CHARACTERIZATION OF TRIMETHOPRIM AND SULPHISOXAZOLE RESISTANT CHLAMYDIA TRACHOMATIS

BY

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Abstract

Chlamydia are obligate intracellular parasites that infect a wide range of host cells and cause a variety of diseases. In an attempt to characterize nucleotide metabolism in *Chlamydia trachomatis* L2, a series of mutants resistant to trimethoprim, sulphonamide, and methotrexate were selected from a wild type population. Each drug targets a step in the thymidylate synthase cycle. Mutants expressed a series of nucleotide pathways designated pathway II, instead of pathway I, which is seen in the wild type. By utilizing a thymidine kinase in pathway II, they had bypassed the necessity of having a thymidylate synthase pathway. Thymidine kinase was chosen as a target for study because of its importance in pathway II while not being expressed in pathway I. The thymidine kinase gene was isolated by complementation and characterized. It was 599 base pairs long with a typical ATG start codon and -35 and -10 promoter regions; there was a typical rho independent terminator sequence downstream of the gene. The GC ratio was 30%. When screening the *Chlamydia trachomatis* L2 genomic library by hybridization, both positive clones that were characterized had a high homology to a chlamydial ribosomal operon. The tRNA gene for glycine ($tRNA^{GLY}$) was found downstream of the thymidine kinase gene. However no homology to this gene was found within the clones. When the probe was divided into the thymidine kinase and $tRNA^{GLY}$ alone, the results were inconclusive. Due to the low GC ratio, the possibility of contamination by mycoplasma was investigated. However, when the gene was used to screen both *Acoliplasma laidlawii* and *Mycoplasma hominis* the result was negative.

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I would like to thank Grant McClarty for his guidance and humour throughout this project. I would also like to express my gratitude towards Pat McNicol and Barb Triggs-Raine who served as members of my committee. Michelle Alfa and Pat Degagne who provided litres of *Mycoplasma hominis*, as well as introducing me to its many peculiarities. Kevin Coombs and the members of his lab who provided the L929 cells. Shiwen Zhang who did the early cloning work. Dr. Brunham and the members of his lab who answered innumerable questions. Graham Tipples and Ling Ling Wang for their friendship and assistance throughout the project. Kristin Buisson for outstanding technical assistance. Portions of this work have been published in Wang *et al*, Molecular Microbiology (1994) **14**(2), 271-281. Sandy Pettigrew who helped me enter the sequences into the computer. Ted Kuschak and Brenda Chan who lent me their printer and pumped me full of coffee in the final hour. Mrs. Banton, Karin and Mike Berry, Lisa Didyk, Raz Dong, Michelle Gordon, Anthony Kiendl, Bela Tiwari, and Kelly Yorkiw for keeping me sane. Sharon Rennie who kept me sane in undergrad. Dr. McAlpine and Ms. Neilson for support and encouragement early in my scientific career. Many thanks to my mother who drew metabolic pathways to explain why I had to eat my vegetables. Thank you for creating an atmosphere where science was never intimidating. To my father, whose knowledge and scientific curiosity inspire me still. Thanks go to my brothers and sister in law who listened patiently while I talked about my project. A final thanks goes to Natalie, who reminds me every day of the reasons I went into science.

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APRT	adenine phoshoribosyltransferase
CH_2FAH_4	5,10 methylene tetrahydrofolate
CTPS	CTP synthetase
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
ds	double stranded
EB	elementary body
FAH_2	dihydrofolate
FAH_4	tetrahydrofolate
GPRT	guanine phosphoribosyltransferase
H_2PtCH_2OPP	6-hydroxymethyl-7,8-dihydropterin pyrophosphate
H_2pteroate	dihydropteroate
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HPLC	high performance liquid chromatography
kDa	kilodalton
LB	Luria-Bertani medium
LGV	lymphogranuloma venereum
LPS	lipopolysaccharide
MI	Mock-infected
MOI	multiplicity of infection
NTP	ribonucleoside triphosphates
pABA	*p*-aminobenzoic acid
RB	reticulate body
RR	ribonucleotide reductase
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
TK	thymidine kinase
TS	thymidylate synthase
Urd^-C	5-fluorouracil resistant CHO K1 cell line
UPRT	uracil phosphoribosyltransferase

1. Introduction

Chlamydia are obligate intracellular eubacterial parasites that infect a wide range of host cells and cause a variety of human, non-human mammalian and avian diseases (Fraiz and Jones, 1988; Storz, 1988). Chlamydia are classified in the order *Chlamydiales*, which has a single family (*Chlamydiaceae*) and genus (*Chlamydia*) (Moulder *et al*, 1984). The genus *Chlamydia* currently contains four species: *Chlamydia trachomatis, Chlamydia pneumonia, Chlamydia psittaci,* and *Chlamydia pecorum*. With the exception of the mouse pneumonitis biovar, *C. trachomatis* is strictly a human pathogen. In developing countries, *C. trachomatis* causes trachoma, the world's leading cause of preventable blindness. In industrialized countries, *C. trachomatis* is the most prevalent sexually transmitted disease, causing urethritis, cervicitis, ectopic pregnancy, and pelvic inflammatory disease. A significant consequence of pelvic inflammatory disease is infertility. *C. pneumoniae* is exclusively a human pathogen, which has recently been identified as an important cause of acute respiratory diseases including pneumonia, bronchitis, and pharyngitis. *C. psittaci* has been isolated from a number of avian and mammalian species, in which they produce a variety of diseases including diarrhoea, pneumonia, abortion, polyarthritis-polyserositis, encephalomyelitis, hepatitis, and conjunctivitis. When humans contract *C. psittaci* from exposure to infected birds, they may develop the disease psittacosis. *C. pecorum* has been isolated from sheep and cattle, where it causes pneumonia, polyarthritis, encephalomyelitis and diarrhoea (Fukushi and Hirai, 1992). Thus, chlamydia are one of the most ubiquitous pathogens in the animal

kingdom.

1.0 Definition of the species

The chlamydia, although heterogeneous, have similar features that define the group (Hackstadt, 1986; Moulder *et al*, 1984; Schachter, 1984). 1) A complex life cycle that includes an infectious extracellular cell type, the elementary body (EB), and a non-infectious, intracellular and replicating cell type, the reticulate body (RB) (Friis, 1972; Schachter and Caldwell, 1980). 2) Disulfide bonding of outer membrane proteins as a mechanism of maintaining structural stability (Bavoil *et al*, 1984; Hackstadt *et al*, 1985; Hatch *et al*, 1984; Newhall and Jones, 1983) in the absence of peptidoglycan (Barbour *et al*, 1982; Garrett *et al,* 1974; Manire and Tamura, 1967; Tamura and Manire, 1967). 3) Replication of the parasite within phagosomes apparently modified by the bacteria to inhibit fusion with the lysosomes (Friis, 1972; Schachter and Caldwell, 1980). 4) Dependence on host cells for the manufacture of high energy adenosine triphosphate metabolites (Moulder, 1991). 5) Chlamydia contain both deoxyribonucleic acid and ribonucleic acid (Starr *et al*, 1960; Collier, 1962), and typical prokaryote ribosomes (Tamura and Iwanaga, 1965; Sarov and Becker, 1968). 6) An outer membrane, similar in some respects to that of gram-negative bacteria, possesses antigenic structures which are common among the chlamydia (Barns, 1989).

1.1 Membrane structure

The outer membrane of the EB protects chlamydia against inactivation caused by antibodies and the complement system in the extracellular environment. Both EBs and RBs are surrounded by a double membrane system as in gram-negative bacteria. The membrane structure of the EB has been intensively investigated. The outer membrane of the EB is composed of protein, phospholipid and lipopolysaccharides as in gram negative bacteria (Jenkin, 1960), but no peptidoglycan has been detected in chlamydia (Barbour *et al* 1982; Garrett *et al*, 1974; Manire and Tamura, 1967; Tamura and Manire, 1967). In gram negative bacteria a fraction of the outer membrane is insoluble in ionic detergents such as sodium dodecyl sulfate or sarkosyl. This fraction is composed of peptidoglycan with covalently bound lipoprotein. Porins such as OmpA and OmpC are associated with the peptidoglycan layer (Benz, 1988; Osborn and Wu, 1980). Though no peptidoglycan layer has ever been detected, a fraction of the chlamydia outer membrane is insoluble in sarkosyl. This fraction is named "chlamydia outer membrane complex" (COMC) (Caldwell *et al,* 1981). The protein components of COMC are: 1) The major outer membrane protein (Omp1) that accounts for 60% of the total protein in COMC (Caldwell *et al*, 1981), 2) two cysteine rich proteins, one at 60 kilodaltons (kDa) (Omp2) that appears as a double band and one at 12-12.5 kDa (Omp3) (Batteiger *et al,* 1985), and 3) a 96 kDa protein which is seen in a coomassie blue-stained SDS-polyacrylamide gel (Newhall, 1988).

2. Characteristics of the species (Table 1)

2.0 *Chlamydia trachomatis*

With the exception of the mouse biovar, *C. trachomatis* is strictly a human pathogen. The preferred site of infection is the squamo-columnar epithelium (ocular, genital, respiratory) for trachoma, the lymph nodes for lymphogranuloma venereum (LGV). The EBs are round, and the inclusions are oval, vacuolar, and contain glycogen.

C. trachomatis is divided into serotypes (Wang and Grayston, 1970). The serotypes A-K are restricted to growth in epithelial cells whereas serovars L1 to L3 grow in lymphatic tissue such as mononuclear cells and macrophages. The DNA homology between the serotypes is nearly 100%.

The serotypes A, B, Ba, and C are the agents of trachoma, the leading cause of preventable blindness that is endemic in the third world. Chlamydia infection of conjunctiva produces a follicular conjunctivitis with diffuse infiltration and pannus that can lead to conjunctival scarring. The conjunctival scarring persists without signs of active trachoma (Schachter and Caldwell, 1980).

The *C. trachomatis* serotypes D-K are a common cause of sexually transmitted genital infection world-wide: cervicitis, endometritis/salpingitis can lead to scarring and agglutination of salpinx with a higher risk of extrauterine pregnancy and infertility.

Table 1: Some characteristics of the species and biovars of the Genus Chlamydia[a]

Characteristic[a]	*C. trachomatis*			*C. psittaci*	*C. pneumonia*[b]	*C. pecorum*[c]
	Trachoma	LGV	Mouse			
Natural Hosts	Humans	Humans	Mice	Birds, Lower mammals	Humans	Sheep, Cattle
Preferred site of Infection	Squamo-columnar epithelium	Lymph Nodes	Lungs	Multiple sites	Squamo-columnar epithelium	Multiple sites
G+C content of DNA (mol %)	40%	42%	43%	41%	40%	39.3%
DNA Homology[c]						
C. trachomatis	>92%	>92%	20%	1-33%	1-7%	1-10%
C. psittaci				14-95%	1-8%	1-19%
C. pneumonia					94-96%	10%
C. pecorum						88-100%
EB Morphology		Round		Round	Pear shaped	N.A.
Inclusion Morphology		Oval, vacuolar		Variable shape, dense	Oval, dense	N.A.
Glycogen in inclusions		+		-	-	-
Folate Biosynthesis		+		-/+	-	N.A.

[a] Modified from Grayston *et al*, 1989

[b] See References Campbell *et al*, 1987; Cox *et al*, 1988; Grayston *et al*, 1986; Kuo *et al*, 1986; Kuo and Grayston, 1988; Wang and Grayston, 1986.

[c] Fukushi and Hirai, 1992

N.A. Not available

The genital infection may cause acute disease, but persistent infection in balance with the host immune system, without clinical signs, can also occur. The persistent infection may become active in connection with induced abortion or child delivery. The child can be infected during the birth leading to conjunctivitis or pneumonia during the first three months of life (Schachter and Osoba, 1983; and Schachter, 1988).

C. trachomatis serotypes L1-L3 are the agents of lymphogranuloma venereum, a sexually transmitted systemic infection. The genital primary lesions of the infection are transmitted to regional lymph nodes that become enlarged and can perforate through the skin. Sepsis may occur with the spread of chlamydia to meninges and joints. Aseptic polyarthritis may occur, which can be treated effectively (Schachter and Osoba, 1983; Birkelund, 1992).

2.1 *Chlamydia psittaci, Chlamydia pneumonia and Chlamydia pecorum*

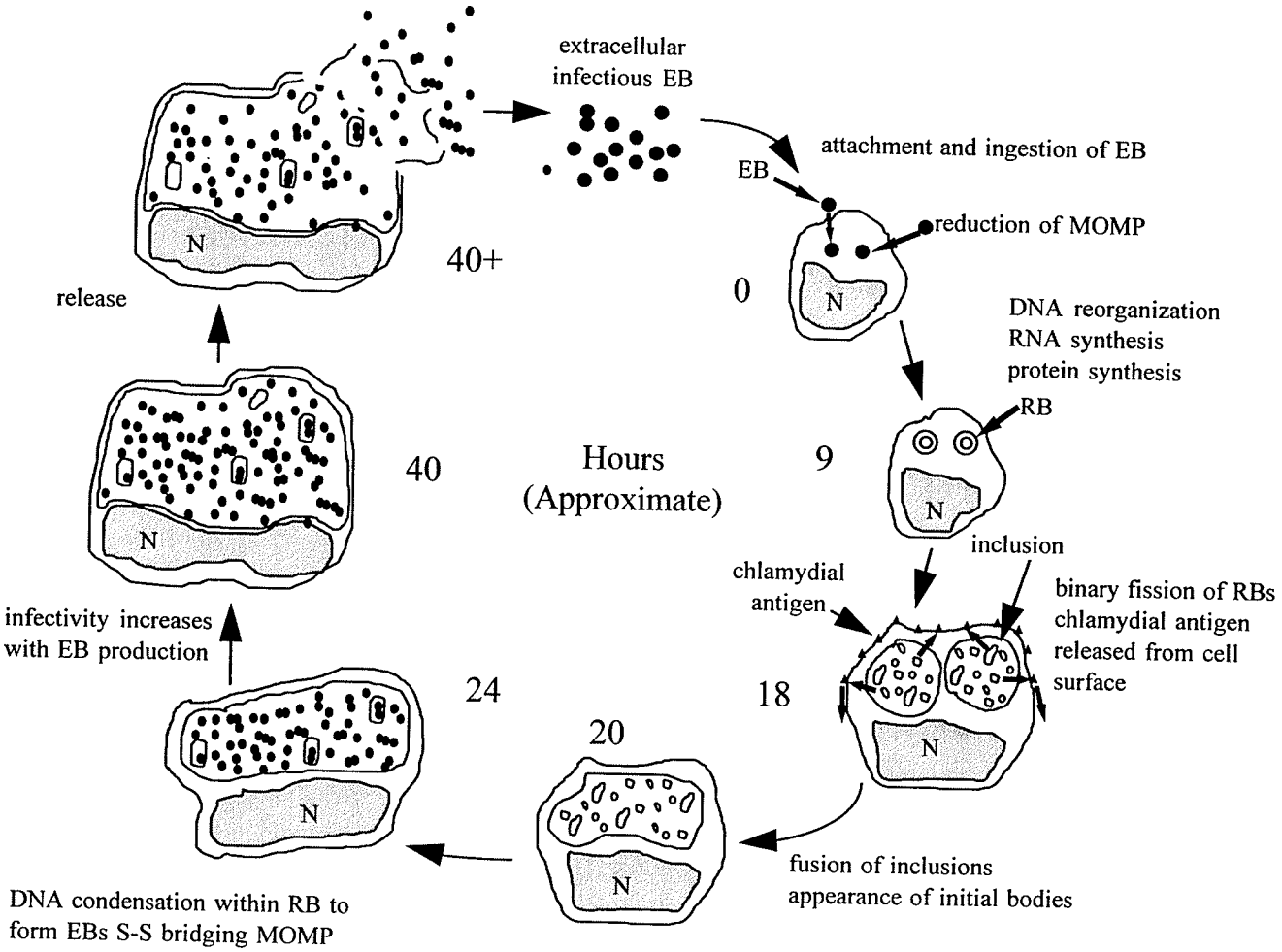
C. psittaci infects birds and mammals at multiple sites. The EBs are round, and the inclusions are of various shapes, dense, and do not contain glycogen. *C. pneumonia* infects humans. The preferred site of infection is squamo-columnar epithelial cells (respiratory). The EBs are pear shaped, and the inclusions are oval and dense (Chi *et al*, 1987). *C. pecorum* infects sheep and cattle at multiple sites. The EBs are round, and the inclusions are oval, dense and do not contain glycogen.

3. The intracellular life cycle (Figure 1)

3.0 *EB to RB transition*

Chlamydia are non-motile, coccoid, intracellular bacteria with a distinctive biphasic life cycle to facilitate their survival in two discontinuous habitats. Chlamydia replicate within a phagocytic vacuole. The unique intracellular developmental cycle of chlamydia begins with infection of a host cell by a spore-like, non-vegetative, elementary body (EB), a uniformly spherical particle 300 nm in diameter (Barns, 1989). The EB initiates infection by attaching to susceptible host cells, by a unique trimolecular mechanism (Zhang and Stephens, 1992). Following ingestion by a host cell, the EB is able to avoid the fusion of its surrounding endosome with primary lysosomes of the parasitized cell, thus allowing it to survive within the endosome. EBs reorganize to RBs during the first 4 to 10 hours of the cycle (Hatch, 1988). Nothing is known about the intracellular environmental signals which trigger this reorganization. It is clearly a period of active protein and RNA synthesis because the peptide and RNA composition of RBs varies considerably from that of EBs: however, the exact synthetic events which take place early in the cycle have not been analyzed because of the difficulty of detecting the activities of a few chlamydial organisms within the background of an infected host cell (Hatch, 1988). The RB is pleomorphic and 800 to 1 200 nm in diameter (Barns, 1989) and has a loosely distributed nucleoid when compared with EBs. RBs grow and divide by binary fission, with a doubling time of approximately 2 hours (McClarty, 1994). The inclusion is apparently formed from host cell membrane upon ingestion of the infecting EB: however the

Figure 1: Diagram of Chlamydial Development



mechanism of growth of the inclusion membrane during further replication is unclear. During most phases of the developmental cycle, both EBs and RBs are found within the same inclusion. By 16-20 hours after infection while some RBs are still replicating, others have begun the process of differentiation back into infectious EBs (Moulder, 1991).

3.1 *RB to EB transition*

The interval between entry and the beginning of reorganization is dependant on the length of the developmental cycle of the particular chlamydia species being studied. However, once started, the series of events leading from RB to EB appear to be much the same for all chlamydia-host cell combinations. Through several divisions of intermediate transitional forms between RBs and EBs, the RB progresses toward the EB by reduction in size, internal condensation, differentiation of electron-dense nucleoids, and formation of rigid cell walls. At all times after intermediate forms first appear, the chlamydial population within a single inclusion consists of a mixture of dividing RBs, intermediate forms, and mature EBs. This lack of synchronicity makes it unlikely that the signal for the differentiation of RB to EB is triggered by the continued multiplication of the RB population. Instead, the signal must originate at different times in each RB (Moulder, 1991).

The transformation of RB to EB, like the change from EB to RB involves the synthesis of

a variety of proteins that are not made at other times in the developmental cycle. In addition to the three cysteine rich outer membrane proteins, proteins that bind to eukaryotic cell membranes (Hackstadt, 1986; Wenman and Meuser, 1986) or to chlamydial DNA (Wagar and Stephens, 1988) are also found in EBs but not in RBs. One of the DNA binding proteins appears to be identical with the largest of the cysteine rich proteins of the outer membranes. The expression of sets of temporally regulated proteins in the developmental cycle is now well established. It has been suggested that this regulation is achieved by a series of promoters specifically recognized in sequence by RNA polymerase either by modified cycle dependant sigma factors or mediated by DNA binding proteins (Engel and Ganem, 1990; Hatch *et al*, 1990; Plaunt and Hatch, 1988).

Growth and differentiation continues until approximately 48-72 hours after infection, when the population is predominantly in the form of EBs. The egress of mature, infectious EBs occurs by lysis of the host cell (Todd and Caldwell, 1985) which initiates a new infection cycle.

4. Metabolism

All bacteria require the essential building blocks (amino acids, nucleotides, nucleotide-sugars and fatty acids) to construct the macromolecules (protein, DNA, RNA, lipids, lipopolysaccharide (LPS) and peptidoglycan) that make up a cell. Since chlamydia is an obligate intracellular parasite, it could dispense with all the biosynthetic pathways

required to make the various metabolites that are available in the host-cell cytoplasm. This would be in keeping with its small genome of approximately $1.0x10^6$ nucleotide pairs (Moulder, 1991). However, two factors influence the access that chlamydia have to the nutrient-rich host cell cytoplasm. One is the competition from the host-cell biosynthetic machinery (Moulder, 1984). In addition to this, many of the metabolites and coenzymes that are available from the host-cell cytoplasm are in forms (e.g. nucleotides, conjugated nucleotides and acetyl CoA) that cannot cross cell membranes without active transport systems. With chlamydia, this permeability barrier includes three membranes: the inclusion membrane, outer membrane and cytoplasmic membrane (McClarty, 1994).

4.0 *Nucleotide metabolism*

Nucleotides play a role in nearly all biochemical processes:

1) They are the activated precursors of DNA and RNA
2) Nucleotide derivatives are activated intermediates in many biosyntheses.
3) ATP, an adenine nucleotide, is a universal currency of energy in biological systems. GTP powers many movements of macromolecules.
4) Adenine nucleotides are components of three major coenzymes: NAD^+, FAD, and Coenzyme A.
5) Nucleotides are metabolic regulators. Cyclic AMP is a ubiquitous mediator of the action of many hormones (Stryer, 1988).

4.0.1 *Dependence on the host*

Most prokaryotic and eukaryotic cells are impermeable to nucleotides because they lack suitable transport systems (Plagemann *et al,* 1988). Various studies with host-free RBs, including the synthesis of RNA in the presence of exogenous ribonucleoside triphosphates (NTPs), suggest that chlamydia can transport NTPs (Hatch, 1988). In contrast, EBs are metabolically inactive and cannot transport NTPs (Hatch, 1988); however, they do have a soluble pool of NTPs that may fuel very early differentiation events (Tipples and McClarty, 1993). These studies are limited, since host-free chlamydia are labile and do not grow, which makes it difficult to study anabolic processes. *In situ* studies, where chlamydia are growing rapidly within their natural environment, the host cell, have assisted in most of the advances in nucleotide metabolism. The most revealing studies have been those where chlamydial-specific nucleotide metabolism has been characterized in a mammalian host cell line with well defined genetic deficiencies.

