Global Transcriptional Analysis of the Chicken Tracheal Response to Virulent and Attenuated Vaccine Strains of Mycoplasma gallisepticum

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Chickens infected with *Mycoplasma gallisepticum*, the primary etiologic agent of chronic respiratory disease (CRD), present with significant respiratory complications. The host response is often immunopathogenic and contributes to increased tracheal thickness due to the chemotaxis of leukocytes, cellular damage at the mucosal surface, and the inability to clear the infection. The response to *M. gallisepticum* is complex and the differences that lead to a maladaptive versus beneficial response are poorly understood. To elucidate the extent of the host response to virulent and attenuated strains, we conducted next-generation sequencing on samples taken directly from the host’s tracheal mucosa after infection with either virulent $R_{\text{low}}$, or laboratory attenuated vaccine strains Mg 7 or GT5.

In the first study, transcriptional analysis of the host response to virulent $R_{\text{low}}$ over a 7-day infection was conducted on samples from days 1, 3, 5 and 7. Data indicated a rapid, pro-inflammatory response, identifying numerous genes involved in immune pathways that peaked 3 days post-infection, and a significant metabolic response observed on days 1 and 3. Further analysis revealed increased expression of host cell receptors that likely heightens their sensitivity to damage- and pathogen- associated molecular patterns and results in the production of cytokines and chemokines that recruit and activate immune cells.
In the second study, comparative transcriptomics identified differences between the early host response to $R_{\text{low}}$ and laboratory vaccine strains, Mg 7 and GT5. Infection with these attenuated strains resulted in a dampened host response involving significantly fewer immune and metabolism-related genes, limited differential expression of host cell receptors, and decreased production of cytokines and chemokines. Future vaccines should aim to target immune genes active in Mg 7 and GT5 infection while avoiding those uniquely differential in $R_{\text{low}}$ infection to induce a protective, and durable adaptive response while avoiding immune-related exacerbation of disease.
Global Transcriptional Analysis of the Chicken Tracheal Response to Virulent and Attenuated Vaccine strains of *Mycoplasma gallisepticum*

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Global Transcriptional Analysis of the Chicken Tracheal Response to Virulent and Attenuated Vaccine Strains of *Mycoplasma gallisepticum*

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# Table of Contents

Chapter 1: Literature Review

- Section 1: Overview
  - Introduction
  - History and Nomenclature

Section 2: Mycoplasmosis within Species

- *Mycoplasma* of Humans
- *Mycoplasma* of Porcines
- *Mycoplasma* of Bovines
- *Mycoplasma* of Caprines and Ovines
- *Mycoplasma* of Rodents
- *Mycoplasma* of Felines
- *Mycoplasma* of Canines
- *Mycoplasma* of Reptiles and Piscines
- *Mycoplasmas* of Avians

Section 3: *Mycoplasma* Host Interaction

- Cytadhesion and Gliding Motility
- Invasion of Host Cells
- Host Cell Receptors
- *Mycoplasma* Lipoproteins

Section 4: Host Immune Response to *Mycoplasma*

- Induction of Innate Immune Response
- The Role of Chemokines and Cytokines
- Adaptive Response to *Mycoplasma* Infection
- Mechanisms of Immune Evasion

Section 5: *Mycoplasma gallisepticum*

- Disease and Significance
- Virulence Factors and Pathogens
Chapter 2: Transcriptional Profiling of the Chicken Tracheal Response to Virulent Mycoplasma gallisepticum Strain R_{low}

Abstract

Introduction

Results

Global Differentially Expressed Genes (DEGs) Between Infected and Control Chickens

Pathway Analysis

Functional Gene Ontology (GO)

Immune Response

Genes with Greatest Differential Expression

Discussion

Materials and Methods

Animals

Chicken Infection

RNA Extraction

Illumina Sequencing

RNA-Seq Analysis

Pathway Analysis

Functional Gene Ontology

Acknowledgements

References

Figures and Legends
Chapter 3: Transcriptional Comparison of the Chicken Tracheal Response to Virulent and Attenuated Vaccine Strains of *Mycoplasma gallisepticum*………………108

Abstract………………………………………………………………………………………………………109

Significance………………………………………………………………………………………………110

Introduction……………………………………………………………………………………………..110

Results………………………………………………………………………………………………………114

Differentially Expressed Genes (DEGs) Between Control and Virulent or Attenuated Strains…………………………………………………………………………………..114

Pathway analysis…………………………………………………………………………………………114

Functional Gene Ontology (GO)………………………………………………………………………115

Immune Pathways……………………………………………………………………………………..116

Genes with Greatest Differential Expression……………………………………………………117

Discussion…………………………………………………………………………………………………118

Materials and Methods…………………………………………………………………………………127

Animals…………………………………………………………………………………………………127

Chicken Infection……………………………………………………………………………………..127

RNA Extraction………………………………………………………………………………………127

Illumina Sequencing……………………………………………………………………………………128

RNA-Seq Analysis……………………………………………………………………………………129

Pathway Analysis……………………………………………………………………………………..129

Functional Gene Ontology…………………………………………………………………………127

Acknowledgements…………………………………………………………………………………………130

References………………………………………………………………………………………………….131

Figures and Legends………………………………………………………………………………………135

Chapter 4: Conclusions and Future Directions……………………………………………………152

Conclusions…………………………………………………………………………………………………152

Future Directions…………………………………………………………………………………………156

References………………………………………………………………………………………………….160
CHAPTER 1: Literature Review

Section 1: Overview

Introduction

*Mycoplasmas* are atypical bacteria belonging to the class of ‘Mollicutes’. They are one of the smallest self-replicating organisms having evolved from gram-positive bacteria with low G + C content through degenerative evolution. As a result, *mycoplasmas* have uniquely small genomes ranging from as low as 580 Kb in *Mycoplasma genitalium* (1) to 1,380 Kb in *M. mycoides* subsp. *mycoides* LC with low G + C content (23-40%) (2), and a high mutation rate likely due to inadequate proof-reading by DNA polymerase enzymes (3, 4). Another unique characteristic is their unusual use of the traditional stop codon UGA to encode the amino acid tryptophan (5, 6). Having limited genomic content has also reduced their biosynthetic capabilities; *mycoplasmas* lack the pathways necessary to synthesize peptidoglycan cell walls and essential molecules including purines and pyrimidines (2). Therefore, *mycoplasmas* rely on close association with host cells to acquire sterols that maintain their cell membrane integrity and essential nutrients such as amino acids, nucleotides, and fatty acids (2).

While several *mycoplasmas* are commensal organisms living as part of the host’s natural flora, most are obligate parasites exhibiting highly specific host and tissue tropism(7, 8). Susceptible host species include humans, animals, reptiles, fish, insects and plants. Infection commonly results in chronic pathology of the respiratory and urogenital tracts, but are also known to cause systemic diseases of the eyes, joints, and mammary glands (2). Very few *mycoplasmas* cause mortality, however, many are associated with significant disease acting as primary, secondary or co-infectious agents. Costs related to the treatment and prevention of
*mycoplasma* infection and subsequent pathology in humans, pets and livestock results in significant economic loss worldwide (2, 9, 10).

