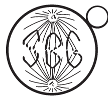

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Research Article

Purine and pyrimidine nucleotide metabolism in Mollicutes

Cristiano Valim Bizarro and Desirée Cigaran Schuck

Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

Abstract

Several mollicute genome projects are underway, offering unique opportunities to study genes and metabolic pathways on a genome-wide scale. Here, we have analyzed the conservation and diversity of purine and pyrimidine metabolism in mycoplasmas. An evaluation of discrepancies between genomic analysis and enzymatic data revealed interesting aspects about these organisms. We found important examples in which enzyme activity was reported without the annotation of a corresponding gene. An interesting example concerns phosphopentomutase. In Mollicutes, we have identified CDSs orthologous to sequences recently identified as new phosphopentomutases in archaeobacteria that are structurally related to phosphomannomutases. It is suggested that these sequences could replace the function of phosphopentomutases in mollicutes lacking the canonical phosphopentomutase gene (*deoB*). Also, the activity of 5'-nucleotidase was reported in mollicutes that do not possess any CDS related to *ushA*. Hypothetical proteins exhibiting domains similar to newly characterized 5' nucleotidases in *Escherichia coli* are proposed as possible CDSs related to this enzymatic activity in Mollicutes. Based on our analysis, the reductive genome evolution of Mollicutes does not appear to result in a minimum set of genes nor a minimum set of metabolic functions shared by all mollicute species.

Key words: mollicutes, purine, pyrimidine, metabolism, metabolic pathways.

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Introduction

Mollicutes are wall-less bacteria found widespread in nature. According to the International Committee on Systematics of Prokaryotes (ICSP), there are 204 described mollicute species, 119 of which belong to the genus *Mycoplasma* (ICSP Subcommittee on the Taxonomy of Mollicutes), which includes pathogens of medical and veterinary importance (Razin *et al.*, 1998). These bacteria, together with *Buchnera* species (Gil *et al.*, 2002) and the archaeobacterium *Nanoarchaeum equitans* (Waters *et al.*, 2003), are among the smallest independently self-replicating cellular organisms, with reduced genome sizes and low GC content. Mycoplasmas present tissue and host-specificity, and are found as free-living or possibly intracellular parasites (Lo *et al.*, 1993; Baseman *et al.*, 1995; Dallo and Baseman, 2000; Momynaliev *et al.*, 2000).

Mollicutes evolved from Gram-positive bacterial ancestors (Woese, 1987) through a genome reduction process, similar to what occurred in *Wolbachia* and *Buchnera* genera (van Ham *et al.*, 2003; Wu *et al.*, 2004). In recent years, we have witnessed the appearance of many mollicute genome projects. There are 14 completely sequenced genomes from 12 different mollicute species deposited in GenBank and another 13 genome sequencing projects from 11 new species in progress (NCBI Microbial Genomes). Thus, this group offers unique opportunities to study genes and metabolic pathways comparatively on a genome-wide scale.

In this work, we studied the purine and pyrimidine metabolism of these bacteria, which is an important topic of research in mollicute biochemistry. Nutritional studies have been hampered by the fastidious nature of these cells and the associated difficulty in developing defined media that enable optimal growth (Pollack, 2002). Nevertheless, defined growth media have been developed for both *Mycoplasma mycoides* subsp. *mycoides* (Rodwell, 1960) and *Acholeplasma laidlawii* (Rodwell and Mitchell, 1979), which became biochemical research models for Mollicutes (Pollack *et al.*, 1997). These studies revealed the general metabolic capabilities of these organisms and some exciting new findings, such as PPi-dependent nucleoside kinase activities, never described previously in any living organism (Tryon and Pollack, 1984; Wang *et al.*, 2001).

Much of this effort was made to try to define the minimum metabolic activities sufficient to support a living cell.

Send correspondence to Cristiano Valim Bizarro. Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Caixa Postal 15005, 91501-970 Porto Alegre, RS, Brazil. E-mail: bizarro@cbiot.ufrgs.br.

More recently, this question was posed in a genomic context, as the minimal genome content necessary to support a living cell. Mushegian and Koonin carried out the first comparative analysis of *Mycoplasma genitalium* and *Haemophilus influenzae* (the only bacterial genomes available at that time) and tried to define the minimal gene set (Mushegian and Koonin, 1996). Later, minimal cell models were developed specifically for purine and pyrimidine tranpsort and metabolism (Castellanos *et al.*, 2004), based on experimentally confirmed essential genes in *M. genitalium* and *Mycoplasma pneumoniae* (Hutchison *et al.*, 1999) and comparative analysis. This kind of approach would benefit considerably from a more comprehensive view of genome and metabolic diversity within mycoplasmas. As we have previously shown (Vasconcelos *et al.*, 2005), the genome reduction process that occurred during the evolution of mycoplasmas has led to the retention of alternate redundant biochemical pathways and not to a single minimal metabolism-related gene set, a finding with important implications for the minimal cell concept. Here, we focus on the conservation and diversity of purine and pyrimidine metabolism in mycoplasmas and we also outline some intriguing and currently unanswered questions about nucleotide metabolic pathways in Mollicutes.

Methods

Using the System for Automated Bacterial Integrated Annotation (SABIÁ) (Almeida *et al.*, 2004), we retrieved clusters of orthologous sequences shared by genome-sequenced mollicutes containing CDSs related to purine and pyrimidine nucleotide metabolism. The annotation of each CDS obtained was confirmed by BLAST similarity searches (National Center for Biotechnology Information - NCBI). The individual confirmation of each sequence retrieved using the SABIÁ system allowed us to identify and discard sequences that clustered together by using the presence of domains unrelated to the enzyme considered. Sequences possessing an e-value greater than E-10 were maintained in the dataset only if a protein domain related to the particular enzyme considered was detected using the CD-search engine (Marchler-Bauer and Bryant, 2004). The set of enzyme activities analyzed in this study was constructed based on BRENDA enzyme database, International Union for Biochemistry and Molecular Biology (IUBMB) Nomenclature Committee Recommendations, the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, 1997; Kanehisa and Goto, 2000) and on an extensive review of biochemical studies in Mollicutes. The lists of annotated CDSs for all sequenced mollicutes (NCBI Microbial Genomes) were inspected for annotated genes related to purine and pyrimidine nucleotide metabolism not included in our primary database. The annotation of each CDS identified by this strategy was confirmed by BLAST similarity searches as mentioned above. Finally, we reconfirmed our ensemble of CDSs retrieved using the Molligen database (Barre *et al.*, 2004).

We systematically analyzed the biochemical studies in which enzyme activities related to purine or pyrimidine nucleotide metabolism were reported for the currently genome-sequenced mollicutes, which includes organisms from the Hominis group (*Mycoplasma hyopneumoniae*, *Mycoplasma mobile*, *Mycoplasma pulmonis* and *Mycoplasma synoviae*), Pneumoniae group (*Mycoplasma gallisepticum*, *M. genitalium*, *M. pneumoniae*, *Mycoplasma penetrans* and *Ureaplasma urealyticum*), *M. mycoides* subsp. *mycoides*, *Mesoplasma florum*, and Onion Yellow Phytoplasma. For each enzyme activity/species combination, a decision was made among the following options: activity detected, no activity detected, not studied, activity suggested, no activity suggested, and structure determined. Proteomic data were also included as a way to validate gene annotations. CDSs with confirmed expression by proteomic studies were assigned as expression was detected.

