Mycoplasma Pyrimidine Deoxynucleotide Biosynthesis

Molecular Characterization of a New Family Flavin-Dependent Thymidylate Synthase

RAHMA WEHELIE
Faculty of Veterinary Medicine and Animal science
Department Molecular Biosciences
Uppsala

Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006

Acta Universitatis Agriculturae Sueciae
2006:76

ISSN 1652-6880
ISBN 91-576-7125-7

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Wehelie, R., 2006. Mycoplasma Pyrimidine Deoxynucleotide Biosynthesis: Molecular Characterization of a New Family Flavin-Dependent Thymidylate Synthase. Doctor's dissertation.
ISSN: 1652-6880, ISBN: 91-576-7125-7

Mycoplasmas (class *mollicutes*) are the smallest self-replicating bacteria. They are obligate parasites in a wide range of organisms, including humans, plants, and animals. This thesis describes studies related to *Mycoplasma mycoides* subsp. *mycoides* Small Colony (MmmSC), the etiological agent of contagious bovine pleuropneumoniae, *Mycoplasma capricolum* (Mcap), causing caprine pneumonia, *Mycoplasma hyopneumoniae* (Mhp), the etiological agent of porcine enzootic pneumonia, *Ureaplasma urealyticum* (Uu), a commensal of human urogenital tract causing nongonococcal urethritis, *Mycoplasma genitalium* (Mg), a causative agent of nongonococcal, chlamydia-negative urethritis, and *Mycoplasma pneumoniae* (Mpn) causing atypical pneumonia and tracheobronchitis.

The first part concerns deoxynucleoside metabolism in *Uu* and growth inhibition of *Uu* and *Mpn* by nucleoside analogs. The uptake and metabolism of the deoxynucleosides, thymidine (dThd), deoxyuridine, deoxycytidine were studied and all these deoxynucleosides were readily taken up by *Uu* and dThd showed the highest rate of uptake. Pyrimidine nucleosides e.g. fluoropyrimidines gave complete inhibition of Uu growth. The addition of dUrd and dThd to the growth medium abolished inhibitory effect. These results suggested a new pathway of thymidylate synthesis in Uu.

The second part deals with identification of a new family of flavin-dependent thymidylate synthases (FDTS) in mycoplasmas. Thymidylate synthases (TS) are essential enzymes in the *de novo* synthesis of thymidylate and are coded either by *thyA* or *thyX* genes. Analysis of the fully sequenced Mycoplasma genomes revealed that there were no TS genes annotated in the genomes of Uu, MmmSC, Mhp, and Mcap. Still, TS activities were detected in Uu and MmmSC extracts. By using a bioinformatics approach together with biochemical methods putative genes for (FDTS) were identified in Uu, MmmSC, Mhp and Mcap genomes and functional verified. New members of this family were also discovered in Mg and Mpn, which also have the *thyA* genes.

These mycoplasma FDTS have similar catalytic activity to the ThyX proteins, but share very little sequence homology to the known bacterial ThyX. The sequence homologies within Mycoplasma FDTS were also very low. Since Mycoplasma FDTS share no sequence or structural homology to ThyA or ThyX this makes them promising targets for future design of new antibiotics.

Keywords: mycoplasmas, ureaplasma, thymidylate synthase, flavin-dependent thymidylate synthase, nucleoside analogs, nucleoside metabolism.

Author's address: Rahma Wehelie, Department of Molecular Biosciences, SLU, BMC box 575, S-751 23, Uppsala, Sweden

To my loving family Rob, Dalmar and Ida-Mona

Contents

Appendix

Paper I-IV

This thesis is based on the following paper, which will be referred to in the text by their Roman numerals.

I. Carnrot, C.*, Wehelie, R.*, Eriksson, S., Bölske, G., and Wang, L. (2003) Molecular characterization of thymidine kinase from *Ureaplasma urealyticum*: nucleoside analogues as potent inhibitors of mycoplasma growth. *Mol Microbiol.* 50, 771-780. (*These authors contribute equally).

II. Rahma Wehelie, Staffan Eriksson and Liya Wang (2004). Effect of Fluoropyrimidines on the Growth of Ureaplasma urealyticum. *Nucleosides, Nucleotides & Nucleic Acids.* 2,1499-1502.

III. Rahma Wehelie, Staffan Eriksson, Göte Swedberg, Göran Bölske and Liya Wang (2006). Thymidylate synthases of *Mycoplasma mycoides* subsp. *mycoides* SC and *Ureaplasma urealyticum* are flavin-dependent (manuscript).

IV. Rahma Wehelie, Staffan Eriksson, Göte Swedberg, Göran Bölske and Liya Wang (2006). A new family of flavin-dependent thymidylate in pathogenic mycoplasmas. (manuscript).

Abbreviations

Enzymes

dAK	deoxyadenosine kinase
dCK	deoxycytidine kinase
dGK	deoxyguanosine kinase
dNMPK	deoxyribonucleoside monphosphate kinase
FDTS	flavin-dependent thymidylate synthase
DHFR	dihydrofolade reductase
NDPK	nucleoside diphosphate kinase
PRT	phosphoribosyltransferases
RR	ribonucleotide reductase
TK	thymidine kinase
TK1	cytosolic thymidine kinase
TK2	mitochondrial thymidine kinase
TS	Thymidylate synthase
ThyX	flavin-dependent thymidylate synthase

Bases, nucleosides and nucleotides

A, Ado, dAdo	adenine, adenosine, and 2′-deoxyadenosine
C, Cyd, dCyd	cytosine, cytidine, and 2′-deoxycytidine
G, Guo, dGuo	guanine, guanosine, and 2′-deoxyguanosine
T, dThd	thymine, and 2′-deoxythymidine
dTTP	2’-deoxythymidine triphosphate
U, Urd, dUrd	uracil, uridine, and 2′-deoxyuridine
PRPP	5-Phosphoribosyl-1-pyrophosphate

Nucleoside analogs

AZT	3′-azido-2′,3′-dideoxythymidine
5-BrdUrd	5-bromo-2′-deoxyuridine
5-FdCyd	5-fluoro-2′-deoxycytidine
5-IdUrd	5-Iodo-2′-deoxyuridine
5-FdUrd	5-fluoro-2′-deoxyuridine
5-FdUMP	5-fluoro-2′-deoxyuridine monophospate
dUMP	2′-deoxyuridine monophospate
5-FUra	5-fluorouracil
5-BrUra	5-bromouracil
MTHF	5,10-methylenetetrahydrofolate
FAD	flavin adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide

General introduction

History of mycoplasmas

The family Mycoplasmataceae, which belongs to the order Mycoplasmatales under the class Mollicutes, contains the genera Mycoplasma and Ureaplasma. Mollicutes, which possess notably small genomes, may have evolved from the Clostridia-bacilli branch of the phylum *Firmicutes* about million years ago by losing considerable regions of the genome (Dandekar *et al*., 2002) (Fig. 1). This group of organisms was considered for a long time to belong to the viruses because they passed through filters, which normally block the passage of bacteria. First recognized from a case of pleuropneumonia in cows, the organism was designed "pleuropneumonia-like organism," or PPLO. This term is still used today, particularly for certain mycoplasma media (Eaton *et al*., 1944; Eaton & Low, 1967).

Mycoplasmas, the smallest self-replicating organism, are highly pleomorphic because they lack a cell wall, which also renders them completely resistant to β-lactam and other cell wall antibiotics (Razin, 1992a).

Mycoplasmas have limited biosynthetic capabilities and are ubiquitous parasites of human, animals and plants, etc. and host specific (Razin *et al.,* 1978; Maniloff *et al*.,1992). They are frequently implicated in respiratory and urogenital tract infections in a variety of mammalian and avian species (Razin, 1992b; Tryon & Baseman, 1992; Adams *et al*., 2000; Baseman *et al*., 1997).

In contrast to other pathogenic bacteria where virulence is mostly determined by toxins, invasins, and cytolysins, pathogenic mycoplasma species appear to have no such primary virulence factors, as revealed by the genomic sequence analysis of eight species that have been completely sequenced (Fraser *et al*., 1995; Himmelreich *et al*., 1996; Glass *et al*., 2000; Chambaud *et al*., 2001; Check *et al*., 2002; Sasaki *et al*., 2002; Papazisiet *et al*., 2003; Jaffe *et al*., 2004; Minion *et al.,* 2004; Wang *et al*., 2004).

Growth requirements, genome size and codon usage

Mycoplasmas are facultative anaerobes, although the growth of some species is improved by aeration. Most species require cholesterol and fatty acids for their growth, as well as for their morphological and osmotic stability. Nutritional requirements are met usually by using a complex medium containing serum and yeast extract. The optimum growth conditions are at temperatures of 36-37°C supplied with 5% CO_2, and at a pH ~ 7 (Moller, 1979; Himmelreich *et al*., 1996; Razin *et al.,* 1998).

Mollicutes possess a single circular chromosome of double stranded DNA and have a G+C content of 24 -33 mol% which is lower than other Gram-positive prokaryotes. The G+C content distribution along the genome is uneven (Altschul *et al*., 1997; Fraser & Fleischmann, 1997; Razin *et al*., 1998). Their genome sizes range from 580 kb of *Mycoplasma genitalium* to 1,358 kb of *Mycoplasma penetrans* (Fraser *et al*., 1995; Sasaki *et al*., 2002).

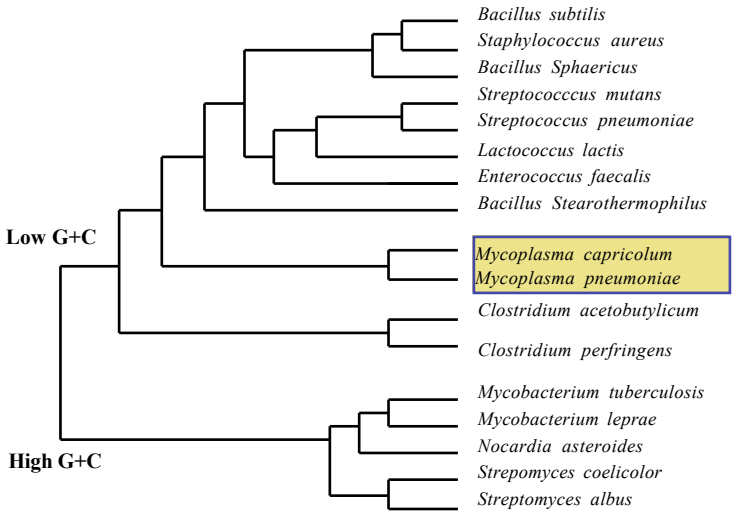

Figure 1. Phylogenetic relationships among Gram-positive bacteria deduced from 16S rRNA.

