



## Regulation of gene expression in Mycoplasmas: Contribution from *Mycoplasma hyopneumoniae* and *Mycoplasma synoviae* genome sequences

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### Abstract

This report describes the transcription apparatus of *Mycoplasma hyopneumoniae* (strains J and 7448) and *Mycoplasma synoviae*, using a comparative genomics approach to summarize the main features related to transcription and control of gene expression in mycoplasmas. Most of the transcription-related genes present in the three strains are well conserved among mycoplasmas. Some unique aspects of transcription in mycoplasmas and the scarcity of regulatory proteins in mycoplasma genomes are discussed.

*Key words:* *Mycoplasma hyopneumoniae*, *Mycoplasma synoviae*, transcription, comparative genomics.

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The recent effort to characterize the minimal gene set required for a living cell on Earth focused on the *Mycoplasma genitalium* genome data, and compared them to that of another parasitic bacteria, *Haemophilus influenzae*. The primary assumption was that those bacteria had shed a number of genes in the process of adaptation to the parasitic lifestyle, without losing their fitness (Koonin, 2000). The complete sequencing of the first mollicute in 1995 surprisingly revealed that *Mycoplasma genitalium* devoted 10 times less genes to regulatory functions than *Haemophilus influenzae* (5 vs. 64 genes, respectively), despite possessing a genome only three times smaller and having a somewhat similar lifestyle (Fraser *et al.*, 1995). This example highlights the contribution of comparative genomics to the understanding of cell function. The comparative approach allows researchers to predict protein functions by transferring information from functionally characterized proteins of model organisms to their uncharacterized homologs and to delineate the functionally critical parts of protein (and RNA) molecules, such as catalytic or binding sites. In that context, comparative genome research has provided new insights into the accurate mechanisms involved in bacterial gene regulation. Genetic data obtained by genome sequencing of several *Mycoplasma* strains in the past decade (Fraser *et al.*, 1995; Himmelreich

*et al.*, 1996; Glass *et al.*, 2000; Chambaud *et al.*, 2001; Sasaki *et al.*, 2002; Papazisi *et al.*, 2003; Jaffe *et al.*, 2004; Minion *et al.*, 2004; Westberg *et al.*, 2004; Vasconcelos *et al.*, 2005) opened an opportunity to explore the functional content of genomes and evolutionary relationships between them at a new qualitative level. Based on recently published data (Vasconcelos *et al.*, 2005), the present report describes the set of genes related to transcription in *Mycoplasma hyopneumoniae* and *Mycoplasma synoviae* strains, in a comparative context with other mollicutes. Using standard bioinformatics tools, we aimed at a better understanding of how these bacteria regulate gene expression, and ultimately, how such mechanisms allow them to thrive in their habitats.

Genes involved in the basic cellular processes of transcription and translation comprise about 20% of the total ORFs in mycoplasmas, similar to results observed in other bacteria (Muto and Ushida, 2002). *M. hyopneumoniae* strains J and 7448 and *M. synoviae* all have 16% of their genes involved in translation, and the percentage of genes involved in transcription in mycoplasmas ranges from 2-3%, based on COG tables available (National Center of Biotechnology Information). Thus, despite the overall conservation of the percentage of genes involved in translation, a process considered the most highly conserved in eubacteria, the absolute number of genes involved in transcription in mycoplasmas is very low, ranging from 11 to 23. Due to the essential biological role of the transcriptional process, evolutionary deletion of genes in this category had

to be limited and selective. Based on the COG database (National Center of Biotechnology Information), the number of genes involved in transcription in other bacteria is usually much higher, such as in *Neisseria gonorrhoeae* (84 genes; 4.2% of total genes), *Haemophilus influenzae* (91; 5.3%), *E. coli* (342; 8.1%) and *Bacillus subtilis* (372; 9%). Data reveal that the ORFs related to transcription in *M. hyopneumoniae* strains J and 7448 and in *M. synoviae* code for the bare-bones proteins involved in transcription in prokaryotes (RNA polymerase holoenzyme, including a single  $\sigma$  factor, elongation and terminator factors), reinforcing the selectiveness of gene saving in mycoplasmas (Table 1). The number of putative transcriptional regulators in these bacteria is minimal, and this drastic saving in genes raises the questions of how they adapt to environmental changes and how they maintain cellular homeostasis.

