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Global Changes in *Mycoplasma gallisepticum* Phase-variable Lipoprotein (vlhA) Gene Expression in the Natural Host

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Global Changes in *Mycoplasma gallisepticum* Phase-Variable Lipoprotein (*vlhA*) Gene

Expression in the Natural Host

Katherine M. Pflaum, PhD

University of Connecticut, 2016

Mycoplasma gallisepticum, the highly transmissible avian pathogen, is the primary etiologic agent of chronic respiratory disease (CRD), a disease largely affecting the respiratory tract of poultry, and causing significant economic losses world-wide. Proteins of the variable lipoprotein and hemagglutinin (*vlhA*) gene family are thought to be important for *M. gallisepticum* host interaction, pathogenesis and immune evasion, but the exact role and overall mechanisms of phase-variation are not well understood. To better understand the phase variation of *M. gallisepticum vlhA* genes, we have conducted a large scale, next generation sequencing analysis of *M. gallisepticum* sampled directly from the tracheal mucosa of experimentally infected chickens as well as those recovered and passaged *in-vitro*.

In the first study, *M. gallisepticum* was recovered from the tracheal mucosa daily over the course of a seven day infection. Of note, the data indicated that, at given time points, specific *vlhA* genes were similarly dominant in multiple independent hosts, suggesting a non-stochastic temporal progression of dominant *vlhA* expression. Additionally we found that *vlhA* expression was not dependent on the presence of exactly 12 GAA repeats, suggesting a previously unrecognized mechanism may be responsible, at least in part, for *vlhA* expression.

In the second study, we report that the *vlhA* profile of *M. gallisepticum* recovered from the trachea of experimentally infected chickens when the input culture expressed an alternate dominant *vlhA*, *vlhA* 2.02. Here we report that even when the culture is dominantly expressing

vlhA 2.02, there is an immediate shift as early as one day post infection to the dominant expression of *vlhA* 3.03, suggesting the expression of this gene may be essential in the earliest stages of the infection process.

**Global Changes in *Mycoplasma gallisepticum* Phase-Variable Lipoprotein (*vlhA*) Gene
Expression in the Natural Host**

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B.S. Salisbury University, 2010

M.S. Towson University, 2012

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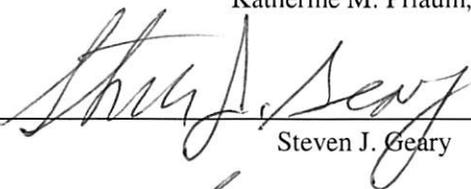
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Doctor of Philosophy Dissertation

Global Changes in *Mycoplasma gallisepticum* Phase-Variable Lipoprotein (*vlhA*) Gene
Expression in the Natural Host

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CHAPTER 1: Literature Review

Section 1: General Features

Introduction

Mycoplasmas are very small selfreplicating Prokaryotes that lack a cell wall. They range in genomic size from 580 Kb as seen in *M. genitalium* (1) to 1,380 Kb as seen in *M. mycoides* subs *mycoides* LC (2) and have very low genomic G+C content (23-40%)(3). They are believed to have evolved from a low G+C containing Gram-positive bacteria through degenerative evolution (4, 5). *Mycoplasmas* also have very limited biosynthetic capabilities, lacking the ability to synthesize many essential molecules, including purines and pyrimidines. and hence must rely on close interactions with host cells for essential nutrients and molecules such as amino acids, nucleotides, and fatty acids. Unique to the *mycoplasmas* is the necessity to acquire sterols from host cells to maintain cell membrane integrity without the support of a rigid cell wall (2). *Mycoplasmas* uncharacteristically use the traditional stop codon, UGA to encode the amino acid tryptophan (6, 7). These organisms tend to have a unusually high mutation rates, likely due to inadequate proof-reading by DNA polymerase enzymes (5).

Mycoplasmas are common pathogens and tend to exhibit strict host specificity over a wide variety of species including humans, animals, reptiles, insects and even plants. They can cause a wide range of diseases, effecting respiratory and reproductive tracts, among other organ systems. Several *mycoplasma* species exist however, as commensals, living with their host species as part of their natural flora. Very few *mycoplasma* species cause mortality, but

many are associated with morbidity and have significant economic impacts around the world resulting from their pathological effects on humans, pets, livestock, and poultry.

History

The first *mycoplasma* was identified by Nocard and Roux in 1898 (8). A semi-permeable nitrocellulose pouch containing sterile peptone broth was inoculated with infectious exudates from a cow exhibiting signs of bovine pleuropneumonia, including distention of the interlobular connective tissue by a large amount of clear-yellowish albuminous serous fluid. This pouch was surgically placed into the peritoneal cavity of a rabbit, along with an uninoculated control and a heat-inactivated control. Approximately 3 weeks later, the broth containing the infectious exudates became opalescent, while the controls remained clear. Eventually, Nocard and Roux were able to cultivate this organism axenically in peptone broth supplemented with serum and experimentally inoculate cattle. When this organism caused disease in experimentally infected cattle, it was deemed the etiologic agent of bovine pleuropneumonia and was therefore named, the pleuropneumonia virus (8). After this initial discovery, much taxonomic contention and confusion would ensue in the years to follow.

Initially this organism was incorrectly classified as a virus. This was the case because the organism was able to pass through a filter, which does not allow the typical bacteria to pass and invisible by light microscopy (9). Additional evidence supporting this theory came when Eaton et. al. (10) isolated, what he termed Eaton's agent, which would later come to be known as *Mycoplasma pneumoniae*, and it appeared to be unaffected by antibiotics. We now

know the antibiotics used (β -lactam) work by disrupting the cell wall formation, a process that *Mycoplasma* species do not undergo, as they lack a cell-wall altogether.

In 1910, Borrel et. al. cultured the organism isolated by Nocard and Roux, that was thought to be a virus at the time, and saw radiating filamentous structures and therefore renamed the organism *Asterococcus mycoides* (11). In retrospect, these structures that were observed were likely due to contamination (9). Just one year later, Martzinovski et. al. cultured the same organism and did not see the filamentous structures, but rather diplococci and streptococci, and therefore renamed the organism for the third time, *Coccobacillus mycoides* (12). Several years later, it was incorrectly characterized as an L-form, or wall-less form, of other known bacteria, such as the *Streptococci* due to their similar morphology (13, 14).

By 1935, this group of organisms had undergone seven different name changes. It was at this time that Klienberger introduced the term ‘pleuropneumonia-like organisms’ (PPLO) to describe these ‘wall-less variants’ of known bacteria (9). It was not until nearly 50 years after the discovery when the Judicial Commission of the International Committee on Bacteriological Nomenclature in 1958 would rule that the name of the etiologic agent of bovine pleuropneumonia was *Mycoplasma mycoides*. It was ten years after that, that the committee agreed on the class *Mollicutes* to describe organisms with ‘the absence of a true cell wall and plasticity of the outer membrane’ (9).

Nomenclature

After many years of contention surrounding the taxonomic classification of these bacteria, the current classification system places the *Mycoplasmas* in the phylum *Tenericutes*

which are phylogenically related to the *Firmicutes* phylum consisting of low G+C content bacteria such as *Bacillus* species (2). The only class within the *Tenericutes* phylum is the *Mollicutes* class, which consists of bacteria that lack a cell wall, have an unusually small genome, and have low G+C genomic content. *Mycoplasmas* belong to the order *Mycoplasmatales* which are known to colonize vertebrates. The *Mollicutes* in the remaining four orders including the *Entomoplasmatales*, *Acholeplasmatales*, *Anaeroplasmatales*, and *Haloplasmatales*, typically tend to colonize insects and plants. *Mycoplasmas* are classified within *Mycoplasmataceae*, the only family found within the *Mycoplasmatales* order. The *Mycoplasmataceae* family is further separated into two genera, the *Mycoplasmas* and the *Ureaplasmas* which are categorized based to their ability to hydrolyze urea as an energy source (15).

Minimal Genome Concept

Since *M. genitalium* harbors the smallest genome for any known self replicating cell in nature (16), the J. Craig Venter Institute (JCVI) has set out to understand and identify the minimal complete set of genes that are necessary for growth in a lab setting, using the *M. genitalium* genome as their foundation. In 2008 JCVI constructed an organism with a synthetically created genome based on that of *M. genitalium* containing what was thought to be the minimal complement of genes for a free living cell (17). This organism, *M. genitalium* JCVI-1.0 contained only 582,970 base pairs.

To further understand the components that are essential to a free living organism, JCVI constructed a synthetic bacterium based on the genome of *M. mycoides* (18), the largest *mycoplasma* genome containing 1,380 Kb (2). This synthetic organism, JCVI-syn1.0

contained 1,079 Kb, and contained all the expected phenotypic properties, including continuous self-replication (18).

Recently, the researchers at JCVI have further defined the minimal complement of genes required for a free living cell by minimizing the JCVI-syn1.0 through a series of cycles of design, synthesis, and testing. They were able to reduce the JCVI-syn1.0 from 1,079 Kbs to 531 Kbs, creating JCVI-syn3.0, an organism with a genome smaller than any known self replicating organism. JCVI-syn3.0 contains only 473 genes and has an approximate doubling time of 180 minutes (19).

This work, conducted by JCVI, has begun to define the genes that are essential for a free living, self-replicating organism using known *Mycoplasma* genomes as their foundation. Further investigations into these minimal organisms will shed more light on the functions of previously unknown *Mycoplasma* genes and the essential components of free living, self-replicating organisms.

Section 2: Infectious *Mycoplasma* species

***Mycoplasmas* of Humans**

Several *Mycoplasma* species are able to infect humans including *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma hominis*, and *Mycoplasma penetrans*. One of the most studied pathogens in this genus is *M. pneumoniae*, which causes community-acquired interstitial pneumonia, commonly referred to as walking or atypical pneumonia (20) and is the predominant cause of pneumonia in school aged children, making this disease a significant burden to public health (20, 21). Additionally, albeit rare, *M. pneumoniae*

infections has been reported to lead to other complications such as encephalitis (22, 23) and pericarditis (24, 25). There is a close association of *M. pneumoniae* infection and acute asthma and Chronic Obstructive Pulmonary Disease (COPD) due to the strong Th2 response elicited by infection with this pathogen (26–28). Aside from the known pathologies of the respiratory tract, infection with *M. pneumoniae* has been linked to renal and gastrointestinal complications and hemolytic anemia (21). *M. pneumoniae* is one of only a few species of *Mycoplasma* that are known to produce a toxin. The *M. pneumoniae* gene, MPN_372, encodes the CARDS (Community-Acquired Respiratory Distress Sndrome) toxin, a ADP-ribosylating and vacuolating cytotoxin (29) that has been shown to bind human surfactant protein A and induce allergic-type inflammation in the airway (30).

Cytadherence is a critical initial step in the infection process, as it prevents clearance by the host. *M. pneumoniae* display a flask shape appearance with a complex tip structure, which is described as a slender protrusion from the cell body with an electron dense core(31). The cytoadhesion molecules must be localized to the tip structure to allow for successful attachment of the organism to host cells. Several proteins are found in the tip structure and are essential for full functionality of *M. pneumoniae* attachment including the primary cytoadhesion P1, proteins B and C, P30 and HMW1, 2, and 3 (32).

The tip structure of *M. genitalium* has been described as very similar to that of *M. pneumoniae* with MgPa (homologous to *M. pneumoniae* P1), the primary cytoadhesion molecule localized to the tip structure (3). *M. gallisepticum* has a similar tip structure where the primary cytoadhesion, GapA (and homolog to *M. pneumoniae* P1) and CrmA are essential for attachment. Pre-incubation of *M. gallisepticum* with Fab fragments reactive to GapA inhibited attachment of *M. gallisepticum* to lung fibroblasts, indicating that it is largely

responsible for cytodherence (33). In addition, an attenuated strain R (Rhigh) lacking both GapA and CrmA demonstrates a significant reduction in attachment (34). Interestingly, complementation of Rhigh with *gapA* did not restore cytodherence, but complementation of with both *gapA* and *crmA* resulted in the restoration of cytodherence to the level of virulent strain R, indicating that these two proteins collaborate in the process of cytodherence (34).

It has been reported that when mice are vaccinated with an avirulent *M. pneumoniae* P30 cytoadhesion mutant, subsequent challenge with a virulent strain surprisingly results in exacerbated disease than unvaccinated control mice (35). This is not the only report of vaccine induced disease exacerbation in experimentally infected rodents (36, 37). These unsuccessful vaccination studies highlight the need to better understand the basic mechanisms and pathogenesis in order to develop effective therapeutics and vaccines.

Mycoplasma genitalium, another well studied human pathogen, is associated urogenital complications in both males and females, commonly causing sexually transmitted non-chlamydial non-gonococcal urethritis in men and pelvic inflammatory disease in women (38–40). *M. genitalium* infections occur globally and, while prevalence rates vary by country and community, human infection has been detected in every country where it has been sought (41). Unfortunately, the exact mechanism and relationship of *M. genitalium* and human disease is still poorly understood. In addition, there is not a single uniform method for clinical detection of infection, creating a large gap in the understanding of how this pathogen truly impacts human health. Despite the availability of several qPCR detection kits, none are FDA approved for clinical/diagnostic use. Additionally, doxycycline, the typical first line antibiotic for urethritis, is not effective against this pathogen, and resistance to macrolides has recently been reported (41) emphasizing the need to further understand the pathogenesis

of this emerging pathogen. Recent technological advances, namely the Nucleic Acid Amplification Test (NAAT) allowing for rapid and accurate detection, has begun to allow scientists to perform more studies to start to elucidate the pathogenic mechanism employed by this emerging sexually transmitted infection (41).