The genome sizes are variable, not only within the same genus but even among strains of the same species. One of the reasons for this variability is the frequent occurrence of repetitive elements, consisting of segments of protein coding genes, differing in size and number of insertion sequences (Citti *et al*., 1997; Razin *et al*., 1998; Dandekar *et al*., 2002; Mrazek, 2006). Based on the analysis of the 16S rRNA sequences, Mycoplasmas have been classified into different groups (Fig. 2).

Mycoplasmas utilize the codon UGA to encode Trp (Dybvig, 1990), and in *E.coli* this codon is recognized as a stop signal, which will result in truncation of mycoplasma proteins expressed from cloned genes. By introducing the opal suppressor allele trpT176 the tRNA produced from this allele is charged with Trp; therefore, it has been possible to express Mycoplasma genes containing UGA codons in *E.coli* (Minion *et al*., 1995). This fact has been an important methodological tool used in this thesis work.

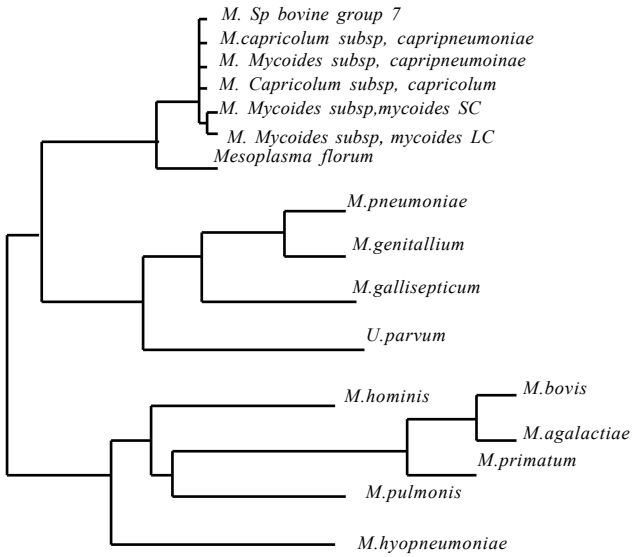

Figure 2. Phylogenetic tree of Mycoplasmas based on the 16S rRNA genes.

Energy metabolism

Mollicutes are divided into two groups, based on their ability to metabolize carbohydrates: fermentative and non-fermentative organisms. Members of the fermentative group produce acids from carbohydrates, decreasing the pH of the medium. Most mollicutes have the ability to use glucose and arginine for ATP synthesis. During their evolution, Mycoplasmas appear to have lost all of the genes involved in amino acid and cofactor biosynthesis, as well as synthesis of the cell wall and lipid metabolism, resulting in a requirement for the full spectrum of substrates and cofactors taken up from the host or from a complex artificial culture medium. (Miles *et al.*, 1991; Himmelreich *et al*., 1996; Razin *et al.*, 1998). The majority of Mycoplasmas is deficient in genes coding for components of intermediary and energy metabolism and thus are dependent mostly on glycolysis as an ATP-generating pathway (Frey, 2002).

The energy metabolism of *Ureaplasmas* presents a special case. *Ureaplasmas*, unique among the Mollicutes, possess a very potent urease, which hydrolyses urea. The resulting intracellular accumulation of ammonia/ammonium ion is coupled to ATP synthesis through a chemiosmotic type of mechanism (Blanchard *et al.*,1988; Neyrolles *et al.*, 1996; Razin *et al*., 1998).

Nucleotide methabolism

Nucleotides have a central role in the physiology of all organisms as building blocks of nucleic acids, storage of chemical energy, carriers of activated metabolites for biosynthesis, structural moieties of coenzymes, and metabolic regulators.

Two fundamentally different pathways are used for the synthesis of nucleotides. One is a *de novo* pathway, in which ribose phosphate, certain amino acids, CO_2, and NH_3 are combined in successive reactions to form the nucleotides. In addition to the *de novo* pathway, cells also have various mechanisms for making use of the free bases and nucleosides released from the breakdown of nucleic acids by converting them back to nucleotides, which is the salvage pathway (Stryer *et al*., 2001) (Fig. 3).

Synthesis of deoxyribonucleotides

Deoxyribonucleotides are building blocks used for DNA synthesis and repair. Deoxynucleotides are produced from ribonucleotides, in the *de novo* pathway, by ribonucleotide reductase (RNR), which reduces ribonucleoside diphosphates to deoxyribonucleoside diphosphates (Brown & Reichard, 1969; Thelander & Reichard, 1979). RNR has been identified in all Mycoplasmas except for Ureaplasmas (Glass *et al.,* 2000) (Fig. 3).

Deoxynucleotides can also be produced in the salvage pathway; deoxyribonucleosides (dN), derived from nutrients and degraded DNA, are taken up and phosphorylated to deoxyribonucleoside monophosphate (dNMP) by deoxyribonucleoside kinases, which are the key enzymes in the salvage of dN (Eriksson *et al*., 1991). Subsequently dNMPs are phosphorylated into diphosphates and triphosphates, which are the precursors of DNA. In mammals there are four deoxyribonucleoside kinases with overlapping specificities (Arner & Eriksson, 1995).

Mycoplasmas have two types of deoxynucleoside kinases, i.e. thymidine kinase (TK) and deoxyadenosine kinase (dAK). TK catalyses the phosphorylation of thymidine and deoxyuridine using a nucleoside triphosphate as phosphate donor. It can be found in most living cells. It is present in two forms in mammalian cells, TK1 and TK2. Certain viruses also have genetic information for expression of viral thymidine kinases. TKs are conserved within Mycoplasmas with sequence identities ~ 50%. Mycoplasma TKs show ~ 25-30 % sequence identities to human TK1 and, thus, belong to the TK1 enzyme family (Carnrot *et al*., 2003). Transposon mutagenesis studies showed that TK is an essential gene and required for the viability of *M.genitalium* (Wasinger *et al*., 2000; Glass *et al*., 2006).

dAK is also present in all mycoplasma species sequenced to date. dAK of M. mycoides has been shown to phosphorylate dAdo, dGuo and dCyd with dAdo as the most efficient substrate (Wang *et al*., 2001). The dAK of *M. mycoides* has 25% sequence homology to the dGK of *U.urealyticum* and *Mycoplasma pneumoniae* and ~25-29 % sequence homology to other bacterial dAK/dCK. Therefore, they belong to the family of mammalian dCK and Herpes TK (Wang *et al*., 2001).

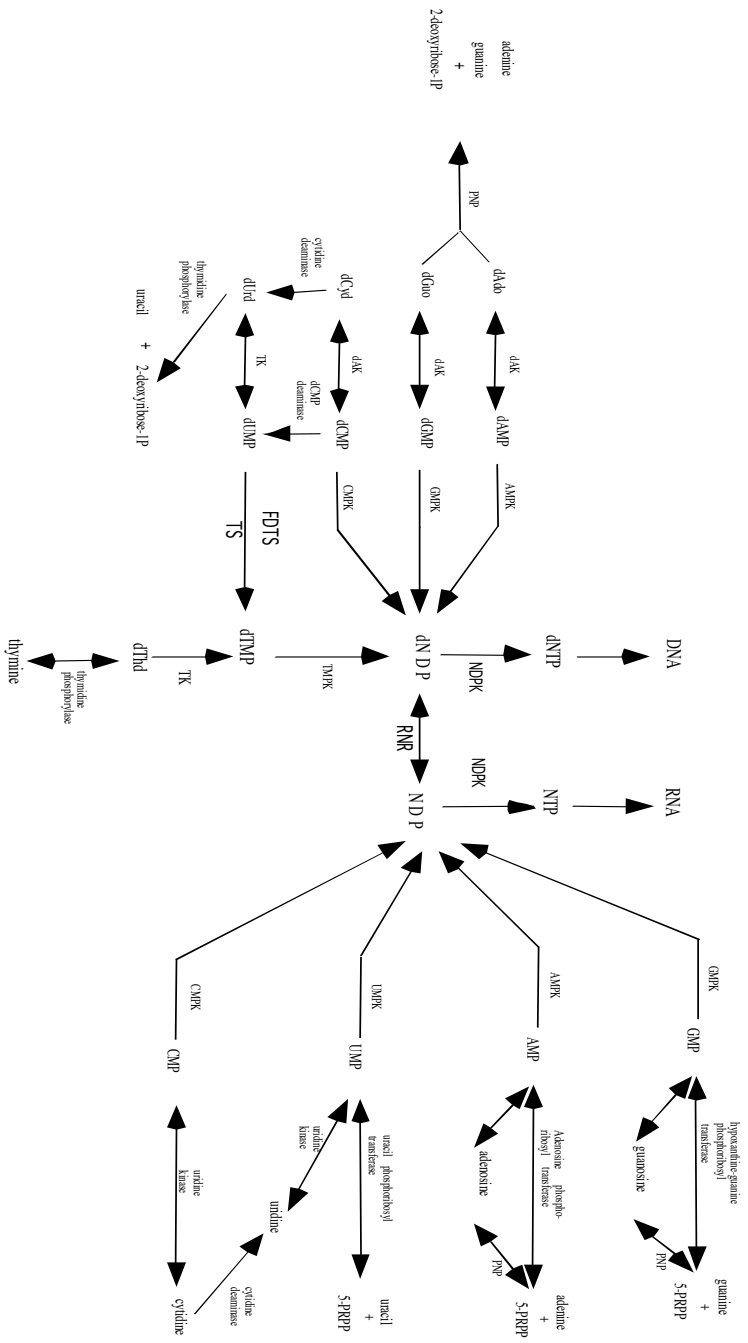

Figure 3. Pathways of nucleotide biosynthesis in Ureaplasma and Mycoplasma. RNR, ribonucleotide reductase; NDPK, nucleoside diphosphate kinase; the newly discovered enzyme - FDTS.