The core RNA polymerase of mycoplasmas resembles that of other eubacteria in subunit structure, encoded by the conserved genes *rpoA* ( $\alpha$  subunit), *rpoB* ( $\beta$  subunit) and *rpoC* ( $\beta'$  subunit), as listed in Table 1. The small, accessory  $\omega$  subunit (*rpoZ*), present in many eubacteria, is absent in mycoplasmas. During gene regulation, the sigma factor ( $\sigma$ ) plays an essential role in the conversion of core

RNA polymerase to holoenzyme, since it directs RNA polymerase to specific promoters, so that transcription initiates at the proper place. As shown in Table 1, the genomes of *M. hyopneumoniae* and *M. synoviae* encode only a single sigma factor, compared to at least 6 in *E. coli* (Blattner *et al.*, 1997) and 18 in *Bacillus subtilis* (Kunst *et al.*, 1997). This marked difference corroborates the findings described in several other mycoplasma strains (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996; Glass *et al.*, 2000; Minion *et al.*, 2004). The single  $\sigma$  factor found in mycoplasmas is homologous to the  $\sigma^{70}$  type factor found in *E. coli* or the  $\sigma^A$  vegetative type of gram-positive bacteria, and preliminary evidence for the presence of a sigma factor inducing mobility has been reported in *M. pneumoniae* (Bornberg-Bauer and Weiner III, 2002). Modulation of promoter selectivity of RNA polymerase by replacement of the sigma subunits is an efficient way of altering the global pattern of gene expression in response to changes in environmental conditions. Thus, the presence of only one sigma factor in mycoplasmas suggests that the level of expression of alternative sigma factors does not control either the response to external stimuli in these organisms (Razin *et al.*, 1998) or promoter selectivity of polymerases with alternative  $\sigma$  fac-

**Table 1** - ORFs related to transcription in *M. hyopneumoniae* J and 7448 and *M. synoviae* strains, based on COG (NCBI) assignment of sequences.

Function	<i>M. hyopneumoniae</i> J		<i>M. hyopneumoniae</i> 7448		<i>M. synoviae</i>	
	Gene	ORFs	Gene	ORFs	Gene	ORFs
DNA-directed RNA polymerase alpha subunit	<i>rpoA</i>	MHJ0164 (1)	<i>rpoA</i>	MHP0168	<i>rpoA</i>	MS0574
DNA-directed RNA polymerase beta subunit	<i>rpoB</i>	MHJ0618 (1)	<i>rpoB</i>	MHP0617	<i>rpoB</i>	MS0485
DNA-directed RNA polymerase beta' subunit	<i>rpoC</i>	MHJ0617 (1)	<i>rpoC</i>	MHJ0616	<i>rpoC</i>	MS0484
DNA-directed RNA polymerase sigma factor	<i>rpoD</i>	MHJ0056 (1)	<i>rpoD</i>	MHP0060	<i>rpoD</i>	MS0455
Exoribonuclease R		MHJ0033 (2)		MHP0037		MS0301
Heat-inducible transcription repressor	<i>hrcA</i>	MHJ0010 (2)	<i>hrcA</i>	MHP0010	<i>hrcA</i>	MS0353
Ribonuclease III		MHJ0411 (1)		MHP0398		MS0675
Serine/threonine protein kinase						MS0121 (1)
Transcriptional accessory protein						MS0548 (2)
Transcription elongation factor	<i>greA</i>	MHJ0667 (1)	<i>greA</i>	MHP0668	<i>greA</i>	MS0379
	<i>nusA</i>	MHJ0586 (1)	<i>nusA</i>	MHP0586	<i>nusA</i>	MS0685
Transcription antitermination protein	<i>nusG</i>	MHJ0637 (2)	<i>nusG</i>	MHP0637	<i>nusG</i>	MS0361
Transcription termination factor		MHJ0158 (1)		MHP0162 MHP0585 (1)		MS0324
Putative transcriptional regulator		MHJ0271 (2)		MHP0279		MS0024
		MHJ0555 (2)		MHP0551		MS0024
		MHJ0515 (1)		MHP0517		MS0195
		MHJ0634 (2)		MHP0633		MS0095
		MHJ0303 (2)		MHP0311		MS0006 (2)
						MS0109 (2)
						MS0127 (2)
						MS0548 (2)