4.1 *Description of the pathways*

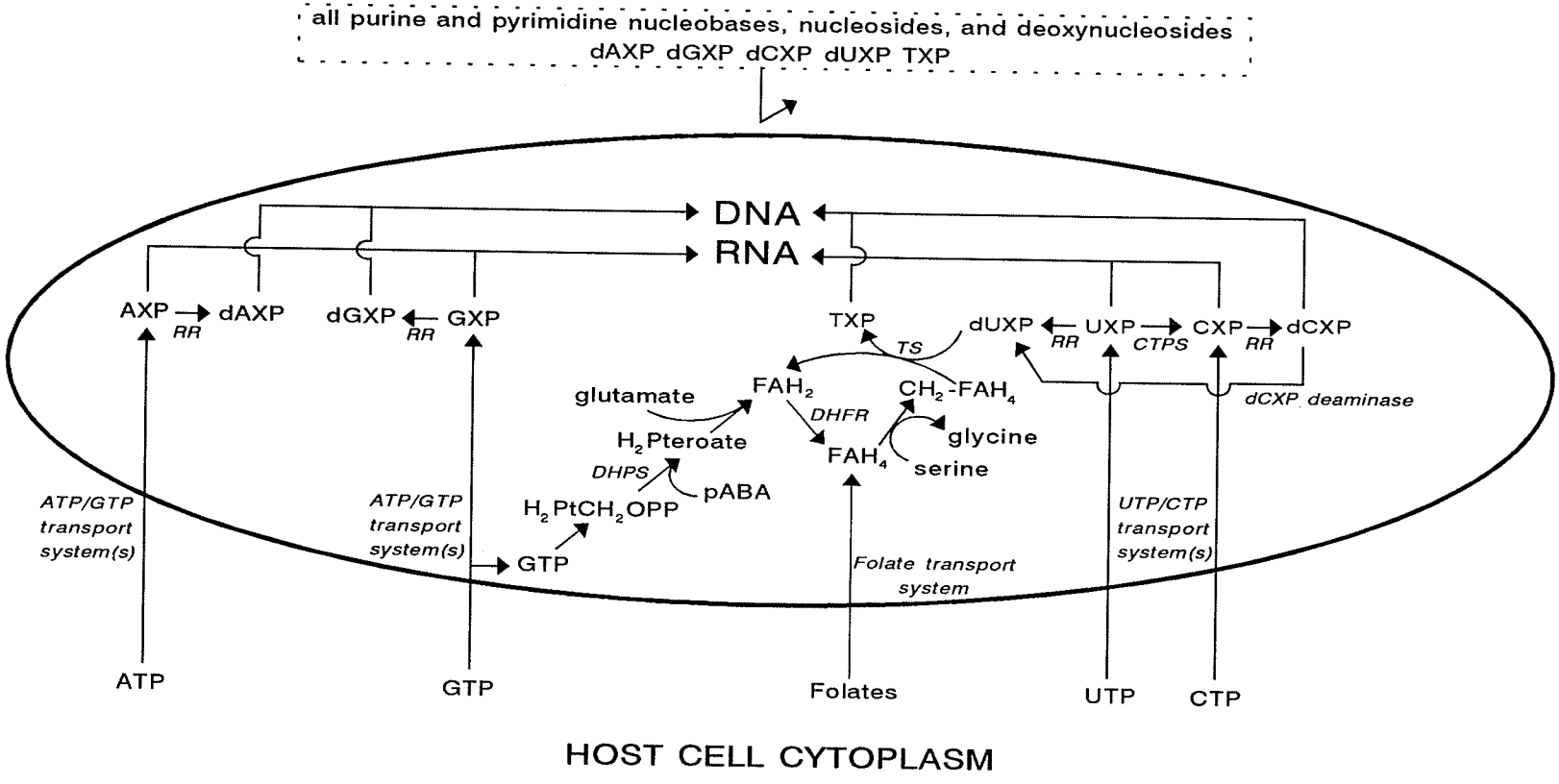
Extensive characterization of the nature of nucleotide metabolism in chlamydia revealed the presence of two different sets of pathways.

Pathway I (Figure 2a) is seen in *C. trachomatis*. With this series of pathways ATP, GTP, UTP and CTP are taken from the host using an undefined transport system. Once inside the cell, they can be incorporated directly into RNA or they can be converted into dNTP

Figure 2a: Pathway I

Abbreviations used: AXP, dAXP, GXP, dGXP, CXP, dCXP, UXP, dUXP, TXP, the appropriate (deoxy)ribonucleoside phosphates; FAH_2, dihydrofolate; FAH_4, tetrahydrofolate; CH_2FAH_4, 5,10 methylene tetrahydrofolate; H_2PtCH_2OPP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; H_2pteroate, dihydropteroate; pABA, *p*-aminobenzoic acid; RR, ribonucleotide reductase; TS, thymidylate synthase; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; CTPS, CTP synthetase.

Figure 2a: Pathway I



via a chlamydial-encoded ribonucleotide reductase. Chlamydia can take up folates via the folate transport system. Folates are used in the thymidylate synthase cycle which catalyses the conversion of dUMP and 5, 10 methylene tetrahydrofolate to dTMP and 7,8 dihydrofolate. The chlamydia also encode a dCXP deaminase which converts dCXP to dUXP, and a CTP synthase which converts UTP to CTP. Chlamydia do not take up any purine or pyrimidine nucleobases, nucleosides or deoxynucleotides when they are expressing Pathway I.

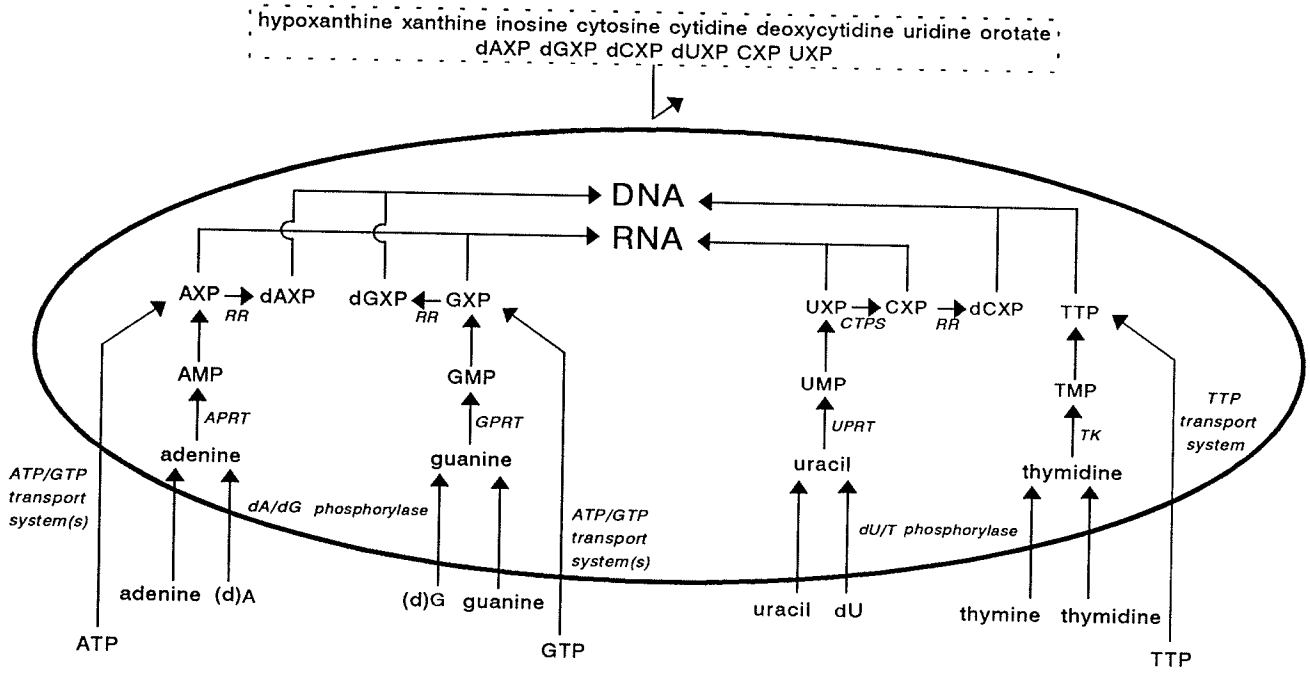
Pathway II (Figure 2b) was first seen in *C. psittaci*. In contrast to Pathway I, ATP, GTP and TTP are taken up by specific transport systems, UTP and CTP cannot enter the cell. Once inside the cell, they can be incorporated directly into RNA or they can be converted into dNTP via a chlamydial-encoded ribonucleotide reductase. The cell is also able to take up compounds that cannot be taken up when the cell is expressing pathway I. Four nucleobases (adenine, guanine, uracil, and thymine) are taken up, as well as four nucleosides (adenosine, guanosine, uridine, and thymidine). Here the thymidylate synthase cycle is no longer expressed, instead the cell salvages thymidine from the host cell, and raises it to the triphosphate level by a previously unexpressed thymidine kinase (Wang *et al*, 1994).

It has been noted that there are two different pathways for the uptake of nucleotides in chlamydia. A critical difference between the two is the differential expression of the

Figure 2b: Pathway II

Abbreviations used: AXP, dAXP, GXP, dGXP, CXP, dCXP, UXP, dUXP, TXP, the appropriate (deoxy)ribonucleoside phosphates; RR, ribonucleotide reductase; CTPS, CTP synthetase; APRT, adenine phoshoribosyltransferase; GPRT, guanine phosphoribosyltransferase; UPRT, uracil phosphoribosyltransferase; TK, thymidine kinase; dA/dG phosphorylase, purine (deoxy)ribonucleoside phosphorylase; dU/T phosphorylase, thymidine phosphorylase.

Figure 2b: *Pathway 2*



thymidylate cycle, and therefore the manner in which the cells obtain thymidine.

5. Methods of obtaining thymidine

There are a number of ways that an intracellular parasite could obtain thymidylate for DNA synthesis. If the organism salvages thymidine it requires only thymidine kinase to obtain the necessary thymidylate. Secondly, the chlamydia could transport uridine phosphates from the host cell's cytoplasm, or synthesize UTP *de novo* from existing amino acids (Speed and Winkler, 1991). UTP can be converted to TTP via a chlamydia-encoded ribonuclotide reductase and a thymidylate synthase.

5.0 *Folates and the thymidylate synthase cycle*

To make thymidine nucleotides *de novo* chlamydia require folates (Fan *et al*, 1991). Since all *C. trachomatis* isolates are sensitive to sulphonamides, a competitive inhibitor of *de novo* folate biosynthesis, and all *C. pneumonia* and *C. psittaci* isolates, with the notable exception of 6BC are resistant, it was assumed that the former synthesize folates while the latter two obtain them from the host (Moulder, 1984). In fact, *C. trachomatis* and *C. psittaci* 6BC can synthesize folates *de novo*, as indicated by their ability to incorporate radiolabelled *p*-aminobenzoic acid into intracellular folates and the presence of dihydropteroate synthase activity in RB extracts (Fan *et al*, 1992). Unexpectedly, *C. psittaci* strain francis (also referred to as *C. psittaci* strain MN or strain Cal 10), a sulphonamide-resistant isolate can also synthesize folates *de novo* and becomes sensitive

to sulphonamide if grown in folate-depleted host cells (Fan *et al*, 1992). It appears that most chlamydial isolates can synthesize folates *de novo*; however, strains differ in their ability to transport pre-formed folates from the host cell. These studies imply that chlamydia are able to regulate their endogenous biosynthetic pathways according to the availability of the end product. They also suggest that despite the fact that chlamydia can transport folates, some chlamydia have retained the genes necessary for *de novo* folate synthesis.

5.0.1 *Thymidylate synthase cycle* (Figure 3)

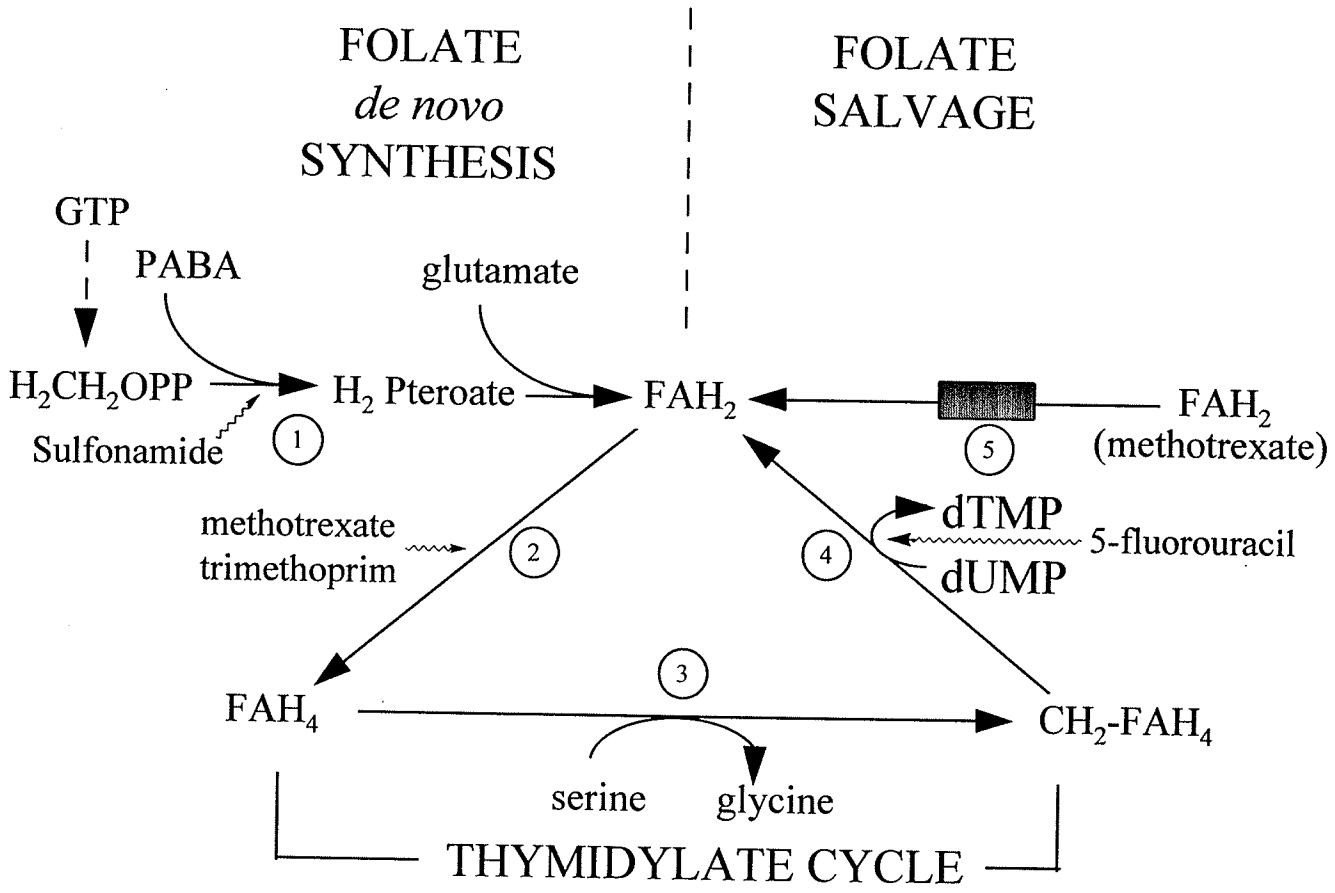
Thymidylate synthase catalyses the conversion of dUMP to dTMP through the transfer of a methylene group from 5, 10 methylene tetrahydrofolate and two reducing equivalents. This reaction produces 7,8 dihydrofolate and dTMP. 5,10 methylene tetrahydrofolate is regenerated by enzymes of the thymidylate synthase (TS) cycle. Dihydrofolate reductase catalyses the NADPH dependent reduction of 7,8 dihydrofolate (H_2 folate) to 5, 6, 7, 8 tetrahydrofolate. Then serine hydroxymethyl transferase catalyses the conversion of serine and 5,6,7,8 tetrahydrofolate to glycine and 5,10 methylene tetrahydrofolate.

The TS cycle is an important target for drug therapy since a block in the cycle will limit the available dTTP and ultimately stop DNA synthesis due to a lack of dTTP. Sulphonamides act by competing with p-aminobenzoic acid (PABA) in the formation of dihydropteroate by the enzyme dihydropteroate synthase. (Brown, 1962). In many

Figure 3: The thymidylate synthase cycle

Squiggly lines represent the action of inhibitors. The numbers represent the following enzymes: 1) dihydropteroate synthase, 2) dehydrofolate reductase, 3) serine hydroxymethyl transferase, 4) thymidylate synthase, 5) membrane transport system for folates. FAH_2-dihydrofolate, FAH_4 -tetrahydrofolate, CH_2FAH_4 - 5,10 methylene tetrahydrofolate.

Figure 3: The Thymidylate Synthase Cycle



bacteria, as well as protozoans and fungi, this inhibition leads to a block in the synthesis of folates and eventually a stop to the TS cycle. Methotrexate and trimethoprim are used to treat cancer and bacterial infections respectively (Schweitzer *et al*, 1990 and Blakeley, 1984). These drugs target dihydrofolate reductase and therefore limit the amount of 5,6,7,8 tetrahydrofolate made. Tetrahydrofolate is an essential carrier of one carbon units in the biosynthesis of thymidylate, purine nucleotides and methyl compounds. The primary mechanism of cytotoxicity of 5-fluorouracil is conseidered to be thymidylate starvation and inhibition of DNA synthesis caused by anabolism of 5'-fluorouracil to its active form 5'-fluoro-dUMP, an irreversible inhibitor of thymidylate synthase (Kaufman, 1989).

5.0.2 *Characterization of resistance mechanisms*

Resistance to drugs targeting enzymes of the TS cycle presents a significant problem. Characterization of the mechanisms of resistance is therefore of significant interest. There is a wealth of knowledge regarding mechanisms of acquired resistance to methotrexate (Schweitzer *et al*, 1990; Blakeley, 1984; and Allegra, 1990). Four mechanisms have been characterized by a study of methotrexate resistant cell lines after *in vitro* selection of cells with stepwise increases in drug concentrations. (Dicker *et al*, 1993).

1) Gene amplification of dihydrofolate reductase.

2) Decreased drug transport of antifolates.

3) Reduced polyglutiamylation of antifolates with decreased retention of the drug.

4) Altered DHFR with decreased affinity for methotrexate.

With these four mechanisms in mind, mutants to trimethoprim, sulphonamide, and methotrexate were selected from a wild type *Chlamydia trachomatis* L2 population. These drugs each target a step in the thymidylate synthase cycle (Figure 3). Each of the mutants was expected to have acquired resistance to the drugs through one or more of the above mechanisms. That was not the case. The mutants acquired the ability to obtain nucleotides via pathway II. By utilizing a thymidine kinase, they had bypassed the necessity of having a thymidylate synthase pathway.

It had been thought that pathway II was a unique characteristic of *Chlamydia psittaci* Cal10. However, the presence of the pathway as a mechanism of resistance in the mutant *Chlamydia trachomatis* L2 population resistant to 100 μM trimethoprim ($L2Tri^R$-100) suggests that this is not the case. The manner in which chlamydia regulate the expression of the enzymes of the two pathways is not understood. It is presumed that it is a response to a change in the environment, with a possible role in the EB/RB transition. A greater understanding of the genes that are encoded in the pathways may shed some light on the nature of their regulation. Thymidine kinase was chosen as the target for cloning because of its central importance in pathway II, while not being expressed in pathway I.

5.1 The regulation of thymidine kinase

Thymidine kinase (TK) is a key enzyme in the salvage pathway of thymidine nucleotides in prokaryotic and eukaryotic cells and many viruses. The enzyme catalyses the production of dTMP from thymidine and ATP. Thymidine kinase shows complex allosteric regulation involving a number of nucleotides, particularly dTTP, which feedback-inhibit the enzyme in a kinetically complex manner (Bresnick and Karjala, 1964, Black and Hruby, 1992). It is intriguing to contemplate the possible mechanisms of regulation in view of the unexpected and interesting method of drug resistance. Each drug, targeting a different gene, gave the same phenotypic change. Could there be a single regulatory protein involved? How would this protein work? An understanding of the regulation of thymidine kinase expression in the mutant *Chlamydia trachomatis* L2Tri^{R}-100 may lead to the characterization of the regulation of the switch between the two pathways.

6. Materials and methods

[2,8-^{3}H] adenine (23 Ci/mmol), [8-^{3}H] guanine (14 Ci/mmol), [8-^{3}H] hypoxanthine (20 Ci/mmol), [6-^{3}H] uridine (21 Ci/mmol), [5-^{3}H] uridine (20 Ci/mmol), [6-^{3}H] uracil (20 Ci/mmol), [methyl-^{3}H] thymidine (65 Ci/mmol) were purchased from Moravek Biochemicals ICN. All tissue culture media and supplements were from Flow Laboratories. Fetal bovine serum was bought from Intergen. Sulphisoxazole, trimethoprim, methotrexate and 5-fluorouracil were purchased from Sigma Chemical Co. All other chemicals were of the highest purity obtainable.

6.0 Media

6.0.1 ***Luria-Bertani medium:*** 10 grams bacto-tryptone, 5 grams bacto-yeast extract, 10 grams NaCl, adjust to 1 litre with dd H_2O (Sambrook *et al*, 1989) and sterilize by autoclaving.

6.0.2 ***SOB:*** 20 grams bacto-tryptone, 5 grams bacto-yeast extract, 0.5 grams NaCl. Adjust to 1 litre with dd H_2O and sterilize by autoclaving. 2.5 mM KCl, and 0.01M $MgCl_2$, which had been autoclaved separately were added just before use.

6.0.3 ***SOC:*** 20 mM glucose was added to the SOB medium.

6.0.4 ***Thymidine kinase selection medium:*** 2% peptone, 1.2% agar, 50 μM uridine, 100

μM 5-fluoro-2'deoxyuridine, and 50 μM thymidine.

15 grams of bacto-agar were added per litre to make plates.

6.0.4 ***B-Broth:*** 4.2 grams of PPLO broth w/o crystal violet (Difco), 0.2 grams yeast extract, 2 ml bromothymol blue (0.4 % w/v) and 170 ml distilled water. This was mixed to dissolve, and autoclaved at 121° C for 15 minutes at 15 psi. The mixture was cooled to 50°C and the following was added: 20 ml of inactivated horse serum, 0.5 ml urea (10 % w/v), 0.2 ml GHL (20 μg/ml), 4.0 ml arginine (10 % w/v), 2.0 ml ampicillin (100 mg/ml) and 1.0 ml nystatin (10 000 u/ml). Solution was mixed thoroughly and the pH adjusted to 6.0 with 2N HCl.