All mycoplasmas lack the orotic pathway for pyrimidine synthesis as well as the enzymatic pathway for *de novo* synthesis of purine bases; therefore, uracil, thymine, and guanine are required for growth. Because of the lack of *de novo* synthesis purine and pyrimidine bases Mycoplasmas synthesize nucleotides by salvage pathways from more complex precursors. A range of salvage enzymatic activities including thymidine phosphorylase and thymidine kinase, have been detected in several Mycoplasma species (Mitchell & Finch, 1977; Mitchell & Finch, 1979; Neale, *et al*., 1984; Pollack, 1995; Wang *et al*., 2001; Carnrot *et al*., 2003) (Fig. 3). Genes coding these salvage enzymes have been identified in all Mycoplasma genomes sequenced.

The synthesis of mononucleotides in mycoplasmas proceeds from nucleobases either with phophoribosylpyrophosphate (PRPP) in a one-step, an essentially irreversible reaction, mediated by phosphoribosyltransferase or in a two-step reversible reaction via the formation of nucleoside (Mitchell *et al.*, 1978; Neale, *et al*., 1983; Pollack & Williams, 1986; Wang *et al*., 2001).

Thymidylate synthase (TS)

Uracil, produced by the pyrimidine synthesis pathway, is not a component of DNA. Rather, DNA contains thymidine, a methylated analog of uracil. Other steps are required to generate thymidylate from uracil. Thymidylate synthase catalyzes the methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to thymidine-5'-monophosphate (dTMP), which is then phosphorylated to thymidine-5'-diphosphate (dTDP) and thymidine-5'-triphosphate (dTTP) and finally incorporated into DNA (Fig. 4). This is a key step in DNA precursor biosynthesis. TS is present in almost all living organisms e.g. bacteria, DNA viruses, protozoa, and animals and is an evolutionary conserved enzymatic step. It has long been used as a key target for antibiotic and chemotherapeutic drugs (e.g. 5-fluorouracil) (Harrap *et al*., 1989; Costi, *et al*., 2005).

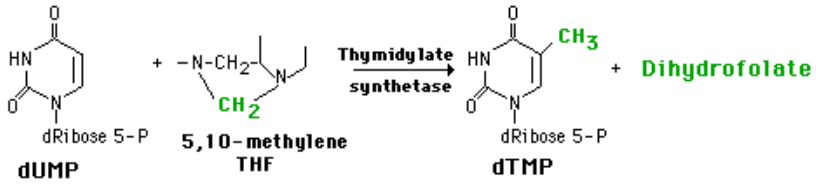

Figure 4. The conversion from dUMP to dTMP by TS.

Classical thymidylate synthase (TS) - ThyA

The first known TS or ThyA is coded by the *thyA* genes. ThyA uses methylene-5,6,7,8-tetrahydrofolate (CH_2H_4folate) as both a carbon (methylene) donor and reductant (hydride) resulting in the formation of dihydrofolate (H_2folate) and dTMP (Fig. 5). This reaction provides the thymidylate necessary for DNA synthesis. Tetrahydrofolate (H4folate), required for various biological functions, is

therefore rapidly regenerated by dihydrofolate reductase (DHFR), which catalyzes the reduction of H_2folate by use of NADPH (Cerreras *et al*., 1992; Shamira *et al*., 1999; Johnson *et al*., 2002).

ThyA can influence the 2'-deoxyadenosine-5'-triphosphate/thymidine triphosphate ratios inside the cell, thus indirectly modifying the incorporation in DNA of the component bases (Berger *et al*., 1984).

Flavin-dependent thymidylate synthase (FDTS) - ThyX

Recent genomic studies revealed that a large number of organisms lack the genes for TS and DHFR, and have an alternative enzyme for the conversion of dUMP to dTMP. This new enzyme is denoted as flavin-dependent thymidylate synthase (FDTS) or ThyX and is encoded by the *thyX* gene (Myllykallio *et al*., 2002).

ThyX catalyzes the conversion of dUMP to dTMP, and the activity depends on NADPH oxidation and CH_2H_4folate is converted to H_4folate (Fig. 5). ThyX activity requires flavin adenine dinucleotide (FAD) which is often tightly bound to the enzyme. (Myllykallio *et al*., 2002; Giladi *et al*., 2002; Graziani *et al*., 2004; Leduc *et al*., 2004; Liu *et al*., 2004; Gattis *et al*., 2005; Griffin *et al*., 2005).

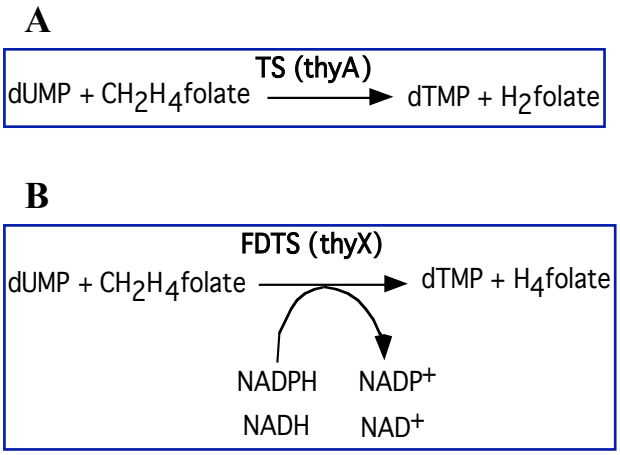

Figure 5. The reaction mechanisms of thymidylate synthase (A) ThyA and (B) ThyX.

ThyX sequences contain a distinctive ThyX motif i.e. (-RHRX$_7$S-), identified through BLAST searches of the GenBank database. Although the vast majority of ThyX proteins are composed of approximately 200-250 amino acid, the ThyX

homologues of *Thermaplasma* and *Chlymadia* species are much longer and contain two fused ThyX domains in the same polypeptide and share very little homology to other ThyX sequences (Mathews *et al*., 2003; Sampathkumar *et al*., 2005; Griffin *et al*., 2005).

The difference of the target site that can be defined most clearly is the covalent inactivation of ThyA by FdUMP. This fluorinated deoxyuridylate analog is formed via the reduction of FUDP by ribonucleotide reductase and dephosphorylation. Alternatively, it can be formed directly from 5-FdUrd by thymidine kinase when this 5-FUrd is regionally infused. However, the inhibition of 5-FdUMP to ThyX or FDTS enzymes is not via covalent complex between 5-FdUMP to ThyX or FDTS enzymes, rather competitive inhibition (Costi *et al.,* 2005).

ThyX proteins contain a flavin group as demonstrated spectroscopically in this thesis as well as by Myllkallio et al. (2002). Flavoproteins are highly reactive with free oxygen (Massey, 2000) and their biochemical reaction is normally studied under anaerobic conditions. The oxygen sensitivity observed in *E.coli ΔThyA* that expressing *thyX* might therefore be attributed to oxygenation of the flavin group by molecular oxygen (Giladi *et al*., 2002).

Many of the FDTS containing organisms are pathogenic or parasitic. ThyA and ThyX share no sequence or structural homology (Fig. 6) and this makes FDTS a promising target for drug design. Information regarding the FDTS reaction mechanisms, 3-D structures and the differences between the classical TS (e.g., human TS) and bacterial ThyX will facilitate future antibiotic development.

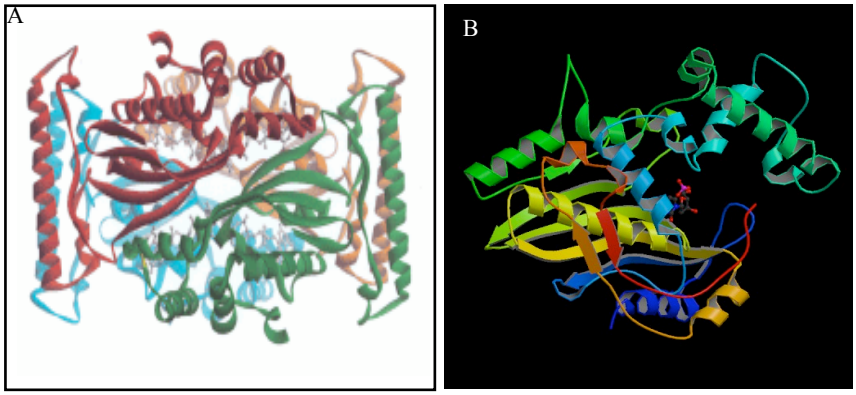

Figure 6. Crystal structure of *Thermotoga maritima* ThyX (A) and *E.coli* ThyA (B).

TS inhibitors

Antimetabolites such as methotexate and fluoropyrimidines are clinically important group of cancer drugs used in the treatment of a variety of solid tumors and hematologic malignancies. The cytotoxicities of the antimetabolites stem from their ability to interfere with key enzymatic steps in nucleic acid metabolism. The discussion below concerns two types of well-studied compounds, the antifolates and the fluoropyrimidines.

Methotrexate (MTX) displays significant tumoricidal activity against a variety of human neoplasms, including acute leukemia, osteogenic sarcoma, choriocarcinoma, breast cancer, head and neck cancers, and others (Bender, 1979; Peters *et al*., 1993). Following uptake in animal cells by the folate transport systems, MTX can bind to and inhibit dihydrofolate reductase (DHFR), which results in depletion of the reduced folate pools essential for thymidylate synthesis. The cytotoxicity of MTX is influenced by intracellular polyglutamation and these polyglutamyl derivatives can also inhibit thymidylate synthase directly (Campbell *et al*., 1991) (Fig. 7).

