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THE NECESSITY OF COMBINING GENOMIC AND ENZYMATIC DATA TO INFER METABOLIC FUNCTION AND PATHWAYS IN THE SMALLEST BACTERIA: AMINO ACID, PURINE AND PYRIMIDINE METABOLISM IN MOLLICUTES

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1. ABSTRACT

Bacteria of the class Mollicutes have no cell wall. One species, *Mycoplasma genitalium* is the personification of the simplest form of independent cell-free life. Its small genome (580 kbp) is the smallest of any cell. Mollicutes have unique metabolic properties, perhaps because of their limited coding space and high mutability. Based on 16S rRNA analyses the Mollicutes *Mycoplasma gallisepticum* is thought to be the most mutable Bacteria. Enzyme activities found in most Bacteria are absent from Mollicutes. The functions of apparently absent genes and enzymes can apparently be fulfilled by other genes and their expression products that have multiple capabilities. Because of these and other properties predictions of their metabolism based only on, *e.g.*, either annotation, enzymatic assay, proteomic studies or structural analyses is problematic. To obtain a more confident appraisal of the functional capabilities of these simplest cells genomic and enzymatic data were combined to obtain a "metabolic consensus". The consensus is represented by a biochemical circuit for central metabolism involving purine and pyrimidine interconversions and their linkages to amino acid metabolism, glycolysis and the pentose phosphate pathway in three human Mollicutes pathogens: *Mycoplasma pneumoniae*, *Mycoplasma genitalium* and *Ureaplasma urealyticum.*

2. INTRODUCTION

In a search for a metabolic model of independent life the simplest microbes were seen as the most profitable examples to study. The bacteria in the class Mollicutes represent these simple microbes. *Mycoplasma genitalium*, a Mollicutes with a genome of 580 kbp and 475 ORFs, has the smallest genome in any free-living cell and is an obvious example of the simplest organism. It is the minimal cell and defines-characterizes, personifies independent cellular life.

All Mollicutes, like *M. genitalium* have no cell wall, are small, passing through 0.45 mμ porosity filters. Some have a cholesterol growth requirement, most have a low 24-33% G+C, they have 1-2 rRNA operons, their 5S rRNAs have 104-113 nucleotides and some use the UGA "universal" stop codon as a tryptophan codon. Their RNA polymerase is rifampicin resistant; they lack cytochromes and the tricarboxylic acid cycle. Unlike other bacteria, some Mollicutes genera have NADH oxidase activity localized in their cytoplasm, some genera do not possess the "ubiquitous" DNA repair enzyme uracil-DNA glycosylase. Some Mollicutes can transport nucleoside monophosphates or have an obligate requirement for pyrophosphate rather than ATP for glycolysis or the phosphorylation of nucleosides. The "household" nucleoside diphosphate kinase (NDPK) gene is not annotated in the four Mollicutes whose genomes are sequenced; NDPK activity was not found in *Mycoplasma pneumoniae.*

Mollicutes have been frequently reported as "unrecognized" contaminants in a large number of established cell lines, and they confound much research because of such intimacy. They infect and cause a

frequently encountered treatable low-mortality respiratory disease of humans. In the U. S. and abroad, they cause enormous economic losses as subtle or severe pathogens of cattle, swine, sheep, goats and poultry. Treatment of large numbers of infected animals or birds by antibiotics is infrequent, sacrifice of the exposed herd or flocks is a preventative solution. Mollicutes also cause commercial losses as pathogens of corn and citrus; their infection of pollinating bees has a high mortality. They are ubiquitous and some are capable of an intracellular existence; they are relatively difficult to eradicate from host tissues.

To understand Mollicutes more fully, we have studied their metabolism in cell-free culture (100). This review continues and expands upon that report but emphasizes Mollicutes amino acid, purine, pyrimidine biochemistry; it primarily correlates enzymatic activity and gene annotations in order to obtain a more complete picture of their metabolism that could be derived from either data base alone. In this manner, a "consensus metabolism" is presented here for the first time. A companion and earlier article have dealt predominantly with Mollicutes carbohydrate metabolism and their respiration (92,100).

We believe that the study of Mollicutes metabolism requires the combination of enzymatic, annotation, gene expression and structural data. The "combinatorial" approach serves two purposes: 1) it validates putative and individual observations that may be derived from only one data base, *i.e*., they are mutually supportive. This occurs, for example, when a gene (Gene C) is annotated in an otherwise recognized metabolic sequence known to contain say 5 components, A, B, C, D and E. In this scenario, enzymatic assays had demonstrated the presence of components A and E. Combining the data suggests more strongly that the entire pathway is present and functional. 2) Further, the reliability or confidence in the annotation of Gene C itself is improved by virtue of the fact that the metabolically linked activities or their genes (*i.e*., A and E) are also known.

In order to compensate for an apparently limited coding capacity and the absence of "essential housekeeping" enzymes, *e.g*., nucleoside diphosphate kinase (NDPK), Mollicutes have a number non-specific enzymatic activities (glycolytic kinases) that apparently replace some or all of the missing functions of NDPK (96). Such "genomic economy" in a small genome may spare both any coding restraint and the elaboration of additional proteins. Genomic economy of another sort is represented by the apparent presence of a multienzyme (single) protein that can carry out more than one enzymatic function, *i.e*., the lactate/malate dehydrogenase (LDH/MDH) protein of *Mycoplasma* spp. (23,24,92). These observations and hypotheses are cautionary in relation to the reliability and accuracy of enzymatic or metabolic predictions (90).

3. METABOLIC LINKAGES TO AMINO ACIDS AND PROTEINS

The study of amino acid and protein metabolism in Mollicutes is associated with the development of chemically defined or semi-defined growth media. Unfortunately, there have been few successes and scant continued study. The circumstances are most probably related to the expense of producing large quantities of such media and the relatively poor growth yields of some of the putatively more "interesting" organisms, *i.e.*, the human pathogens: *M. pneumoniae*, *M. genitalium* and *U. urealyticum*.

Smith (123) and Rodwell (109-111) and Miles (74) in their reviews, among others, on the nutrition of the Mollicutes described a synthetic medium (Medium E) for *M. mycoides*, strain Y, lacking protein, *e.g.*, containing no BSA or casein digests. Tourtellotte *et al*. developed a synthetic medium for *Acholeplasma. laidlawii* B described in reference 110. Synthetic media for *Spiroplasma* spp. (20,48) and *Anaeroplasma intermedium* (Medium D, with amino acids, vitamins, and phosphatidylcholine) have also been reported (108).

Metabolic studies are complicated by the necessary culture of Mollicutes in undefined complex-protein rich media (references are cited in 35,73,110). The complications involve the incompletely defined nutritional needs of the organism and the less appreciated possibility that some "too-rich" media formulations are inimical to optimal growth. Gardella and DelGuidice and others emphasized that some Mollicutes are sensitive to inhibitors present in conventional protein-yeast-serum rich Mollicutes media (43,119).

The reported amino acid requirements for the growth of various Mollicutes have been listed in detail (35). Other studies document the role of Mollicutes proteases that cleave growth medium proteins to small peptides and amino acids that then become available for transport (1,19,120,137). Miles has reviewed protease activities reported in Mollicutes (75). Various peptidase activities possibly related to arthritic disease were reviewed by Gabridge *et al.* (38). Various proteases and peptidases have been putatively annotated in *M. pneumoniae*, *M. genitalium*, *M. pulmonis* and *U. urealyticum* (18,26,37,45,50). Regula *et al*. (107) identified a protease in proteomic expression studies of *M. pneumoniae.*

4. AMINO ACIDS: TRANSPORT

Genomic or enzymatic evidence of amino acid transport or synthesis in Mollicutes is limited. The apparent absence of genes for the synthesis of amino acids suggested that there would be a large number of transporters, but such expectations in *M. genitalium* and *M. pneumoniae* were not confirmed by annotation, although histidine, glutamine, oligopeptide and general amino acid permease transporters were annotated (37,50). In *M. pulmonis*, there is an annotated arginine-ornithine antiporter (*pot*E, NT02MP1513) (18). Wasinger *et al*. (136) identified an oligopeptide transport permease in proteomic expression studies of *M. genitalium*. Five amino acid permeases and a protease were putatively annotated in *M. pneumoniae* (26). Presumed or apparent insufficiency has suggested to some authors that amino acid transport is associated with

transporters that are not specific (106), an opinion resonant with our view of genomic economies in Mollicutes.

Paulsen *et al*. reported the identification of 2 or 3 secondary transporters of the amino acid-polyamine-organocation family (APC), in *M. genitalium* and *M. pneumoniae* (87). These authors also identified the presence of members of the MPS and ABC transporter families. These transporters are involved in the transport of a variety of compounds that speculatively may include amino acids. The APC transporter identified in *M. genitalium* as a conserved hypothetical protein (MG226) is also speculated to have low amino acid transport specificity (I. Paulsen, personal communication). There are only a few *in aqua* studies on amino acid transport, and they involve histidine and methionine (103,134).

5. AMINO ACIDS: BIOSYNTHESIS AND METABOLISM

The enzymes involved in the synthesis of amino acids in *M. genitalium* and *M. pneumoniae* are believed absent, but this may be an exaggerated opinion. In *M. pneumoniae*, a fermentative non-arginine utilizing Mollicutes, the genes coding for enzymes that synthesize citrulline and ornithine in the process of catabolizing arginine were annotated: carbamate kinase (MPN307) (*arcC*); ornithine transcarbamylase (MPN306) (*argl*); and three arginine deiminase genes (MPN305, MPN304 and MPN560) (*arcA*) (26,50,107). Of these, only MPN560 (arginine deiminase) was identified as translated following proteomic analysis (2-D gel-electrophoresis and mass spectroscopy) (107). However, no enzyme or activity associated with these putative genes has been reported in *M. pneumoniae* (6,50,107). These genes have not been annotated in *M. genitalium* or *M. pulmonis*. In *U. urealyticum*, it was reported that synthesis of citrulline does occur; the authors suggested that ornithine transcarbamylase and carbamoyl phosphate synthase activities are present, however, no annotation for these genes in this *Ureaplasma* have been reported (45,121).

