Pathogenicity & virulence of Mycoplasma hyopneumoniae

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ABSTRACT

Mycoplasma hyopneumoniae: is the etiological agent of porcine enzootic pneumonia (EP), a disease that impacts the swine industry worldwide. Pathogen-induced damage, as well as the elicited host-response, contribute to disease. Here, we provide an overview of EP epidemiology, control and prevention, and a more in-depth review of M. hyopneumoniae pathogenicity determinants, highlighting some molecular mechanisms of pathogen-host interactions relevant for pathogenesis. Based on recent functional, immunological, and comparative "omics" results, we discuss the roles of many known or putative M. hyopneumoniae virulence factors, along with host molecules involved in EP. Moreover, the known molecular bases of pathogenicity mechanisms, including M. hyopneumoniae adhesion to host respiratory epithelium, protein secretion, cell damage, host microbicidal response and its modulation, and maintenance of M. hyopneumoniae homeostasis during infection are described. Recent findings regarding M. hyopneumoniae pathogenicity determinants also contribute to the development of novel diagnostic tests, vaccines, and treatments for EP.

Introduction

Mycoplasma hyopneumoniae is the etiological agent of porcine enzootic pneumonia (EP), a chronic respiratory disease that affects pigs [1]. Infections with M. hyopneumoniae are highly prevalent worldwide, causing major economic losses to the swine industry [2] due to costs of treatment and vaccination, decreased feed conversion rate, and increased mortality resulting from secondary infections [3]. Immunodiagnosis of M. hyopneumoniae infections and vaccination against EP are also challenging due to the still limited repertoire of well-characterized antigens. Some diagnostic and/or vaccinal antigens have been characterized immunologically, i.e., with an assessment of their antigenicity and immunogenicity [4–8], and functionally, with an assessment of their roles in M. hyopneumoniae physiology and pathogen-host interactions [9–15].

In the context of the damage-response framework of microbial pathogenesis [16], EP pathology is mainly determined by the damage caused by interactions between M. hyopneumoniae and the swine host. It is known that M. hyopneumoniae infection leads to epithelial damage of the swine respiratory tract, either directly, considering possible bacterial cytotoxicity [14], or indirectly, by causing a strong and damaging inflammatory response. The damage is usually restricted to the bacterium-caused loss of cilia and cell death, and those caused by the host inflammatory response against the pathogen [2]. The exact mechanisms underlying the immunopathogenesis of M. hyopneumoniae, however, are not clear. It is reasonable to assume that the onset of EP in swine depends on M. hyopneumoniae pathogenicity determinants, classically described as virulence factors, as well as on the triggered host responses, immunological or otherwise. M. hyopneumoniae pathogenicity determinants are considered the bacterial factors that allow the pathogen to override the host defense mechanisms and comprehend molecules that mediate processes such as cell adhesion to the host, response to host environment stress, and immunomodulation. On the other hand, host factors contributing to EP include molecules and processes that mediate innate and adaptive defenses against the pathogen, but also cause damage host tissues, such as lung lesions resulting from exacerbated inflammatory responses.
In the last decade, comparative analyses between pathogenic and nonpathogenic *M. hyopneumoniae* strains, and between *M. hyopneumoniae* and the closely related species *Mycoplasma flocculare*, have considerably improved the knowledge of *M. hyopneumoniae* pathogenesis. *M. hyopneumoniae* strains may differ in pathogenicity or virulence, ranging from the nonpathogenic type strain J (ATCC 25,934) to virulent strains isolated from EP outbreaks, such as the Brazilian strain 7448 or the American strain 232 [17-20]. *M. flocculare*, in turn, is genetically closely related to *M. hyopneumoniae* but is a commensal species, with its presence in the host being virtually asymptomatic. The “omics” prospective and comparative studies with these strains and species, discussed in a later section, have improved our knowledge on the factors that determine *M. hyopneumoniae* pathogenicity.

Host EP determinants, on the other hand, have been less assessed, aside from some punctual studies regarding innate and adaptive immunological response and cell death [21-24]. Nevertheless, differences between pathogenic and nonpathogenic strains or closely related species may also depend on the triggered host responses. Recently, comparative studies regarding the interactions between *M. hyopneumoniae* or *M. flocculare* and the swine host demonstrated differences in intracellular and secreted protein repertoires of swine cells infected by these mycoplasma species [25,26].

This review includes recent data regarding EP epidemiology, prevention, and control of this disease, and a comprehensive overview of the main *M. hyopneumoniae* and swine molecular mechanisms and cellular processes underlying the disease. Moreover, it also surveys recent advances toward the identification of both the bacterial and host repertoires of EP determinants, including the most recent efforts involving functional and comparative “omics” approaches to elucidate why some *M. hyopneumoniae* strains are more virulent than others and why *M. flocculare* is nonpathogenic despite of sharing most of the virulence factors with *M. hyopneumoniae*. Interactions between *M. hyopneumoniae* and swine cells are discussed in the context of their intimate contact during infection, emphasizing molecular/cellular mechanisms related to cell adhesion, biofilm formation, host cell invasion, secretion and signaling, cytotoxicity and apoptosis, immunomodulation and stress response.

**Epidemiology, prevention and control of EP**

**Epidemiology**

As far as is known, *M. hyopneumoniae* is a specific pathogen of domestic pigs (*Sus scrofa domesticus*) and wild boars (*Sus scrofa scrofa*), and has a worldwide geographical distribution [27]. Specific data on *M. hyopneumoniae* prevalence by country are scarce in the literature, as EP does not require mandatory notification in many countries and does not limit commercial trade [2]. An average *M. hyopneumoniae* prevalence of 30–80% has been reported in domestic pig herds worldwide [28]. In South America, the estimated *M. hyopneumoniae* prevalence was 48% for pigs in the Mendoza province, Argentina, based on molecular diagnosis [29], and prevalence varying from 52% (based on serology of non-vaccinated animals) to more than 90% (based on molecular diagnosis of slaughtered animals) was reported for Southeastern and Southern Brazil [30,31]. A lower *M. hyopneumoniae* prevalence, near 10%, was reported for pigs in Africa (Uganda) [32]. For wild boars, the most recent studies carried out in European countries, such as Sweden and Italy, have shown seroprevalences of *M. hyopneumoniae* of 24.8% and 21.12%, respectively [33,34]. In the last decade, the wild boar population has increased in Europe, which increases the likelihood of its potential contact with domestic pigs, and, thus, the risk of transmission of *M. hyopneumoniae* and other pathogens [34].

*Mycoplasma hyopneumoniae* transmission dynamics within swine herds depend on the intensity of the production system used, as recently reviewed by Maes et al. [2]. The first exposure events occur during the lactation period, when piglets are in contact with dams shedding the microorganism [35,36]. At weaning age, colonization with *M. hyopneumoniae* is important in segregated production systems, since pigs are transferred to clean facilities for the growing and finishing phases [37,38]. Subsequent transmission of *M. hyopneumoniae* among pen-mates is slow, and a clear understanding of *M. hyopneumoniae* transmission in the field is still needed to improve infection models used in experimental researches [39-41]. A critical aspect of the epidemiology of *M. hyopneumoniae* is its long pathogen persistence [42,43], but the factors that determine such persistence are still poorly understood.

An additional complicating factor regarding *M. hyopneumoniae* epidemiology is the occurrence of distinct strains, with different degrees of virulence, circulating in the field [2]. Therefore, the identification and characterization of the *M. hyopneumoniae* strains circulating within a herd or geographical region are of utmost importance. Discrimination among *M. hyopneumoniae* strains usually rely on partial sequencing of the P146 gene [45], multilocus sequence typing (MLST) [46], and multiple-locus variable number tandem repeat analysis (MLVA) [47-49]. More recently, Betlach et al. [49] reviewed published
information on *M. hyopneumoniae* variability in pathogenicity and at the antigenic, proteomic, transcriptomic, and genomic levels, and proposed the variable number of tandem repeats (VNTR)-based common terminology and classification. This VNTR-based system is expected to avoid discrepancies and allow to make inferences across the literature.

**Prevention and control**

Enzootic pneumonia control and prevention are based on the optimization of management conditions, vaccination and treatment with antibiotics. Factors such as management, biosecurity practices and housing conditions should be optimized within the herd [2]. Decrease of infection levels and/or improvement of the clinical outcome of *M. hyopneumoniae* infections can be achieved with strategies involving management practices such as all-in/all-out pig flows, medicated and segregated early weaning, and multisite operations [27]. Preventing strategies to avoid the introduction of the pathogen in farms free of *M. hyopneumoniae* are also important. Garza-Moreno et al. [50] presented different *M. hyopneumoniae* monitoring strategies of incoming gilts and recipient herds and proposed a farm classification based on their health status. According to clinical signs, lung lesions, and ELISA and PCR results, farms and incoming replacements can then be classified into negative, provisional negative and positive.