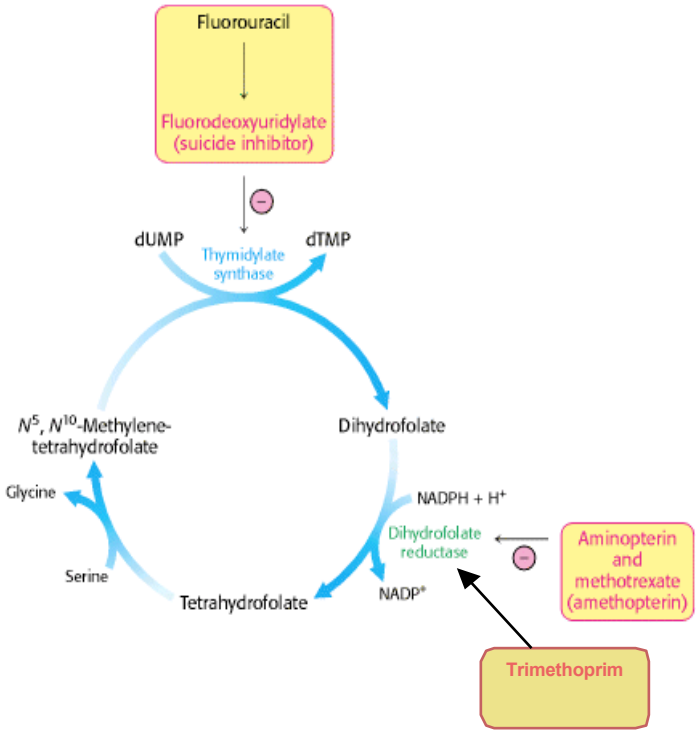

Figure 7. Inhibition of thymidylate synthase (TS) by 5-FdUMP and dihyrofolate reductase by aminopterin, methotrexate, and trimethoprim. (modified from Stryer *et al.,* 2001).

Trimethoprim, a folate analog, has potent antibacterial and antiprotozoal activity because Trimethoprim binds specifically to DHFRs of microorganisms and 10^5-fold less tightly to mammalian DHFRs. The combination of trimethoprim and sulfamethoxazole (an inhibitor of folate synthesis) is presently widely used to treat bacterial infections (Fig. 7).

Fluoropyrimidines e.g. 5-fluorouracil (5-FU) have been used extensively in the treatment of gastrointestinal carcinomas since their introduction in the late 1950s. Mechanisms of action are including inhibition of TS by 5-fluoro-2'-deoxyuridine 5'-monophosphate (5-FdUMP), incorporation of the fluorouracil base into RNA with resultant inhibition of processing and function, and incorporation into DNA (Fig. 7). Additionally, the incorporation of 5-fluorouridine triphosphate into RNA correlated with cytotoxicity in some systems (Houghton *et al*., 1986). Resistance to 5-FU is often caused by alterations in enzymes involved in fluoropyrimidine metabolism, particularly those enzymes associated with the conversion of 5-FU to FdUMP. Furthermore, changes in TS level or its affinity for 5-FdUMP are associated with 5-FU resistance (Au *et al*., 1982; Keyomars & Moran, 1988; Mini *et al*., 1990) (Fig. 7).

Clinical manifestations of Mycoplasma diseases: general information on Mycoplasma species studied in this thesis

Mycoplasma mycoides subsp. *mycoides* Small Colony *(MmmSC)*

MmmSC is the etiological agent of contagious bovine pleuropneumoniae (CBPP), a severe infectious disease causing major losses of livestock (Cheng *et al*., 1995). This organism has been used as a model to investigate the molecular basis of mycoplasmal virulence. MmmSC is an extracellular pathogen with a genome size of 1,211kb and lives in close association with host cells (Westberg *et al*., 2004). The rationale for the use of this species as a model is high virulence as well as the fact that it is clearly established as the etiological agent of CBPP (Vilei *et al*., 2001). Furthermore, this severe cattle disease is of extraordinary socioeconomic importance to livestock production in countries that currently suffer CBPP outbreaks. Countries that are free from MmmSC are continuously threatened by reemerging infections. Recently, it has been suggested that MmmSC might release toxic intermediates or side products as virulence determinants (Pilo *et al*., 2003).

Ureaplasma urealyticum (Uu)

Uu has been implicated in infertility, spontaneous abortion, stillbirth, premature birth, low birth weight, and perinatal morbidity and mortality (Yoon *et al*., 1989). Vaginal colonization of Uu has not been associated with preterm birth (Cassell *at el*., 1993; Glass *et al*., 2000), while the presence of Uu in the amniotic fluid is associated with a robust host response in fetal, amniotic, and maternal compartments and subsequent preterm birth (Domingues *et al*., 2003; Kim *et al*., 2003; Deguchi *et al*., 2004). It is not known why this microorganism invades the

amniotic cavity only in some women, despite heavy colonization of the vagina (Gerber *et al*., 2002; Povlsen *et al*., 2002). Recently, the species previously classified as *Ureaplasma urealyticum* was separated into two new species: *U. parvum* (previously *U. urealyticum biovar* 1) and *U. urealyticum* (previously *U. urealyticum biovar* 2) (Kong *et al*., 2000; Martinez *et al*., 2001). *Ureaplasma urealyticum* is present in the lower genital tract of 40-80% of pregnant women and is strongly associated with chorioamnionitis during pregnancy (Hillier *et al*., 1991). The rate of vertical transmission from the mother to full term infants and preterm infants ranges from 18-55% and 29-55% respectively (Harrison *et al*., 1983; Sperling *et al*., 1988; Lockwood & Kuczynski, 1999). Respiratory tract colonization by *Ureaplasma urealyticum* is associated with increased incidences of pneumonia (Shapiro *et al*., 1980; Heggie *et al*., 2001).

Mycoplasma capricolum (Mcap)

This bacterium is primarily a pathogen of goats, causing caprine pneumonia, though it has also been found in sheep and cows. In goats, M. capricolum is highly destructive, causing significant mortality and morbidity. The primary clinical manifestation is severe arthritis (polyarthritis). The infection progresses in the form of septicemia, with severe lesions in the joints leading to permanent lameness. In young goats it also causes fever (Wesonga *et al*., 2004).

Mycoplasma hyopneumoniae (Mhp)

Mhp is the etiological agent of porcine enzootic pneumonia, a chronic respiratory disease that causes significant economic losses to the swine industry (Minion *et al,* 2004). Infections are established via colonization of porcine respiratory epithelia, a process initiated by the adherence of bacterial cells to host cilia (Chen *et al*., 2003). Successful colonization results in ciliostasis and shedding of cilia from the epithelial surface, thereby disrupting the mucociliary escalator and leaving the host susceptible to secondary infections (Haesebrouck *et al*., 2004; Minion *et al*., 2004). In spite of widespread vaccination with the currently marketed vaccines (bacterins) and/or antibiotic therapy, pigs are not completely protected from either *M. hyopneumoniae* infection or secondary infection (Thacker *et al*., 2000; Thanawongnuwech *et al*., 2001; Menon *et al*., 2002; Haesebrouck *et al*., 2004).

Mycoplasma genitalium (Mg)

Mg is an important, emerging sexually transmitted bacterial pathogen capable of eliciting a wide range of symptomatologies. In men *M. genitalium* has been identified as a causative agent of nongonococcal, chlamydia-negative urethritis (Colman *et al*., 1990; Fraser *et al*., 1995; Svenstrup, *et al*., 2003). In women with urogenital symptoms, *M. genitalium* was detected in the cervix and vagina. Recently, *M. genitalium* has been strongly associated with cervicitis, endometritis, salpingitis, pelvic inflammatory disease, and tubal factor infertility or serological criteria (Manhart *et al*., 2003; Yoshida *et al*., 2003). Furthermore, *M. genitalium* was detected in rectal, respiratory tract, and synovial fluid specimens and has been

linked to a range of pathologies, such as arthritis, pneumonia, AIDS progression, chronic fatigue, autoimmune disorders, and encephalitis (Horner *et al*., 1993).

Mycoplasma pneumoniae (Mpn)

This bacterium colonizes human respiratory tract epithelial cells to cause tracheobronchitis and atypical pneumonia, which is the more acute form of pneumoniae, which progresses quickly with severe early symptoms. Acute forms of respiratory diseases caused by *Mycoplasma pneumonia* have been described in all age groups (Himmelreich *et al*., 1996; Chabot *et al*., 1998). However, older children and young adults are at greatest risk of developing the infection (Harris *et al*., 1998; Waites & Talkington, 2004). Sometimes the disease develops symptoms outside of the lungs, such as anemia and rashes, as well as neurological syndromes (meningitis, myelitis, and encephalitis). Tetracyclines, macrolides, ketolides, and fluoroquinolones are active against *M. pneumoniae*. Most of these agents are primarily bacteriostatic for *M. pneumoniae*, and only the fluoroquinolones have been shown to be bactericidal.

Present investigation

Aim

The work described in this thesis is aimed to enhance our understanding of nucleotide metabolism in Mycoplsamas more specifically the synthesis of dTTP. Thereby, I hope to contribute by identifying novel genes in Mycoplasmas, which can be used as targets to develop new antibiotics against myoplasma infection. The main contribution of this thesis was the identification and primarily characterization of a new family of flavin dependent thymidylate synthase in Mycoplasmas.

Results and discussion

Paper I: Pyrimidine deoxynucleoside metabolism in U. urealyticum and growth inhibition of U.urealyticum and M. pneumoniae by nucleoside analogues.

Mycoplasmas lack the *de novo* synthesis of purines and pyrimidines and they totally rely on salvage pathway for DNA/RNA precursor synthesis. Using radiolabelled deoxynucleosides, i.e. thymidine (dThd), deoxyuridine (dUrd), and deoxycytidine (dCyd), we have studied the uptake and metabolism of these nucleosides. All these nucleosides are readily taken up by *U. urealyticum* and dThd had the highest rate of uptake than the other deoxynucleosides studied. The radioactivity from [^{3}H]-dThd was distributed as nucleosides, nucleotides and nucleic acids with the highest ~ 68% as thymidine monophosphate (dTMP) and ~ 20% incorporated into DNA. We could not detect any radiolabelled thymidine triphosphate (dTTP). The high level of dTMP detected in Uu supported the thymidine kinase (TK) mediated uptake and metabolism pathway.

The radioactivity from [^{3}H]-dCyd was recovered ~ 7% as dCyd, 42% as dUMP, 31% as dCMP, and ~ 2% as dCDP. There was ~ 18% of the radioactivity was incorporated into DNA. Again no dCTP or dTTP was detected. [^{3}H]-dUrd was recovered as dUMP and also high degree incorporation into DNA. This observation strongly suggested the presence of TS-like enzyme in Uu, which converted dUMP to dTMP despite the lack of a thyA or thyX homolog in the genome.

Some radioactivity originating from [^{3}H]-5-FdUrd or [^{3}H]-5-FUra was recovered as nucleotides, e.g. ~ 2% as 5-FdUMP or ~ 3% as 5-FUMP. No incorporation into DNA could be detected. The enzyme responsible for the phosphorylation of dThd and dUrd i.e. thymidine kinase of *U.urealyticum* was cloned and characterized in detail in this paper, but the main focus of this thesis is

thymidylate synthase of mycoplasmas, and therefore these results will not be described further.