Another infectious *Mycoplasma* species that is able to colonize the human urogenital tract is *M. penetrans* (38). This pathogen was the first identified invasive member of the *Mycoplasma* genus and has been reported to penetrate Eukaryotic cell membranes using its tip structure (42). A correlation was established between *M. penetrans* and HIV infection where the rate of *M. penetrans* infection was significantly higher in patients with HIV, suggesting they are potential co-pathogens (42). However, subsequent studies have reported that there is no distinct pathology associated with this infection and no compelling evidence to suggest that they act do as co-pathogens (43). Host immune evasion is believed to be achieved in this organism through the phase variation of the Mpl lipoproteins (44, 45), agglutination of red blood cells, lymphocytes and monocytes (42) and it has been demonstrated that *M. penetrans* can bind IgA, serving as a defense mechanism evading clearance by IgA specific antibodies (46).

M. hominis, another human pathogen that can inhabit the urogenital tract, is associated with bacterial vaginosis and have been linked to pre-term births (38). *M. hominis* induces the production of inflammatory cytokines and chemokines that are thought to be responsible for pre-term births and potential brain damage commonly observed in infections (47–49). A recently identified gene, *goiC*, appears to be associated with virulence, specifically related to the bacterial density in the amniotic fluid and pre-term births as

isolated mutant strains lacking this gene, show a significant decrease in bacterial density in the amniotic fluid and reduction in pre-term births (50).

Taken together, these data demonstrate the lack of complete understanding of the pathogenic mechanisms employed by these *Mycoplasma* species that infect humans and highlight the need for a more complete understanding of these organisms and their virulence factors.

***Mycoplasma* of Porcines**

Two main *Mycoplasma* species are known to infect swine and cause significant economic losses to farmers around the world. *M. hyopneumoniae* is the causative agent of porcine enzootic pneumonia, a highly contagious and infectious disease of swine. *M. hyopneumoniae* can contribute to the super infection known as the porcine respiratory disease complex (51). Recent genomic comparisons of a pathogenic strain with a high-passage attenuated strain identified genes and gene families that are important in the virulence of the organism, including cytoadhesins, cell envelope proteins (P95), cell surface antigens (P36), chaperone proteins (DnaK), and genes involved in metabolism (52). A significant portion of the pathogenesis can be attributed to the ability to attach to swine respiratory cells via the P97 cytoadhesion (53) and to bind host glycosaminoglycan heparin sulfate (54, 55).

M. hyorhinis, spread by respiratory secretions, can cause serofibrinous to fibrinopurulent polyserositis and arthritis in swine (56). Of note P37, a surface exposed lipoprotein that binds pyrophosphate (57) has been shown to induce tumor transformation of Eukaryotic cells (58–60). It has recently been reported that the P37 lipoprotein can transform

normal gastric cells to cancer cell through activation of the PI3-kinase-AKT signaling pathway, initially stimulated through the epidermal growth factor receptor (EGFR) (61).

Mycoplasmas of Bovines

The Select Agent, *Mycoplasma mycoides* subs. *mycoides* Small Colony (MMMSC) was the first *Mycoplasma* described by Nocard and Roux in 1898 (62) and is a highly contagious respiratory pathogen of cattle that can cause fatal pleuropneumonia (63). This organism is one of the few species of *mycoplasma* that causes mortality. MMMSC has been described as the single most important threat to the cattle industry in Africa and economically the most important mycoplasmal pathogen in the world (3). The United States has been free of this pathogen since 1893 (64), however re-emergences of this pathogen has been reported in Europe as recently as that late 19th century with confirmed cases in Portugal and Italy and sporadic outbreaks in France and Spain (3). Currently, there are commercially available live attenuated vaccines, however they provide inadequate protection, require annual boosting, and can revert to virulence, demonstrating the need for improved vaccines (65). It has been speculated that MMMSC can evade the host immune system via the phase variable Vmm lipoproteins, a trait shared with many other *mycoplasma* species (66). Of interest and related to the virulence of MMMSC, is the ability to ferment glycerol via the *glpO* gene (67) resulting in the production of hydrogen peroxide (68, 69) a reactive oxygen species, that is known to cause cellular damage to host Eukaryotic cells.

M. bovis, another *mycoplasma* pathogen of cattle, has been reported to cause mastitis, (70) pneumonia, (71, 72) and contributes to Bovine Respiratory Disease Complex (73) which can lead to broncopneumonia. *M. bovis* has been reported to evade host immune responses

though the phase and antigenic variation of the surface exposed *vsp* genes. Of significance is the membrane associated polysaccharide is an inflammatory toxin that causes mastitis in cattle when inoculated into the udder (74).

Mycoplasmas of Caprines

M. capricolum subs. *capripneumoniae*, formally known as the F-38 group of *mycoplasma*, is a significant pathogen of goats and the causative agent of Contagious caprine pleuropneumonia a frequently fatal disease. It has also been reported to cause mastitis and arthritis (75). This Select Agent is particularly a problem in Africa and Asia, causing devastating economic losses (3). Little is understood about the pathogenesis and virulence mechanisms of this organism, but genomic comparisons of the highly virulent and frequently fatal *M. capricolum* subs. *capripneumoniae* and *M. capricolum* subs *capricolum* show that the *lppA* lipoprotein gene is truncated in *M. capricolum* subs. *capricolum*, suggesting it may be involved in the virulence and pathogenesis (76).

M. agalactiae causes mastitis resulting in the marked decrease of milk production in small ruminants, causing a significant economic impact (77). The organism is often isolated from milk samples of infected animals and can be a significant contaminant in milk storage tanks (78). *M. agalactiae* can be vertically transmitted from doe to kid through direct contact, during suckling (77). Virulence determinants of this pathogen include biofilm formation (79), hydrogen peroxide production (80), and a putative cystine desulfurase, *nifS* which is essential for growth in Eukaryotic co-culture (81).

Mycoplasmas of Rodents

M. pulmonis, a respiratory pathogen of mice causes a disease similar to atypical pneumonia in humans, and can induce allergic airway disease and airway hyper-reactivity (82), making this organism an excellent model to study the role of both the innate and the adaptive immune response in the respiratory tract (3). Virulence of this organism is related to immune evasion via phase variation of the *vsa* variable lipoproteins (83), which also have been report to be associated with biofilm formation(84). Additionally, *M. pulmonis* produces a hemolysin, nuclease, and a glycoprotease which is of particular interest, as hydrogen peroxide production has been linked to virulence in other *mycoplasma* species (85).

M. arthritidis has been linked to polyarthritis in rats and chronic proliferative arthritis in mice, but has also been reported to have been isolated from animals without arthritis (3, 86). *M. arthritidis* hydrolyzes arginine to produce energy (86) and contains multiple variable lipoproteins speculated to be involved in immune evasion (87). Of particular interest, is the superantigen, *M. arthritidis* T-cell Mitogen (MAM) (88) which acts by binding the alpha chain of the MHC II molecule on B cells and the variable Beta chains on T helpers cells, thereby connecting them (89–91). This linkage results in lymphocyte activation in an antigen independent fashion leading to the uncontrolled production of inflammatory cytokines and polyclonal antibodies (92, 93).

Mycoplasmas of Felines

M. felis was originally isolated from a cat with severe conjunctivitis (94) and has seen been linked with pneumonia (95). Interestingly, it has also been reported to cause pleuritis

(96) and lower respiratory tract diseases in horses (97). *M. gateae* has been reported to cause erosive polyarthritis and synovitis in felines (98, 99).

Mycoplasma of Canines

There have been 15 species of *mycoplasma* isolated from or detected in dogs, (100) however their roles in respiratory infections is not well understood. They are thought to be part of the normal flora of the upper respiratory tract but have been observed colonizing the lower respiratory tract during infections such as pneumonia (101). *M. edwardii* and *M. spumans* have been associated with respiratory diseases in dogs. *M. cynos* has been reported to cause respiratory disease in young dogs and has been associated with canine infectious respiratory disease (CIRD) (100) and *M. canis* causes urinary tract infections and can lead to infertility (102). These two pathogens in addition to *M. molare*, secrete sialidase, a feature unique to these species in the genera, as other *mycoplasma* species have membrane bound sialidase (103). Sialidase has been associated with virulence in other species, such as *M. gallisepticum*, where a transposon insertion mutation in the sialidase gene demonstrated reduced lesions and recovery in experimentally infected animals (104). Sialidase has also been associated with virulence in *M. synoviae* where it was reported that those strains demonstrating higher virulence exhibited significantly higher levels of sialidase activity suggesting an enzymatic basis for virulence (105). However, to date, the role of secreted sialidase in the virulence of these canine pathogens still remains unknown.

Mycoplasmas of Reptiles and Piscines

In 1995, an epidemic of acute multisystemic inflammatory disease was discovered in captive alligators in Florida. Four years later it was confirmed that the etiologic agent was the flesh-eating bacterium, *M. alligatoris* (106). Infection results in interstitial or fibronectin pneumonia with fibrinous polyserositis and multifocal arthritis (107). Identified potential virulence factors in this organism include hyaluronidase and sialidase which have been shown to induce the CD-95 signaling pathway, resulting in apoptosis of Eukaryotic cells (108).

The closely related *M. crocodyli* (98% 16s rRNA similarity) shows a comparatively reduced virulence in crocodiles, which could be due, at least in part, to the lack of sialidase (109). It has been reported that while *M. alligatoris* contains both hyaluronidase and sialidase, genome comparisons suggest that *M. crocodyli* contains only hyaluronidase and lacks sialidase (109). This further emphasizes the link between the enzymatic activity of sialidase in *mycoplasmas* and virulence. Another *mycoplasma* pathogen of reptiles, *M. agassizii*, emerged during the late 1900's causing rhinitis with palpebral edema and serous nasal and ocular discharge in the desert tortoise (110, 111).

The only known *mycoplasma* species to infect fish, *M. mobile*, can cause severe necrosis of the gills of infected fish (112, 113). Interestingly, this species is the fastest motile *mycoplasma* and is considered to be the model organism for gliding motility in which *M. mobile*, and other motile *mycoplasma* species, are able to glide on solid surfaces without containing any flagella, pili, or gene homologs to any known motility systems. *M. mobile* is able to move by gliding motility and is explained through a model termed the 'centipede model' (114). During motility, the tip structure protein Gil349 attaches to sialic acid residues

on host cells and ATP is hydrolyzed, providing the necessary energy. This causes a conformational change, which physically moves the mycoplasma forward. The next sialic acid is released and the process is repeated (114).

Mycoplasmas of Avians

M. synoviae has been implicated in infectious synovitis, respiratory lesions, and airsacculitis in poultry (115). Virulence determinants likely include immune evasion via the phase variable *vlhA* lipoproteins (116) and sialidase activity (104). However, reliable methods for specific gene mutations have not yet been developed for *M. synoviae*, making it difficult to directly assess how a specific gene is related to the virulence of the organism. Despite these potential virulence factors, the disease is well controlled in poultry with the use of a live attenuated vaccine (117–119).

M. iowae has been reported to cause airsacculitis, embryo mortality, and physical abnormalities in infected poultry (115). It is resistant to bile salts (120) and commonly isolated from the digestive tract of infected embryos (121). Interestingly, *M. iowae* is able to both ferment glucose and hydrolyze arginine, two processes that are usually not seen together in an individual *mycoplasma* species (122).

Several other avian *mycoplasma* species have been identified including *M. gallinarium*, which has been reported to cause mild respiratory signs in geese when concurrently infected with parvovirus, *M. columbinasale* which causes rhinitis and pharyngitis in pigeons, and *M. anseris* which has been reported to cause lesions in geese in conjunction with *M. cloacale*. Additionally *M. meleagridis* has been reported to cause sinusitis, airsacculitis, and infection of the genital tract of turkeys (123).

Spiroplasmas and Phytoplasmas

Spiroplasmas and *Phytoplasmas*, belonging to the class *Mollicutes*, are mostly plant pathogens that have a dual host life cycle dependent on sap-feeding insects for transmission between plant hosts. The sap-feeding insect vectors, including planthoppers, leafhoppers, and psyllids, acquire the bacterium by ingesting the phloem of infected plants (124). Upon ingestion, the bacteria reach the mid-gut lumen and must traverse the gut epithelial cell layer, by aligning the tip structure between the microvilli, to reach the circulatory system. To be transmitted to the new plant host, the organism must reach the salivary glands and become incorporated into the salivary secretions of the insect host (124). Little is understood about the molecular mechanisms of pathogenesis in these plants pathogens as they are un-cultivable in axenic media, but it has been demonstrated that this dual host lifestyle requires the organism to successfully pass many physical barriers and requires several specific protein-protein interactions between the bacterium and insect host.