6.0.5 ***Ampicillin*** was made up in stocks of 50 mg/ml. The working concentration was 50μg/ml.

6.1 Bacterial strains

6.1.1 Thymidine kinase deficient bacteria: *Escherichia coli* KY895. (*F*$^-$, *tdk*$^-$, 1-*ilv*).

6.1.2 Wild type bacteria: *Escherichia coli* XL1-blue (obtained from Stratagene) *endA1,hsd*R17(*rk*$^-$*mk*$^+$), *supE11, thi-1, lambda*$^-$, *recA1, gyrA96, relA1 F'proAB, lac1qZ*ΔM15, *Tri10(tet*^{r}*)*

7. Methods

7.0 *Conditions for the selection of Chlamydia trachomatis L2 mutants*

7.0.1 *Cell lines and culture conditions*

The wild type mouse fibroblast L 929 cell line was provided by K. Coombs, Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada. The mutant mouse cell line deficient in hypoxanthine-guanine phosphoribosyltransferase activity (HGPRT $^-$) and in adenine phosphoribosyltransferase activity (APRT$^-$) (line A-9; catalogue number GM00346B) and the mutant mouse cell line deficient in thymidine kinase activity (TK$^-$) and APRT activity (line B-82: catalogue number GM00347A) were purchased from the National Institute of General Medical Sciences Human Genetic Human Cell Repository, Camden, N.J. The mutant chinese hamster ovary (CHO) K1 cell line deficient in dihydrofolate reductase activity (DHFR$^-$) (Urlaub and Chasin, 1980) was kindly provided by R. Johnson, University of Calgary, Calgary Alberta, Canada. All four cell lines were routinely cultured on the surface of plastic tissue culture flasks (Corning Glass Works). The wild-type mouse L cells, the HGPRT$^-$/APRT$^-$ mouse cells and the APRT$^-$/TK$^-$ mutant mouse L cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum, 0.2 mM glutamine, 0.3 proline, 0.3 mM glycine, 30 μM hypoxanthine, and 30 μM thymidine. For logarithmically growing cultures, approximately 10^5 cells were plated onto 5-cm dishes and grown for 36 to 40h at 37°C in 5 ml of α–minimal essential medium to a density of approximately 10^6 cells (McClarty and Tipples, 1991). All cell lines used in this study were routinely checked for

mycoplasma contamination by culture.

7.0.1 *Chlamydia trachomatis strains and propagation*

Chlamydia trachomatis L2/434/Bu obtained from R. Brunham, Department of Medical Microbiology, University of Manitoba, Winnipeg, was used throughout this study. Confluent monolayers ($3x10^6$ to $4x10^6$ cells per 5-cm plate) of host cells were infected at a multiplicity of infection (MOI) of 3 to 5 infection-forming units per cell, which resulted in 90 to 100% infection with little host cell toxicity (McClarty and Tipples, 1991). Unless otherwise indicated 1 μg of cycloheximide per ml was present in the post infection (p.i.) growth medium. Mock-infected (MI) host cell cultures were treated in the same fashion as infected cultures except that chlamydia were not added. Cycloheximide (1 μg/ml) was always present in MI cell culture medium. All chlamydial stocks were routinely checked for free-living bacterial contamination.

7.0.3 *Selection for trimethoprim and sulphisoxazole resistant Chlamydia trachomatis*

Since trimethoprim and sulphisoxazole have little or no effect on mammalian cells (Hitchings, 1983), wild-type mouse L 929 cells were used as host for the selection of *Chlamydia trachomatis* isolates resistant to these two drugs. Starting with a non-mutagenized population of *Chlamydia trachomatis* L2/B434/Bu, a series of L2 isolates was selected in a stepwise manner in the presence of the following concentrations (micromolar) of trimethoprim: wild type 2→5→10→20→50→60→75→87.5→100 or in the presence of the following concentrations (micromolar) of sulphisoxazole: wild

type→1.25→2.5→5→7.5→10→15→25→50→75→100. To start the selection, confluent monolayers (75-cm^2 flask; Corning Glass Works) of mouse L 929 cells were infected with *Chlamydia trachomatis* L2 at a multiplicity of infection of 5 inclusion forming units per cell, which resulted in 90 to 100% infection with little host cell toxicity (McClarty and Tipples, 1991). Immediately following infection complete medium supplemented with 1μg cyclohexamide per ml plus the initial concentration of trimethoprim (2 μM) or sulphisoxazole (1.25 μM) was added. The infected cultures were incubated at 37° C for 3 days, by which time some inclusions would be seen. The cultures were harvested and passed 1:1 onto fresh confluent monolayers of mouse L 929 cells. The cultures were again incubated at 37°C for 2 days in the presence of 2 μM trimethoprim or 1.25 μM sulphisoxazole. This infection, growth, and 1:1 passage was continued for whatever number of cycles was required (usually >10) to produce a highly infectious inoculum (i.e. a passage of at least 1:10 could be made). At this point the drug concentration was increased to the next step (i.e. 5 μM trimethoprim or 2.5 μM sulphisoxazole) and the process of infection, growth and passage was repeated. At each selection step an aliquot of EBs, for both trimethoprim and sulphisoxazole, was retained and stored at -70° C.

7.0.4 *Selection for 5-fluorouracil resistant Chlamydia trachomatis L2Tri^R-100*

In contrast to wild-type *C. trachomatis* L2, the mutant *C. trachomatis* L2 population

resistant to 100 μM trimethoprim (L2Tri^R-100) is sensitive to 5-fluoruracil, when Urd$^-$C cells are used as host (see Results). The L2Tri^R-100 isolate was used as the starting inoculum for the selection of a mutant population resistant to 5-fluorouracil and trimethoprim. The 5-fluorouracil mutant isolation procedure utilized took advantage of the fact that we had a 5-fluorouracil resistant CHO K1 cell line (Urd$^-$C) as host to support *C. trachomatis* L2Tri^R-100 growth. Starting with the mutant L2Tri^R-100 population of *C. trachomatis*, a series of L2Tri^R-100 population were sequentially selected in a stepwise manner in the presence of the following (micromolar) concentrations of 5-fluorouracil: 10→20→30→40→50→60. To start the selection, confluent monolayers (75-cm^2 flask; Corning Glass Works) of Urd$^-$C cells were infected with L2Tri^R-100 at a multiplicity of infection of 5 inclusion forming units per cell, which resulted in 90 to 100% infection with little host cell toxicity (McClarty and Tipples, 1991). Immediately following infection, complete medium supplemented with 30μM uridine, 0.3μM proline and 1μg cyclohexamide per ml plus the initial concentration of 5-fluorouracil (10 μM) was added. The infected cultures were incubated at 37° C for 3 days, by which time some inclusions would be seen. The cultures were harvested and passed 1:1 onto fresh confluent monolayers of Urd$^-$C cells. The cultures were again incubated at 37°C for 2 days in the presence of 10 μM 5-fluorouracil. This infection, growth, and 1:1 passage was continued for whatever number of cycles was required (usually >10) to produce a highly infectious inoculum (i.e. a passage of at least 1:5 could be made). At this point the drug concentration was increased to the next step (i.e. 20 μM 5-fluorouracil) and the process of

infection, growth and passage was repeated. At each selection step an aliquot of EBs was retained and stored at -70° C.

7.0.5 *Incorporation of radiolabelled precursors into chlamydial nucleic acids*

Radiolabelling experiments were performed as described previously (McClarty and Tipples, 1991; McClarty and Qin, 1993). Briefly, isotope experiments were done with duplicate dishes (5-cm dishes, 5 ml of medium) of stationary-phase cultures (3 x 10^6 to 4 x 10^6 cells per plate in the presence of 1μg of cycloheximide per ml) MI or infected with wild type or mutant *Chlamydia trachomatis*. For chlamydia-infected cultures incorporation of precursor into nucleic acid was measured at 22 h p.i., a time when parasite RNA and DNA synthesis are maximal (McClarty and Tipples, 1991). A particular radiolabelled precursor was added without dilution to achieve a final concentration of 0.3 μM. Incubation in the presence of isotope was continued for 2 h. For determining incorporation into total nucleic acid (RNA and DNA) 2 ml of 0.6 M trichloroacetic acid was added directly to the cell monolayer. After standing at 4° C for 2h the cell monolayer was scraped off with a rubber spatula and the precipitate was collected on a Whatman GF/B filter and analyzed for radioactivity by liquid scintillation counting. For determining incorporation into parasite specific DNA the cell monolayer was dissolved in 0.45 N NaOH and then incubated at 37 ° C for 16 hours to degrade RNA. Previous studies have shown that this treatment will degrade RNA completely in 3 hours (Nicander and Reichard, 1983). DNA was precipitated by adding concentrated

trichloroacetic acid to 0.6M, then the precipitate was collected on Whatman GF/B filters and analyzed for radioactivity by liquid scintillation counting.

7.0.6 *Acid hydrolysis of nucleic acids and subsequent nucleobase analysis*

In order to determine which nucleobases were labelled in DNA and RNA isolated from cultures pulsed with radioactive uracil or thymidine, the following procedures were carried out. Cell monolayers were prepared and infected as described above. At 22 hours postinoculation [6-^{3}H] uracil or [methyl-^{3}H] thymidine was added without dilution to achieve a final concentration of 0.3 μM, and incubation was continued at 37°C for 2 hours. Reactions were terminated on ice, medium was aspirated, and the cell monolayer was washed 3 times with ice-cold phosphate buffered saline (PBS). The cells were then resuspended in 200 μl lysis solution (20mM ethylenediamide tetraacetic acid (EDTA):5% 2-mercaptoethanol: 0.5% n-lauryl sarcosine) (Fukushi and Hirai, 1989), the dishes scraped, and the suspension transferred to a microfuge tube. The protein was then degraded with proteinase K (200μg/ml, Sigma) samples were extracted with phenol, and phenol:chloroform (1:1), and then nucleic acid was precipitated with ethanol. The resulting nucleic acid was hydrolyzed to free bases by boiling in 11.3 N perchloric acid for 1 hour. The acid-hydrolyzed samples were neutralised with 10 N KOH and subjected to high-performance liquid chromatography (HPLC) analysis. Isotope incorporation into nucleobases was measured by on-line radioactive flow detection (Beckman 171 radiodetector) after separation of the pyrimidines by HPLC on a reverse phase C18

column (Whatman) using 20% ammonium phosphate [pH 3.4] 2.5 % acetonitrile/80% H_20 with a flow rate of 1 ml/min. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} (Beckman 1066 wavelength detector) of known nucleobase standards. All data were plotted and processed with an IBM PC50 and Beckman System Gold software.

7.1 *Cloning the thymidine kinase gene from Chlamydia psittaci* Cal10 *isolate by complementation*

7.1.0 *Competent cells*

Ten ml of SOC was inoculated with a single TK^- KY895 colony and allowed to grow over night at 37°C with shaking. The next morning, this culture was used to inoculate 1 litre of SOB medium. The culture was allowed to grow until the OD_{600} was between 0.5 and 1.0, when the culture was put on ice for 10 - 15 minutes. The culture was distributed to three chilled 500 ml centrifuge bottles, and centrifuged in a Beckman J2-21, with a JA-10 rotor at 4000 rpm for 15 minutes at 4°C. The pellet was resuspended in ice cold sterile double distilled H_2O, and centrifuged. The washing step was repeated twice, and the final pellet was resuspended in 10 ml 10 % glycerol and redistributed to 50 ml Falcon tubes. The cultures were centrifuged at 3000 rpm in a Beckman 32-21 for 10 minutes, and resuspended in a final volume of 2 ml of 10% glycerol. The competent cells were distributed in aliquots of 160 µl each, and stored at -70 °C.

7.1.1 *Electroporation*

A 1 µl (250ng) aliquot of *C. psittaci* Cal10 *Eco*R1 partial digest genomic library in pUC19 (kindly provided by Huizhou Fan, Department of Medical Microbiology, University of Manitoba) and 40µl of competent cells were added to the 0.1 cm electroporation cuvette (BioRad). The mixture was pulsed at 1.8 volts on the BioRad gene pulser for 1 second. One ml of SOC media was added to the cuvette, and the mixture transferred to a test tube. The cells were then incubated for one hour at 37 °C with shaking, and 0.5 ml of the culture was plated on Luria-Bertani plus ampicillin (LB+amp) plates. The plates were incubated overnight at 37°C. Colonies were picked to fresh Luria-Bertani plus ampicillin plates and incubated overnight.

7.1.2 *Selection by complementation*

Colonies that grew on LB+amp plates were picked to thymidine kinase selection media and incubated at 37°C overnight. The next morning colonies were picked to a fresh thymidine kinase selection plate and incubated overnight.

7.1.3 *Plasmid isolation*

Plasmids were isolated according to Sambrook *et al*, 1989. Briefly, 3ml cultures were grown overnight at 37°C, and then spun down at 3000 rpm in a Beckman 32-21 for 10 minutes. They were resuspended in 200 µl of solution one (50mM glucose:25mM Tris-HCl:10mM EDTA), transfered to a microfuge tube (Eppendorf), and 200 µl of

solution two (0.2N NaOH:1%SDS) was added. The solution was incubated on ice for ten minutes. 200µl of ice cold solution three (3M potassium acetate:11.3% glacial acetic acid) was added, and the solution left on ice for 10 minutes. The tubes were spun at 14,000 rpm in a BiofugeA (Baxter-Canlab) at 4°C for 10 minutes. The supernate was transferred to a fresh microfuge tube, and extracted with phenol, phenol-chloroform (1:1), and chloroform. Plasmid DNA was precipitated with 500 µl of 95% ethanol, washed with 70% ethanol and resuspended in TE buffer (50mM Tris:20mM EDTA, pH 8.0).

7.1.4 *PCR directed dideoxynucleotide sequencing*

PCR directed sequencing was done using the ds DNA cycle sequencing system (BRL) as per package directions. Briefly, the primer was labeled by mixing 2 µl of primer (10ng), 1µl of 5x Kinase buffer, 0.5µl γ–^{32}P-ATP (ICN), 1µl of Kinase, and 0.5µl ddH_2O and incubating at 37°C for 30 minutes, and then at 55°C for 10 minutes. A prereaction mixture was made up by mixing 4.5 µl of 10x Taq polymerase buffer, 0.5µl Taq polymerase, 5µl of the labelled primer mixture, 2µl of template DNA (100ng), and 26µl of ddH_2O. 8 µl of the prereaction mix was added to 2 µl of each of the ddNTP termination mixes. The PCR program used was 94°C for 4 minutes then [95°C 30 seconds, 55°C 30 seconds, 72°C 60 seconds] for twenty cycles, and a final extention step of 72°C for ten minutes. The product was run on a 6% [acrylamide:bisacrylamide (29:1 w/w)] gel as described in Sambrook *et al*, 1989.

7.2 *PCR cloning of the Chlamydia trachomatis L2 and L2Tri^R-100 thymidine kinase gene.*

Primers, binding sites, and PCR conditions for the L2 and L2Tri^R-100 thymidine kinase gene (designated whole TK-tRNA^{GLY}) are shown in Table 2.

7.2.0 *PCR amplification*

All PCR reactions were done according to the following protocol. A 1 µl aliquote (250 ng) of template DNA was added to the PCR reaction mix. The reaction mix consisted of 1 µl (200 ng) of the upstream and downstream primers, 0.8 µl dNTP (100mM, pH7.5), 10µl of 10x PCR buffer (500mM KCl:100mM Tris-HCl (pH 8.4), 15mM $MgCl_2$:0.22% gelatin), 0.5µl Taq polymerase (5 unit/µl), and 85.7µl ddH_2O. In each PCR reaction, a positive and a reagent control were run. The positive control was the whole TK-tRNA^{GLY} primers with the original thymidine kinase clone (designated TKpUC19E(2)), the reagent control did not have any template DNA added.

7.3 *Obtaining the genomic clone of Chlamydia trachomatis L2 thymidine kinase*

*C. trachomatis Hind*III partial digest genomic library in pUC19 kindly provided by Graham Tipples, Department of Medical Microbiology, University of Manitoba.

Table 2: Primers and PCR conditions used to make the probes used in screening for the thymidine kinase gene

Probe name	Upstream primer[a]	Downstream primer	Expected size	PCR conditions
whole TK-tRNA^{GLY}	CCCGAATTCAGGACAT ATGTAT bp -9 to +6[b]	TCAGCCTTCCAAGCTGGATCCCAG bp 660 to 674	681 bp	(94°C 1 min, 55°C 1 min, 72°C 2 min) 25 cycles, 72°C 10 min.
internal thymidine kinase (TK)	GCCAAGAATTGATTCTAG bp 129 to 146	TCTGCACCTTGATTCGTA bp 540 to 557	400 bp	(94°C 1 min, 55°C 1 min, 72°C 2 min) 25 cycles, 72°C 10 min.
whole tRNA^{GLY}	TCTGCAAGTGTAGTTTAA bp 633 to 650	GGTCAAATTTGATAT bp 708 to 722	89 bp	(94°C 1 min, 45°C 2 min, 72°C 3.5 min) 35 cycles, 72°C 10 min.

[a]all primers made on the Beckman Oligo1000 as per manufacturers instructions
[b] binding sites shown on Figure 4

Figure 4: Primers used to clone Thymidine Kinase and $tRNA^{GLY}$

```
-87   AAA TAG ATT TTT TTA TTA CTT AAA TAT TTT TCA AAA TTA TTT TTT
      TTT ATC TAA AAA AAT AAT GAA TTT ATA AAA AGT TTT AAT AAA AAA
                                                      TK5'
-42   TGT TTA CAA TTT TAT TTA CTC TAT AAC TTA AAA GGA CAA ACT ATG
      ACA AAT GTT AAA ATA AAT GAG ATA TTG AAT TTT CCT GTT TGA TAC

+4    TAT AAA AAA TTT TCA GAT GGA ACT ATT GAA GTA ATT ACT GGT CCC
      ATA TTT TTT AAA AGT CTA CCT TGA TAA CTT CAT TAA TGA CCA GGT

49    ATG TTT TCG GGA AAA AGT GAT GAA GTA ATT AAA AGG ATT AGA ACA
      TAC AAA AGC CCT TTT TCA CTA CTT CAT TAA TTT TCC TAA TCT TGT
                                                   TK 5'2
94    TTA TCT TAT GCT AAT GTC AAA ACT TTA GCT GTA AAG CCA AGA ATT
      AAT AGA ATA CGA TTA CAG TTT TGA AAT CGA CAT TTC GGT TCT TAA

139   GAT TCT AGA TTT TCT ACA AAT GAA ATA GTT TCC CGT GCT GGT ACT
      CTA AGA TCT AAA AGA TGT TTA CTT TAT CAA AGG GCA CGA CCA TGA

184   AAA ATT CCT ACA TAT GTA GTA GAA ACA GTA GCA GAT ATA AAA AGT
      TTT TAA GGA TGT ATA CAT CAT CTT TGT CAT CGT CTA TAT TTT TCA

229   TTA TTT GAA AAA TCA AAA TAC AAA GCA ATC GCA ATA GAT GAA GCT
      AAT AAA CTT TTT AGT TTT ATG TTT CGT TAG CGT TAT CTA CTT CGA

274   CAA TTT TTT GAT AAA GAT TTA GTA CCT TAT GTT GAA GAA TTA GCT
      GTT AAA AAA CTA TTT CTA AAT CAT GGA ATA CAA CTT CTT AAT CGA

339   AAT CAA GGA ATT AGA GTG ATT ATT TGC GGT TTA GAC CAA GAT TAT
      TTA GTT CCT TAA TCT CAC TAA TAA ACG CCA AAT CTG GTT CTA ATA

384   TTA AGA CGT CCT TTC GGA GTA ATG CCT AGT TTA TTA GCG ATG GCT
      AAT TCT GCA GGA AAG CCT CAT TAC GGA TCA AAT AAT CGC TAC CGA

429   GAA CAT ATT ACA AAA TTG CAA GCT ATT TGC GTA ATT TGT AAA AAT
      CTT GTA TAA TGT TTT AAC GTT CGA TAA ACG CAT TAA ACA TTT TTA

474   GCA GCT TCT ACA ACT TTT AGA ACT ATT AAG TCC AAT AAA TTG AAA
      CGT CGA AGA TGT TGA AAA TCT TGA TAA TTC AGG TTA TTT AAC TTT
                                      TK3'2
519   GTT ATT GGA GAT TTA GAC GAA TAC GAA GCA AGG TGC AGA ATT TGT
      CAA TAA CCT CTA AAT CTG CTT ATG CTT CGT TCC ACG TCT TAA ACA

564   CAC AAT AAA GGA TTA AAT AAT TTA GAA AAA AAT TAA CAA TTT TAT
      GTG TTA TTT CCT AAT TTA TTA AAT CTT TTT TTA ATT GTT AAA ATA

609   TTT TTT CTG TTA TAA TTT AAT TAA TCT GCA AGT GTA GTT TAA TGG
      AAA AAA GAC AAT ATT AAA TTA ATT AGA CGT TCA CAT CAA ATT ACC
                TK3'                        tGly5'1
654   CAG ACT TCA GCC TTC CAA GCT GAT TGT AAG GGT TCG ATT CCC TTC
      GTG TGA AGT CGG AAG GTT CGA CtA ACA TTC CCA AGC TAA GGG AAG
                      tGly3'1
699   ACT TGC TCC ATA TCA AAT TTG ACC AAT GAG CTT TTT GCT CAT TTT
      TGA ACG AGG TAT AGT TTA AAC TGG TTA CTC GAA AAA CGA GTA AAA
```

7.3.0 *Colony lift*

The *C. trachomatis HindIII* partial digest genomic library in pUC19 was plated on Luria-Bertani plates containing ampicillin, and incubated overnight at 37°C. Nylon Biotrans membranes (ICN) were placed in sterile ddH_2O, blotted dry, and placed carefully on the chilled plate. Filters were then peeled from the plates using blunt ended forceps, and placed colony side up on 10% sodium dodecyl sulfate (SDS) impregnated 3MM paper (Canlab) and left for 3 minutes. They were then transferred to denaturing solution (0.5N NaOH:1.5MNaCl), neutralizing solution (1.5M NaCl:0.5M Tris-HCl), and 2x SSC (0.3 M sodium chloride: 0.03M sodium citrate) impregnated paper, and incubated for 5 minutes in each. Filters were allowed to dry, and baked at 80°C for 1-2 hours under vacuum.