Since clinically important nucleoside analogues used for this study have known function and inhibit specific enzymes, we wanted to examine their growth inhibitory effect with *U. urealyticum* and *M. pneumoniae*. *Uu* culture with an inoculum of 10^4 cfu/ml was killed by 0.8 µM 5-FdUrd, even after prolonged incubation time there was no detectable survival of the bacteria. When 5-FdCyd was added to *Uu* culture with inoculum 10^4 cfu/ml, similar results were obtained. Most of the 5-halogenated deoxyuridine and uracil analogs inhibited the *Uu* growth during the first 24 hrs, and the color changes of the culture media were observed after 72-hrs incubation, indicating survival of the bacteria. Other nucleoside analogs such as AZT and ACV were less potent inhibitors as compare to the fluoropyrimidine analogs.

The EC_{50} values for 5-FdUrd, 5-FdCyd and 5-FUra were determined with different Uu inoculum sizes. 5-FdUrd had the lowest EC_{50} values as compared to 5-FdCyd and 5-FUra with the same inoculum size. 5-FdUrd inhibited also the growth of Mpn, but was less potent as compared with that of Uu.

Paper II: Mechanism of Growth inhibition of Ureaplasma urealyticum with nucleoside analogs

Fluoropyrimidines are known to inhibit thymidylate synthase (TS) activity and thereby interfere with DNA synthesis. Metabolic labelling with radiolabelled dThd and dCyd showed that they were efficiently taken up by Uu cells and converted to the corresponding nucleotides. In addition dCyd could be deaminated leading to the formation of dUMP (Paper I). To understand the mechanism of inhibition by 5-FdUrd, we tested if addition of dUrd or dThd could protect Uu from the inhibitory effect of 5-FdUrd. Normally an inoculum of 10^5 cfu/ml of Uu was killed by 1.2 µM 5-FdUrd; however, when dUrd was added to the growth medium in a ratio of 1:1 (dUrd:5-FdUrd), the EC_{50} value was 3 µM. The inhibitory effect of 5-FdUrd was completely abolished by a 10-fold excess of dUrd. When dTHU, a potent inhibitor of cytidine deaminase, was added to the growth medium the inhibitory effect of 5-FdCyd was drastically reduced, indicating that it is the deamination product 5-FdUrd, which inhibited Uu growth.

Growth inhibition effect of 5-FdUrd and 5-FdCyd was reversed by addition of dUrd, dThd, and dTHU added to the growth medium, suggesting that the inhibitory of effect 5-FdUrd and 5-FdCyd occur via the pyrimidine salvage pathway, a competition between the natural dUMP and the analog 5-FdUMP.
It has been shown that mycoplasmas require pyrimidine bases for growth e.g. uracil. In the presence of dUrd, Uu culture showed about twice the growth rate as compared to the culture without dUrd. This observation suggested that Uu growth is limited by the availability of pyrimidines in the media.

Paper III: *Flavin dependent thymidylate synthase (FDTS)*

The studies in paper I, and II suggested the presence of TS-like enzymes in Uu. Therefore, the goal of this work was to identify the alternative yet unknown TS enzyme in Mycoplasma.

We cultured Uu and MmmSC in large scale and total proteins were extracted. A flavin dependent thymidylate synthase (FDTS) activity was detected in the protein extracts by using radiolabelled substrate and HPLC method to analyze the reaction products. FPLC and anion-exchange chromatography techniques were used to purify the Uu and MmmSC FDTS proteins. Both Uu and MmmSC FDTS proteins showed complete conversion of dUMP to dTMP using 5,10-methylenetetrahydrofolate (CH_2THF) as methyl donor, and requiring FAD and NADPH. We have used native gel electrophoresis to identify the presence of FDTS in MmmSC extract, after electrophoresis gel pieces were excised and mixed with reaction mixture with or without FAD. No activity was detected in the reaction without FAD. Thus, FAD coenzyme is not covalently bound to the enzyme and MmmSC FDTS activity is FAD dependent. We estimated the molecular mass of MmmSC-FDTS in the native form ~ 200 kDa. Fluorescent emission spectra demonstrated that MmmSC and Uu-FDTS were flavin-proteins and enzyme-bound FAD was essential for activity. Kinetic studies with MmmSC-FDTS showed negative cooperative for dUMP and CH_2THF with apparent K_m values of 17.3 μM (dUMP), 69 μM (CH_2THF) and 103 μM (NADPH), respectively. 5-FdUMP inhibited MmmSC FDTS competitively with a K_i value of 1.5 μ M.

All mycoplasmas have *thyA* gene except for Uu, MmmSC, Mcap and Mhp where no *thyA* homologs have been identified. Recently, a new family of FDTS (ThyX) was discovered, which is present in many pathogenic bacteria such as *Chlamydia* and *Helicobacter*. These FDTSs share a common thyX motif ($RHRX_8S$-) and sequence homology to each other. However, *Fermaplasma* and *Chalymadia* thyXs have two thyX motifs, one common thyX motif and another within their group. Using *Fermaplasma* thyX as query, we identified UU572 and MSC0676 as putative FDTS.

UU572 and MSC0676 were cloned and the recombinant plasmids were transformed to an *E. coli* thyA mutant ISM612. UU572 and MSC0676 could rescue the thymidine auxotrophic phenotype of the TS deficient ISM612 grown on M9 minimal medium plates lacking thymidine. Recombinant UU572 and MSC0676 were purified and showed to have similar properties as the native enzymes. The low (< 13%) sequence identity to either ThyX or ThyA suggested that UU572 and MSC0676 belong to a new family of flavin-dependent thymidylate synthase. The unique properties of Uu and MmmSC TS make them attractive targets for design of new antibiotics against these pathogenic bacteria.

Paper IV: A New family of flavin-dependent thymidylate synthase in pathogenic mycoplasmas.

ThyA homologues are presence in many Mycoplasmas species e.g. *M. genitallium* (Table. 1). The ThyA sequences are conserved within Mycoplasmas, ~ 50-70% sequence identities, while sequence homology to other bacterial ThyA is only ~ 30-40%. As expected, Mycoplasmas, which have the *thyA* genes, possess also genes for DHFR, which is required for reduction of the H_2folate to H_4folate.

M. pneumoniae and M. genitalium share an identical set of pyrimidine metabolism pathways and other proteins (Inamine *et al*., 1989) as compared to other mycoplasmas and both *M. genitalium* (Mg) and *M. pneumoniae* (Mpn) possess *thyA* genes (Table. 1). However, studies by Glass *et al*. showed that Mg *thyA* gene is not essential since its interruption did not affect Mg growth (Glass *et al*., 2006). Using Uu FDTS sequence as query sequence, we identified an open reading frame in Mg (Mg255) and Mpn (MPN358), which showed significant homology to Uu FDTS, ≈ 26%.

We also identified homologous genes e.g. Mcap620 in Mcap genome and Mhp655 in Mhp genome by using MmmSC FDTS as query sequence. We cloned these four genes by using PCR method using genomic DNA as template. MPN358, Mcap612 and Mhp655 could functionally replace the *thyA* gene of an *E. coli* strain ISM612 that is TS deficient and suitable for expression of mycoplasma proteins. Recombinant Mhp655 protein was expressed and purified using anion exchange chromatography. Purified mhp655 protein showed FDTS activity and the elution profile of Mhp655 was similar to that of UU572 and MSC0676. Fractions containing TS activity displayed yellow color.

Thus, we have identified a new family of flavin dependent thymidylate synthase. Since these Mycoplasma favin dependent thymidylate synthases have no sequence and possibly structural similarity to ThyA proteins, and they are attractive targets for the design of new antibiotics against these pathogenic bacteria.

Table1. Thymidylate synthase genes (thyA and thyX) in Mollicutes.

Mollicutes	thyA	thyX
M.gallisepaticum R	+	–
M.synoviae	+	–
M.mobile	+	–
M. penetran	+	–
M.pneumoniae	+	+
Onion yellow phytoplasma	+	–
Mesoplasma florum	+	–
M.genitalium	+	+
M.pulmonis	+	–
M.mycoides SC	–	+
M.hyopneumoniae	–	+
M.capricolum	–	+
U.realyaticum	–	+

Concluding remarks and future perspectives

There are only a few studies on the nucleotide metabolism of Mollicutes, but together with the information derived from Mollicute genome analysis, a wealth of information was provided regarding the pathways of biosynthesis and degradation of pyrimidine and purine nucleotides. The nucleotide biosynthetic enzymes are potential targets for antimicrobials drugs designed selectively inhibit the growth of these organisms.

There are several important differences between mycoplasma and mammalian cells in the overall biosynthesis, transport and catabolism of nucleosides and nucleotides. Of particular interest in the present study is the biosynthesis pathway for dTMP from dUMP, in which the enzymes catalyzing the reaction and the reaction mechanism are completely different. A better understanding of the nucleotide metabolism of mycoplasmas is of fundamental interest in microbiology and may help in the development of new anti-mycoplasma therapies.

Mollicutes posses pyrimidine nucleotides salvage enzyme and this pathway is essential for the growth and survival of the *Mollicutes*. This is due to the limited biosynthetic capacity and the absence of key enzymes of *de novo* pathways.

The discovery of an alternative flavin-dependent mechanism for thymidylate synthesis described in this thesis has revealed a plausible explanation for the unexpected observation that life without two essential enzymes ThyA and FolA is still possible. It is also very important to understand folate metabolism in bacteria using ThyX for thymidylate synthesis, which undoubtedly aid the evolution of intermediary metabolism, as well as in designing new compounds for inhibiting microbial growth.

The studies carried out in this thesis have demonstrated that targeting the pyrimidine salvage pathway may present valuable opportunities for the development of antimicrobial agents selective against *Mollicutes*. The practical potential of the pathways of pyrimidine synthesis as therapeutic targets for antibacterials specific to *Mollicutes* remains to be tested. To achieve this it is necessary to establish the basic principals of the metabolism of nucleotides in the *Mollicutes*, which this thesis is aimed to contribute to.

