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MOLECULAR PATHOGENESIS OF MYCOPLASMA ANIMAL RESPIRATORY PATHOGENS

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1. ABSTRACT

Mycoplasmas contain the smallest genomes and are the smallest known free-living organisms, yet little is known about the molecular details of their pathogenic mechanisms. This review focuses on pathogens of production animals, but related species colonize and cause disease in humans, fish and other animals, plants and insects. The general lack of genetic tools and the inability to apply the few that are available to some mycoplasma-host systems has hindered studies of this nature. During the last decade, which was characterized by unparalleled advances in the understanding of bacterial virulence, studies of mycoplasma pathogenesis has languished behind other experimental systems. The one exception has been studies on mycoplasma antigenic variation. The explosion of studies in this area has been due primarily to the fact that they can be performed in vitro without genetic tools and with simple well developed biochemical approaches. Not withstanding that antigenic variation may play an important role in disease, there have been few studies establishing the importance of this phenomenon in vivo for a variety of reasons. The same is true for cell invasion as it has been defined in cell culture systems, which if it occurs in vivo may change the way we think about mycoplasma disease. These advances give insight to an extraordinary group of organisms that interact with their hosts in unique and intriguing ways.

2. INTRODUCTION

Scientists have studied the unusual cell wall-less bacteria in the taxonomic class *Mollicutes* more commonly referred to as mycoplasmas for over a century. Once

considered to be viruses (1), it was decades before their true nature as free-living bacteria was realized (2). Then for three more decades they were thought to be L-forms of common bacteria. Today they are considered appropriately as the simplest self-replicating organisms known. In fact, their simplicity led to *Mycoplasma genitalium* being one of the first genomes sequenced (3). Further studies to begin to define the minimal gene complement needed for autonomous growth has also been performed with this species (4). Despite their genetic simplicity, the members of the class *Mollicutes* are considered as major animal and plant pathogens world-wide. Interestingly, the only disease caused by a bacterium on the A-list of communicable animal diseases of the Office International des Epizootics (OIE) and two of the five bacteria on the United States Department of Agriculture Restricted Animal Pathogens list are mycoplasmas. *Mycoplasma mycoides* subsp. *mycoides* small colony (SC), which causes contagious bovine pleuropneumonia, is on both lists. *Mycoplasma agalactiae* is the second mycoplasma on the United States Department of Agriculture list. For the purposes of this article, I will focus on pathogens of production animals, primarily ruminants (cattle and sheep), swine and poultry, recognizing that there are many other important production animals that are subject to mycoplasma infections. I will also confine myself to studies of pathogenesis or molecular biology leaving to others diagnostics, epidemiology and immunology. There are also other publications that one can refer to for additional information (5-10).

There have been several reviews of mycoplasma pathogenesis in recent years, the most comprehensive being the four volume series edited by M. F. Barile, S. Razin, J. G. Tully and R. F. Whitcomb (8). Though dated in many respects, this reference still contains the most comprehensive description of animal mycoplasmal diseases available. This aspect of mycoplasma pathogenesis has not changed much over the past 20 years. More recently, research has focused more on adherence mechanisms, surface topography (antigen variability) and diagnostic reagents although there are notable exceptions to this generalization. The excellent book Mycoplasmas: Molecular Biology and Pathogenesis (7) and the article by Razin et al. (11) are also excellent sources of information. In addition, a new book on Molecular biology and Pathogenesis will be released shortly (10). For readers who are more interested in microbial physiology, I recommend the Pollack et al. review (12).

3. CLASSIFICATION

Grouped originally by the trait that all members are cell wall-less, it is now clear that mycoplasmas represent a large diverse group of organisms. The minimal standards for classification as a mycoplasma are set out by the International Committee on Systematic Bacteriology, Subcommittee on Taxonomy of Mollicutes (13) and include common characteristics such as permanent lack of a cell wall, tendency to form fried egg colonies on solid media, passage through membrane filters of 450 nm and 220 nm pore size, low genome G + C content (23-40 mol %), and small genome size (0.58 – 2.2 Mbp). Classification of these organisms has always been problematic because of the few biochemical or physiological properties that can be used for differentiation. Included among these are morphology, host origin, optimal growth temperature, biochemical and cultural characteristics such as urea utilization, fermentation of glucose or arginine. Serological methods have been used extensively for classification, but there are only a few laboratories in the world with the capacity to serotype mycoplasmas given the large number of antisera needed today. There are over 200 species of the class *Mollicutes*, and a hundred or so species of mycoplasmas, many of which are animal pathogens (14). Antigenic variation may also complicate analyses based upon antibody reactivity. Phylogenetic analysis using 16S RNA sequences has become the defining phylogenetic tool (15, 16), although in some instances, 16S RNA sequence alone is not able to completely differentiate closely related species (17). The Mollicutes are most closely related phylogenetically to the Gram positive bacteria with low G + C mol % content in their genomes, the Clostridium-Streptococcus-Lactobacillus group. The hemotrophic bacteria *Haemobartonella* spp. and *Eperythrozoon* spp. are now considered *Mollicutes* (18) and are most closely related to the *Mycoplasma pneumoniae* group. Further information regarding the taxonomic status of the *Mollicutes* can be obtained from one of the many recent reviews and articles on the subject (12, 14, 17, 19-22).