Vaccination is today still regarded as the most effective way to control *M. hyopneumoniae* infections. Gilt replacement acclimation procedures against *M. hyopneumoniae* in positive farms in Europe and North America showed that vaccination is the main strategy to avoid EP [50]. Anti-*M. hyopneumoniae* vaccines are used worldwide and consist mainly of inactivated, adjuvanted whole-cell preparations that are administered intramuscularly [27]. Presently, there are at least 26 vaccines approved and commercially available worldwide to prevent *M. hyopneumoniae* infection [51].

Vaccinated animals present reduced clinical signs and lung lesions, improved performance and reduced number of microorganisms in the respiratory tract [2,38,52,53]. Although vaccination confers overall beneficial effects in most infected herds, the results are often variable [54–56]. These variations in the outcomes of vaccination may be due to many factors, including different infection levels, diversity of the circulating *M. hyopneumoniae* strains, and unknown aspects of the induced immune responses, along with technical issues, such as improper vaccine storage conditions and administration, and lack of vaccination compliance [44,57]. Furthermore, thus far, commercially available anti-*M. hyopneumoniae* vaccines have conferred only a limited reduction in the transmission ratios [41,58].

The exact mechanisms of protection needed to avoid *M. hyopneumoniae* infection are not yet fully understood, but constant efforts are invested in the development of new vaccines that may confer better protection. The latest efforts toward the development of more efficient vaccines against EP have been recently reviewed by Tao et al. [51]. These efforts focus on genetically engineered vaccines and some novel combined vaccines. The most recent genetically engineered vaccines are based on adhesins, such as P97, P95, P46, P42, and P36 delivered as recombinant vectors or recombinant subunits [8,59–62]. However, of a total of 24 genetically engineered vaccines studied over the years, only eleven were tested for their efficacies in pigs.

As *M. hyopneumoniae* infection can predispose swine to secondary infections, combined vaccines have gained attention, as they prevent multiple diseases at the same time. Combined vaccines developed thus far for EP and other swine respiratory infections consist of a mixture of *M. hyopneumoniae* bacterin and live attenuated viruses, such as porcine reproductive and respiratory syndrome virus and porcine circovirus 2 (PCV2) [63–65], or genetically engineered antigens from *M. hyopneumoniae* and other porcine pathogens [66]. There have been studies demonstrating the efficacy of a combined vaccine against *M. hyopneumoniae* and PCV2 [61,67], and their promising results suggest that bivalent or multivalent vaccines may present advantages over monovalent vaccines.

When EP control by the improvement of management and biosecurity and the implementation of vaccination fails, the clinical disease occurs. Then, the treatment of affected animals with antibiotics is required to maintain animal health and welfare. Treatment of *M. hyopneumoniae* infections can be accomplished using medication with antibiotics against *M. hyopneumoniae* and major secondary invading bacteria [68]. Potentially active antibiotics against *M. hyopneumoniae* include tetracyclines, macrolides, lincosamides, pleuromutilins, amphenicols, aminoglycosides, aminocyclitols and fluoroquinolones [27,69]. *M. hyopneumoniae* strains with high minimal inhibitory concentration (MIC) values for some antibiotics have been reported, along with a description of resistance mechanisms [70,71]. Therefore, even with the use of antibiotics, improvements in management and/or housing conditions are still necessary to ensure long-lasting effects during and after antimicrobial treatment.

**M. hyopneumoniae** “omics” studies

The “omics” era started for *M. hyopneumoniae* in 2004, when the first strains (the pathogenic strains 232 and 7448, and the nonpathogenic type strain J) had their
whole genomes sequenced [18,19]. Since then, the genomes of several other strains have been sequenced, and, presently, 21 whole sequenced *M. hyopneumoniae* genomes are available, 10 already assembled and annotated, and 11 still not fully assembled (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/190/). Apart that of *M. hyopneumoniae* J strain, the available sequenced genomes are from American, Brazilian, European, Korean, and Chinese pathogenic strains. These genomes are 0.86–0.96 Mb in size, and in each of them there are 528 to 691 protein-encoding genes. Interestingly, despite their small sizes, up to 30% of their gene contents are still of unknown function [72]. Moreover, 20 to 30% of the *M. hyopneumoniae* genes code for surface proteins (many of them also of unknown function) [72], therefore pointing out to a complex and still poorly characterized scenario of pathogen-host interactions.

More recently, functional “omics” studies, including transcriptomic, proteomic, and metabolomic, have followed the pioneering descriptive and comparative genomics research. These studies were enriched by data generated for the close relative species *M. flocculare* and have provided insights into *M. hyopneumoniae* pathogenicity determinants. *M. hyopneumoniae* strains and *M. flocculare* share most (>85%) of the genes that code for known (or predicted as such) pathogenicity determinants, including most adhesins, proteases and antioxidant proteins [19,72]. Moreover, they also share at least 90% of the genes coding for surface proteins. These findings have raised questions regarding the gene products and mechanisms that effectively underlie the differences in pathogenicity or virulence between *M. hyopneumoniae* strains, and between them and *M. flocculare*. Therefore, comparative studies between *M. hyopneumoniae* and *M. flocculare* have focused on possible differences in gene expression and protein abundances. Transcriptomics, proteomics, secretomics, and metabolomics approaches have been carried out as attempts to correlate differential *M. hyopneumoniae* transcripts, proteins or metabolites to pathogenicity or virulence [73–76]. In the following sections the main findings of these functional, comparative “omics” studies are integrated with those from several other complementary studies and discussed, to provide an overview of *M. hyopneumoniae* virulence factors and both bacterial and host EP determinants.

### *M. hyopneumoniae* adhesion to swine epithelial cells

In EP, *M. hyopneumoniae* adhesion to ciliated epithelial cells of the respiratory tract is the initial event in host colonization. This adhesion process is complex, dynamic, and not fully understood, but it is known to involve several surface-displayed molecules of both the pathogen and the host cells [77,78]. The available information on *M. hyopneumoniae* and host cell adhesion determinants are discussed in the next sub-sections and are schematically represented in Figure 1.

### *M. hyopneumoniae* adhesion determinants

The interaction between pathogenic bacteria and host cells during colonization is a critical process for pathogen survival and disease development [79]. Upon inhalation, *M. hyopneumoniae* must cope with the mucociliary apparatus in the swine respiratory tract, as the so-called lung mucociliary clearance (MCC) is the primary defense mechanism against respiratory pathogens [80]. It is the result of the coordinated interaction of the mucus and a low viscosity periciliary layer with ciliated epithelial cells. The mucus entraps inhaled pathogens and the low viscosity periciliary layer lubricates the airway surface, facilitating the ciliary beating that propels pathogens and particles out of host airways.

*Mycoblastema hyopneumoniae* attachment to the cilia of the respiratory epithelium allows the pathogen to overcome MCC. It has been long known that the pathogen adhesion causes ciliostasis and subsequent cilium loss and epithelial cell death [81,82]. More recently, *in vitro* infection assays provided evidence that *M. hyopneumoniae* infection disrupts the mucociliary function of respiratory epithelial cells by transiently reducing the amounts of mucin 5B secreted in the respiratory epithelium [83]. This reduction causes an uneven distribution of this mucin and might lead to the damage to the epithelial structure, including loss of cilia, observed upon *M. hyopneumoniae* adhesion to ciliated respiratory cells.

A repertoire of at least 35 *M. hyopneumoniae* proteins have been previously associated with cell adhesion, including several related to the P97/P102 paralog families and other surface proteins that moonlight as adhesins [78,84,85] (Table 1). However, the number of *M. hyopneumoniae* adhesins can be higher, considering that its surfaceome includes more than 290 proteins [72,75,86] and that many uncharacterized surface-displayed proteins may bear adhesion properties. Moreover, *M. hyopneumoniae* pathogenic and nonpathogenic strains, and the nonpathogenic *M. flocculare*, which differ in pathogenicity and were classically described as having differential adhesion capacities [87], share almost their entire repertoires of known adhesins, according to more recent comparative genomics and proteomics studies [72,75,87], also
pointing out to differences beyond the mere sets of known adhesins.