Main conclusion of the thesis work

♦Nucleoside analogs, especially fluoropyrimidine nucleoside analogues e.g. 5-FdUrd strongly inhibited the growth of Uu and Mpn. The mechanism of inhibition is most likely due to blocked dTMP synthesis.

♦Flavin dependent thymidylate synthase activities were detected in the extracts of Uu and MmmSC and the corresponding genes were identified.

♦ FDTS genes were identified in those mycoplasma genomes (Uu, Mhp, Mcap and MmmSC), which lack *thyA* as well as in those Mycoplasma genomes (Mpn and Mg), which possess *thyA*.

♦Mycoplasma FDTS sequences have little sequence homology to ThyX from other bacteria and, thus, belong to a new family of flavin dependent thymidylate synthase.

♦ Future structure and function studies of with recombiant proteins are needed to explain the minimal sequence identities found between the ThyX and the new family of Mycoplasma FDTS.

♦The unique properties of Mycoplasma FDTS make it an important target for future design of specific antibiotic against these pathogenic bacteria.

References

Adams, M. M., Elam-Evans, L. D., Wilson, H. G. and Gilbertz, D. A. 2000. Rates of and factors associated with recurrence of preterm delivery. *JAMA* 283, 1591-1596.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25, 3389-3402.

Arnér, E. S. J. and Eriksson, S. 1995 .Mammalian deoxcyribonucleoside kinases. *Pharmac. Ther*. 67, 155-186.

Au JL., Rustum YM. and Ledesma EJ. 1982. Clinical pharmacological studies of concurrentinfusion of 5-fluorouracil and thymidine in treatment of colorectal carcinoma. *Cancer Res*. 42, 2930-7.

Baseman, J. B. and J. G. Tully. 1997. Mycoplasmas: sophisticated, reemerging, and burdened by their notoriety. *Emerg. Infect*. Dis. 3, 21-32.

Bender RA. 1979. *The membrane transport of methotrexate.* In *high dose methotrexate, pharmacology, toxicity and chemotherapy* (ed. P Periti), Editrice Guintina, Florece, p.23-35.

Berger SH. and Hakala MT. 1984. Relationship of dUMP and free FdUMP pools to inhibition to thymidylate synthase by 5-fluorouracil. *Mol Pharmacol*. 25, 303-9.

Blanchard, A., Razin, S., Kenny, G. E. and Barile, M. F. 1988. Characterization of the Ureaplasma Urealyticum urease. *J. Bacteriol*. 170, 2629-2697.

Brown, N. C. and Reichard, P. 1969. Role of effector binding in allosteric control of ribonucleoside diphosphate reductase. *J Mol Biol*. 46, 39-55.

Campbell IG., Jone TA., Foulkes WD. and Trowsdale J. 1991. Folate-binding protein is a marker for ovarian cancer. *Cancer Research*. 51, 5329-38.

Carnrot, C., Wehelie, R., Eriksson, S., Bölske, G. and Wang, L. 2003. Molecular characterization of thymidine kinase from Ureaplasma urealyticum: nucleoside analogues as potent inhibitors of mycoplasma growth. *Mol Microbiol*. 50, 771-780.

Carreras, C. W., Climie, S. C. and Santi, D. V. 1992. Thymidylate synthase with a C-Terminal deletion catalyzes partial reaction but unable to catalyze thymidylate formation. *Biochemistry*. 31, 6038-6044.

Cassell, G. H., K. B. Waites, H. L. Watson, D. T. Crouse. and R. Harasawa. 1993. Ureaplasma urealyticum intrauterine infection: role in prematurity and disease in newborns. *Clin. Microbiol. Rev*. 6, 69-87.

Chabot, F., Mitchell, J. A., Gutteridge, J. M. and Evans, T. W. 1998. Reactive oxygen species in acute lung injury. *Eur. Respir. J.* 11, 745-757

Chambaud, I., Heilig, R., Ferris, S., Barbe V., Samson, D., Galisson, F., Moszer, I., Dybyig, K., Wroblewski, H., Viari, A., Rocha, E. P. C. and Blanchard, A. 2001. The complete genome sequence of the murine respiratory pathogen Mycoplasma pulmonis. *Nucleic Acids Res*. 29, 2145-2153.

Check, E. 2002. Venter aims for maximum impact with minimal genome. *Nature* 420, 350.

Chen, YL., Wang, SN., Chen, YJ., Lin, HH. and Shiuan, D. 2003.Expression and immunogenicity of Mycoplasma hyopneumoniae heat shock protein antigen P42 by DNA vaccination. *Infect Immun*. 71, 1155-60.

Cheng, X., Nicolet, F., Poumarat, J., Regalla, F., Thiaucourt, C. and Frey. J. 1995. Insertion element IS1296 in *Mycoplasma mycoides* subsp. *mycoides* small colony identifies a European clonal line distinct from African and Australian strains. *Microbiology*. 141, 3221-3228.

Citti, C., and R. Rosengarten. 1997. Mycoplasma genetic variation and its implication for pathogenesis. *Wien. Klin. Wochenschr*. 109, 562-568.

Colman SD., Hu PC., Litaker W., and Bott KF. 1990. A physical map of the Mycoplasma genitalium genome. *Mol Microbiol*. 4, 683–687.

Costi, M. P., Ferrari, S., Venturelli, A., Calo, S., Tondi, D. and barlocco, D. 2005. thymidylate synthatse structure, function and implication in drug discovery. *Curr Med Chem*. 12, 763-771.

Dandekar, T., Snel, B., Schhmidt, S., Lathe, W., Suyama, M., Huynen, M. and Bork, P. 2002. *Comparative genome analysis of the Mollicutes. In Molecular Biology and Pathogenicity of Mycoplasmas*, ed. Razin &Herrmann, Kluwer. Academic/plenum Publishers, New York, p.255-278.

Deguchi, T., Yoshida, T., Miyazawa, T., Yasuda, M., Tamaki, M., Ishiko, H. and S. Maeda. 2004. Association of Ureaplasma urealyticum (biovar 2) with nongonococcal urethritis. *Sex. Transm*. Dis. 31, 192-195.

Domingues, D., Tavora, L., Tavira, L., Duarte, A., Sanca, A., Prieto, E. and Exposto, E. 2002. Ureaplasma urealyticum biovar determination in women attending a family planning clinic in Guinea-Bissau, using polymerase chain reaction of the multiple-banded antigen gene. *J. Clin. Lab. Anal*. 16, 71-75.

Dybvig K. Mycoplasmal genetics. 1990. *Annu Rev Microbiol.44*, 81–104.

Eaton, M. D. and Low, I. E. 1967. Propagation of Mycoplasma pneumoniae and other fastidious strains of PPLO. *Ann. N. Y. Acad. Sci*. 143, 375-383.

Eaton, M. D., Meiklejohn, G. and Herick, W. 1944. Study of the etiology of primary atypical pneumoniae. A filterable agent transmissible to cotton rats, hamster, and chicken embryos. *J. Exp. Med*. 79, 649-668.

Eriksson, S., Kierdaszuk, B., Munch-Petersen, B., Öberg, B. and Johansson, N. G. 1991. Comparison of the substrate specificities of human thymidine kinase 1 and 2 and deoxycytidine kinase toward antiviral and cytostatic nucleoside analogs. *Biochem Biophys Res commun*. 176, 586-92.

Fraser, C. M. and Fleischmann, R. D. 1997. Strategies for whole microbial genome sequencing. *Electrophoresis*. 18, 1207-1216.

Fraser, C., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., Bult, C. J., Kerlavage, A. R.., Sutton, G. and Kelley, M 1995. The minimal gene complement of Mycoplasma genitalium. *Science*. 270, 397- 403.

Frey, J. 2002. *Mycoplasmas of animals*. In *Molecular biology and pathogenicity of mycoplasmas*. S. Razin and R. Herrmann (ed.), Kluwer Academic/PlenumPublishers, New York, N.Y. p. 73-90.

Gattis, S. and Palfey, B. 2005. Direct observation of the participation of flavin product formation by thyX-encoded Thymidylate synthase. *J. Am Chem Soc*. 127, 832-833.

Gerber, S., Vial, Y., Hohlfeld. and Witkin, S. S. 2003. Detection of Ureaplasma urealyticum in second trimester amniotic fluid by polymerase chain reaction correlates with subsequenct preterm labor and delivery. *J. Infec. Dis*. 187, 518-521.

Giladi, M., Bitan-Banin, G., Mevarech, M., and Ortenberg, R. 2002. Genetic evidence for a novel thymidylate synthase in the halophilic archaeon Halobacterium salinarum and in Campylobacter jejuni. *FEMS Microbiol Lett*. 216, 105-109.

Glass, J., Lefkowitz, E., Glass, J., Heiner, C., Chen, E. and Cassell, G. .2000. The complete sequence of the mucosal pathogen Ureaplasma urealyticum. *Nature*. 407, 757-762.

Glass, J. I., Assad-Carcia., Alperovich, N., Yooseph, S., Lewis, M. R., Maruf, M., Hutchison, C. A., Smith, H. O. and Venter, C. J. 2006. Essential genes of a minimal bacterium. *PNAS*. 103, 425-430.

Graziani, S., Xia, Y., Gurnon, J., Van Etten, J., Leduc, D. and Skouloubris, S., *et al.* 2004. Functional analysis of FAD-dependent thymidylate synthase ThyX from Paramecium bursaria Chlorella virus-1. *J Biol Chem.* 279, 54340-54347.

Griffin, J., Roshick, C., Iliffe-Lee, E. and McClarty, G. 2005. Catalytic mechanism of Chlamydia trachomatis flavin-dependent thymidylate synthase. *J Biol Chem.* 280, 5456-5467.

Haesebrouck, F., Pasmans, F., Chiers, K., Maes, D., Ducatelle, R. and Decostere, A. 2004. Efficacy of vaccines against bacterial diseases in swine: what can we expect? *Vet Microbiol*. 100, 255-68.

Harrap, K., Jackman, A., Newell, D., Taylor, G., Hughes, L. and Calvert, A. 1989. Thymidylate synthase: a target for anticancer drug design. *Adv Enzyme Regul.* 29, 161-179.

Harris, J. A., Kolokathis, A., Campbell, M., Cassell, G. H. and Hammerschlag, M. R. 1998. Safety and efficacy of azithromycin in the treatment of community-acquired pneumonia in children. *Pediatr. Infect. Dis. J*. 17**,** 865-871.