**History and Nomenclature**

Cases of pleuropneumonia in cattle have been documented since 1693, but it was not until 1765 that Claude Bourgelat made what has been credited as the first clinical description of Bovine pleuropneumonia (11). This was assumed to be a viral disease, though they had not been able to successfully pass the organism in broth or solid media, nor had they been able to observe it through light microscopy (11). In 1898, the etiologic agent of Bovine pleuropneumonia was identified by Nocard and Roux (12). They obtained infectious exudate from a cow with distention of the interlobular connective tissue due to accumulation of a clear-yellow albuminous serous fluid; characteristic of pleuropneumonia. A semi-permeable nitrocellulose pouch containing sterile peptone broth was inoculated with the exudate and surgically placed into the peritoneal cavity of a rabbit, along with an uninoculated and heat-inactivated controls. After 15-20 days, the broth containing the infectious exudate became opalescent, with no visible changes observed in the controls(12). Upon further cultivation of the organism in peptone broth, they were able to experimentally inoculate cattle. This resulted in the development of pleuropneumonia; deeming this microbe the causative agent.

In 1910, Bordet discovered filaments, vibrios, spirochaetes, and globules after culturing and staining the pleuropneumonia ‘virus’. He believed this was due to ‘metamorphosis’ of the virus during prolonged incubation, and that these different morphologies occur during developmental stages of the organism. Bordet proposed the new name *Asterococcus mycoides*
because of the radiating filaments that enveloped the organism while in the streptococci form, though retrospectively, these observations were likely due to contamination (11, 13). The ‘virus’ was then renamed again by Martzinovski in 1911, when he failed to replicated Bordet’s findings. He concluded that the microbe existed primarily as coccobacillus and therefore, proposed the name *Coccobacillus mycoides* (11, 12).

It was incorrectly characterized as an L-form of bacteria due to its resemblance to *Streptococci* in 1925. when Bridré and Donatien associated the ‘virus’ with polyarthritis, mastitis, and agalactia in sheep and goats (11, 14). Observational differences found after filtering and culturing led them to concluded that there was a second microbe belonging to this group. By 1935, this ‘group’ had seven different names largely because of the efforts made to explain the passage of these particles through filters that were impermeable to known bacteria. Klieneberger then created the term ‘pleruopneumonia-like organisms’ (PPLO) to describe the wall-less group of bacteria, though this did not alleviate the confusion surrounding these organisms (11, 14).

In 1944, the ‘virus’ termed Eaton’s agent, now known as *Mycoplasma pneumoniae*, was discovered in humans suffering from atypical pneumonia. Passage of this ‘virus’ was successful in chick embryos, and was detected by the development of pulmonary lesions in hamsters and rats, post-inoculation (15). The agent was incorrectly classified as a virus because it was not affected by antibiotics. It is known today that the antibiotic β-lactam works by disrupting the synthesis of bacterial cell wall components, a process that is absent in *mycoplasmas* (11).

A classification system was created by Edward and Freundt in 1956, which consisted of the order: *Mycoplasmatales* (alternative 1) and *Mollicutes* (alternative 2), family: *Mycoplasmataceae*, and Genus: *Mycoplasma* (16). It was not until 1958, that the etiologic agent
of bovine pleuropneumonia was officially named *Mycoplasma mycoides* by the Judicial Commission of the International Committee on Bacteriological Nomenclature. Nearly ten years later, the committee agreed that organisms with the ‘absence of a true cell wall and plasticity of the outer membrane’ belong to the class ‘*Mollicutes*’ (11, 17). Today, classification of species as *Mollicutes* includes 16S rRNA gene sequence analysis, DNA-DNA hybridization tests, directed serology and phenotypic data (11). There are five orders of *Mollicutes*; the *Mycoplasmatales, Entomoplasmatales, Acholeplasmatales, Anaeroplasmatales,* and *Haloplasmatales,* which belong to the phylum *Tenericutes.* This phylum is related to the *Firmicutes* phylum consisting of other bacteria with low G + C content (18). *Ureaplasmas,* determined by their ability to hydrolyze urea as an energy source, and *Mycoplasma* comprise the *Mycoplasmataceae* family. There are 117 known species in the *Mycoplasma* genus, seven in the *Ureaplasma* genus, as well as several within other families of *Mollicutes* that are considered legitimate species of *Mycoplasmas* (11).

**Section 2: Mycoplasmosis within Species**

*Mycoplasma* of Humans

Humans may become infected with several different *Mycoplasmas* including *Mycoplasma pneumoniae, M. genitalium, M. hominis, M. penetrans, M. fermentens,* and *M. salivarium,* and others. The most studied of these is *M. pneumoniae,* the pathogen responsible
for causing community-acquired interstitial pneumonia, also known as atypical pneumonia or ‘walking’ pneumonia (19). Clinical manifestations of atypical pneumonia often develop slowly, initially presenting with sinus congestion, pharyngitis, and otitis media. While the lower respiratory tract is eventually affected, it notably lacks lobar consolidation (15, 19). *M. pneumoniae* has also been found in association with asthma and Chronic Obstructive Pulmonary Disease (COPD) (20, 21), and less commonly, it has been found to lead to extrapulmonary conditions of the central nervous system including encephalitis and meningoencephalitis, cardiovascular conditions such as pericarditis, as well as conditions of the gastrointestinal system, renal system and the skin (22, 23).

It has been estimated that *Mycoplasma pneumoniae* causes up to 40% of community-acquired pneumonias (CAP). Active *M. pneumoniae* infection can be found in the nose, throat, trachea, and sputum, and is transmitted through aerosols (19, 22) with the incubation period lasting as long as 3 weeks. Respiratory illness develops slowly with a dry cough typically being the first symptom resulting from tracheobronchitis (19). While it affects people of all ages, infection develops into bronchopneumonia most frequently in school-aged children (22). Approximately 50% of asthma patients have evidence of *M. pneumoniae* infection, during which the number of mast cells, bronchial hyperresponsiveness, and Th2 cytokines are found to be increased (24). COPD patients are affected by *M. pneumoniae* in a similar manner; Th2 cytokines, specifically IL-4, is thought to exacerbate COPD by increasing the presence of immune cells and inducing goblet cell hyperplasia. This subsequently results in mucous gland hyperplasia and increases mucus secretion (24, 25). In addition to immunopathology, *M. pneumoniae* produces a toxin termed the Community-Acquired Respiratory Distress Syndrome (CARDS) toxin that has been shown to exacerbate allergic-type inflammation in the airway (26).
This ADP-ribosylating and vacuolating cytotoxin (27) acts by binding to, and thereby inactivating, human surfactant protein A (SP-A) primarily produced by type II pneumocytes (26).

Extrapulmonary manifestations of *M. pneumoniae* occur in as many as 25% of infected people. PCR testing has confirmed the presence of *M. pneumoniae* in other body sites including blood, synovial fluid, cerebrospinal fluid, pericardial fluid, and the skin (22). However, the frequency of its dissemination into tissues is unknown. It is likely that many cases result from autoimmune reactions due to sequence similarity of adhesin proteins and glycolipids found in the cell membranes of *M. pneumoniae* with those in mammalian tissues (22, 28, 29). Encephalitis and meningoencephalitis are the most common extrapulmonary complications, with encephalitis occurring most frequently in children less than 10 years of age (30).