These pathogens can infect a wide range of plants (3, 124–126) causing a significant economic impact around the world and have been linked to horseradish brittle root disease, citrus stubborn disease, and grass stunt disease among others (125, 127, 128). The Aster Yellows phytoplasma strain Witches' Broom infection leads to phyllody (conversion of flowers to leaf-like structures), virescence (greening of floral organs), and Witches' Broom (increased proliferation of stems). These changes are particularly beneficial for the bacteria, as they make the plants more attractive to the insect vectors, enhancing the spread of the bacteria (124, 126, 128). While little is understood about the pathogenic mechanisms of these organisms, it has been reported that the Aster Yellows phytoplasma strain Witches' Broom is

able to release effector proteins inside the phloem of the plant, including the recently identified Sap54, which has been linked to the observed pathologies. This novel virulence factor functions by inhibiting floral development by promoting the degradation of the plant MADS transcription factors which have an essential role in flower development (124).

Section 3: *Mycoplasma gallisepticum*

Disease and Significance

Mycoplasma gallisepticum is largely a respiratory and reproductive pathogen of poultry. Infection with virulent strains, such as strain Rlow, lead to clinical manifestations including tracheal lesions, airsacculitis, inflammation, metaplasia, loss of cilia, and thickening of the tracheal mucosa (129). Early lesions are typically composed of heterophils and macrophages, while later lesions contain large numbers of lymphocytes with high proportions of T-cells (130). The clinical impacts of this include coughing, sneezing, respiratory rales, and nasal and ocular discharge (131). If this pathogen is not cleared by the host, it can cause a chronic disease state (2).

M. gallisepticum contributes to Chronic Respiratory Disease (CRD), a polymicrobial syndrome largely affecting the respiratory tract, yet also affecting other organ systems, such as the reproductive tract (132, 133). Concurrent infections with Avian Influenza virus (AIV) (134), Newcastle disease virus, infectious bronchitis virus (135), pathogenic *E. coli*, *Haemophilus*, and avian rhinotracheitis virus (136) have been reported to enhance clinical signs associated with CRD. The exact sequence and pathogenesis of co-infecting organisms

and CRD is poorly understood at this time. However, Sid et al., recently reported that co-infection with *M. gallisepticum* and AIV promoted bacterial growth, inhibited antiviral gene expression and affected AIV attachment to the host cell by desialylation of α -2,3 linked sialic acids (134), beginning to elucidate the biological mechanisms involved in co-infection.

M. gallisepticum is transmitted throughout the flock via horizontal transmission from inhalation of respiratory droplets from infected birds or through conjunctival exposure (136, 137). Additionally, *M. gallisepticum* can spread via vertical transmission from the layer to the egg (138).

Infection does not usually result in mortality, especially in older chickens, but the morbidity has significant adverse effects to poultry industries around the world due to the necessity to cull positive flocks and condemn and downgrade carcasses, decreased egg production and hatchability, decreased egg quality, and a substantial reduction in weight gain (139, 140). The economic losses to the poultry industry are substantial as it is estimated to be \$588 million per year lost in broiler industries alone in the United States with an additional \$132 million lost from the egg laying industry (115, 141, 142).

Because of this and other significant poultry diseases, the United States Federal Government implemented the National Poultry Improvement Plan (NPIP) in the 1960's, a collaboration between both state and federal departments of agriculture to use new technology to improve poultry and poultry products throughout the United States by establishing guidelines for control of *M. gallisepticum* (and other significant poultry pathogens such as *M. synoviae*, *Salmonella*, and avian influenza) in primary breeder flocks (143). Because of the severe economic impact, rapid spread, and chronicity of this infection, *M. gallisepticum* was determined to be one of the top 3 most significant avian pathogens to

U. S. poultry producers at the USDA ARS/CSREES National Animal Health Program Planning Workshop (144).

The current strategies employed by commercial farms include strict biosecurity measures, antibiotic therapy, and vaccination programs to attempt to prevent and/or control disease. However, medications cannot effectively be used to eliminate infection from an entire flock and therefore is not an adequate long-term solution. In severe cases, culling of *M. gallisepticum* positive flock is also used to control infection and spread of this important disease (145).

M. gallisepticum has been shown to cause significant pathology in other species causing infectious sinusitis in turkeys and conjunctivitis in house finches (2, 146) which could promote the spreading, especially between back-yard flocks. Biosecurity measures have been put in place on many farms to prevent such spread (147). Of particular interest is the emergence of a new strain of *M. gallisepticum* that was identified in house finch (*Carpodacus mexicanus*) populations in the eastern United States in 1994 (146). *M. gallisepticum* has also been isolated from many other species of passerine birds such as purple finches, blue jays, and American goldfinches (148–150). This *M. gallisepticum* strain was shown to be genotypically different from the poultry strains by Random Amplification of Polymorphic DNA (RAPD) and Amplified-fragment length polymorphism (AFLP) analysis (151) and caused severe conjunctivitis in the house finches (149). Interestingly, only minimal to mild disease was displayed when these house finch *M. gallisepticum* strains were used to experimentally infect poultry (152).

However, over the course of the epizootic event, the house finch-related *M. gallisepticum* strains demonstrated increased virulence in the house finch host, but a

significant reduction in virulence in experimentally challenged poultry, indicating possible reciprocal evolution of house-finch related *M. gallisepticum* virulence in house finch and poultry hosts (S. Geary, unpublished data). Of note, is a recent study comparing the genomic changes in the *M. gallisepticum* house finch strains between the initial index case in 1994 and seven other isolates separated both spatially and temporally (153). The data indicated that the most dramatic genomic differences were observed in the phase-variable lipoprotein hemagglutinin genes *vlhA* in both presence and genomic location, suggesting a potentially important role of these genes in the virulence of this pathogen (153)

Current *M. gallisepticum* Vaccines

Over the years, several attempts have been tried to develop an efficacious and safe vaccine to protect poultry from *M. gallisepticum* infection. To date, two types of commercially available vaccines exist, bacterins and live attenuated vaccines. Bacterins, which are inactivated suspensions of whole organisms, initially seemed promising in the field as vaccinated and challenged birds showed a lack of clinical signs and reduced air sac lesions upon gross examinations (154). Furthermore, it was demonstrated that the use of bacterins as vaccines reduced the decline in egg production associated with *M. gallisepticum* infection (154–157) and prevented vertical transmission (158). Unfortunately, later reports showed that the reduction in bacterial load was only minimal, at best (159) and microscopic lesions, while less severe, were still present (160). Furthermore, the use of bacterins did not protect against infection with heterologous *M. gallisepticum* strains (154–157, 161).

Meanwhile the live attenuated vaccines were being evaluated and compared to the bacterins, showing potentially more promising results. The live F strain vaccine derived from a naturally attenuated strain, was first identified in 1956 (162). Since it was first identified, several safety and efficacy studies have been conducted in both chickens and other poultry. In chickens, the live F strain vaccine elicits a strong serological response and, in fact, that response is the strongest of any commercially available *M. gallisepticum* vaccine (163). That serum antibody response, however, is not comparable in magnitude to that of a chicken challenged with pathogenic *M. gallisepticum* strain Rlow (164). Vaccination with live F strain provides good protection for layers while inducing no lesions (141, 165, 166). This vaccine strain is able to displace the virulent R strain from infected flocks (167) which has been suggested to a possible mechanism of protection as the F strain out-competes the virulent strains in the airway, reducing the bacterial load (168). Of note, the F strain vaccine has been demonstrated to be readily differentiated from field strains through the use of Western blotting and RAPD fingerprinting (169, 170). Most importantly, vaccination with the live F strain prevented the development of lesions and disease in birds when vaccinated birds were subsequently experimentally challenged with virulent strain Rlow (139, 171, 172). However, the live F strain vaccine was not optimal in all conditions. Vaccination caused severe lesions and disease in turkeys and young chickens (173) and demonstrated the ability to pass vertically to the egg (174). In addition, vaccination with the live F strain has been linked to adverse effects in the reproductive performance in layers, causing a reduction in total egg production due to a delay in lay onset (165, 175, 175). Furthermore, transmission to naïve poultry has been reported (176) which is of concern to farmers, as chickens and turkeys

are often kept in close proximity, creating the possibility of transmission from a vaccinated chicken (177).

Despite the negative impact the F strain vaccine has on the layers, it has been reported that F strain vaccinated birds that have been subsequently challenged with a virulent Rlow strain, have a higher and more successful laying rate and display less vertical transmission than their unvaccinated counterparts (141, 161). Despite the obvious safety concerns surrounding the live F strain vaccine, it still elicits a strong antibody response and provides complete protection against challenge, fairing far better than any other commercially available vaccine (163).

Another live attenuated vaccine, the ts-11, a temperature sensitive mutant has been generated through chemical mutagenesis (178). This strain, which is administered via eye drop displayed a temperature sensitive phenotype with a maximum growth temperature of 33°C, well below the body temperature range of the chicken (40.6° to 41.7°C) and is unable to permanently colonize the lower respiratory tract. The ts-11 strain is, however, able to colonize the upper respiratory tract of the chickens, where the temperature is lower, and stimulate a protective immune response. ts-11 has been demonstrated to be safe in both chickens and turkeys as it did not cause clinical signs or disease. Minimal lesions were observed in less than 10% of animals and egg production remained normal (179–181). However, while a safer option, ts-11 does not always provide protection from challenge and the level of protection is variable (180, 181). When assessed, it was determined that the magnitude of the antibody response was very low, which was likely the key reason for the inconsistent protection reported (180, 182, 183). The ts-11 strain has been shown to persist in vaccinated animals for several months (179) with limited horizontal transmission (183) and

no reported vertical transmission to eggs (184). The egg quality is not adversely effected by vaccination (185) and in fact layers demonstrate an increase in egg production when vaccinated and subsequently challenged, compared to their unvaccinated counterparts (184). While the ts-11 strain is unable to displace the virulent Rlow strain in infected birds, it has been reported to be able to displace the F strain (186). Even though the live attenuated, temperature sensitive ts-11 vaccine strain may not be able to provide as much protection as the live F strain vaccine, it is the safer option, especially for turkeys and young chicks.

The final commercially available, live attenuated vaccine is the 6/85 vaccine which was generated though serial passage *in-vitro* (163, 179). This vaccine strain was found to be safe in both chickens and turkeys demonstrating the formation of only few mild lesions in a small percentage of experimental animals (187) with no effect on egg production or egg quality (188). However, the efficacy of the vaccine in birds challenged with a virulent strain is variable and weak (187) and elicits minimal to no detectable serum antibody response (163). The vaccine strain is unable to prevent the spread of the challenge strain from vaccinated animals or displace virulent strains from infected birds (186, 189). More concerning is the report of 6/85-like strains being recovered from un-vaccinated animals, suggesting the possibility of reversion of this vaccine to virulence (190). However, this reversion has not yet been successfully reproduced experimentally (191) and the isolated strain was unable to produce disease in experimentally infected animals (190).

The availability of efficacious and safe vaccines that can be administered to an entire flock of animals is significantly lacking at this point in time. On one side there is the live F strain vaccine, which elicits high antibody titers and provides sufficient protection, but is virulent in young chickens and turkeys. On the other side, there is the ts-11 and 6/85 vaccine

strains which are much safer and cause mild to no adverse effects, but do not provide sufficient protection to the flock against virulent *M. gallisepticum* strains. This demonstrates the need for the development of a single efficacious and safe vaccine to protect poultry from *M. gallisepticum* infections.

The Center of Excellence for Vaccine Research (CEVR) at Uconn has produced two live attenuated vaccine strains to protect chickens from infection. One of the vaccines was created by complementing an avirulent strain with a cytoadhesin molecule, while the other strain was developed by disrupting a known virulence factor in a virulent strain. The first live attenuated vaccine was created by complementing the avirulent Rhigh strain with the cytoadhesin gene *gapA*. This resulting strain was termed GT5. It was demonstrated that GT5 was safe at high doses and prevented lesions when vaccinated birds were challenged with virulent strain, Rlow. The same study reported increased production of *M. gallisepticum* specific IgG but surprisingly not IgA (192). It was reported that lymphocytes were arranged in organized follicles and both *M. gallisepticum* specific IgA and IgG mucosal plasma cells were produced as early as four days post-vaccination (193).

Mg7 was the second live attenuated vaccine developed by CEVR. It was created by disrupting the metabolic gene *lpd* via transposon mutagenesis in the virulent strain, Rlow. This vaccine displayed complete attenuation in experimentally infected animals (194). Much like GT5, this vaccine strain was safe at high doses and prevented colonization and therefore lesion formation when challenged with the virulent Rlow strain. Similarly these vaccinated birds produced increased amount of *M. gallisepticum* specific IgG (168).

Unfortunately these live attenuated vaccine strains were created using transposon insertions, containing a gentamicin selectable marker, making them unsuitable for

commercial production in their current forms. Despite that, both vaccines have passed the “proof-of-concept” stage of development and are superior in performance to ts-11, 6/85 and the F in laboratory-controlled studies (168). Geary et. al. believe that the need to develop a rationally designed, safe, and efficacious vaccine still exists and that further knowledge of the pathogenic mechanisms employed by this organism are necessary to accomplish that goal.