7.4 Purification of PCR product for hybridization

The PCR product was purified by electroelution. Each band of interest, was cut out of the gel and eluted using the following protocol. The electroelution tank (model UEA, Unidirectional Electroeluter, Kodak) was filled with 0.5x TBE (0.045M Tris-Borate: 0.0001 M EDTA) and all the bubbles were removed from the elution chamber. 3M Sodium acetate with 1% bromophenol blue was added to the bottom of the chamber, and the gel containing the DNA was added. After running for one hour at 100 volts, UV light (260nm, Mineralight lamp, UVP Inc.) was used to confirm that the DNA had run out of the gel. A 200 µl aliquot of the 3M sodium acetate buffer was removed from the

chamber, and extracted with phenol-chloroform:chloroform. The probe was precipitated with 95% ethanol, washed with 70% ethanol, and resuspended in TE buffer.

7.4.0 *Hybridization*

The prehybridization solution contained 6x SSC (0.9M sodium chloride:0.09M sodium citrate), 5x Denhardt's solution (5g Ficoll (type 400, Pharmacia):5g polyvinyl pyrrolidone:5g bovine serum albumin (FractionV, Sigma): ddH_20 to 500 ml), 0.5% SDS, 0.1 mg/ml denatured fragmented salmon sperm DNA. The probe was labelled using the Random Primer kit (Gibco BRL) according to manufacturers instructions. Briefly, 25 ng of the probe DNA plus 18 µl ddH_2O were boiled for 10 minutes, and placed on ice for 5 minutes. The following was added to the DNA mixure: 15 µl of Random Primer mixture, 2µl of each of the following dCTP, dGTP, dTTP, and Klenow and finally, 4µl (~50µCi) of α^{32}P-ATP (ICN). After incubating at 37°C for one hour, boiling for 10 minutes and placing the probe on ice for 5 minutes, it was ready to be added to the hybridization solution. The filters were hybridised for 16 hours at 65° C. The filters were washed three times at 65° C in 2x SSC for 15 minutes each, three times with 0.2x SSC for 15 minutes, and once with 0.1x SSC for 15 minutes. Filters were then exposed to Kodak Scientific Imaging film X-OMAT-AR overnight at room temperature.

7.4.1 *Hind*III *digest*

A 500 quantity ng of plasmid DNA (isolated as described above) was digested with 15

units of *Hind*III (Pharmacia), and 1x of Pharmacia Phos-4-all buffer. The digest was left at 37 °C for 3 hours, and then run out on a 1% agarose gel.

7.4.2 *Southern Blot*

The southern blots were set up as described in Sambrook *et al*, 1989. Briefly, the gel was placed in denaturing solution (1.5M NaCl:0.5N NaOH) for 45 minutes with gentle agitation. After a brief rinse in ddH_2O, the gel was neutralized in 1.0MTris-HCl:1.5M NaCl for 30 minutes. The nylon Biotrans membranes were supplied by ICN. The transfer buffer was 10x SSC.

7.5 *Isolation of Mycoplasma hominis DNA*

A 1.2 litre culture of *M. hominis* was grown up to midlog phase (as judged by the color of the bromothymol blue indicator in the medium) and harvested by centrifugation (Beckman, J2-21) at 10,000 x g for 60 minutes with a JA-10 rotor in polycarbonate bottles. The supernate was removed by aspiration and the pellet was resuspended in 1 ml of TE (pH8.0) and distributed to microfuge tubes. SDS was added to a concentration of 1%, and 11µl proteinase K (200µg/ml, Sigma) was added. The mixture was then incubated at 55°C for two hours. The supernate was divided between two microcentrifuge tubes and the residual protein was extracted with chloroform-isoamyl alcohol (24:1). The high molecular weight DNA was precipitated with 1/10 volume 3M sodium acetate (pH 5.0) and 2 volumes of 95% ethanol, washed with 70% ethanol, and resuspended in a

small volume of water (Michelle Alfa, personal communication).

8. Results

8.0 *Characterization of resistance to sulphisoxazole and trimethoprim*

Chlamydia trachomatis L2 growth is inhibited by trimethoprim (Fan *et al.*, 1992), an inhibitor of bacterial dihydrofolate reductase (Woemser and Keusch, 1983), and sulphisoxazole, and inhibitor of bacterial dihydropteroate synthase (Anand, 1983). A series of *Chlamydia trachomatis* isolates were sequentially selected in the presence of increasing concentrations of trimethoprim and sulphisoxazole for the ability to proliferate in normally bactericidal drug concentrations. The phenotypic properties of all of the various drug-selected L2 populations have not been studied in detail; however, three populations were chosen for in-depth study because they showed interesting phenotypic properties in a preliminary screening. The three isolates chosen were: a *Chlamydia trachomatis* isolate that readily grew in the presence of 100 μM sulphisoxazole (L2Sulf^R-100, the final concentration in the sulphisoxazole selection protocol), a *Chlamydia trachomatis* isolate that readily grew in the presence of 60 μM trimethoprim (L2Tri^R-60, an intermediate step in the trimethoprim selection protocol), and a *Chlamydia trachomatis* isolate that readily grew in the presence of 100 μM trimethoprim (L2Tri^R-100, the final concentration in the trimethoprim selection protocol). Chlamydial growth was monitored by measuring the incorporation of [2,8 ^{3}H] adenine into DNA in the presence of cyclohexamide (McClarty and Tipples, 1991). The effects of trimethoprim and sulphisoxazole on the growth of wild type *Chlamydia trachomatis* L2,

L2Sulf^R-100, L2Tri^R-60, and L2Tri^R-100 are shown in Figures 5 and 6, respectively. In keeping with earlier findings (Fan *et al*, 1992), both trimethoprim and sulphisoxazole were effective inhibitors of wild type *Chlamydia trachomatis* L2 growth. The concentration of trimethoprim and sulphisoxazole required to inhibit DNA synthesis by 50% (ID_{50}) were 1.7 μM and 0.8 μM respectively. The L2Tri^R-60 isolate showed substantial resistance to trimethoprim, but was just as sensitive to sulphisoxazole as wild type *Chlamydia trachomatis* L2. The L2Tri^R-100 and L2Sulf^R-100 isolates were highly resistant to both trimethoprim and sulphisoxazole, being essentially unaffected by concentrations of either drug up to 60 μM.

8.0.1 *Sulphisoxazole and trimethoprim resistant mutants are cross resistant to methotrexate*

Wild type *Chlamydia trachomatis* L2 is sensitive to the dihydrofolate reductase inhibitor methotrexate (Fan *et al.*, 1992). Since methotrexate is also an inhibitor of mammalian cell growth (Blakley, 1984) it is necessary to do these experiments in a cell line that is resistant to methotrexate, so that host and bacterial effects of the inhibitor can be distinguished. For this purpose a DHFR-deficient CHO cell line is used as host to support chlamydial growth (Fan *et al.*, 1992). The effect of methotrexate on wild-type *Chlamydia trachomatis* L2, L2Sulf^R-100, L2Tri^R-60, and L2Tri^R-100 growth is shown in Figure 7. The concentration of methotrexate required to inhibit *Chlamydia trachomatis* L2 and L2Tri^R-60 growth by 50% in this cell line was 2.0 μM and 28 μM, respectively. The

Figure 5: Trimethoprim Killing Curve.

The effect of trimethoprim on [2,8^{3}H]-adenine incorporation into DNA of wild-type *C. trachomatis* L2 (■), L2Tri^R-60 (▲), L2Tri^R -100 (✱), L2Sulf^R-100 (+), and L2Tri/5-FU (●) infected wild-type mouse cells (3.0 x 10^6 cells per plate cultured in the presence of 1μg ml^{-1} cyclohexamide). The indicated concentrations of trimethoprim were added immediately after infection with chlamydia, i.e. 2h p.i. Radiolabelled adenine (final concentration 0.3μM) was added at 22 h p.i. Cell culture conditions, chlamydia infection procedure, and ^{3}H-labelling procedure are as described in the methods. All analyses were made on duplicate dishes, with results varying by less than 10%. The amount of radiolabel incorporated into DNA in the presence of inhibitor is expressed as a percentage of the uninhibited control value. The following are 100% control values: wild-type *C. trachomatis* L2 infected cultures 169 353 dpm/10^6, L2Tri^R-60 infected cultures 183 968 dpm/10^6, L2Tri^R -100 infected cultures 202 741 dpm/10^6, L2Sulf^R-100 infected cultures 189 652 dpm/10^6, and L2Tri/5-FU-infected cultures 174 903 dpm/10^6 cells.

Figure 5: Trimethoprim killing curve

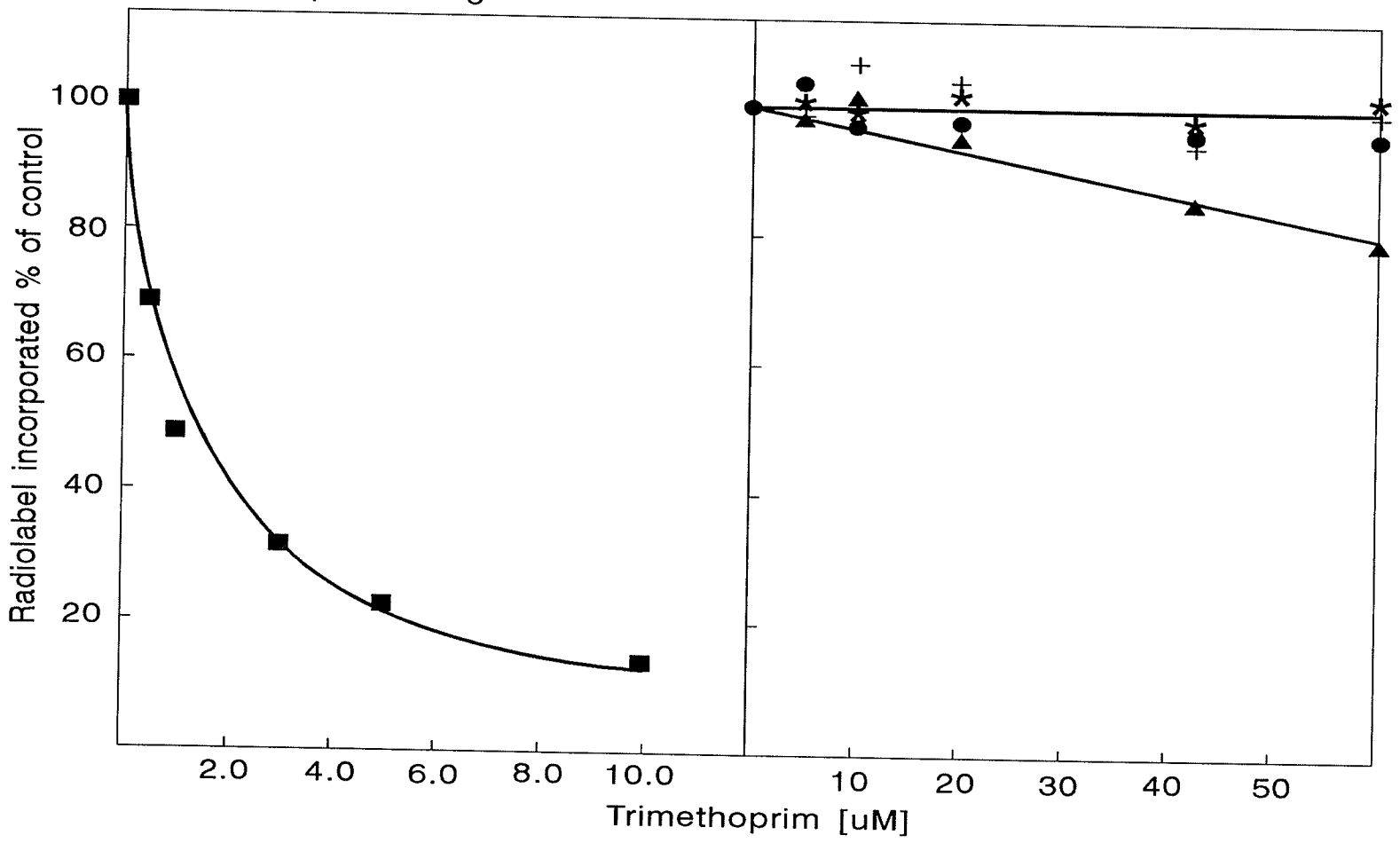


Figure 6: Sulphisoxazole Killing Curve.

The effect of sulphisoxazole on [2,8^{3}H]-adenine incorporation into DNA of wild-type *C. trachomatis* L2 (■, L2Tri^R-60 (▲), L2Tri^R -100 (✱), and L2Sulf^R-100 (+), and L2Tri/5-FU (●) infected wild-type mouse cells (3.0 x 10^6 cells per plate cultured in the presence of 1μg ml^{-1} cyclohexamide). The indicated concentrations of sulphisoxazole were added immediately after infection with chlamydia, i.e. 2h p.i. Radiolabelled adenine (final concentration 0.3μM) was added at 22 h p.i. Cell culture conditions, chlamydia infection procedure, and ^{3}H-labelling procedure are as described in the methods. All analyses were made on duplicate dishes, with results varying by less than 10%. The amount of radiolabel incorporated into DNA in the presence of inhibitor is expressed as a percentage of the uninhibited control value. The following are 100% control values: wild-type *C. trachomatis* L2 infected cultures 163 285 dpm/10^6, L2Tri^R-60 infected cultures 170 653 dpm/10^6, L2Tri^R -100 infected cultures 197 458 dpm/10^6, L2Sulf^R-100 infected cultures 173 618 dpm/10^6, and L2Tri/5-FU-infected cultures 201 580 dpm/10^6 cells.

Figure 6: Sulphisoxazole killing curve

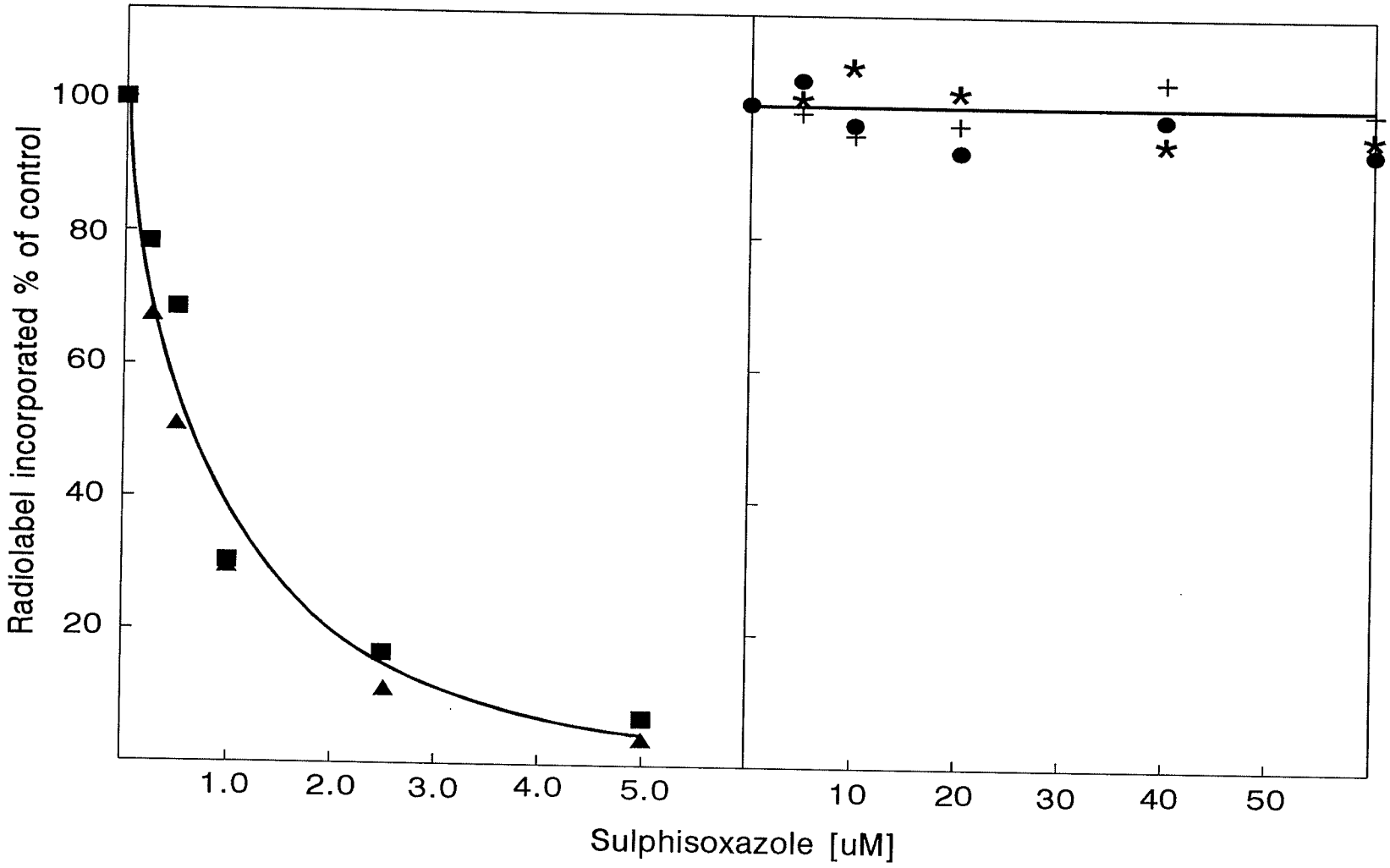
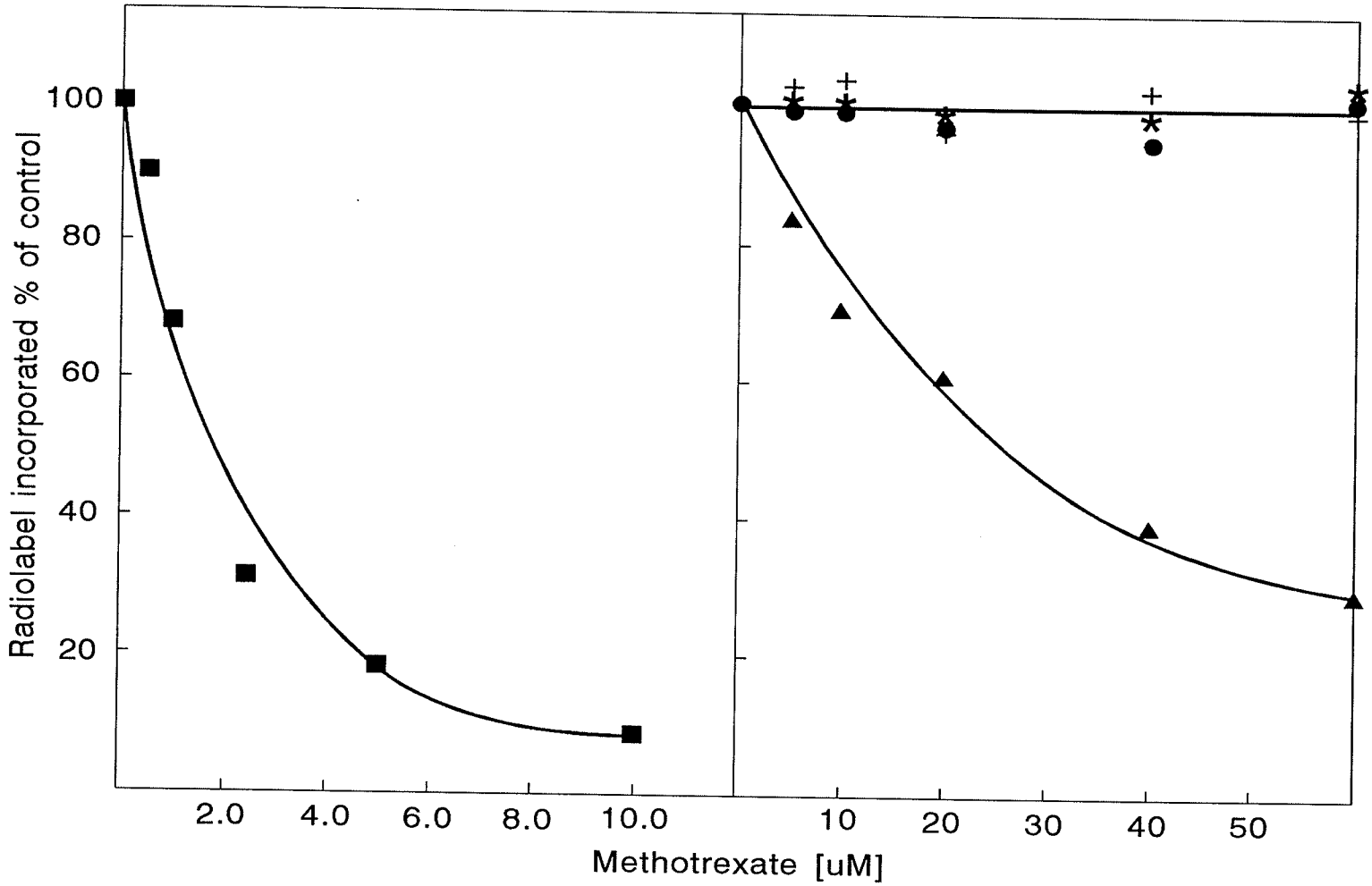


Figure 7: Methotrexate Killing Curve.

The effect of Methotrexate on [2,8^{3}H]-adenine incorporation into DNA of wild-type *C. trachomatis* L2 (■), L2Tri^R-60 (▲), L2Tri^R-100 (✱), and L2Sulf^R-100 (+) and L2Tri/5-FU (●), infected DHFR deficient CHO K1 cells (4.0 x 10^6 cells per plate cultured in the presence of 1μg ml^{-1} cyclohexamide). The indicated concentrations of methotrexate were added immediately after infection with chlamydia, i.e. 2h p.i. Radiolabelled adenine (final concentration 0.3μM) was added at 22 h p.i. Cell culture conditions, chlamydia infection procedure, and ^{3}H-labelling procedure are as described in the methods. All analyses were made on duplicate dishes, with results varying by less than 10%. The amount of radiolabel incorporated into DNA in the presence of inhibitor is expressed as a percentage of the uninhibited control value. The following are 100% control values: wild-type *C. trachomatis* L2 infected cultures 123 836 dpm/10^6, L2Tri^R-60 infected cultures 131 472 dpm/10^6, L2Tri^R -100 infected cultures 159 956 dpm/10^6, L2Sulf^R-100 infected cultures 148 385 dpm/10^6, and L2Tri/5-FU-infected cultures 151 238 dpm/10^6 cells.

Figure 7: Methotrexate killing curve



L2Sulf^R-100, and L2Tri^R-100 isolates were highly resistant to methotrexate, being essentially unaffected by the concentrations tested.

The stability of the L2, L2Sulf^R-100, L2Tri^R-60, and L2Tri^R-100 drug resistant phenotypes was assessed after passage in the absence of sulphisoxazole or trimethoprim for ten growth cycles. After this time, there was essentially no change in drug sensitivity of any of the three isolates.

