



Comparative genome analysis of proteases, oligopeptide uptake and secretion systems in *Mycoplasma* spp

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Abstract

Mycoplasmas are very fastidious in their nutritional requirements for *in vitro* growth and have limited biosynthetic capacity, a reflection of their reduced genomes. As a result, these bacteria depend upon external metabolites for nutrition and growth and have developed dependence on their hosts for survival and maintenance. Protein degradation and peptide importation play an important role in *Mycoplasma* spp. nutrition, and proteases can play a role in host adaptation and pathogenicity. Here, we present a general survey on the genes involved in protein degradation, secretion and importation, comparing all available Mollicute genomes.

Key words: *Mycoplasma*, proteases, minimal genomes, secretion systems.

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Introduction

Mycoplasmas are considered the smallest cells capable of propagation in cell-free medium, and some species are pathogenic to humans, animals and plants. In spite of their reduced genomes, due to significant gene loss through evolution/adaptation, mycoplasmas are a very successful group of organisms, as judged by their large number of species and habitats (Razin *et al.*, 1998). The 'minimum cell' life style of mycoplasmas became possible by the adoption of a parasitic mode of life, exploiting nutrients not synthesized by themselves, and evolving systems to invade and to persist in their hosts (van Ham *et al.*, 2003).

Over the last few years, the genomes of nine *Mycoplasma* species were sequenced, reinforcing comparative genome studies that allow a better understanding of their metabolism and the relations with their hosts. Mycoplasmas evolved from gram-positive bacteria and, through evolution, lost the cell wall and many metabolic pathways for the synthesis of macromolecule building blocks. Mycoplasmas possess no complete routes for amino acids synthesis and degradation, implying that these monomers must be acquired either from their hosts or from a culture medium, depending upon membrane transporters (Vasconcelos *et al.*, 2005). Exogenous peptides are an important source of amino acids. Indeed, bacteria have evolved peptide transport systems that also assist in responses to envi-

ronmental changes, mediating functions such as quorum sensing, sporulation, pheromone transport, and chemotaxis (Wang *et al.*, 2004).

Despite the presence of a complete set of genes responsible for essential cell activities such as replication, transcription and translation, genes involved in post-translational protein modifications are not readily disclosed by the annotation of the mycoplasma genomes. Some of these processes, such as protein maturation and localization, are intrinsically dependent on proteases. Microbial proteases may also play important roles in pathogenicity and nutrition.

Bacterial development is also dependent on the secretion of proteins with a plethora of functions. One of the major transport routes, the so-called Sec pathway, is conserved in all domains of life and is the only system found in mycoplasmas by genome surveys (Stephenson, 2005).

In this work, we present a general survey on the genes involved in protein metabolism, based on the available mycoplasma genomes.

Material and Methods

The complete genome sequences of the *Mycoplasma* spp. used in this work were retrieved from the NCBI data base (<http://www.ncbi.nlm.nih.gov>), as available in October, 2005. Primary searches were conducted using BLAST search tools (Altschul *et al.*, 1990), or based on annotated genome files. The search for *Opp* (oligopeptide transport genes) and secretion systems was conducted using InterPro entries for *Bacillus subtilis* components (<http://www.ebi>).

ac.uk/interpro, Mulder *et al.*, 2005). The classification and analysis of proteases were done according to the MEROPS peptidase database (<http://merops.sanger.ac.uk>, Rawlings *et al.*, 2004).

Results and Discussion

Oligopeptide importing

Mycoplasma genomes possess a diversity of ABC transporters predicted to be involved in the uptake of several inorganic and organic substrates. One class of ABC transporters, the peptide/opine/nickel uptake transporter family (3.A.1.5.1), is involved in oligopeptide uptake with high affinity for tripeptides (Transport Classification Database, <http://www.tcdb.org>).