Salih *et al.* examined the isoenzymes of the fermentative non-arginine utilizing (hydrolyzing) *A. laidlawii* (10 strains), *Acholeplasma granularum* and *Acholeplasma oculi* by horizontal starch gel electrophoresis (115). They reported 12 enzymes that had not been previously recognized in acholeplasmas, including: arginase, arginine deiminase and carbamyl (*sic*) phosphor-kinase. They did not detect ornithine transcarbamylase (OCT) and considered that the inability of acholeplasmas to utilize arginine resided in the absence of OCT activity. No genomic annotations for these activities in acholeplasmas have been reported. The 46 kDa arginine deiminase isolated from *Mycoplasma orale* is reported as unusually resistant to proteinase K (16). The presence and activity of the arginine deiminase pathway in Mollicutes deserves more study.

Parenthetically, the arginine deiminase enzyme from mycoplasmas and arginine have been studied for their roles as anti-tumor agents, inhibitors of human mononuclear proliferation, ability to arrest cell cycle in G1, ability to induce apoptosis, and the inhibition of the proliferation of human leukemia cells (28,47,60,125). As arginine deiminase inhibits the proliferation of human leukemia cells, it has been directly suggested as a possible therapy for leukemia (46). The relationship, if any, of Mollicutes and these *in vitro* properties to pathologic processes or mechanisms or any form of treatment is either untested or unknown.

The metabolism of methionine is relevant to the pathogenicity of *M. genitalium*. Dhandayuthapani *et al*. (31) described studies of the anti-oxidant repair enzyme methionine sulfoxide reductase. An *M. genitalium* mutant was incapable of growing in hamsters and was hypersensitive to H_2O_2. The response was apparently due to the mycoplasma's inability to reduce deleterious methionine sulfoxide to methionine. The enzyme is considered to protect mycoplasmal proteins from oxidative damage and is characterized as a virulence determinant.

Glutamate dehydrogenase (GDH) activity was reported in Mollicutes (119, 133), but not in *U. urealyticum* (121) or annotated in *M. pulmonis* (18). GDH mediates the oxidative deamination of glutamate and NAD^+ or $NADP^+$ to a-ketoglutarate (2-oxoglutarate) (AKG) and NADH or NADPH and ammonia. The synthesis of AKG is perhaps of considerable import. The reaction is reversible. In the oxidative mode towards AKG the reaction leads to the synthesis of pyruvate and ATP, in the opposite reductive direction it leads to glutamate with the reoxidation of NADH or NADPH to NAD^+ or $NADP^+$ this occurs without the production of lactate (100). Of additional and equal importance, these reactions may be both a locus where intracellular levels of $NAD^+/NADP^+$ are maintained when lowered oxygen levels limit NADH oxidase activity and also act as a source of NADPH for those Mollicutes lacking the oxidative portion of the pentose phosphate pathway. The limiting factor might be ammonia.

Glutamate has a significant role in other biochemistries and may have as well in the Mollicutes. Glutamate by a number of different paths, some short, is convertible to arginine, ornithine and proline. These amino acids are characterized as the glutamate-family. Further, glutamate is a reactant in other known biosyntheses, *e.g.*, the synthesis of serine from G3P; in other cells, serine is convertible to glycine. In *M. genitalium*, Bang *et al*. have reported that the peptidyl-prolyl *cis*/*trans* isomerase activity catalyzes the isomerization of prolyl-peptide bonds and can act as a chaperone (5). They stated that this is Mollicutes' only folding isomerase enzyme activity, the "trigger-factor" (5).

In *M. genitalium* and *M. pulmonis*, there is a putative gene for the folate dependent enzyme that mediates the conversion (synthesis) of serine and glycine: serine [glycine] hydroxymethyl transferase, *glyA* (18,37). In other cells, the enzyme is also known to catalyze the conversion of glycine with acetaldehyde to threonine. In *U. urealyticum*, an asparagine synthase (UU363, *asnA*) and an S-adenosylmethionine synthase (*metK*) are annotated (45). Asparagine synthase mediates the conversion of aspartate

and glutamine + ATP to glutamate + PPi and asparagine. *U. urealyticum*, coincidently, has pyrophosphatase activity (27) that may drive the reaction by favorably converting the PPi to 2 Pi. However, expression of or enzymatic activities for these examples are not reported in Mollicutes.

A cysteine desulfurase (CD) gene (*nifS*) has been annotated in *M. genitalium* (MG336), *M. pneumoniae* (MP355), *M. pulmonis* (MYPU1720) and *U. urealyticum* (UU454). This "nitrogen fixing protein"-class V, is a pyridoxal phosphate transferase and in other cells catalyzes the removal of sulfur and selenium atoms from L-cysteine, L-cystine and L-selenocysteine to produce L-alanine (83,145). The transference of S is involved in the formation of Fe/S metalloclusters (with *nifU*), selenide delivery and the biotin synthase reaction (63,60,83). CD has not been directly studied in Mollicutes. There is a report suggesting the presence of Fe/S centers in some Mollicutes including *M. pneumoniae* FH and *M. pulmonis* JB (95).

There is more direct evidence for amino acid synthesis or interconversion in Mollicutes. Transamination has been documented in *Acholeplasma*, *Anaeroplasma* and *Mycoplasma* species (66,115 and as cited in 35 and 122). Only alanine and aspartate transaminases have been detected. Fischer *et al*. depict a role for transaminase activity in *Acholeplasma* that primarily involves the synthesis of the aromatic amino acids tyrosine and phenylalanine (35).

6. AMINO ACIDS: AROMATIC AMINO ACID SYNTHESIS IN *ACHOLEPLASMA-ANAEROPLASMA*

Berry *et al.* (10) were the first to describe the synthesis of aromatic amino acids in Mollicutes, they studied *A. laidlawii*, and later, others described aromatic amino acid synthesis in species of *Anaeroplasma* (88). Fischer *et al.* describe in some detail the *de novo* synthesis of aromatic amino acids in these Mollicutes and consider that *Spiroplasma melliferum* might also be similarly capable (35). No enzymatic activity of this pathway is reported in any other Mollicutes; neither was gene annotation for any enzyme of this pathway reported in any Mollicutes.

The biosynthetic pathway to tyrosine and phenylalanine is also called the shikimate (or shikimic acid) pathway (17). Fischer *et al*. emphasized the following distinguishing features of the Mollicutes metabolic sequence when compared to other cells, in Mollicutes: 1) the dehydroshikimate reductase utilizes only NADH rather than NADPH, 2) phenylalanine synthesis occurs by the phenylpyruvate route and 3) there are unique patterns of allosteric control on the activity of DAHP synthase (35). DHAP synthase is the first reaction in the pathway and requires G3P and erythrose-4-phosphate (E4P) as reactants to form the 7-carbon product (3-deoxy-D-*arabino*-heptulosonate 7-phosphate) that continues towards the *de novo* synthesis of tyrosine and/or phenylalanine. The requirement for E4P indicates the involvement of the pentose phosphate pathway (see above) and the activities of its transaldolase and its enzymatic antecedents, activities also reported in *Acholeplasma* and *Anaeroplasma* spp.

In effect, Mollicutes are able to synthesize or interconvert some amino acids. More precise knowledge of the source of Mollicutes nitrogen is needed.

7. PURINE METABOLISM: TRANSPORT

Mollicutes salvage and interconvert but do not synthesize *de novo* purines or pyrimidines. The source of their nucleic acids has been reviewed extensively (34,100). A current view holds that Mollicutes nucleic acid metabolism relies on the import of nucleosides and nucleobases (less likely nucleotides) and perhaps small oligonucleotides. The major source of these components, particularly nucleobases, is nutritional. Individual or combinations of ingredients such as yeast extract, yeast dialysate, RNA, DNA and oligonucleotides supply the precursors necessary for Mollicutes growth. Nutritional studies involving purines pioneered by Razin and co-workers led to the development of more defined media (102,104,105). Rodwell (109,111), as noted before, described two synthetic (completely defined) media, one for *A. laidlawii* and another for *M. mycoides* subsp. *mycoides*, in the latter case, he demonstrated that the combination of guanine, uracil and thymine were sufficient to act as nucleic acid precursors.

It is also assumed that the oligonucleotides and nucleic acids in media are processed by Mollicutes nucleases that are surface-bound, transported or released from injured or dying cells. The nuclease action produces smaller transportable units (perhaps, monomers) that can be assimilated by growing cells. There is experimental support for these possibilities (57,76,93,105). The calculation that 37% of the genome of *M. pneumoniae* codes for putative exported/secreted proteins seems relevant (114).

Mollicutes nucleases may have pathogenetic potential. Paddenberg *et al*. (85) suggested that internucleosomal DNA fragmentation in a human pancreatic adenocarcinoma cell line was caused by *Mycoplasma hyorhinis* endonucleases. They later showed that the cell free purified nuclease of *M. hyorhinis* led to the internucleosomal DNA-degradation (84). Working with the invasive *Mycoplasma penetrans*, Bendjennat *et al.* (8) found that a purified endonuclease(s) induced internucleosomal fragmentation of chromatin, they suggested that such ability may contribute to host cell DNA degradation and act, therefore, as a potential pathogenic determinant. The same authors reported that *in vitro* studies of the purified endonuclease ("P40") at 10^{-7} M was lethal to cell lines (LD_{100} at 24 hr); they re-emphasized their belief in the major role of mycoplasmal nucleases as potential pathogenic determinants (9).