Discrepancies in which an enzyme activity was reported and no gene annotation was found were selected for detailed analysis. We screened mollicute genomes for domains related to the enzyme activity of interest using the Conserved Domain Architecture Retrieval Tool (CDART) (Geer *et al.*, 2002), which finds protein similarities using sensitive protein domain profiles. Using the same approach, sequences included in the same COG or sharing a pfam entry in mollicute genomes were retrieved and analyzed. We also reviewed the literature to find cases of other organisms in which a protein belonging to a structurally novel class was involved in the same reaction pathway. When these cases were encountered, we searched for orthologous sequences in Mollicutes.

The combined and curated data were used to construct models for the purine and pyrimidine nucleotide pathways possibly present in each studied organism. These graphical representations of metabolic pathways were used to evaluate the differences and similarities presented by Mollicutes in the purine and pyrimidine nucleotide metabolism. These data were assembled to create a general representation of reaction pathways possibly present in at least one completely sequenced mollicute.

Results and Discussion

Nucleotide precursor's uptake

Most mollicutes are unable to synthesize *de novo* purine and pyrimidine bases (Mitchell and Finch, 1977). A possible exception is *M. penetrans*, which has an orotate-related pathway for converting carbamoyl-phosphate to UMP (Sasaki *et al.*, 2002). This limited metabolic capability made mycoplasma cells dependent on environmentally-derived nucleotide precursors. However, there are fewer transporters in mycoplasmas than in most bacteria

(Fraser *et al*., 2000). It was suggested that the reduction in the number of transporters has been compensated by the presence of transporters with broad substrate specificity (Saurin and Dassa, 1996). No nucleobase or nucleoside transporter was found in *M. genitalium* and *M. pneumoniae* genomes (Paulsen *et al*., 2000). It was suggested that transporters with a wide variety of substrates identified in both species could be involved in nucleic acid precursor import, including 11 ATP-binding cassette (ABC) and one Major Facilitator Superfamily (MFS) primary active transporter (Pollack, 2002).

External membrane-associated nuclease activities may be important for nucleotide precursor uptake, and were found in all 20 mycoplasma species tested (Minion *et al.*, 1993). Later, the first membrane nuclease gene, *mnuA*, was cloned and isolated in *M. pulmonis* (Jarvill-Taylor *et al.*, 1999). We found *mnu*A orthologous sequences in *M. hyopneumoniae* (232, J and 7448 strains), *M. gallisepticum*, *M. pneumoniae*, *M. penetrans* and *U. urealyticum* (Table S1 in supplementary online material - see Internet Resources). Besides a possible involvement in the pathogenic process (Bendjennat *et al.*, 1999), the host DNA and RNA degradation mediated by mycoplasma membrane nucleases perhaps provided the bacterial cell with small oligonucleotides and free bases, suggested as important routes for nucleotide precursor uptake (Finch and Mitchell, 1992). Extracellular dephosphorylation of medium nucleotide monophosphates was perhaps achieved by ecto 5'nucleotidases, followed possibly by deribosylation of nucleosides.

Purine and pyrimidine nucleotide pathways in Mollicutes

Based on a previous nutritional study showing that guanine was the unique purine precursor required for *M. mycoides* subsp. *mycoides* growth (Rodwell, 1960) and on experiments of incorporation of labeled nucleotide precursors into RNA, Mitchell and Finch (1977) proposed pathways for purine nucleotide biosynthesis. Later studies have offered a more complete picture of purine nucleotide interconversions in Mollicutes (Pollack *et al.*, 1997). A schematic diagram of proposed purine and pyrimidine pathways in Mollicutes is presented in Figures 1 and 2. These pathway representations include any activity related to purine or pyrimidine metabolism described in at least one genome-sequenced mollicute plus genome annotation data. In Tables S1 and S2 (supplementary online material - see Internet Resources), each reaction path is associated with gene annotation and references for enzyme activity or expression data from proteomic studies for all genome-sequenced mollicutes.

Nucleobases, ribo- and deoxyribonucleosides are imported by the bacterial cell, where purine and pyrimidine nucleosides are deribosylated to nucleobases by the purine nucleoside phosphorylase activity (PNP) or by the pyrimidine nucleoside phosphorylase (thymidine phosphorylase) activity, respectively. Deoxynucleosides can also be directly phosphorylated by deoxyribonucleoside kinases, generating the corresponding deoxyribonucleoside monophosphates (dNMPs). Cytoplasmic nucleobases can be converted to the corresponding nucleoside monophosphates by the activities of adenine phosphoribosyltransferase (APRT), hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and uracil phosphoribosyltransferase (UPRT). Conversion of (d)NMPs to (d)NDPs is achieved by nucleoside monophosphate (NMP) kinases. The ultimate step, phosphorylation of (d)NDPs to (d)NTPs, constitutes a major gap in the understanding of mollicute nucleotide metabolism, as no nucleoside diphosphate kinase (*ndk*) gene was identified in any genome from Mollicutes. It is possible that, in the absence of a genuine NDP kinase activity, low substrate-specificity kinases could provide the necessary (d)NTPs required for normal growth and reproduction (Pollack *et al*., 2002). Interestingly, adenylate kinase was identified as the enzyme responsible for the NDP kinase activity detected in *Escherichia coli ndk* mutants (Lu and Inouye, 1996). Later, it was found that adenylate kinase from *Mycobacterium tuberculosis* also has NDP kinase activity (Meena *et al.*, 2003). It would be interesting to functionally assay adenylate kinase from Mollicutes for NDP kinase activity.

The conversion of ribonucleotide precursors to the corresponding deoxyribonucleotides could be achieved by the activity of nucleoside diphosphate reductase (NDR). *M. penetrans* also contains a gene coding for a ribonucleotide-triphosphate reductase (RTR) and an RTR activating protein NrdG. Importantly, a novel route for deoxynucleotide synthesis was proposed based on enzymatic studies of purine and pyrimidine nucleoside phosphorylases, PNP and PyNP, respectively (McElwain and Pollack, 1987). PNP and PyNP can interconvert nucleosides and nucleobases. When converting nucleobases into nucleosides, these enzymes can accept a deoxyribose-1-phosphate instead of ribose-1-phosphate, generating deoxyribonucleosides. It was also found that mycoplasmas have the ability to phosphorylate all natural deoxynucleosides via deoxynucleoside kinases with ATP-dependent activities but also, in some species, NMP-dependent (phosphotransferases) and PPi-dependent activities (Wang *et al*., 2001).

