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Research Article

Identification of the GTPase superfamily in *Mycoplasma synoviae* and *Mycoplasma hyopneumoniae*

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Abstract

Mycoplasmas are the smallest known prokaryotes with self-replication ability. They are obligate parasites, taking up many molecules of their hosts and acting as pathogens in men, animals, birds and plants. *Mycoplasma hyopneumoniae* is the infective agent of swine mycoplasmosis and *Mycoplasma synoviae* is responsible for subclinical upper respiratory infections that may result in airsacculitis and synovitis in chickens and turkeys. These highly infectious organisms present a worldwide distribution and are responsible for major economic problems. Proteins of the GTPase superfamily occur in all domains of life, regulating functions such as protein synthesis, cell cycle and differentiation. Despite their functional diversity, all GTPases are believed to have evolved from a single common ancestor. In this work we have identified mycoplasma GTPases by searching the complete genome databases of *Mycoplasma synoviae* and *Mycoplasma hyopneumoniae,* J (non-pathogenic) and 7448 (pathogenic) strains. Fifteen ORFs encoding predicted GTPases were found in *M. synoviae* and in the two strains of *M. hyopneumoniae*. Searches for conserved G domains in GTPases were performed and the sequences were classified into families. The GTPase phylogenetic analysis showed that the subfamilies were well resolved into clades. The presence of GTPases in the three strains suggests the importance of GTPases in `minimalist' genomes.

Key words: Mycoplasma, GTPase superfamily, genome.

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Introduction

Mycoplasmas are a genus of obligate parasites belonging to the Mollicutes class, the smallest known prokaryotes with self-replication ability (Razin *et al.*, 1998). They present a very small genome evolved to the minimalist status by losing non-essential genes, including those involved in cell wall synthesis, as well those related to catabolic and metabolic pathways (Himmelreich *et al.*, 1996). The two species, *Mycoplasma hyopneumoniae* and *Mycoplasma synoviae*, are responsible for significant economic impact on animal production. *M. hyopneumoniae* is the infective agent of swine mycoplasmosis (DeBey and Ross, 1994), which increases the susceptibility to secondary infections (Ciprian *et al.*, 1988). *M. synoviae* is responsible for subclinical upper respiratory infections, but may also result in airsacculitis and synovitis in chickens and turkeys (Kleven, 1997; Allen *et al.*, 2005).

Many crucial functions for life are provided by a single versatile mechanism that has evolved to fulfill many roles. A prime example is the GTPase superfamily of proteins that occurs in all domains of life, regulating functions such as protein synthesis, cell cycle and differentiation (Bourne *et al.*, 1990). Despite this extraordinary functional diversity, all GTPases are believed to have evolved from a single common ancestor, a fact which resulted in the conservation of their action mechanism, of the core structure and of sequence motifs (Bourne, 1995).

GTPases are often described as molecular switch proteins because of their particular mode of action. Each GTPase specifically binds and hydrolyzes GTP in a cyclic mechanism that activates and inactivates the GTPase protein (Bourne *et al.*, 1991). In this cycle, a GTPase passes through three conformational states. Initially, the GTPase is inactive and is not bound to any nucleotide. After binding GTP, the protein becomes active and changes its conformation, and as such its affinity to effector molecules or other enzymes. GTP is then hydrolyzed simultaneously, with an effect being generated in the GTPase target. Subsequently, GDP is released from the inactive GTPase, returning the protein to the empty state. This cycle allows the active GTPase to interact periodically with a target and, in this

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way, to act as a timed switch in the cell (Bourne *et al.*, 1990).

That cyclic reaction usually involves several other factors that either catalyze the hydrolysis step of the GTPase cycle or catalyze the release of bound GDP from the inactive state of the GTPase (Bourne, 1995). Each GTPase cycle appears to be unique. The rate of switch turnover is dependent on specific interaction factors, as well as on the intrinsic properties of each GTPase. Additionally, some GTPases interact with many different effectors and targets and, in that way, can coordinate cellular responses (Bourne *et al.*, 1990; Bourne, 1995). A core domain that is able to bind either GTP or GDP confers the characteristic switch mechanism of GTPases. The folding of this domain is a defining feature of GTPases (Jurnak *et al.*, 1990). In fact, X-ray crystallography of diverse GTPases shows that the folding of this G-domain is nearly invariant throughout the GTPase superfamily. GTPases can consist solely of the G-domain or may have additional domains on the amino- and carboxyl-terminal ends of the proteins (Sprang, 1997).