To date, therapeutic and vaccine studies have been unsuccessful. Early attempts at vaccine development included formalin inactivated *M. pneumoniae* and the creation of attenuated strains through repeated passage; however, these approaches were unable to provide adequate protection (31). Interestingly, a vaccination study in mice reported disease exacerbation upon challenge even when vaccinating with a P30 cytadhesin mutant with the reduced ability to bind erythrocytes and inability to glide: two functions critical to the infection process (32, 33). Better understanding of the pathogenesis of *M. pneumoniae* is needed to develop effective therapeutics and vaccines.

*Mycoplasma genitalium* is associated with many urogenital infections including nongonococcal urethritis, cervicitis, endometritis, and pelvic inflammatory disease (PID), and has also been found in association with infertility (34). HIV patients have been shown to have higher rates of infection, though aside from these patients being immunocompromised, the
association between these two infections is poorly understood (35). \textit{M. genitalium} is very difficult to culture as it requires cocultivation in Vero cells and may take months to grow (36). Diagnosis can be performed with either PCR or transcription-mediated amplification assays, however, there are currently no commercially available, FDA-approved diagnostic tests on the market. (37–39). The difficulties with culturing and lack of standardized detection has negatively affected efforts made to understand \textit{M. genitalium}’s mechanisms of pathogenesis and impacts on human health (40). Coinfection of \textit{M. genitalium} with \textit{Chlamydia trachomatis} or \textit{Neisseria gonorrhoeae} is common (41). Women with \textit{M. genitalium} infection or coinfection present with inconsistent symptoms including pelvic pain, inflammation, and mucopus (42). Those suspected to be infected with \textit{M. genitalium} often receive one of the recommended syndromic therapies for nongonococcal urethritis or cervicitis such as doxycycline, though this is not very effective. Azithromycin has been preferred due to high cure rates, however bacterial resistance to this treatment has rapidly emerged (39, 43).

Another \textit{Mycoplasma} capable urogenital colonization is \textit{M. hominis}. While this organism has been found to be present in the absence of disease, it has been associated with bacterial vaginosis, infertility, pre-term births, and has been found present in PID (44, 45). Pro-inflammatory cytokine and chemokine levels have been found to be increased in amniotic fluid when \textit{M. hominis} infection is associated with pre-term births, and is also thought to play a role in subsequent brain damage of the infant (46, 47). IL-4, a Th2 inducing cytokine, has also been found at higher concentrations in amniotic fluids when compared to other infections such as \textit{U. urealyticum}. Differences in cytokine activation between \textit{U. urealyticum} and \textit{M. hominis} may suggest the means by which \textit{M. hominis} is able to evade immune destruction and allow it to persist (48). The \textit{M. hominis} gene \textit{goiC}, has recently been found in association with higher
bacterial loads and pre-term birth compared to isolated mutant strains lacking this gene, suggesting a potential role in virulence (47). Some studies have found *M. hominis* in other areas of the body including the lungs of transplant patients (49, 50), the skin and soft tissue of an immunocompromised pediatric patient (51), however further study is needed.

*Mycoplasma penetrans* is capable of infecting the urogenital and respiratory tracts (44, 52). First isolated from the urine of an HIV infected patient, subsequent studies have shown a strong association between the incidence and progression of both *M. penetrans* and HIV infections (53). *M. penetrans* first adheres to host cells, binds fibronectin and then induces cytoskeleton rearrangement through aggregation of tubulin and α-actinin resulting in their internalization within host cells (54). The protein MYPE9110 from *M. penetrans* has ADP-ribosyltransferase activity similar to the CARDs toxin found in *M. pneumoniae*, but has shown differences in activity, persistence and host cell targets. Within the urogenital tract this protein acts as a toxin resulting in ammonium chloride-dependent host-cell vacuolization (55). *M. penetrans* is thought to have a few of different mechanisms to avoid clearance by the host. In human serum, *M. penetrans* has shown to bind IgA, suggesting that it may act to prevent the Fc receptor of the IgA from binding to the CD89 receptor on neutrophils; therefore, avoiding host recognition and phagocytosis (56). Lipid associated membrane proteins (LAMPs) of *M. penetrans* has been shown to mediate apoptosis in mouse macrophages through NFκB activation (57). As found in other *Mycoplasmas* including *M. hyorhinis, M. bovis, M. pulmonis, M. gallisepticum, M. synoviae, and M. agalactiae*, *M. penetrans* is capable of antigenic variation by altering the gene expression of their surface lipoproteins. Because they are major surface antigens, it has been suggested that this contributes to the survival of *M. penetrans* by avoiding recognition by the host immune system (52).
Mycoplasma fermentans was first isolated from the urogenital tract, but has since been associated with conditions of the respiratory tract, has also shown to play a role in rheumatoid arthritis (RA) (58, 59). M. fermentans binds plasminogen, fibronectin, heparin and laminin; aiding in cell adhesin capabilities (60). LAMPs from M. fermentans have been shown to induce leukopenia and production of granulocyte-macrophage colony-stimulating factor (GM-CSF), presumably by macrophages, in the serum of BALB/c mice (61) (61-63). Expression of cytokines IL-6, IL-8, MCP-1, and CXCL1 was increased in human lung fibroblasts exposed to M. fermentans, however the reason for the selective upregulation of these cytokines and not others remains unknown (59). Lipoproteins of M. fermentans have been shown to induce ATP release and increase membrane permeability of lymphocytes and monocytes resulting in apoptosis (62). The M. fermentans lipid-antigen (GGPL-III) has been shown to be a potential virulence factor. This antigen was detected in nearly 40% of RA patients and resulted in the increased production of inflammatory cytokines TNF-α and IL-6 in mononuclear cells, and was also shown to induce the proliferation of synovial fibroblasts (63). Further study of this pathogenic mechanism may assist in the development of immunotherapeutics for RA patients.

Mycoplasma salivarium, typically part of the normal oral microbial flora residing in dental plaques and gingival sulci, increases in incidence and presence with the progression of periodontal disease, ostitis, pericoronitis and temporomandibular disorders (64). Recently, it has also been found intracellularly in epithelial cells of oral leukoplakia (65). Little is known about the pathogenic capabilities of M. salivarium, however studies have suggested its association with the progression of periodontal diseases. Similar to M. penetrans, M. salivarium was found to contain proteins capable of binding the Fc fragment of human IgG, suggesting it may prevent the binding of IgG with the Fc receptors on neutrophils (66). M. salivarium has also been shown to
play a role in inflammation. Exposing human gingival fibroblasts resulted in increased production of IL-6 and IL-8, two pro-inflammatory cytokines that may contribute to inflammatory response seen in periodontal diseases (67). The oxidant scavenging capacity of *M. salivarium* is another potential self-preservation mechanism by reducing the levels of reactive nitrogen and reactive oxygen species that are normally elevated during infection and disease (68). Activation of inflammasomes in dendritic cells and macrophages by *M. salivarium* was recently confirmed. This suggests *M. salivarium* likely contributes to the increased IL-1β levels, a pro-inflammatory cytokine associated with the death of matrix-producing cells in periodontal tissues resulting in increased destruction of alveolar bone and periodontal connective tissue (64).