8.0.2 *Incorporation of various nucleic acid precursors into Chlamydia trachomatis* L2, L2Sulf^R-100, L2Tri^R-60, and L2Tri^R-100 *DNA*

Preliminary experiments suggested that both trimethoprim (L2Tri^R-100) and sulphisoxazole (L2Sulf^R-100) resistant forms of *C. trachomatis* showed identical patterns of nucleotide metabolism, which differed significantly from the original L2 strains, yet agreed with the pathways seen in *C. psittaci* (McClarty and Qin, 1993). In these experiments a variety of precursors and host cells were used (Table 3). The first series of experiments were done in wild type host cells. When the cells were pulsed with adenine or guanine the host cell raised the nucleoside to the triphosphate form, ATP and GTP respectively. Both trimethoprim and sulphisoxazole resistant strains incorporated ATP and GTP, as did the wild type L2 and the intermediate form of the mutant Tri0.6. To recall, mutants were selected through an extensive stepwise selection procedure. The

Table 3: Incorporation of nucleic acid precursors into various *C. trachomatis* isolates DNA in wild type and H^-/A^- mouse L cells[a]

Cell line	Precursor added [b]	Nucleotide(s)[c] Available	Incorporation of precursor into chlamydial DNA (x 10^3 d.p.m./10^6 cells)[d] wild type L2	L2Tri^R-60	L2Tri^R-100	L2Sulf^R-100	L2Tri/5-FU
wild type	[2,8-^{3}H] Adenine	ATP, GTP	126	105	200	206	172
	[8-^{3}H] Guanine	GTP	51	47	69	75	63
	[2,8-^{3}H] Hypoxanthine	ATP, GTP	102	89	186	207	168
H^-/A^-	[2,8-^{3}H] Adenine	-	2	3	215	261	193
	[8-^{3}H] Guanine	-	<MI[e]	0.2	53	65	47
	[2,8-^{3}H] Hypoxanthine	-	0.5	0.9	<MI	<MI	<MI

a. Duplicate cultures of wild-type mouse cells, TK^-/A^-, and H^-/A^- mouse cells were seeded, cultured and infected with various *C. trachomatis* L2 isolates as described in the methods.
b. The various ^{3}H-labelled purine and pyrimidine precursors were added at 22h p.i. to achieve a final concentration of 0.3 μM, and incubation was continued for 2h.
c. The primary nucleoside triphosphate(s) synthesized, from the added precursor, by wild type, TK^-/A^-, or H^-/A^- mouse cells. -, precursors not metabolized by the host cell.
d. Incorporation of radiolabel into chlamydial DNA was determined as described in the methods. All analyses were made on duplicate dishes, with results varying by less than 10%. <MI, radiolabel incorporated by these chlamydial-infected cultures was less than that of an identically treated MI control.
e. MI, mock infected control

organisms are grown in the presence of the drug, and passed to higher concentrations. L2Tri^{R}-60 (Tri0.6) represents the population that readily grew at a concentration of 60μM trimethoprim. When the host cells were pulsed with radioactive cytidine and uridine, where CTP and UTP would be available, only the wild type L2 and Tri0.6 incorporated them into nucleic acid. The resistant strains had lost the ability to incorporate labelled cytidine and uridine. Another stark contrast is in the uptake of TTP. When the cells were pulsed with thymidine, the host cell raised it to TTP, and though the wild type L2 and Tri0.6 did not incorporate thymidine, the mutant L2 L2Sulf^{R}-100 and L2Tri^{R}-100 were able to. Uracil which is not anabolized by the wild type host cell, was not taken up by wild type L2 or Tri0.6. However, the resistant L2Sulf^{R}-100 and L2Tri^{R}-100 strains were able to utilize uracil. These contrasts lead to further experimental work with host cells that were deficient in thymidine kinase and adenine phosphoribosyltransferase (TK^{-}/A^{-}) (Table 4). The cells were pulsed with uridine, the host cell raised it to the triphosphate state and incorporated into RNA. Both the wild type L2 and Tri0.6 were able to incorporate the uridine, while the resistant strains were not. In addition, when the cells were pulsed with thymidine, the host cell was unable to raise it to the triphosphate state. The wild type L2 and Tri0.6 did not incorporate the thymidine, however the resistant L2Sulf^{R}-100 and L2Tri^{R}-100 were able to. This suggests that the mutant chlamydia may produce a thymidine kinase.

Table 4: Incorporation of nucleic acid precursors into various *C. trachomatis* isolates DNA in wild-type and TK$^-$/A$^-$ mouse cells[a]

Cell line	Precursor added [b]	Nucleotide(s)[c] Available	wild type L2	L2Tri^R-60	L2Tri^R-100	L2Sulf^R-100	L2Tri/5-FU
Wild type	[5-^{3}H] Cytidine	CTP	103	76	<MI	<MI	54
	[*methyl*-^{3}H] Thymidine	dTTP	1	2	385	297	350
	[6-^{3}H]Uracil	-	0.5	1	31	29	11
	[6-^{3}H]Uridine	UTP, CTP	203	174	3	2	61
	[5-^{3}H] Deoxycytidine	dCTP	<MI	<MI	<MI	<MI	<MI
TK$^-$/A$^-$	[*methyl*-^{3}H] thymidine	-	<MI	3	286	295	276

a. Duplicate cultures of wild-type mouse cells and TK$^-$/A$^-$ mouse cells were seeded, cultured and infected with various *C. trachomatis* L2 isolates as described in the methods.

b. The various ^{3}H-labelled pyrimidine precursors were added at 22h p.i. to achieve a final concentration of 0.3 μM, and incubation was continued for 2h.

c. The primary nucleoside triphosphate(s) synthesized, from the added precursor, by wild type and TK$^-$/A$^-$ mouse cells. -, precursors not metabolized by the host cell.

d. Incorporation of radiolabel into chlamydial DNA was determined as described in the methods. All analyses were made on duplicate dishes, with results varying by less than 10%. <MI, radiolabel incorporated by these chlamydial-infected cultures was less than that of an identically treated MI control.

8.0.3 *Further characterization of uptake pathway*

The small amount of uracil/uridine incorporated into DNA by L2Tri^R-100 and L2Sulf^R-100 suggested that they were not synthesizing thymidine nucleotides *de novo*. To determine if this was the case, L2Sulf^R-100 and L2Tri^R-100 were labelled with [5-^{3}H]-uracil and [6-^{3}H]-uracil. If thymidylate synthase is present and [5-^{3}H]-uracil is used as a precursor, deoxycytidine phosphates are labelled but thymidine phosphates are not, since isotope is lost from the pyrimidine ring and recovered as ^3H_2O in the medium during the formation of dTMP. In contrast, with [6-^{3}H]-uracil as precursor all pyrimidine deoxyribonucleotides are labelled, because the isotope is retained with the pyrimidine ring. Both L2Sulf^R-100 and L2Tri^R-100 readily incorporated [5-^{3}H]-uracil and [6-^{3}H]-uracil, but not uridine, into parasite RNA. Furthermore, both mutants incorporated [5-^{3}H]-uracil into DNA as efficiently as [6-^{3}H]-uracil suggesting that uracil is not a precursor of thymidine in these isolates (Table 5). To confirm this result, we used HPLC to analyse the distribution of [6-^{3}H]-uracil into pyrimidine bases of acid hydrolyzed nucleic acids, isolated from L2Sulf^R-100 and L2Tri^R-100 infected wild-type mouse L-cells. In agreement with the above findings, these experiments showed that radiolabel was detected in uracil and cytosine, but not thymidine (Figure 8). To prove this, L2 and L2Tri^R-100 infected TK$^-$/A$^-$ cells were incubated with [*methyl*-^{3}H] thymidine. The samples were treated as described above. Results showed that radioactivity was detected in thymine from [*methyl*-^{3}H] thymidine in L2Tri^R-100 (Figure 9). This provides additional support to our hypothesis that L2Tri^R-100 expresses a thymidine kinase.

Table 5: Incorporation of pyrimidine nucleotide precursors into various *C. trachomatis* isolates nucleic acids in wild-type mouse L cells[a]

Incorporation into:	Precursor added[b]	Nucleotide Available[c]	Incorporation of Precursor into Chlamydial Nucleic Acid (x 10^3 d.p.m./10^6 cells)[d]		
			L2Tri^R-100	L2Sulf^R-100	L2Tri/5-FU
RNA	[5-^{3}H]-Uracil	-	199	186	135
	[6-^{3}H]-Uracil	-	206	174	142
	[5-^{3}H]-Uridine	UTP, CTP	2	5	78
	[6-^{3}H]-Uridine	UTP, CTP	5	3	81
	[5-^{3}H]-Cytidine	CTP	<MI	<MI	66
DNA	[5-^{3}H]-Uracil	-	30	26	14
	[6-^{3}H]-Uracil	-	26	29	12
	[5-^{3}H]-Uridine	UTP, CTP	2	2	28
	[6-^{3}H]-Uridine	UTP, CTP	3	1	33

a. Duplicate cultures of wild-type mouse cells were seeded, cultured and infected with various *C. trachomatis* L2 isolates as described in the methods.

b. The various ^{3}H-labelled pyrimidine precursors were added at 22h p.i. to achieve a final concentration of 0.3 μM, and incubation was continued for 2h.

c. The primary nucleoside triphosphate(s) synthesized, from the added precursor, by wild type, TK$^-$/A$^-$, or H$^-$/A$^-$ mouse cells. -, precursors not metabolized by the host cell.

d. Incorporation of radiolabel into chlamydial DNA was determined as described in the methods. All analyses were made on duplicate dishes, with results varying by less than 10%. <MI, radiolabel incorporated by these chlamydial-infected cultures was less than that of an identically treated MI control.

Figure 8: [6-^{3}H] Uracil Uptake

Incorporation of [6-^{3}H] Uracil into nucleic acid deoxynucleosides of mock infected (—), *C. trachomatis* L2 (- - -), and L2Tri^{R}-100 (—) infected TK^{-} cells. Mock infected and chlamydia-infected cells incubated with [6-^{3}H] uracil . The position of deoxynucleoside standards are indicated by arrows.

Figure 8: [6-^{3}H] uracil uptake

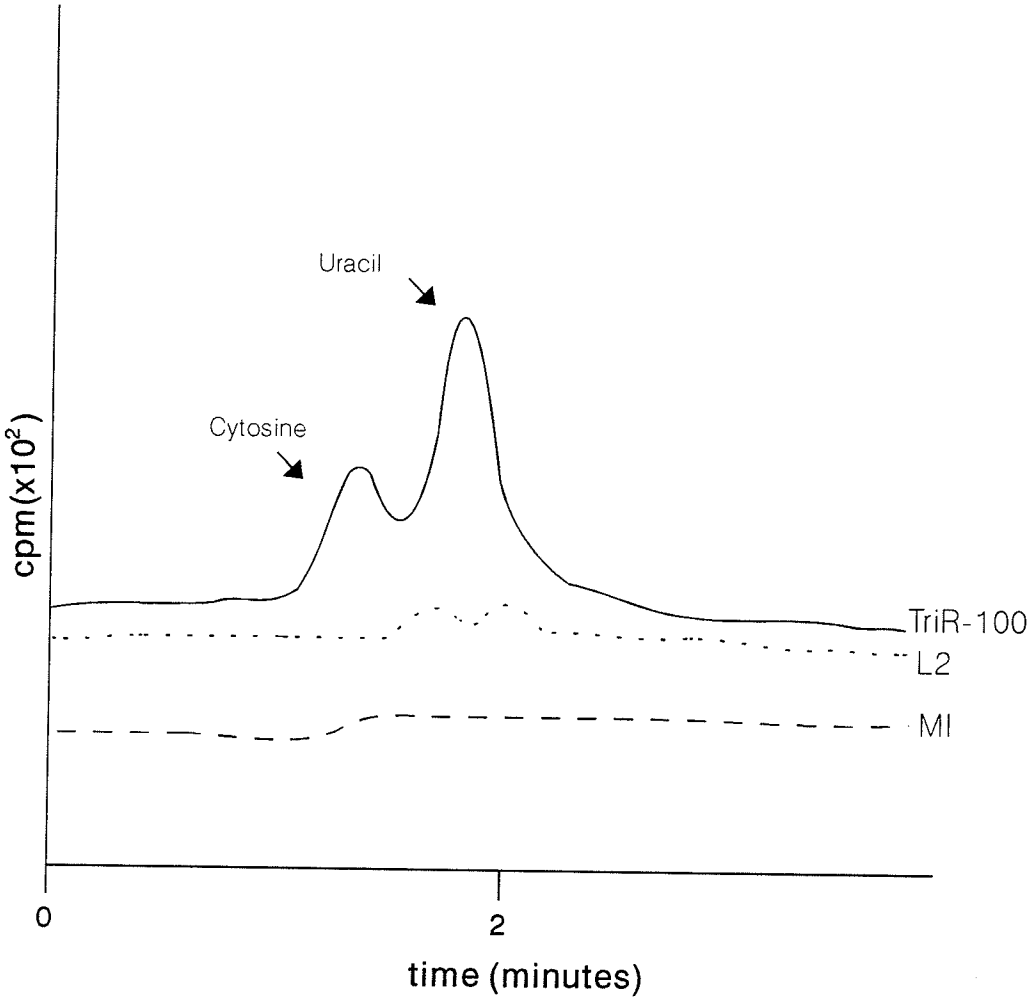
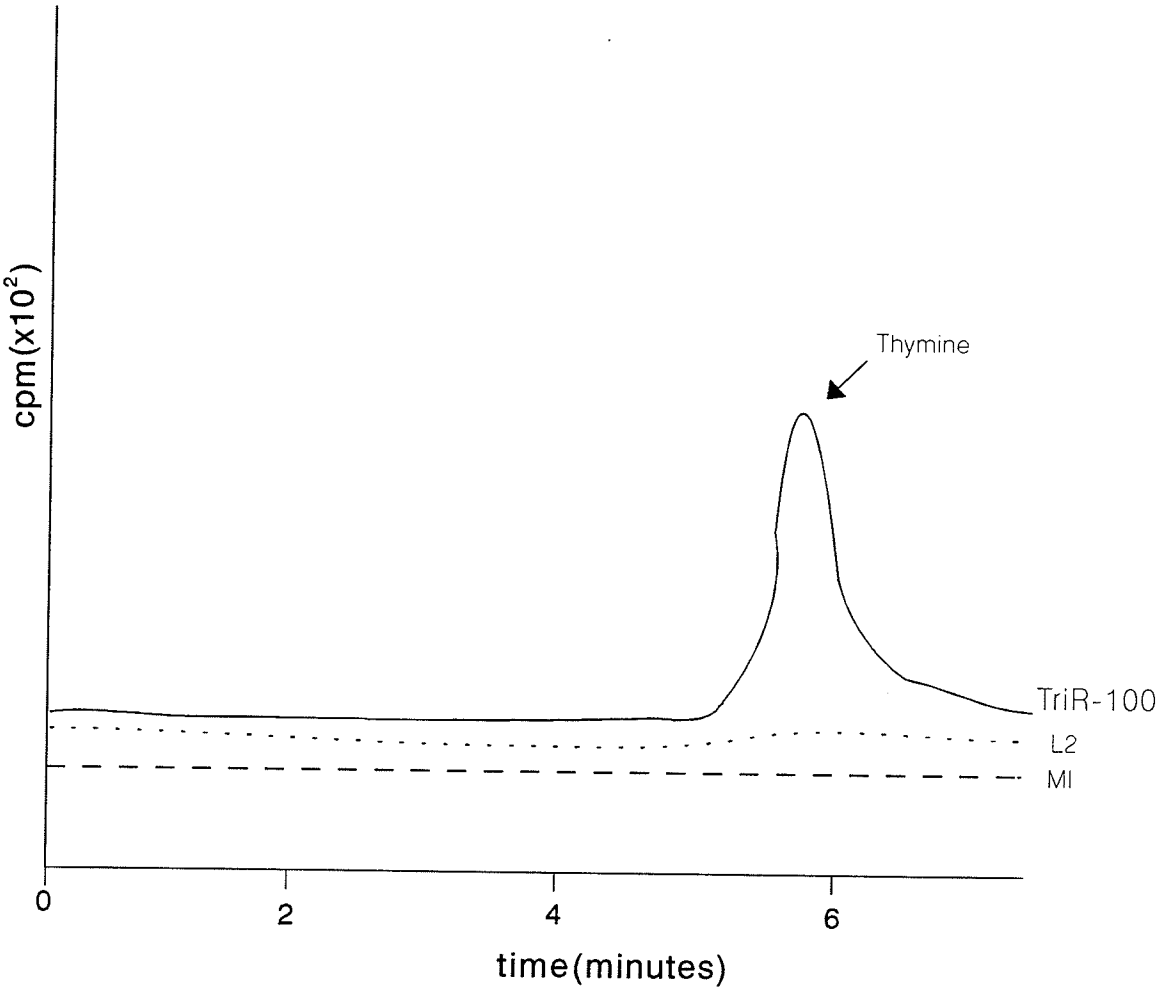


Figure 9: Uptake of [*methyl*-^{3}H] thymidine

Incorporation of [*methyl*-^{3}H] thymidine into nucleic acid deoxynucleobases of mock infected (– –), *C. trachomatis* L2 (- - -), and L2Tri^R-100 (—) infected TK$^-$ cells. Mock infected and chlamydia-infected cells incubated with [*methyl*-^{3}H] thymidine. The position of deoxynucleobases standards are indicated by arrows.

Figure 9: [*methyl*-^{3}H] Thymidine uptake



As L2Tri^R-100 and L2Sulf^R-100 incorporated exogenous uracil and thymidine into parasite-specific nucleic acid, we tested their sensitivity to the pyrimidine antimetabolite 5-fluorouracil (5-FU). 5-fluorouracil did inhibit L2Tri^R-100 and L2Sulf^R-100 growth, in both wild-type mouse L cells (5-FUTP available to the parasite) and Urd$^-$C CHO cells, which lack orotate phosphoribosyltransferase (5-FU available to the parasite) (data not shown). As L2Tri^R-100 and L2Sulf^R-100 appeared to be phenotypically identical, just L2Tri^R-100 was used for the next series of experiments.

Since 5-fluorouracil inhibited L2Tri^R-100 growth by targetting the thymidylate synthase cycle an attempt was made to isolate a revertant (i.e. a population that displayed the wild type *C. trachomatis* L2 nucleotide acquisition phenotype). A series of L2Tri^R-100 populations were sequentially selected in the presence of increasing concentrations of 5-fluorouracil for the ability to proliferate in normally cytotoxic drug concentrations, as described in the experimental procedures. As they are resistant to the cytotoxic effects of 5-fluorouracil (Patterson, 1980), Urd$^-$C CHO cells were used as a host to support chlamydial growth, during the selection procedure. Following selection, one 5-FU resistant population was chosen for further study. The one chosen, L2Tri^R-100/5-FU^R-50, grows well in the presence of 50 μM 5–FU. For simplicity, this population will be called L2Tri/5-FU. With wild-type mouse cells as host, 5-fluorouracil was a potent inhibitor of all three populations showing a similar ID_{50} of approximately 1-3 μM (data not shown). The effect of increasing concentrations of 5-FU on wild-type *C. trachomatis* L2, and the

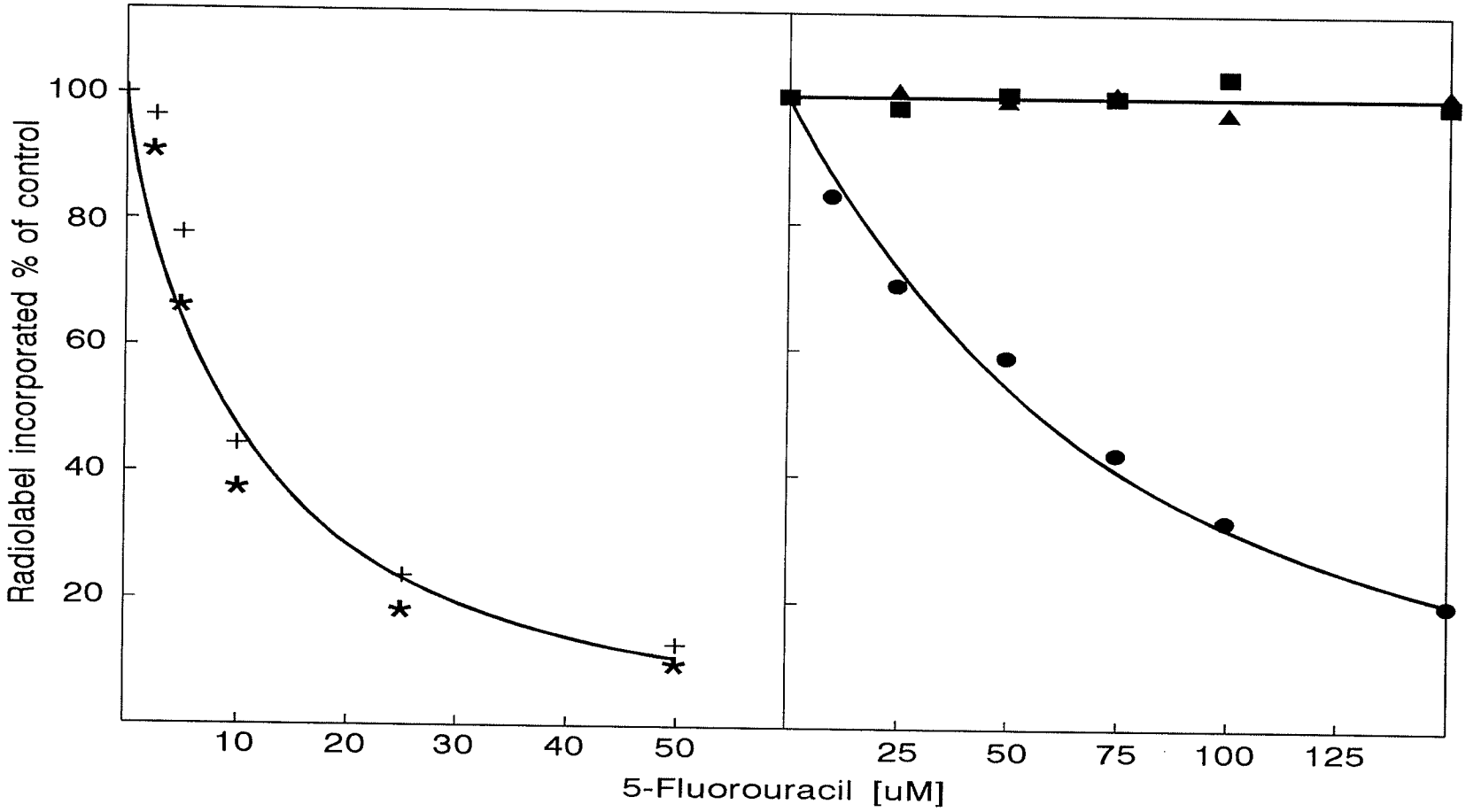
various drug-resistant mutants grown in Urd$^-$C CHO cells is shown in Figure 10. With Urd$^-$C cells as host, *C. trachomatis* L2 was completely resistant to 5-FU, L2Tri^R-100 and L2Sulf^R-100 had an ID_{50} of approximately 8 μM, and L2Tri/5-FU showed intermediate sensitivity, with an ID_{50} of about 60 μM.

