC and GltT transporters which allow net gain of glucose-6-P (6 carbons) and glutamate (5 carbons) respectively (McClarty, 1999; Weber *et al.*, 1995).

Studies have indicated that eukaryotes as well as several prokaryotes regulate glycogen biosynthesis in response to carbon changes in the growth environment (Gaudet *et al.*, 1992; Kiel *et al.*, 1994; Neidhardt, 1990; Preiss, 1996; Slock and Stahly, 1974; Stryer, 1988). It is well known that when blood glucose levels become elevated in

humans, glycogen synthetic enzymes are activated by a series of regulated reactions and glycogen is synthesized and stored. Conversely, when blood glucose levels become depleted, glycogen synthesis is inhibited, degradation enzymes are activated, and blood glucose levels are restored. *E. coli* controls glycogen metabolism at both the protein and transcriptional level (Preiss, 1996). In rich media the activities of the glycogen synthetic enzymes are suppressed, but when nitrogen becomes limiting, the enzyme activities are increased 5-12 fold and glycogen is accumulated provided carbon is available. In minimal media, *E. coli* senses that nutrients are limiting and responds by increasing glycogen synthetic enzyme activities (AGP, GS, BE) during the exponential phase in order to accumulate glycogen (Preiss, 1996). Bacillus is also responsive to its environment and accumulates glycogen under conditions where nitrogen is limited but carbon is available (Cooney and Freese, 1976; Slock and Stahly, 1974).

In contrast to most free-living organisms (Cooney and Freese, 1976; Neidhardt, 1990; Preiss, 1996; Slock and Stahly, 1974), *C. trachomatis* does not increase its rate of synthesis or yield of glycogen when excess carbon is available. That is, the yield of glycogen attributed to the infection grown under glucose-rich conditions is approximately the same as that found when grown under normal conditions (1mg/ml glucose)(Table 17). These results suggest that some reaction in the pathway required for glycogen biosynthesis in *C. trachomatis* could be fully saturated when grown under non-limiting carbon conditions. Although at this time it is not known what that step is, it may be at the level of glucose phosphate availability from the host or transport into the chlamydial vacuole or RB. It has been shown that *C. psittaci* infection increases host cell surface expression of the glucose transporter, Glt1, presumably to meet the increased demand for

glucose placed on the host cell by the infection (Ojcius *et al.*, 1998). This coupled with the fact that we see dramatic increases in host cell glycogen accumulation in response to excess glucose in the medium suggest that the amount of glucose phosphate available in the host cell cytoplasm is unlikely limiting. Furthermore, results from RT-PCR experiments showed that there was no obvious increase in the level of expression of *uhpC* when chlamydiae infected cells were cultured in medium containing excess glucose. Taken together these results suggest that glycogen accumulation in chlamydiae may be limited by glucose phosphate transport. The lack of responsiveness to excessive glucose also suggests that glycogen in *C. trachomatis* may serve other or additional purposes besides storage and glucose sequestration.

Cyanobacteria has been suggested to use glycogen synthesis and degradation to control osmotic pressure in cells (Bruton *et al.*, 1995). Similarly, *C. trachomatis* may use the dynamics of glycogen synthesis and degradation to control osmotic pressure within the inclusion. It may be that a certain amount of glycogen is needed to obtain the osmotic balance, and an increase in glycogen stores may upset this dynamic putting the integrity of the inclusion in jeopardy. Under this scenario however, the glycogen metabolizing enzymes would have to be secreted from RBs in order to control the size of the glycogen store within the inclusion and glucose availability. The presence of these enzymes in the inclusion may be advantageous to chlamydiae, as it could allow for the sequestration of glucose from the host and storage in an osmotically favorable form. Subsequent parasite controlled breakdown of glycogen would provide glucose 1-P which could be directly transported into a RB via the *uhpC* gene product. Another possible function for glycogen in *C. trachomatis* would be to play a role in EB to RB differentiation. Glycogen

accumulation and breakdown has been implicated in sporogenesis in Bacillus (Preiss, 1996), and has also been suggested to play a role in morphological differentiation in the gram positive bacteria streptomycetes (Martin *et al.*, 1997). *C. trachomatis* RBs may store glycogen to sequester glucose from the host and then use it to help fuel transformation into EBs. Similarly, glycogen in EBs may be used as a source of glucose phosphate to fuel very early EB/RB differentiation events. This may be especially critical if EBs lack glucose phosphate transporters.