One of the possible explanations for the differences in adhesion capacity between *M. hyopneumoniae* strains and their nonpathogenic counterpart *M. flocculare* would be differences in the amount of adhesins presented at the cell surface. Different adhesins may vary in abundance at the cell surface between mycoplasma strains or species, due to differential transcriptional rates of the respective genes, and/or to differential translational rates of the corresponding mRNAs. However, few differences have been found in the expression of ortholog adhesins from *M. hyopneumoniae* and *M. flocculare* at transcriptional and proteomic levels [75,76]. Taken together, the available comparative transcriptomic and proteomic results suggest that there are no major overall differences in the repertoires of known adhesins between *M. hyopneumoniae* pathogenic and nonpathogenic strains and *M. flocculare* due to gene expression regulation at the transcriptional and translational levels.

Alternative explanations for the observed differences in the adhesion capacity of different *M. hyopneumoniae* strains and *M. flocculare* can be found in post-translational events, including their export to the cell membrane and proteolytic processing. Regarding protein export, the most typical *M. hyopneumoniae* and *M. flocculare* adhesins, such as members of the P97 and P102 adhesin families, have predicted signal-peptides and are likely exported by the general secretory Sec pathway [74]. Other less typical adhesins, such as some enzymes that moonlight as adhesion proteins at
the cell surface, lack a signal-peptide and would be exported by non-classic pathways. However, differential export efficiency apparently is not a major determinant of the differences in the abundance of adhesins in the cell surface, as both M. hyopneumoniae and M. flocculare surfaceomes are similarly enriched with adhesins [75].

Regarding proteolytic processing, it has been described that many adhesins may be targets of endoproteolytic post-translational processing [15,78,-78,88–93] (Table 1). These proteolytic processing events can shape the bacterial surface architecture [78,94], generating several adhesin proteoforms, that may be aimed to different locations and exert alternative functions. At least some of them are displayed at the cell surface, while others may stay in the cytoplasm or be released from the cell membrane to the extracellular milieu. Apart from those adhesin proteoforms

Table 1. M. hyopneumoniae surface adhesins and adhesion-related proteins and their host ligands.

| Protein name | M. hyopneumoniae strains a | Host ligands b | Endoproteolytic processing c | References |
|--------------|---------------------------|---------------|-----------------------------|------------|
| 46 kDa surface antigen (p46) | mhp511 MH7448_0513 MHJ_0511 | Fibronectin, heparin | Y | [78,94] |
| ABC transporter xylose-binding lipoprotein | mhp623 MH7448_0604 MHJ_0606 | Fibronectin, heparin | Y | [78,94] |
| Acetate kinase | mhp505 MH7448_0508 MHJ_0505 | Heparin | Y | [78] |
| Adenine phosphoribosyltransferase | mhp266 MH7448_0114 MHJ_0110 | Fibronectin, heparin | Y | [78] |
| Adhesin-like protein P146 | mhp684 MH7448_0663 MHJ_0663 | Fibronectin, plasminogen, heparin, porcine epithelial cilia | Y | [78,170] |
| ATP-dependent zinc metalloprotease FtsH | mhp175 MH7448_0206 MHJ_0202 | Fibronectin, heparin | Y | [78] |
| Chaperone protein DnaK (HSP70) | mhp072 MH7448_0067 MHJ_0063 | Fibronectin, heparin | Y | [78,94] |
| Dihydrolipoamide dehydrogenase | mhp504 MH7448_0507 MHJ_0504 | Fibronectin, heparin | Y | [78] |
| Elongation factor Tu (EFTu) | mhp540 MH7448_0523 MHJ_0524 | Fibronectin, heparin | Y | [78,171] |
| Glyceraldehyde 3-phosphate dehydrogenase | mhp036 MH7448_0035 MHJ_0031 | Fibronectin | Y | [78] |
| Hexose-6-phosphate synthase | mhp441 MH7448_0438 MHJ_0436 | Heparin | Y | [78] |
| L-lactate dehydrogenase (LDH) | mhp245 MH7448_0137 MHJ_0133 | Fibronectin, heparin | Y | [78] |
| Leucyl aminopeptidase | mhp462 MH7448_0464 MHJ_0461 | Plasminogen, heparin | Y | [10] |
| Lipoprotein | mhp390 MH7448_0378 MHJ_0374 | Porcine epithelial cilia | NR | [139] |
| Lpp protein | mhp384 MH7448_0372 MHJ_0368 | Heparin, porcine epithelial cilia | Y | [78,90] |
| M42 glutamyl aminopeptidase | mhp252 MH7448_0129 MHJ_0125 | Plasminogen, heparin | Y | [95] |
| Oligoendopeptidase F | mhp520 MH7448_0521 MHJ_0522 | Heparin | Y | [78] |
| P97-copy 1 | mhp183 MH7448_0198 MHJ_0194 | Fibronectin, plasminogen, heparin, porcine epithelial cilia | Y | [78,91,93,94] |
| P102-copy 1 | mhp182 MH7448_0199 MHJ_0195 | Fibronectin, plasminogen, porcine epithelial cilia | Y | [100] |
| P97-copy 2 | mhp271 MH7448_0108 MHJ_0105 | Fibronectin, heparin, porcine epithelial cilia | Y | [98] |
| P102-copy 2 d | mhp272 MH7448_0107 MHJ_0104 | ND | NR | [18,19] |
| P97-like protein | mhp107 MH7448_0272 MHJ_0264 | Fibronectin, plasminogen, heparin, porcine epithelial cilia | Y | [99] |
| P102-like protein | mhp108 MH7448_0271 MHJ_0263 | Fibronectin, plasminogen, porcine epithelial cilia | Y | [110] |
| Periplasmic sugar-binding protein | mhp145 MH7448_0234 MHJ_0227 | Fibronectin, heparin | Y | [78] |
| Putative MgPa-like protein d | mhp005 MH7448_0005 MHJ_0005 | ND | NR | [19,172] |
| Putative P76 membrane protein (P159) | mhp494 MH7448_0497 MHJ_0494 | Fibronectin, heparin, porcine epithelial cilia | Y | [78,173] |
| Putative P216 surface protein | mhp493 MH7448_0496 MHJ_0493 | Heparin, porcine epithelial cilia | Y | [15,94,102] |
| Putative prolipoprotein P65 | mhp677 MH7448_0656 MHJ_0656 | Fibronectin, heparin | Y | [78] |
| Pyruvate dehydrogenase | mhp264 MH7448_0116 MHJ_0112 | Fibronectin, heparin | Y | [78] |
| Pyruvate dehydrogenase E1-alpha subunit | mhp265 MH7448_0115 MHJ_0111 | Fibronectin, heparin | Y | [78] |
| Uncharacterized protein | mhp009 MH7448_0009 MHJ_0009 | Heparin | Y | [78] |
| Uncharacterized protein | mhp165 MH7448_0216 MHJ_0212 | Heparin | Y | [78] |
| Uncharacterized protein | mhp347 MH7448_0335 MHJ_0326 | Fibronectin, heparin | Y | [78] |
| Uncharacterized protein | mhp385 MH7448_0373 MHJ_0369 | Heparin, porcine epithelial cilia | Y | [78,90] |
| Uncharacterized protein | mhp683 MH7448_0662 MHJ_0662 | Heparin, porcine epithelial cilia | Y | [78,89] |

aNCBI accession numbers corresponding to the genes annotated in the M. hyopneumoniae 232, 7448 and J (RefSeq NC_006360.1, NC_007332.1 and NC_007295.1, respectively).

bHost ligands according to published data available for at least one M. hyopneumoniae strain; ND, not determined.

cEndoproteolytic processing with published experimental evidence available for at least one M. hyopneumoniae strain. Y, yes; NR, not reported.

dP102 copy-2 and putative MgPa-like protein adhesins were predicted as such based on their paralogy/orthology with M. hyopneumoniae P102 copy-1 and Mycoplasma genitalium MgPa adhesins, respectively.
with bona fide transmembrane domains, it is not known how processed adhesin proteoforms are anchored in the mycoplasma cell membrane. While some of these proteoforms may interact with and be retained by glycosaminoglycans (GAGs) [15,89,90], others may stay only transiently in the cell surface and then be released as soluble secretion products [74].