4. INCIDENCE

Mycoplasmas are found worldwide and are considered major pathogens in animal production units in every country of the world. Most species are universally distributed with some notable exceptions; for instance, *M. mycoides* subsp. *mycoides* SC and *M. agalactiae* are not found in the United States. In many instances, mycoplasmas and the diseases they cause are ubiquitous. Vaccines provide some protection against disease and lessen the economic burden to producers, but almost universally, they fail to prevent colonization, and disease outbreaks still occur at unacceptable frequencies. For many farmers in third world countries, vaccines are not available, so the economic impact to these individuals is much greater than to a large producer in the developed world with access to vaccines. One of the most contagious and devastating mycoplasmal diseases, contagious bovine pleuropneumonia (CBPP) caused by *M. mycoides* subsp. *mycoides* SC, is found only in Africa and possibly in some areas of Asia. It has been described as the most economically important mycoplasma disease in the world despite its limited distribution (5). European countries and others have mounted considerable efforts to eliminate this species. *Mycoplasma hyopneumoniae*, on the other hand, is found worldwide wherever pigs are raised resulting in the loss of hundreds of millions of dollars each year to large and small producers alike. It has not been possible to completely eliminate *M. hyopneumoniae* from pig producing countries although some European countries have expended considerable effort in this regard. Most diagnoses are dependent on serological responses, which are not dependable indicators of infection with this species. Vaccines lessen the economic burden, but they do not eliminate the organism and do not prevent spread of the disease.

5. GENETICS

Over the last decade, there has been remarkable progress in understanding the molecular basis of pathogenesis of many bacterial pathogens. This has been largely due to two reasons, genetic systems to study pathogenesis at the cellular and molecular levels are well developed, and technological advances have provided insight into pathogen interactions not possible just a few years ago. For most mycoplasma animal pathogens, genetic approaches to study specific cellular and pathogenic processes are not available. The initial report of transformation in mycoplasmas did not occur until 1987 (23), and thus, it is a relatively young field in mycoplasmas. Although the original paper studied *Acholeplasma laidlawii* and *Mycoplasma pulmonis*, additional publications appeared on mycoplasma transformation shortly thereafter. The review article by Kevin Dybvig is a succinct review of the state-of-the-art at that time (24). For *M. gallisepticum*, however, transformation or genetic manipulation did not occur until several years later. In the early 1990s, several studies describing the development of genetic tools for use in mycoplasmas and their use in *M. gallisepticum* were published. The initial paper describes the development of Tn*4001lac* reporter tools and their use in *A. oculi* and *M. gallisepticum* (25). These studies were significant in that they were the first demonstration of transcriptional genetic tools and the first use of *lac* fusions in mycoplasmas. It has not been until recently that these tools have been used in animal pathogens with the description of the role of GAA repeats in regulation of expression of pMGA genes in *M. gallisepticum* (26). Two additional papers describe the transformation of *M. gallisepticum* with double stranded and single stranded plasmids containing Tn*916* and Tn*4001* and the use of integrative vectors to deliver genes to the mycoplasma chromosome (27, 28). A study on the use of the conjugative properties of Tn*916* to introduce the transposon into *M. gallisepticum* has also been published (29). This latter report was especially significant because they were able to sequence directly from the chromosomal DNA to identify the insertion site of the transposon. A methods manual for transforming mycoplasmas is available (30).

The single most important advance in the field of mycoplasmas will be genome sequencing. The genomes of four mycoplasma species have been reported, *M. pneumoniae* (31), *M. genitalium* (3), *Ureaplasma urealyticum* (32), and *M. pulmonis* (33). There are several sequencing projects nearing completion including *M. hyopneumoniae* (Minion, unpublished), *M. gallisepticum* (Steve Geary, personal communication) and *M. mycoides* subsp. *mycoides* SC (Karl-Erik Johansson, personal communication). Genome sequencing will revolutionize the way that scientists study mycoplasmas, particularly those that are difficult to study because of poor growth or lack of genetic tools.

6. MYCOPLASMAL DISEASES OF RUMINANTS

6.1. *Mycoplasma mycoides subsp. mycoides SC*

The agent for contagious bovine pleuropneumonia was the first mycoplasma cultured which occurred approximately 100 years ago (34). It had been studied for some years before isolation, and studies with this organism played a key role in developing the idea of prophylactic immunization in the nineteenth century. The physician Louis Willems was a key figure in the development of the idea of prophylactic immunity during the nineteenth century (35). His interest in mycoplasmas arose from the fact that his father was a cattleman, and he understood the nature of the disease from an early age. His astute observations that animals surviving the infection were immune to further disease bolstered the arguments of the "contagionists". Further, he was able to show that injection of serous fluid from the lungs of infected cattle offered a significant degree of protection although it did lead to pronounced lesions at the site of injection and sometimes death. By inoculating cattle at the tip of the tail, he was able to reduce the side effects while still providing protection. It is these side effects that are the major impediment to an effective vaccine today.