Regardless of the source or supply of adequately sized nucleic acid precursors, there is a significant body of enzymatic evidence, reviewed primarily for *M. mycoides* subsp. *mycoides* by Finch and Mitchell (34), that some phosphorylated nucleosides are transported by Mollicutes. In most other cells, nucleotides are dephosphorylated to nucleosides presumably by phosphatase or nucleotidase-like activity before uptake. However, in *M. mycoides* subsp.

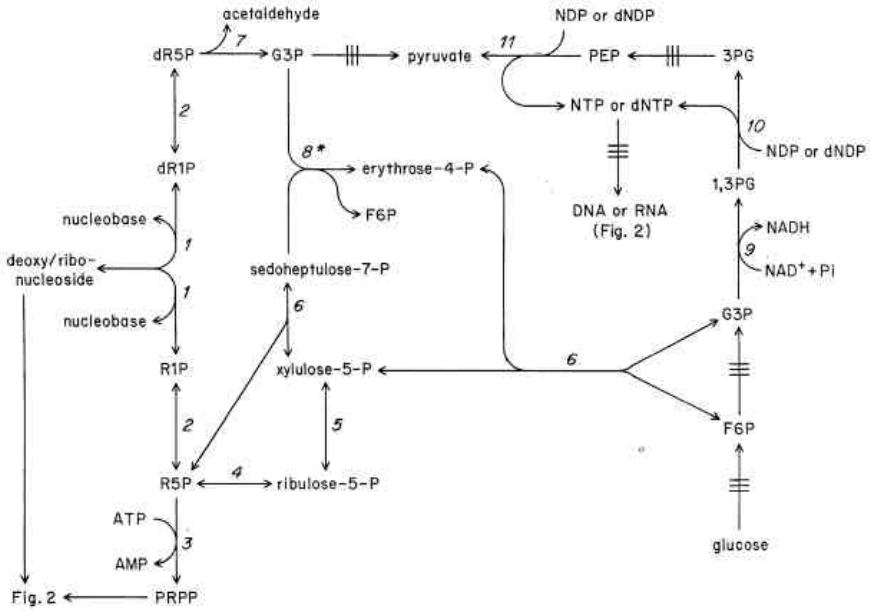

Figure 1. Proposed consensus purine precursor-pentose phosphate-glycolysis pathway for *Ureaplasma urealyticum* (*parvum*), *Mycoplasma pneumoniae* M129 and FH, and *Mycoplasma genitalium* G37. Reactions: 1. ribo- and deoxyribo- hypoxanthine and guanine phosphorylase, 2. phosphopentomutase, 3. ribose phosphate pyrophosphokinase, 4. ribose-5-phosphate isomerase, 5. ribulose-5-phosphate 3-epimerase, 6. transketolase, 7. deoxyribose-5-phosphate aldolase, 8. transaldolase, 9. glyceraldehyde-3-phosphate dehydrogenase, 10. phosphoglycerate kinase, 11. pyruvate kinase. Modified after Pollack (91).

mycoides, the mononucleotide phosphoryl group is transported attached to the nucleoside and enters the cell as a typical nucleoside monophosphate (NMP) (81). This system, unique for Mollicutes, mediates the transfer of the four deoxy- and ribo- NMPs. It was suggested by Neale (cited in reference 34) that *M. mycoides* subsp. *mycoides* additionally possessed a nucleoside transport system. Convincing identification by putative annotation or expression of nucleic acid precursor transporters in *M. genitalium*, *M. pneumoniae* and *U. urealyticum* is limited. Paulsen *et al*. (87) did not identify any specific nucleoside or nucleobase transporter of the 20 or 22 total identified in *M. genitalium* or *M. pneumoniae*, respectively. However, 11 ABC and one MFS primary active transporters were identified in each species, as these are involved in the transport of a wide variety of molecules (see, Section 4), they may conceivably be involved in nucleic acid precursor (purine and pyrimidine) transport. Speculatively, the MFS transporter identified in *M. genitalium* as a conserved hypothetical protein (MG294) and members of the ABC system (MG289, MG290, MG291) may be involved in nucleic acid precursor transport (I. Paulsen, personal communication). Chambaud *et al*. (18) have annotated 83 transport and binding proteins in the genome of *M. pulmonis* (<http://genolist.pasteur.fr/MypulList/>).

An earlier study by McIvor and Kenny (71) is particularly relevant. These authors reported the transport of nucleobases and nucleosides into growing cells of *M. pneumoniae* (2 strains), *Mycoplasma putrefaciens*, *Mycoplasma gallisepticum*, *Mycoplasma hyorhinis*, *Mycoplasma arginini*, *Mycoplasma hominis*, a bovine isolate and *A. laidlawii*. Adenine, guanine and uracil were incorporated by all, cytosine by none. Thymine was incorporated by the bovine strain and *M. pneumoniae*, and its incorporation was stimulated in other strains by the presence of deoxynucleosides. Arginine utilizing species, the non-fermentors *M. hominis* and *M. arginini*, did not incorporate nucleosides (guanosine, adenosine, thymidine, uridine and cytidine) the other Mollicutes did (71).

However, experimental evidence of transport from the media is limited. There are no homologues in *M. genitalium* or *M. pneumoniae* for the secondary transporters of the nucleobases:cation symporter-1 or -2 families, or nucleoside uptake permease family, *i.e.*, NCS1, NCS2, NUP, respectively (87). There are no other known nucleoside/base transporter families in these mycoplasmas, channel system proteins seem unlikely. Further, other than components of the PTS-system proteomic analyses have not revealed any proteins ascribed to purine-pyrimidine transporters (107,136).

8. PURINE METABOLISM: INTERCONVERSIONS

In Mollicutes the metabolism of nucleic acid precursors are characterized as interconversions rather than syntheses. Their composite pathways are a series of "salvage" pathways. The pathways are terminated by the synthesis of the deoxyribo- and ribo- purine and pyrimidine trinucleotides required for DNA and RNA syntheses. They are "rooted" in glycolysis and the pentose phosphate (shunt) pathway (PPP) and linked to them by key enzymes as, *e.g.*, PRPP synthase, phosphopentomutase and non-specific (promiscuous) phosphorylases and kinases (Figure 1). Parenthetically, pathways mediated by enzymes with

crucial linkages to various metabolic routes and with broad specificity are indicative of ancient origins (7,55,65). The convolutions of biochemical pathways involving purines and pyrimidines in different cells are complex (73,77).

Mollicutes purine and pyrimidine metabolism is apparently simpler with significantly fewer components. Figure 1 describes the involvement of glycolysis and the pentose phosphate pathway. Figures 2 and 3 describe more explicitly purine and pyrimidine salvage, respectively. In particular emphasis, Figures 1 and 2 include two newly and significantly effecting observations: the putative substitution of nucleoside diphosphate kinase activity by components of glycolysis (96) and hitherto unknown aspects of deoxyribonucleoside metabolism (135).

The purine and pyrimidine pathways of Mollicutes have been described and diagrammed in detail (34,74,100). However, those studies were based almost entirely upon *in aqua* assays that left equivocal gaps in our understanding since not every prospective enzymatic step was or could be demonstrated. Many of these apparently missing steps were identified (annotated) in genomic, proteomic and structural reports (37,45,50,51,135). The combination of these findings with the enzymology data lead us to a consensus model, although not entirely proven, that not only lends support to the probable enzymatic expression of each of the annotated genes but also the presence and activity of the complete pathway. It is hypothesized that this "consensus" model (Figure 1 and also 2 and 3) may be an accurate representation of the "functional" *in vivo* metabolism of the human Mollicutes.

9. PURINE METABOLISM: INTERVENTION OF THE PENTOSE PHOSPHATE PATHWAY AND GLYCOLYSIS

A consensus analysis of the "mix" of purine-precursor-pentose-phosphate-glycolysis and metabolism is shown in Figure 1 and is patterned after a *U. urealyticum* report (91), but is significantly modified. (Note: In the publication cited as reference 91, in its Figure 2 the reaction leading from NMPs to nucleosides is mislabeled, it should be labeled 5'-nucleotidase).

The hypothetical model in that study is now extended here (Figure 1) to include the fermentative *M. pneumoniae* and *M. genitalium.* However, this Figure 1 probably cannot be taken as representative of *Acholeplasma laidlawii* and certainly not of the non-fermentative Mollicutes, *e.g.*, *Mycoplasma arginini.*

To prepare Figure 1, Reactions #1-10 were recorded in a Worksheet, similar to Table 1 for pyrimidine metabolism, to be discussed later, as either enzymaticaly detected or annotated or both in the three human Mollicutes (*M. genitalium*, *M. pneumoniae* and *U. urealyticum*) using many references cited in 91 and 100 and TIGR-Comprehensive Microbial Source, COG, ExPasy and TrEmbl data bases. Some enzymes have been enzymaticaly detected or annotated in only one or two of the three Mollicutes species, *e.g.*, phosphopentomutase was only detected enzymaticaly in *U. urealyticum* and annotated (fragment) in *M. pulmonis.* (*M. pulmonis* is not included in Figure 1 because of the absence of sufficient enzymatic data). Also, R5P isomerase, ribulose-5-phosphate epimerase, and transaldolase were neither detected nor annotated in *U. urealyticum*, similarly G3P dehydrogenase, in *M. pneumoniae*. Nevertheless, Figure 1 presently assumes that these "missing" activities are operative in the three human *Mycoplasma* spp. by virtue of their annotation or the enzymatic presence of recognized metabolic connecting steps in at least one them and knowledge of their compatible metabolic activities. The most problematic steps in the scheme that must be viewed speculatively are the reactions for transaldolase (Reaction #8) and with less doubt the phosphopentomutase (Reaction #2). Noteworthy reactions are #2, 3, 8, 10 and 11 and require comment.