Numbers in parentheses represent (1) proteins whose function was determined experimentally in or purified from other bacteria; (2) proteins whose function was deduced from sequence comparison using NCBI's BLASTp.

tors. However, the genome sequences of *M. pneumoniae*, *M. genitalium*, *M. gallisepticum*, and *U. urealyticum* contain a gene homologous to the  $\delta$  subunit or  $\sigma^E$  (*rpoE*), as deduced from their gene sequences, that is required for the expression of several stress-response genes in *E. coli* and some other bacteria (Muto and Ushida, 2002).

The genomic sequences of both *M. hyopneumoniae* and *M. synoviae* lack several major regulators of gene expression, including two-component regulatory systems and multiple  $\sigma$  factors. Gene promoters of *M. hyopneumoniae* and *M. synoviae* contain two distinct sequence motifs that reside, respectively, -10 and -35 regions upstream of the transcriptional start site. Primer extension studies conducted by Weiner III *et al.* (2000) have shown that, although a strong consensus -10 region (TATA/TATTTTTT AAAATTAA) could be seen in *M. pneumoniae*, there was only a weak consensus in the -35 region (CATAATTTT ATTTTTGAAATA/TTTT). It was also observed that there are a high proportion of transcripts with heterogeneous 5' ends derived from initiation of transcription at reduced levels between 1 and 4 bases 5' to the major starting point. In addition to this apparently unique feature, in *M. pneumoniae* a high proportion of transcripts lacked an untranslated 5' leader region that could contain a ribosomal binding site (Weiner III *et al.*, 2000). Such leaderless transcripts are rarely seen in other bacterial species and seem to be an accurate regulatory mechanism of gene transcription in *Mycoplasma*, although there is no confirmation of this role so far. The unusual A+T content of intergenic regions in mycoplasma (Muto and Osawa, 1987) and the weak -35 consensus (Weiner III *et al.*, 2000) prevents an efficient prediction of promoters by current bioinformatics tools. Moreover, the likely occurrence of cryptic operons with dysfunctional promoters resulting from mutations in the regulatory regions complicates matters further. *In vivo* analysis of promoter activity in mycoplasmas is presently difficult; as a plasmid transformation system is not in place, a more expedite understanding of this fundamental aspect of transcription initiation is not possible.

It is well known that there are two different mechanisms of transcription termination in *E. coli*, one rho-dependent and one rho-independent (Opperman and Richardson, 1994). Such regulatory events are characterized by the presence of a hairpin loop in the secondary structure of mRNA, in rho-independent termination, while in the rho-factor-dependent process transcription is terminated by the interaction of the rho-factor protein with RNA polymerase. These general mechanisms are used for transcriptional regulation in a wide range of prokaryotes, although no mollicute genome sequenced so far seems to possess the rho protein. Transcription termination modulators NusA and factor G (*greA* gene), elongation factors, and NusG,

which is involved in antitermination, are proteins involved in transcription in *M. hyopneumoniae* strains J and 7448 and in *M. synoviae* (Table 1). Gene homologs for *nusA*, *greA* and *nusG* are also found in nine other sequenced mollicute genomes (Fraser *et al.*, 1995; Himmelreich *et al.*, 1996; Glass *et al.*, 2000; Chambaud *et al.*, 2001; Sasaki *et al.*, 2002; Papazisi *et al.*, 2003; Jaffe *et al.*, 2004; Minion *et al.*, 2004; Westberg *et al.*, 2004), with the exception of *nusG* in *M. genitalium*. In contrast, *nusB*, a transcription terminator that acts by modulating the efficiency of transcriptional antitermination, is present only in *M. mycoides*, *M. penetrans* and *U. urealyticum/parvum*. Washio *et al.* (1998) suggested that *M. pneumoniae* and *M. genitalium* may not rely on hairpin formation in transcription termination, based on calculations of free energy values around the stop codons over the entire genome. Nevertheless, it should be noted that the lack of apparent free energy drop does not necessarily indicate the complete absence of hairpins, since some of the species may have only small or weak hairpin formation at the transcription termination sites. In fact, *in vitro* analysis of transcription in the *M. capricolum* tRNA<sup>Trp</sup> gene cluster indicated that termination takes place at the T-stretch region soon after the dyad-symmetrical structure that leads to the hairpin loop formation (Yamao *et al.*, 1988).