In recent years, the interaction of *M. mycoides* subsp. *mycoides* SC with host immune cells has been studied in hopes of better understanding the mycoplasma-host interactions. Pathogenic strains could induce TNF-alpha in cultured bovine alveolar macrophages while nonpathogenic strains could not (36). This correlated well with the idea that TNF-alpha plays an important, if not crucial role in pathogenesis of this mycoplasma species.

The antigenic profile of *M. mycoides* subsp. *mycoides* SC is not as well understood as it is in other species (see below). One of the problems has been the lack of well-defined monoclonal antibodies to dissect the nature of the surface architecture and a general lack of understanding of the genome structure and the genes coding for surface antigens. This will change once the genome sequence is published. Studies have been performed comparing antigenic profiles of different strains of *M. mycoides* subsp. *mycoides* SC and attempts have been made to use these results to categorize the strains tested (37). Without an understanding of the genes involved or whether size variation or phase switching occurs in this species, characterization schemes based upon immunoblot analysis can be misleading. Studies of this nature, however, are able to identify constitutively expressed proteins and possible variable proteins, which should be targets for future studies.

Only recently has there been interest in genomic structure of this species. A new insertion sequence has been identified, IS*1634*, a relative of IS*1549* from *Mycobacterium smegmatis* (38). These particular elements seem to form a new insertion sequence class. IS*1634* was found in about 30 copies in the genome and was specific for *M. mycoides* subsp. *mycoides* SC.

6.2. *Mycoplasma bovis*

6.2.1. Pathogenesis

Mycoplasma bovis infections of cattle are an important disease worldwide. *Mycoplasma bovis* causes pneumonia, arthritis and mastitis in cattle and can be isolated from a variety of tissues including all areas of the reproductive tract and the eye (39). During natural infections, *M. bovis* can be easily observed lining the epithelial surfaces of the respiratory tract (40). Often lung

abscesses appear which can be encapsulated. Interestingly, *M. bovis* antigen is often seen only at the periphery of necrotic areas suggesting that the organism is not actively involved in the necrosis itself. During infections, macrophages and neutrophils are actively recruited to infected areas (41). *Mycoplasma bovis* antigen can be detected within epithelial cells, inflammatory cells in the airway and in alveolar walls. The organism is not limited to cattle. Experimental infections in goats have also been reported (42).

Mycoplasma bovis is highly invasive and is not confined to the initial area of colonization, the respiratory tract. Consequently, organisms rapidly gain access to multiple organ systems. This suggests that in order to survive, *M. bovis* may alter its gene expression as it encounters different host environments and different selective pressures. Among those genes thought to be regulated to the advantage of the microbe and to the detriment of the host would be adhesins. In this way, specific adhesins could be expressed only when needed, preventing an early host immune response that could block colonization of specific tissues. Adherence of *M. bovis* to epithelial cells is a complex process. Early studies showed that the mechanism of adherence to embryonic bovine lung cells may involve interactions with sialic acid-containing receptors or sulfated lipids (43). One potential adhesin was identified as a 32 kDa antigen, P26, based upon inhibition of adherence by monoclonal antibody (Mab) 4F6. The adherence was sensitive to trypsin, neuraminidase and temperature (44). Purified P26 was shown to block mycoplasma adherence in competition assays. A second Mab directed against a common epitope of the Vsp family, Mab 1E5, also showed adherence-blocking activity suggesting that one of the Vsp antigens may also be involved in adherence to embryonic bovine lung cells. To further examine this possibility, peptides representing different repeating units of four Vsp proteins were used to map reactivity of convalescent sera and to study adherence of *M. bovis* to embryonic bovine lung cells (45). The effectiveness of several of the peptides in blocking adherence suggests that Vsp proteins are involved in cytadherence.

6.2.2 Antigenic variation

There has been a great deal of interest in the antigenic variation observed in *M. bovis*. David Yogev at The Hebrew University and his colleagues published the initial papers on antigenic variation in this species (46, 47). In their initial studies, the family of lipid-modified variable surface proteins, Vsps, consisted of at least four classes, VspA-D, but this has now been enlarged to at least 13 different members (48). These proteins exhibit both phase switching (ON-OFF states) and size variation. These proteins differ from other mycoplasma size variant proteins in that their immunoblot patterns display irregular periodic spacing, suggesting that the mechanism responsible for the variation may differ from other mycoplasma species (46). Each of the members of this group could be distinguished using monoclonal antibodies directed against unique antigenic epitopes (46, 49), but they also share a common antigenic epitope found on the PvpA protein of *M. gallisepticum* (50). This epitope was expressed in 246 of 250 different *M. bovis* strains (51). Hybridization analysis with oligonucleotides sharing sequences among the vsp genes indicate that the *vsp* repertoire varies in size and composition among different isolates (51). All of the Vsp proteins were organized on the surface of the mycoplasma in evenly distributed clusters (52). In addition, *vsp* homologues have been identified in *M. agalactiae* (53). An oligonucleotide representing a conserved region in all *vsp* genes reacted to multiple bands upon Southern hybridization. Oligonucleotides representing unique distinct *vsp* regions failed to react indicating that the *vsp* family in this species was analogous but distinct from the *vsp* family in *M. bovis*.