Studies of human mycoplasmosis have indicated commonalities between certain species related to colonization, pathogenesis and survivability. This has aided in the understanding of their association with disease and virulence factors, however, further understanding of their pathogenic mechanisms, interaction with, and subsequent host response is needed to develop efficacious therapeutics and vaccines.

**Mycoplasma of Porcines**

Mycoplasmosis of swine results in significant economic loss worldwide even though mortality rates are low. *M. hyopneumoniae* and *M. hyorhinis* are the two most commonly known *Mycoplasmas* of swine. Highly contagious *M. hyopneumoniae* is the primary agent of catarrhal bronchopneumonia and results in poor feed conversion and slow growth. Infection results in epithelial cell hyperplasia and accumulation of mononuclear cells including lymphocytes and plasma cells (10). Known to adhere to ciliated epithelial cells, studies
assessing cytadherence of *M. hyopneumoniae* have found multiple membrane proteins including P159 and two repeat regions, R1 and R2 of P97, that mediate adherence by binding to heparin (69–71). An inactivated, adjuvanted whole-cell bacterin is frequently used in efforts to control infection, however the efficacy of the vaccine is dependent upon many factors including the age of infection and variation between isolates and herds (72, 73). Experimental studies in mice on a recently developed chimeric protein composed of four *M. hyopneumoniae* antigens; P97R1, P46, P95, and P42 resulted in the production of specific IgG antibodies, warranting further study as a vaccine candidate (74).

*M. hyorhinis* is typically found in the mucous membranes of the upper respiratory tract and the tonsils. While it can be present without disease, *M. hyorhinis* sometimes spreads to the lungs and contributes to pneumonia in piglets (10, 75). More commonly, however, it is known to cause serofibrinous to fibrinopurulent polyserositis and arthritis in swine (10). Interestingly, *M. hyorhinis*, once thought to be non-pathogenic in humans, has been detected in human gastric, lung, and pancreatic cancers. Furthermore, it has been shown to contribute to epithelial cell transformation (76) and promote cell migration and invasion through activation of the PI3-kinase-AKT signaling pathway upon binding of lipoprotein P37 and the production of IL-1β and LI-18 due to NLRP inflammasome activation (77, 78). A recent study assessing the metabolism of swine *Mycoplasmas* has suggested that the wider carbohydrate uptake of *M. hyorhinis* may contribute to its ability to colonize different body sites and hosts (79).
Mycoplasmas of Bovines

First described by Nocard and Roux in 1898, *Mycoplasma mycoides* subs. *Mycoides* Small Colony (MMMSC) is the etiological agent of contagious bovine pleuropneumonia (CBPP) (13, 80). CBPP is the only animal disease belonging to the A list of most severe infectious animal diseases, can spread rapidly, and is responsible for significant socio-economic loss in Africa, impacts international trade, and remains a threat internationally (81). Commercial vaccines currently available are live attenuated strains that may revert to virulence and require yearly boosting, therefore, efforts to develop improved vaccines continues (82). MMMSC possesses variable surface proteins (Vmm), that undergo high-frequency phase variation (83), as seen in many *Mycoplasma* species, and that different Vmm-type proteins are shown to elicit antibody responses of varying strengths (84). A recent clinical trial used a recombinant antigen vaccine candidate containing of Vmm and four immunogenic lipoproteins, however, only a weak humoral response was observed (85). L-α-glycerophosphate oxidase (GlpO) is a membrane-located enzyme of MMMSC involved in the metabolism of glycerol that yields toxic by-products such as H$_2$O$_2$ resulting in epithelial cell death (86, 87). Further assessment of the *glpO* gene suggested that removing its binding site would likely attenuate the cytotoxic capabilities of MMMSC, and has offered a point of research for future vaccine development (88).

*Mycoplasma bovis* causes chronic pneumonia and polyarthritis syndrome (CPPS), mastitis, conjunctivitis, otitis media, and meningitis, resulting in significant economic loss in North America and Europe (89, 90). *M. bovis* contains a membrane associated polysaccharide that acts as an inflammatory toxin which results in eosinophilic mastitis when inoculated into the udder (91). It has also been reported that *M. bovis*, like many *Mycoplasmas*, possess variable
surface lipoproteins. However, their \textit{vsp} gene complex is modified, providing them with a greater capacity for antigenic variation (92). It is thought that \textit{M. bovis} does not induce neutrophil extracellular traps which likely serves as a survival mechanism (93). Most of the current vaccines available for \textit{M. bovis} are bacterins or recombinant proteins that offer varied levels of protection. Recent research on vaccine candidates utilizing different antigens in combination with adjuvants is ongoing (94).

\textbf{Mycoplasmas of Caprines and Ovines}

\textit{Mycoplasma capricolum} subsp. \textit{capripneumoniae} commonly causes contagious caprine pleuropneumoniae (CCPP) resulting in high mortality rates in goats. It is also known to cause arthritis, mastitis, and septicemia, causing significant economic loss in Africa and Asia (95, 96). Belonging to the \textit{Mycoplasma mycoides} cluster, \textit{M. capricolum} subsp. \textit{capripneumoniae} is closely related to the less pathogenic \textit{M. capricolum} subsp. \textit{capricolum}. Both subspecies are genetically very similar to other members of the \textit{M. mycoides} cluster which has made detection/identification difficult. Genetic evaluation determined differences found within the lipoprotein gene \textit{lppA} is truncated in subsp. \textit{capricolum} suggesting it may be a virulence factor (96).

\textit{Mycoplasma agalactiae} causes mastitis, arthritis, keratoconjunctivitis, and pneumoniae, known as contagious agalactia, in sheep and goats (95, 97). \textit{M. agalactiae} is transmitted vertically from doe to kid during suckling, and is frequently isolated from milk samples of infected animals (98). Attempts to control \textit{M. agalactiae}/contagious agalactia through the use of vaccines and antibiotics have been able to reduce the appearance of new clinical signs and
mycoplasmal excretion, however, they are unable to prevent transmission (99). Known virulence factors of *M. agalactiae* include biofilm formation (100), production of hydrogen peroxide (101), and two homologues of nitrogen fixation proteins, NifS and NifU, which may enable adaptation to different growth conditions (97).

**Mycoplasmas of Rodents**

*Mycoplasma pulmonis* is a respiratory pathogen of rats and mice that causes respiratory mycoplasmosis, similar to atypical pneumonia in humans, and can induce changes associated with allergic airway disease and airway hyper-reactivity due to production of Th2 cytokines (102, 103). Characteristics of *M. pulmonis* that likely contribute to survivability within the host include biofilm formation (104, 105), and phase variation of surface lipoproteins (Vsa) to assist in evading the host immune response (106) and modulate adherence to pulmonary epithelial cells (107). Predicted virulence factors, based on the ability of other Mycoplasmas to produce hydrogen peroxide, include the secretion of the nuclease hemolysin, and glycoprotease (108). Studies on the immune response to *M. pulmonis* have indicated the importance of Toll-like receptor 2 (TLR-2) signaling in both the pathogenesis and clearance of the organism; it appears to be critical for airway mucin expression (20), but also results in decreased bacterial load when compared to TLR-2 knockout mice (109).