A recent comparative analysis of endoproteolytic processing between *M. hyopneumoniae* and *M. flocculare* adhesins [94] demonstrated that the five most abundant of them in the *M. hyopneumoniae* pathogenic strain 7448 are differentially processed compared to their corresponding orthologs in the non-pathogenic *M. hyopneumoniae* J strain and *M. flocculare*. Most of the analyzed surface-displayed adhesins from the pathogenic strain were more proteolytically processed (i.e., cleaved at more sites) than the orthologs from the non-pathogenic counterparts, which is consistent with the observed enrichment of several aminopeptidases and endoproteases at the surface of this pathogenic *M. hyopneumoniae* strain [75]. There is also evidence that adhesins can be differentially proteolytically processed in the cytoplasm, during or after their translation, and/or in the cell membrane, during or after their translocation to the cell surface [94]. Moreover, some adhesin proteoforms generated by proteolytic cleavage were observed in both cytoplasmic and surface compartments, indicating that at least some of the involved proteolytic events occur primarily in the cytoplasm, prior to the translocation of the resulting proteoforms to the cell surface.

The differential adhesin proteoforms generated by proteolytic processing may play differential roles. Those retaining adhesive domains, may retain adhesion properties (i.e., their capacity to bind to host cell or extracellular matrix ligand molecules) and contribute to the overall mycoplasma adhesion capacity. Alternatively, proteoforms devoid of adhesive domains may exert moonlighting functions. As many of the generated proteoforms are potentially antigenic [94], and may be presented in the cell surface or even secreted as soluble antigens [74], it is likely that they contribute, at least to some degree, to the mycoplasma strategies of immunomodulation or immunoevasion (discussed in Host immune response and immunomodulation during *M. hyopneumoniae* infection). Therefore, the mechanism of post-translational proteolytic processing of adhesins (including proteases and their target adhesins) would be a main *M. hyopneumoniae* pathogenicity determinant, of utmost relevance for EP determination. It would lead to differential presentations of adhesin proteoforms at the cell surface of *M. hyopneumoniae* strains (and *M. flocculare*), resulting in, and explaining, at least in part, the observed differential adhesion capacities of these strains and species.

Besides the canonical adhesins and derived proteoforms, other proteins, with different primary function, may moonlight as adhesins. For instance, some typical cytosolic proteins that are also consistently displayed at the cell surface likely exert alternative functions in this ectopic compartment. They may exert moonlighting functions by interacting with host components and contributing to host colonization. Among these surface-displayed cytosolic proteins, there are glycolytic enzymes, proteases, chaperones and translation factors that have been characterized as adhesins in different mycoplasma species, including *M. hyopneumoniae* [10,84,85,95,96]. Interestingly, several of these known moonlighting proteins are overrepresented at the surface of the pathogenic *M. hyopneumoniae* strain 7448 in comparison to the non-pathogenic J strain and *M. flocculare* [75]. This overrepresentation also likely contributes to the differences in adhesion capacity observed among these strains and species.

**Host cell adhesion determinants**

The interaction between *M. hyopneumoniae* adhesins (or adhesin proteoforms) and host cells also depends on the corresponding host ligands on the cell surface or in the extracellular matrix (ECM). Preliminary studies demonstrated that some swine ciliary glycolipids could act as receptors for *M. hyopneumoniae* attachment [97], but the bacterial adhesins involved in these pathogen-host interactions remain unknown. In the last decade, it has been demonstrated that adhesive proteoforms from the *M. hyopneumoniae* P97/P102 adhesin family can bind to GAGs from proteoglycans exposed on the cilia surface, which thereby could act as receptors [89,90]. Indeed, adhesins and adhesin proteoforms of the P97/P102 family display short linear motifs enriched in positively charged amino acids, which promote their binding to anionic molecules, such as GAGs and heparin [78,90,93,98–100].

Apart from the cilia-exposed glycans, some swine ECM molecules, such as fibronectin and plasminogen, also provide binding sites for surface adhesins of *M. hyopneumoniae*, contributing to host colonization [78,93,98,101,102]. Moreover, interactions between ECM molecules and bacterial surface proteins have been described for several mycoplasma species, including *Mycoplasma pneumoniae*, *Mycoplasma gallisepticum* and *Mycoplasma bovis*, among others [103–107]. Interestingly, the fibronectin-binding ability of *M. hyopneumoniae* may mediate the adherence to swine respiratory cilia and can provide a mechanism for host cytoskeleton rearrangements, which may
facilitate bacterial internalization [108]. Additionally, the plasminogen-binding ability of *M. hyopneumoniae* may facilitate its traffic via the circulatory system and penetration into host organs, such as liver, kidneys and spleen, from which it has already been isolated [109,110].

More recently, it was also demonstrated that extracellular actin is used as a surface receptor by different proteoforms of *M. hyopneumoniae* P97 adhesin and another 143 proteins, including lipoproteins, glycolytic enzymes, chaperones and translation factors, among others [77]. Interestingly, anti-actin antibodies inhibit 90% of the ability of *M. hyopneumoniae* to adhere and colonize PK-15 swine cells, indicating that extracellular actin is an important receptor for *M. hyopneumoniae* infection. Surface proteins of *M. hyopneumoniae* also interact with other cytoskeletal proteins besides extracellular actin, such as vimentin, keratin, tubulin, myosin, and tropomyosin [77,111].

**M. hyopneumoniae** biofilm formation, host cell invasion and systemic trafficking across the porcine respiratory epithelium barrier

Direct contact of *M. hyopneumoniae* cells with each other and with host cells or ECM molecules may render the pathogen capable of forming biofilms. Biofilm formation is a strategy used by many bacteria, including other mycoplasma species [112–114], to cope with host immune response or with antimicrobial effects, thereby rendering bacteria extremely adaptive and thus contributing to virulence. Recent studies have shown that at least some *M. hyopneumoniae* strains are indeed capable of forming biofilms, in experimental *in vitro* conditions, on abiotic surfaces or on host cell monolayers, and within the respiratory tract of experimentally infected swine [115,116]. Biofilm formation makes *M. hyopneumoniae* more resistant to antibiotics, at least *in vitro* [116], providing evidence of the importance of biofilm formation for pathogen survival. The molecular interactions and cellular processes underlying *M. hyopneumoniae* biofilm formation are thus far mostly unknown. However, at least *in vitro*, biofilm formation involves the generation of a subpopulation of unstable large cell variants that may contribute to the release of extracellular DNA, essential for forming biofilms on abiotic surfaces [115].

Along with biofilm formation capacity, another valuable strategy for mycoplasma survival and pathogenesis is the ability to invade host cells, which is well described for *Mycoplasma penetrans* and *M. pneumoniae*, for example [117,118]. *M. hyopneumoniae* is usually regarded as an extracellular mycoplasma. However, the demonstration that it binds to fibronectin and plasmin at the site of infection [100,110,119,120], an interaction classically associated to host cell invasion by bacteria [121,122], suggested that it could also penetrate porcine cells. Indeed, it was recently demonstrated that *M. hyopneumoniae* could invade *in vitro*-infected host-derived epithelial cells [108]. Host cell invasion is mediated by endocytic pathways, which are initiated by interactions between mycoplasma surface proteins and host fibronectin and integrin β1. Remarkably, within porcine cells, at least some bacterial cells can survive phagolysosomal fusion and escape into the cytosol, providing evidence of an alternative intracellular form for *M. hyopneumoniae*. Moreover, infected host porcine cells could function as a source of pathogen cells for re-infection, as internalized, dormant *M. hyopneumoniae* could eventually leave to the extracellular environment. In line with that, evidence that *M. hyopneumoniae* may influence the endosomal trafficking and modulate the maturation of early endosomes was provided by proteomic analyses of swine epithelial cells infected with this pathogen [25]. In such a scenario, infected host cells could also function as a reservoir for the traffic of *M. hyopneumoniae* within the respiratory tract, thereby contributing to the chronic infection status.

As it is mostly recovered from trachea and lung lesions from infected pigs, *M. hyopneumoniae* has been considered an exclusive respiratory pathogen [109]. However, at least in experimentally infected pigs, *M. hyopneumoniae* cells have been re-isolated from inner organs, such as liver, spleen, brain, kidneys, and lymph nodes, although at frequencies lower that of the respiratory tract [109,123,124]. These findings demonstrate that *M. hyopneumoniae* is able to disseminate to extrapulmonary sites within the swine host, but the mechanisms that allow the systemic trafficking of the bacterium across the respiratory epithelial barrier remain elusive. One possible mechanism mediating this *M. hyopneumoniae* trafficking might involve its cell invasion capacity, discussed above. As it has been recently demonstrated that *M. hyopneumoniae* could also, at least *in vitro*, invade porcine macrophages and avoid phagocytosis [125], it can be speculated that, by invading these and possibly other immune cells, the bacterium could be spread to other organs.