The known genomic organization of the *Opp* operon in Mollicutes is shown in Table 1. Proteins encoded by this operon are anchored in the cell membrane; they transport oligopeptides from the extracellular milieu and represent an important form of nutrition (Detmers *et al.*, 1998). *Mesoplasma florum* is the only species with no sequences related to the *Opp* system, as far as predicted by the annotation. The remaining genomes vary from one to two copies of the operon and also scattered single cistron copies. This distribution does not follow the division *hominis/pneumoniae* clades. The complete *Opp* operon (*OppABCDF*) was annotated only in *M. mycoides* and in *Phytoplasma*, and is present in two copies. It is noteworthy that in one of the *M. mycoides* operons the cistron order is altered from *ABCDF* to *BCDFA*. Moreover, in both species there are scattered copies of single components elsewhere in the genome. Two copies of the incomplete operon (lacking *OppA*) are present in three species (*M. pulmonis*, *M. penetrans* and *M. hyopneumoniae*). The same incomplete operon is present, as single copies, in all five genomes; however, in *M. synoviae*, the cistron order is different, and in *M. mobile* an extra copy of *OppF* is present.

The function of *OppA* as substrate-binding protein (oligopeptide recognition) is well recognized in bacteria (LeDeaux *et al.*, 1997; Detmers *et al.*, 1998). In *Mycoplasma hominis* (genome sequence not available), *OppA* functions as the P100 adherence-associated lipoprotein, and the operon is organized as *OppABCDF* (Henrich *et al.*, 1999). Therefore, an important role for *OppA* in oligopeptide uptake could be expected in other Mollicutes. However, the *OppA* gene was found only in two Mollicute genome sequences (Table 1). This raises the question if *OppA* is really a necessary component of the oligopeptide uptake systems in these bacteria. Nevertheless, the low conservation of this protein could hinder its annotation. In addition, the habitat broadness of Mollicutes could result in strong selection/adaptation, especially for proteins involved in the recognition (binding) of oligopeptides, expected to be variable in different habitats. Also, lipoproteins are among the most prominent components of

Table 1 - Genomic organization of *Opp* uptake system in mycoplasmas. The five components are disposed in clusters or as isolated ORFs in the genome.

Microorganism	Clusters		Scattered	
	I	II		
<i>Mycoplasma genitalium</i>	<i>OppBCDF</i>	- ^a	-	-
<i>Mycoplasma pulmonis</i>	<i>OppBCDF</i>	<i>OppBCDF</i>	-	-
<i>Mycoplasma penetrans</i>	<i>OppBCDF</i>	<i>OppBCDF</i>	-	-
<i>Mycoplasma pneumoniae</i>	<i>OppBCDF</i> ^b	-	-	-
<i>Ureaplasma urealyticum</i>	<i>OppBCDF</i>	-	-	-
<i>Mycoplasma gallisepticum</i>	<i>OppBCDF</i>	<i>OppCDF</i>	<i>OppF</i>	<i>OppF</i>
<i>Phytoplasma</i> sp.	<i>OppABCDF</i>	<i>OppABCDF</i>	<i>OppF</i>	<i>OppA</i>
<i>Mycoplasma mycoides</i>	<i>OppBCDFA</i>	<i>OppABCDF</i>	<i>OppF</i>	<i>OppF</i>
<i>Mycoplasma mobile</i>	<i>OppBCDF</i> ^c	-	<i>OppF</i>	-
<i>Mycoplasma hyopneumoniae</i> ^d	<i>OppBCDF</i>	<i>OppBCDF</i>	-	-
<i>Mycoplasma synoviae</i>	<i>OppBCFD</i>	-	-	-

^anot found. ^b*OppC* also known as *AmiD*. ^c*OppD* annotated as *pgk*, but contains the classical ATP-binding domain of the *opp* family. ^dstrains J, 7448 [P], and 232.

mycoplasma cell membranes (Razin *et al.*, 1998), and the substrate recognition function of *OppA* could be fulfilled by one of these proteins.

Proteases

Bacterial development is dependent on a plethora of proteolytic activities involved in diverse functions, such as protein homeostasis, pathogenicity and nutrient acquisition. Mycoplasma genomes analysis revealed a complex distribution of these enzymes (Table 2). ATP-dependent proteases, such as Lon and FtsH, that degrade aberrant proteins, were found in all genomes analyzed here. Lon is a DNA-binding protease that degrades regulatory and abnormal proteins and has both the proteolytic and the ATPase domains. FtsH is a membrane protease that degrades membrane and cytoplasmic proteins. However, other important proteases involved in abnormal protein degradation, such as ClpPX and HslUV, were not annotated in five Mycoplasma species analyzed previously. This wider *in silico* survey of 12 Mollicute genomes supports the hypothesis outlined by Wong and Houry (2004) that the protein homeostatic process in these organisms has shifted through evolution towards favoring protein degradation rather than protein folding. Lon-defective *Escherichia coli* mutants remain phenotypically stable when overproducing HslU and HslV proteases, denoting a probable substrate overlapping among these proteins under certain physiological conditions (Wu *et al.*, 1999). This suggests that the lack of the HslUV system in mycoplasmas could be surpassed by the presence of Lon. FtsH is an endopeptidase, dependent on ATP and Zn²⁺, that degrades abnormally-folded proteins