Harrison, H. R., Alexander, E. R., Weinstein, L., Lewis, M., Nash, M. and Sim, D. A. 1983. Cervical Chlamydia trachomatis and mycoplasmal infections in pregnancy. Epidermiology and outcomes. *JAMA*. 250, 1721-1727.

Heggie, A. D., Bar-shain, D., Boxerbaum, B., Fanaroff, A. A., O'Riodan, M. A. and Robertson, J. A. 2001. Identification and quantification of ureaplasmas colonizing the respiratory tract and assessment of their role in the development of chronic lung disease in preterm infants. *Pediatr. Infect. Dis. J.* 20, 854-859.

Hillier, S. L., Krohn, M. A., Kiviat, N. B., Watts, D.H. and Eschenbach. 1991. Microbiologic causes and neonatal outcomes associated with chorioamnion infection. *Am. J. Obstet. Gynecol*. 165, 955-961.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B. C. and Herrmann, R. 1996. Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Res*. 24, 4420-4449

Horner, P. J., Gilroy, C. B., Thomas, B. J., Naidoo, R. O. M. and Taylor-Robinson, D. 1993. Association of Mycoplasma genitalium with acute non-gonococcal urethritis. *Lancet.* 342, 582-585.

Houghton J. A., Weiss K. D. and Williams L. G. 1986. Relationship between 5-fluoro-2-deoxyuridylate, 2-deoxyuridylate, and thymidylate synthase activity subsequent to 5-fluorouracil administration in xenografts of human colon adenocarcinomas. *Biochem Pharmacol*. 35, 1351-8.

Inamine JM., Loechel S., Collier AM., Barile MF., Hu, PC. 1989. Nucleotide sequence of the MgPa (mgp) operon of Mycoplasma genitalium and comparison to the P1 (mpp) operon of Mycoplasma pneumoniae. *Gene*. 82, 259–267.

Jaffe, J. D., Stange-Thomann, N., Smith, C., DeCaprio, D., Fisher, S., Butler, J., Calvo, S., Elkins, T., FitzGerald, M. G., Hafez, N., Kodira, C. D., Major, J., Wang, S., Wilkinson, J., Nicol, R., Nusbaum, C., Birren, B., Berg, H. C. and Church, G. M. 2004 The complete genome and proteome of *Mycoplasma mobile*. *Genome Res.* 14, 1447-1461

Johnson, E. F., Hinz, W., Atreya, C. e., Maley, F., and Anderson, K. 2002. Mechanistic characterization of Toxoplasma gondii thymidylate synthase (TS-DHFR)-dihydrofolate reductase. *J Biol Chem*. 277, 43126-43136.

Keyomarsi, K and Moran, R. 1988. Mechanism of the cytotoxi synergism of fluoropyrimidines and folic acid in mouse leukemic cells. *J Biol Chem*. 263, 14402-14409.

Kim, M., Kim, G., Romero, R., Shim, S. S., Kim, E. C. and Yoon, B. H. 2003. Biovar diversity of Ureaplasma urealyticum in amniotic fluid: distribution, intrauterine inflammatory response and pregnancy outcomes. *J. Perinat. Med*. 31, 146-152.

Kong, F., James, Ma, G., Gordon, S., and Gilbert, G. L. 2000. Species identification and subtyping of Ureaplasma parvum and Ureaplasma urealyticum using PCR-based assays. J. Clin. Microbiol. 38:1175- 1179.

Leduc, D., Graziani, S., Meslet-Cladiere, L., Sodolescu, A., Liebl, U., and Myllykallio, H. 2004 Two distinct pathways for thymidylate (dTMP) synthesis in (hyper)thermophilic Bacteria and Archaea. *Biochem Soc Trans.* 32, 231-235.

Liu, X. and Yang, J. 2004. Bacterial thymidylate synthase with intein, group II Intron, and distinctive ThyX motifs. *J Bacteriol*. 186, 6316-6319.

Lockwood, C. J. and E. Kuczynski. 1999. Markers of risk for preterm delivery. *J. Perinat. Med*. 27, 5-20..

Manhart, L. E., Critchlow, C. W., Holmes, K. K., Dutro, S. M., D. A. Eschenbach, D. A., Stevens, C.E. and Totten, P. A. 2003. Mucopurulent cervicitis and Mycoplasma genitalium. *J. Infect. Dis*.187, 650-657.

Maniloff, J., McElnaney, R. N. and Baseman, J. B. 1992. *Mycoplasmas: molecular biology and pathogenesis*. American Society for Microbiology, Washington, D.C. p.14-59

Martinez, M., Ovalle, A. A., Santa-Cruz, A., Barrera, B., Vidal, R. and Aguirre, R. 2001. Occurrence and antimicrobial susceptibility of Ureaplasma parvum (Ureaplasma urealyticum biovar 1) and Ureaplasma urealyticum (Ureaplasma urealyticum biovar 2) from patients with adverse pregnancy outcomes and normal pregnant women. *Scand. J. Infect. Dis*. 33, 604-610.

Massey, V. 2000. The chemical and biological versatility of riboflavin. *Biochem Soc Trans*. 28, 283-296.

Mathews, I., Deacon, A., Canaves, J., McMullan, D., Lesley, S., Agarwalla, S. and Kuhn, P. 2003. Functional analysis of substrate and cofactor complex structures of a thymidylate synthase-complementing protein. *Structure*. 11, 677-690.

Menon, SA., Wannemuehler, MJ., Mahairas, GG. and Minion, FC. 2002. Mycobacterial ESAT-6 protein enhances mouse IFN-gamma responses to Mycoplasma hyopneumoniae P71 protein. *J Interferon Cytokine Res*. 22, 807-13.

Miles, R. J., Taylor, R. R. and Varsani, H. 1991. Oxygen uptake and H_2O_2 production by fermentative Mycoplasma spp. *J. Med. Microbiol*. 34, 219-223.

Mini E, Trave F., Rustum YM. and Bertino, JR. 1990. Enhancement of the antitumor effects of 5-fluorouracil by folinic acid. *Pharm Ther*. 47, 1-19.

Minion, F. C., E. J. Lefkowitz, M. L. Madsen, B. J. Cleary, S. M. Swartzell. and Mahairas, G. G.. The genome sequence of *Mycoplasma hyopneumoniae* strain 232, the agent of swine mycoplasmosis. *J. Bacteriol*. 186, 7123-7133.

Minion, F., VanDyk, C. and Smiley, B. 1995. Use of an enhanced Escherichia coli opal suppressor strain to screen a Mycoplasma hyopneumoniae library. *FEMS Microbiol Lett*. 131, 81-85.

Mitchell, A. and Finch, L. R. 1977. Pathways of Nucleotide Biosynthesis in Mycoplasma mycoides subsp. mycoides. *J Bacteriol*. 130, 1047–1054.

Mitchell, A. and Finch, L. R. 1979. Enzymes of Pyrimidine Metabolism in Mycoplasma mycoides subsp. mycoides. *J Bacteriol*. 137, 1073–1080.

Mitchell, Al., Sin, I L. and Finch, L R. 1978. Enzymes of Purine Metabolism in Mycoplasma mycoides subsp. mycoides. *J Bacteriol*. 134, 706–712.

Moller, B. R. 1979. A modification of the mycoplasma serum-drop growth inhibition test. *J. Appl. Bacteriol.* 47, 97-104.

Mrazek. J. 2006. Analysis of Distribution Indicates Diverse Functions of Simple Sequence Repeats in Mycoplasma Genomes. *Mol. Biol. Evol*. 23, 1370 - 1385.

Myllykallio, H., Lipowski, G., Leduc, D., Filee, J., Forterre, P., and Liebl, U. 2002. An alternative flavin-dependent mechanism for thymidylate synthesis. *Science* 297, 105-107.

Neale GA., Mitchell A. and Finch LR. 1983. Pathways of pyrimidine deoxyribonucleotide biosynthesis in Mycoplasma mycoides subsp. mycoides. *J Bacteriol*. 154, 17–22

Neale GA., Mitchell A. and Finch LR. 1984. Uptake and utilization of deoxynucleoside 5'-monophosphates by Mycoplasma mycoides subsp. mycoides. *J Bacteriol*.158, 943–947.

Neyrolles, O., Ferris, S., Behbahani, N., Montagnier, L., and Blanchard, A. 1996. Organazation of Ureaplasma urealyticum urease gene cluster and expression in a suppressor strain of Escherichia coli. *J. Bacteriol*. 178, 647-655.

Papazisi, L., Gorton, T. S., Kutish, G., Markham, P. F., Browning, G. F., Nguyen,, D. K., Swartzell, S., Madan, A., Mahairas, G. and Geary, S. J. 2003. The complete genome sequence of the avian pathogen Mycoplasma gallisepticum strain R (low). *Microbiology* 149, 2307-2316.

Peters GJ., Schornage J. and Milano GA. 1993. Clinical pharmacokinetics of antimetabolites. *Cancer Survey*. 17, 123-56

Pilo, P., Martig, S., Frey, J. and Vilei, E. M. 2003. Antigenic and genetic characterisation of lipoprotein LppC from Mycoplasma mycoides subsp. mycoides SC. *Vet. Res*. 34, 761-775.

Pollack, J. D. 1995. Methods for testing metabolic activities in mollicutes. In *Molecular and diagnostic procedures for mycoplasmology*, S. Razin and J. G. Tully (ed.) vol. 1. Academic Press, Inc., New York p.277–286

Pollack, J. D. and Williams, M. V. 1986. PPi-dependent phosphofructotransferase (phosphofructokinase) activity in the mollicutes (mycoplasma) *Acholeplasma laidlawii*. *J. Bacteriol*. 165**,** 53–60.

Povlsen, K., P. Thorsen. and I. Lind. 2002. Relationship of Ureaplasma urealyticum biovars to the presence or absence of bacterial vaginosis in pregnant women and to the time of delivery. *Eur. J. Clin. Microbiol. Infect. Dis.* 20, 65-67.

Razin, S; D. Yogev. and Y. Naot. 1998. Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev*. 62, 1094-1156.

Razin, S. 1992a. Peculiar properties of mycoplasms: the smallest self-replicating prokaryotes. *FEMS Microbiol. Lett*. 100, 423-432.