***M. gallisepticum* Virulence Factors**

Adequate understanding regarding the mechanisms of pathogenesis of *M. gallisepticum* are lacking, but significant strides have been made in elucidating the genes and pathways related to virulence, especially after the complete genome was sequenced (195) and compared to Rhigh, an avirulent high passage mutant (196). There were several genes identified as absent in Rhigh, including the major cytoadhesin *gapA* and the gene encoding the cytoadhesin associated molecule CrmA (196). An essential first step in colonization of any pathogen is cytoadhesion as it prevents mechanical clearance by the host. Despite some differences, GapA and CrmA are homologs of the well characterized P1 and B/C adhesion molecules of *M. pneumonia* (33, 34, 197) . As previously discussed, complementation of Rhigh with *gapA* or *crmA* alone did not restore cytoadherence or virulence of the organism to wild-type levels. However, complementing with both *gapA* and *crmA* did in fact restore both cytoadherence and virulence of the organism (34). Additionally, it has been demonstrated that transposon insertion mutations in the virulent Rlow *gapA* and *crmA* genes abolish the

cytadherence capabilities and significantly diminish the virulence of the organism in the natural host (198) further linking these two genes to virulence of *M. gallisepticum*.

Two additional genes that were found to be lacking from the avirulent Rhhigh strain were the genes responsible for fibronectin binding, *hlp3* and *plpA*(196). These products were identified when the peptides that exhibited similarity to known fibronectin binding proteins were shown to bind the gelatin/heparin-binding domain of fibronectin (199). PlpA demonstrated homology to the well-characterized cytoadhesin associated protein P65 of *M. pneumoniae* (*pneumoniae*-like protein A) and Hlp3 demonstrated homology to the cytoskeletal protein of *M. pneumoniae*, HMW3 (HMW3-like protein) (199).

Virulence has been linked to proteins involved in metabolism in many pathogens (67, 68). The metabolic protein, dihydrolipoamide dehydrogenase (*lpd*) is a part of the pyruvate dehydrogenase complex pathway, a pathway responsible for the production of ATP during glycolysis. When a transposon mutant was generated via an insertion in the *lpd* gene of *M. gallisepticum*, the organism was completely attenuated and was no longer able to be recovered from experimentally infected birds (194). This attenuation was likely the result of the inability of the organism to produce sufficient amounts of ATP and therefore energy for survival in the host (194).

Another metabolism-related gene, *malF* has been reported to be essential for the persistence of *M. gallisepticum* in experimentally infected birds. When a transposon mutation was made in this predicted ABC sugar transport permease gene, the organism was unable to be recovered from experimentally infected bird or cause disease, suggesting its necessity for persistence (200).

MslA is an immunogenic mycoplasma-specific lipoprotein that exhibits reduced expression in the live attenuated F strain vaccine (201). When a transposon mutant disrupting *msla* was created, the organism showed reduced recovery and attenuated virulence in experimentally infected chickens (201). Since homologs of this lipoprotein are only found in *mycoplasma* species, it was coined *mycoplasma*-specific lipoprotein A (*msla*). While its relationship to the virulence of *M. gallisepticum* has been demonstrated, the exact role it plays is not yet completely understood. However, it has been hypothesized that MslA binds Toll-like receptors (TLRs) which in turn activate NF- κ B, leading to the production of cytokines and chemokines that result in inflammation (201).

The *M. gallisepticum* gene, MGA_0329, a homolog of sialidase in *M. pneumoniae* has been linked to virulence in *M. gallisepticum*. A transposon mutant disrupting the gene, resulted in loss of sialidase activity and when used to experimentally infect chickens, showed significantly less severe tracheal lesions and mucosal thickening in addition to a significant reduction in bacterial recovery (104). However, complementation restored wild-type levels of sialidase activity, but did not restore the virulence. This was surprising as sialidase production has been shown to correlate with virulence in the closely related, *M. synoviae* (105) as strains with increasing levels of sialidase activity showed increased virulence *in-vivo* while those strains with less sialidase activity produced mild to no respiratory lesions in chickens (105). It has been hypothesized that since sialylation protects against the hydrolysis of glycosidic bonds on the host cell surface and degradation of the host cell extra-cellular matrix, removal of these sialic acid residues can expose new host antigens and play a role in autoimmune complications of infections (202). These data suggest that the link between

sialidase activity and virulence is more complicated than originally thought in *M. gallisepticum*, and not completely understood at this time.

Several *Mycoplasma* species (*M. bovis* for example) have the ability to ferment glycerol and produce hydrogen peroxide, a potent reactive oxygen species known to cause cytotoxicity in Eukaryotic cells (67, 68). Surprisingly, when transposon mutants were created in the *M. gallisepticum* *glpO*, *glpK*, and *glpF* genes of the glycerol transport and utilization pathway, the organism still retained virulence as evidenced by typical lesions in the tracheas of infected chickens, suggesting glycerol metabolism and hydrogen peroxide production is likely not a virulence factor in *M. gallisepticum*, as previously hypothesized. These data demonstrate that the function of this pathway is not yet understood in *M. gallisepticum*.

Potentially tied to the virulence of *M. gallisepticum* is the phase-variable lipoprotein hemagglutinin gene family (*vlhA*) (153). This gene family, further discussed in detail below, has been reported to display significant genomic changes in size and location between strains with varying levels of virulence suggesting they may play a role in the virulence of *M. gallisepticum* (153).

Section 4: Phase and Antigenic Variation in *Mycoplasma*

Mycoplasma species have demonstrated the ability to establish chronic infection in a wide range of host species, even when faced with strong immune pressure. This success may be due, at least in part, to the ability of these organisms to rapidly change the expression and size and even the structure of their surface exposed proteins. These events lead to a dynamic

and plastic cell membrane allowing for rapid adaptations to the environment, enabling the bacteria to escape host immune pressures and enhance host interactions providing the bacteria with optimal chances for survival (203).

Phenotypic variation encompasses both phase (the on/off expression of a given antigen) and antigenic variations (the expression of alternate forms of a given antigen)(203). At least four molecular strategies have been described in *mycoplasma* species that affect the expressed antigens. They include (1) on/off switching via DNA slippage and point mutations, (2) surface antigenic variation via DNA rearrangement (site-specific DNA inversions), (3) domain shuffling via homologous recombination, and (4) epitope masking and unmasking (203).

DNA slippage

Among *mycoplasmas*, the first antigenic variation mechanism described was the *vlp* genes of the porcine pathogen, *M. hyorhinis*. These genes are control by a molecular switch involving DNA slippage (slipstrand mutations) in the promoter region (204–206). The *vlp* gene family contains 3-8 genes designated A-G, each representing a single transcription unit with a poly-A tract in the promoter region (207, 208). Elongation or contraction of this polyA tract by a single nucleotide is sufficient to abolish transcription (206). The change in length causes structural alternations in the DNA curvature, no longer presenting an optimal conformation for transcription initiation (206). These genes also demonstrate size variation through intragenic expansion and contraction of tandem repeat regions, wherein it has been reported that variant cells expressing a long *vlp* appear to be resistant to antibody mediated growth inhibition, while the short versions are susceptible (209).

A similar mechanism has been reported for the phase-variable cytoadhesin gene, *maa2* in *M. arthritidis* where the gene is only expressed when it is preceded by exactly 16 thymine residues (210). Similarly, phase variation of the *vmm* gene of *M. mycoides* subs. *mycoides* SC and the *vmc* of *M. capricolum* subs. *capricolum* are under the control of reversible promoter mutations (66, 211). These genes are expressed when the poly-TA tract contains exactly 10 di-nucleotide TA repeats (66, 211). Additionally, the expression of the *vaa* gene of *M. hominis* depends on the insertion/deletion of adenine residues in the poly-A tract, where mutations result in the production of an in-frame stop codon and truncated protein (212).

M. gallisepticum contains the phase-variable lipoprotein hemagglutinin gene family (*vlhA*, previously referred to as pMGA) which consists of 43 closely related genes distributed across 5 loci, totaling just over 10% of the genome in strain Rlow (195). Previous studies initially indicated that only one *vlhA* gene was expressed at any one time, however subsequent studies have shown that more than one gene can be expressed simultaneously in a population. (213, 214). *vlhA* expression has been reported to be dependent on the presence of exactly 12 GAA tri-nucleotide repeats upstream of the expressed gene and is believed to change via slip-stranded mutations in the promoter regions (117, 214–216). It has also been hypothesized that *vlhA* gene expression is stochastic, driven by anti-VlhA mediated negative selection and resulting in random outgrowth populations (117). The exact function of the VlhAs are still unknown, but has been speculated to involve immune evasion during the infection process (217) and variation has been loosely associated with virulence in other *M. gallisepticum* strains (153). Additionally it has been suggested that the ability of the gene products to bind red blood cells could be a factor to aid the dissemination of the organism through the infected host (218). Reports have also suggested that VlhAs may function in a

weak initial attachment of *M. gallisepticum* to target host cells. Despite these data, with recent technology a current study, described in detail in chapter 2, suggests that another mechanism is, at least in part, responsible for the control of *vlhA* gene expression (219).

DNA rearrangement

Many *mycoplasma* species can achieve phase variation by DNA inversion via site specific recombination, which is processed by a specific recombination enzyme. This ‘cut-and-paste’ process uses two specific and inverted DNA sequences to alternate silent genes behind a functional promoter (203). There are three well described systems where a single gene is placed behind a functional promoter while all other genes are silent and they include the *vsa* gene family of *M. pulmonis* (220, 221), the *vsp* genes family of *M. bovis* (222, 223) and the *vpma* gene family of *M. agalactiae* (224, 225). All of these bacteria have a site-specific recombinase that recognizes a specific short DNA sequence as a target for rearrangement lying adjacent to the phase-variable locus (226).

Homologous recombination

The third type of genetic mechanisms that has been described is homologous recombination which can be non-reciprocal, reciprocal, or intra-chromosomal (203). Non-reciprocal recombination or gene conversion has been demonstrated in the phase-variable *vlhA* gene family of *M. synoviae* and the MgPa encoding gene of *M. genitalium* while reciprocal recombination has been reported in the MgPar elements of *M. genitalium* (227–231). The *vlhA* family of *M. synoviae* consists of a single transcriptionally active unit and multiple homologous pseudogenes, lacking a coding sequence, located 5’ to the *vlhA* gene.

Antigenic variation occurs from unidirectional recombination between the donor pseudogene and the functional *vlhA*, resulting in the duplication of the pseudogene and total loss of the previously expressed gene. (227, 232). Intrachromosomal recombination has been reported for the *vsp* locus of *M. bovis*, which results in the generation of a chimeric variable lipoprotein (223). Each potential *vsp* is preceded by a homologous sequence that serves as the region for site specific DNA inversion (223).

Epitope masking and unmasking

The fourth mechanism of phenotypic variation, epitope masking and un-masking demonstrates how a constitutively expressed gene could contribute to the plasticity of the cell surface (203). This process involved one constitutively expressed antigen and a phase variable counterpart which may not actually be the target of the host immune response. During this process, the phase variable, surface exposed component can physically mask another surface antigen. This has been described for the P56 and P120 proteins of *M. hominis* where the phase variable P120 is able to mask the constitutively expressed P56 (203).

This process has also been described in detail for the previously discussed phase variable *vlp* genes of *M. hyorhinae*. The products of three genes, *vlpA*, *B*, and *C* are subjected to size variation through the intragenic expansion and/or contraction of tandem repeat regions, resulting in long and short variation of *M. hyorhinae*. Those expressing the long variants are completely resistant to immune serum antibodies where the short version variants are not. The target of the serum antibodies are not the *vlp* genes, but rather another neighboring surface exposed molecule that is masked by the expression of the long *vlp* variant and exposed when the short *vlp* variant is expressed (209).

These changes, regardless of the specific mechanisms, allow for the rapid adaptations to the changing environment, helping the organisms evade host immune responses and express the most advantageous suite of genes to best persist in the current environment.

Section 5: *Mycoplasma* Transcriptomics

In order to further elucidate the pathogenic mechanisms employed by these infectious *mycoplasma* species, there has been significant effort in the field to understand the transcriptomics of the organism, both *in-vitro* and *in-vivo*. The lack of obvious homologs of traditional bacterial transcriptional regulators and signaling factors had initially led to the thought that mycoplasmas were unable to regulate transcription by conventional mechanisms. This absence, combined with the single identified sigma factor, has led to the supposition that differences in gene expression in mycoplasma species are due to population selection and heterogeneity rather than more traditional mechanisms.

Using microarray analysis, it has been demonstrated that the human pathogen, *M. pneumoniae* up-regulates the expression of 47 genes, including the conserved heat shock genes *dnaK*, *ionA*, and *clpB* when the organism was incubated in heat shock conditions (43°C) for 15 minutes (233). It has also been reported that the porcine pathogen, *M. hyopneumoniae* differentially expresses 27 different genes when the organism is exposed to low iron growth conditions (234) and undergoes significant transcriptional changes in response to growth in heat shock conditions and in the presence of oxidative stress (235,

236). Of particular importance, was a study conducted by Cecchini et al (237) where RNA was extracted from *M. gallisepticum* cells co-cultured with MRC-5 human lung fibroblast cells for one hour. In comparison to control cells, *M. gallisepticum* displayed 58 genes that were differentially expressed when interacting with the human lung cells. The incubation time of this study was only one hour, a timeframe less than that of the generation time of a *mycoplasma* cell, suggesting that the observed changes in gene expression were, in fact, a result of exposure to lung cells and not a result of the outgrowth of a subpopulation.