The enzyme of reaction #2 in Figure 1, phosphopentomutase, presumably mediates the interconversion of D-ribose 1-phosphate and D-ribose 5-phosphate and their deoxyribose forms. In effect, its activity serves or can serve to connect glycolysis, transamination, pentose phosphate, purine, pyrimidine metabolism (not pictured in Figure 1) and DNA and RNA metabolism. Phosphopentomutase activity has not been reported in *M. pneumoniae* and *M. genitalium* but was reported in *U. urealyticum* (21). *The* enzyme was not annotated in the three human *Mycoplasma* spp. but was in *M. pulmonis* (18).

In these Mollicutes the absence of the oxidative portion of the pentose phosphate pathway (91) leading to ribulose-5-phosphate and subsequently R5P, requires then that (d)R5P must arise from (d)R1P by the action of phosphopentomutase. Hence, its presence (activity) here is strongly assumed although not proven.

Reaction #3 (ribose phosphate pyrophosphokinase or PRPP synthase) in Figure 1 was enzymaticaly detected in *M. pneumoniae* FH and *A. laidlawii* (unpublished studies noted in reference 100), its gene was annotated in *M. pneumoniae* (MPN073) (50), *M. genitalium* (MG058) (37), *U. urealyticum* (*parvum*) (UU193) (45) and *M. pulmonis* (MYPU1270) (18). PRPP synthase was identified in proteomic analyses of *M. pneumoniae* (107). PRPP synthase mediates essential activities that are presumed to be present in all Mollicutes; linking the pathways in Figure 1 to the phosphoribosyltransferase activities of purine metabolism in Figure 2, that then feed back to components in Figure 1.

The inclusion of reaction #8 the transaldolase (TA) in Figure 1 is problematic. TA has not been annotated in any Mollicutes. Activity has been detected in only one direction in extracts of *M. pneumoniae* and *M. genitalium* (29). However, these experimental findings may be misleading. Although enzymatic activity is clearly present, a homologue of any of the near 400 *bona fide* transaldolases cited in various public data bases was not found. Therefore, a TA protein with sequence similarity or identity to established TAs may be absent in these *Mycoplasma* spp. I speculate that the aldol cleavage mediated by "classical" TA, if indeed necessary for the mycoplasmal path, may be conducted by another protein with unrecognized multifunctional activity or by a protein

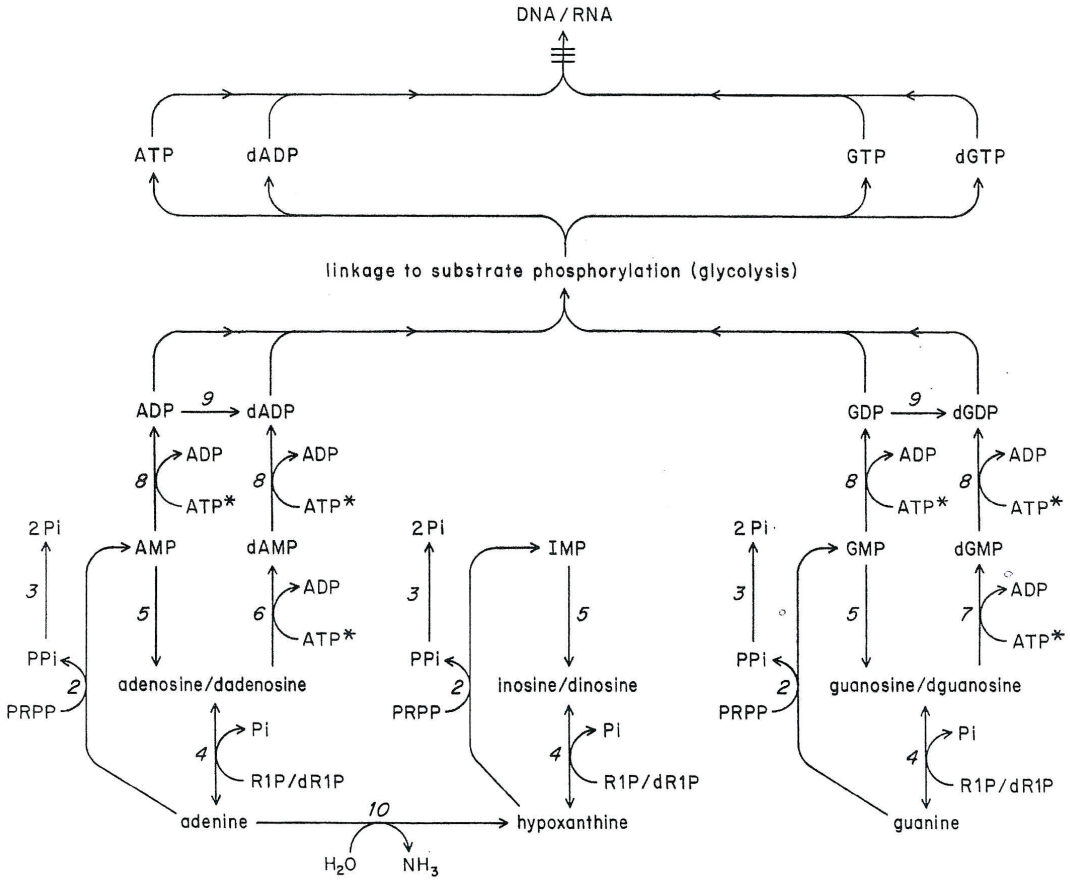

Figure 2. Proposed consensus purine salvage pathway for *Ureaplasma urealyticum* (*parvum*), *Mycoplasma pneumoniae* M129 and FH, and *Mycoplasma genitalium* G37. Reactions: 1(2). adenine phosphoribosyltransferase, 2. hypoxanthine-guanine phosphoribosyltransferase, 3. pyrophosphatase, 4. purine phosphorylase(s), 5. 5'-nucleotidases, 6. adenosine kinase, 7. deoxyguanosine kinase, 8. adenylate-guanylate kinase(s), 9. nucleoside diphosphate reductase, 10. adenine deaminase. *Perhaps other deoxyribo- or ribo- NTPs may substitute for ATP (see text).

whose sequence shows little or no homology to recognized TAs. TA-like activity is necessary, otherwise it is quite unclear how the sequence that involves sedoheptulose-7-P and G3P to yield E4P and F6P occurs. TA enzyme activity was reported in extracts of other Mollicutes genera: Acholeplasma and Anaeroplasma spp. (29,89).

Reaction #10 is the phosphoglycerate kinase (PGK) and Reaction #11, the pyruvate kinase (PK) of glycolysis (92,96); their activity is an integral part of the hypothetical scheme. PGK and PK enzyme activities are reported in all Mollicutes and annotated in the four human species whose genomes have been sequenced. The reaction of both towards ATP and pyruvate is exergonic and thermodynamically favorable. The reactions are generally described as requiring ADP and either PEP for the PK or 1,3-bisphosphoglycerate for PGK activity. It is recently discovered that the activities of both of these kinases and 6-phosphoglycerate kinase (which could also have been included in Figure 1) can proceed without the involvement of ADP (96). ADP can be replaced by a variety of purine and pyrimidine deoxyribo- and ribo- nucleoside diphosphates resulting in the synthesis of the concomitant trinucleotides. The reactions are not classifiable as examples of non-orthologous gene displacement since the proteins and their reaction are both different. This property may be a fortuitous characteristic of the human Mycoplasma spp., perhaps others, since they lack an annotated gene for the presumably essential nucleoside diphosphate kinase (NDPK) activity that mediates the phosphorylation of dinucleotides to trinucleotides. In further support of this hypothesis, attempts to demonstrate any NDPK activity in M. pneumoniae have failed (96). The scheme (Figure 1), if confirmed, links glycolysis, purine (and pyrimidine) salvage and the pentose phosphate pathway, supports the existence of multifunctional or "replacement" or "substitute" enzymes and the concept of genomic economy in small genome bacteria that is personified by the Mollicutes.

These observations do not preclude a role for ribonucleoside di- or tri- phosphate reductase activities (EC 1.17.4.1-2). The diphosphate gene has been annotated in *M. pneumoniae* (*nrdF*) and *M. pulmonis* (MYPU5410). If this enzyme is active it may equally contribute to the production of deoxyribonucleoside diphosphates from their ribo- forms.

The conjecture that activities of glycolytic kinases replace the missing NDPK function in *Mycoplasma* spp. may be related or supportive of the "patchwork" hypothesis regarding the genesis of metabolic pathways (55,143). The "patchwork" hypothesis suggests that metabolic pathways were organized by the association of primitive non-specific enzymes, that is, those capable of reacting with many substrates. Such enzymes would enable primitive cells to circumvent primordial coding limitations. Changes in the gene and enzymatic function occurring in subsequent duplications resulted in a family of homologous enzymes with different less broad specificities. The relationship of this hypothesis to our preliminary observations on the replacement-activity of glycolytic kinases in *Mycoplasma* spp. is unclear. No inference is intended that *Mycoplasma* cells are "primitive". Their relatively small genomes and theoretical calculations suggested that only a maximum of ? of their DNA codes for functional genes (52). The supposed constraint may impose metabolic limitations that require, lead or force the development of genomic economies. Our working hypothesis is that most likely some or all missing NDPK functions in *Mycoplasma* spp. is/are replaced by the activities of their glycolytic kinases.