Due to the importance of the mycoplasmas, complete genome projects have been reported in the last years (Himmelreich *et al.*, 1996; Hutchison *et al.* 1999; Glass *et al.*, 2000; Chambaud *et al.*, 2001; Papazisi *et al.*, 2003; Sasaki *et al.*, 2002; Jaffe *et al.*, 2004; Minion *et al.*, 2004; Westberg *et al.*, 2004). Complete genomes of *M. synoviae* (strain 53), *M. hyopneumoniae* pathogenic strain (7448) and non-pathogenic strain (J [ATCC25934]) were recently described (Vasconcelos, *et al.*, 2005) and the data are available in databases. The objective of this work is the identification and classification of the GTPase superfamily in the three complete genomes of *M. synoviae* strain 53 and *M. hyopneumoniae* (strains J and 7448).

Material and Methods

By using data from the complete genome of *M. synoviae* and *M. hyopneumoniae,* strains J and 7448 associated to BLAST search tools we have identified 15 ORFs encoding GTPase superfamily homologs in *M. synoviae*, as well as 15 ORFs in both strains of *M. hyopneumoniae*. Classification of the GTPase families and their putative function has been performed by using Pfam interface and InterPro homepage. Search for G-domains in mycoplasma GTPases was performed by alignment of described *Escherichia coli* GTPases sequences (Caldon *et al.*, 2001) with those of *M. synoviae* and *M. hyopneumoniae* (strains J and 7448). Multiple sequence alignments were generated using the ClustalX 1.81 software (Thompson *et al.*, 1997).

The phylogenetic relationships within the GTPase superfamily were inferred from all 33 sequences from *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448. A phylogenetic tree was constructed by multiple sequence alignments using the Clustal X program and visualized by using the Tree View software. Trees were constructed by using the neighbor-joining method (Saitou and Nei, 1987). Robustness of branches was estimated by using 100 bootstrap replicates.

Results and Discussion

Structural analysis of the GTPases superfamily

Searches for GTPases performed on *M. synoviae* and *M. hyopneomoniae* strains J and 7448 genome databases revealed the presence of 15 GTPase orthologs. These GTPases were classified into subfamilies, and the results are shown in Table 1. ORFs were classified as belonging to the Elongation factor, the Era, the FtsY/Ffh and the Obg/YchF subfamilies, or were annotated as unclassified proteins related to GTPases or GTP binding proteins.

Searches for the G-domain, described in all GTPase subfamilies, was performed by using the deduced protein sequences encoded by the identified ORFs presented in Table 1. Figure 1 presents the alignment of the G1-G4 motifs of the cited GTPases. The G-domain is divided into four G motifs: G1 (G/AXXXXGKT/S), G2 (not conserved), G3 (DXXG) and G4 (NKXD) sequence motifs, where X denotes any amino acid (Caldon, *et al.*, 2001). The G1, G2 and G3 motifs were found in all mycoplasma GTPase subfamilies (Figure 1). The G4 motif was found in the EF-G, EF-Tu, IL-2, LepA, Era, EngA, ThdF/TmE, and OBG subfamilies. In the YchF, FtsY and Ffh subfamilies, the region of the G4 motif, although present, was not well conserved (Figure 1).

Functions ascribed to G-motifs include the mediation of interactions with the guanine nucleotides and effector proteins. It has been suggested that G1, G3 and G4 motifs could have evolved to bind and hydrolyze guanosine triphosphate and also for interacting with the cofactor mg^{2+} (Bourne *et al.*, 1991). The non conserved G2 motif is described as the effector domain that undergoes a conformational change necessary for GTPase function (Bourne, *et al.*, 1995, Sprang, 1997).

Elongation factor subfamily

The elongation factor subfamily (EF) is composed of the Elongation factor - G (EF-G), Elongation factor-TU (EF-TU), Initiation factor-2 (IF-2) and GTP-binding protein LepA (LepA), (Caldon, *et al.* 2001). The EF family from bacteria is composed of multidomain GTPases with essential functions in the elongation and initiation phases of translation. EF-Tu catalyzes binding of aminoacyl-tRNA to the ribosomal A-site, while EF-G catalyses the translocation of peptidyl-tRNA from the A-site to the P-site (Rodnina *et al.*, 2000; Nilsson and Nissen, 2005). The initiation factor-2 (IF-2) may be involved in introducing the initiator tRNA into the translation machinery and in performing the first step in the peptide chain elongation cycle (Kyrpides and Woese, 1998). ORFs encoding all elongation factor members were present in *M. synoviae* and *M. hyopneumoniae* J and 7448 (Table 1). All G1-4 motifs were

Table 1 - ORFs encoding GTPases and GTP binding proteins from *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448, with putative functions.