Combining genomic and enzymatic data: A powerful tool for data mining in purine and pyrimidine nucleotide metabolism of Mollicutes

A more reliable model of the metabolic potential of an organism can be achieved by combining genomic analysis with enzymatic data. A systematic evaluation of discrepancies between the two datasets will reveal interesting aspects about these organisms and prompt further research. The differences found between enzymatic studies reported in the literature and gene annotation for the 12 mollicutes

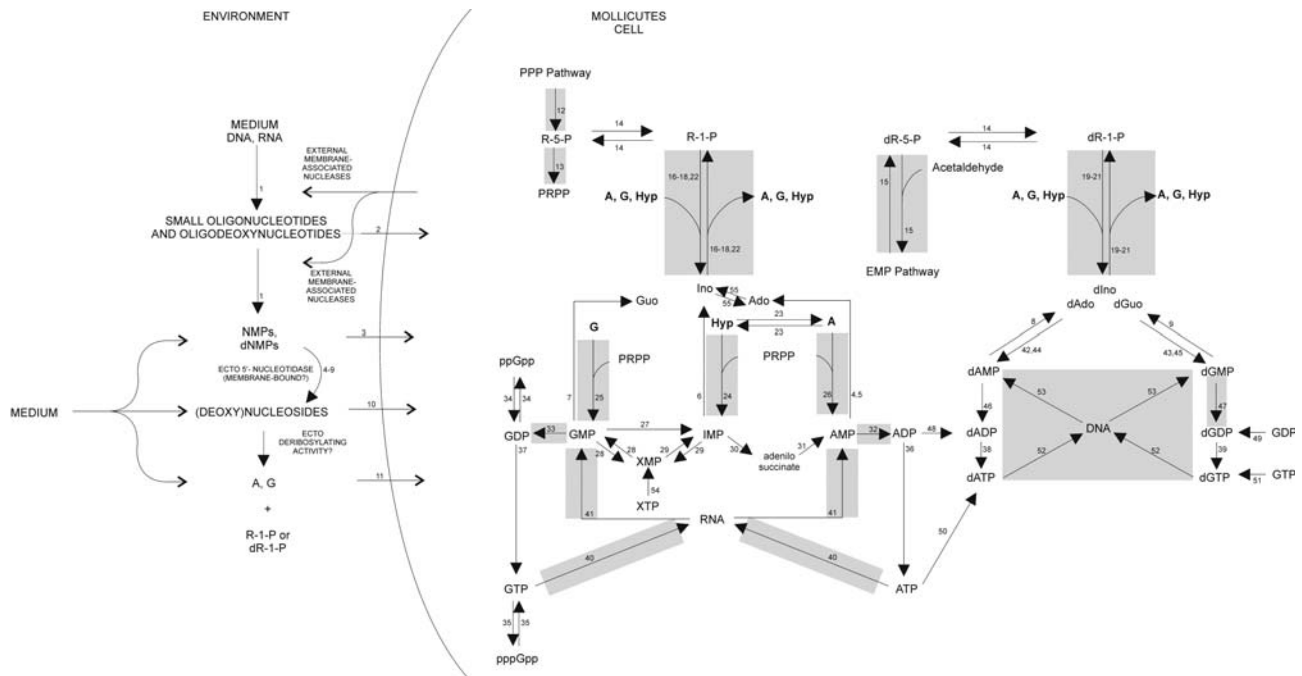


Figure 1 -Schematic representation of purine nucleotide metabolic pathways found or suggested to be present in Mollicutes. The reaction pathways added in this representation were experimentally reported and/or a gene coding the putative corresponding enzyme was annotated in at least one of the 12 genome-sequenced mollicutes analyzed in this work. For reactions shown in gray we have found the corresponding CDS in all mollicutes except Onion Yellow Phytoplasma (for some of these CDSs an orthologue was also identified in this Phytoplasma). Enzyme activities: 1, membrane-associated nucleases (RNAses and DNAses); 2, oligo(deoxy)nucleotide transporter; 3, (d)NMP uptake; 4, AMP phosphatase (non-specific); 5, AMP phosphatase; 6, IMP phosphatase; 7, GMP phosphatase; 8, dAMP phosphatase; 9, dGMP phosphatase; 10, (deoxy)nucleoside uptake; 11, nucleobase uptake; 12, Ribose-5-phosphate isomerase; 13, Ribose-phosphate pyrophosphokinase; 14, phosphopentomutase; 15, Deoxyribose-5-phosphate aldolase; 16, adenosine phosphorylase; 17, guanosine phosphorylase; 18, inosine phosphorylase; 19, deoxyadenosine phosphorylase; 20, deoxyguanosine phosphorylase; 21, deoxyinosine phosphorylase; 22, inosine nucleosidase (without PO4); 23, adenine deaminase; 24, hypoxanthine phosphoribosyltransferase; 25, guanine phosphoribosyltransferase; 26, adenine phosphoribosyltransferase; 27, GMP reductase; 28, GMP synthase; 29, inosine 5'-monophosphate dehydrogenase; 30, adenylosuccinate synthetase; 31, adenylosuccinate lyase; 32, AMP kinase (adenylate kinase); 33, GMP kinase (guanylate kinase); 34, ppGpp 3'-pyrophosphohydrolase; 35, GTP diphosphokinase; 36, ADP kinase; 37, GDP kinase; 38, dADP kinase; 39, dGDP kinase; 40, RNA polymerase; 41, RNAse; 42, deoxyadenosine kinase (ATP-dependent); 43, deoxyguanosine kinase (ATP-dependent); 44, deoxyadenosine kinase (PPi-dependent); 45, deoxyguanosine kinase (PPi-dependent); 46, dAMP kinase; 47, dGMP kinase; 48, ribonucleoside-diphosphate reductase - ADP reductase; 49, ribonucleoside-diphosphate reductase - GDP reductase; 50, ribonucleoside-triphosphate reductase (RTR) - ATP reductase; 51, ribonucleoside-triphosphate reductase (RTR) - GTP reductase; 52, DNA polymerase; 53, DNAse; 54, xanthosine triphosphate pyrophosphatase; 55, adenosine deaminase.

with sequenced genomes are found in Tables 1 and 2 for purine and pyrimidine nucleotide pathways, respectively. Tables S1 and S2 (supplementary online material - see Internet Resources) depict the complete datasets constructed in this study, integrating gene annotation, enzyme activity studies and proteomic analysis.

An interesting example concerns phosphopentomutase, an enzyme that interconverts deoxyribose-1-phosphate (dR1P) and deoxyribose-5-phosphate (dR5P) (Figures 1 and 2), and which is considered crucial to the development of an acceptable integrated scheme linking RNA, DNA, glycolysis and the pentose phosphate pathway (Pollack, 2001). A gene coding for this enzyme (*deoB*) was found in all sequenced mycoplasmas from Hominis group (*M. hyopneumoniae*, *M. mobile*, *M. pulmonis* and *M. synoviae*) but it was not found in any other mollicute (Table 3). However, phosphopentomutase activity was reported in both *U. urealyticum* and *M. mycoides* subsp. *mycoides* extracts (Cocks *et al.*, 1985), which do not possess any gene related to *deoB*. Phosphopentomutases (COG1015) are included in a domain family together with the glycolytic enzyme 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (pfam01676). We searched for pfam01676-containing CDSs in all mollicute genomes. However, all the sequences retrieved after a BLAST search using CDART (see Methods) were part of the already annotated *deoB* or *pgm* genes, coding for phosphopentomutase and phosphoglycerate mutase (COG0696), respectively.