Mycoplasma bovis demonstrates significant interstrain variability within the *vsp* loci at both the protein and DNA levels (54). The ON-OFF phase switch for the *vsp* genes involves DNA rearrangements or inversions possibly involving a recombinatorial mechanism (55). These inversions sometimes result in the creation of chimeric genes and new Vsp phenotypes (48). In some cases, two site-specific DNA inversions between inverted 35-base pair sequences were needed for phase variation (56). These inversions resulted in the juxtaposition of a promoter-like sequence to a silent *vsp* gene promoting transcription initiation.

Size variation in the Vsp proteins is due to repetitive sequences that sometimes make up 80% of the coding sequence (57). When expressed, these sequences produce periodic polypeptide structures. Eighteen distinct repetitive domains of different lengths and amino acid sequences have been identified (57). The sequences are arranged in blocks of similarity and the number of repeats can vary between strains. It is thought that slipped strain mispairing at high frequency during DNA replication causes the unequal copy of the number of repetitive sequence units producing siblings with a different number of repeat units. Since there are multiple repetitive domains of different lengths within each Vsp, replication errors result in the irregular periodic spacing seen in the immunoblot patterns.

In the presence of antibodies from experimentally-infected or naturally-infected calves, selective pressure of specific Vsp phenotypes *in vitro* resulted in the repression or shortening of some Vsp proteins while others appeared in the cell population (58). When the antibody was removed, the cells reverted to the original phenotype. This shows that there is a preferred Vsp phenotype during *in vitro* growth and that selective pressure from the host humoral response can alter or modulate that phenotype (58). Whether this modulation is operative *in vivo* where other selective pressures such as adherence receptors exist to enhance maintenance of specific phenotypes has yet to be determined. In addition, it is not known if variation in the Vsp phenotype can affect tissue tropism and enhance invasiveness within the host.

A second Vsp unrelated protein that underwent phase switching and size variation, pMB67, was also

identified (59). Unlike the Vsp proteins, pMB67 was not lipid modified and did not contain the Vsp-like repetitive domains. It appeared to be a predominant antigen recognized during *M. bovis* infections and could be used as a diagnostic reagent or used in a vaccine.

6.3. *Mycoplasma dispar*

Mycoplasma dispar is frequently isolated from normal and pneumonic lungs of cattle and is often considered a commensual organism (39). The most interesting aspect of this organism relative to this review is the studies of its capsule (60). The capsule is related to polygalacturonic acid (pGalU) polymer (61, 62), but there are significant differences between the capsule and pGalU. They are similarly immunologically, but not identical because antibodies raised against pGalU fail to recognize the capsule, but antibodies raised against the capsule do recognize pGalU (63). Both electron microscopic and antibody-based studies confirmed the fact that the capsule produced *in vitro* was the same immunologically as that produced during infections (60). During lung infections, IgM and IgA antibodies reactive to the purified polysaccharide could be seen in lung secretions (63). In addition, the capsule demonstrated anti-phagocytic properties with bovine alveolar macrophages (64). The effect on the macrophages seemed to be focused on its anti-phagocytic abilities since purified capsule prevented activation of cultured macrophages (63). The capsular polysaccharide induced IgM antibodies in mice, but no secondary response was observed. Also, the capsule was not mitogenic. The capsule is lost during *in vitro* passage, but can be regained with a single passage on bovine lung fibroblast cells (60). Since expression of the genes needed for production of this material is under tight regulatory control, it is unlikely that selection for capsule positive siblings would occur rapidly in tissue culture in the absence of selective pressure.

7. MYCOPLASMAL DISEASES OF SWINE

7.1. *Mycoplasma hyorhinis*

Mycoplasma hyorhinis is an occasional agent of polyserositis and arthritis of swine (39). Its importance to this review is not its pathogenicity, but rather its historical significance in being the first mycoplasma in which antigenic variation was identified (65-68). This seminal publication has resulted in renewed interest in the general scientific community and an explosion of publications on phase switching and antigenic variation in mycoplasmas. Along with these early publications came the concept of a constantly changing surface architecture and antigenicity, which changed our general concept of the mycoplasma membrane and how it interacts with the host (69), It was immediately obvious that these variable proteins in *M. hyorhinis* were attached to the membrane through lipid linkages. Soon it became apparent that size variation was due to the loss or gain of repetitive intragenic coding sequences while retaining a conserved domain for membrane insertion and processing (70). The nature of the phase switch was not readily apparent as no changes could be identified in restriction fragment patterns ruling out long range changes such as DNA inversions or genomic rearrangements (70). The only changes detected during phase switching were in a polymeric adenine tract upstream of the structural gene in the space between the putative –10 and –35 regulatory binding sites. A single base change (loss or gain) in this region was sufficient to eliminate expression of a particular *vlp* (variable lipoprotein) gene. These changes affected transcription of the downstream gene in a manner reminiscent to tight regulatory control (71). In addition, the level of protein product was associated with the length of the transcript or gene sequence (71). The number of *vlp* genes within the genome is not known, but at least six different *vlp* loci have been identified in strain GDL-1 in comparison to the three loci of strain SK76 (72). Additional variability in Vlp antigenic structure is possible through genetic mutations, which alter the reading frame of each *vlp* contributing to a greater diversity of antigenic surface molecules than was previously reported (72).