Reactions leading from PEP to carbohydrate transport, and from pyruvate to acetylCoA and acetate or oxaloacetate and then aspartate have been reported in Mollicutes and in some cases the three human species emphasized in this report. These reactions are not included in the Figure 1. A more detailed review of these aspects of Mollicutes metabolism is found elsewhere (92).

The combination of enzymatic, genomic and other metabolic data suggest that much if not all of the path in Figure 1 is operative in *M. genitalium*, *M. pneumoniae*, *M. pulmonis* and *U. urealyticum* (*parvum*).

10. PURINE METABOLISM: METABOLIC CONSENSUS

Metabolic consensus figures, as those in Figs. 1 and 2, represent a first approximation of the general metabolic features by which these three pathogenic Mollicutes metabolize purines and pentoses with the involvement of glycolysis. Figure 2 in particular is a consensus pathway for purine-salvage metabolism in: *U. urealyticum* (*parvum*), *M. pneumoniae* and *M. genitalium.* Figure 2 is much altered from an earlier version studying only *U. urealyticum* (91).

Figure 2 was constructed after selective combination of genomic, expression, structural and enzymatic data. The analyses included over 20 Enzyme Commission (EC) proteins identified as purine salvage enzymes in the three human pathogenic Mollicutes and the gene annotations of *M. pulmonis*. The components of Figure 2 were selected by virtue of the unanimity of the available data for these Mollicutes and their apparent "metabolic sense" as it appears to this analyst. However, the scheme does not mean to infer completeness. There may be other unrecognized activities of equal importance not yet included.

The number of enzymatic reactions comprising the combined enzymatic-annotation-expression hypothesis represented by Figure 2 is about 12-30 activities. The number depends on whether one believes, *e.g.*, that the 11-12 activities identified in these three human Mollicutes as purine nucleoside phosphorylases are conducted by separate enzymes or more likely by very few with broad specificities (69,100). In this presentation my position is that Mollicutes have a distinctively rich collection of multifunctional or substitute proteins. They have enzymes accepting a variety of related substrates for reaction (non-specific enzymes), *e.g.*, a single protein with dehydrogenase activity acting upon both lactate and malate, the gene and enzyme identified as LDH (24). Other examples are: the nucleoside phosphorylase(s) acting upon a variety of deoxyribo- and ribo- purine and pyrimidines and their respective nucleosides and various 5´-nucleotidase(s) activities (69,100).

In Figure 2, Reactions #1 and 2 are adenine and hypoxanthine-guanine phosphoribosyltransferases (APRT and HGPRT), respectively. They were both annotated in each of the three human Mollicutes and *M. pulmonis*. The scheme suggests these PRTs are primarily responsible for the synthesis of ribomononucleotides, less so for deoxyribomononucleotides. PRT enzyme activity was reported in *M. genitalium*, *M. pneumoniae* and *U. urealyticum* (18,37,45,50,69).

Reaction #3, inorganic pyrophosphatase (iPPi-ase) activity is linked to PRTase activities and has been detected in *U. urealyticum*, annotated in *M. genitalium*, *M. pneumoniae* and *M. pulmonis* and expressed in *M. pneumoniae* (18,37,50,107). iPPi-ase activity is a relatively strong exergonic reaction (-33.5 ? Gº´) (54) that may be a major thermodynamic driving force of purine and pyrimidine salvage.

Purine nucleoside phosphorylase (PNP) (Reaction #4) activity has been reported for each of the three human Mollicutes with a variety of reactant nucleobases and nucleosides, the gene(s) has also been putatively annotated in these as well as in *M. pulmonis* (18, 37,50,107). Of unrecognized significance at the time of these reports was the observation that the phosphorylases were equally and surprisingly active in either direction and could use as substrates either dR1P or R1P and a variety of nucleobases to produce the deoxyribo- or ribo- nucleoside or in the "back" direction release the ribose from the nucleoside (69,70).

5'-Nucleotidase enzyme activity (Reaction #5) has been identified in the three human Mollicutes for each of three substrates: AMP, IMP, GMP (69). In *M. pulmonis* 5'-nucleotidase membrane activity and an annotated and cloned gene associated with this activity were reported (53,76). It is presumed that a few or a single protein with broad specificities mediates the 5'-nucleotidase reaction.

5'-Nucleotidase activity may have survival value related to deficiencies in nucleoside phosphate transport. However, the ability to transport purine NMPs into the three human Mollicutes is apparently absent, transport of the nucleoside is much more probable. Nucleotidase activity external to the cell would be of survival value because external non-transportable nucleotides could be converted to the more transportable nucleosides. Once inside the cell the nucleoside is deribosylated by phosphorylase in order to salvage R1P/dR1P.

The transport of monophosphate nucleotides (NMPs) has been reported in *M. mycoides* subsp. *mycoides* (81). This is not necessarily a conflict to the hypothesis here as this species is not a human pathogen nor am I certain that it is a *bona fide Mycoplasma* spp. having a variety of phylogenetic and metabolic affinities to another group of Mollicutes the spiroplasmas (100).

Johnson and Pitcher (57) have reported the presence of ecto 5'-nucleotidases on *Mycoplasma* spp. At pH 8.5, ecto 5'-nucleotidase activity was found on some strains of *M. fermentans* and a *M. pulmonis*. A non-specific phosphatase-like activity was found in *Mycoplasma buccale* and *U. urealyticum*, but no dephosphorylating activity was found in other *Mycoplasma* spp. that they studied including *M. pneumoniae* and *M. genitalium*. This is in conflict with the report of McElwain *et al*. (69) who found 5'-nucleotidase in washed cell extracts of these two *Mycoplasma* spp. The differences, presumably technical or related to localization, need resolution.

The findings of Jarvill-Taylor *et al*. (53) also emphasize the perimeter cellular location of 5'-nucleotidase. They reported the activity as membrane associated and apparently localized on the membranes' internal surface. In culture it is possible that internally localized nuclease and or protease activities are released into the medium and then act to convert inassimilable medium components to transportable ones.

An unannotated although metabolically related inosine nucleosidase activity has been reported in *U. urealyticum*, it was not annotated in the other three *Mycoplasma* spp. and it is not included in the consensus figure (100).

The genes and enzyme activities in Mollicutes, Bacteria and Eukarya associated with kinases and reactions #6 and 7 (deoxyriboadenosine (dAK) and deoxyribo-guanosine kinase (d)GuoK or dGK) are the focus of much study (4,33,77,135). Numerous reports and reviews have described the purine and pyrimidine kinase activities in different genera of Mollicutes and their obligatory requirements for either ATP or PPi as phosphate donors (69,70,89,94,98-100,135).

However, our primary interest concerns those Mollicutes whose genomes have been sequenced and annotated. The dGK gene (*dgk*) has been annotated in *M. pulmonis* (MYPU5520) (18) and *U. urealyticum* (UU086) (45). McElwain *et al*. (69) did not detect ribo- or deoxyribo- adenosine or guanosine kinase activity in dialysed 250K x *g* supernatants of hypotonically lysed or explosively decompressed *U. urealyticum*, *M. genitalium* and two strains of *M. pneumoniae*. In the same study we did detect phosphorylation of the four purine substrates using PPi but not ATP in extracts of *M. hominis* 1620 and *Asteroleplasma intermedium. Spiroplasma citri* extracts were also studied and had identical responses with PPi, further, extracts of *S. citri* like those of its relative *Mycoplasma mycoides* subsp. *mycoides* SC could additionally use ATP as a phosphate donor for the phosphorylation of deoxyriboguanosine in producing dGMP.

Contrary to our negative findings with the FH and M129 strains of *M. pneumoniae* (69), Wang *et al*. (135) discovered in 15K x *g* supernatants of lysed *M. pneumoniae* FH high levels of ATP-dependent dGK and deoxyriboadenosine kinase activity. This is a significant observation because it demonstrates a route to purine deoxyribomononucleotides not previously recognized and resolves some metabolic perplexities. The kinase activities they detected in *M. pneumoniae* FH indicate that deoxyribo- adenosine and guanosine are phosphorylated by ATP to yield dAMP and dGMP, respectively (Reaction #6 and 7). Subsequently, these monophosphates may further be phosphorylated to the respective diphosphate: dADP or dGDP (Reaction #8). Previously, the assumption was that the only recognized route from nucleobase flowed via the phosphoribosyltransphosphorylase to the NMP (Reaction #1), then to NDP by a kinase (Reaction #8) and then to dNDP by the mediation of a reductase (Reaction #9). This may still be so, however, the study of Wang *et al*. suggests another sequential route to dNDPs: Reactions #4, 6 and then 8. The combined data clarifies to some extent the roles of the purine phosphorylases, the path to the production of deoxyribo-nucleosides and emphasizes the existence of routes possibly reserved for the preferential synthesis of either ribo- or deoxyribo- mononucleotides but not both.

Wang *et al*. (135) also cloned and expressed the *M. mycoides* subsp. *mycoides* dGK-like enzyme in *E. coli*. The enzyme phosphorylated dAdo, dGuo and dCyd. They suggested that the active enzyme (a 50 kDa dimer) be named deoxyguanosine kinase. They consider that mycoplasmal dAK and TK activities support the presumably large dATP and dTTP pools required for the replication of the AT-rich mycoplasmal genomes.