Table 2 - Distribution of putative proteases in mycoplasmas.

Group	Microorganism ^a	Mge	Mpu	Mpe	Mpn	Mga	Mmy	Mmo	Mhy	Msy
	Peptidase									
A	Signal peptidase I	- ^b	+ ^c	-	-	+	-	-	+	+
B	Prolipoprotein signal peptidase II (SpaseII)	+	+	+	+	+	+	-	+	+
C	C1A subfamily peptidase	-	-	-	-		-	-	-	+
D	Family C39 (SunT protein) - ABC trans., Leucyl peptidase Heat shock ATP-dependent protease (Lon) Methionine aminopeptidase (map) Xaa-proaminopeptidase (pepP) o-sialoglycoprotein endopeptidase FtsH peptidase Putative glycoprotein endopeptidase	+	+	+	+	+	+	+	+	+
E	ATP-dependent serine protease-binding protein	+	-	-	-	-	+	+	+	-
F	Aminopeptidase	-	+	-	-	-	-	-	-	-
G	Subtilisin-like serine protease	-	+	-	-	-	-	-	+	+
H	Proline dipeptidase	-	+	+	-	+	+	-	+	+
I	Oligoendopeptidase F (pepF)	+	+	+	-	-	+	+	+	+
J	Prolyl aminopeptidase	+	-	+	+	-	-	-	-	-
K	Aminopeptidase C Collagenase Zn-dependent protease	-	-	+	-	+	-	-	-	-
L	Endopeptidase O	-	-	+	-	-	+	-	-	+

^aAbbreviations: Mge, *Mycoplasma genitalium*; Mpu, *Mycoplasma pulmonis*; Mpe, *Mycoplasma penetrans*; Mpn, *Mycoplasma pneumoniae*; Mga, *Mycoplasma gallisepticum*; Mmy, *Mycoplasma mycoides*; Mmo, *Mycoplasma mobile*; Mhy, *Mycoplasma hyopneumoniae*; Msy, *Mycoplasma synoviae*.
^bnot present. ^cpresent.

and the proteolysis products that otherwise cause cellular abnormalities. The absence of the HslUV system and the presence of Lon and FtsH appear to be conserved among mycoplasmas, except for *M. florum* that does not possess FtsH. The same applies to the absence of ClpPX, observed in Mollicute genomes except the Onion Yellow *Phytoplasma*, which possesses ClpX (Table 2).

Protease secretion in order to obtain peptides from the milieu is a common feature of bacteria (Morales *et al.*, 2001). Most subtilisin-like and other serine proteases are secreted endopeptidases with little specificity for their substrates. The subtilisin-like serine proteases in the mycoplasma genomes belong to the subfamily S8A, which are endopeptidases. It is important to note that the gram-negative bacterium *Dichelobacter* has one subtilisin-like serine protease directly implicated in pathogenesis. Microbial pathogens often utilize secreted proteases as virulence factors, which may contribute largely to their pathogenicity. These proteases participate in tissue destruction, inactivation of host defense molecules, activation of key regulatory proteins or peptides and in nutrient acquisition. Some bacterial proteases can also activate bacterial toxins, thus triggering toxigenic pathogenesis. These proteases are also capable of degrading immunoglobulins and components of the complement system assisting infection propagation. Microbial proteases are very critical in enhancing

pathogenesis of many severe diseases. However, only three out of the nine mycoplasmas analyzed here possess putative genes coding for serine proteases (Table 2).