Evidence of another possible mechanism that could mediate *M. hyopneumoniae* trafficking to alternative host sites was revealed more recently by experiments using an *in vitro* air-liquid culture system of porcine bronchial epithelial cells [83]. With that, it was shown that *M. hyopneumoniae* cells could migrate across the epithelial barrier by the paracellular route, but not by the transcellular route. In this *in vitro* model, *M. hyopneumoniae* reversibly disrupts the tight junctions between epithelial cells, increasing the
permeability and damaging the integrity of the epithelial barrier. In line with that, several studies have demonstrated that *M. hyopneumoniae* infection facilitates the activation of plasminogen to plasmin, which contributes to the degradation of several ECM and cellular junction components [10,95,100,110,120]. Such disruption of the porcine respiratory epithelial barrier would contribute to the extrapulmonary dissemination of *M. hyopneumoniae* and to the persistence of infection. Overall, further studies are needed to elucidate how and to what extent *M. hyopneumoniae* disseminates to different extrapulmonary niches in natural infections.

**Protein secretion of *M. hyopneumoniae* and its impact on pathogen-host interactions**

Protein secretion is a vital process for all organisms and has a particular role in the pathogenesis of bacterial infections. Gram-negative and Gram-positive bacteria have at least 6 reported secretion/translocation systems [126]. The general secretory (Sec) pathway comprises an essential, ubiquitous and universal export machinery for most proteins that translocate through (soluble secretome) or integrate into (insoluble secretome) the cell membrane [127]. In mycoplasmas, however, the Sec secretory pathway is apparently incomplete, as some genes coding for components of this pathway were not found in their genomes. In the *M. hyopneumoniae* genome, only the genes coding for SecY, SecG, SecD/F, YidC and SecA proteins were identified, and the absence of the SecE protein may indicate an incomplete SecYEG transmembrane channel [18,19,72]. However, the secretome analysis of *M. hyopneumoniae* provided evidences that the Sec secretory pathway is functional, as several detected proteins in the soluble secretome fraction were predicted to be secreted by this secretion pathway [74]. This evidence suggests that other more divergent and still unknown proteins may be part of the Sec pathway, which is consistent with the fact that ~35% of the *M. hyopneumoniae* genome has thus far no functional annotation.

In contrast, most of the detected proteins in the *M. hyopneumoniae* soluble secretome were predicted as secreted by Sec-independent secretion pathways [74]. This finding suggests that *M. hyopneumoniae* may present alternative secretion mechanisms not yet identified due to their lack of conservation with other well-characterized secretion pathways. In line with that, protein secretion in extracellular vesicles was observed in several mycoplasma species [128,129], suggesting that *M. hyopneumoniae* may alternatively use a vesicle system to secrete proteins.

The soluble secretome of *M. hyopneumoniae* can be considered a reservoir of virulence factors (Table 2), being composed of several adhesins, lipoproteins, and nucleases [26,74]. The detection of adhesins in the soluble secretome fraction may result from the extensive proteolytic processing of these proteins on the *M. hyopneumoniae* cell surface (discussed in *M. hyopneumoniae* adhesion determinants), eventually resulting in the release of some adhesin proteoforms in the extracellular milieu. These adhesin proteoforms may act as extracellular antigens that could trigger the host immunological defenses (discussed in Host immune response and immunomodulation during *M. hyopneumoniae* infection). The potential relevance of the *M. hyopneumoniae* secretome for pathogenesis is evident by comparative analyses with *M. flocculare*, the secretome of which is less complex and presents fewer orthologs of known *M. hyopneumoniae* virulence factors.

In addition to the differences in the virulence factor content, the overall protein content and abundance in *M. hyopneumoniae* and *M. flocculare* secretomes are differential. Both species possess the genes coding for the Sec secretory pathway components, although the partial Sec secretory machinery may be supplemented by unknown proteins not shared by these mycoplasma species, leading to differential secretion efficiencies. Moreover, *M. hyopneumoniae* and *M. flocculare* may use differential secretion signals and secretion pathways, which resulted in the observed differences in secretome contents. More studies are needed to elucidate the secretion mechanisms used by these and other mycoplasma species.

The insoluble secretome (or surfaceome) of *M. hyopneumoniae* also acts as a reservoir of virulence factors (Table 2), which strongly contribute to EP establishment. Proteomic studies focused on surface proteins have demonstrated that this cell fraction is enriched with adhesion proteins (discussed in section 4.1), lipoproteins, and proteases, among others [75,86]. The roles of these proteins in immunomodulation and cell damage are discussed in the following sections.

**Host immune response and immunomodulation during *M. hyopneumoniae* infection**

Upon *M. hyopneumoniae* adherence in the swine respiratory tract (discussed in section 4.1), the host immune response starts to adjust in order to cope with infection. Histopathological changes during *M. hyopneumoniae* infection are frequently observed in the host respiratory tract [130]. They are characterized by a prominent accumulation of mononuclear cells
and infiltration of lymphocytes, plasma cells, and neutrophils in the alveolar lumina and septa. Infected lungs present bronchoalveolar exudate, enlargement of alveolar septa and lymphoreticular hyperplasia of the bronchus-associated lymphoid tissue (BALT). Moreover, several studies have demonstrated that the accumulation of immune cells increases the production and secretion of different pro-inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-8, and IL-18 [120,131,132]. The resultant peribronchiolar and perivascular infiltration of mononuclear leukocytes, along with the increase of cytokine production, are, therefore, associated with immunopathological EP lesions. Altogether, these observations support the idea that the host immune cell response against *M. hyopneumoniae* can be considered one of the main causes of the lung lesions observed in EP (Figure 2).

Despite of the acute host inflammatory immune response elicited by *M. hyopneumoniae* infection, EP is considered a chronic disease. The persistence of this pathogen in infected pigs is associated with its capacity to modulate and/or evade of host defenses (Figure 2). This is supported by comparative studies on the porcine immune responses elicited by *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* (which also colonizes the porcine respiratory tract) revealing that *M. flocculare* and *M. hyorhinis* induce the secretion of higher levels of TNF-α and IL-12, respectively, than *M. hyopneumoniae* [131]. Moreover, *M. hyopneumoniae* infection also induces the expression of high levels of IL-10 but decreases the levels of IL-12 and interferon-γ (IFN-γ) expression in dendritic cells [133]. In line with that, it was demonstrated that a surface protein from *M. hyopneumoniae* elicited the secretion of high levels of IL-10 in murine splenocytes [134]. These findings suggest that *M. hyopneumoniae* infection modulates the host defenses to favor the Th2 immune response, which was also reported in dendritic cells against mycoplasmal infection [133], promoting the development of a chronic disease. On the other hand, one study reported a specific systemic humoral immune response found to be predominately involving the IgG2 subclass, suggesting a dominant Th1-mediated immune response to *M. hyopneumoniae* [135]. In fact, there are several inconsistencies regarding the type of predominant immune response induced by *M. hyopneumoniae* [133, 135–137]. Such inconsistencies observed in the published literature suggest that *M. hyopneumoniae* can induce mixed Th1/Th2 responses, but more studies are necessary to confirm this dual response and to understand how it is elicited.

The functional modulation of porcine antigen-presenting cells, such as dendritic cells, was also reported in *M. hyopneumoniae* infections [133]. It was demonstrated that *M. hyopneumoniae* down-regulates the expression of the CD1a protein in porcine dendritic cells. Since this protein is responsible for displaying antigenic lipids to T cell receptors, its down-regulation by the pathogen (by a still unknown mechanism), decreases the host dendritic cell capacity for antigen presentation. Moreover, *M. hyopneumoniae* also reduces the overall populations of dendritic and T cells in the porcine nasal cavity in long-term infections, weakening the immune function in the upper respiratory tract [133].

The ability of *M. hyopneumoniae* to reduce the populations of immune cells may be associated with the cytotoxic potential of some bacterial proteins displayed at its surface (Figure 2). *M. hyopneumoniae* cytotoxicity has been considered a virulence mechanism, as the bacterial infection induces apoptosis of immune cells, weakening the immune system, and apoptosis of respiratory epithelial cells, resulting in physical damage to the host tissue. Lipid-associated membrane proteins (LAMPS) from *M. hyopneumoniae* are known to be crucial for *M. hyopneumoniae* cytotoxicity to host cells, as they induce cell death by apoptosis or necrosis and modulate the inflammatory response [21,22,138]. It was demonstrated that these LAMPS induce apoptosis in porcine peripheral blood mononuclear cells, alveolar macrophages and lung epithelial cells *in vitro* by increasing the levels of nitric oxide (NO) and reactive oxygen species (ROS) and by caspase-3 activation. Besides LAMPS, the *M. hyopneumoniae* P68 surface lipoprotein was also identified as an inflammatory and pro-apoptotic mediator to swine immune cells [139]. In this context, apoptosis induction in host immune cells may elicit an immunosuppressive effect, which likely plays an important role in immunomodulation and evasion.