Adenylate kinase (ADK) (reaction #8, AMP to ADP), guanylate kinase (GUK) (#8, GMP to GDP) have been annotated in each of the three Mollicutes but their enzyme activities have not been studied; in this ecuchemical approach they are included in the consensus Figure 1. GUK was reported as expressed in *M. genitalium* and *M. pneumoniae* (107,136). Studies with *Mycoplasma arginini* and *A. laidlawii* are discussed elsewhere (135).

There is no gene annotation in any of the four sequenced Mollicutes genomes for nucleoside diphosphate kinase (NDPK). This is the "household" enzyme supposedly essential and ubiquitous in nature that specifically phosphorylates NDPs to NTPs, like ADP to

ATP, GDP to GTP, CDP to CTP, or dCDP to dCTP, etc. It was initially assumed, without experimental data to support the opinion, that in the absence of NDPK that ADK and GUK (Reactions #8), presumably different proteins, substituted.

Recent studies suggest an alternate and entirely different scenario (96). We found that in crude or only partially purified extracts of *M. pneumoniae*, *M. genitalium*, *M. capricolum* subsp. *capricolum* and *M. fermentans* the kinases of glycolysis: pyruvate kinase, phosphoglycerate kinase and 6-phosphofructokinase could proceed without ATP-ADP. Their assays showed that a variety of ribo- and deoxyribo- purine and pyrimidine nucleoside diphosphates could substitute for adenylates. In extracts of each of four *Mycoplasma* spp. the products of their three kinases were the trinucleotides: ATP, dATP, TTP, GTP, dGTP, CTP and dCTP. It was postulated that these sources, and perhaps others, of trinucleotides replace the activity of the apparently absent NDPK. This hypothesis is included in Figure 2. The degree of uncertainty in our hypothesis is related to the possibility that purified (homogeneous) preparations of these twelve kinases would not exhibit the same broad specificity found in the cruder preparations used in the study. The findings strongly link glycolysis to purine and pyrimidine metabolism.

The presence of ADP/GDP reductase (rNDP reductase) enzyme activity (Reactions #9, ADP or GDP to dADP or dGDP) has not been reported. However, the gene(s) putatively coding for this activity is(are) reported in *M. pneumoniae* and *M. genitalium* and expressed in *M. pneumoniae* (37,50,107). In *U. urealyticum the* conserved hypothetical gene (UU176) may be a substitute for rNDP reductase and is discussed more fully in reference 91.

Reaction #10 represents adenine deaminase activity for which no gene has been reported in any Mollicutes and unverified activity only once in *U. urealyticum* (55). By this limited notice, the activity should not be included in Figure 2; however, it is the only recognized link between the hypoxanthine-IMP loop and the adenine-AMP or guanine-GMP loops in these three Mollicutes. In *in vitro,* IMP-inosine-HPX components have been identified. We have detected in all three Mollicutes: 1) purine phosphoribosyl-transferase enzyme activity (Reaction #2) using either HPX or guanine, 2) 5'-nucleotidase activity (Reaction #5) against IMP and GMP and 3) phosphorylase activity (Reaction #4) with inosine and guanosine (100). Enzymaticaly the IMP-inosine-hypoxanthine loop seems entirely functional, that is *in vitro*. Genes putatively coding for HGPRT and purine phosphorylase have also been identified in all three Mollicutes but no other associated genes have been annotated. Additionally, in *U. urealyticum* inosine nucleosidase (inosine to HPX + R1P) activity was reported, but no gene coding for this activity has been annotated in *U. urealyticum* or in any other Mollicutes.

However, there is no nutritional or any immediately recognizable role for IMP, inosine or HPX in these Mollicutes. This is the consequential point. Except for traces of activity in *A. laidlawii* (130) and only conjectured involvement of IMP in the synthesis of adenylosuccinate (67), the role of HPX, inosine and IMP in *U. urealyticum*, *M. pneumoniae* and *M. genitalium* metabolism is apparently minimal or negligible, likely it is absent. Hence, the omission of the entire IMP-inosine-HPX loop from Figure 2 on these grounds might be reasonable assuming the enzymology is correct. This issue also emphasizes the need to look beyond the immediate enzymatic assay or annotation and consider the likelihood of the metabolic role the component may or may not play.

In the Mollicutes represented in Figure 2 the apparent metabolic isolation of ATP and GTP synthesis accentuates the consequences of inhibiting either loop, for in so doing the purine-salvage synthesis of ATP and/or GTP would be impaired and reasonably effect growth. We have discussed the clinical ramifications of inhibiting purine salvage involving adenylate or guanylate on the growth of Mollicutes (91). As AMP and GMP can also be synthesized in mammalian host tissues by other routes it is conceivable that drugs inhibiting the APRT or HGPRT sites or the metabolism of nucleosides might be inimical to Mollicutes and less toxic if at all to the host.

Tham *et al*. reported the presence of genes in *Mycoplasma pirum* for purine phosphorylase (*deoD*) and deoxyriboaldolase (*deoC*) (128). *DeoC* was functionally identified by complementation in *E. coli* (128). In the same study, the genes were considered to be clustered with others suggesting an operon organization. The other genes (non-purine) were: thymidine phosphorylase (*deoA*), cytidine deaminase (*cdd*), phosphomannomutase (*cpsG*) and triose phosphate isomerase (*tpi*).

Related to the role of guanosine and noteworthy here is the report identifying in *M. genitalium* a gene coding for a tRNA-methyltransferase that mediates the synthesis of 1-methylguanosine-(m(1)G37) (11). The enzyme acts to maintain proper reading frame and is considered primordial, present before the “last-common-community” (LCC) a term preferable to last-common-ancestor. The authors consider it to be part of the minimal gene set and essential for all life.

In summary, the cellular pathway (Figure 2) to purine nucleoside triphosphates in the fermentative human *Mycoplasma* and *Ureaplasma* spp. is envisioned to begin as a sequence involving, in approximate order: extracellular dephosphorylation of medium nucleotides, possible deribosylation of nucleosides, the transport of nucleobases or ribo- or perhaps deoxyribo- nucleosides, the internal deribosylation of nucleosides to nucleobases (adenine, guanine), phosphorylation of ribo- and deoxyribo-adenosine and guanosine. Internal nucleobases may be ribosylated or deoxyribosylated to nucleosides by phosphorylase(s). Nucleobases may also be irreversibly converted to mononucleotides (AMP or GMP) and PPi by a pyrophosphate cleavage reaction involving PRPP. The reaction is driven towards the riboNMP by the thermodynamically favorable hydrolysis of PPi mediated

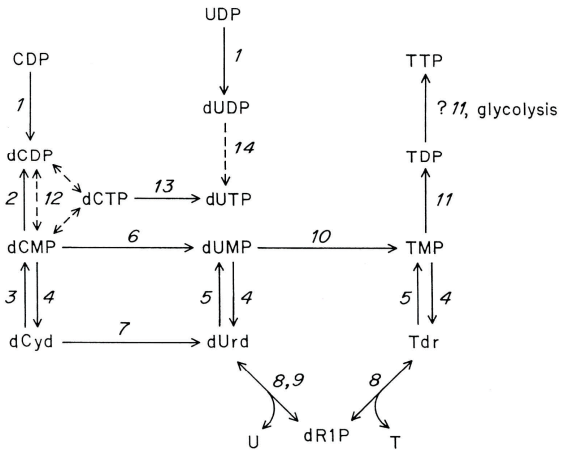

Figure 3. Proposed consensus pyrimidine deoxyribonucleotide pathway for *Ureaplasma urealyticum* (*parvum*), *Mycoplasma pneumoniae* M129 and FH, and *Mycoplasma genitalium* G37. Reactions:1. ribonucleoside diphosphate reductase, 2. nucleoside diphosphate kinase, 3. thymidylate kinase, 4. thymidine kinase, 5. deoxycytidine kinase, 6. 5'-pyrimidine nucleotidase, 7. thymidine phosphorylase, 8. uridine phosphorylase, 9. (deoxy)cytidine deaminase, 10. dCMP deaminase, 11. dCTPase, 12. dUTPase, 13. thymidylate synthase, 14. cytidylate kinase. Modified after Williams and Pollack (140).

by the pyrophosphatase. Ribo- or deoxyribo- nucleosides are also transformed to the respective ribo- or deoxyribo-mononucleotide by the action of specific ATP-dependent kinases. The "loss" of mononucleotides by the "back" phosphorolysis of NMPs to nucleosides mediated by 5'-nucleotidases is unclear. The (d/r)NMPs are convertible to (d/r)NDPs by kinase reactions. NDPs may be reduced to dNDPs. Speculatively, (d/r) NDPs are converted by kinases of glycolysis to the respective (d/r)NTP.

11. PYRIMIDINE METABOLISM: METABOLIC CONSENSUS

Mollicutes pyrimidine metabolism has been reviewed (34,138,140). A very detailed and thoroughly comprehensive figure has been offered by Finch and Mitchell depicting not only the individual components of most Mollicutes pyrimidine metabolism, especially *M. mycoides* subsp. *mycoides*, but also the activators and effectors of each reaction (34). We constructed a consensus pathway (pyrimidine ribonucleotide and pyrimidine deoxyribonucleotide) for *U. urealyticum*, *M. pneumoniae* and *M. genitalium* following the same analyses as for the purine-pentose phosphate consensus pathway described above but using as a pattern a relatively simpler diagram drawn for deoxyribonucleotide pyrimidine metabolism (140).