Razin, S. 1992b. Mycoplasma taxonomy and ecology. In *Mycoplasmas: molecular biology and pathogenesis*. Maniloff, J., McElnaney, R. N., Finch, L. R. and Baseman, J. B. (ed). American Society for Microbiology, Washington, D.C. p. 2-22.

Razin, S. 1972. Reconstitution of biological membrane. *Biochim. Biophys. Acta.* 265, 241-296

Shamira, S., Zhang, K., Jiang, L. and Rathod, PK. 1999. Essential protein-protein interactions between plasmodium falciparum thymidylate synthase anhd dihyrofoate reductase domains. *J Biol Chem*. 274, 37781-37786.

Shalom, S., Zhang, K., Jiang, L. and rathod, P. K. Essential protein-protein interactions between Plasmodium faciparum thymidylate synthase and dihydrofolate reductase domains.1999. *J Biol Chem*. 274, 37781-37786.

Sampathkumar, P., Turley, S., Ulmer, J., Rhie, H., Sibley, C. and Hol, W. 2005. Structure of the Mycobacterium tuberculosis flavin dependent thymidylate synthase (MtbThyX) at 2.0A resolution. *J Mol Biol* 352, 1091-1104.

Shapiro, S., McCormick, M. C., Starfield, B. H., Krischer, J. P. and Bross. D. 1980. Relevance of correlates of infant deaths for significant morbidity at 1 year of age. *Am. J. Obstet. Gynecol*. 136, 363-373.

Sasaki, Y., Ishikawa, J., Yamashita, A., Oshima, K., Kenri, T., Furuya, K., Yoshino, C., Horino, A., Shiba, T., Sasaki, T. and Hattori, M. 2002. The complete genomic sequence of Mycoplasma penetrans, an intracellular bacterial pathogen in humans. *Nucleic Acids Res*. 30, 5293-5300.

Sperling, R. S., Newton, E. and Gibbs, R. S. 1988. Intraamniotic infection in low birth-weight infants. *J. Infect. Dis*. 157, 113-117.

Svenstrup HF., Fedder J. and Abraham-Peskir J. 2003. *Mycoplasma genitalium* attaches to human spermatozoa. *Hum Reprod* 18, 2103–9.

Stryer, L., Berg, J. M. and Tymoczko, J. L.2001. *Biochemistry.* W. H. Freeman and Company, New York p. 704-707.

Thacker, EL., Thacker, BJ., Kuhn, M., Hawkins, PA. and Waters, WR. 2000. Evaluation of local and systemic immune responses induced by intramuscular injection of a Mycoplasma hyopneumoniae bacterin to pigs. *Am J Vet Res*. 61, 1384-9.

Thanawongnuwech, R., Young, TF., Thacker, BJ. and Thacker, EL. 2001.Differential production of proinflammatory cytokines: in vitro PRRSV and Mycoplasma hyopneumoniae co-infection model. *Vet. Immunol. Immunopathol*. 79, 115-27.

Thelander, L. and Reichard, P. 1979. Reduction of ribonucleotides *Annu. Rev. Biochem*. 48, 133-158.

Tryon, V. V. and Baseman, J. B. 1992. *Pathogenic determinants and mechanisms*, In J. Maniloff, R. N. McElhaney, L. R. Finch, and J.B. Baseman (ed.), *Mycoplasmas: molecular biology and pathogenesis*. American Society for Microbiology, Washington, D.C. p.457-471.

Waites, K. B. and Talkington, D. F. 2004. Mycoplasma pneumoniae and Its Role as a Human Pathogen. *Clin. Microbiol. Rev*. 17, 697 - 728.

Wang, L., Westberg, J., Bölske, G. and Eriksson, S. 2001. Novel deoxynucleoside-phosphorylating enzymes in mycoplasmas: evidence for efficient utilization of deoxynucleosides. *Mol Microbiol.* 42, 1065-1073.

Wang, J., Wilkinson, R., Nicol, C., Nusbaum, B., Birren, H., Berg, C. and G. M. Church, M. G. 2004 The complete genome and proteome of Mycoplasma mobile. *Genome Res.* 14, 1447-1461.

Wasinger, V., Pollack, J. and Humphery-smith, I. 2000. The proteome of mycoplasma genitalium. Chaps-soluble component. *Eur J Bioch*. 267, 1571-1582.

Wesonga, HO., Bölske, G., Thiaucourt, F., Wanjohi, C. and Lindberg, R. 2004. pleuropneumonia: a long term study on the course of infection and pathology in a flock of goats infected with Mycoplasma capricolum subsp. capripneumoniae. *Acta Vet Scand*. 45, 167-79.

Westberg, J., Persson, A., Holmberg, A., Goesmann, A., Lundeberg, J., Johansson, K. E., Pettersson, B. and Uhlen, M. 2004. The genome sequence of Mycoplasma mycoides subsp. mycoides SC type strain PG1T, the causative agent of contagious bovine pleuropneumonia (CBPP). *Genome Res*. 14, 221-227.

Vilei, E. M and J. Frey. 2001. Genetic and biochemical characterization of glycerol uptake in Mycoplasma mycoides subsp. mycoides SC: Its impact on H_2O_2 production and virulence. *Clin. Diagn. Lab. Immunol*. 8, 85-92.

Yoon, B. H., Romero, R., Park, J. S., Chang, J. W., Kim, Y. A., Kim, J. C. and Kim, K. S. 1998. Microbial invasion of the amniotic cavity with Ureaplasma urealyticum is associated with a robust host response in fetal, amniotic, and maternal compartments. *Am. J. Obstet. Gynecol*. 179, 1254-1260.

Yoshida, T., Maeda, S., Deguchi, T., Miyazawa, T. and Ishiko, H. 2003. Rapid detection of Mycoplasma genitalium, Mycoplasma hominis, Ureaplasma parvum, and Ureaplasma urealyticum organisms in genitourinary samples by PCR-microtiter plate hybridization assay. *J. Clin. Microbiol*. 41, 1850-1855.

Acknowledgements

The studies presented in this thesis were carried out at the Department of Molecular bioscience and was founded by the AgriFunGen program at the Swedish University of Agricultural Sciences and by grants from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning, and the Swedish Research Council.

I would like to express my sincere gratitude and appreciation to everyone who has helped, supported me in any way during these years and made this thesis possible, but in particular the following:

Staffan Eriksson, my supervisor, for introducing me to the field of nucleotide metabolism, for your support and great enthusiasm on my project, for your open mind during discussion and consistently with your positive response to my new ideas (which was sometimes out of place!), for your patience in discussing manuscript, and most important for your understanding my other commitment besides being PhD student, and for the yearly barbecue group meeting together with your wife Lena in your garden which letter become at your apartment. ***Liya Wang***, my supervisor, for your guidance and support in the lab, for your never-ending enthusiasm for science and your depth of knowledge for always having an answer for my questions. The discovery of FDTS of mycoplasma has been challenging, but fruitful and will produce many interesting results in the future, your support, encouragement and advice made possible to achieve my goal. Beside the science we talked also about life in general, especially about kids. **Thank you both for all these exciting years and for being excellent supervisors!**

Göte Swedberg, thanks for sharing your knowledge of folate metabolism, and your contribution for the FDTS project.

Past and present of members of the group:

Svetlana, I do admire your patient both in life and science, our lunch discussion about life and your help in the lab. Thanks for company for long, cold and unforgettable trip to Hemavan. ***Ashraf,*** for teaching me many molecular biology techniques in the lab especially old techniques which always better the new ones. ***Cecilia,*** for great company to those conferences in USA, Cambridge, and Holland, for all discussion about our problems as PhD student and for being good office mate. ***Louise***, for interesting conversations regarding science as well as life and lately about kids. Welcome to motherhood! ***Elena,*** thanks for teaching me so many things about horse, you are true expert of horses. ***Gunnar F,*** thanks for being my cameraman and taking so many pictures of my plates, please, don't

forget to bring your camera! ***Jay***, good luck with mycoplasma FDTS, you will definitely find a way to beat mycoplasmas. ***Hanan***, too bad I didn't get a chance to know you, good luck.

Past and present members of department:

Kerstin, Thanks for helping me in lab and for keeping the spirit high and filling the lab with laughter.

Piotr, you certainly beat me for being computer expert, thank you for all the help and always having a time to joke no matter how busy you are.

Gunnar P, Anna B, Ingemar, Tiago, Magnus G, Stefan, My, Hanna, Jan, Gunnar M, Charlotte ,Siwei, Maria, Anna R, Osama, Margareta, Ida, Sara, Magnus Å, Göran H, Tommy L, Elena T, Frida, Maud, Kersti and Mikeal K, For all the helps during these years and for contributing to the pleasant and inspiring environment at the department.

Structural biology group:

Hasse, Martin, Urszula and Nisse, for interesting discussion about structures and journal club.

My proteomic mentors:

Birger, Henrik, and Anders, thank you for introducing me the world of proteomics, too bad my project never worked the way I hoped for.

Members of mycoplasma lab in SVA:

Göran Bölske, for your support and teaching many things about mycoplasma and being excellent collaborator. ***Virginia and Stefan,*** for always providing me mycoplasma cells despite of your overload work, the difficult to grow mycoplasma and supplying me on time. ***Agneta*** and **Zora,** for helping to find my way around the lab.

I would like to thank to people who supported me over the years just for being good friend. ***Rosemary,*** for you honesty and care. ***Chantal***, for always being there whenever I need to talk, no matter how far we are from each other, and for coming to visiting me here in Sweden even when I am no longer living in

Canada. ***Suzett, and Yasmin,*** for always being there especially when my mum was sick. It has been very long time since we met each other, I promise to come to visit you in Canada.

To my family: ***mum***, for always want me to have the best of all, All my sisters and brothers especially my dearest brother, ***Aziz,*** I do admire your believe in education and always persuade me to come back to North America. ***Yasmin***, my sister in-law, thank you for your encouragement and support.

Last, but not least my beloved children ***Dalmar and Ida-Mona***, for all the laughter we have shared together and your endless love. No matter how tired I am when I come home, you always lifted my spirit, and make me smile.

Rob, my dearest husband, your love, support, patience and encouragement made this thesis possible. You are my friend, companion in life, and thank you for being there for me always. I love you, waan ku jeclahay♥