GTPase Family	ORF Product	EC /Cellular process involvement	ORFs encoding GTPases found in Mycoplasmas		
			Mycoplasma synoviae 53	*Mycoplasma hyopneumoniae -J*	*Mycoplasma hyopneumoniae -7448*
Elongation factor subfamily					
EF-G	Elongation factor EF-G	3.6.1.48 / protein biosynthesis	MS0047	MHJ0071	MHP0075
EF-TU	Elongation factor Tu	3.6.1.48 / protein biosynthesis	MS0667	MHJ0524	MHP0523
IF-2	Translation initiation factor IF-2	- / Binding / protein biosynthesis	MS0686	MHJ0585	MHP0584
LepA	GTP-binding protein LepA	- / Protein biosynthesis	MS0489	MHJ0069	MHP0073
Era subfamily					
Era	GTP-binding protein Era	- / ATP Binding / nucleic acid binding	MS0387	MHJ0152	MHP0156
EngA	GTP-binding protein EngA	- / 70s ribosome stabilization	MS0142	MHJ0066	MHP0070
ThdF/TrmE	Thiophene and furan oxidation protein ThdF	- / tRNA processing - indirect Ribosome function	MS0362	MHJ0205	MHP0209
FtsY/Ffh subfamily					
FtsY	Cell division protein FtsY	- / Cell division	MS0145	MHJ0008	MHP0008
Ffh	Signal recognition particle, subunit FFH/SRP54	- / Protein targeting to membrane	MS0021	MHJ0053	MHP0057
Obg and YchF					
OBG	GTP-binding protein Obg	- / Ribosome maturation.	MS0168	MHJ0037	MHP0041
YchF	GTP-binding protein YchF	- / Putative ATP Binding	MS0663	MHJ0284	MHP0293
Unclassified	GTP-binding protein	- / Cell division	MS0650 - YihA	MHJ0446 - YihA	MHP0449 - YihA
	Cell division protein FtsZ	- / Cell division	MS0340 - FtsZ	MHJ0406 - FtsZ	MHP0393 - FtsZ
	Probable GTPase EngC	EC 3.6.1.- / unknown	MS0120 - EngC	MHJ0148 - EngC	MHP0152 - EngC
	Putative GTP-binding protein	- / ATP Binding	MS0664 - YlqF	MHJ0083 - YlqF	MHP0087 - YlqF

found in the ORFs encoding EF GTPases from both mycoplasma species (Figure 1), suggesting that the proteins can be functional in these organisms. Two truncated hypothetical EF-G proteins were also found in the *M. synoviae* genome. The ORFs present high homology to the 3' region of the complete EF-G ORF found in this organism, suggesting that they are not functional genes, in accordance with the 'minimal genome' characteristic of mycoplasmas.

Era subfamily

This family is comprised of the GTP binding protein ERA (ERA), the GTP binding protein EngA (EngA), as well as the Thiophene and furam oxidation protein (ThdF). Both *M. synoviae* and *M. hypneumoniae* (J and 7448) present ORFs related to the Era subfamily. The Era member of the Era subfamily is an essential GTPase that probably regulates the cell cycle (Gollop and March, 1991; Britton *et al.*, 1998) and is involved in regulating carbon (Lerner and Inouye, 1991) and nitrogen (Powell *et al.*, 1995) metabolism. A second member of this group, EngA, has been suggested to be essential for growth in *Neisseria gonorrhoeae* (Mehr *et al.*, 2000). ThdF may be involved in tRNA modification and in the direct or indirect regulation of ribosome function (Caldon, *et al.*, 2001). The presence of all Era subfamily members (Table 1) with all G1-G4 motifs (Figure 1) in *M. synoviae* and *M. hyopneumoniae* (J and 7448) suggests that those ORF products are active and play biological functions in the analyzed organisms.