Table 1 is a representative Worksheet for the development of the consensus figure (Figure 3) for deoxyribonucleotide pyrimidine metabolism in *U. urealyticum* (*parvum*), *M. pneumoniae* and *M. genitalium*. The figure and table exclude some data for ribonucleotide pyrimidine metabolism that are reviewed elsewhere (34,140). When not specifically cited the enzymatic data are referenced in 100. In Table 1, the ✓ marked items are: #1 nucleoside diphosphate reductase, #2 dCMPkinase, #3 deoxycytidine kinase, #4 5´-nucleotidase, #5 thymidine kinase, #6 dCMP deaminase, #7 (d)cytidine deaminase, #8 thymidine phosphorylase, #9 (d)uridine phosphorylase, #10 thymidylate synthase and #11 thymidylate kinase. These activities are included in the consensus Figure 3 because each has been found in the Mollicutes spp. either by enzymatic assay or putative annotation and each can fit into a metabolically appropriate position in an otherwise recognized pathway. The unmarked items are problematic. They concern some nucleotide aspects of the cytidylate and particularly the uridylate metabolism of the Mollicutes.

Mycoplasma spp. are not known to synthesize *de novo* UMP, nor have the involved enzymes been annotated. The source for uridylates, except for scavenging or deamination of cytidylates is uncertain. In the general scheme shown in Figure 1, when UMP or CMP are available they may be phosphorylated by reported kinase activity to UDP or CMP and subsequently reduced to dUDP and dCDP (Reaction #1) by ribonucleoside reductase that has been annotated in *M. pneumoniae* and *M. pulmonis*.

The next generally envisioned and probable sequence for dUDP involves nucleoside diphosphate kinase (NDPK, the problematic Reaction #14). However, NDPK, as noted before, has not annotated in any Mollicutes and is undetectable in *M. pneumoniae* (96). Therefore, how is dUDP phosphorylated to dUTP? Perhaps glycolytic kinases could perform the phosphorylation of pyrimidinic (d)NDPs, as was already noted. However, the quantitative data presented in that unpublished study suggests that the level of pyrimidine nucleoside triphosphates synthesized might be too low. Even if the level of dUTP was "adequate" the absence of dUTPase reported by others (140) (the gap between dUTP and dUMP in Figure 3 and Reaction #15 in Table 1) in these Mollicutes spp. preclude the formation of the dUMP this way. dUMP is needed to synthesize TMP. Therefore, this sequence from UDP to dUMP is apparently absent.

Cytidylates generally arise in both Bacteria and Eukarya from uridylates by amination mediated by CTP synthase (Eq. 1), it is uncertain as to their synthetic origin in the Mollicutes, perhaps scavenging is involved.

$$\text{UTP} + \text{glutamine} + \text{ATP} + H_2O \leftrightarrow \text{CTP} + \text{glutamate} + \text{ADP} + \text{Pi} \quad \text{(Eq. 1)}$$

The conversion of dCDP to dCMP and the involvement of dCTP (Reaction(s) #12) are also uncertain. Presumably a kinase or a phosphatase is the effector. dCTPase activity is assumed leading to dCTP. The phosphorylation of dCMP to dCDP is known (Reaction #2). In an effort to unravel the nature of this locus and the source of dCTP we attempted but failed to detect any dCTP deaminase activity (Reaction #13) in *M. pneumoniae* and *M. genitalium* (unpublished data).

Table 1. Consensus Worksheet for Figure 3: A Partial List of Deoxyribonucleotide Pyrimidine Enzyme Activities and Gene Annotations Reported for *Ureaplasma urealyticum* (*parvum*) serovar 3, *Mycoplasma genitalium* G37, *Mycoplasma pneumoniae* M129 and *Mycoplasma pulmonis* UAB-CTIP

	Enzyme	Gene	U. urealyticum (parvum)		M. genitalium		M. pneumoniae		M. pulmonis[a]
			Enzyme activity	Annotation UU-(45)	Enzyme activity	Annotation MG-(37)	Enzyme activity	Annotation MPN-(50)	Annotation MYPU-(18)
1✓	Nucleoside diphosphate reductase	nrdE/ nrdF	NS[b]	NYA[c]	NS	231/229	NS	324/322	NYA
2✓	DeoxyCMP kinase	cmk	Yes	NYA	Yes	NYA	Yes	NYA	0890
3✓	Deoxycytidine kinase	NYA (cmk?)	Yes	NYA	Yes	NYA	Yes	NYA	NYA
4✓	5'Nucleotidase (pyrimidine)	NYA	NS	NYA	NS	NYA	NS	NYA	0550 (frag.)
5✓	Thymidine kinase	tdk	Yes	594	Yes	034	Yes	044	1450
6✓	DeoxyCMP deaminase	NYA	SNo	NYA	Yes	NYA	Yes	NYA	NYA
7✓	Deoxycytidine/cytidine deaminase	cdd	Yes	531	Yes	052	Yes	065	*0842[e]
8✓	Thymidine phosphorylase	deoA	Yes	368	Yes	051	Yes	064	*1475[e]
9✓	Uridine phosphorylase (deoxy-)	NYA	Yes	NYA	Yes	NYA	Yes	NYA	NYA
10✓	Thymidylate synthase	thyA	NS	NYA	NS	227	NS	320	5380
11✓	Thymidylate kinase	tmk	Yes	020	Yes	006	Yes	006	0520
12	DeoxyCTPase[f]	NYA	?[g]	NYA	?	NYA	?	NYA	NYA
13	DeoxyCTP deaminase	NYA	?	NYA	SNo	NYA	SNo	NYA	NYA
14	Unknown kinase activity	?	?	?	?	?	?	?	?
15	DeoxyUTPase	NYA	SNo(138)	NYA	SNo(138)	NYA	SNo(138)	NYA	NYA

[a]No enzyme activity has been reported for 1-15, [b]NS = not studied, [c]NYA = not yet annotated, [d]SNo = studied but no, activity detected, *[e]TIGR annotation: NTO2MP-*, [f]? = existence is questioned, ✓see text for explanation.

Figure 3 shows with more experimental confidence the interactions of dCMP and dUMP leading to thymidylates. The two pyrimidine nucleoside monophosphates may arise from dcytidine and duridine by the mediation of dcytidine kinase (dCK) and thymidine kinase (TK) (69,100,135,140) (Reactions #3 and #5). The reverse dephosphorylations are known through the action of 5´-nucleotidase(s) (Reaction #4), as are the two deaminases (reactions #6 and #7) leading to dUMP and duridine. Reactions #8 and #9 are phosphorylases involved in the reversible phosphodeoxyribosylation of uracil and thymine to duridine and thymidine with dR1P. Finch and Mitchell have convincingly postulated and supported the view that the central issue in Mollicutes pyrimidine metabolism is the conservation and scavenging of dR1P (66).

The synthesis of TMP proceeds in Mollicutes by two routes: phosphorylation by thymidine kinase (TK, Reaction #5) and from dUMP by thymidylate synthase (TySy, Reaction # 10). The thymidylate synthase (TySy) (Reaction #10) gene *thyA* was annotated in the genomes of *U. urealyticum* (*parvum*), *M. pneumoniae*, *M. genitalium* and *M. pulmonis* (18,37,45,50). TySy mediates the biochemically unique and complex "one-step" methylation of dUMP to dTMP (Eq. 2).

dUMP + N^5,N^{10}-methylenetetrahydrofolate ↔ dTMP + dihydrofolate (Eq. 2)

The presence of TySy activity in Mollicutes has been questioned and its absence was assumed to be related to some folate deficiency (79,102). There has been one report of TySy enzyme activity in *Mycoplasma hominis* (124). TySy has been annotated in *M. pneumoniae*, *M. genitalium* and *M. pulmonis* (18,37,50,51).

Enzymes related to the TySy function and involving folate related methyl-transfer metabolism have been annotated in the three Mollicutes: dihydrofolate reductase, N^5,N^{10}-methylene-tetrahydrofolate dehydrogenase and S-adenosylmethionine synthase (37,45,50). Serine hydroxymethyltransferase (*glyA*) has been annotated in *M. pneumoniae* (MPN576) and *M. genitalium* (MG394). Pyridoxal phosphate is presumably required for this latter activity. Mollicutes folic acid metabolism is discussed in detail elsewhere (34).

A second relevant reason to consider the ramifications of TySy activity is that when and if active it obviates or reduces the role of an alternate 4-step synthesis of TMP from dUMP involving "ribose salvage". In these four steps, dUMP is first dephosphorylated to dUrd (Reaction #4). The dUrd is deribosylated and the dR1P product is used to ribosylate transported thymine producing Tdr (Reactions #8 and #9). The Tdr is phosphorylated to dTMP (Reaction #5) (37,45,50,107,136). Many of these activities were reported in *M. mycoides* subsp. *mycoides* (34).

The reduction of dUMP pools by TySy activity and 5´-nucleotidase (Reaction #4) has additional consequences. Reduction of dUMP levels by the action of TySy is believed to reduce dUTP levels that are associated with Mollicutes mutability (139). This reasoning involves the role of the "missing" dUTPase (Table 1, Enzyme #15). dUTPase is included in Table 1 although it has not been detected nor annotated in these three Mollicutes, or in any *Mycoplasma* spp., it is placed there for convenience. The role of dUTPase is consequential in discussing its role in TySy activity, DNA repair, and Mollicutes mutability.