While the role of Vlps in viability, pathogenicity or survival is not known, the host can profoundly affect the repertoire of expressed Vlps (73). Hypothetically, high frequency switching of Vlp surface lipoproteins in the host ensures that a wide variety of antigenic architectures are present in the host at any one time, although there is no direct evidence of this. It is thought that as the host immune response to one Vlp type develops to control the organism, additional Vlp types begin to appear through random switching and outgrowth of that antigenic type. Inhibition of growth by antibody is a well-characterized phenomenon, but unexpectedly when tested *in vitro* with convalescent swine sera, the length of the Vlp products determined the susceptibility of *M. hyorhinis* SK76 to antibody inhibition (73). The Vlp products themselves were not the targets of the antibody, but rather underlying surface antigens appeared to be protected by the “protein shield” provided by the long chain Vlps. These antigens have yet to be identified (73).

Mycoplasma lipoproteins from *Mycoplasma fermentans* have been identified as potent modulators of macrophage function (74-77). Lipopeptides from VlpA and VlpC of *M. hyorhinis* have been shown to have these characteristics as well (78). Through the construction of lipopeptide variants, it was possible to identify the reason for the extremely high macrophage stimulating activity shown by mycoplasma lipopeptides. The lack of N-acyl groups within the lipopeptides explains their extraordinary macrophage activating activity. It is thought that these molecules are the main agents inducing inflammatory reactions in the host (78).

The ease with which *M. hyorhinis* can be grown *in vitro* was an important contributor to the studies described above. It is also a common contaminant of cell culture lines whose presence is often not detected. The presence of *M. hyorhinis* in cell cultures can have profound affects on cell function and viability. Early studies of translocation of cell surface markers to mycoplasma membranes were some of the first studies in mycoplasma pathobiology that focused on the organism’s ability to conscript host macromolecules for its own purposes (79,

80). Alteration of host cell function through modification of host cell surface structures may also be an important component of the pathogenic process of mycoplasmas. Recent studies on the ability of *M. hyorhinis* to induce apoptosis in cell cultures has led to the observation that mycoplasma coded nucleases play an important role in this process (81). Further analysis of these nucleases showed that they were readily found in culture supernatants and in nuclei from infected cells. In addition, partially purified nucleases were able to fragment chromosomal DNA of nuclease-negative nuclei at internucleosomal sites (82).

7.2. *Mycoplasma hyopneumoniae*

7.2.1. Pathogenesis

In contrast to *M. hyorhinis*, *M. hyopneumoniae* is exclusively a respiratory pathogen. As the agent of enzootic pneumonia in pigs and a primary component of Porcine Respiratory Disease Complex (PRDC), *M. hyopneumoniae* has become one of the most important diseases in the swine industry. While *Actinobacillus pleuropneumoniae* and *Pasteurella multocida* can be found associated with PRDC, *M. hyopneumoniae* is most frequently identified (83). In fact, the majority of severe PRDC cases examined at the Iowa State Veterinary Diagnostic Laboratory had microscopic lesions typical of *M. hyopneumoniae* infection. Lesions of enzootic pneumonia (well-demarcated dark red to purple or tan-gray areas of cranioventral consolidation) are consistently observed in >50% of swine at slaughter. Use of serum herd profiling has revealed strong and widespread seroconversion to *M. hyopneumoniae* in many groups of growing and finishing swine after escalation of coughing and other clinical signs of chronic pneumonia (83).

Mycoplasma hyopneumoniae induces a slow and ineffective immune response as measured by slow seroconversion and low levels of antibodies in the respiratory tract (84-86). A major component of *M. hyopneumoniae* disease is immunopathologic changes such as decreased phagocytic activity of alveolar phagocytes (87), decreased responsiveness of lymphocytes to non-specific mitogens (88), and production of proinflammatory cytokines IL-1, IL-6 and TNF-alpha (85, 89, 90). Recent studies have shown that IL-8, IL-10 and IL-12 levels are also increased in bronchial alveolar lavage fluids. While inflammation is a proper response toward respiratory pathogens, it appears to be an important factor in *M. hyopneumoniae* disease and in the potentiation of porcine reproductive and respiratory syndrome virus-induced pneumonia during dual infections (91).

This reemerging mycoplasma disease problem is occurring in spite of the availability of antibiotics and bacterins for control of bacterial pneumonia in swine. Veterinarians and swine producers indicate that current control measures for enzootic porcine pneumonia are inadequate. The vaccines are reported to reduce pneumonia in swine, improve weight gains, reduce feed required and reduce the number of days to market. However, these vaccines do not provide protection against development of lesions or prevent establishment of *M. hyopneumoniae* infection in the lung. Moreover, studies of uncomplicated, experimental mycoplasmal pneumonia have revealed that none of the currently available drugs provide appreciable therapeutic benefit (91). The potential for adequate control of this important disease complex through the therapeutic use of antibiotics seems limited for many reasons with cost being a major factor. In addition, there is growing public concern over the prophylactic use of antibiotics in animal feeds, and it is reasonable to conclude that regulations restricting such usage may be forthcoming in the not too distant future.