Recently, Benders *et al.* (2005) established a detailed transcriptional profile of the phylogenetically conserved *ftsZ* cell division gene cluster in both *M. genitalium* and its closest relative, *M. pneumoniae*. Initiation and termination points were determined for the cell division cluster that comprises just four genes, with the *ftsZ* gene at the 3' end. Transcription of this cluster in both these organisms was shown to be highly strand-specific. While the four genes in this cluster are cotranscribed, their transcription unit also includes two additional genes of close proximity, yet with related function. Two amino acid permeases belonging to the same COG are separated from the *ftsZ* cluster by only 45 bp (*M. genitalium*) or 42 bp (*M. pneumoniae*). A transcription initiation point immediately upstream of these two genes was detected in *M. genitalium*, but not in *M. pneumoniae*. In *M. pneumoniae*, transcription of these genes that comprise the cell division cluster terminates at a poly(U)-tailed hairpin. In *M. genitalium*, this transcription terminates at two closely spaced points by an unknown mechanism. The authors concluded that groups of functionally related genes in *M. genitalium* and *M. pneumoniae* are often preceded by promoters, but rarely followed by terminators. This causes functionally unrelated genes to be commonly cotranscribed in these organisms. Due to the presence of few signals for discrete and efficient transcription termination, many genes may be transcribed by "run-on" transcription from upstream promoters. Real-time reverse transcription-PCR analysis of the *ftsZ* gene cluster in *M. pneumoniae* showed that the mRNA levels for all six genes

vary at most fivefold and form a gradient of decreasing quantity with the increasing distance from the promoter at the beginning (Benders *et al.*, 2005).

Although limited to one gene cluster comprising six genes, Benders *et al.* (2005) provided a new understanding of the coordination of gene expression in mycoplasmas. Based on the results from *M. pneumoniae* and *M. genitalium*, as expected, these bacteria cotranscribe genes with related functions. However, these bacteria also cotranscribe genes that are not functionally related, and it has been proposed that this may be the rule rather than the exception in these organisms. If there are few terminators in the genomes of mycoplasmas, as previously proposed (Washio *et al.*, 1998), then the position of these elements may not be relevant to the functional grouping of genes. Regardless of whether two genes are cotranscribed, a promoter between the two may indicate that they are not functionally related. For instance, cotranscription evidence suggests that the ribosomal protein in the *hmw* cluster is part of an operon with upstream genes. However, it has its own promoter and is the 3'-terminal gene in the cluster (Benders *et al.*, 2005).

It has further been proposed that *M. genitalium* makes heavy use of operon systems, potentially reducing the number of regulatory elements required for controlling the transcription of genes (Peterson *et al.*, 1993). The arrangement of ORFs is such that there are rarely more than a few nucleotides between the stop codon of one ORF and the methionine of the next. Data taken from a recently developed operon database (Alm *et al.*, 2005) confirm the assumption of heavy use of operons in mycoplasmas. Operon prediction data for *M. hyopneumoniae* strains J and 7448 and *M. synoviae* reveal that 70%, 74% and 79% of their genes, respectively, are organized in operons. These numbers compare to the 74% of operon usage in *M. pneumoniae* and are significantly higher than in other bacteria, such as *E. coli* (63%) and *Bacillus subtilis* (58%). For genes to be included in the database as part of an operon, prediction of gene pairs uses statistical inference based on distance, comparative features (how often their orthologs are near each other - within 5 kb - in other genomes), functional similarity (whether their predicted functions are in the same COG category), and similarity of CAI (codon adaptation index), a measure of synonymous codon usage (Price *et al.*, 2005).