In a comprehensive RNA sequencing analysis, using a combination of spotted arrays, strand-specific tiling arrays, and transcript sequencing, looking at differential gene expression in *M. pneumoniae*, Guell et al reported differential gene expression in a wide variety of conditions including increased temperature, varying pH, minimal media, and antibody pressure (238). Of interest, it was reported that there were frequent antisense and alternate transcripts and multiple regulators per gene indicating a very dynamic and complex transcriptome that is similar to conventional bacteria such as *Escherichia coli* and *Bacillus subtilis*. (238).

Additional transcriptomic studies in *mycoplasma* species have found heat shock proteins up-regulated in *M. gallisepticum* upon exposure to increased temperatures (239), up-regulation of genes important in the virulence (*cysP*, *nanH*, *vlhA*, and a nuclease) of *M. synoviae* upon co-incubation with chicken chondrocytes (240), and differential expression of lipoproteins and nutrient acquisition genes in *M. hominis* upon exposure to human dendritic cells (241).

A recent study was conducted by Ron et. al., to identify candidate target proteins associated with infection by using *in-vivo* induced antigen technology (IVIAT). This study

showed that there were 13 proteins preferentially expressed during *in-vivo* infection including previously identified virulence factors, GapA, PlpA, Hlp3, VlhA 1.07, and VlhA 4.01. In addition it was also found that several transport and translation related proteins and the chaperone protein GroEL were also preferentially expressed in an *in-vivo* infection (242).

Collectively these data demonstrate that *mycoplasma* species do, in fact, have the ability to alter their transcription in response to external stimuli and may undergo significant changes, including the up-regulation of virulence related genes, in response to changes in the environment and exposure to potential host cells.

Hypothesis and Specific Aims

Hypothesis

The phase-variable *vlhA* gene expression profile of *M. gallisepticum* will change over the course of early infection in the natural host in a coordinated, non-random manner.

Specific Aims

Specific aim 1: Investigate and characterize the *vlhA* gene expression profile of *M. gallisepticum* before and during the course of early infection in the natural host.

Specific aim 2: Determine the changes in the *vlhA* gene expression profile in *M. gallisepticum* recovered directly from the trachea mucosa of infected chickens and serially passed *in-vitro* in a nutritive medium. The second goal of this aim will be to determine the necessity of exactly 12 GAA trinucleotide repeats for the expression of any given *vlhA*.

Specific aim 3: Determine and characterize the *M. gallisepticum vlhA* transcriptional profile over the course of early infection when expressing a different initial predominant *vlhA* at time of challenge.

CHAPTER 2:

Global changes in *Mycoplasma gallisepticum* phase-variable lipoprotein gene (*vlhA*) expression during *in vivo* infection of the natural chicken host

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Abstract

Mycoplasma gallisepticum is the primary etiologic agent of Chronic Respiratory Disease in poultry, a disease largely affecting the respiratory tract and causing significant economic losses worldwide. Immunodominant proteins of the variable lipoprotein and hemagglutinin (*vlhA*) gene family are thought to be important for mechanisms of *M. gallisepticum*-host interaction, pathogenesis, and immune evasion, but their exact role and the overall nature of their phase variation are unknown. To better understand these mechanisms, we assessed global transcriptomic *vlhA* gene expression directly from *M. gallisepticum* populations present on tracheal mucosae during a seven day experimental infection in the natural chicken host. Here we report differences in both dominant and minor *vlhA* gene expression throughout the first week of infection and starting as early as day one post infection, consistent with a functional role not dependent on adaptive immunity for driving phase variation. Notably, data indicated that, at given timepoints, specific *vlhA* genes were similarly dominant in multiple independent hosts, suggesting a non-stochastic temporal progression of dominant *vlhA* gene expression in the colonizing bacterial population. The dominant expression of a given *vlhA* gene was not dependent on presence of 12-copy GAA trinucleotide repeats in the promoter region and did not revert back to the predominant *vlhA* when no longer faced with host pressures. Overall, these data indicate that *vlhA* phase variation is dynamic throughout the earliest stages of infection and that the pattern of dominant *vlhA* expression may be nonrandom and regulated by previously unrecognized mechanisms.

Introduction

Mycoplasma gallisepticum, the primary etiologic agent of Chronic Respiratory Disease (CRD) in poultry, is highly transmissible and causes severe inflammation of trachea, lungs, and air sacs, ultimately resulting in an overall reduction in weight gain and egg production in infected chickens. *M. gallisepticum* is pathogenic in other species, causing infectious sinusitis in turkeys and conjunctivitis in house finches (2, 146). Aside from primary attachment proteins GapA and CrmA, fibronectin binding proteins PlpA and Hlp3, sugar transport permease MalF, and dihydrolipoamide dehydrogenase Lpd, little is understood about specific bacterial factors affecting survival and persistence of this important avian pathogen in its natural host (34, 194, 199, 200).

The lack of obvious homologs of traditional bacterial transcriptional regulators and signaling factors had previously led to the thought that mycoplasmas were unable to regulate transcription by conventional mechanisms. However, *M. gallisepticum* exhibits differential gene expression in response to co-incubation with MRC-5 human lung fibroblasts(237) and in response to various stress conditions (239, 243). These data suggest that changes in transcription are indeed occurring as mycoplasmas respond to external stimuli and that the regulation is, in fact, more dynamic than originally hypothesized, with genes potentially affected by multiple regulators (238).

Potentially important for *M. gallisepticum* pathogenesis is the variable lipoprotein and hemagglutinin (*vlhA*) family, consisting of 43 genes distributed across 5 loci and totaling just over 10% of the entire genome in strain R_{low} (195). Previous studies have indicated that *M. gallisepticum vlhA* expression is dependent on the presence of exactly 12 GAA trinucleotide repeats upstream of the gene (213–216). It has also been hypothesized that *M.*

gallisepticum vlhA phase variation is stochastic, driven by anti-VlhA antibody-mediated negative selection of the initial population and resulting in random outgrowth of bacterial subpopulations expressing alternate *vlhA*(117). If that were correct, we would expect to see varying patterns of *vlhA* expression from the individual chickens at each time point.

The function of VlhA proteins is still unknown, but it has been speculated to involve immune evasion during infection (116), and variation in *vlhA* gene content has been loosely associated with virulence (153). While there have been efforts to understand the antigens expressed by *M. gallisepticum in-vivo* (242), there has not yet been a comprehensive global scale, *in-vivo* transcriptome study of *M. gallisepticum* in response to the natural host over the course of early infection. Previous studies have assessed selected *in-vivo vlhA* gene expression changes using monoclonal antibodies, however methods for comprehensive *vlhA* analysis were not yet available (213, 214). Now using next-generation sequencing, which enables comprehensive gene expression assessment at the nucleotide level, we present results indicating changes in *vlhA* expression of *M. gallisepticum* sampled directly from the tracheas of the experimentally infected chickens over the course of a seven-day infection.

Materials and Methods

Animals

Four-week-old female specific-pathogen-free White Leghorn chickens (SPAFAS, North Franklin, CT, USA) were received and divided randomly into groups, placed in HEPA-filtered isolators, and allowed to acclimate for one week. Non-medicated feed and

water were provided *ad libitum* throughout the experiment. All animal studies were performed in accordance with approved UConn IACUC protocol number A13-001.

Chicken infection

Stocks of *M. gallisepticum* strain R_{low} (passage 17) were grown overnight at 37°C in complete Hayflick's medium until mid-log phase was reached as indicated by a color shift from red to orange. Bacterial concentrations were determined by the OD₆₂₀ and 10-fold serial dilutions were conducted to confirm viable color changing unit titers. Bacteria were pelleted by centrifugation at 10,000 x g for 10 min and resuspended in Hayflick's complete medium. Chickens were challenged intratracheally as previously described (194) with 1 x 10⁸ CFU/200 µl.

RNA extraction

Five infected chickens were humanely sacrificed daily for a total of seven days. After sacrifice, tracheas were excised and total RNA was extracted from each individual trachea by washing the lumen with 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was then purified using the Zymo Direct-zol RNA Miniprep Kit (Zymo Research Corporation, Irvine, CA, USA) and standard PCR was conducted to ensure the RNA preps were free of any DNA. RNA was quality-checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and high quality samples with RNA integrity numbers (RIN) >8 were utilized to construct cDNA libraries.

To enrich for bacterial RNA, total RNAs were subjected to a polyA depletion step to remove the eukaryotic mRNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA). Briefly, 5 µg of total RNA combined with an equal volume of bead binding buffer was bound to the poly-T oligo-attached magnetic beads at 65°C for five minutes. The remaining supernatant was collected, cleaned and concentrated using the Zymo clean and concentrator -25 kit (Zymo Research Corporation) and eluted in 25µl RNase-free water.

Both prokaryotic and eukaryotic ribosomal RNA were removed from 2.5 µg of poly-A depleted RNA using the RiboZero Magnetic Gold kit (Epidemiology) (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions. Each rRNA depleted RNA sample obtained after cleaning and concentrating with the Zymo clean and concentrator -25 kit (Zymo Research Corporation) was eluted in 25 µl of RNase free water and used to create a cDNA library.

Illumina Sequencing

The cDNA libraries were created using the Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions starting at "Make RFP" (step 14, p.56) of the Illumina TruSeq RNA Sample Preparation v2 (HT) protocol. Briefly, 10-400 ng of purified mRNA was fragmented and used to synthesize first-strand cDNA using reverse transcriptase and random hexamer primers. Second-strand cDNA synthesis was performed using dUTP, DNA polymerase, and RNase. The products were amplified by PCR and purified after end repair and adaptor ligations.

The cDNA libraries were assessed for quantity using the Qubit 2.0 fluorometer (Invitrogen) and correct fragment size (~260 bp) using the Agilent TapeStation 2200 (Agilent Technologies). Libraries were then normalized to two nM, pooled, denatured, and sequenced on a NextSeq500 Sequencing platform (Illumina Inc.) using a 75 bp paired-end approach.

RNA-seq Analysis

Fastq data were assembled and mapped, and differential gene expression assessed, using Rockhopper, an RNAseq analysis program with algorithms specifically designed for the bacterial gene structures and transcriptomes (<http://cs.wellesley.edu/~btjaden/Rockhopper/>) (244, 245). Bowtie2 parameters allowing 0 mismatches were used to map sequence reads to the R_{low} genomic template (195). The data were normalized by the standard method of determining the ratio of reads per kilobase of transcript per million reads mapped (RPKM), allowing for comparisons both within and between samples. The fold change was determined from the \log_2 transformation of the RPKM between two samples. The differential gene expression was determined by pair-wise comparisons between the normalized expression values of a given *vlhA* gene between two different days. The program-generated p-value was used to determine the significance of the differential gene expression by calculating q-values based on the Benjamini–Hochberg correction with a false discovery rate <1%. Differences in expression values were considered significant when $q < 0.02$ (245).

Sequencing of culture-passed bacteria

Mycoplasma cultures recovered from tracheas of the experimentally infected birds at day seven postinfection were passed five or ten times in Hayflick's complete medium. Cultures were then grown to mid-log phase for DNA extraction using Chelex reagent (BioRad, Hercules, CA, USA) or RNA extraction, enrichment, and sequencing as was described with the trachea samples above.

Targeted sequencing of *vlhA* 2.02 gene promoter sequences to query GAA repeat copy number was performed directly on specific PCR amplicons. PCR primers targeting the *vlhA* 2.02 promoter region designed using the *M. gallisepticum* R_(low) genomic sequence (GenBank accession no. AE015450) (195) were as follows: forward 5'-GATGAGATTATCAAGCTTTTAGATG-3' and reverse 5'-CTGAACGAATCAAAGAGTTACAGC-3'. PCR was performed using Amplitaq (Invitrogen), 20 pmol of each primer and 200-300 ng DNA with the following cycling conditions: 94°C for 5 min, followed by 40 cycles at 94°C for 15 sec, 54°C for 30 sec, and 72°C for 1 min with a final extension of 72°C for 10 min. Amplicons were purified using the MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA, USA) to remove excess primers.

Sequencing was performed using 2 µl BigDye Terminator Mix (Invitrogen), 10 pmol of primer, and 300 ng of DNA (246). Excess dye terminators were removed with AutoSeq G-50 spin columns (Amersham Biosciences, Piscataway, NJ, USA), and the sequencing was performed by the University of Connecticut Biotechnology Center. Using Sequencher software (Gene Codes, Ann Arbor, MI, USA), the sequences were compared to the R_{low} genome and the number of GAA trinucleotide repeats upstream of the gene start codon were assessed.