As with the other mutant chlamydia, the L2Tri/5FU-resistant phenotype was stable when passed in the absence of a selective agent for extended periods of time (data not shown). Like the parental population, L2Tri^R-100, L2Tri/5-FU was completely resistant to the cytotoxic effects of trimethoprim (Figure 5), sulphisoxazole (Figure 6), and methotrexate (Figure 7). The L2Tri/5-FU population displayed an interesting nucleotide acquisition phenotype. Like all other *C. trachomatis* populations tested, it could utilize exogenous adenine, guanine and hypoxanthine (Table 3) but not deoxycytidine (Table 4) with wild-type mouse L cells as host. L2Tri/5-FU was similar to L2Tri^R-100 and L2Sulf^R-100 in that it could utilize adenine and guanine but not hypoxanthine when HGPRT$^-$/APRT$^-$ cells were used as host (Table 3) and it readily incorporated uracil and thymidine when growing in wild-type or TK$^-$ host mouse cells (Table 4). Furthermore, uracil was not a precursor for thymine in L2Tri/5-FU (Table 5). We know this because the incorporation of [5-^{3}H] uracil and [6-^{3}H] uracil is similar. If uracil were a precursor for thymidine, incorporation of [5-^{3}H] uracil would be low, while [6-^{3}H] uracil would be high. This is because the isotope would be lost from the pyrimidine ring of [5-^{3}H] uracil and recovered as ^3H_2O in the medium during the formation of thymidine. In contrast to

Figure 10: 5-fluorouracil Killing Curve.

The effect of 5-fluorouracil on [2,8^{3}H]-adenine incorporation into DNA of wild-type *C. trachomatis* L2 (●), L2Tri^R-60 (▲), L2Tri^R-100 (✱), L2Sulf^R-100 (+) and L2Tri/5-FU (●) infected URD$^-$C CHO K1 cells (4.0 x 10^6 cells per plate cultured in the presence of 1μg ml^{-1} cyclohexamide). The indicated concentrations of trimethoprim were added immediately after infection with chlamydia, i.e. 2h p.i. Radiolabelled adenine (final concentration 0.3μM) was added at 22 h p.i. Cell culture conditions, chlamydia infection procedure, and ^{3}H-labelling procedure are as described in the methods. All analyses were made on duplicate dishes, with results varying by less than 10%. The amount of radiolabel incorporated into DNA in the presence of inhibitor is expressed as a percentage of the uninhibited control value. The following are 100% control values: wild-type *C. trachomatis* L2 infected cultures 176 978 dpm/10^6, L2Tri^R-60 infected cultures 183 276 dpm/10^6, L2Tri^R -100 infected cultures 193 617 dpm/10^6, L2Sulf^R-100 infected cultures 202 642 dpm/10^6, and L2Tri/5-FU-infected cultures, 185 316 dpm/10^6 cells.

Figure 10: 5-fluorouracil killing curve



L2Tri^{R}-100 and L2Sulf^{R}-100, but like *C. trachomatis* L2 and L2Tri^{R}-60, L2Tri/5-FU was capable of incorporating exogenous uridine and cytidine into both RNA and DNA, when grown in wild-type mouse L cells (Tables 4 and 5). Results from HPLC experiments analyzed the distribution of [6-^{3}H]-uridine or [6-^{3}H]-uracil into pyrimidine bases of acid-hydrolyzed DNA, isolated from L2Tri/5-FU infected wild-type mouse L cells, indicated that radiolabel was detected in cytosine but not in thymine (data not shown).

8.1 *Cloning the thymidine kinase gene from Chlamydia psittaci Cal10*

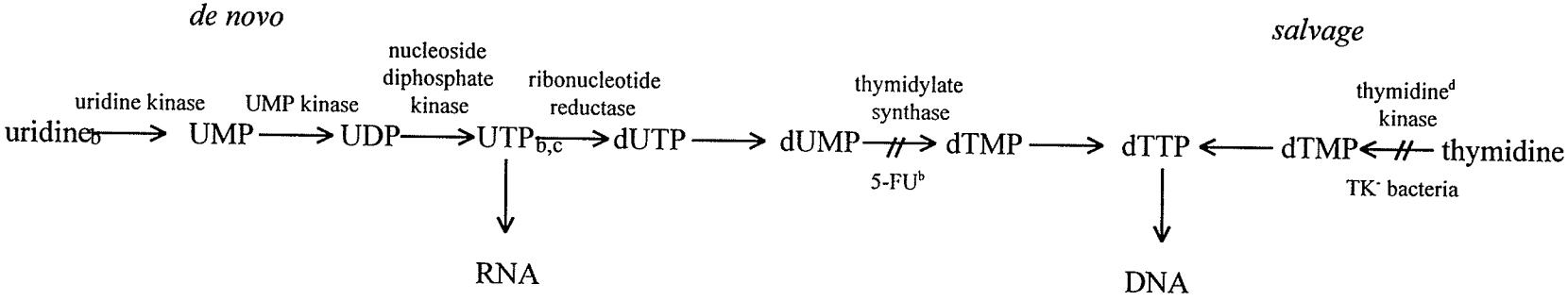
8.1.0 *Cloning via complementation*

Interspecies complementation has proven to be a useful strategy for cloning metabolic pathway genes and cDNAs from bacteria, fungi, and mammals (Peoples and Sinskey, 1989; Dougherty *et al*, 1992; Williams and Kantrowitz, 1992; and Kaslow and Hill, 1990). Using a *C. psittaci* C10 *Eco*R1-digested genomic DNA library, in the multi-copy plasmid pUC19 the libraries were screened for complementation of the thymidine kinase gene in a thymidine kinase deficient *E. coli*.

8.1.1 *Mechanism of complementation*

Since TTP can be obtained via *de novo* synthesis and salvage pathways, the selection system for thymidine kinase involves more than simply deleting the thymidine kinase gene in the host bacteria (Figure 11). 5'-Fluorodeoxyuridine inhibits thymidylate synthase by forming a ternary complex with thymidylate synthase, 5'-fluorodeoxyuridylate and

Figure 11: Selection by complementation[a]



a-TK$^-$ host bacteria
b- components provided in the media
c- UTP provided in limiting quantities to ensure protein synthesis takes place
d- the only way cells could be rescued is to have an active thymidine kinase gene. This will take thymidine to dTTP for DNA synthesis

5,10 methylene tetrahydrofolate (Kaufman, 1989) thereby preventing the synthesis of dTMP from host dUMP pools. Uridine is provided in the media to insure that the host cells do not die because of 5'fluoro-UTP incorporation into RNA. Under these conditions, cells cannot obtain dTTP except via thymidine kinase. The presence of TK in its active form allows DNA synthesis to take place and the host cell to survive.

8.2 *Thymidine kinase of Chlamydia psittaci*

Both *Chlamydia trachomatis* L2 and *Chlamydia psittaci* Cal10 were screened by complementation. The first positive was obtained in *C. psittaci* Cal10, and was then used for further analysis. *E. coli* cells containing the *C. psittaci Eco*RI partial digest genomic library were plated on thymidine kinase selection media. Several colonies were picked to fresh selection media and incubated at 37°C overnight. Colonies that grew on the selection media and therefore were thought to contain the correct insert were grown up, and their plasmids isolated and sequenced by PCR directed dideoxynucleotide sequencing (Figure 12). The gene had a typical ATG start codon (+1) with -35 and -10 promoter regions. The terminator region has a TAA stop codon (599) with a rho-independant terminator. The gene has a GC ratio of 30%. An interesting feature of the gene organization is the presence of a second gene, a tRNA gene for glycine ($tRNA^{GLY}$) (bp 636-707). Here the -35 and -10 promoter regions are found within the rho independent terminator of the thymidine kinase gene.

Figure 12: Sequence of thymidine kinase and $tRNA^{GLY}$ genes

```
-87   AAA TAG ATT TTT TTA TTA CTT AAA TAT TTT TCA AAA TTA TTT TTT

          -35 region                          -10 region                    TK Start
-42   TGT TTA CAA TTT TAT TTA CTC TAT AAC TTA AAA GGA CAA ACT ATG
                                                                          met

+4    TAT AAA AAA TTT TCA GAT GGA ACT ATT GAA GTA ATT ACT GGT CCC
      tyr lys lys phe ser asp gly thr ile val asp ile thr gly pro

49    ATG TTT TCG GGA AAA AGT GAT GAA GTA ATT AAA AGG ATT AGA ACA
      met phe ser gly lys ser asp glu val ile lys arg ile arg thr

94    TTA TCT TAT GCT AAT GTC AAA ACT TTA GCT GTA AAG CCA AGA ATT
      leu ser leu ala asn val lys thr leu ala val arg pro arg ile

139   GAT TCT AGA TTT TCT ACA AAT GAA ATA GTT TCC CGT GCT GGT ACT
      asp ser arg phe ser thr asn glu ile val ser arg ala gly thr

184   AAA ATT CCT ACA TAT GTA GTA GAA ACA GTA GCA GAT ATA AAA AGT
      lys ile pro thr tyr val val glu thr val ala his ile lys ser

229   TTA TTT GAA AAA TCA AAA TAC AAA GCA ATC GCA ATA GAT GAA GCT
      leu phe glu lys ser lys try lys ala ile ala ile asp glu ala

274   CAA TTT TTT GAT AAA GAT TTA GTA CCT TAT GTT GAA GAA TTA GCT
      gln phe phe asp lys asp leu val pro tyr val glu glu leu ala

339   AAT CAA GGA ATT AGA GTG ATT ATT TGC GGT TTA GAC CAA GAT TAT
      asn gln gly ile arg val ile ile cys gly leu asp gln asp tyr

384   TTA AGA CGT CCT TTC GGA GTA ATG CCT AGT TTA TTA GCG ATG GCT
      leu arg arg pro phe gly val met pro ser leu leu ala met ala

429   GAA CAT ATT ACA AAA TTG CAA GCT ATT TGC GTA ATT TGT AAA AAT
      glu his ile thr lys trp gln ala ile cys val ile cys lys asn

474   GCA GCT TCT ACA ACT TTT AGA ACT ATT AAG TCC AAT AAA TTG AAA
      ala ala ser thr thr phe arg thr ile lys ser asn lys leu lys

519   GTT ATT GGA GAT TTA GAC GAA TAC GAA GCA AGG TGC AGA ATT TGT
      val ile gly asp leu asp glu tyr glu ala arg cys arg ile cys

                          Rho independant terminator  -35 region
564   CAC AAT AAA GGA TTA AAT AAT TTA GAA AAA AAT >TAA CAA TTT TAT <
      his asn lys gly leu asn asn leu glu lys asn stop
                  -10 region                          tGly Start
609   TTT TTT CTG TTA TAA TTT AAT TAA TCT GCA AGT GTA GTT TAA TGG

654   CAG ACT TCA GCC TTC CAA GCT GAT TGT AAG GGT TCG ATT CCC TTC

            tGly Stop
699   ACT TGC TCC ATA TCA AAT TTG ACC AAT GAG CTT TTT GCT CAT TTT
```

8.3 *PCR directed cloning of Chlamydia trachomatis L2 and L2Tri^R-100 thymidine kinase gene*

8.3.0 *Thymidine kinase of Chlamydia psittaci seems to be conserved across the species*

The deduced thymidine kinase amino acid sequence showed a high degree of homology with other thymidine kinases (Figure 13). With this in mind, primers were designed (Table 2) which bound as shown in Figure 4. The primers were used with *C. trachomatis* L2 and TriR genomic DNA and gave a product of 681 bp. The clones which were in pUC19, were sequenced and the sequences compared (Figure 14). There is a high degree of homology (95%) between thymidine kinases of the two isolates obtained by PCR cloning.

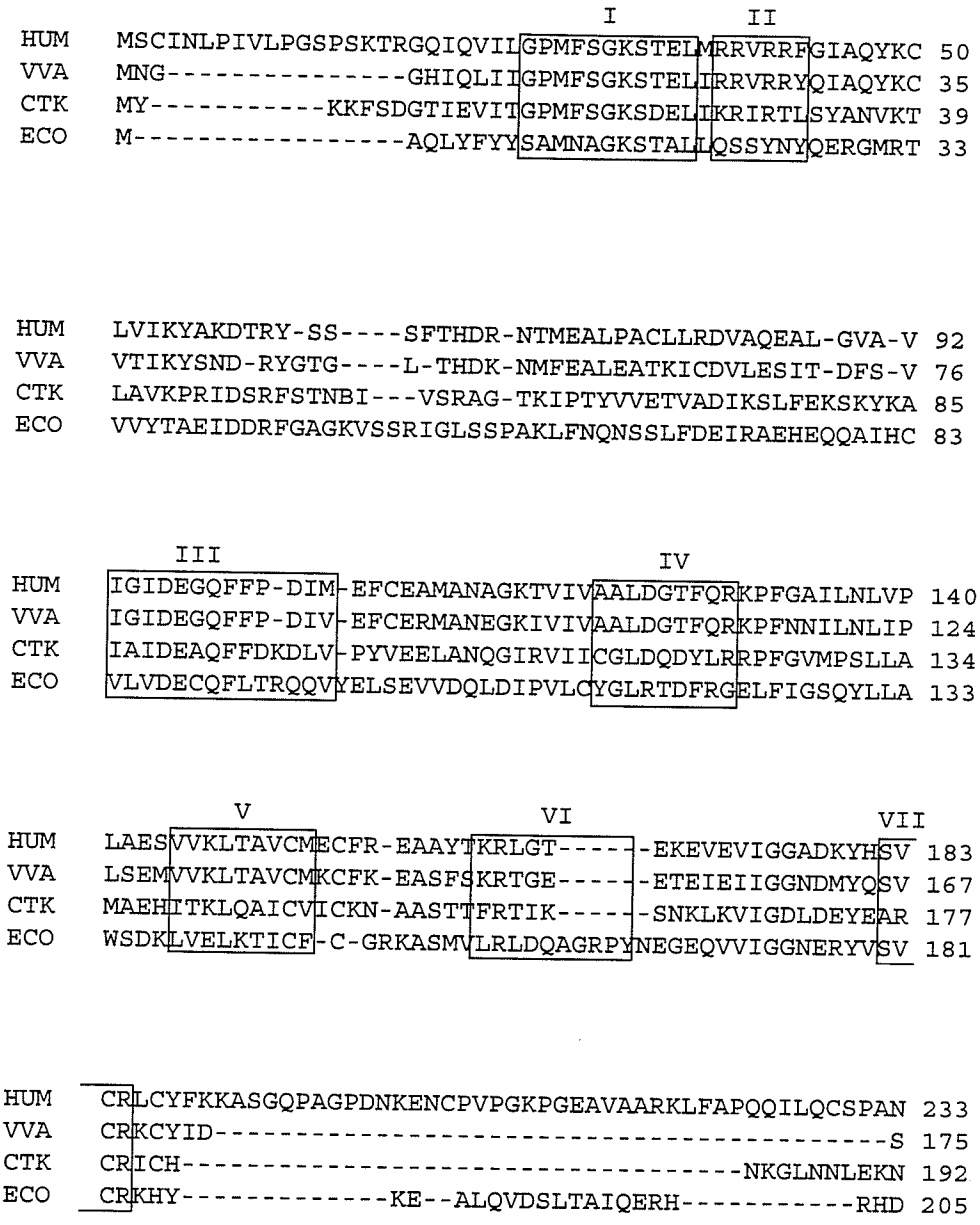
8.3.1 *Obtaining a genomic clone of thymidine kinase from Chlamydia trachomatis*

In colony hybridization, cultures are transformed with the multicopy plasmid pUC19 that contains the *C. trachomatis* L2 *Hind*III partial digest library. The pUC19 plasmid contains an ampicillin gene which serves as a marker. Only colonies that contain the pUC19 plasmid are able to grow on the selective media containing ampicillin. These colonies are picked to a second plate containing ampicillin, and the colonies that remain positive are blotted to a nylon membrane. The nucleic acids are fixed to the membrane and screened by colony hybridization. Here, the whole TK-tRNA^{GLY} probe is used to locate colonies containing DNA homologous to a portion of the thymidine kinase gene probe.

Figure 13: Alignment of thymidine kinase amino acid sequences

Alignment of the amino acid sequence of human TK (HUM), vaccinia virus TK (VVA), *Chlamydia trachomatis* TK (CTK), and *Escherichia coli* TK (ECO). Boxes I to VII represent those domains that have been identified previously as being completely conserved between vaccinia virus, monkey pox, variola virus, chicken, mouse and human thymidine kinases (Black and Hruby, 1990). Function of the regions is discussed in the text. Standard single amino acid nomenclature is used (Black and Hruby, 1991).

Figure 13: Alignment of Thymidine Kinase Amino Acid Sequences



10.8% homology

Figure 14: Analysis of Clones obtained by PCR amplification

```
CPTK   ATGTATAAAAAATTTTCAGATGGAACTATTGAAGTAATTACTGGTCCCAT 50
TRITK  ATGTATAAAAAATTTTCAGATGGAACTATTGAAGTAATTACTGGTCCAAT 50
L2TK   ATGTATAAAAAATTTTCAGATGGAACTATTGAAGTAATTACTGGTCCAAT 50

CPTK   GTTTTCGGGAAAAAGTGATGAAGTAATTAAAAGGATTAGAACATTATCTT 100
TRITK  GTTTTCGGGAAAAAGTGATGAACTAATTAAAAGGATTAGAACATTATCTT 100
L2TK   GTTTTCGGGAAAAAGTGATGAACTAATTAAAAGGATTAGAACATTATCTT 100

CPTK   ATGCTAATGTCAAAACTTTAGCTGTAAAGCCAAGAATTGATTCTAGATTT 150
TRITK  ATGCTAATGTCAAAACTTTAGCTGTAAAGCCAAGAATTGATTCTAGATTT 150
L2TK   ATGCTAATGTCAAAACTTTAGCTGTAAAGCCAAGAATTGATTCTAGATTT 150

CPTK   TCTACAAATGAAATAGTTTCCCGTGCTGGTACTAAAATTCCTACATATGT 200
TRITK  TCTACAAATGAAATAGTTTCCCGTGCTGGTACTAAAATTCCTACATATGT 200
L2TK   TCTACAAATGAAATAGTTTCCCGTGCTGGTACTAAAATTCCTACATATGT 200

CPTK   AGTAGAAACAGTAGCAGATATAAAAAGTTTATTTGAAAAATCAAAATACA 250
TRITK  AGTAGAAACAGTAGCAGATATAAAAAGTTTATTTGAAAAATCAAAATACA 250
L2TK   AGTAGAAACAGTAGCAGATATAAAAAGTTTATTTGAAAAATCAAAATACA 250
```

Consensus length: 694
Identity: 660 (95.1%)

Several colonies were isolated using this protocol, however, upon rescreening, only six remained positive. Each colony was picked from a separate plate, and therefore represents an independent transformation event. Each plasmid (designated 1, 2, 3, 4b, 5, and 6b) was isolated and digested with *Hind*III, the enzyme used to create the genomic library (Figure 15, top and bottom). The *Hind*III digested plasmids were southern blotted and hybridized with the whole TK-tRNA^{GLY} probe (Figure 16).Lanes c, d, and e on the top blot, and lanes b, c and d on the bottom blot represent the six independent clones obtained by colony hybridization. These clones were designated 4b, 5, 6b and 1, 2, 3 respectively. Five of the six were positive with Southern hybridization with the whole TK-tRNA^{GLY} probe, clone 5 was the only one that was negative. The controls run in the hybridization were TKpUC19E(2)se.time (the original *C. psittaci* full length clone), and the pUC19 plasmid which served as positive (lane e, bottom blot) and negative (lane b, top blot) controls respectively. Since at this point we are only interested in the presence or absence of a signal, a molecular weight marker was not hybridized.

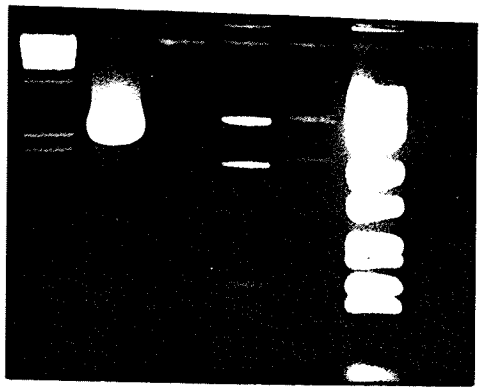
8.3.2 *Characterization of Genomic Clone*

Inserts 4b and 6b were sequenced by PCR-directed dideoxynucleotide sequencing. Early comparisons with the Gene Bank data base showed that the 5' end of the insert was highly homologous to the 16s ribosomal gene of *Chlamydia trachomatis* (Figure 17), while the 3' end was a portion of the 23s ribosomal gene.