Nitric oxide and ROS are important players in host immune response, contributing to inflammation and microbicidal response. NO is used as a signaling molecule involved in the regulation of vascular hemodynamics and mediates interaction and recruitment of immune cells during infection [140–142]. ROS are key toxic metabolites to kill bacterial pathogens [143]. These molecules are produced intracellularly by phagocytic cells, such as neutrophils and macrophages, eliminating phagocytized bacteria in a process called respiratory burst response [144]. However, bacterial pathogens can subvert these microbicidal responses and induce apoptosis in host cells by increasing the host production and release of NO and ROS [142,145]. Indeed, NO and ROS are also crucial signal molecules for host cell apoptosis, being implicated in the apoptotic cascade and in the activation of initiator
and effector caspases [146]. Porcine cells treated with *M. hyopneumoniae* LAMPS presented high levels of NO and superoxide anion radicals, which form peroxynitrite, and lead to oxidative stress and activation of the apoptotic cascade [21,22,138]. This cytopathogenic mechanism was also observed in other mycoplasma species, including *M. hyorhinis*, *Mycoplasma synoviae*, and *M. pneumoniae* [147–149].

The success of host colonization by several mycoplasma species depends on their ability to rapidly alter the antigenic repertoire of their surface, using different genetic systems, such as those of phase- or antigenic variation caused by DNA slippage [150]. However, for *M. hyopneumoniae*, no evidence of such a mechanism has been found. Instead, the differential proteolytic processing resulting in the presence of differential proteoforms on the *M. hyopneumoniae* surface (discussed in *M. hyopneumoniae* adhesion determinants) may contribute to antigenic variation, which can be associated with immune response modulation and/or evasion. In this sense, *M. hyopneumoniae* surface-associated proteases would be indirectly involved in

### Table 2. Putative virulence factors found in the *M. hyopneumoniae* secretome and/or surfaceome.

| Protein name | *M. hyopneumoniae* strain | Function associated to pathogenicity | Subcellular localization | References |
|--------------|---------------------------|-------------------------------------|--------------------------|------------|
| 46 kDa surface antigen (p46) | mhp511 MHP7448_0513 MHJ_0511 | Adhesion | Se/Su | [26,78] |
| Adhesin like-protein P146 | mhp684 MHP7448_0663 MHJ_0663 | Adhesion | Se/Su | [26,170] |
| Aminopeptidase | mhp252 MHP7448_0129 MHJ_0125 | Proteolytic processing, immunomodulation, adhesion | Su | [75,95,151] |
| ATP-dependent protease binding protein | mhp278 MHP7448_0101 MHJ_0098 | Heat shock protein, proteolytic processing | Su | [75,78] |
| ATP-dependent zinc metalloprotease FtsH | mhp175 MHP7448_0206 MHJ_0202 | Proteolytic processing, adhesion | Su | [75,78] |
| Chaperone protein DnaJ | mhp073 MHP7448_0068 MHJ_0064 | Chaperone, post-translational processing | Su | [75,169] |
| Elongation factor Tu (EFTu) | mhp540 MHP7448_0523 MHJ_0524 | Immunomodulation, adhesion | Su | [75,156] |
| Hemolysin C | mhp663 MHP7448_0643 MHJ_0643 | Cytotoxicity | Su | [75,174] |
| Leucyl aminopeptidase | mhp462 MHP7448_0464 MHJ_0461 | Proteolytic processing, adhesion | Su | [10,75] |
| Lipoprotein | mhp164 MHP7448_0217 MHJ_0213 | Cytotoxicity | Se/Su | [21,26,75,138] |
| Lipoprotein | mhp302 MHP7448_0505 MHJ_0502 | Cytotoxicity | Se/Su | [21,26,75,138] |
| Lipoprotein | mhp378 MHP7448_0367 MHJ_0363 | Cytotoxicity | Se/Su | [21,74,75,138] |
| Lipoprotein | mhp345 MHP7448_0333 MHJ_0324 | Cytotoxicity | Su | [21,75,138] |
| Lipoprotein | mhp377 MHP7448_0366 MHJ_0362 | Cytotoxicity | Su | [21,75,138] |
| Lipoprotein | mhp379 MHP7448_0368 MHJ_0364 | Cytotoxicity | Su | [21,75,138] |
| L-lactate dehydrogenase (LDH) | mhp245 MHP7448_0137 MHJ_0133 | Adhesion | Se/Su | [26,78] |
| Lon protease (ATP-dependent protease La) | mhp524 MHP7448_0524 MHJ_0524 | Proteolytic processing | Su | [75] |
| Lpp3 protein | mhp384 MHP7448_0372 MHJ_0368 | Adhesion | Se/Su | [26,74,90] |
| Membrane nuclease, lipoprotein | mhp597 MHP7448_0580 MHJ_0581 | Surface nuclease, cytotoxicity, immunomodulation | Se/Su | [11,26,74] |
| Oligoendopeptidase F | mhp520 MHP7448_0521 MHJ_0522 | Proteolytic processing, immunomodulation, adhesion | Su | [9,75,151] |
| Outer membrane protein-P95 | mhp280 MHP7448_0099 MHJ_0096 | Surface antigen | Se/Su | [169; 26, 75] |
| P192-copy 1 | mhp182 MHP7448_0192 MHJ_0195 | Adhesion | Se/Su | [26,74,100] |
| P192-copy 2 | mhp272 MHP7448_0107 MHJ_0104 | Adhesion | Se/Su | [26,74] |
| p37-like ABC transporter substrate-binding lipoprotein | mhp371 MHP7448_0360 MHJ_0356 | Cytotoxicity | Su | [21,75,138] |
| P60-like lipoprotein | mhp364 MHP7448_0353 MHJ_0348 | Cytotoxicity | Se/Su | [21,74,138] |
| P97-copy 1 | mhp183 MHP7448_0198 MHJ_0194 | Adhesion | Se/Su | [26,74,93] |
| P97-copy 2 | mhp271 MHP7448_0108 MHJ_0105 | Adhesion | Se/Su | [26,74,98] |
| Phosphoglyceromutase | mhp221 MHP7448_0161 MHJ_0157 | DNA damage response | Se/Su | [175] |
| Protein GpE (HSP-70 cofactor) | mhp011 MHP7448_0011 MHJ_0011 | Chaperone, post-translational processing | Se/Su | [169; 26, 75] |
| Putative lipoprotein | mhp390 MHP7448_0378 MHJ_0374 | Cytotoxicity | Se/Su | [21,26,138] |
| Putative lipoprotein | mhp640 MHP7448_0621 MHJ_0622 | Cytotoxicity | Su | [21,75,138] |
| Putative MgpA like-protein | mhp005 MHP7448_0005 MHJ_0005 | Adhesion | Se/Su | [74] |
| Putative P216 surface protein | mhp493 MHP7448_0496 MHJ_0493 | Adhesion | Se/Su | [15,26,102] |
| Putative P76 membrane protein (P159) | mhp494 MHP7448_0497 MHJ_0494 | Adhesion | Se/Su | [26,173] |
| Putative prolipoprotein P65 | mhp677 MHP7448_0656 MHJ_0656 | Adhesion | Se/Su | [26,78] |
| Signal-peptidase I | mhp028 MHP7448_0026 MHJ_0022 | Proteolytic processing, citotoxicity | Su | [13,14] |
| Thioredoxin | mhp283 MHP7448_0096 MHJ_0093 | Antioxidant protection | Se | [12,26] |
| Thioredoxin | mhp396 MHP7448_0384 MHJ_0380 | Antioxidant protection | Se | [26] |
| Trigger factor | mhp233 MHP7448_0149 MHJ_0145 | Chaperone, post-translational processing | Se/Su | [169; 26, 75] |
| XAA-PRO aminopeptidase | mhp680 MHP7448_0659 MHJ_0659 | Proteolytic processing, immunomodulation | Se | [9,75] |

**Notes:**

- **NCBI accession numbers corresponding to the genes annotated in the *M. hyopneumoniae* 232, 7448 and J sequenced genomes (RefSeq NC_006360.1, NC_007332.1 and NC_007295.1, respectively).
- **Function(s) associated to pathogenicity** predicted in *in silico* or according to functional data available for at least one *M. hyopneumoniae* strain.
- **Subcellular localization** according to published proteomic data available for at least one *M. hyopneumoniae* strain. Se, secreted; Su, surface-displayed.
The modulation of the host immune response, by generating proteoforms of adhesins (and possibly of other surface proteins) presenting different epitope sets.