Interestingly, a structurally novel phosphopentomutase was characterized in *Thermococcus kodakaraensis*, which is similar to phosphomannomutases within COG1109 (Rashid *et al.*, 2004). We searched for COG1109-related sequences in genome-sequenced mollicutes and analyzed the genomic position of each retrieved sequence. The results are summarized in Table 3. Within the four sequenced mycoplasmas from Hominis

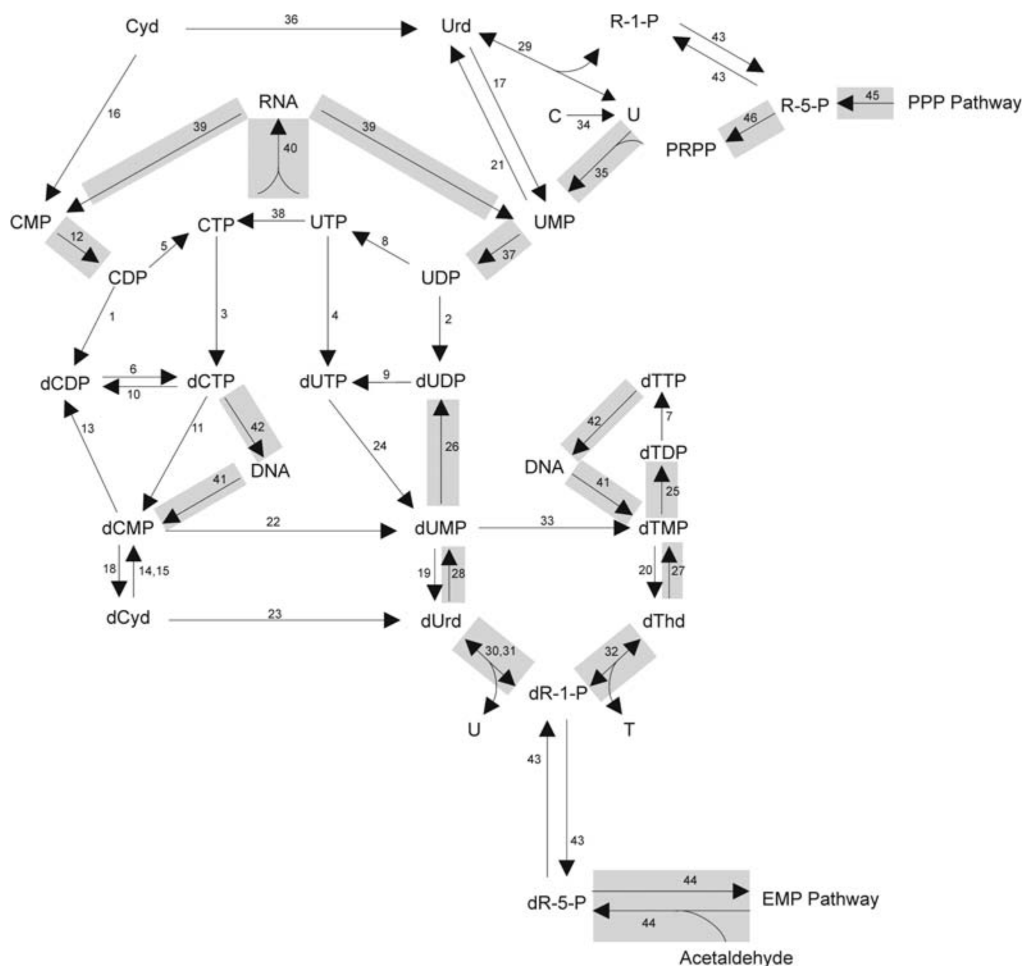


Figure 2 -Schematic representation of pyrimidine nucleotide metabolic pathways found or suggested to be present in Mollicutes. The reaction pathways added in this representation were experimentally reported and/or a gene coding the putative corresponding enzyme was annotated in at least one of the 12 genome-sequenced mollicutes analyzed in this work. For reactions shown in gray we have found the corresponding CDS in all mollicutes except Onion Yellow Phytoplasma (for some of these CDSs an orthologue was also identified in this Phytoplasma). Enzyme activities: 1, ribonucleoside-diphosphate reductase - CDP reductase; 2, ribonucleoside-diphosphate reductase - UDP reductase; 3, ribonucleoside-triphosphate reductase (RTR) - CTP reductase; 4, ribonucleoside-triphosphate reductase (RTR) - UTP reductase; 5, CDP kinase; 6, dCDP kinase; 7, dTDP kinase; 8, UDP kinase; 9, dUDP kinase; 10, dCTPase (dCTP to dCDP) (ATP-insensitive); 11, dCTPase (dCTP to dCMP) (ATP-insensitive); 12, CMP kinase; 13, dCMP kinase; 14, deoxycytidine kinase (ATP-dependent); 15, deoxycytidine kinase (PPi-dependent); 16, cytidine kinase; 17, uridine kinase; 18, dCMP phosphatase; 19, dUMP phosphatase (AMP-insensitive); 20, dTMP phosphatase; 21, UMP phosphatase; 22, dCMP deaminase; 23, deoxycytidine deaminase; 24, dUTPase; 25, thymidylate kinase; 26, dUMP kinase; 27, thymidine kinase; 28, deoxyuridine kinase; 29, uridine phosphorylase; 30, deoxyuridine phosphorylase (uridine phosphorylase enzyme); 31, deoxyuridine phosphorylase (pyrimidine nucleoside phosphorylase enzyme); 32, thymidine phosphorylase (pyrimidine nucleoside phosphorylase enzyme); 33, thymidylate synthase; 34, cytosine deaminase; 35, uracil phosphoribosyl transferase; 36, cytidine deaminase; 37, uridylate kinase; 38, CTP synthetase; 39, RNAse; 40, RNA polymerase; 41, DNAse, 42, DNA polymerase; 43, phosphopentomutase; 44, Deoxyribose-5-phosphate aldolase; 45, Ribose-5-phosphate isomerase; 46, Ribose-phosphate pyrophosphokinase.

group, all containing a *deoB* gene, *M. hyopneumoniae* and *M. synoviae* do not possess any COG1109-related sequence while *M. pulmonis* and *M. mobile* contain two and three genes, respectively. These sequences are not colinear with any gene related to the metabolism of (d)R1P or (d)R5P. Nevertheless, *M. mycoides*, *M. florum* and all the mycoplasmas from the Pneumoniae group, which do not contain the phosphopentomutase *deoB* gene, possess only one sequence related to COG1109. This sequence is located between *deoA* and *deoC* genes in both *M. mycoides* and *M. florum,* while in the Pneumoniae group it is found adjacent to the *cdd* gene. The structural organization of the region containing the COG1109-related sequence, *cdd*, *deo*A, *deo*C and *deo*D genes in Pneumoniae group suggest the presence of an operon. It should be noted that *deoA*, *deoC* and *deoD* gene products are metabolically linked to phosphopentomutase, as the corresponding reactions performed by thymidine phosphorylase (*deoA*), deoxyribose-5-phosphate aldolase (*deoC*) and purine nucleoside phosphorylase (*deoD*) involve (d)R1P or (d)R5P (Figures 1 and 2). We propose that sequences containing COG1109 in *M. mycoides*, *M. florum* and the Pneumoniae group code for phosphopentomutases and represent a new example of non-orthologous gene displacement (NOD). A functional

Table 1 - Differences between enzyme activity assays and gene annotation in purine nucleotide metabolism[1].