Figure 15: *Hind*III digestion of the clones of interest

Clones obtained by screening L2 *Hind*III:pUC19 partial digest library with L2 thymidine kinase PCR product. The fragments were run on a 1.5% agarose gel. Lanes are marked as follows; Top Gel--a- lambda *Hind*III molecular weight marker, b-pUC19, c-clone 4b, d-clone 5, e-clone 6b, f-CT47. Bottom Gel--a-*Hind*III molecular weight marker, b-clone 1, c-clone 2, d-clone 3, e-TKpUC19E(2)se.time, the original clone.

a b c d e f



a b c d e

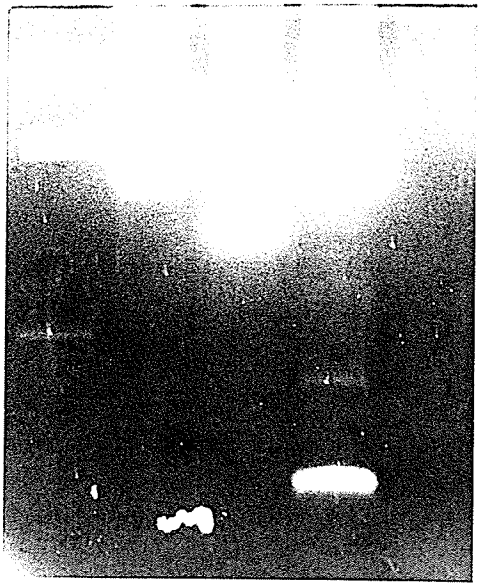
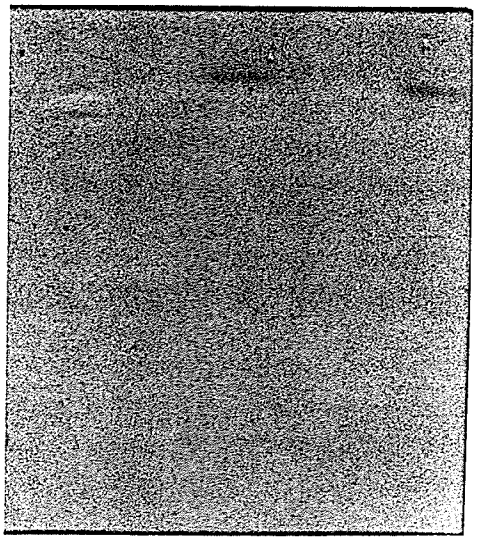


Figure 16: Hybridization of Clones with the whole TK·tRNA^{GLY} Probe

Blots were done together under identical conditions, as described in the methods section. TK·tRNA^{GLY} was used as a probe. Lanes are marked as follows; Top Blot--a- lambda *Hind*III molecular weight marker, b-pUC19, c-clone 4b, d-clone 5, e-clone 6b. Bottom Blot--a-*Hind*III molecular weight marker, b-clone 1, c-clone 2, d-clone 3, e-TKpUC19E(2)se.time, the original clone.

a b c d e



a b c d e

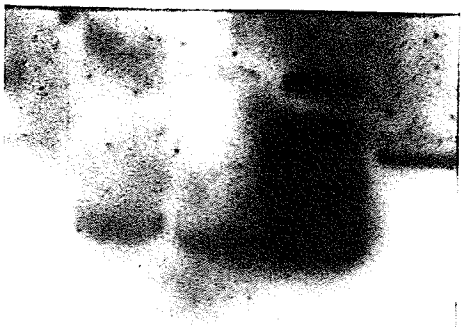


Figure 17: Clones contain 16S ribosomal gene

```
6bpUC19    GAACCCGTTAATAAATCAAAGTATATATTTTTCTGAGAATTTGATCTGT
CHTDAA                                  ATTTTTCTGAGAATTTGATCTGT

6bUC19     TCAGATTGAACGCTGGCGGCGTGGATGAGGCATGCAAGTAG-ACGGAGC
CHTDAA     TCAGATTGAACGCTGGCGGCGTGGATGAGGCATGCAAGTCGAACGGAGC

6bUC19     AATTGTTTCGGCAATTGTTT-GTGGCGGAAGGGTTAGTAATGCATAGAA
CHTDAA     AATTGTTTCGGCAATTGTTTAGTGGCGGAAGGGTTAGTAATGCATAG-A

6bUC19     TAATTTGTCCTTAACTTGGGAATAACGGTTGGAAACGGCCGCTAATACC
CHTDAA     TAATTTGTCCTTAACTTGGGAATAACGGTTGGAAACGGCCGCTAATACC

6bUC19     GAATGTGGCGATA-TTGGGCATCCGAGTAACGTTAAAGAAGGGGATCT-
CHTDAA     GAATGTGGCGATATTTGGGCATCCGAGTAACGTTAAAGAAGGGGATCTT

6bUC19     -AGACCTTTCGGTTAAGGGAGAGTCTATGTGATATCAGCTAGTTGGTGG
CHTDAA     AAGACCTTTCGGTTAAGGGAGAGTCTATGTGATATCAGCTAGTTGGTGG

6bUC19     GGTAAAGGCCTACCAAGGCTATGACGTCT-GGCGGATTGAGAGATTGGC
CHTDAA     GGTAAAGGCCTACCAAGGCTATGACGTCTAGGCGGATTGAGAGATTGGC

6bUC19     CGCCAA-ACTGACTAG
CHTDAA     CGCCAACACTGACTGAGACACTGCCCAGACTCCTACGGGAGGCT
```

CHTDAA-*C. trachomatis* 16S ribosomal RNA gene.
97.0% identity in 334 nt overlap.

A plasmid known to contain the 16s and 23s ribosomal genes from *C. trachomatis* designated CT47 (kindly provided by Tom Hatch, Department of Microbiology and Immunology, University of Tennessee, Memphis, Tennessee) was run concurrently with the clones of interest (Figure 18). Colony hybridization with the whole TK-tRNA^{GLY} probe indicates some homology within the target gene. Since tRNA genes are frequently seen in ribosomal gene operons (Fournier and Ozaki, 1985) the interspacer region was sequenced in an attempt to clarify the hybridization results (Figure 19). No homology to any tRNA genes was seen.

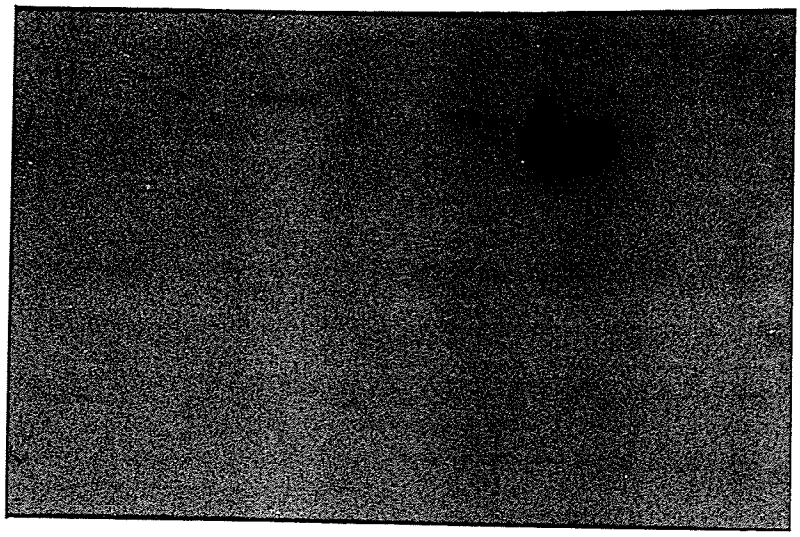
To determine which portion of the whole TK-tRNA^{GLY} probe is binding within the clones and CT47, primers were made to amplify the internal TK gene and the whole tRNA^{FLY} gene separately (Table 2). The location of the primer binding is shown in Figure 4. The sequence of the probes was checked by PCR directed dideoxynucleotide sequencing. When the blot was reprobed using the whole tRNA^{GLY} probe (Figure 20), only the original clone remained positive (lane e, bottom blot). The internal TK probe (Figure 21) continued to hybridise with 6b (lane e, top blot) and CT47 (lane f, top blot) while 4b (lane c, top blot) went from positive to negative. However the negative control (pUC19) has gone from negative to positive (lane b, top blot).

Chlamydia are obligate intracellular parasites, there is no cell free growth system. This leaves the researcher vulnerable to contamination by foreign organisms and viruses. The

Figure 18: Hybridization of Clones with the whole TK-tRNA^{GLY} Probe

Blots were done together under identical conditions, as described in the methods section. Whole TK-tRNA^{GLY} was used as a probe. Lanes are marked as follows; Top Blot--a-lambda *Hind*III molecular weight marker, b-pUC19, c-clone 4b, d-clone 5, e-clone 6b, f-CT47. Bottom Blot--a-*Hind*III molecular weight marker, b-clone 1, c-clone 2, d-clone 3, e-TKpUC19E(2)se.time, the original clone.

a b c d e f



a b c d e

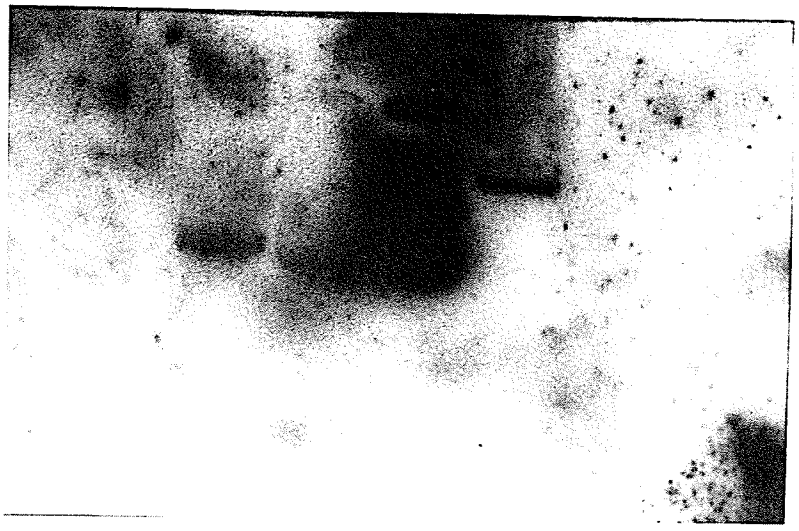


Figure 19: Sequence of the interspacer region of the 4b clone

16srRNA5'1

1 **TGA TGA CTA GGA TGA AGT CG**T AAC AAG GTA GCC CTA CCG GAA GGT GGG

48 TTG GAT CAC CTC CTT TTA AGG ATA AGG AAG AAG CCT GAG AAG GCC CTT

96 CTG AAC TAG GTT GGG CAA GCA TTT ATA TGT TAA GAG CAA GCA TTC TAT

144 TTA TTT GTG TTG TTA AGA GTA GCG TGG TGA GGA CGA GAC ATA TAG TTT

192 GTG ATC AAG TAT GTT ATT GTA AGG AAA TAA TCA TGG TAA CAA GTA TAT

240 TTC ACG CAT AAT AAT AGA CGT TTA AGA GTA TTT GTC TTT TAG GTG AAG

288 TGC TTG CCA TGG ATC TAT AGA AAT TAC AGA CCA AGT TAA TAA GAG CTA

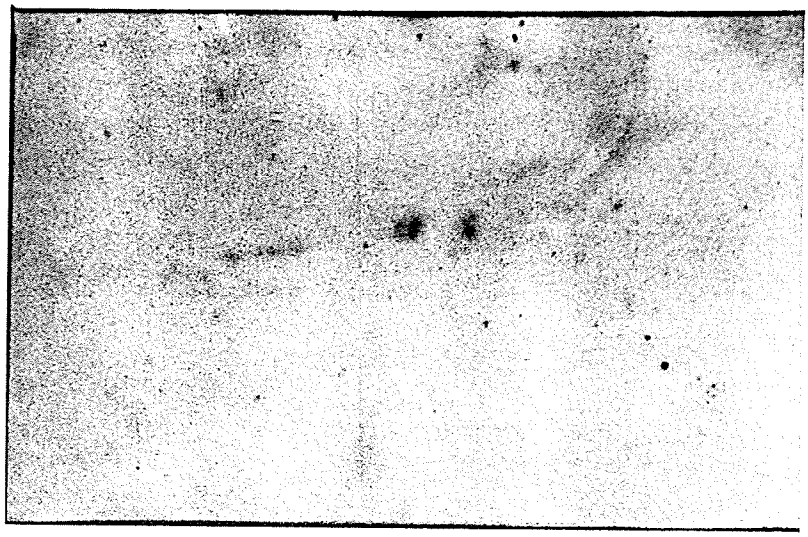
336 TTG GTG GAT G

No homology to $tRNA^{GLY}$ seen

Figure 20: Hybridization of Clones with the whole $tRNA^{GLY}$ Probe

Blots were done together under identical conditions, as described in the methods section. whole $tRNA^{GLY}$ was used as a probe. Lanes are marked as follows; Top Blot--a-lambda *Hind*III molecular weight marker, b-pUC19, c-clone 4b, d-clone 5, e-clone 6b, f-CT47. Bottom Blot--a-*Hind*III molecular weight marker, b-clone 1, c-clone 2, d-clone 3, e-TKpUC19E(2)se.time, the original clone.

a b c d e f



a b c d e

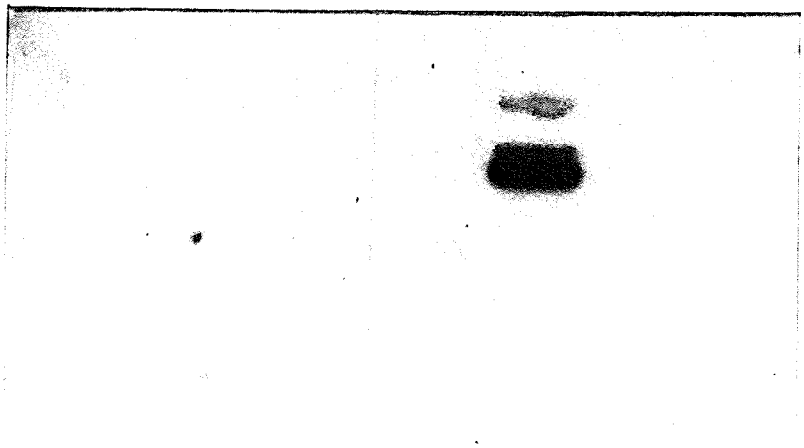
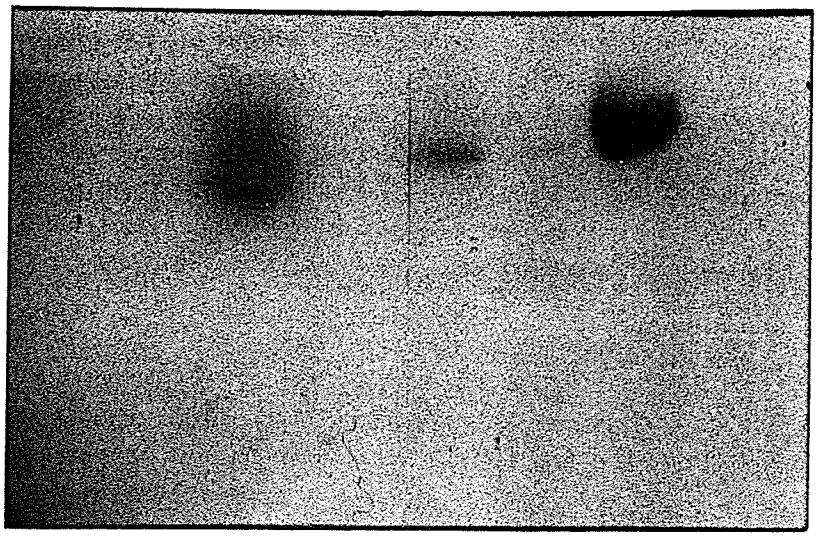


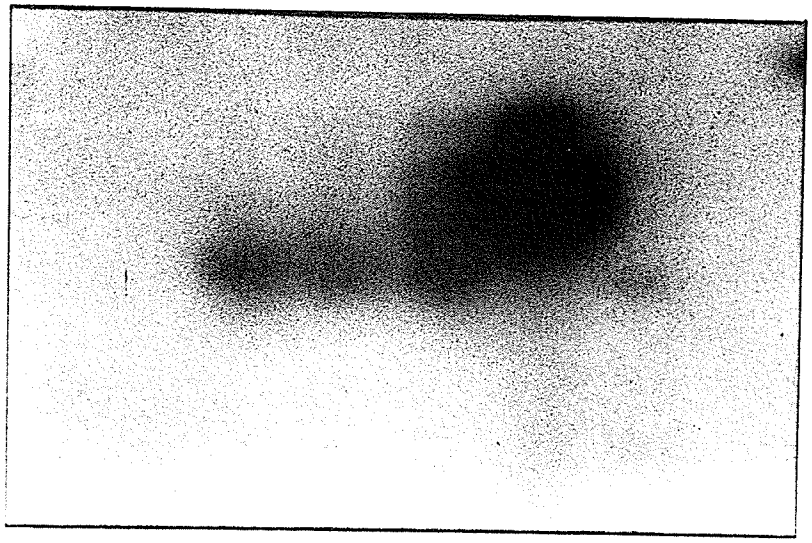
Figure 21: Hybridization of clones with internal TK probe

Blots were done together under identical conditions, as described in the methods section. Internal thymidine kinase was used as a probe. Lanes are marked as follows; Top blot--a- lambda *Hind*III molecular weight marker, b-pUC19, c-clone 4b, d-clone 5, e-clone 6b, f-CT47. Bottom blot--a- lambda *Hind*III molecular weight marker, b-clone 1, c-clone 2, d-clone 3, e-TKpUC19E(2)se.time, the original clone.

a b c d e f



a b c d e



30% G-C ratio of the thymidine kinase gene cloned suggests that it may have originated from mycoplasma a common tissue culture contaminant. Chlamydia has an G-C ratio of 39.3% to 43% (Table 1) whereas mycoplasma is much lower, ranging from 23% to 40% (Table 6). However, when the thymidine kinase gene and the $tRNA^{GLY}$ was screened against two common tissue culture contaminants, *Acholiplasma laidlawii* and *Mycoplasma hominis* only *C. psittaci* C10 and *C. trachomatis* L2 were positive (Figures 22-25).

Table 6: Taxonomy and properties of mycoplasmas (class *Mollicutes*)[a]

Classification	Current no. of recognized species	Mol % G+C of DNA	Distinctive Properties	Habitat
Mycoplasmatales				
Mycoplasmataceae				
Mycoplasma	92	23-41		Humans, animals, plants, insects
Ureaplasma	5	27-30	urease positive	Humans, animals
Spiroplasmataceae				
Spiroplasma	11	25-31	helical filaments	Arthropods (including insects), plants
Acholeplasmatales				
Acholeplasmataceae				
Acholeplasma	12	27-36		Animals, plants, insects
Anaeroplasmatales				
Anaeroplasmataceae				
Anaeroplasma	4	29-33	obligate anaerobe	Bovine-ovine rumen
Asteroleplasma	1	40	obligate anaerobe	Bovine-ovine rumen
Uncultivated, unclassified MLOs		23-29	Uncultivated	Plants, insects

a. Modified from Razin, S. (1992).

Figure 22: Hybridization of *A. laidlawii* with the whole $tRNA^{GLY}$ probe

Hybridization conditions were as described in the methods. DNA digested with *Hind*III. Lanes are as follows: a-molecular weight markers b,e-*C. trachomatis* L2 c,f-*C. psittaci* C10 d,g-*A. laidlawii*.

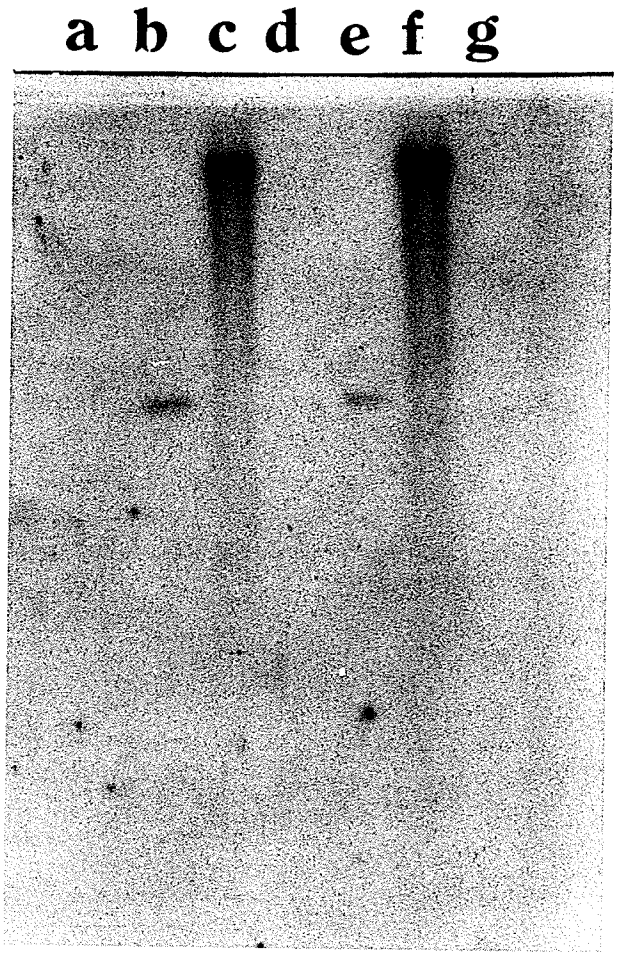

a b c d e f g

Figure 23: Hybridization of *A. laidlawii* with the internal TK probe

Hybridization conditions were as described in the methods. DNA digested with *Hind*III. Lanes are as follows: a-molecular weight markers b,e-*C. trachomatis* L2 c,f-*C. psittaci* C10 d,g-*A. laidlawii*.