*Mycoplasma arthritidis* is a natural pathogen found in rats that causes severe polyarthritis, and has been shown experimentally to induce chronic proliferative arthritis in mice (110). Perhaps the most well-known characteristic of *M. arthritidis* is the *M. arthritidis* mitogen (MAM): considered to be a model superantigen (Sag). MAM acts on multiple immune cells
including T-cells, B-cells, NK cells and macrophages, presented via MHC class II on accessory cell surfaces (111, 112). This Sag plays an important role in toxicity and dermal necrosis; it has been shown that mouse strains with highly susceptible lymphocytes, when activated by MAM, develop a suppurative abscess which may lead to coagulative necrosis of the dermis(112). It is likely that the interaction of MAM with TLR-2 and TLR-4 differentially upregulates co-stimulatory molecule B7-1 on T-cells and therefore alters the cytokine response to *M. arthritidis* (113).

**Mycoplasmas of Felines**

*Mycoplasma felis* was originally isolated from a severe case of conjunctivitis in a cat (114). While this *Mycoplasma* can be found in normal conjunctival tissue, it is recommended that cats presenting with conjunctivitis who test negative for *Chlamydophila felis* and feline herpesvirus (FHV-1) should be further assessed for *M. felis* (115). *M. felis* is also associated with severe respiratory disease (116). Both diseases appear to occur more commonly in kittens than adult cats (115, 116). More recently, *M. felis* was isolated from bronchoalveolar lavage fluid samples of healthy cats as well as those suffering from feline asthma and chronic bronchitis. Further study is needed to determine whether *M. felis* may contribute to the exacerbation of these diseases, or whether this mycoplasma may represent a commensal organism of the lower respiratory tract of felines (117).

Erosive polyarthritis in cats is associated with *Mycoplasma gateae*. The organism was isolated strictly from the synovial fluid of affected cats, however, its pathogenesis and potential role in disease progression is not well known (118).
**Mycoplasmas of Canines**

There are over 15 species of *Mycoplasmas* that have been isolated from canines over the past 70 years. They form part of the normal bacterial flora in the upper respiratory tract, however *Mycoplasma cynos*, is the only species found in the lungs of dogs that is thought to exacerbate canine infectious respiratory disease (CIRD) (119, 120). A recent study on the prevalence of *M. cynos* in shelter dogs has shown that a high percentage of asymptomatic dogs harbor CIRD-associated pathogens including *M. cynos* (121). Other species isolated from the dog during pneumonia include *M. bovigenitalium, M. canis, M. edwardii, M. feliminutum, M. gateae*, and *M. spumans* (120). *M. canis* is also associated with urogenital disease and infertility and has been isolated from the prostate, epididymis and the bladder wall even after antibiotic therapy. Anemia in dogs is associated with *M. haemocanis* infection in immune-compromised animals. *Mycoplasma* sp. Strain HRC689 is part of the normal flora found within the colon, though it is questioned whether it may play a role in colitis (119). Little is known about the pathogenicity and virulence factors of canine mycoplasmas, though it has recently been shown that *M. cynos, M. canis*, and *M. molare*, secrete sialidase by catalyzing the hydrolysis of α-(2-3)-, α-(2-6)-, and α-(2-8)- glycosidic linkages of terminal sialic residues. This is a unique characteristic of these species, as other species such as *M. gallisepticum* and *M. synoviae* contain membrane-bound sialidase (122, 123). Sialidase activity was first thought to contribute to virulence after transposon insertion mutation in the sialidase gene of *M. gallisepticum* resulted in decreased lesions and recovery of the organism (124). Association with virulence was also been shown in *M. synoviae* where strains with greater sialidase activity were more virulent (125).
Further analysis is needed to determine whether secreted sialidase from these canine  
mycoplasmas contributes their pathogenesis.

Mycoplasmas of Reptiles and Piscines

In 1995, there was an epidemic in captive American alligators of pneumonia, pericarditis,  
and multifocal arthritis. It was not until four years later that an experimental infection study  
identified flesh-eating Mycoplasma alligatoris as the etiologic agent of the epidemic (126).  
Pathologic changes associated with M. alligatoris include fibrinous polyserositis and  
polyarthritis, with the severe and sudden inflammation leading to death (126). M. alligatoris was  
found to have genes encoding hyaluronidase and sialidase. These likely act as spreading factors  
when degrading the extracellular matrix (ECM), have been shown to promote CD-95 mediated  
fibroblast apoptosis, and therefore contribute to the virulence of this organism (127, 128).

Mycoplasma crocodyli, sharing 98% 16S rRNA gene similarity with M. alligatoris,  
causes necrotizing synovitis, and in some cases, subacute pneumonia in Nile crocodiles (129,  
130). This mycoplasma shows reduced virulence compared to M. alligatoris which may, in part,  
be due to the lack of sialidase activity (130). Another reptilian Mycoplasma identified around  
the same time as M. alligatoris is M. agassizii, which causes an upper respiratory tract disease in  
the desert tortoise (131, 132).

Mycoplasma mobile, the only known mycoplasma to infect fish, causes necrosis of the  
gills (133). This mycoplasma is one of 12 known species to have gliding motility, though the  
speed of its motility is considerably faster than the average speed of others including M.  
pulmonis, M. pneumoniae, M. genitalium, and M. gallisepticum (134). The molecule Gli349,
distributed around the cell neck of the organism, is responsible for cytadherence during gliding (135) as described by the ‘centipede model’ which relies on the ATP-dependent binding and subsequent conformational change that moves the mycoplasma forward (136). Further analysis of their gliding motility has identified Gli521 as the protein involved in the generation of a tilted propulsive force with a curved trajectory (137, 138).

**Mycoplasmas of Avians**

*Mycoplasma synoviae* is known for causing infectious synovitis in chickens and turkeys. It has also been associated with respiratory disease, though, depending on the strain, these infections are usually subclinical (139, 140). Decreased egg production and the observation of Eggshell Apex Abnormality (EAA) has motivated research on the potential association whether *M. synoviae* infects the oviducts and affects eggshell formation, though little evidence currently exists to support this notion (141). Infectious arthritis is still poorly understood, though it was recently shown that *M. synoviae* results in apoptosis of chondrocytes by upregulating genes involved in nitrous oxide production, caspase 3 activation, and mitochondrial inactivation (142). Virulence factors likely include phase variable *vlhA* lipoproteins which may assist in evading the host immune response (143), and sialidase activity (124). While the pathogenesis of this organism is not completely understood, a live attenuated vaccine (strain MS-H) was developed that elicits a systemic, humoral antibody response which prevents colonization (144, 145).

*Mycoplasma iowae*, a significant pathogen in turkey breeder hens, causes late embryo mortality, reduced hatchability, leg abnormalities, and may be involved in chondrodystrophy (146–148). Upon experimental infection of chickens and turkeys, airsacculitis, tenosynovitis,
arthritis, rupture of digital flexor tendons, and cartilage erosion was observed (148). Interestingly, dead-in-shell embryos showed colonization in the genital tract, respiratory tract and bursae, indicating the capability of *M. iowae* to survive in multiple organ systems (149). As seen in numerous mycoplasmas, *M. iowae* is capable of phenotypic variation which may contribute to host immune evasion and its ability to establish chronic infection (149). It has also been found that *M. iowae* encodes the gene *katE*, an active catalase that is known to be capable of degrading H$_2$O$_2$, which may increase its survivability (150, 151). When found in the intestinal tract, *M. iowae* does not cause disease; this may be due to the decreased expression of a CARDS-like toxin producing gene that occurs in when colonizing tissues with low atmospheric oxygen (150).