Discussion about an essential role for glycogen in chlamydial growth and development has to take into account that, unlike *C. trachomatis,* neither *C. psittaci* nor *C. pneumoniae* have been found to accumulate glycogen (Moulder, 1991) even though all three species contain the same complement of glycogen metabolizing genes (Kalman *et al.*, 1999; Read *et al.*, 2000; Stephens *et al.*, 1998; http:www.tigr.org/). Possibly, the lack of glycogen accumulation by *C. pneumoniae* and *C. psittaci* is not due to an absence of functional enzymes, but rather the dynamics of glycogen synthesis and degradation are relatively equal such that glycogen does not accumulate in appreciable amounts. Alternatively, glycogen accumulation may be influenced by species-specific inclusion properties such as fusibility. For example, several investigators have demonstrated that *C. trachomatis* inclusions fuse with each other within an infected-cell, whereas *C. psittaci* generally form multiple inclusions within a single cell (Hackstadt, 1999a; Matsumoto *et al.*, 1991; Rockey *et al.*, 1996). Another study demonstrated that when a cell is multiply infected with more than one chlamydial species, the inclusions do not fuse suggesting unique inclusion properties specific to each chlamydial species (Matsumoto *et al.*, 1991). Another possible reason for the lack of glycogen found in *C. pneumoniae* or *C. psittaci*

may be the lack of the 7.5 kb plasmid strictly conserved in the *C. trachomatis* species (Comanducci *et al.*, 1988; Palmer and Falkow, 1986; Sriprakash and Macavoy, 1987). A role for the 7.5 kb plasmid in glycogen accumulation has been proposed in *C. trachomatis* as plasmid-less isolates no longer accumulate glycogen (Matsumoto *et al.*, 1998). Further studies on the dynamics of glycogen metabolism in *C. psittaci* and *C. pneumoniae* are warranted, to address this fascinating issue of chlamydial biology.

RT-PCR analysis indicates that the expression patterns of all the *C. trachomatis* L2 central metabolism genes examined are similar during the developmental cycle. Generally, transcripts are at a maximum in the middle of the cycle when most chlamydiae are in RB form and are less prominent at the earlier and later stages when fewer RBs are present. A closer look at the genes involved in glycogen metabolism suggests that chlamydial glycogen biosynthesis may be delayed relative to glycogen degradation as GS transcript is not prominent until mid-cycle (Fig. 39A). A recent study by Vandahl *et al.* (2001) reported the presence of GLGP protein, but not GLGA protein in *C. pneunomiae* EBs further suggesting that glycogen degradation may occur at the start of the developmental cycle, where as biosynthesis may be delayed until EBs have transformed into RBs later in the cycle. In contrast, a study by Shaw *et al.*, (2000) reported the presence of *glgC* transcript, another gene involved in glycogen biosynthesis, as early as 2 h p.i. Assuming that the detection of transcript implies protein production, then the presence of GS and GP transcripts at the same time points in the chlamydial developmental cycle suggests that glycogen metabolism in *C. trachomatis* is a dynamic process where glycogen synthesis and degradation may be occurring simultaneously. Interestingly, a recent study by Belanger *et al.*, (1999) suggests that the biosynthetic and

degradative steps of glycogen metabolism in *Mycobacterium smegmatis* is an on-going process where carbon flows preferentially through the glycogen biosynthetic pathway and is synthesized into glycogen before it is then used in cellular metabolism and energy production. Further studies on glycogen metabolism in *C. trachomatis* is needed to clarify whether the proteins involved are functional, and whether they are developmentally regulated.