FtsY/Ffh subfamily

The FtsY/Ffh subfamily is represented by the cell division protein FtsY, termed FtsY, and by the signal recognition particle FFH/SRP54, termed Ffh. ORFs encoding for the two proteins of this subfamily have been reported in the *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448 (Table 1). The G1-G3 motifs were found in the deduced amino acid sequences for FtsY and Ffh of *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448, when compared with *E. coli* FtsY/Ffh sequences (Figure 1). The sequence corresponding to the G4 motif was found in the three analyzed mycoplasmas, even though this motif was not well conserved (NKXD). The amino acids K and D are present in mycoplasma FtsY and Ffh sequences in comparison to the *E. coli* ortholog predicted proteins. These proteins are described as essential to *E. coli* since Ffh/SRP mutants present a lethal phenotype and SRP subunit mutants present growth defects (Lu, *et al.*, 2001).

OBG and YchF subfamily

The comparative analysis of *M. synoviae* strain 53, *M. hyopneumoniae* (strains J and 7448) showed the presence of the same ortholog ORFs encoding OBG and YchF proteins (Table 1). G1-G3 motifs were found in all ORF products. The G4 motif was found in the OBG member, but not in the YchF ORF product (Figure 1). Similarly, this motif was also not found well conserved in the *E. coli* YchF protein.