Furthermore, *M. hyopneumoniae* surface proteases have also been associated with immunomodulation through the proteolytic degradation of pro-inflammatory peptides, such as bradykinin, kininogen, substance P, neurokinin A, and neuropeptide Y [9,151]. It is known that bradykinin and kininogen are involved in the host innate immune response, being associated with bronchoconstriction, MCC and cough induction [152]. Moreover, substance P, neurokinin A and neuropeptide Y are known as inducers of the inflammatory response, eliciting the secretion of high levels of pro-inflammatory cytokines and chemokines [153,154]. Therefore, the proteolytic degradation of these peptides can be considered a virulence mechanism associated with ciliostasis, impairing MCC, and modulation of host immune response.

*Mycoplasma hyopneumoniae* also displays the ability to evade cellular immune responses. Recently, it was demonstrated that *M. hyopneumoniae* evades the phagocytic uptake by porcine alveolar macrophages *in vitro*, although the mechanism underlying this resistance to phagocytosis is still unclear [125]. The presence of convalescent sera, as a source of specific antibodies and complement components for opsonization, did not improve *M. hyopneumoniae* phagocytosis by cell. This result indicates that, although *M. hyopneumoniae* can induce the production of antibodies in the host [155], they are not protective. Another mechanism of host immune evasion by *M. hyopneumoniae* may be the inhibition of the complement pathway. Indeed, it has recently shown that several *M. hyopneumoniae* surface-displayed proteins bind complement factor H, namely elongation factor thermo unstable (EF-Tu), P146, pyruvate dehydrogenase (acyetyl-transferring) E1 component subunit alpha (PdhA), P46, pyruvate dehydrogenase E1 component subunit beta (PdhB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and three different hypothetical proteins [156]. Factor H binding to the *M. hyopneumoniae* cell surface can confer to the bacterium the ability to inactivate the C3b eventually deposited on the *M. hyopneumoniae* surface, thereby avoiding the consequences of complement activation [157]. The binding of factor H by EF-Tu also contributed to decrease C3 deposition on the *M. hyopneumoniae* surface and increased *M. hyopneumoniae* adhesion to epithelial cells [156].
By counteracting complement activation effects on *M. hyopneumoniae*, these mechanisms contribute to immune evasion and allow more efficient colonization of the respiratory epithelium by the pathogen.

Moreover, it has been shown that *M. hyopneumoniae* is also able to evade neutrophil/macrophage extracellular traps (NETs or METs, respectively), at least *in vitro* [11,158]. In line with that, it has also been demonstrated that the surface nuclease MnuA is responsible for the degradation of NETs, allowing *M. hyopneumoniae* to escape the host immune defense [11].

Overall, despite fostering a strong immune response, *M. hyopneumoniae* can persist for several months in the swine host [42,43]. In this context, the ability of *M. hyopneumoniae* to modulate the host immune responses is an important feature that impacts virulence and disease progression. The entire set of immunomodulation mechanisms used by *M. hyopneumoniae*, however, remains elusive and is worthy of future investigation.

**Cell damage induced by *M. hyopneumoniae* infection**

The mechanisms underlying *M. hyopneumoniae* pathogenesis described thus far are bacterium-induced loss of cilia and cell death, which can also be correlated to the damage caused by the intense host microbicidal response against this pathogen [2]. On the other hand, there were additional studies that described the presentation of cytotoxic proteins at the bacterial cell surface and the release of toxic bacterial metabolites as a *M. hyopneumoniae* virulence mechanism causing damage in swine tissues as will be discussed below.

As discussed in Host immune response and immunomodulation during *M. hyopneumoniae* infection, the cytotoxicity of proteins displayed at the *M. hyopneumoniae* cell surface has been associated with immunosuppression because, at least in some cases, these proteins induce apoptosis in host immune cells. In addition, they have also been associated with epithelial cell death, causing physical damage to the swine respiratory epithelium (Figure 3), which may contribute to the lung lesions observed in swine with EP. For instance, *M. hyopneumoniae* LAMPs induce apoptosis in porcine lung epithelial cell line by activating caspase 3, caspase 8, cytochrome C, Bax, and p38 MAPK pathways [22]. Besides LAMPs, the MnuA nuclease and a putative type I signal peptidase (SPase I) from *M. hyopneumoniae* also showed pro-apoptotic effects on porcine epithelial cells [11,14]. MnuA is also secreted and, extracellularly, contributes to both NETs degradation and to host immunomodulation (as discussed in Host immune response and immunomodulation during *M. hyopneumoniae* infection). The putative SPase I, in turn, is likely involved in the secretion of many proteins, including at least some adhesins, to the cell surface or extracellular milieu (as discussed in Protein secretion of *M. hyopneumoniae* and its impact on pathogen-host interactions). The additional roles of *M. hyopneumoniae* MnuA and SPase I in *M. hyopneumoniae* biology may be the result of the specific evolutionary history of Mollicutes, which might have led to the acquisition of additional functions by some proteins to compensate in part for the observed genome reduction events [159].

Mycoplasmas can also produce ROS, by which they can induce cell death and tissue damage [160,161]. In line with that, it was demonstrated that *M. hyopneumoniae* can produce hydrogen peroxide (H₂O₂) when glycerol is available as a carbon source, while *M. flocculare* is unable to produce detectable amounts of this toxic molecule [162]. Notably, the gene encoding glycerol-3-phosphate oxidase (glpO), one of the enzymes responsible for H₂O₂ production in other mycoplasmas, is present in the genomes of multiple *M. hyopneumoniae* strains, but is absent in the *M. flocculare* genome [72]. However, the exact mechanism of H₂O₂ production in *M. hyopneumoniae* strains is still unknown. Moreover, the production of H₂O₂ by *M. hyopneumoniae* has the potential to cause oxidative stress to host cells and, at least *in vitro*, was associated with the antioxidant response elicited in swine respiratory epithelial cells [163].

*In vitro* experimental infections of a swine respiratory epithelial cell line with *M. hyopneumoniae* have also shown that several genes related to ciliary motility, ciliogenesis and ciliary polarization were down-regulated in infected host cells [163]. This evidence suggests a scenario in which *M. hyopneumoniae* infection could modulate the expression of genes related to the mucociliary apparatus, explaining, at least in part, the ciliostasis and loss of cilia observed in infected host tissues.

Besides the cilia damage, *M. hyopneumoniae* infection causes apoptosis-related cell events involving endoplasmic reticulum (ER) stress (Figure 3). Proteomic analyses of a tracheal swine cell line infected with *M. hyopneumoniae* revealed that infection induces dysregulation of Ca²⁺ homeostasis and ER stress [25]. It was also shown that ER stress leads to activation of the cytoprotective unfolded protein response (UPR), which, despite triggering signal transduction events associated with host defenses, is also associated with host cell apoptosis and, consequently, with damage to the ciliated respiratory epithelium. On the other hand, it was demonstrated that *M. hyopneumoniae* infection could also suppress one of the UPR pathways (the NF-κB
pathway), counteracting at least in part the pro-apoptotic effects and favoring the survival of infected cells [164]. These apparently antagonistic effects of *M. hyopneumoniae* infection on UPR may represent alternative mechanisms favorable to the pathogen at different times of infection. An anti-apoptotic effect may be interesting in the initial steps of infection, to allow bacterial adherence and colonization of the host respiratory tract. The pro-apoptotic effect would be more prominent later on, contributing to tissue damage.

Additional evidences of swine epithelial cell death triggered by *M. hyopneumoniae* infection were also provided by secretome analyses of cells infected in vitro with pathogenic and nonpathogenic strains and with *M. flocculare* [26]. Cell death-related proteins were detected only in culture supernatants of swine cells infected with a *M. hyopneumoniae* pathogenic strain, suggesting a specific host response to pathogenic *M. hyopneumoniae*. Among these secreted cell death-related proteins, there were danger-associated molecule patterns (DAMPs), known to be secreted by host cells undergoing apoptosis during pathogen infections to alert the immune system and trigger a pro-inflammatory immune response.