Enzyme activities			Mollicute species[2]					
			Mga	Mge	Mpn	Uur	Mmy	Mfl
AMP phosphatase	Enzyme activity	3.1.3.5	**AD**[3, 4]	NAD[5]	NAD[5]	NAD[5]	NS	NS
(5'-nucleotidase)				**AD**[6]	**AD**[6, 7]	**AD**[6]		
	Gene annotation	*ushA*	**NCA**	**NCA**	**NCA**	**NCA**	NCA	NCA
IMP phosphatase	Enzyme activity	3.1.3.5	NAD[3, 4]	**AD**[6]	**AD**[6]	**AD**[6]	NS	NS
(5'-nucleotidase)	Gene annotation	*ushA*	NCA	**NCA**	**NCA**	**NCA**	NCA	NCA
GMP phosphatase	Enzyme activity	3.1.3.5	NAD[3, 4]	**AD**[6]	**AD**[6]	**AD**[6]	NS	NS
(5'-nucleotidase)			**AD**[6]					
	Gene annotation	*ushA*	**NCA**	**NCA**	**NCA**	**NCA**	NCA	NCA
dAMP phosphatase	Enzyme activity	3.1.3.5	**AD**[3]	**AD**[6]	**AD**[6]	**AD**[6, 8]	**AD**[9]	NS
(5'-nucleotidase)	Gene annotation	*ushA*	**NCA**	**NCA**	**NCA**	**NCA**	**NCA**	NCA
dGMP phosphatase	Enzyme activity	3.1.3.5	NAD[3]	NS	NS	**AD**[8]	**AD**[9]	NS
(5'-nucleotidase)	Gene annotation	*ushA*	NCA	NCA	NCA	**NCA**	**NCA**	NCA
phosphopentomutase	Enzyme activity	5.4.2.7	NS	NS	NS	**AD**[10]	**AD**[10]	NS
	Gene annotation	*deoB*	NCA	NCA	NCA	**NCA**	**NCA**	NCA
adenine deaminase	Enzyme activity	3.5.4.2	NAD[4]	NS	NS	**AD**[11]	NS	NS
	Gene annotation		NCA	NCA	NCA	**NCA**	NCA	NCA
adenylosuccinate synthetase	Enzyme activity	6.3.4.4	**AD**[4]	NS	NS	NS	AS12	NS
							AD13	
	Gene annotation	*purA*	**NCA**	NCA	NCA	NCA	MSC_0850	Mfl074
adenylosuccinate lyase	Enzyme activity	4.3.2.2	**AD**[4]	NS	NS	NS	AS12	NS
	Gene annotation	*purB*	**NCA**	NCA	MPN639	NCA	MSC_0849	Mfl075
deoxyguanosine kinase	Enzyme activity	2.7.1.113	NS	**NAD**[6]	AD[14]	**NAD**[6]	AD[14]	**AD**[15]
					NAD[6]			
	Gene annotation	dAK/dGK	MGA_0174	**MG268**	MPN386	**UU086**	MSC_0388	**NCA**
			MGA_0175					
deoxyadenosine kinase	Enzyme activity		NAD[3]	NAD[6]	NAD[6, 14]	NAD[6]	**AD**[14]	NS
(PPi-dependent)	Gene annotation		NCA	NCA	NCA	NCA	**NCA**	NCA
deoxyguanosine kinase	Enzyme activity		NAD[3]	NAD[6]	NAD[6, 14]	NAD[6]	**AD**[14]	**AD**[15]
(PPi-dependent)	Gene annotation		NCA	NCA	NCA	NCA	**NCA**	**NCA**

[1]Items in boldface correspond to differences found between enzyme activity studies and gene annotation data. [2]Abbreviations: Mga, *Mycoplasma gallisepticum*; Mge, *Mycoplasma genitalium*; Mpn, *Mycoplasma pneumoniae*; Uur, *Ureaplasma urealyticum*; Mmy, *Mycoplasma mycoides*; Mfl, *Mesoplasma florum*; AD, activity detected; NAD, no activity detected; NS, not studied; NCA, no CDS annotated. [3]McElwain and Pollack, 1987. [4]Tryon and Pollack, 1985. [5]Johnson and Pitcher, 2000. [6]McElwain *et al.*, 1988. [7]Hamet *et al.*, 1980. [8]Cocks *et al.*, 1988. [9]Neale *et al.*, 1983a. [10]Cocks *et al.*, 1985. [11]Davis *et al.*, 1984. [12]Mitchell and Finch, 1977. [13]Mitchell *et al.*, 1978. [14]Wang *et al.*, 2001. [15]Pollack *et al.*, 1996.

analysis of the corresponding sequences will be fundamental to confirm this hypothesis.

Another important disagreement between activity studies and gene annotation found in this work involves 5'-nucleotidase. Despite its importance in nucleotide metabolism, the prokaryotic 5'-nucleotidases are poorly characterized. It is generally assumed that the *ushA* gene product is responsible for the 5'-nucleotidase activity found in bacteria, including the intracellular interconversion of all (d)NMPs to the corresponding (deoxy)nucleosides. However, *E. coli* UshA is a periplasmic protein that also possesses UDP-sugar hydrolase activity (Neu, 1967) whose major physiological role is the degradation of exogenous UDP-glucose and 5'-nucleotides for internal utilization of reaction products (Glaser *et al.*, 1967). We found that the 5 genome-sequenced mollicute species for which a nucleotidase activity was reported do not possess any CDS related to *ushA* (Figure 1). Also, there are reports of nucleotidase activity detection towards some (d)NMPs but not others, indicating different substrate specificities (Tryon and Pollack, 1985).

Proudfoot *et al.* (2004) identified three uncharacterized *E. coli* proteins containing nucleotidase activity, SurE, YfbR, and YjjG, which exhibit different substrate

Table 2 - Differences between enzyme activity assays and gene annotation in pyrimidine nucleotide metabolism[1].