Results

***M. gallisepticum* input *vlhA* expression**

In broth grown *M. gallisepticum* strain R_{low} input cultures, data here indicated that 31 *vlhA*s were expressed with an average normalized expression value of 719. *vlhA* 3.03 was the predominant *vlhA* expressed in this culture, with an RPKM value >12,000 (Fig.1). This *vlhA* is preceded by exactly 12 GAA repeats (11), a characteristic previously thought to be necessary for expression (12-15). *vlhA* 3.03 was not the only *vlhA* expressed, however, as several other *vlhA*s, including 2.02, 5.06, and 4.07, were expressed at levels (RPKM of 1500 – 2300) above the average RPKM (1455) of all genes in the genome that were expressed. This indicated that, while *vlhA* 3.03 expression predominated, additional *vlhA*s that were not preceded by 12 GAA repeats were expressed at minor levels in the cultured experimental input population.

***M. gallisepticum* *vlhA* expression over the course of infection**

Over the course of the seven-day infection, the *vlhA* expression profile changed dramatically. The expression of *vlhA* 3.03 showed an initial increase at day one postinfection and then decreased daily throughout the experiment (Fig 2). Over the course of infection, there was a 34-fold total decrease in *vlhA* 3.03 expression ($q < 0.0001$), with the largest change being a 4.5-fold decrease between days 5 and 6 postinfection ($q < 0.0001$).

While expression of *vlhA* 3.03 decreased early in infection, expression of *vlhA* 4.07 and its tandem repeat, *vlhA* 4.07.6 began to increase as early as one day postinfection. These expression levels peaked at two days postinfection (3.7-fold higher than input culture) before

decreasing over the remainder of the seven-day infection (Fig 2). Similarly, as *vlhA* 4.07 and 4.07.6 decrease in expression, *vlhA* 4.08 and its tandem repeat, *vlhA* 4.07.1 showed increased expression until peaking at seven days postinfection with 31-fold higher ($q < 0.0001$) expression than levels of input cultures (Fig. 2). Finally, there was an initial increase in *vlhA* 5.05 expression at one day postinfection, followed by decreasing expression through the time course. The *vlhA* expression profile observed here at day seven postinfection was consistent with that of a previous pilot experiment (data not shown). Taken together, these changes in *vlhAs* expression were not completely random, and appeared to be relatively coordinated as similar *vlhA* profiles were seen in five independently infected birds at each time point postinfection.

***M. gallisepticum vlhA* expression in recovery cultures**

From infected birds sacrificed seven days postinfection, *M. gallisepticum* was recovered directly from tracheas into Hayflick's complete medium and serially passed *in-vitro*. After either five or 10 passages, *M. gallisepticum* populations were predominantly expressing *vlhA* 2.02 with an average RPKM value of 8463, a 4.4-fold increase relative to *vlhA* 2.02 expression observed directly on tracheal mucosae at seven days postinfection ($q < 0.0001$) (Fig 3A). The *M. gallisepticum* recovered after one passage *in-vitro* displayed a transitional *vlhA* profile with *vlhA* 3.03 and 2.02 increasing in expression and *vlhA* 4.07.1 and 4.08 decreasing in expression (Data not shown). These data suggest the expression of *vlhA* 2.02 is favorable for the pathogen after recovery from the host trachea and passage in a nutritive medium. Several other *vlhAs*, including *vlhA* 3.03, were also expressed in the recovery cultures, but at levels much lower than *vlhA* 2.02. Interestingly, *vlhA* 3.03 was

expressed at a much lower, non-dominant level (4.5-fold) than in the initial input culture (Fig 3A).

When the promoter region upstream of the highly expressed *vlhA* 2.02 gene from the recovery cultures was PCR amplified and sequenced, data revealed only a single GAA trinucleotide upstream of the start codon, suggesting that 12 GAA repeat copies might not be essential for the expression of a given *vlhA*, at least in the *in-vitro* culture following direct recovery from the trachea (Fig 3B). Furthermore, we observed no changes in the genomic nucleotide sequence upstream of *vlhA* 2.02 in the cultures recovered from the infected animals and passed *in-vitro* compared to the broth grown input culture.

Discussion

This study has demonstrated the global population changes in *M. gallisepticum vlhA* gene expression assessed directly from tracheas of experimentally infected chickens over the course of a seven day infection. It was important to recover the *M. gallisepticum* RNA directly from the trachea into the TRIzol reagent to minimize the time between collection and extraction, as we observed significant changes in the gene expression profile of *M. gallisepticum* after just one single passage in culture medium (data not shown). This study design allowed for the capture of the most accurate representation of the *vlhA* profile *in-vivo* at each time point during the infection process.

These observed changes in expression are likely not antibody mediated, as the decrease in expression of *vlhA* 3.03 is seen as early as two days postinfection, well before the formation of specific antibodies(193). This finding is in agreement with a previous study that demonstrated that *vlhA* expression in infected chickens switches prior to the host mounting

an antibody response (214). However, this study greatly expands on those initial observations to include the expression status of all of the *vlhAs* over the early course of infection.

These findings are significant as they imply that the driving force behind the shift in *vlhA* expression is not controlled entirely by the adaptive immune system. These data suggest that another mechanism is, at least in part, controlling changes in expression of the *vlhA* genes *in-vivo*.

It is possible that early phase variation could be driven, in part, by the alterations in the cellular architecture of the tracheas during infection. As the infection progresses, the host cells experience denuding of cilia, squamous cell metaplasia, and eventual destruction of the host cell membranes. It is possible that the *M. gallisepticum vlhA* expression is changing in response to these alterations and the pathogen is expressing a specific set of *vlhAs* to best persist in the current environment. Consistent with the hypothesis that *M. gallisepticum vlhA* expression is nonrandom and follows progression of pathogenesis in the host, *vlhA 1.07* and *4.01* reported by Ron et al. (242) to express significant antigens at two to five weeks postinfection were not predominantly expressed within one week postinfection as reported here.

Data here indicate that despite expressing different *vlhAs* in the host at day seven postinfection, *vlhA 2.02* is the predominant *vlhA* expressed in the *M. gallisepticum* population following recovery and passage in Hayflick's complete medium. Unanticipated was the dominance of *vlhA 2.02* and the lack of reversion to predominant *vlhA 3.03* expression observed in the broth grown input culture. However, in these recovery cultures, *vlhA 2.02* was also accompanied by the expression (albeit at lower levels) of several other *vlhAs* in the population, including *vlhA 3.03* and *5.13*, suggesting that more than one *vlhA* is

expressed at a given time point in the population. These data also indicate that *M. gallisepticum* may favor the expression of several *vlhA*s (*vlhA* 3.03, 2.02, and 5.13) when no longer faced with the pressures of surviving in the airway of the host

Data here also demonstrated that 12 GAA trinucleotide repeats are not essential for the expression of a given *vlhA*, since the *vlhA* 2.02 highly expressed in the recovery samples does not contain 12 GAA repeats in upstream sequences. It had previously been hypothesized that 12 GAA repeats were necessary for expression of a given *vlhA* because it allowed for the correct spacing between flanking sequences for necessary accessory factors to bind, as *M. gallisepticum* does not possess a robust consensus sequence at -10 and -35 (216). The observed expression of *vlhA* genes without the 12 GAA repeats upstream suggest that there may be another mechanism at play that is responsible, at least in part, for the regulation of the *vlhA* expression in *M. gallisepticum*.

These results, revealing global *vlhA* expression changes over the course of early infection, are important in elucidating mechanisms of persistence, colonization, and overall pathogenesis of *M. gallisepticum* in the natural host. Full and complete understanding of these mechanisms will provide information likely to be critical for development of rationally designed *M. gallisepticum* therapeutics and vaccines.

Figure 1. The *vlhA* expression profile of the broth grown *M. gallisepticum* input cultures as determined from RNA sequencing.

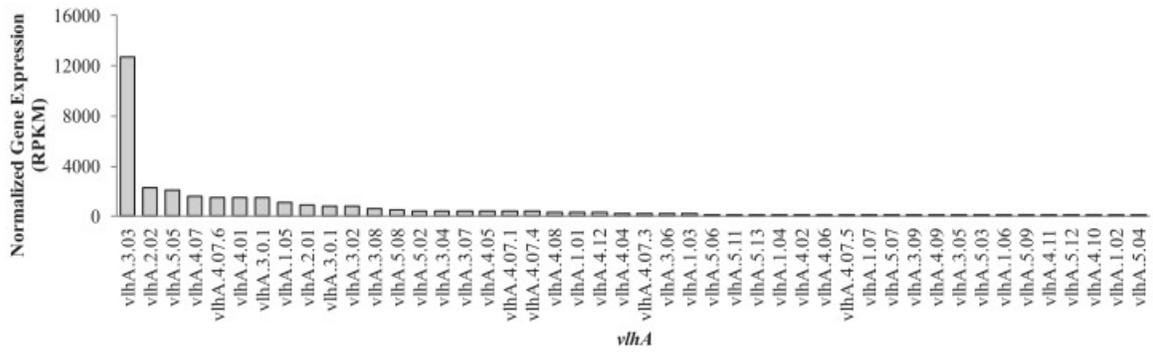


Figure 2. The *vlhA* expression profile of *M. gallisepticum* extracted directly from tracheas of experimentally infected birds over the course of the seven day infection as determined through RNA sequencing. Each data point is an average RPKM value from five animals (with the exception of the broth sample). Error bars show SEM. Key statistically significant changes between two time points are indicated by paired upper and lower case letters for the following genes: *vlhA* 3.03 (A/a and A'/a'), and *vlhA* 4.07.1/4.08 (B/b).

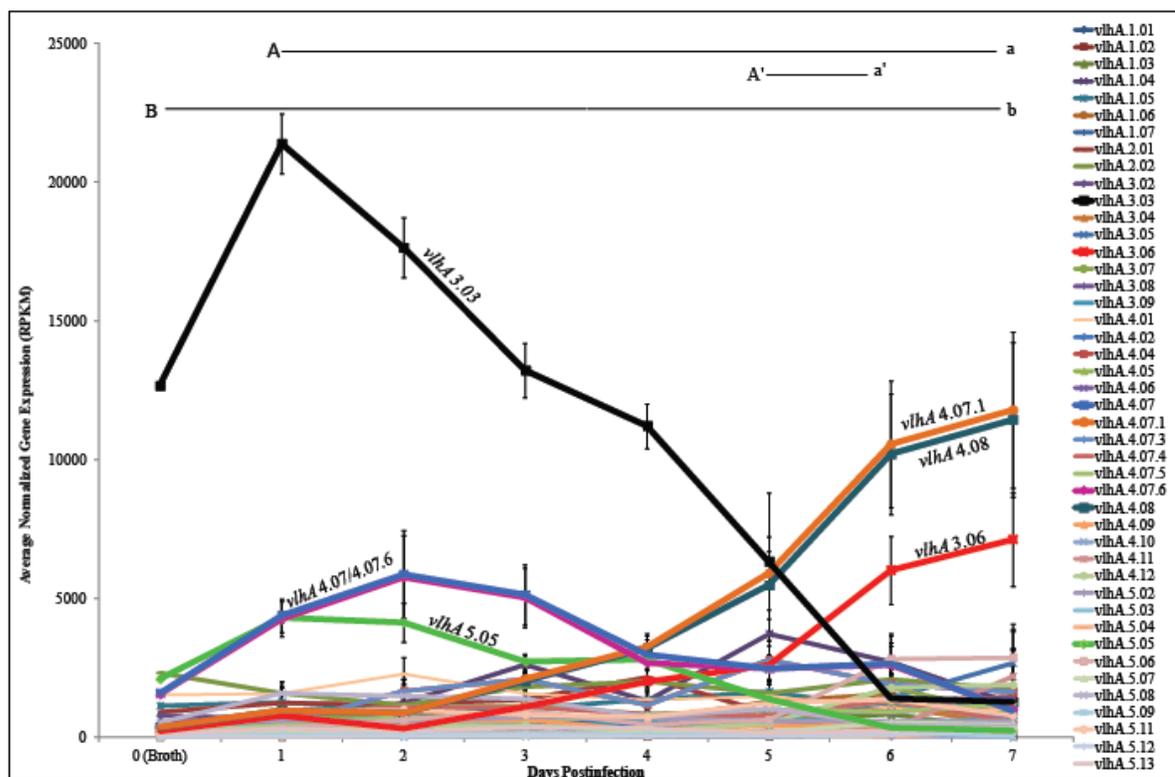
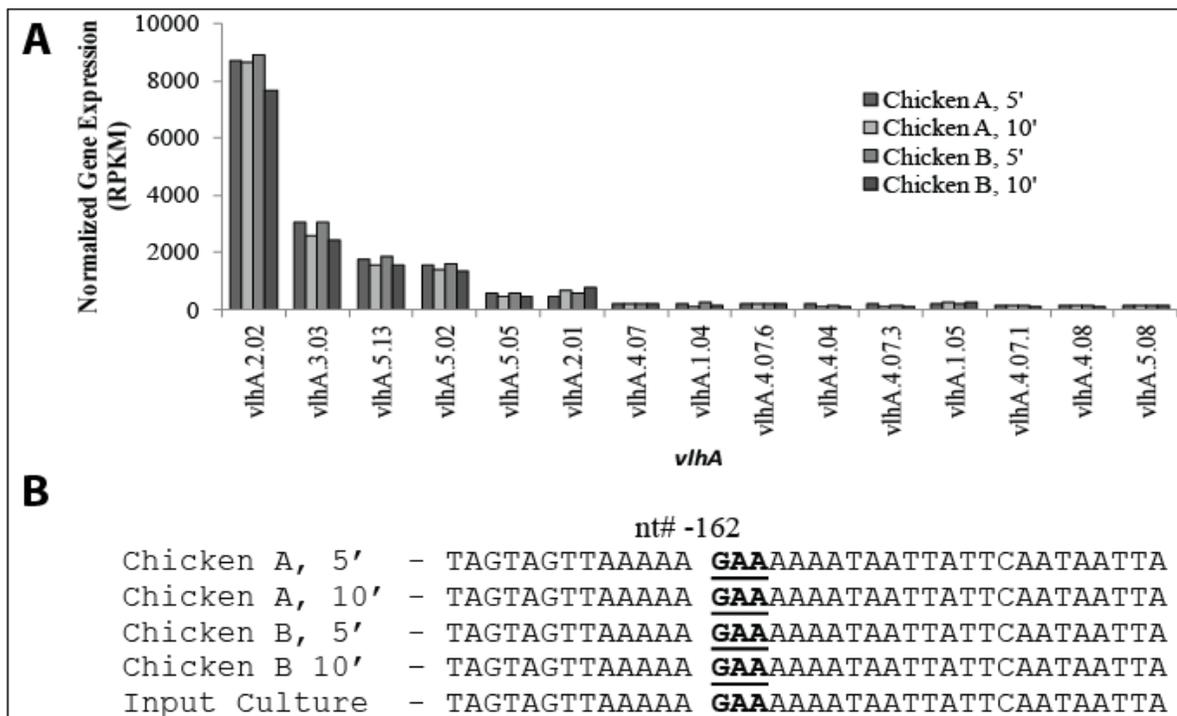