All of nature's cells are reputed to possess dUTPase except *Mycoplasma* and *Ureaplasma* spp. (139,140). The exception is *M. mycoides* subsp. *mycoides* that like its relative *Spiroplasma* spp. has dUTPase activity (80). dUTPase reduces the cell pool of dUTP by converting

it to dUMP + PPi. This conversion prevents DNA polymerase from substituting dUTP for dTTP. In fact, the DNA polymerase of *M. capricolum* subsp. *capricolum* can incorporate dUTP into DNA at the same rate and to the same level as dTTP (77). When dUTP is incorporated into DNA a second enzyme: uracil-DNA glycosylase (UNG) removes residues of uracil from the DNA resulting in the release of uracil and the creation of an apyrimidinic (AP) site (140). This is thought to lead to mutagenesis because the repair of AP-sites normally by the base-excision repair system is error-prone and such faults result in point, deletion and frameshift mutations. The effect of this process on Mollicutes has led to the opinion that the absence of dUTPase, and in some other Mollicutes UNG as well is contributory if not responsible for the A:T biased mutation pressure and tachytelic evolution observed in the Mollicutes (139). *Mycoplasma gallisepticum* is thought to be the most rapidly evolving Mollicutes, perhaps Bacteria, based on analyses of 16S rRNA (112,142), it is one of the very few Mollicutes that lacks both dUTPase and UNG (140).

12. PREDICTING METABOLISM

This report emphasizes the utility, the necessity of combinatorial analyses for the prediction of the presence and role of metabolic pathways and their components: annotation, expression, structural and enzymatic data and protein-protein interaction assessment (22,129).

Predicting the existence of enzymatic function or the presence of metabolic pathways using only annotation or expression data or for that matter only enzymatic data appears reasonable and tempting but is perhaps misleading or incorrect (3,12,13,40-42, 49,59,62,63,68,90,91,130). The number of annotation errors are claimed to be calculable and even higher than usually believed (30).

Sequencing error is not uncommon and subsequent annotation is only putative and may also be incorrect (15,40). The varieties of error have been critically evaluated for genomic analyses: enzymes with the same function in different species can be encoded by distantly related or paralogous genes (*i.e.*, non-orthologous gene displacement) or totally unrelated enzymes may catalyze a similar reaction (analogous enzymes). There may be post-translational modifications resulting in enzyme inactivation; some functions may require protein-protein interactions or the interaction of larger complexes (modularity); multienzyme proteins are a confounding influence. In Mollicutes as already noted are: the possible presence of cryptic or silent or apparently missing genes, *e.g.*, certain transporters, 5'-nucleotidase, etc.; unique, unrecognized or unknown activities, as the PPi-dependent purine nucleoside kinase activity of the acholeplasmas; and PPi-dependent deoxynucleosides kinase activity of *M. mycoides* subsp. *mycoides* SC. These “errors” or misapprehensions exacerbate the problems of pathway prediction (23,100,130,131,135).

A surprising explanation of genomic error was made by Early and Britt (32). These authors presented data that suggested that enzyme sequence similarities are not solely a function of evolutionary relationship. They studied G3P dehydrogenase, phosphoglycerate and pyruvate kinases from a yeast, bacteria, mammals and a bird. They suggested that species distantly related evolutionarily could have some sequence similarity if they metabolize at the same temperature. They also posed the opposite possibility: closely related organisms that subsist at different temperatures may possess sequences with less similarity that masks their evolutionary relationship. They suggested that those sequences that maintain enzymes in their environment be “subtracted out” in establishing evolutionary relationships.

Regulatory proteins may have dozens of cellular functions that vary from one cell to another (36). The presence of unrecognized functionally in-activating single amino acid substitutions is a challenge to any effort to predict metabolism. A consideration that may gain more support is that these putative essential functions are met by proteins whose capabilities are of a general non-specific nature, *i.e.*, these enzymes with broad specificities can use a variety of substrates to produce products that themselves have multiple metabolic roles, *e.g.*, the kinases of glycolysis, as noted before. Such enzymes are both multifunctional and commonplace (117). Their presence creates some if not major uncertainties in annotation and in pathway prediction and obviously in describing minimal cell functions and components.

Enzymatic assays are not without inherent error. Cultural conditions (temperature, pH, medium components, etc.) may preclude expression of activities of interest; preparation for and fractionation of cells may destroy or separate necessary components; faulty cell-free extract preparation (*e.g.*, loss of essentials during dialysis, oxidation of labile components, improper storage, etc.). The choice of assay is critical as many procedures have modifications that are not comparable or equally reliable; conditions of assay (pH, ionic strength, co-factors, etc.) are critical and may necessarily vary with different microbes; insensitive assay procedures may result in false negative results; improper controls may bias conclusions; and misinterpretations of such unsuspectedly tainted findings lead to erroneous views of metabolism.

As suggested above, metabolic inconsistencies that arise from sequencing, as annotation, or enzymatic analyses are perhaps less likely when additional data, *e.g.*, structural data is also part or the basis for functional assignment - but, in this case perhaps not. Wise *et al*. (141) studied 3-keto-L-gulonate 6-phosphate decarboxylase (KGPDC) and orotidine 5´-monophosphate decarboxylase (OMPDC) in *E. coli.* These functionally distinct enzymes, in different metabolic pathways, similar in sequence and structure were claimed unequivocally to be derived from the same common ancestor: in this case, the mechanistically diverse family having the most common $(ß/a)_8$-barrel fold. These observations led the authors to conclude that “evolution can be opportunistic so that any structural feature of an active site can be conscripted for the development of new enzymatic functions”. In other words, inferring metabolism or minimal metabolism strictly on the basis of either annotation, sequence, enzymatic analyses or even structural homology can be incorrect.

A goal of metabolic investigation seems to be to "…bridge the gap between genotype and phenotype" (12). However, some problems associated with phenotype are significant impediments. Firstly, to rigorously and confidently define phenotype is not easy, as it has become quite clear even in contemporary molecular terms that stress and the environment exert a marked influence on phenotype. Tao *et al*. (126) performed transcript assays for 4290 *E. coli* proteins encoding genes and showed clear differences in gene expression depending on whether cells were grown in rich or minimal media. Suggestion of differential expression was also observed in a proteome analysis of *M. genitalium* (136). In that work the authors noted that many presumably active proteins expressed in log growth phase were absent during late exponential growth phase. Further, the genome (in *E. coli*) is highly dynamic even over a time scale of 10,000 generations (86). In these experiments, almost every individual in the population after 10,000 generations had a different genetic fingerprint. These findings accentuate the need for careful description and consideration of many effectors in the study of enzymatic content and genomic expression leading to annotation and pathway prediction.

In describing or defining such effectors, it is reasonable to consider first: under what conditions of growth or growth phase are cells to be phenotypically characterized? As emphasized, predicting metabolic pathways based on genomic data alone may not be a reflection of immediate needs or represent essentiality or minimal metabolism, but may represent putative perhaps irrelevant metabolic function not yet discarded from the cells genome during its evolution (90).

Metabolic prediction and the terms "essential" or "minimal" are inextricably linked to "environment". Our immediate necessity then is to create an environment that is definable. Most Mollicutes seem "locked-in" to a relatively rich complex growth milieu containing, *e.g.*, proteins, yeast and serum, an unfortunately indefinable environment. Some Mollicutes like the molecularly related (16S rRNA) phytoplasmas and the newly recognized haemotropic *Eperythrozoon* and *Haemobartonella* spp. are entirely uncultivable in a cell free environment (56,66,72,82). Kaeberlein *et al*. (58) reported the isolation of "uncultivable" microorganisms by simulating their natural environment in an approach that may have relevance to the study of Mollicutes.

An interesting and promising approach in the study of the Mollicutes metabolism employs co-culture with eukaryotic cells with aphidicolin, an inhibitor of eukaryotic DNA polymerase alpha. The technique was reported using *Mycoplasma hyorhinis* by Schaeffer *et al*. (116). Dallo and Baseman (25) also used aphidicolin in a study of intracellular survival and long term growth of pathogenic *Mycoplasma* spp. The procedure may have significant and general potential in studying other areas of Mollicutes metabolism. The procedure is similar to that employed for the *in vitro* propagation and study of *Chlamydia trachomatis* in cycloheximide protein-synthesis inhibited host cells.

Mollicutes metabolism *in situ* seems to be a burgeoning area of research. Mollicutes as intracellular parasites can penetrate, persist and presumably modulate their host metabolically (2,14,25,44,127). The natural parasitism of eukaryotic cells, in this case *Trichomonas vaginalis* parasitized by *Mycoplasma hominis*, is a striking example where the metabolic interplay of a symbiotic relationship may be explored (101). Somewhat perplexing metabolically, but perhaps most consequential is the report of Zhang *et al*. (144). These authors reported that *M. fermentans* and *M. penetrans* induced malignant transformations in mouse embryo cells. The transformation was permanent and did not need the presence of the mycoplasmas to maintain malignancy in the cell line. Further, in these transformed mycoplasma-free cells no evidence by representational difference analysis and PCR enrichment was found for gene fragments of mycoplasmal origin. The role of Mollicutes metabolism in the generation of this striking finding is unclear but certainly suspect. Diverse metabolic processes of Mollicutes that have been related *mechanistically* to host cell pathology are reviewed elsewhere (39,97,106,113,118,132).

Mollicutes have a special place in studies attempting to relate genomic sequence to metabolism and the requirements for an independent life. Mollicutes are relatively simple Bacteria: small in size, with small genomes, having a lower biological limit of functional components, enzymes and the number proteins. They replicate and grow *in vitro* and their unique wall-less character make them easily fractionated and manipulated. They cause significant diseases in humans and also in animals, birds, plant and insect populations of great socio-economic value. The study of Mollicutes is a practical and societally relevant choice; they are one of the most logically exploitable cell models.

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Abbreviations: Phenylmethylsulfonylfluoride: PMSF

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