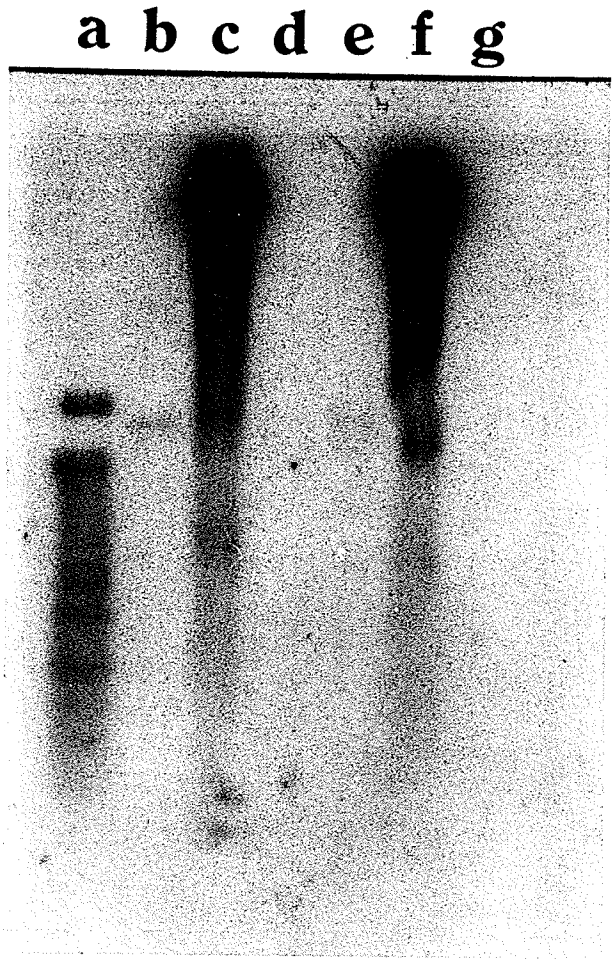

a b c d e f g

Figure 24: Hybridization of *M. hominis* with the whole $tRNA^{GLY}$ probe

Hybridization conditions were as described in the methods. DNA digested with *Hind*III. Lanes are as follows: a-molecular weight markers b,e-*C. trachomatis* L2 c,f-*C. psittaci* C10 d,g- *M. hominis.*

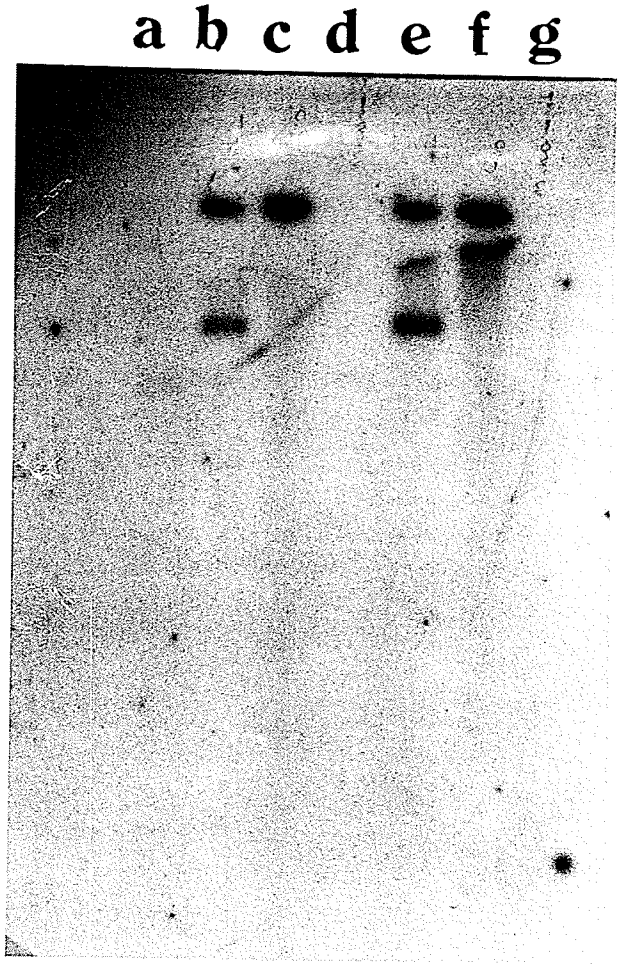
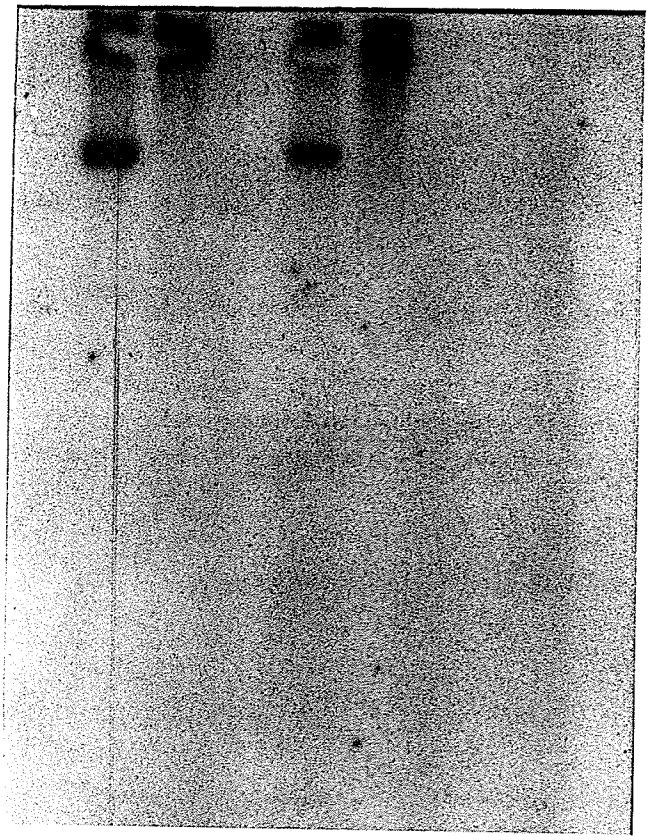

a b c d e f g

Figure 25: Hybridization of *M. hominis* with the internal TK probe

Hybridization conditions were as described in the methods. DNA digested with *Hind*III. Lanes are as follows: a-molecular weight markers b,e-*C. trachomatis* L2 c,f-*C. psittaci* C10 d,g- *M. hominis.*

a b c d e f g



9. Discussion

9.1 *Nucleotide acquisition pathways displayed in wild type and mutant Chlamydia trachomatis*

The goal of this project was to obtain evidence for the existance of a thymidine kinase gene in *C. trachomatis* and to clone and characterize it. Since no cell-free growth or gene transfer systems exist for chlamydia, the isolation and characterization of mutant organisms is very useful for identifying and characterizing metabolic capabilities. The following is a summary of the nucleotide acquisition patterns displayed by wild-type *C. trachomatis* L2 and the various mutant chlamydia isolated. *C. trachomatis* L2 and L2Tri^{R}-60 can take all four NTPs from the host cell; can synthesize all four deoxyribonucleotides through the combined action of ribonucleotide reductase and thymidylate synthase and; cannot salvage nucleosides or nucleobases. Mutants L2Tri^{R}-100 and L2Sulf^{R}-100 can take ATP, GTP, and dTTP, but not UTP or CTP, directly from the host cell; cannot make thymidine *de novo;* and can salvage adenine, guanine, uracil and thymidine.

It is intriguing that the nucleotide acquisition pattern displayed by L2Tri^{R}-100 and L2Sulf^{R}-100 appears to be identical to that of *C. psittaci* Cal10 (McClarty and Fan, 1993; McClarty and Qin, 1993). At the time it was assumed that the phenotype displayed by *C. psittaci* Cal10 represented an alternative to the ribonucleotide auxotrophy displayed by

most chlamydial isolates. Clearly, the results of this study with isogenic strains indicate that the genes encoding enzymes necessary for both nucleotide acquisition phenotypes are present in *C. trachomatis* L2.

There are five well recognized mechanisms for becoming resistant to sulphonamide and trimethoprim (Hitchings, 1983): a) synthesis of a mutationally altered enzyme with a decreased affinity for the inhibitor; b) increased synthesis of a wild-type enzyme; c) decreased cellular permeability to the inhibitor (very rare event); d) development of dependence on exogenous reduced folate (can only occur in organisms that have the capacity to transport reduced folates); and f) development of dependence on exogenous thymine or thymidine (can only occur in organisms that have a thymidine kinase). The first two mechanisms are the most common cause of resistance in clinical isolates. Thymine dependence commonly occurs when selecting for resistance to trimethoprim *in vitro*, however, it is rarely found with clinical isolates (<1%). This is because the level of thymine (thymidine) in body fluids is too low to support the growth of these organisms *in vivo* (Bushby, 1983).

The results presented suggested that the L2Tri^{R}-60 isolate likely synthesizes a mutant dihydrofolate reductase with decreased affinity for trimethoprim (category a) or over-expresses wild-type enzyme (category b). This suggestion is supported by the following lines of evidence. L2Tri^{R}-60 is highly resistant to trimethoprim, cross

resistance to methotrexate, sensitive to sulphisoxazole and displays a nucleotide acquisition phenotype identical to that of the parental *C. trachomatis* L2 isolate. Since the increase in resistance to trimethoprim is much greater than to methotrexate, it seems most likely that a mutant dihydrofolate reductase is being synthesized by L2Tri^{R}-60, however, conclusive evidence awaits characterization of the dihydrolate reductase enzyme from L2Tri^{R}-60.

All evidence suggests that the mutant isolates L2Tri^{R}-100 and L2Sulf^{R}-100 are phenotypically identical. Both are highly resistance to trimethoprim, sulphisoxazole, methotrexate, and are sensitive to 5-fluorouracil. In addition, both isolates exhibit a unique, but similar, nucleotide acquisition phenotype that can adequately explain their resistance characteristics. Since L2Tri^{R}-100 and L2Sulf^{R}-100 can salvage thymidine (a category d resistance mechanism) and no longer synthesize it *de novo* from uracil containing nucleotides, they are therefore resistant to all inhibitors that prevent *de novo* thymidine synthesis.

9.1.0 *A possible explanation for the existence of two sets of nucleotide acquisition pathways*

What is the underlying genetic mutation in these isolates that allows for expression of the different phenotypes? Why has chlamydia retained the ability to salvage nucleotides in this manner? Although definitive answers are not available at this time, the following

represents a reasonable hypothesis. It is generally accepted that EBs are metabolically inert and are incapable of nucleotide transport (Hatch, 1988; Moulder, 1991). The ability to transport ribonucleotides no doubt requires specialized transport system(s) that may well be specific to RBs. There would be a significant demand for ribonucleotides in the early stages of differentiation, to fulfill the need for mRNA synthesis required for the production of early developmental stage specific proteins. It is possible that all the specific transport protein(s) required for NTP uptake are not present in the EB cytoplasmic membrane and, as a result, the need for net nucleotide production must be met via salvage pathways. Furthermore, there may also be a short period of time during RB→EB differentiation when the NTP transporters cannot meet the nucleotide demand required for late stage mRNA synthesis and ultimately, storage of a NTP pool. In wild-type *C. trachomatis* L2 the salvage enzymes would be expressed in the very early stage of EB→RB reorganization. Some of the earliest proteins synthesized would be NTP transporters. Soon after the transporters become functional, the synthesis of the salvage pathway enzymes would be repressed. The signal for repression may be as simple as an adequate supply of NTPs obtained via direct transport. The reverse may happen at late RB→EB differentiation. NTP transporters would no longer be synthesized, NTP pools would drop, and salvage pathways would be derepressed.

To become resistant to trimethoprim and/or sulphonamide all that is required is the ability to utilize exogenous thymidine (i.e. express a thymidine kinase), however, both

L2Tri^R-100 and L2Sulf^R-100 express an entire set of nucleotide salvage enzymes (uracil, adenine, and guanine phosphoribosyltransferases; and thymidine kinase). This suggests that expression of all the salvage enzymes are under the control of a common regulator. It may be that mutants like L2/Tri^R-100 and L2Sulf^R-100 are regulatory mutants, with a mutation in the regulator that controls the switching from salvage pathways to NTP transport. Clearly, this model is over-simplified because the phenotype displayed by the different mutant isolates is mixed, that is, parts of both pathways (NTP transport and nucleotide salvage) are expressed.

In an attempt to characterize, at the molecular level, the mechanism of resistance, thymidine kinase was chosen to be cloned. Thymidine kinase shows complex allosteric regulation involving a number of nucleotides, particularly dTTP, which feedback-inhibit the enzyme in a kinetically complex manner. Thymidine kinase is expressed in pathway II but not in pathway I. This makes it an ideal target for the characterization of the switch between these two pathways.

9.2 *Cloning the thymidine kinase gene*

The original thymidine kinase gene was cloned from *Chlamydia psittaci* Cal10 using complementation. Several groups have reported success in isolating chlamydial genes in *E. coli* expression systems (Kaul and Wenman, 1985; Nano and Caldwell, 1985; Palmer and Falkow, 1986; Sardinia *et al*, 1989). It appears that there is a subset of chlamydial

promoters that function in *E. coli.* In complementation, only bacteria containing an active thymidine kinase gene will be able to synthesis DNA, and therefore survive. The thymidine kinase gene is 579 base pairs (bp) long (Figure 12). It has a typical ATG start codon and TAA stop codon. At the 3' end there is a rho independent terminator like structure, consisting of a dyad symmetrical sequence. Overlapping with this rho independent terminator is the -35 and -10 regions of a second gene, a tRNA gene for glycine ($tRNA^{GLY}$). The thymidine kinase gene cloned using a *C. psittaci* genomic library showed 24% homology with known thymidine kinase genes from vaccinia virus and human thymidine kinase (Figure 13).

9.2.0 *Interspecies sequencing*

PCR cloning was used to amplify the thymidine kinase genes from *C. trachomatis* L2 and *C. trachomatis* L2-Tri^R100. The sequences were compared and there was a high degree of homology (95.1%) between the three (Figure 14). It is not surprising to see a high degree of homology between *C. trachomatis* L2 and *C. trachomatis* L2TriR-100 since TriR was derived from L2. However, between *C. trachomatis* and *C. psittaci* the DNA homology is between 1 and 33 per cent (Fukuski and Hirai, 1992), and it is unexpected that one gene would be so perfectly conserved. It is possible that this gene represents carryover of *C. psittaci* DNA, contaminating all chlamydial preps. A second possible, though disturbing, explanation would be a contaminant, present in each lab strain. It would not be

impossible to detect an extremely low level of contamination using powerful techniques such as PCR.

9.3 *Characterization of the genomic clone*

By using sensitive techniques such as hybridization, under stringently controlled conditions, one has to believe that non-specific clones will be screened out during the course of the procedure. However the data suggests that this is not the case. Two clones (4b and 6b) were obtained that remained strongly positive through three rounds of screening (data not shown). Preliminary sequencing showed that the clones were from the same location in the genome, since they were identical at their 3' end.

Sequence comparison showed that the insert contained a ribosomal operon, with the 23s and 16s ribosomal genes. A common structural arrangement is to find tRNA genes within ribosomal operons (Fournier and Ozaki, 1985). The next step would be to check if there was a tRNA gene for glycine, and therefor an explanation for the hybridization. The interspacer region was sequenced, however, no homology to $tRNA^{GLY}$ was found. The original thymidine kinase gene was separated into its components using PCR. Primers were designed (Table 2) to amplify the thymidine kinase and $tRNA^{GLY}$ genes separately. The separate genes were confirmed by PCR directed dideoxynucleotide sequencing. When the probe was divided into its components, and the blots probed separately, the pattern of hybridization was indicative of the thymidine kinase gene being

present in some form within the clones. The whole TK-tRNA^{GLY} probe hybridized to five of six clones isolated, as well as to itself, and a plasmid known to contain a chlamydial ribosomal operon. The difference in the strength of the signal can be partially explained by overloading in the CT47 lane (Figure 18). The tRNA^{GLY} gene hybridized only to itself, and to the plasmid known to contain a ribosomal operon. A possible explanation for the lower intensity of the signal could be because the gene is very small, and the specific activity generated by random primer labelling will be lower than that of the larger probe. However, when the thymidine kinase gene was used alone, it hybridized with pUC19, thereby invalidating the experiment. All three probes were confirmed by PCR directed dideoxynucleotide sequencing (data not shown). None had any homology to pUC19. However, despite the fact that the probes were gel purified, contamination with pUC19 cannot be ruled out. This could be confirmed by repeating the experiment and amplifying the probes in the presence of a radioactive dNTP. This would label the probe alone.

To determine where the DNA probe is binding, a possible experiment would involve the techniques of DNA footprinting. Though this technique is seen in the study of protein:DNA interactions, the basic premise is the same. Double stranded DNA is protected from digestion, while single stranded DNA is not. Here the thymidine kinase probe will be hybridized with the 6b clone. This will leave single stranded DNA ends which could be digested by S1 nuclease (Darnell *et al*, 1990). Under carefully controlled conditions, flanking DNA regions could be preserved to serve as landmarks in the known

6b sequence. The TK probe would act as a primer for Klenow fragment, and the double stranded section could be ligated into a suitable plasmid and sequenced. This would show the location of the hybridization.

9.4 *Further suspicion of contamination*

Since the G-C ratio is so low within the cloned thymidine kinase gene, the possibility that the gene is in fact from another source cannot be overlooked (Mattsson and Johansson, 1988). Bacterial and fungal contamination may cause some concern, but are usually obvious and easily detected. Insidious infection caused by mycoplasma (McGarrity *et al*, 1985; Hay, 1986), walless procaryotes which are difficult to detect and even more difficult to eradicate, often leads to erroneous results or causes the loss of unique cell lines. Many reports describe deleterious consequences of mycoplasma infection. 1) False negatives with HIV isolation (Vasudevachari, *et al*, 1990), 2) increased invasive behaviour of tumour cells (Schmidhauser *et al*, 1990). 3) New immunosuppressive properties thought to be produced by thymus derived T cells (Teh *et al*, 1988). Indirect methods of detection are much more efficient than direct methods, since the organisms are fastidious in nature, and often difficult to cultivate.

A variety of techniques have been developed to detect and to isolate mycoplasma from infected cell cultures. These techniques include microbiological, microscopic, radioisotopic, and enzymatic procedures. Some of the procedures that have been

developed and used are microbiological culture, DNA fluorescence staining, immunofluorescence, uridine phosphorylase, uridine-uracil ratio, electron microscopy, autoradiography, and RNA speciation (Rodwell and Whitcomb, 1983). Until 1973 it was believed that careful, quality controlled microbiological assays with aerobic and anaerobic incubation would isolate and detect all cell culture mycoplasma. In that year Hopps and colleagues (Hopps *et al.*, 1973) reported on a strain of *Mycoplasma hyorhinis* that would not propagate in cell-free media. It is now apparent that a significant percentage of *M. hyorhinis* do not grow well or at all on cell free media. There are over 100 different species of mycoplasma, but many cannot be grown in cell free media. Because of this, indirect methods must be incorporated into the screening procedures. One of these methods is a biochemical assay, which can be incorporated into the experimental design. The fact that cell cultures infected with mycoplasma exhibit uridine phosphorylase activity (i.e. uridine + PO_4^{-3} $\leftrightarrow$ uracil + ribose phosphate) (Levine, 1972; Becker and Levine, 1976; Levine and Becker, 1978) makes simultaneous screening possible within the bounds of the current experimental design. In all the experiments, the level of uridine incorporation was very low in the mock infected cultures, suggesting that the level of mycoplasma infection is significantly below the level of sensitivity of the test. However, there have been several reports (Levine, 1972; Levine and Becker, 1978) of "cryptic" mycoplasma variants that do not incorporate exogenous tracer nucleosides. It therefore can be said that both the sensitivity, and the specificity of the tests available may lead to the erroneous conclusion that a cell culture is "mycoplasma free".

Two of the most common contaminants of tissue culture are *Acholiplasma laidlawii* and *Mycoplasma hominis*. An unusual and perhaps characteristic feature of mycoplasma gene expression in *E. coli* was revealed in a study that identified mycoplasmal translation products expressed in *E. coli* from a cloned *M. hyorhinis* gene (Notarnicola *et al*, 1990). Products were synthesized that could only have been generated by promiscuous translation initiation at multiple sites on a single, mycoplasma-encoded message. This finding contrasted with a commonly invoked alternative mechanism, whereby truncated proteins result from TGA codon usage differences. Several mycoplasma species use TGA to encode tryptophan rather than a translational stop (Notarnicola *et al*, 1991). These observations suggest that mycoplasma promoters are easily recognised in *E. coli*.

Southern blots were done with *C. trachomatis* L2 and *C. psittaci* C10, along with genomic DNA of each of *A. laidlawii*, and *M. hominis*. Each were probed with the thymidine kinase (Figure 23,25) and the $tRNA^{GLY}$ (Figure 22,24) genes. Despite the sensitivity of the protocol, hybridization to chlamydia alone was observed on all the blots. This suggests that neither of the organisms are involved in the contamination of the stocks.

The only viable method of cultivating Chlamydia is to grow them within a host cell. No cell free system has been developed. This leaves the researcher vulnerable to

contamination of their chlamydial stocks with foreign organisms. Careful technique, coupled with sonication which disrupts the cell wall of the mycoplasma, has protected the researcher from gross contamination. However, techniques as powerful as PCR and complementation, which can detect one copy of the gene of interest, do not differentiate between the thymidine kinase gene of *Chlamydia trachomatis* and that of *Mycoplasma spp.*

Therefore, methods for recognizing genes from foreign organisms must be developed. The structure, DNA homology, and G-C ratios all provide vital information as to the origin of the gene. When the tRNA gene was sent to GeneBank, homology to *M. capricolum* was detected (Figure 26), though the thymidine kinase gene is not upstream of the $tRNA^{GLY}$ in *M. capricolum*. This suggests that the gene may be from a species of mycoplasma, though no hybridization was observed with $tRNA^{GLY}$ alone. Tan *et al*, cloned the RNA polymerase α-subunit operon from *Chlamydia trachomatis* (Tan *et al*, 1993). The gene was later proven to be mycoplasma in origin (Tan *et al*, 1995). The cloned genes showed a high A/T bias in the third position of codons, characteristic of genes from *Mycoplasma* species. Since this is also the case in the thymine kinase gene we have cloned, it is an undeniable possibility that the source is species of mycoplasma. However, it has also been shown that all strains of *C. trachomatis* that have been examined possess homologous plasmids (Hatt *et al*, 1988). Some strains of *C. psittaci* also have a plasmid of similar size, that shares some homology to the *C. trachomatis*

Figure 26: $tRNA^{GLY}$ gene of *Chlamydia psittaci* vs *Mycoplasma capricolum*

```
          680         690         700         710         720         730
CPTK  TATTTTTTTCTGTTATAATAAATTAATCTGCAAGTGTAGTTTAATGGCAG-ACTTCAGC
MCTR                                          CAGGTGTAGTTTAATGGTAGAACTTCAGC
                                                        10          20          30

            740         750         760         770
CPTK  CTTCCAAGCTGATTGTGAGGG-TCGATT-CCTTCACTTGCTCC
MCTR  CTTCCAAGCTGATTGTGAGGGTTCGATTCCCTTCACCTGCTCCA
               40          50          60          70
```

Mycoplasma capricolum transfer RNA-Gly (UCC) 91.8% identity in 73 nt overlap

plasmid (Joseph *et al,* 1986). The plasmid was found to have a GC content of 36.3% compared to the genomic composition of 42.2% GC for *C. trachomatis.* This example of varying GC ratios in Chlamydia serves as a possible explanation for the GC ratio of 30% seen in the thymidine kinase gene.

There are three possible origins for the thymidine kinase gene cloned. Mycoplasma, as suggested by the GC ratio, and the tRNA homology. An unknown bacterial contaminant, which would explain why the source of the gene cannot be confirmed. The most compelling evidence is the hybridization results (Table 7) which suggest that Chlamydia is the source.

Table 7: Summary of hybridization results

Target DNA	Probe Used[a]		
	Thymidine Kinase-$tRNA^{GLY}$	Thymidine Kinase	$tRNA^{GLY}$
TKpUC19 E(2) se. time	Positive	Positive	Positive
pUC19	Negative	Positive	Negative
Clone 1	Positive	Positive	Negative
Clone 2	Positive	Positive	Negative
Clone 3	Positive	Positive	Negative
Clone 4b	Positive	Negative	Negative
Clone 5	Negative	Positive	Negative
Clone 6b	Positive	Positive	Negative
CT47	Positive	Positive	Positive
L2 genomic DNA	N.A.	Positive	Positive
C10 genomic DNA	N.A.	Positive	Positive
A. laidlawii	N.A.	Negative	Negative
M. hominis	N.A.	Negative	Negative

a. Sequence of the probes was verified by dideoxy sequencing (data not shown).

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