**M. hyopneumoniae** homeostasis maintenance during host colonization

The persistence of *M. hyopneumoniae* infection relies not only on its ability to modulate and evade the host immune response, but also on its arsenal of protective mechanisms to cope with different stress conditions imposed by the swine host. Moreover, *M. hyopneumoniae* survival also depends on the uptake of nutrients provided by host cells. In this final section, these aspects, which are essential for *M. hyopneumoniae* cell homeostasis, will be presented.

As discussed in Host immune response and immunomodulation during *M. hyopneumoniae* infection and Cell
damage induced by *M. hyopneumoniae* infection, *M. hyopneumoniae* infection is marked by the production of ROS by both host and mycoplasma cells. However, it is not yet clear how this pathogen can protect itself from endogenously and host produced toxic metabolites. The *M. hyopneumoniae* anti-toxic metabolites arsenal is limited, as its genome lacks genes encoding important antioxidant proteins [19,72]. Thus far, four antioxidant enzymes have been identified, namely thioredoxin, thioredoxin reductase, NADH-oxidase and peroxiredoxin, and all of them were overrepresented in the *M. hyopneumoniae* 7448 pathogenic strain in comparison to the nonpathogenic strain J and *M. flocculare* [75]. This evidence provided a clear link between protection against oxidative stress and *M. hyopneumoniae* pathogenicity. Furthermore, the *M. hyopneumoniae* peroxiredoxin (MhPrx) was functionally characterized and shown to protect DNA from ROS-mediated damage *in vitro* [12,165]. Therefore, MhPrx may play an essential role in pathogen survival.

In functional “omics” studies, virtually no changes in transcript or protein levels were observed for *M. hyopneumoniae* genes/proteins classically involved in oxidative stress between stress and control conditions [166–168]. At the proteome level, it was observed that three of the four known *M. hyopneumoniae* proteins involved in the oxidative stress response (thioredoxin, NADH-oxidase and MhPrx) were among the top 20 most abundant proteins, even in the absence of oxidative stress conditions [167]. Altogether, these transcriptomic and proteomic results suggest that the anti-oxidative stress arsenal of *M. hyopneumoniae* is constitutively expressed, enabling the pathogen being always prompt to respond to this kind of stress. Interestingly, pathogenic and nonpathogenic strains of *M. hyopneumoniae* presented similar abundances of antioxidant proteins under oxidative stress [167]. On the other hand, the protein repertoire of *M. hyopneumoniae* pathogenic strain 7448 was enriched with potential virulence factors, as adhesins, nucleases and lipoproteins, upon exposure to oxidative stress in comparison to control conditions (absence of oxidative stress) and to the *M. hyopneumoniae* nonpathogenic J strain.

*Mycoplasma hyopneumoniae* must also cope with temperature shifts in the host environment due to the release of pyrogenic cytokines as a mechanism of the immune response [48,132]. A transcriptomic study regarding the expression of heat stress-related genes demonstrated that several genes, including those coding for the heat shock proteins DnaK, DnaJ, Lon proteases, and ATP-dependent serine proteinase were affected by temperature shifts [166]. However, quantitative proteomic analyses did not reveal significant differences in the abundance of these proteins between heat stress and control conditions [167]. As discussed above for the response to oxidative stress, in temperature stress conditions, the heat stress-related proteins were found to be among the most abundant, while some potential virulence factors were more abundant in the *M. hyopneumoniae* pathogenic 7448 strain under heat stress.

Taken together, the transcriptomic and the proteomic findings suggest that *M. hyopneumoniae* may require the constitutive presence of proteins involved with stress protection. These findings also suggest that the synthesis of at least some virulence factors, including adhesins, nucleases, and lipoproteins, can be triggered by oxidative and temperature stresses, which would imply increased virulence in infection (stress) conditions. Further studies will be required to improve our understanding of this relationship between stress response and virulence, and also to identify, among the non-annotated genes of *M. hyopneumoniae*, those possibly encoding so far unknown stress-response proteins.

Along with the *M. hyopneumoniae* capacity to respond to stress conditions, its metabolic capacity is essential for survival in the host environment and may contribute to virulence. The *M. hyopneumoniae* transcription unit coding for myo-inositol catabolism proteins provides a good example of that (and thus far the only one characterized regarding this aspect). *In silico* metabolic reconstructions for swine respiratory mycoplasmas demonstrated that the metabolic capacities of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* are similar [73]. However, among mycoplasma species, *M. hyopneumoniae* is the only one with a transcription unit coding for myo-inositol catabolism proteins in its genome [19,73], suggesting that it can use inositol as an alternative carbon source [169]. Indeed, it was demonstrated that *M. hyopneumoniae* strains uptake myo-inositol from culture medium, while *M. hyorhinis* and *M. flocculare* are unable to do so [162]. This capacity to uptake and metabolize the myo-inositol from the host system would allow *M. hyopneumoniae* to persist longer than *M. hyorhinis* and *M. flocculare* in the host respiratory tract [176,177].

**Conclusion**

Regardless of its inherent simplicity, as a small, wall-less bacterium with reduced genome, *M. hyopneumoniae* utilizes several pathogenicity mechanisms, some of which are not fully understood. These mechanisms include the adherence to host ciliated epithelial cells, modulation of immune response and other host defenses, and induction of cell damage by both pathogen and host components. Moreover, comparative “omics” studies between *M. hyopneumoniae* and a nonpathogenic closely related species, *M. flocculare*, have provided evidence of proteins
Figure 4. Schematic representation of *M. hyopneumoniae* pathogenicity determinants and host responses involved in EP establishment. Infection success depends on *M. hyopneumoniae* capacity to escape from the host mucociliary clearance and to adhere to ciliated cells of the porcine respiratory epithelium. Upon adhesion, *M. hyopneumoniae* causes ciliostasis and may form biofilms or invade host epithelial cells. *M. hyopneumoniae* also has a cytotoxic effect on host cells, causing cell damage and epithelial and immune cell death by apoptosis. This activity contributes to the modulation of the induced host immunological responses and may lead to lung lesions. Cell damage and epithelial cell death is also an outcome of host microbicidal and epithelial response to *M. hyopneumoniae* infection. Immunomodulation may also be associated with bacterial antigenic variation generated by proteolytic processing of surface adhesins and other surface proteins, and with the secretion of several virulence factors, as well as host pro-inflammatory peptides. During infection, *M. hyopneumoniae* may maintain its homeostasis using myo-inositol from lung tissue as an alternative carbon source. *M. hyopneumoniae* must also deal with stressing conditions, and its exposure to the host environment triggers the expression of several virulence factors. *M. hyopneumoniae* pathogenicity mechanisms, host response mechanisms and EP pathogenesis outcomes are shown in orange, green and red rectangles, respectively. Molecular processes and molecules involved in each mechanism or outcome are pointed out by gray arrows. Associations among *M. hyopneumoniae* pathogenicity mechanisms, host responses and EP pathogenesis outcomes are pointed out by black arrows.
and functions associated with pathogenicity, as the production of differential adhesin proteoforms presented in the cell surface, the secretion of differential repertoires of proteins, and the overrepresentation of proteases, antioxidant proteins, and adhesion-related proteins in pathogenic M. hyopneumoniae strains. A schematic representation of the M. hyopneumoniae pathogenicity determinants discussed in this review is presented in Figure 4. In this complex scenario of pathogen-host interplay, more studies are needed to corroborate the available evidence and to elucidate further aspects of M. hyopneumoniae pathogenicity, especially those related to the in vivo infection process. Overall, the identification of M. hyopneumoniae pathogenicity determinants and their mechanisms of action are of utmost relevance to discover new and efficient targets for the development of novel diagnostic methods, therapeutic drugs, and preventive vaccines against EP.

Acknowledgements

FMALZ was a recipient of a Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil) Ph.D. fellowship (140742/2015-8) and a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) PDSE fellowship (PDSE 19/2016 - 88881.133246/2016-01). JAP received a CAPES post-doctoral fellowship (CAPES-Biologia Computacional - Process Number: 611 23038.010043/2013-02), and is a recipient of a CAPES post-doctoral fellowship (PNPD – 88887.464453/2019-00).

Disclosure statement

The authors declare no conflict of interest.

Funding

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico [140742/2015-8]; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior [PNPD – 88887.464453/2019-00]; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior [PDSE 19/2016 - 88881.133246/2016-01]; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES Biologia Computacional - Process Number: 611 23038.010043/2013-02).

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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