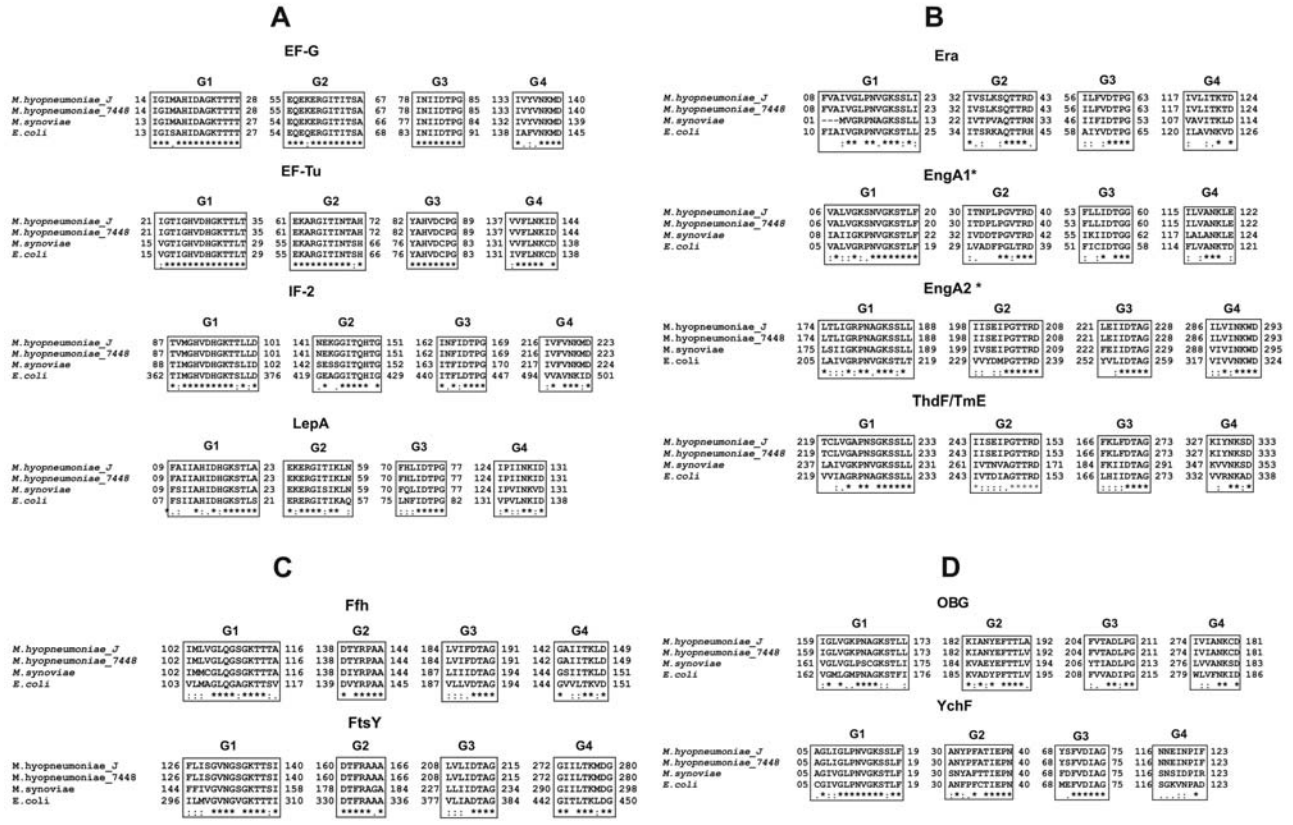

Figure 1 - Alignment of G1, G2, G3 and G4 motifs of the GTPase subfamilies. Panel A: Elongation factor subfamily. Panel B: Era subfamily. Panel C: FtsY/Ffh subfamily. Panel D: OBG YchF subfamily. The sequences used in the alignment are listed in Table 1 and were obtained from: *M. hyopneumoniae J* (*Mycoplasma hyopneumoniae J* GenBank accession number NC-007295), *M. hyopneumoniae 7448* (*Mycoplasma hyopneumoniae 7448*, GenBank accession number NC-007332), *M. synoviae* (*Mycoplasma synoviae* GenBank accession number NC-007294) and *E. coli* (*Escherichia coli*, GenBank accession number NC-000913). The positions of the G1-G4 motifs were obtained by comparison with the most highly conserved regions of *E. coli* orthologs.
*EngA1 and EngA2 refer to the two different G-domains found in all EngA orthologues.

The function of the OBG subfamily remains elusive, although there is evidence for its involvement in the initiation of chromosome replication (Kok *et al.*, 1994), in bacterial sporulation (Trach and Hoch, 1989; Vidwans *et al.*, 1995), and in the activation of a transcription factor that controls the general stress response (Scott and Haldenwang, 1999). The YchF members of the OBG/YchF subfamily are also distributed in all domains of life, (Mittenhuber, 2001), but the biological function of this protein has not been elucidated.

Unclassified GTPases

The GTPases found in the genomes of mycoplasmas which were not classified as belonging to one of the 11 universally conserved bacterial GTPases (Caldon, *et al.*, 2001) were described here as unclassified. Four ORFs from *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448 were identified in this group: EngC, YlqF, FtsZ and YihA. The *E. coli* ortholog EngC is a GTPase with a predicted role as a regulator of translation (Daigle and Brown, 2004). The putative GTP binding protein YlqF is described as necessary for growth of *Streptococcus pneumoniae* and *Staphylococcus aureus* and may be involved in ribosomal assembly (Zalacain *et al*., 2003).

The cell division protein FtsZ was also found in *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448. This protein appears to act at the earliest step in cell septation and is required at the final steps of cytokinesis (Ma, *et al.*, 1996; Jensen, *et al.*, 2005). The GTPase YihA has been described as an essential gene of the bacterial "minimal genome' , even though it seems to be dispensable in some organisms, as described for *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Treponema pallidum*, *Borrelia burgdorferi* and *Synechocystis sp.* (Dassain *et al.*, 1999).

GTPase amino acid sequence relationships

To visualize the amino acid sequence relationship of Mycoplasma GTPase subfamilies, a phylogenetic tree was constructed by using the neighbour-joining method (Saitou and Nei, 1987). A total of 33 deduced amino acid sequences encoding GTPases from *M. synoviae*, *M. hyopneumoniae* J and *M. hyopneumoniae* 7448 were aligned using the CLUSTAL X program (Thompson *et al.*, 1997). Robust-