RT-PCR analysis indicates that the expression patterns of the *C. trachomatis* central metabolism genes examined are relatively unaltered in response to changes in the amount and/or type of carbon found in the media. For example, when infected cells were cultured in the presence of radiolabeled glutamate and cycloheximide, there was essentially no incorporation of radioactivity into glycogen suggesting that there was limited glucose 1-phosphate available for glycogen synthesis. Despite this glycogen synthase was expressed just as it was when excess glucose was present and glycogen was accumulated. A similar expression ratio occurred when cells were cultured in glutamate minus cycloheximide only all the genes were expressed at a lower level, a result consistent with the reduced EB yield under this condition. Furthermore, even under conditions when little or no glycogen is available for degradation glycogen phosphorylase is expressed. This inability to respond to the environment may be due to the fact that chlamydiae has few homologues of components of global response systems including key components (adenylate cyclase, cAMP receptor protein) of the catabolite repression system found in most free-living organisms (Saier *et al.*, 1996). The elimination of catabolite repression may be linked to the evolution of the obligate parasitic lifestyle of chlamydiae that requires much less versatility in response of the

parasite to environmental changes than its free-living counterparts. In short, the hosts cell cytoplasm likely represents a relatively static nutrient environment.

The one exception to this general observation was found when chlamydiae-infected cells were cultured in the absence of both glucose and gluconeogenic substrates. In this case *euo*, an early gene that is expressed within 2 hr of infection was present suggesting that EB to RB differentiation was initiated, however expression of *omcB*, a gene expressed 16 hr after infection was absent suggesting that RB to EB differentiation was blocked. In agreement with this, it was recently reported that cultivation of chlamydiae in glucose free medium gave rise to abnormal chlamydial forms that were non-infectious (Harper *et al.*, 2000a). Together these results imply that, at least under the condition tested, EBs do not sense whether the host cell environment is permissive for growth. Interestingly, Harper *et al.* (2000a) showed that the abnormal developmental forms induced by glucose deprivation could give rise to infectious EBs if glucose was reintroduced into the medium, a result that indicates that the abnormal forms remain viable. Previously, Scidmore *et al.* (1996b), showed that if chlamydial transcription or translation are inhibited from the onset of infection chlamydial EB to RB differentiation was blocked and the EBs were eventually delivered to lysosomes. It may be that even if host cell nutrient conditions are not conducive for supporting a complete chlamydial growth cycle, initial EB differentiation is absolutely necessary as a survival mechanism for preventing destruction of the parasite via trafficking to lysosomes.

In total, the results presented on central carbon and glycogen metabolism indicate that *C. trachomatis* can survive and undergo limited growth with gluconeogenic substrates as sole carbon source. However, under these conditions glycogen does not

accumulate. Furthermore, in contrast to other organisms, *C. trachomatis* does not accumulate additional glycogen in response to excess glucose. Unlike most free-living bacteria (Kiel *et al.*, 1994; Preiss, 1996; Takata *et al.*, 1997), chlamydiae do not appear capable of regulating the expression of genes encoding key enzymes of central metabolism at the transcriptional level. Presumably in the intracellular environment in which chlamydiae have evolved to survive this type of regulation is no longer advantageous. This may also explain why, unlike most bacteria, genes encoding enzymes from biosynthetic pathways are often dispersed around the chromosome rather than being arranged in operons.

4. Summary

The work in this thesis demonstrates that chlamydia contains biologically active, energy-producing enzymes in the glycolytic (GAPDH, PGK, PK) and pentose phosphate pathways (ZWF). In addition, the *C. trachomatis* D genome sequence indicates that chlamydia encode enzymes for a complete Embden-Meyerhof-Parnas pathway, pentose phosphate pathway and partial TCA cycle. In fact, the genome sequence indicates that chlamydiae have biosynthetic and energy generating capacity beyond those present in other free-living small genome organisms such as *Mycoplasma genitalium* (Fraser *et al.*, 1995), *Treponema pallidum* (Fraser *et al.*, 1998) and *Borrelia burgdorferi* (Fraser *et al.*, 1997). This information necessitates a major change in the way we view central metabolism in chlamydiae. For several decades, it was accepted that chlamydia was an "energy parasite" and lacked the machinery to generate its own energy. The work in this