Pathogenic mechanisms for *M. hyopneumoniae* are not well understood. This mycoplasma species is difficult to study because of its fastidious growth requirements, poor overall growth on agar surfaces, and difficulty in establishing infections in swine with laboratory grown organisms. Because of these difficulties, many of the early studies focused on descriptive studies of infected tissues (92-94) and the interactions with various cell types in vitro (95-97). The following sections will focus on adherence and ciliostasis mechanisms.

7.2.2. Adherence

Attachment to host tissue is essential for colonization by most mucosal pathogens, and this has been studied in *M. hyopneumoniae* as well. *Mycoplasma hyopneumoniae* closely adheres to the cilia of swine respiratory epithelium both in vivo and in vitro (92, 98). Unlike many mycoplasma species that recognize and bind cell receptors found on different cell types, however, *M. hyopneumoniae* displays a remarkable binding specificity. The *M. hyopneumoniae* cell receptors are found exclusively on cilia of epithelial cells in the respiratory tract of swine. This unique binding specificity limited the study of the molecular basis of mycoplasma-cell interactions until the development of an in vitro adherence assay (99) and concomitant development of adherence-blocking monoclonal antibodies (100). This led to a series of studies beginning with the identification of the protein involved in adherence, P97, by immunoblot analysis (100). The molecular basis for adherence was not determined until the gene for the cilium adhesin, P97, was cloned, and the preliminary genetic analysis on the coding sequence could be performed (101, 102). These studies were notable because they represented the first time that a mycoplasma adhesin was expressed in *E. coli* and shown to have the same binding characteristics as the mature protein in the organism. In addition, they also were the first time that transposon mutagenesis was used to define regions within a cloned mycoplasma gene sequence. It is now clear that a repetitive sequence in P97 designated R1 mediates adherence to swine cilia (102). The repeat region consists of a 5 amino acid repeat, and it was not until the repeat region was reconstructed in a β-galactosidase fusion system in *E. coli* that the number of units of the R1 repeat sequence needed for adherence was defined as eight (103). Adherence-blocking monoclonal antibodies recognized as few as three repeats (103). There is variability in the number of repeats in different *M. hyopneumoniae* field strains (104), and even in the nonadhering strain J, the binding epitope is fully functional (102).

Two other interesting aspects of the adhesin story arose from the studies of Zhang et al. (100). First, P97 demonstrated a multiple banding pattern by immunoblot analysis with adherence blocking monoclonal antibodies (100). Until recently, the nature of this multiple banding pattern was not clear. The gene sequence for P97 gave no evidence that variation within the two repeat regions of the P97 could account for the type of pattern observed. Normally, a ladder-like pattern is seen with size variant lipoproteins in mycoplasmas, but this was not the type of pattern observed with this protein. P97 is not a lipoprotein even though it possesses an N-terminal hydrophobic domain. In addition, there was no evidence that there were multiple copies of P97 in the genome capable of producing the products observed in the immunoblot analyses (105). The protein undergoes proteolytic cleavage at amino acid 195 to generate what was once thought of as the "mature" cilium binding protein. It is now clear, however, that P97 undergoes further proteolytic processing on the mycoplasma surface resulting in fragments with potentially different functions (Steve Djordjevic, personal communication). The cilium-binding fragment containing R1 seems to be held to the membrane through a bridge of unknown composition (100). Other fragments of P97 are found elsewhere in the cell or in the extracellular matrix (Minion, unpublished). Their functions are unknown, but it is likely that they contribute to the overall pathogenesis of the organism.

7.2.3. Ciliostasis

Following attachment of *M. hyopneumoniae in vivo*, there is extensive loss of cilia from the epithelial cells of the trachea, bronchi and bronchioles (92). Ciliostasis and ciliary damage can be induced in tracheal ring cultures by inoculation with low-passage *M. hyopneumoniae* grown in broth culture supplemented with sterile, irradiated lung homogenate or lung homogenate from a pig infected with *M. hyopneumoniae* (98). This correlates exactly with the results of swine challenge studies when comparing *in vitro* passaged organisms and *in vivo* grown organisms in infected lung homogenate (106). *Mycoplasma hyopneumoniae* grown in broth have reduced virulence when compared with organisms grown in the presence of porcine lung tissue suggesting that a component of lung homogenate is needed for continued expression of a necessary virulence determinant. Such a determinant may be lost upon normal in vitro passage but whose expression is maintained in the presence of lung homogenate.

One mechanism by which *M. hyopneumoniae* could initiate cilial damage is by altering cell signaling pathways. Early studies by Debey et al. showed that *M. hyopneumoniae* could alter the calcium concentrations within activated neutrophils (107), but it was not until recently that similar results were confirmed in porcine ciliated epithelial cells (108). The mechanism by which this occurs is unknown, but it seems to involve surface proteins (or their fragments) either by activating cell surface receptors or by direct interaction with pathway constituents. Calcium seemed to be released from intracellular stores through the PLC pathway. Neither *M. hyopneumoniae* strain J or *Mycoplasma flocculare*, both of which fail to bind cilia, could induce the increased intracellular calcium (108).