Figure 3. The *vlhA* expression of the *M. gallisepticum* recovered and cultured at five (5') or ten (10') passes from the tracheas of the two different infected birds at seven days postinfection (A). Only *vlhAs* with an average RPKM >100 are displayed. The sequence of the region upstream of the highly expressed *vlhA* 2.02 in the cultures recovered from the infected birds (B).



CHAPTER 3:

The *vlhA* profile of *Mycoplasma gallisepticum* predominantly expressing an alternate *vlhA* over a two day *in-vivo* infection

Abstract

Mycoplasma gallisepticum is the primary etiologic agent of Chronic Respiratory Disease in poultry, a disease largely affecting the respiratory tract and causing significant economic losses worldwide. Proteins of the variable lipoprotein and hemagglutinin (*vlhA*) gene family are thought to be important for mechanisms of *M. gallisepticum*-host interaction, pathogenesis, and immune evasion, but their exact role and the overall nature of their phase variation are unknown. To better understand these mechanisms, we assessed global transcriptomic *vlhA* gene expression directly from *M. gallisepticum* populations present on tracheal mucosae during an experimental infection in the natural chicken host with a strain expressing an alternate dominant *vlhA*. Here we report that the *in vivo* *vlhA* expression profile of *M. gallisepticum* when chickens are infected with a culture predominantly expressing *vlhA* 2.02 is similar to that seen when the *M. gallisepticum* predominantly expressing *vlhA* 3.03 is used for infection during early stages of infection. This indicates that the progression of *vlhA* expression, especially the initial predominantly expression of *vlhA* is likely important during the earliest stages of the infection process.

Introduction

Mycoplasma gallisepticum, a highly transmissible avian pathogen, contributes to chronic respiratory disease (CDR), a pathological syndrome largely affecting the respiratory tracts of poultry causing inflammation of the air sacs, lungs, and trachea causing significant monetary losses world-wide (2). *M. gallisepticum* has also been linked to infectious sinusitis in turkeys and severe conjunctivitis in house finch(2, 247).

Despite much effort, little is completely understood about the mechanisms of survival and persistence or pathogenic mechanisms employed by this organism (34, 194, 237, 199, 200, 242). Potentially important in the virulence of the organism is the phase-variable lipoprotein hemagglutinin gene family (*vlhA*)(195) as they have been shown to differ in both size and genomic location in strains varying in levels of virulence (153). Initial studies have reported that *vlhA* expression is stochastic, and due to random outgrowth after anti-VlhA antibody mediated negative selection (117). It has also been reported that the expression of a single *vlhA* gene occurs only when that gene is preceded by exactly 12 GAA trinucleotide repeats in the promoter region (213, 215, 216, 224). However, despite this report, a recent study has shown that at given timepoints, specific *vlhA* genes were similarly dominant in multiple independent hosts, suggesting a non-stochastic temporal progression of dominant *vlhA* gene expression and that the expression was not completely dependent on the presence of 12 GAA trinucleotide repeats (248).

Here, using next-generation sequencing, we extend the understanding of *vlhA* gene expression, specially the importance of the *vlhA* 3.03, the initial immuno-dominant *vlhA* at one

day post-infection. We report the results of the global *vlhA* gene expression profile when the input population is expressing an alternate dominant *vlhA*, *vlhA* 2.02.

Materials and Methods

Animals

Four-week-old female specific-pathogen-free White Leghorn chickens (SPAFAS, North Franklin, CT, USA) were received and divided randomly into groups, placed in HEPA-filtered isolators, and allowed to acclimate for one week. Non-medicated feed and water were provided *ad libitum* throughout the experiment. All animal studies were performed in accordance with approved UConn IACUC protocol number A13-001.

Chicken infection

M. gallisepticum R strain cultures stabilized to express an alternate *vlhA* (*vlhA* 2.02) have been identified (248) were used as the input cultures in. As a positive control, *M. gallisepticum* strain R_{low} (passage 17) were also used for inoculation. 10-fold serial dilutions were conducted to confirm viable color changing unit titers. Bacteria were pelleted by centrifugation at 10,000 x g for 10 min and re-suspended in Hayflick's complete medium. Chickens were challenged intratracheally as previously described (248).

RNA extraction

Four infected chickens from each group were humanely sacrificed at day 1 and day 2 post infection. After sacrifice, tracheas were excised and total RNA was extracted from each individual trachea by washing the lumen with 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was then purified using the Zymo Direct-zol RNA Miniprep Kit (Zymo Research Corporation, Irvine, CA, USA) and standard PCR was conducted to ensure the RNA preps were free of any DNA. RNA was quality-checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and high quality samples with RNA integrity numbers (RIN) >8 were utilized to construct cDNA libraries.

M. gallisepticum RNA was enriched as previously described (248). Briefly, total RNAs were subjected to a polyA depletion step to remove the eukaryotic mRNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA). Briefly, 5 µg of total RNA combined with an equal volume of bead binding buffer was bound to the poly-T oligo-attached magnetic beads at 65°C for five minutes. The remaining supernatant was collected, cleaned and concentrated using the Zymo clean and concentrator -25 kit (Zymo Research Corporation) and eluted in 25µl RNase-free water.

Both prokaryotic and eukaryotic ribosomal RNA were removed from 2.5 µg of poly-A depleted RNA using the RiboZero Magnetic Gold kit (Epidemiology) (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions. Each rRNA depleted RNA sample obtained after cleaning and concentrating with the Zymo clean and concentrator -25 kit (Zymo Research Corporation) was eluted in 25 µl of RNase free water and used to create a cDNA library.

Illumina Sequencing

The cDNA libraries were created using the Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions starting at "Make RFP" (step 14, p.56) of the Illumina TruSeq RNA Sample Preparation v2 (HT) protocol. Briefly, 10-400 ng of purified mRNA was fragmented and used to synthesize first-strand cDNA using reverse transcriptase and random hexamer primers. Second-strand cDNA synthesis was performed using dUTP, DNA polymerase, and RNase. The products were amplified by PCR and purified after end repair and adaptor ligations.

The cDNA libraries were assessed for quantity using the Qubit 2.0 fluorometer (Invitrogen) and correct fragment size (~260 bp) using the Agilent TapeStation 2200 (Agilent Technologies). Libraries were then normalized to two nM, pooled, denatured, and sequenced on a NextSeq500 Sequencing platform (Illumina Inc.) using a 75 bp paired-end approach.

RNA-seq Analysis

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comparisons between the normalized expression values of a given *vlhA* gene between two different days. The program-generated p-value was used to determine the significance of the differential gene expression by calculating q-values based on the Benjamini–Hochberg correction with a false discovery rate <1%. Differences in expression values were considered significant when $q < 0.02$ (245).

Results

When chickens are experimentally challenged with an *M. gallisepticum* strain predominantly expressing an alternate *vlhA*, the *vlhA* expression profile of the bacteria recovered from the tracheal mucosa is very similar to that of a wild type strain expressing *vlhA* 3.03 predominantly (Fig 1).

In the *M. gallisepticum* recovered from experimentally infected chickens, we observed a sharp increase in the expression of the *vlhA* 3.03 at one day post infection with a 14.8-fold increase in expression compared to the *vlhA* 3.03 gene expression in the input population. Followed by this sharp increase, was a 6.1-fold decrease in expression of *vlhA* 3.03 by two days post infection (Fig 1 and Table 1). This pattern was very similar to that of the initial wild-type input culture in which that population was predominantly expressing *vlhA* 3.03 (see chapter 2).

Additionally, we observed a similar pattern of expression with *vlhA* 4.07 and its tandem repeat *vlhA* 4.07.6, where there was a 80.1-fold and 78.7-fold increase compared to the input population, respectively, in the expression at day one post infection. This was then followed by a 5.8- and 5.4-fold decrease in expression by day two post infection (Fig1 and

Table 1). This pattern is also observed when the wild type input cultures are predominantly expressing *vlhA* 3.03 (see chapter 2).

Surprisingly, *vlhA* 2.02, the predominant *vlhA* expressed in the challenge culture, stayed relatively stable through one day post infection displaying only a slight 1.1-fold increase in expression. However, the expression level of *vlhA* 2.02 showed a 9.7-fold reduction in expression level by two days post infection (Fig 1 and Table 1). This differs from the *vlhA* expression profile of wild type *M. gallisepticum* initially expressing *vlhA* 3.03 predominantly. When *vlhA* 3.03 was predominantly expressed in the input population, *vlhA* 2.02 did not show an expression level above background at any point during the seven day infection study (see chapter 2).

Discussion

Here we report the initial *vlhA* expression profile of *M. gallisepticum* predominantly expressing *vlhA* 2.02 recovered from the chicken tracheas during the early course of experimental infection. The *vlhA* expression profile was very similar to that of the wild type *M. gallisepticum* strains with a sharp increase in the expression of *vlhA* 3.03 at one day post infection and a decrease in expression by two days post infection.

These data suggest that *vlhA* 3.03 is likely very important in the early infection process of *M. gallisepticum* as it is highly expressed at one day post infection regardless if it was predominant in the initial culture used for challenge or not.

Additionally, we observed an increase in the expression of *vlhA* 4.07 and 4.07.6 at one day post infection, a pattern also observed when the initial input culture was predominantly expressing *vlhA* 3.03. This suggests that, while not the predominant *vlhA*

genes expressed, they still may play a vital role for *M. gallisepticum* in the earliest stages of infection.

It is important to note that, while the input population used in this second study was predominantly expressing *vlhA* 2.02, *vlhA* 3.03 was still expressed, albeit at a much lower level, in this population. It is possible that the perceived increase in the expression of *vlhA* 3.03 at day one post infection is due to the fact that only those *M. gallisepticum* expressing *vlhA* 3.03 were able to colonize the trachea at that time. Either way, these data emphasize the importance of the predominant expression of *vlhA* 3.03 in the earliest stages of infection.

In totality these data demonstrate that even when the predominant *vlhA* expression is altered, *M. gallisepticum* still undergoes the same non-stochastic temporal progression of expression over the earliest stages of infection. This suggests that progression of *vlhA* expression is likely important in the initial stages of the infection process in the chicken trachea.

Further insight into these patterns of expression may lead to a more complete understanding of the pathogenic mechanisms employed by *M. gallisepticum* during early stages of the infection process.

Figure 1. The *vlhA* gene expression profile of the *M. gallisepticum* strain predominantly expressing *vlhA* 2.02 from the tracheal mucosa of experimentally infected birds over a two-day infection.