Enzyme activities			Mollicute species[2]				
			Mga	Mge	Mpn	Uur	Mmy
CDP kinase (nucleoside diphosphate kinase)	Enzyme activity	2.7.4.6	NS	NS	NS	**AD**[3]	**AS**[4, 5] **AD**[6]
	Gene annotation	*ndk*	NCA	NCA	NCA	**NCA**	**NCA**
dCDP kinase (nucleoside diphosphate kinase)	Enzyme activity	2.7.4.6	NS	NS	NS	NS	**AS**[5] **AD**[6]
	Gene annotation	*ndk*	NCA	NCA	NCA	NCA	**NCA**
dTDP kinase (nucleoside diphosphate kinase)	Enzyme activity	2.7.4.6	NS	NS	NS	NS	**AS**[5] **AD**[6]
	Gene annotation	*ndk*	NCA	NCA	NCA	NCA	**NCA**
UDP kinase (nucleoside diphosphate kinase)	Enzyme activity	2.7.4.6	NS	NS	NS	NS	**AS**[4, 5] **AD**[7]
	Gene annotation	*ndk*	NCA	NCA	NCA	NCA	**NCA**
dUDP kinase	Enzyme activity	2.7.4.6	NS	NS	NS	NS	**AS**[5]
(nucleoside diphosphate kinase)	Gene annotation	*ndk*	NCA	NCA	NCA	NCA	**NCA**
dCTPase (dCTP to dCDP) (ATP-insensitive)	Enzyme activity	3.6.1.12	NS	NS	NS	NAD[3]	**AS**[5] **AD**[3, 6]
	Gene annotation		NCA	NCA	NCA	NCA	**NCA**
dCTPase (dCTP to dCMP)	Enzyme activity	3.6.1.12	NAD[11]	NS	NS	NAD[3]	**AD**[3, 6]
(ATP-insensitive)	Gene annotation		NCA	NCA	NCA	NCA	**NCA**
deoxycytidine kinase (ATP-dependent)	Enzyme activity	2.7.1.74	NS	**AD**[8, 9]	**AD**[8, 9, 10]	**AD**[3, 8, 9]	**AS**[5] **AD**[6, 10]
	Gene annotation		NCA	**NCA**	**NCA**	**NCA**	**NCA**
deoxycytidine kinase	Enzyme activity	2.7.1.74	NS	NS	NAD[10]	NS	**AD**[10]
(PPi-dependent)	Gene annotation		NCA	NCA	NCA	NCA	**NCA**
dCMP phosphatase	Enzyme activity	3.1.3.5	NS	NS	NS	**AD**[3]	NAD[6]
(5'-nucleotidase)	Gene annotation	*ushA*	NCA	NCA	NCA	**NCA**	NOA
dUMP phosphatase (AMP-insensitive) (5'-nucleotidase)	Enzyme activity	3.1.3.5	NAD[11]	NS	NS	NAD[3]	**AS**[5] **AD**[6] NAD[3]
	Gene annotation	*ushA*	NCA	NCA	NCA	NCA	**NCA**
dTMP phosphatase (5'-nucleotidase)	Enzyme activity	3.1.3.5	NS	NS	NS	**AD**[3]	**AS**[5] **AD**[6]
	Gene annotation	*ushA*	NCA	NCA	NCA	**NCA**	**NCA**
UMP phosphatase	Enzyme activity	3.1.3.5	NS	NS	**AD**[12]	NS	NS
(5'-nucleotidase)	Gene annotation	*ushA*	NCA	NCA	**NCA**	NCA	NCA
dCMP deaminase	Enzyme activity	3.5.4.12	AD[11]	**AD**[8, 9]	**AD**[8, 9]	NAD[3, 8, 9]	AS[5] AD[6]
	Gene annotation		MGA_0701	**NCA**	**NCA**	NCA	MSC_0581
dUTPase	Enzyme activity	3.6.1.23	**NAD**[9, 11]	NAD[8, 9]	NAD[8, 9, 13]	NAD[3, 8, 9]	**AS**[5], **AD**[3, 6, 9]
	Gene annotation		**MGA_0994**	NCA	NCA	NCA	**NCA**
uridine phosphorylase	Enzyme activity	2.4.2.3	**AD**[14, 15]	**AD**[8]	**AD**[8, 12, 15]	**AD**[3, 8, 15, 16]	**AS**[4] **AD**[7, 16]
	Gene annotation		**NCA**	**NCA**	**NCA**	**NCA**	**NCA**
thymidylate synthase	Enzyme activity	2.1.1.45	NS	NS	NS	**AS**[17]	NAS[5]
	Gene annotation	*thyA*	MGA_0699	MG227	MPN320	**NCA**	NCA
cytosine deaminase	Enzyme activity	3.5.4.1	NS	NS	NS	NS	**NAS**[4]
	Gene annotation		NCA	NCA	NCA	NCA	**MSC_0049**

[1]Items in boldface correspond to differences found between enzyme activity studies and gene annotation data. [2]Abbreviations: Mga, *Mycoplasma gallisepticum*; Mge, *Mycoplasma genitalium*; Mpn, *Mycoplasma pneumoniae*; Uur, *Ureaplasma urealyticum*; Mmy, *Mycoplasma mycoides*; Mfl, *Mesoplasma florum*; AD, activity detected; AS, activity suggested; NAD, no activity detected; NS, not studied; NCA, no CDS annotated. [3]Cocks *et al.*, 1988 [4]Mitchell and Finch, 1977. [5]Neale *et al.*, 1983a. [6]Neale *et al.*, 1983b. [7]Mitchell and Finch, 1979. [8]McElwain *et al.*, 1988. [9]Williams and Pollack, 1990. [10]Wang *et al.*, 2001. [11]Williams and Pollack, 1985. [12]Hamet *et al.*, 1980. [13]Williams and Pollack, 1984. [14]McElwain and Pollack, 1987. [15]McGarrity *et al.*, 1985. [16]Cocks *et al.*, 1985. [17]Carnrot *et al.*, 2003.

specificities. These new nucleotidases belong to different enzyme superfamilies, possibly involving distinct catalytic mechanisms: SurE-like family (SurE), HD domain family (YfbR), and haloacid dehalogenase (HAD)-like superfamily (YjjG). We did not find orthologous sequences to these *E.coli* genes in Mollicutes. However, we found sequences containing HD domain or (HAD)-like domains (but not SurE-like) in different domain architectures (data not shown). These sequences could be considered as candidates for novel 5'-nucleotidases and could be experimentally evaluated in further efforts to characterize the proteins exhibiting nucleotidase activity in Mollicutes.

Uridine phosphorylase activity was detected in 5 genome-sequenced mycoplasmas, but no gene related to *udp* was annotated in any mollicute (Table 2). It is possible that thymidine phosphorylase (*deoA*) is responsible for the uridine phosphorylase activity detected, as it was found for *Giardia lamblia*, in which thymidine, uridine and deoxyuridine phosphorylase activities remained associated throughout the enzyme purification process (Lee *et al.*, 1988).