thesis demonstrates that chlamydia are not energy parasites, at least not in the strict sense because in addition to obtaining ATP from the host cell (via ATP/ADP translocator), they have the functional capacity to produce some of their own energy and reducing power. The genome sequence also indicates that *C. trachomatis* also has all the components for a complete respiratory chain and ATP synthase complex to regenerate NAD and produce ATP respectively. It also contains the genetic capacity to synthesize, store and degrade glycogen. This glucose containing polysaccharide has the potential to serve as a carbon and energy source during various stages in the chlamydial developmental cycle, particularly in early differentiation. Thus hypothetically, it appears that chlamydia could generate enough energy to fulfill its life cycle. Glycogen could serve as an early carbon and energy source to fuel initial EB to RB differentiation and the stored metabolic enzymes and ATP could carry out initial biosynthetic and energy producing reactions. Energy could be supplied throughout the rest of the life cycle through chlamydia's biochemical machinery until RBs differentiate back into EBs. At this stage, glycogen, ATP and metabolic enzymes could again be stored in EBs to allow the EBs to under go another round of infection.

One of the problems with this senario is that despite the ability of chlamydia to produce its own energy, the genome sequence indicates that chlamydia lacks homologues of genes in the *de novo* nucleotide synthesis or nucleotide salvage pathways indicating that chlamydia cannot synthesize its own nucleotides. This inability to synthesize nucleotides may be a key as to why chlamydiae are obligate intracellular parasites because sufficient concentrations of these high-energy metabolites would only be available from nutrient rich environments like the host cell cytoplasm. Thus, whether

chlamydia contains unique enzymes involved in nucleotide biosynthesis and can survive without host NTPs, particularily ATP, or can live off stored ATP which it could potentially recycle will help provide insight into whether chlamydia is truly an obligate "energy-parasite". Further analysis of the enzymes involved in central metabolism in chlamydia will provide a better understanding of the metabolic relationship chlamydiae has with its host cell.

Overall, the data presented in this thesis provides considerable insight into the central carbon and energy metabolism in chlamydia. This work provides a basis for continued studies in a number of directions. For example: Is chlamydia an obligate energy parasite or can it survive without host NTPs transported by the ATP/ADP transporters? Is the ATP synthase complex and respiratory chain in chlamydia functional? Does chlamydia encode for ATP- and PPi- dependent PFK enzymes that are reversible and are they allosterically regulated? Does aldolase have unique properties in chlamydia? Is PEP carboxykinase the key linker between the EMP and TCA cycle and does it have any regulatory properties? Are the glycogen metabolizing enzymes in *C. trachomatis*, *C. pneumoniae* and *C. psittaci* functional? What is the biological function of glycogen in chlamydia? Information generated from these types of studies will be essential for the development of a cell free growth system for chlamydiae which would greatly simplify studies on their metabolism and assist in the development of a system for genetic transformation.

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APPENDIX

Abbreviations

p.i.	post infection
MI	mock-infection
NTP	ribonucleoside triphosphate
RB	reticulate body
EB	elementary body
MOMP	major outer membrane protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
PGK	phosphoglycerate kinase
PK	pyruvate kinase
ZWF	glucose-6-phosphate dehydrogenase
PPi-PFK	pyrophosphate-dependent phosphofructose kinase
ATP-PFK	adenosine triphosphate-dependent phosphofructose kinase
NAD(P)+	nicotinamide adenine dinucleotide (phosphate) [oxidized form]
NAD(P)H	nicotinamide adenine dinucleotide (phosphate) [reduced form]
FAD	flavin adenine dinucleotide [oxidized form]
FADH	flavin adenine dinucleotide [reduced form]
PEP	phosphoenolpyruvate
F26BP	fructose-2,6-bisphosphate
rRNA	ribosomal ribonucleic acid
LGV	lymphogranuloma venereum

PG	peptidoglycan
POMP's	polymorphic outer membrane proteins
Hsp	heat shock protein
GAG	glycosaminoglycan
HS	heparan sulfate
PPP	pentose phosphate pathway
TCA	tricarboxylic acid cycle
G3P	glycerol-3-phosphate
3PGA	3-phophoglycerate