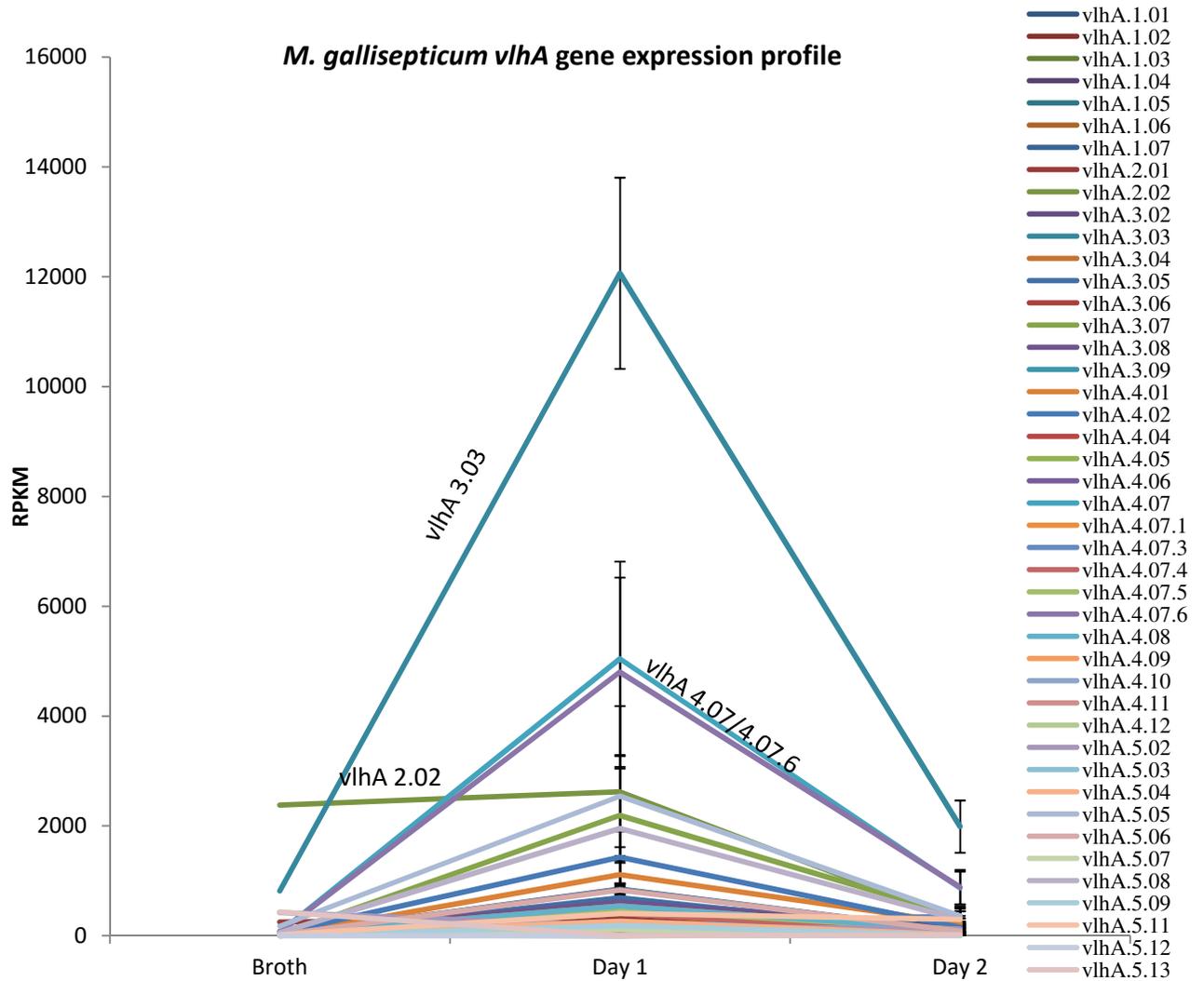


Table 1. The fold change of select *vlhA* genes over from the *M. gallisepticum* strain predominantly expressing *vlhA* 2.02 from the tracheal mucosa of experimentally infected birds over a two-day infection.

<i>vlhA</i> gene	Fold Change	
	Broth vs Day 1	Day 1 vs Day 2
<i>vlhA</i> 3.03	+14.8	-6.1
<i>vlhA</i> 4.07	+80.1	-5.8
<i>vlhA</i> 4.07.6	+78.7	-5.4
<i>vlhA</i> 2.02	+1.1	-9.7

CHAPTER 4

CONCLUSIONS AND DISCUSSION

Collectively, these data significantly further our understanding of the *M. gallisepticum* phase-variable *vlhA* gene expression, showing that the *vlhA* genes exhibit a non-stochastic temporal progression of expression and altering the starting *vlhA* does not alter the predominant expression of *vlhA* 3.03 at one day post infection.

We observed changes in *vlhA* gene expression as early as 1-2 days post infection, well before the formation of specific antibodies (193), suggesting that the changes observed at these earliest stages of infection are not influenced by the adaptive immune system of the host,. However, it is very likely that as the disease progresses, the host adaptive immune response will play a role in the *vlhA* genes expression patterns as it has been demonstrated that a change in *vlhA* expression can be forced *in-vitro* by the addition of specific antibodies (249). These data suggest that another mechanism is, at least in part, responsible for the changes in expression of the *vlhA* genes *in-vivo* during the earliest stages of the infection process.

It is possible that these changes in *vlhA* expression could be in response to the alterations in the cellular architecture of the tracheas during infection. As the infection progresses, the host cells experience denuding of cilia, squamous cell metaplasia, and eventual destruction of the host cell membranes. It is possible that the *M. gallisepticum vlhA* expression is changing in response to these alterations and the pathogen is expressing successive specific sets of *vlhAs* to best persist in the current environment.

This is similar to what has been reported in *Wolbachia* species, a rickettsial bacterium which infects invertebrate organisms lacking adaptive immune systems. *Wolbachia* variable surface proteins are involved in host cell attachment and vary their expression in response to non-immune environmental pressures (250).

As the function of VlhAs is still unknown at this time, it is also possible that these gene products could be functioning in sensing the current environment and relaying messages into the cell. It is also possible that these gene products could be involved in the transport of essential nutrients and molecules, as these VlhAs are surface exposed. *M. gallisepticum* could be altering expression of *vlhAs* in response to a change in nutrient availability in the current environment.

Data here also indicated that the *vlhAs* exhibit a non-stochastic, temporal progression of expression, as *vlhAs* were similarly dominant in multiple independent hosts in repeated studies. This is particularly interesting as previous studies have hypothesized that that *vlhA* expression was stochastic, and due to random outgrowth after anti-VlhA antibody mediated negative selection (214).

Unanticipated was the dominance of *vlhA* 2.02 and the lack of reversion to predominant *vlhA* 3.03 expression observed in the broth grown input culture. However, in these recovery cultures, *vlhA* 2.02 was also accompanied by the expression (albeit at lower levels) of several other *vlhAs* in the population, including *vlhA* 3.03 and 5.13, suggesting that more than one *vlhA* is expressed at a given time point in the population. These data also indicate that *M. gallisepticum* may favor the expression of several *vlhAs* (*vlhA* 3.03, 2.02, and 5.13) when no longer faced with the pressures of surviving in the airway of the host.

Data here also demonstrated that 12 GAA trinucleotide repeats are not essential for the expression of a given *vlhA*, since the *vlhA* 2.02 highly expressed in the recovery samples does not contain 12 GAA repeats in upstream sequences. It had previously been hypothesized that 12 GAA repeats were necessary for expression of a given *vlhA* because it allowed for the correct spacing between flanking sequences for necessary accessory factors to bind, as *M.*

gallisepticum does not possess a robust consensus sequence at -10 and -35 (216). The observed expression of *vlhA* genes without the 12 GAA repeats upstream suggests that there may be another mechanism at play that is responsible, at least in part, for the regulation of the *vlhA* expression in *M. gallisepticum*.

Additionally, we found that *M. gallisepticum* strains expressing an alternate dominant *vlhA* still display a very similar expression pattern over the earliest stages of the infection, showing a sharp increasing the expression of *vlhA* 3.03 at one day post infection, followed a decrease by two days post infection. Additionally the initial increase in the expression of *vlhA* 4.07 and its tandem repeat, 4.07.6 was observed, followed by the decline in expression by two days post infection. These data suggest that *vlhA* 3.03 may play a critical role in the infection process of *M. gallisepticum* in the trachea of the chicken host as it is consistently highly and predominantly expressed at day one post infection regardless of the predominant *vlhA* expressed in the input culture. Additionally, *vlhAs* 4.07 and 4.07.6 are likely important during the earliest stages of the infection process as their increased expression is repeatedly observed at day one post infection, regardless of the predominantly expressed *vlhA* in the challenge culture.

Taken together these data demonstrate that even when the predominant *vlhA* expression is altered, *M. gallisepticum* still undergoes the same non-stochastic temporal progression of expression over the earliest stages of infection. This suggests that progression of *vlhA* expression is likely important in the initial stages of the infection process in the chicken trachea.

Specific *vlhA* gene products are likely to have specific functions; however, the exact roles are unknown at this time. These gene products could be involved in attachment to host

cells to prevent clearance, sensing of the surrounding environment, or transport of nutrients into the cell. Regardless of the function of these products, *M. gallisepticum* expresses these genes in a coordinated, non-random manner over the course of early infection in the chicken trachea. This non-stochastic *vlhA* expression pattern is still displayed *in-vivo* even when the initial challenge culture expression pattern is altered.

The data suggest that these gene products are important in early infection in the natural host and each VlhA (or specific set of VlhAs) may have a unique function. *M. gallisepticum* is likely expressing a specific subset of *vlhA* genes in order to best persist in the current environment. If there were no change in the environment *M. gallisepticum* is persisting in, it appears there is little to no change in the predominant *vlhA* expressed. For example when *M. gallisepticum* is passed numerous times *in-vitro*, the predominant *vlhA* gene expression remains stable. However, when it is exposed to the cells of the chicken trachea, the *vlhA* profile begins to change immediately, as early as day one post infection, suggesting that *M. gallisepticum* is responding to the change in environment and likely expressing a subset of *vlhAs* to best persist in that environment.

Furthermore, once *M. gallisepticum* is recovered from the trachea of experimentally infected chickens and passed *in-vitro*, the *vlhA* expression profile changes again, suggesting, yet again, that *M. gallisepticum* is expressing a very specific subset of *vlhA* genes to best survive in the current environment.

Overall, these data indicate that *vlhA* phase variation is dynamic throughout the earliest stages of infection and that the patterns of dominant *vlhA* gene expression are nonrandom and regulated by a currently unknown mechanism(s). These data also stress the

importance of the expression of *vlhA* 3.03 during the initial stages of infection, furthering our understanding of phase variation in *M. gallisepticum*.

These results, revealing global *vlhA* expression changes over the course of early infection, are important in elucidating mechanisms of persistence, colonization, and overall pathogenesis of *M. gallisepticum* in the natural host. We can now begin to further understand *vlhA* gene expression and phase variation in *M. gallisepticum*. Full and complete understanding of these patterns of expression will help to provide insight that may be critical for the development of rationally designed therapeutics and vaccines.

Future Directions

Although beyond the scope of this project, it will be beneficial to determine if there is tissue specificity for different *vlhA* genes. For example, it is possible that different *vlhA* genes are dominantly expressed when exposed to varying cell types in tissues of the host, allowing for optimal survival in the different tissues. Additionally, as infection with *M. gallisepticum* progresses in the respiratory tract, the architecture of the trachea changes as well as the cell type predominating as metaplasia occurs. The *M. gallisepticum* could be altering the predominant *vlhA* expressed as a response to the changing cell type in the chicken trachea at a given time point and expressing the best *vlhA* to persist in that current environment. Examining the global *vlhA* profile in various host tissues including, air sacs, lungs, and oviducts and understanding the driving force behind the changes in *vlhA* gene expression in the trachea would provide valuable information into the specificity of the *vlhA* expression profile.

Given that *vlhA* 3.03 appears to be important in the initial stages of the infection process, as it is the predominant *vlhA* at day one post infection, regardless of what the starting predominant *vlhA* is in the input culture, it will be beneficial to assess the virulence of mutants lacking *vlhA* 3.03 in the natural host. Screening our transposon mutant library, a *vlhA* 3.03 mutant could be identified and used to experimentally challenge chickens and assess the virulence of the mutant and to determine the plasticity of the *vlhA* profile. These data would indicate the necessity (or lack thereof) of the expression of *vlhA* 3.03 to attach, colonize, and cause pathology in the respiratory organs of chickens.

In conjunction with this study, the lab is also sequencing the host RNA obtained from the same samples used in this study over the course of early infection. This will allow a glimpse into the interplay between the pathogen and its natural host over the initial stages of the infection process, furthering our understanding of the pathogenesis of *M. gallisepticum* and how the host responds to that challenge. Assessing these data concurrently could provide insight into the host factors that could be playing a role in the non-stochastic temporal progression of *vlhA* expression.

Additionally, examining the *vlhA* profile of live attenuated vaccine strains will enhance our understanding of the progression and importance of *vlhA* gene expression. These strains do not cause the same level of pathology in the trachea as wild-type Rlow strains, and therefore, may display a different *vlhA* profile over the initial stages of infection. Preliminary data suggest that the *in vivo* *vlhA* expression profile of the live attenuated vaccine strains Mg7 and GT5 is different than that of wild-type Rlow, with the expression of *vlhA* 3.03 continually increasing over the first two days of infection. This could be due to the fact that the vaccine strains do not cause significant damage to the tracheal mucosa as severe as Rlow,

therefore the environment is not changing significantly, and the *M. gallisepticum* is not stimulated to change predominant expression from *vlhA* 3.03.

Understanding these changes in *vlhA* expression and the effects those genes have on the virulence and persistence of the pathogen will be important in elucidating mechanisms of colonization, persistence, and overall pathogenesis of *M. gallisepticum* in the natural host and will provide information likely to be critical for development of rationally designed *M. gallisepticum* therapeutics and vaccines.

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