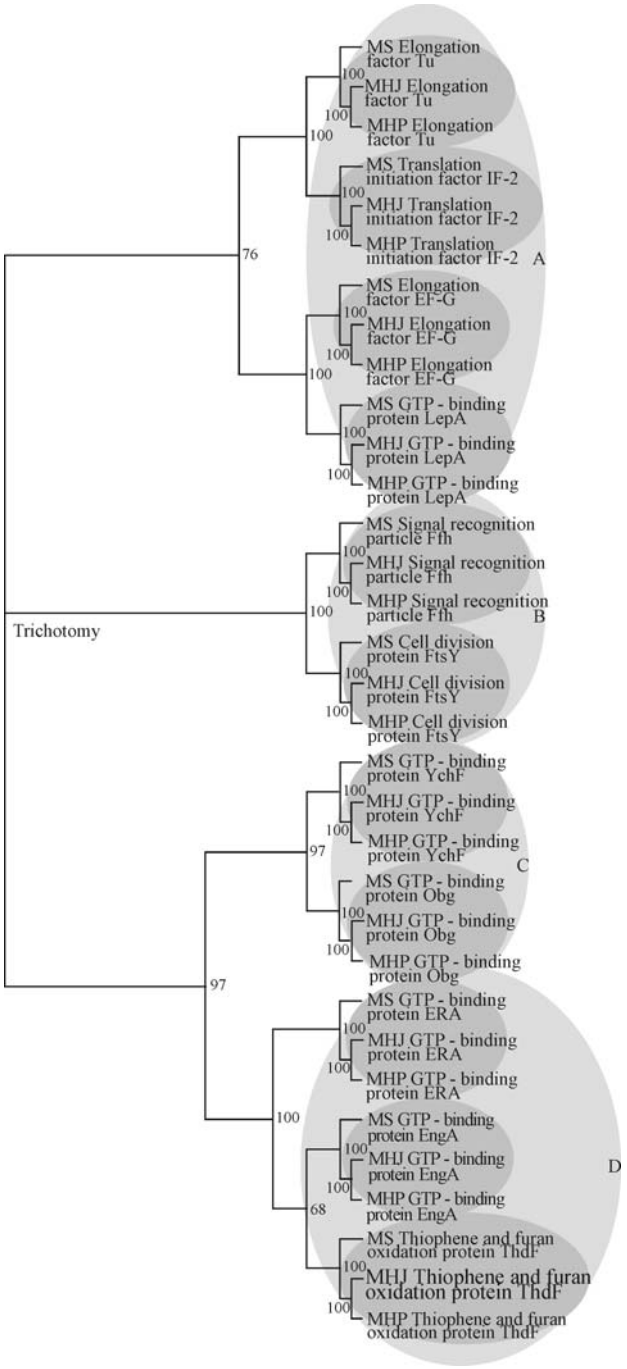

Figure 2 - Amino acid sequence relationship of the GTPase superfamily. (A) Elongation factor subfamily. (B) FtsY/Ffh subfamily. (C) OBG/YchF subfamily. (D) Era subfamily. The numbers on the branches are bootstrap values obtained with 100 replications. Members of each family are described as MS for *M. synoviae* strain 53, MHJ for *M. hyopneumoniae* strain J and MHP for *M. hyopneumoniae* strain 7448.

ness of branches was estimated by using 100 bootstrap replicates. By using the Tree View software a deduced phylogeny was visualized and is shown in Figure 2. A close relationship among amino acid sequences of proteins which belong to the same subfamily can be observed in the three Mycoplasma species. GTPases that have similar functions were clustered into the same clade, suggesting a metabolic conservation in reactions involving GTPases. The bootstrap values reveal the high homology among the subfamilies of proteins of *M. synoviae* strain 53 and *M. hyopneumoniae* strains J and 7448. GTPases are classified into subfamilies based on the presence of different G-domains (G1, G2, G3 and G4). Since unclassified GTPases do not present conserved G-domains, and were not classified by Caldon *et al*. (2001), they were not included in our phylogenetic analysis.

Concluding Remarks

The GTPase superfamily, present in all domains of life, is related to many functions such as protein synthesis, cell cycle and differentiation. The presence of orthologs for all the subfamily members described in prokaryotes in the complete genome of *M. synoviae* and *M. hyopneumoniae* strains J and 7448, evidences the essential functions of GTPases in these 'minimalist' organisms.

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Abbreviations

EF-G (Elongation factor G).
EF-TU (Elongation factor Tu).
IF-2 (Translation initiation factor 2).
MHJ (*Mycoplasma hyopneumoniae* strain J).
MHP (*Mycoplasma hyopneumoniae* strain 7448).
MS (*Mycoplasma synoviae* strain 53).
ThdF (Thiophene and furan oxidation protein).

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Internet Resources

M. synoviae complete genome database, http://www.brgene.lncc.br/finalMS/.

M. hyopneumoniae strain J and *M. hyopneumoniae* strains 7448 complete genome databases, http://www.genesul.lncc.br.

BLAST tools, http://www.ncbi.nlm.nih.gov/blast.

Database of protein families (Pfam), http://www.sanger.ac.uk/Software/Pfam/.

InterProScan software, http://www.ebi.ac.uk/InterProScan/.

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