8. MYCOPLASMAL DISEASES OF BIRDS

The poultry industry is affected by several different species of mycoplasmas including *M. gallisepticum*, *Mycoplasma synoviae* and *Mycoplasma meleagridis* only two of which will be discussed here. Of the three, only *M. gallisepticum* and *M. synoviae* have been studied to any depth from a molecular pathogenesis and cell biology standpoint.

8.1. *Mycoplasma gallisepticum*

8.1.1. Introduction

Mycoplasma gallisepticum has been a favorite disease model system to study for many years. It has many features that make it attractive to the scientist including its ease of growth and inexpensive host, and it binds to many different cell types. *Mycoplasma gallisepticum* is transmitted both horizontally and vertically (109) creating unique problems in the poultry industry. Management of *M. gallisepticum* disease occurs primarily through rigorous screening of egg laying operations and depopulation of infected flocks. Vaccines are available, the most effective being attenuated mutants ts-11 and F (110). These mutants are not without problems, however. Both are virulent for turkeys and should not be used in regions where turkeys are raised in close proximity to chickens. The F strain is still slightly virulent for chickens as well. Interestingly, the ts-11 strain is capable of replacing the F strain in flocks vaccinated with the F strain (111). This offers an opportunity for eradication of *M. gallisepticum* in flocks.

8.1.2. Adherence

Some of the earliest studies of *M. gallisepticum*-cell interactions used erythrocytes as models for adherence (112, 113). Classical biochemical approaches were initially used to define the mycoplasma protein involved in hemagglutination (114). These early studies used affinity chromatography to isolate a 75 kDa protein. Eventually, hemagglutination-blocking monoclonal antibodies were used to define the protein involved in the interaction with erythrocytes in a different strain of *M. gallisepticum* (115). Assisted by a three monoclonal antibodies, Markham et al. identified a 67-kDa protein, designated pMGA, as the hemagglutinin in strain S6. The affinity-purified protein was digested with proteases and the resulting peptides sequenced to provide probes used in cloning the gene sequences (116). Unexpectedly, the probes identified gene sequences that were related to pMGA, but were not the pMGA gene itself. This was located downstream of the initial clones. These were the first studies to show unambiguously that *M. gallisepticum* contains a family of related genes that appear to be transcriptionally and translationally functional (116). Hybridization studies showed that the number of pMGA family members varied between strains with 32 members in the avirulent vaccine strain F and 70 members in the virulent strain R (117). If the average size of the pMGA family members is approximately the size of pMGA1.1, then 16% of the

genome of strain R or 168 kb of DNA codes for members of this family. The genes seem to be arranged in four different regions of the genome, mostly likely arrayed as tandem repeats (117). As individual family members are identified and studied, they have been given numbers as pMGA1.1, pMGA1.2, etc. Strains differ in their pMGA sequences (118).

Further analysis of this family of genes showed that variation in expression of each member of the family appears to be regulated at the transcriptional level (119). The control of pMGA expression lies within a 3 base pair GAA repeat sequence found in the intergenic region. The initial observations compared pMGA1.1 sequences in expressing and nonexpressing strains. When 12 repeats were found in the intergenic region, the pMGA gene downstream was expressed. This also occurred in pMGA1.2 and pMGA1.9 genes (120). To further examine the mechanism of regulation, Liu et al. constructed a *lac* fusion with the GAA repeat region, the promoter and the translational start codon in Tn*4001* and then inserted it into *M. gallisepticum* by transformation. ON-OFF variants were identified by screening on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-containing media. The GAA region was then PCR amplified and sequenced in different ON-OFF variants. The only changes were in the number of GAA repeats; those containing 12 repeats were always ON (Lac^+), those containing greater or fewer GAA repeats were always OFF (Lac^-)(26).

Related pMGA genes have been identified in other avian mycoplasma pathogens, but not in closely related species (121). In *M. synoviae* the *vlhA* gene family is the corresponding family, but there were differences in the gene structure and in the regulatory mechanism (122). Regions of VlhA were found in two other phase-variable proteins that exhibited coordinate phase variation. Further analysis of *vlhA* family members showed that the promoter and 5´ regions occurred as a single copy, but that the remainder of the sequence was found in multiple, tandemly-arrayed copies. These regions lacked the putative promoter and the 5´ regions of the expressed gene suggesting that the promoter and 5´ sequences form an expression site. Variation occurs as a result of gene conversion, duplicative recombination of sequences from the tandem repeat region with the concomitant loss of the corresponding sequences in the expression site (123). *Mycoplasma imitans* also contains a family of genes related to pMGA (121). In this species, the GAA repeats seemed to control expression in similar fashion to *M. gallisepticum*. Thus, a large family of related hemagglutinins is found in three different avian pathogens, but their mechanism of regulation varies between species.