Comparative analysis of purine and pyrimidine pathways in Mollicutes

For each genome-sequenced mollicute, we constructed schematic representations of purine and pyrimidine nucleotide pathways by integrating data from gene annotation and enzymatic studies (data not shown). In an effort to represent potential metabolic pathways presented by these mollicutes, an inclusive approach was adopted. A reaction was considered to be present if we detected an annotated gene coding for a protein orthologous to enzymes known to catalyze the corresponding reaction in other organisms, or if the reaction itself was experimentally validated in this organism. However, even if a particular reaction was not studied and no gene annotation was found, there still remains the possibility that the product of an uncharacterized CDS could possess the corresponding enzyme activity. As shown in Tables 1 and 2, we found important examples in which enzyme activity was reported without the annotation of a corresponding gene. These unknown enzymes may have orthologs in related mycoplasmas for which no enzyme activity study was carried out. This possibility was included in each pathway model as an undefined reaction.

It appears that *M. hyopneumoniae*, *M. mycoides* and *U. urealyticum* are not able to synthesize thymidylate derivatives using a *thyA*-coded thymidylate synthase. As we were not able to find any sequence related to the alternate ThyX thymidylate synthase (data not shown), the dTMP pools would then be dependent on direct uptake, as already characterized for *M. mycoides* (Neale *et al.*, 1984), or by the concerted action of thymidine phosphorylase and thymidine kinase activities. In this scenario, phosphopentomutase activity would be crucial in the dTMP metabolism of these three species, a possibility that could be experimentally evaluated. Conversely, Onyon Yellow Phytoplasma seems to rely exclusively on thymidylate synthase activity to regulate the dTMP pool. There is no gene annotated for either thymidine phosphorylase or phosphopentomutase. Moreover, no COG1109-related sequence was found in this species, as was the case for the other mollicutes for which no phosphopentomutase gene was found (Table 3). A gene coding for thymidine kinase is present in Onyon Yellow Phytoplasma. However, its product is probably a bifunctional enzyme, exhibiting both thymidine and deoxyuridine kinase activities. The remaining mollicutes studied appear to possess both pathways for dTMP biosynthesis.

There are different pathways for interconversion of cytidine and uridine derivatives in Mollicutes in both pyrimidine ribonucleotide and deoxyribonucleotide metabolism. In fact, *M. mycoides*, *M. florum* and Onyon Yellow Phytoplasma possess a gene coding for cytosine deaminase, an enzyme that directly converts cytosine into uracil nucleobase. The comparative analysis of routes for both CTP and UTP nucleotide biosynthesis in Mollicutes reveal an intricate pattern of alternate retention of redundant metabolic pathways (Figure 3). UTP biosynthesis can proceed from uracil and PRPP by the activity of uracil phosphoribosyltransferase (UPRT), followed by nucleoside monophosphate kinase (NMK) and nucleoside diphosphate kinase (NDK) activities. CTP may be produced from UTP by the activity of CTP synthase. However, both pyrimidine nucleotides may be produced by alternate routes from the corresponding nucleosides using a bifunctional enzyme, cytidine/uridine kinase. Genome-sequenced mollicutes can be divided into 5 categories according to the presence of these three enzymes (Figure 3). *M. gallisepticum*, *U. urealyticum*, *M. mycoides* and *M. florum* belong to the first category, presumably possessing the three enzyme activities. These organisms exhibit redundant metabolic pathways for both CTP and UTP nucleotide biosynthesis. The phylogenetically related *M. genitalium* and *M. pneumoniae* possess only UPRT and cytidine/uridine kinase enzymes. These organisms retained alternate ways to produce UTP but rely only on cytidine kinase activity to produce CTP. However, interconversion of cytidine and uridine precursors is also occurring, as both mycoplasmas possess a cytidine deaminase gene. In the third category, Onion Yellow Phytoplasma has lost the UPRT gene, exhibiting only one route for UMP synthesis but retaining redundancy in the cytidine nucleotide pathway. Four species (*M. penetrans*, *M. hyopneumoniae*, *M. penetrans* and *M. hominis*) do not exhibit alternate ways to produce both UTP and CTP nucleotides. These mollicutes do not possess cytidine/uridine kinase but have UPRT and CTP synthase enzymes. *M. synoviae* was placed in an isolated category as this organism does not possess any gene related to cytidine/uridine kinase or CTP synthase. It is currently not

Table 3 -Putative novel phosphopentomutase in Mollicutes[1].

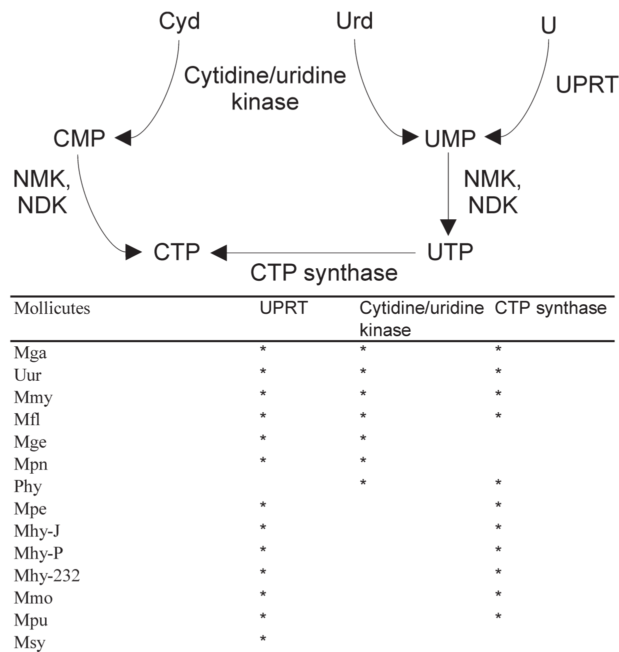
Species	COG1015 (phosphopentomutase)		COG1109 (phosphomannomutase or novel phosphopentomutase)			
	Accession number	Locus tag	Accession number	Locus tag	Colinearity[2]	Proposed activity
Mhy-J	YP_278959	MHJ_0157	NCA			
Mhy-P	YP_287558	MHP7448_0161	NCA			
Mhy-232	AAV27772	mhp221	NCA			
Mmo	YP_016016	MMOB3190	YP_016221	MMOB5240	not colinear	phosphomannomutase
			YP_015960	MMOB2630	not colinear	phosphomannomutase
			YP_015899	MMOB2020	not colinear	phosphomannomutase
Mpu	NP_326108	MYPU_2770	NP_326540	MYPU_7090	not colinear	phosphomannomutase
			NP_326315	MYPU_4840	not colinear	phosphomannomutase
Msy	YP_278215	MS53_0083	NCA			
Mga	NCA		NP_853364	MGA_0358	MGA_0358-*cdd-deoA-deoC-deoD*	novel phosphopentomutase
Mge	NCA		NP_072713	MG053	MG053-*cdd-deoA-deoC-deoD*	novel phosphopentomutase
Mpn	NCA		AAB95736	MPN066	MPN066-*cdd-deoA-deoC-deoD*	novel phosphopentomutase
Mpe	NCA		NP_757495	MYPE1070	MYPE1070-*cdd-deoA-deoD*	novel phosphopentomutase
Uur[3]	NCA		NP_078368	UU530	UU530-*cdd*	novel phosphopentomutase
Mmy[3]	NCA		NP_975802	MSC_0829	deoC-MSC_0829 *deoA*[4]	novel phosphopentomutase
Mfl	NCA		YP_053360	Mfl120	*deoA -Mfl120-deoC*	novel phosphopentomutase
Phy	NCA		NCA			