There are several other avian mycoplasma species that have been identified including *M. gallinarum* that infects chickens, pheasants, geese and sparrows, and *M. gallopavonis* that infects turkeys: both resulting in mild respiratory symptoms. *M. meleagridis* infection of turkeys causes sinusitis, airsacculitis and infection of the genital tract. In pigeons, *M. columbinasale* and *M. columborale* results in rhinitis and pharyngitis (152).

**Section 3: Mycoplasma Host Interaction**

*Mycoplasmas* have the ability to colonize and often establish chronic infection in a variety of tissues within different hosts despite having small genomes and limited metabolic capabilities. Their survivability is highly dependent on the interaction with the host, for which
they have various strategies used in disease pathogenesis and persistence while exposed to the host immune response.

**Cytadhesion and Gliding Motility**

Cytadhesion of *mycoplasmas* to host cells is a prerequisite for colonization and is important in the motility, virulence and pathogenesis of the organism. The structure is also important to adherence capabilities as *mycoplasmas* are generally flask shaped with cytadhesion occurring at ‘tip organelle’ located on the leading end. This organelle is composed of an electron dense core and contains a high concentration of adhesin molecules (2, 153, 154). The cytadhesion capabilities of *M. pneumoniae* have been the most extensively studied to date. The primary adhesin molecule protein P1 is assisted by P30, a protein that is known to be important in cell development, both of which are required for *M. pneumoniae* to adhere to respiratory epithelia (155–158). It is important to note that both P1 and P30 elicit strong immunological responses. Additionally, P30 shares sequence homology with eukaryotic structural proteins such as fibrinogen, keratin, and myosin, suggestive of molecular mimicry which may result in autoimmunity (155, 156). The P1 and P30 molecules are localized in the tip organelle, along with other molecules thought to be associated with cytadhesion. These include P40 and P90, and high-molecular-weight cytoskeleton proteins HMW1-HMW3 known to maintain proper distribution of the adhesin proteins. Their role was further elucidated when mutants lacking any one of these proteins were unable to cytadhere (2, 153, 159, 160).

Several *mycoplasmas* are known to possess cytadhesion molecules including *M. hyopneumoniae* (69–71), *M. mobile* (135), *M. agalactiae* (100), and *M. hominis* (161). However,
only *M. gallisepticum* and *M. genitalium* contain molecules with significant sequence homology to P1 of *M. pneumoniae*; GapA and MgPa, respectively (162–164). GapA is the primary cytadhesion molecule of *M. gallisepticum* and an important factor in virulence, as strains lacking the molecule were found to be avirulent in comparison (164, 165). Further sequence analysis revealed another molecule (CrmA) encoded within the *gapA* operon that is essential for cytadherence and pathogenesis (166). Interestingly, *crmA* shares sequence homology with the *ORF6* gene of *M. pneumoniae* which encodes a protein that is cleaved into cytadhesion molecules P40 and P90 (167). Other mechanisms that have been implicated in assisting cytadhesion include the ecto-ATPase activity of cytadhesin OppA in *M. hominis* (168).

Cytadherence is also involved in the immune response to *mycoplasmas*. *M. pneumoniae* was shown to induce cytadherence-dependent production of pro-inflammatory cytokines such as TNF-α and IL-1β due to *M. pneumoniae* lipoproteins such as P1, ligating TLR-2 receptors of monocytes *in vitro* (169). Lipoproteins isolated from *M. gallisepticum* have also been shown to increase the production of proinflammatory cytokines through the ligation of TLR-2 (170). Additionally, it has been observed that cytadhesion of *M. pneumoniae* induces autophagy-dependent TLR-4 signaling (171). Studies on vaccine development for *mycoplasma* infections have included mimic epitopes of *M. genitalium* and recombinant adhesion proteins of *M. suis* in attempt to induce a protective immune response (172, 173).

The ability to cytadhere coupled with the flask-like morphology enables *mycoplasmas* to move on solid surfaces in a unique process known as gliding motility (155, 174). Not all pathogenic *mycoplasmas* have gliding motility, however, those that do including *M. pneumoniae*, *M. genitalium*, *M. penetrans*, *M. mobile* and *M. gallisepticum*, motility has been indicated as an important virulence factor (174, 175). Adhesion molecules P1 and P30 of *M. pneumoniae* were
shown to be important in gliding motility as seen when anti-P1 monoclonal antibodies and deletions or substitutions within the P30 domain had inhibitory effects on motility (137, 176). MG200 and MG249 of M. genitalium (177), Gli521 and Gli349 of M. mobile (138), and GapA and CrmA of M. gallisepticum (178) are critical proteins involved in their motility. Most mycoplasmas utilize energy from ATP hydrolysis as reflected by the fact that depletion of ATP inhibited M. mobile motility (179). However, M. penetrans may be unique as it has shown to be able to use energy derived from temperature: in an environment depleted cellular ATP, increasing temperature increased the speed of gliding motility (180). While not yet identified, this suggests the potential for polarized polymerization of the cytoskeleton (180). Cytadhesion and gliding motility enable mycoplasmas to escape mucociliary clearance and to maintain close proximity to host cells, and therefore, play significant roles in the pathogenesis of these organisms.

**Invasion of Host Cells**

Mycoplasmas are generally considered to be extracellular pathogens as they are found to cytadhere and colonize the mucosal surfaces of their hosts. However, several mycoplasmas have been found in multiple locations/organ systems, and some have mechanisms for entering non-phagocytic host cells (155). These include M. bovis, M. suis, M. agalactiae, M. fermentens, M. hyorhinis, M. hominis, M. penetrans, M. genitalium, and M. gallisepticum (181–194). The first mycoplasma thought to invade host cells was M. penetrans as it was found to survive within host cells of the urogenital tract in AIDS patients (192), and has recently been shown to invade and survive in HeLa cells (185, 195). M. gallisepticum was shown to invade human epithelial cells
and chicken embryo fibroblasts in vitro (182). After experimental aerosol inoculation of chickens with M. gallisepticum, virulent strain R\textsubscript{low} was re-isolated from the heart, brain, liver, spleen, and kidney (189) and was also detected within erythrocytes sampled directly from the bloodstream (190). This suggests, that once M. gallisepticum crossed the mucosal barrier, it was able to disseminate intercellularly through the blood stream. While eukaryotic cell invasion was not directly observed, there was a positive correlation between respiratory infection and reisolation from internal organs (189). Most recently it was demonstrated that components of the host extracellular matrix (ECM) including type IV collagen, plasminogen and fibronectin, were necessary for M. gallisepticum to invade host cells (196). While these components are likely exposed during the pathogenesis of M. gallisepticum within the chicken trachea, evidence of tracheal epithelial cell invasion has not been seen.

**Host Cell Receptors**

Sialoglycoconjugates on the surface of the host cell have been established as receptors for M. pneumoniae, M. genitalium, M. synoviae, and M. gallisepticum (197). The adhesion of M. pneumoniae via adhesin molecule P1 to cultured erythrocytes and lung fibroblasts is mediated through sulfated glycolipids and glycoproteins containing α2-3-linked sialic acid (198, 199).