Other than the pMGA phase variable hemagglutinins, *M. gallisepticum* contains other proteins that have been linked to cell adherence. A 64 kDa protein has been implicated in attachment to chicken tracheal rings (124). A gene with sequence similarity to the *M. pneumoniae* P1 and *M. genitalium* MgPa cytadhesins has also been identified (125). This gene, designated *mcg1*, codes for a 150-kDa protein. A homologue of the P30 adherence-associated protein of *M. pneumoniae* and its P32 homologue from *M. genitalium*, *mcg2*, has been reported as well (126). A second gene from *M. gallisepticum* with homology to the P1 cytadherence protein of *M. pneumoniae* has also been described (127). This gene, *gapA*, codes for a protein of 105 kDa. It has a closer homology to *P1* than *mcg1*. Unlike *P1*, there is a single copy of *gapA* in the genome. Anti-GapA Fab fragments blocked adherence of *M. gallisepticum* to chicken tracheal rings suggesting it plays a role in cytadherence (127). A high-passage strain of *M. gallisepticum* R deficient in adherence activity was missing three proteins, GapA, P116 and P45 (128). Restoration of GapA expression did not restore cytadherence activity, however. The gene for P116, designated *crmA*, is the downstream gene of *gapA* in the two-gene operon. Since *crmA* expression was not restored in the *M. gallisepticum* GapA (+) strain, it was suggested that CrmA may play an important role in cytadherence along with GapA.

8.1.3. Antigenic variation

The first studies to show variation in expression of membrane proteins in *M. gallisepticum* were those of Garcia et al. (129) and Yogev et al. (50). Garcia et al. used a panel of monoclonal antibodies to study colony lifts demonstrating phase switching in several of the proteins. Yogev et al. reported that a common epitope of three different Vsp proteins of *M. bovis* was shared with a protein of *M. gallisepticum* designated PvpA. This protein also underwent high frequency phase variation like the Vsp proteins of *M. bovis*, but unlike the Vsp proteins, it was not lipid modified. The gene sequence of *pvpA* showed no sequence homology to that of the *vsp* genes from *M. bovis* although it was antigenically related (50). PvpA had two, fifty two amino acid repeats, a fourteen tetrapeptide repeat (Pro-Arg-Pro-X) and varied in size between strains (130). Size variability was due to short deletions within the two direct repeated sequences. Expression of *pvpA* was controlled by five GAA codons within the N-terminal region of the gene, which was subject to frequent mutation to a UAA stop codon (130). The protein was localized to the membrane surface in the terminal tip region of the cell suggesting that the protein was actively involved in cytadherence. Convalescent serum from infected chickens has also used to identify phase variable lipoproteins in *M. gallisepticum* (50).

The role of pMGA and many other phase variable proteins in the mycoplasma is unknown, although some appear to function as hemagglutinins or cytadhesins. It is clear that pMGA proteins are major antigens on the membrane surface. It is thought that variation in pMGA expression results in evasion of the immune response. This is supported by the observation that growth of *M. gallisepticum* in the presence of polyclonal and monoclonal antibodies reactive to a specific pMGA protein resulted in its repression and expression of a second antigenically distinct pMGA protein (131). When the antibodies were removed, colonies became sectored as siblings began expressing the original pMGA protein. A second study monitored expression of two phase variable proteins during growth *in vivo* (132). This study also demonstrated a rapid

change in the expression profile of PvpA and p67a further supporting the hypothesis that *M. gallisepticum* has evolved a unique antigen variation mechanism different from other mycoplasma species. In a similar series of studies, Gorton et al. grew *M. gallisepticum* in the presence of anti-LPP64 antibodies. This produced a population of cells lacking LP64, but now expressing new unique membrane proteins of 91, 42, 41, 38, 37 and 18 kDa (133). These studies suggest that selective pressure can select subpopulations of *M. gallisepticum* cells expressing an alternative array of membrane proteins lacking the antigenic epitope(s) of the original parental population.

8.1.4. Invasion

For many years, mycoplasmas were believed to be capable of host cell invasion where they would access to the pools of amino acids, purines and pyrimidines needed for growth. Much of the evidence suggesting such an existence was circumstantial, electron micrographs suggesting an intracellular position, but unconvincing because of experimental artifacts common to electron microscopy. It was not until 1995 that clear evidence of intracellular existence was obtained using confocal microscopy and long term passage in cell culture (134). The initial observations were made with the human mycoplasmas *Mycoplasma penetrans*, *M. pneumoniae* and *M. genitalium*, but recent studies with *M. gallisepticum* have rekindled the interest in cell invasion and intracellular growth of mycoplasmas (135). Using chicken embryo fibroblasts and a gentamicin selection protocol, Winner et al. were able to establish the kinetics of intracellular invasion of *M. gallisepticum* and to demonstrate that *in vitro* passage number was inversely correlated with invasion ability. Passage of invasion-negative high passage cells in cell culture significantly increased their ability to invade tissue culture cells (135). Since these observations have been made with tissue culture cells, one critical question remains, does cell invasion occur *in vivo* during infections? Once that has been confirmed, interest in mycoplasma invasion will increase significantly.

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