[1]Abbreviations: Mhy, *Mycoplasma hyopneumoniae* (strains J, 7448 [P], and 232); Mmo, *Mycoplasma mobile*; Mpu, *Mycoplasma pulmonis*; Msy, *Mycoplasma synoviae*; Mga, *Mycoplasma gallisepticum*; Mge, *Mycoplasma genitalium*; Mpn, *Mycoplasma pneumoniae*; Mpe, *Mycoplasma penetrans*; Uur, *Ureaplasma urealyticum*; Mmy, *Mycoplasma mycoides*; Mfl, *Mesoplasma florum*; Phy, Onion Yellow Phytoplasma; NCA, no CDS annotated. [2]Identified CDSs containing COG1109 are represented here when the sequence is colinear to genes whose products are involved in (deoxy)ribose-1-phosphate and/or (deoxy)ribose-5-phosphate metabolism. [3]Phosphopentomutase activity detected (Cocks *et al*., 1985). [4]*deoA* gene is located on the opposite strand.

known how *M. synoviae* could obtain cytidylates, except for a direct uptake from the environment.

Differences in the ability to interconvert guanine, inosine and adenine nucleotides are interesting to consider when devising defined media for Mollicutes. Based on nutritional studies using *M. mycoides*, it was proposed that adenine nucleotides could be formed from guanine nucleotides, but that GMP could not be formed from IMP (Mitchell and Finch, 1977). Taking into account these studies on *M. mycoides*, guanine nucleotide pathways were considered the "Achilles heel of the Mollicutes" because of the supposed dependence on phosphoribosyltransferase activity or transport of preformed guanine derivatives (Pollack, 2002). The genomic data from *M. mycoides* corroborated nutritional studies. This organism possesses a GMP reductase, which converts GMP into IMP, adenylosuccinate synthase and adenylosuccinate lyase, both involved in the formation of AMP from IMP. Moreover, no genomic sequence was found related to either GMP synthase or inosine 5' -monophosphate dehydrogenase, the nucleotide pathway from IMP to GMP through an XMP intermediate. However, a more comprehensive analysis of combined genomic and enzymatic data indicates that generalizations based on *M. mycoides* studies can be misleading when applied to other mollicutes.

From the 12 genome-sequenced mollicutes, no organism other than *M. mycoides* possesses the same set of enzymes involved in GMP to AMP conversion presented by *M. mycoides*. All the species from the Hominis group plus *U. urealyticum*, *M. pneumoniae, M. genitalium* and Onion Yellow Phytoplasma do not possess any enzyme involved in interconversion of GMP and IMP or conversion of the latter into AMP. A predictable consequence of this observation is that these organisms would not be able to grow in a medium containing only guanine as a preformed purine nucleobase, as is the case for *M. mycoides* (Rodwell, 1960). *M. gallisepticum* is presumably unable to interconvert GMP and IMP, but can generate AMP from IMP. Adenylosuccinate synthase and adenylosuccinate lyase activities were reported for *M. gallisepticum* (Tryon and Pollack, 1985) but no related gene was found. Further work will be required to validate these experimental data. Curiously, there is a GMP synthase annotated but we have not found any sequence related to IMP dehydrogenase in this organism. It is possible that the GMP synthase gene from *M. gallisepticum* represents a relict from an ancestral pathway no longer present in this species. *M. penetrans* and *M.*



Mollicutes	UPRT	Cytidine/uridine kinase	CTP synthase
Mga	*	*	*
Uur	*	*	*
Mmy	*	*	*
Mfl	*	*	*
Mge	*	*	
Mpn	*	*	
Phy		*	*
Mpe	*		*
Mhy-J	*		*
Mhy-P	*		*
Mhy-232	*		*
Mmo	*		*
Mpu	*		*
Msy	*		

Figure 3 -Pathways for CTP and UTP synthesis in Mollicutes. Abbreviations: Cyd, cytidine; Urd, uridine; U, uracil; CMP, cytidine monophosphate; UMP, uridine monophosphate; CTP, cytidine monophosphate; UTP, uridine monophosphate; NMK, nucleoside monophosphate kinase; NDK, nucleoside diphosphate kinase; Mga, *Mycoplasma gallisepticum*; Uur, *Ureaplasma urealyticum*; Mmy, *Mycoplasma mycoides*; Mfl, *Mesoplasma florum*; Mge, *Mycoplasma genitalium*; Mpn, *Mycoplasma pneumoniae*; Phy, Onion Yellow Phytoplasma; Mpe, *Mycoplasma penetrans*; Mhy, *Mycoplasma hyopneumoniae* (strains J, 7448 [P], and 232); Mmo, *Mycoplasma mobile*; Mpu, *Mycoplasma pulmonis*; Msy, *Mycoplasma synoviae*.

florum possess the complete set of enzymes related to interconvertion of GMP to IMP and convertion of IMP to AMP.

Our data is consistent with the view that a reduction in the metabolic repertoire accompanied the reductive evolution of mycoplasmas' genomes. However, in our view, this reduction process, which occurred in parallel in many lineages, does not seem to lead to an unequivocal minimum set of genes or even to a minimum set of metabolic functions. It is apparent from the examples described in this work that the metabolic repertoire displayed by *M. genitalium*, the smallest known mollicute, does not represent a minimum set of enzyme activities shared by the other mollicutes. Instead, different minimum sets of enzymatic functions could be generated through reductive evolution from an ancestral organism displaying redundant activities. We think it would be worth considering this hypothesis in further studies.

Concluding Remarks

In this work, we have systematically analyzed enzyme activity reports on purine and pyrimidine nucleotide metabolic pathways from the currently completely sequenced mollicutes. These data were combined with the corresponding gene annotation to generate a more complete description of nucleotide metabolism in Mollicutes. We found that members of a structurally novel family of phosphopentomutases recently characterized in the archaeobacterium *Thermococcus kodakaraensis* seem to be present in Mollicutes lacking the conventional *deoB* gene but displaying the corresponding enzyme activity.

The predicted purine and pyrimidine nucleotide pathways for each species were analyzed comparatively, and revealed the extent of conservation and diversity in the nucleotide metabolism of Mollicutes. Based on the comparative analysis, it is suggested that our ability to offer generalizations about mollicute biochemistry based on well-studied species, like *M. mycoides* and *A. laidlawii*, is rather limited. A complex pattern of redundancy and alternate retention of redundant pathways seems to emerge and the limitations of the minimum genome concept were discussed in this context.

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Supplementary Online Material

Tables S1 and S2: This material is part of the electronic version at: http://www.scielo.br/gmb and in (http://www.genesul.lncc.br/GMB/MS2006-503_supplmaterial.pdf)

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