Cilia also plays an important role in the adhesion and pathogenesis of mycoplasmas in the respiratory epithelium. M. hyopneumoniae, known to colonize the surface of ciliated cells in the trachea and bronchi, bind host glycolipid receptors (200) with outer membrane adhesion molecules P97, P159, Mhp384, and Mhp385 (201, 202). The importance of cilia was further supported when M. pneumoniae accessed the base of the cilia utilizing gliding motility to
penetrate the mucuosal barrier. Tethered mucins where shown to not only support the mucus barrier, but enabled better gliding motility once the initial barrier was breached (203). Cytadhesion was markedly reduced upon squamous metaplasia, or the exposure of basal cells during cellular growth/turnover or as a host defense mechanism (203, 204). However, several surface exposed proteins of mycoplasmas have shown the ability to bind components of the ECM including plasminogen, fibronectin, and heparin, which may become exposed during the pathogenesis of mycoplasma infection (205). In M. gallisepticum, for example, has shown to bind fibronectin with their HMW3 homologue (206), binds heparin through an OsmC-like protein (207), and fibronectin with PlpA (208). While mycoplasmas have been shown to bind these components, definitive evidence that they contribute to cytadherence and subsequent colonization is still needed.

Mycoplasma have also been shown to result in ciliostasis; likely allowing better access to the base of the cilia (209). Further damage to cilia in the presence of cytadherent mycoplasma results in loss of cilia may benefit the host by limiting suitable binding cites (209), the typical host defense mechanisms including mucus production and ciliary clearance have therefore been shown to be ineffective at clearing mycoplasmas.

Mycoplasma Lipoproteins

Mycoplasmas are only enveloped by a plasma membrane, and therefore, do not contain lipopolysaccharides (LPS), lipotechoic acid, peptidoglycan, or flagella: the typical pathogen associated molecular pattern (PAMP) molecules that are frequently target by and interact with host cell receptors. Instead, mycoplasmas contain numerous lipoproteins, also known as lipid
associated membrane proteins (LAMPs), anchored within the plasma membrane (155, 210). A significant percentage of the mycoplasma genome encodes lipoproteins; in M. gallisepticum, variable lipoprotein hemagglutinins (VlhA) encoding genes accounts for 10.4% of their total genome (167). Several LAMPs are typically expressed by mycoplasmas at any one time. Many of these LAMPs are in close contact with host cells, likely acting as antigens, and may serve as immunomodulins contributing to pathogenesis (211). Ligation of pattern recognition receptors (PRRs) of the innate immune system include Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (212).

Numerous studies have implicated mycoplasma lipoproteins in the activation of the innate immune response and subsequent production of chemokines and cytokines. Lipoproteins are well known to ligate TLRs 1, 2, 6, (213) and TLR 15 specific to avian and reptilian species (214). Lipoprotein macrophage activating lipopeptide-2 (MALP-2) of M. fermentans and a 44 kDa lipoprotein from M. salivarium were the first found to interact with host TLR and activate the immune response as evidence by production of macrophage inflammatory protein-1α (MIP-1α) (215, 216). The structure of mycoplasma lipoproteins has also proven to be important. Triacylated lipoproteins ligate TLR-1/2 heterodimers and diacylated lipoproteins ligate TLR-2/6 heterodimers, sometimes requiring CD14, all indicating the importance of the MyD88-dependent signaling pathway. Additionally, the N-terminal lipid moiety was found to be responsible for activation of NF-κB and subsequent production of pro-inflammatory cytokines (53, 169, 217–220). The greater understanding of the interaction between mycoplasma lipoproteins and TLRs promoted research in several mycoplasmas such as M. genitalium, M. bovis, M. fermentans, M. pneumoniae, M. synoviae, and M. gallisepticum which further elucidated the role of cytokines and chemokines in the pathogenesis (28, 170, 218, 221–225). In chickens, in vivo analysis
showed that experimental infection of *M. gallisepticum* within the trachea induced the production of pro-inflammatory cytokines and chemokines such as IL-6, IL-8, CCL20, CXCL-13, CXCL-14, and RANTES (224), this pro-inflammatory response was further substantiated when LAMPs from *M. gallisepticum* resulted in the upregulation of IL-1β, IL-6, IL-8, IL-12p40, CCL20, and NOS-2 in cultured tracheal epithelial cells through an NF-κB dependent pathway via TLR-2 (170).

Ligation of mycoplasma lipoproteins to TLRs has also been shown to induce apoptosis and necrosis of host cells. *M. salivarium* and *M. fermentans* lipoproteins induced necrotic cell death in lymphocytic and monocytic cell lines, and induced necrosis or apoptosis of monocytic cell lines. Both functions were found to be dependent on caspase activation and TLR-2 ligation (62, 226, 227). The induction of ATP release from host cells after lipoprotein ligation has also been shown to result in host cell apoptosis in *M. salivarium, M. fermentans,* and *M. hominis.* Extracellular ATP (ATPε) binds purinergic receptors such as P2X7 and P2Y1 on host cells that activate leukocytes (62, 227–229) and epithelial cells (230). Binding of ATP to these receptors results in the release of activated caspase 1 in the inflammasome and subsequent release of mature IL-1β (231, 232). This indicates another pathway induced and pathogenic mechanism of mycoplasma lipoproteins.
Section 4: Host Immune Response to Mycoplasma

Induction of Innate Immune Response

The host’s initial encounter with mycoplasmas typically occurs with the epithelial cells of the mucosal tissues of the respiratory, reproductive or conjunctiva. As discussed above, gliding motility and/or the capability to cytadhere to host cell receptors enables mycoplasmas to successfully colonize and often times result in pathogenesis. The proximity to the host cells enables lipoproteins anchored within the membrane of mycoplasmas, acting as PAMPs, to ligate PRRs such as TLRs and NLRs important in recognizing the presence of the bacteria subsequent pro-inflammatory signaling (233).

Factors such as nuclear or cytosolic proteins and ATP are also involved in the innate immune response by acting as DAMPs that ligate to nucleotide binding oligomerization domain (NOD) -like receptors (NLRs). Ligation and subsequent activation of the NLR pathway can result in the production of chemokines and pro-inflammatory cytokines, as well as activation of the inflammasome. Extracellular release of ATP has been shown to occur in the presence of mycoplasmas resulting in the activation of the Nlrp-3 inflammasome due to the ligation of ATP to the P2X7 purinergic receptor. Activation of the inflammasome resulted in increased production of IL-1β (62, 228, 229). Inflammasome activation during host defense typically occurs in macrophages, as was seen when lipoproteins of M. fermentans and M. salivarium and the presence of extracellular ATP acted synergistically to activate macrophage inflammasomes (62). However, inflammasomes have been shown to become activated in respiratory epithelium (234, 235). Recently, M. pneumoniae CARDS toxin was shown to regulate inflammasome
activity through bacterial ADP-ribosylation, resulting in activation of the Nlrp-3 inflammasome (236).