Many rRNA and tRNA genes in bacteria are clustered forming operons. Like other eubacteria, the rRNA genes of most mycoplasmas are organized in an operon and in the same order, 16S-23S-5S (Muto and Ushida, 2002). However, in *Mycoplasma hyopneumoniae* strains J, 7448 and 232, only genes for 16S and 23S are in the same operon, and the 5S gene is some 100 kb further downstream. Detailed organization of these genes as well as of others involved in translation in *M. hyopneumoniae* strains J and 7448 and *M.*

*synoviae* is presented in an accompanying paper (Santos *et al.*, 2006).

The regulatory mechanisms involved in the control of prokaryotic gene expression are limited, since the bacterial genome is associated directly with the protein synthesizing machinery. In general, every step that is required to make an active gene product can be the focus of a regulatory event, but in practice most bacterial regulation occurs at the transcriptional level. In the last decade, complete genome sequencing of several mycoplasma strains and elaborate experimental data revealed some unique features of mycoplasma gene expression regulation (Weiner III *et al.*, 2003; Benders *et al.*, 2005). A survey of the mycoplasma genomes sequenced to date reveals a lack of several major gene expression regulators, including two-component regulatory systems and, as seen above, multiple  $\sigma$  factors. In addition, the high A+T content of their genomes has been found to result in adventitious promotion of transcription when fragments of their genomic DNA were introduced into *E. coli*. (Weiner III *et al.*, 2003). The apparent scarcity of regulatory genes functioning as sensors to environmental stimuli and of genes encoding transcriptional factors suggests, but does not rule out, that adaptation of mycoplasmas to the changing environment is not *per se* a response to signals (Rottem, 2003). In the genome of *M. hyopneumoniae* strain 232, no PROSITE AraC, LysR, GntR, LuxR or sigma 54 interaction domains were found (Minion *et al.*, 2004). Also, a search for helix-turn-helix (HTH) motifs revealed numerous AraC motifs with low scores, and consequently these proteins have a low probability of having a regulatory function. On the other hand, Papazisi *et al.* (2003) found 21 proteins (excluding sigma 70, recombinases, helicases and other enzymes involved in nucleic acid modification) that contained HTH motifs similar to the PROSITE AraC, LysR, GntR and LuxR motifs, even though the degree of identity is not presented.

The number of putative regulatory proteins present in the two strains of *M. hyopneumoniae* and in *M. synoviae* is very low (Table 1), based on COGs assigned to sequences. In either strain of *M. hyopneumoniae*, only five proteins possessing DNA-binding regulatory motifs were found compared to eight found in *M. synoviae*. Searching for regulatory proteins in the genome of other completely sequenced mycoplasmas showed the presence of *fur* (ferric uptake regulator), *fruR* (transcriptional repressor fructose operon) and a sucrose PTS repressor in *M. mycoides* (Westberg *et al.*, 2004); a transcription regulator of the MarR family in *M. penetrans* (Sasaki *et al.*, 2002); and *trsE* (transfer complex protein) in *M. pneumoniae* (Dandekar *et al.*, 2000). No homologs of such genes were found in *M. hyopneumoniae* or in *M. synoviae*.

The minimal set of regulatory proteins identified in completely sequenced mycoplasma genomes, including *M. synoviae* and *M. hyopneumoniae* strains J and 7448, could be either the result of overlooking by usual computational genome analysis, the result of a remarkable adaptation to constant environments, or both. The price for the drastic saving in genes in mycoplasmas can be questioned, as this reduction may render them less adaptable to environmental changes. If so, the mechanisms for maintaining cellular homeostasis in mycoplasmas are not obvious. Answering these questions and unraveling the subtleties of the interconnected regulation mechanisms in mycoplasmas is expected to be a demanding enterprise (Razin *et al.*, 1998). Comprehensive comparative analysis of genomic sequences and the proteins they encode is an absolute prerequisite to further advances in our understanding of cell biology. It is expected that novel mechanisms of transcription initiation and/or a refinement of the current understanding of the transcriptional process in mycoplasmas will be achieved in the coming years, as more elaborate techniques for the study of gene expression in mycoplasmas are being employed. Ultimately, increased knowledge of gene expression in these minimalist organisms should help us understand how they maintain their lifestyle.

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### Internet Resources

National Center of Biotechnology Information, <http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi> (October 20, 2005).

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