Intracellular peptidases were also found in the present genome survey (Table 2). Bacterial leucyl aminopeptidases are involved in processing and maintaining a regular turnover of intracellular proteins and peptide breakdown products generated by intracellular proteases (Jenal and Hengge-Aronis, 2003). Methionyl aminopeptidase (Map) degrades “Ala/Ser-Pro” dipeptides, avoiding their accumulation, which could become toxic to the cell. Furthermore, oligopeptidase F (functions) acts in the degradation of intracellular oligopeptides generated from cell protease activity and can also cleave signal peptides. The main role of Map is to remove the initial methionine of many proteins during translation. The Xaa-Pro aminopeptidases, that hydrolyse “Xaa-Pro” dipeptides, and the prolyl dipeptidases, that release N-terminal residues from peptides, preferably (but not exclusively) a proline, were annotated in mycoplasma genomes (Table 2). The o-sialoglycoprotein endopeptidases cleave heavily o-sialoglycosylated proteins. These enzymes do not possess identifiable signal peptides and, therefore, are probably not secreted, establishing themselves as intracellular proteins. The involvement of these enzymes in pathogenicity remains to be demonstrated.

Protein secretion

The first description of secretion systems in mycoplasmas came with the genomes of *Mycoplasma genitalium* (Fraser *et al.*, 1995) and *M. pneumoniae* (Himmelreich *et al.*, 1996). These authors referred to the incomplete Sec translocation machinery in mycoplasmas compared to *Haemophilus influenzae* and *E. coli*, as a result of the lower complexity of the mycoplasma cell membrane. Now, the characterization of secretion systems in phylogenetically closer bacteria (Tjalsma *et al.*, 2000; van Wely *et al.*, 2001) and the availability of several Mollicute genomes allow a more accurate comparison. A survey of mycoplasma genomes, based on similarity searches using conserved domains of the Sec translocation proteins, yielded near-complete secretion systems in all individuals (Table 3). The absence of identifiable SecB protein is in agreement to the Sec translocation machinery of *B. subtilis*, in which other chaperone-like protein(s) would function as SecB-like protein (Tjalsma *et al.*, 2000). The most intriguing fact is that some mycoplasmas are devoid of annotated pore-forming transmembrane proteins SecG or SecE.

Signal peptidases, such as signal peptidase I (Spase I) and signal peptidase II (Spase II), are responsible for cleaving off the hydrophobic N-terminal signal peptide regions of proteins to be exported or held in specific parts of the cell, such as the cytoplasmic membrane. Several types of Spase I from gram-negative and gram-positive bacteria have clear differences concerning gene size, gene copy number and substrate specificity, although there are substantial sequence similarities, as indicated by six different

Table 3 - Sec translocon pathway secretion system components among known mycoplasma genomes.

Gene	SecA, SecY, Ffh, ftsY, scRNA	YajC, SecB	SecDF	SecE	SecG
Microorganism ^a					
Mge	+ ^b	- ^c	-	+	-
Mpu	+	-	+	-	-
Mpe	+	-	+	+	+
Mpn	+	-	-	+	+
Uur	+	-	-	+	+
Mga	+	-	-	+	+
Phy	+	-	+	+	-
Mmy	+	-	+	+	-
Mmo	+	-	+	-	-
Mhye	+	-	+	-	+
Msy	+	-	+	-	-

^aAbbreviations: Mge, *Mycoplasma genitalium*; Mpu, *Mycoplasma pulmonis*; Mpe, *Mycoplasma penetrans*; Mpn, *Mycoplasma pneumoniae*; Uur, *Ureaplasma urealyticum*; Mga, *Mycoplasma gallisepticum*; Phy, *Phytoplasma*, Mmy, *Mycoplasma mycoides*; Mmo, *Mycoplasma mobile*; Mhye *Mycoplasma hyopneumoniae*; Msy, *Mycoplasma synoviae*. ^bpresent. ^cnot present.

regions with conserved amino acids. Besides that, Spases type I are essential for cell life. The distribution of type I and type II Spases was found to be quite different in the mycoplasmas analyzed here (Table 2). Signal peptidases are thought to be fundamental in the pathogenicity of mycoplasmas, since their activities were found to be involved in the processing of a cilium adhesin from *M. hyopneumoniae* (Djordjevic *et al.*, 2004) and *M. pneumoniae* and hence its role in pathogenicity.

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