The role of type-1 macrophages as mediators of inflammation through antigen presentation has shown to be limited during mycoplasma infection. Early studies showed that peritoneal macrophages attached to M. pulmonis without hindering the growth of the M. pulmonis population (237), while others showed that M. pulmonis was in fact phagocytized by peritoneal macrophages (238). A study assessing the virulence of M. pulmonis strains based it on the ability of the organism to survive in the respiratory tract, induce pneumonia, and persist in the peritoneal cavity. These factors were largely based on the ability of these strains to resist killing by macrophages. Strains that evaded phagocytosis typically resulted in exacerbated disease (239). A recent study showed that the ability of M. pulmonis to evade alveolar macrophages was dependent on the length of their Vsa lipoproteins: long Vsa lipoproteins containing tandem repeats prevented macrophage binding (240). They further explained that the previous studies were not able to replicate this observation in part because they exposed macrophages to M. pulmonis that had been grown in a biofilm, which yields shorter lipoproteins (240).

Mycoplasma induced lesions in the early course of infection largely consist of neutrophils/heterophils, depending on the host species. Previous studies assessing the interplay between neutrophils and M. pneumoniae, M. hominis, and M. salivarium suggested that neutrophils spontaneously bind mycoplasmas through complement receptors, activate C1 of the complement system, but without the prior opsonization by antibody, they remained viable within the neutrophil upon phagocytosis (241). An important role that neutrophils play during microbial infection is the release of neutrophil extracellular traps (NETs) composed of
antimicrobial peptides and enzymes. Assessment of mammary glands infected with *M. agalactiae* confirmed that NETs were released during *mycoplasma* infection, and further determined that this could be induced by the interaction of *mycoplasma* lipoproteins with neutrophils (242). Production of reactive oxygen species (ROS) by neutrophils is thought to be a necessary preliminary step prior to the release of NETs. ROS production was found to be prevented upon exposure to *M. bovis* which also prevented the NET release (243). Furthermore, it has been observed that *M. bovis* enhances neutrophil apoptosis, inhibits nitric oxide production, and increases neutrophil elastase production known for degrading the extracellular matrix which may represent an attempt to clear the pathogen (244). However, long-term survival of *M. bovis* has been found in necrotic lesions despite the presence of neutrophils and macrophages (245). Interestingly, *M. pneumoniae* was shown to induce the release of a cathelin-related antimicrobial peptide (CRAMP) from neutrophils which may be able to kill the organism. However, *M. pneumoniae* produces Mpn491, a nuclease that has been shown to degrade other components of NETs (246). These studies indicate that neutrophils may not be very effective at eliminating *mycoplasma* infection, and due to the release of histamines as seen in *M. pneumoniae* infection, neutrophils may further contribute to lung and airway inflammation (247).

Natural killer (NK) cell activity has been enhanced by some species of *mycoplasma*. The lytic capability if human NK cells was augmented both directly and indirectly due to cytokines produced by T cells (248). *M. pulmonis* infection resulted in splenic and pulmonary NK cell activity and indicated that exacerbation of disease occurred upon NK cell depletion (249). However, it was found that NK cell depletion at the time of a nasal-pulmonary vaccine against *M. pulmonis* resulted in a stronger adaptive immune response, and the presence of NK cells at the time of vaccination skewed cytokine production resulting in higher IL-4, IL-13 and IL-17
Activation of TLR-2 signaling with *M. fermentans* lipoprotein MALP-2 was not able to induce NK cell activation (251).

**The Role of Chemokines and Cytokines**

Colonization of the mucosal epithelium by pathogens results in the production of chemokines and cytokines by epithelial cells in contact with the pathogen. This results in the recruitment and activation of innate immune cells including neutrophils (heterophils in avian species), macrophages, natural killer (NK) cells, dendritic cells, eosinophils and basophils to the mucosa (233). The intended purpose of these cytokines and chemokines is host defense, however in many instances, as seen in the response to mycoplasmas, they contribute to the manifestation and immunopathology observed in chronic disease.

Human cultured endocervical epithelial cells exposed to *Mycoplasma genitalium* were found to produce IL-6, IL-7, IL-8, MCP-1 and GM-CSF during early infection, and IL-8, MIP-1β and MCP-1 during late stage infection (220, 252, 253). Experimental infection of pigs with *M. hyopneumoniae* showed increased expression if IL-5 and IL-13 in both epithelial and mononuclear cells (254). Patients infected with *M. pneumoniae* showed increased expression of IL-8 and RANTES in nasal epithelial cells, while *in vitro* analysis using adenocarcinomic human alveolar basal epithelial cells (A549) showed increased expression of IL-1β, IL-8 and TNF-α (255). Cultured tracheal epithelial cells exposed to *M. gallisepticum* LAMPs resulted in the transient upregulation of several cytokines and chemokines including IL-1β, IL-6, IL-8, IL-12p40, CCL20, and NOS-2 (170). The cytokines and chemokines observed in these studies are well known for the activation and recruitment of leukocytes.
Mycoplasmas have also induced the production of chemokines and/or cytokines by inflammatory cells, further contributing to disease outcome. LAMPs were shown to induce production TNF-α, IL-1β and MIP-1β upon infection with mycoplasmas including *M. pneumoniae*, *M. gallisepticum*, *M. fermentans* and *M. penetrans* (53, 169, 171, 217, 221, 256). The chemotaxis of inflammatory cells into the lamina propria often results in gross inflammatory lesions and subsequent increased production of cytokines including, but not limited to IL-1β, IL-4, IL-5, IL-6, IL-17A, IFN-γ, and TNF-α as seen in infection with *M. pulmonis* and *M. mycoides* subsp. *mycoides* (257–259). The cytokines and chemokines produced can significantly impact the balance between Th1, Th2, and Th17 responses. A predominant Th1 response is associated with a strong pro-inflammatory response associated with cell mediated immunity and production of cytokines such as IFN-γ capable of exacerbating disease. A Th2 response is associated with humoral immunity and allergic inflammatory responses through the production of cytokines including IL-4. Th17 results in a pro-inflammatory response associated with bacterial infections, autoimmunity, and the production of cytokines such as IL-23 and IL-17 (260). *M. pneumoniae* infection has been shown to induce a significant increase in IL-4, suggesting a Th2 response (261), though vaccine-mediated exacerbation upon *M. pneumoniae* infection yielding increased IL-17 and eosinophilia (33). Furthermore, while IL-6, IL-10, IL-17A and TGF-β were increased using the mouse model for *M. pneumoniae*, it was suggested that Treg and IL-10 are involved in the suppression of IL-17A production which may interrupt the Th1/Th2/Th17 balance (262). While another study showed that *M. pneumoniae* induced macrophage production of IL-23 was required for IL-17 production by CD4+ T cells (260). Patients with pneumonia due to infection with *M. pneumoniae* showed increased expression of both Th1 and Th2 cytokines including IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ (261, 263). In contrast, *M. gallisepticum*, known to
cause chronic respiratory disease in chickens, results in increased expression of Th1 cytokine
genes including those resulting in IL-1β, IL-6, IL-8, and IL-12p40 (170, 224). These data further
emphasize the importance of cytokine and chemokine production as a result of *mycoplasma*
infection and the role they can play in host defense or the development of immunopathologic
conditions.

**Adaptive Response to Mycoplasma Infection**

Activation of the adaptive immune response is a complex and specialized process
involving antigen presentation initiated by cells of the innate immune system, including
macrophages and dendritic cells, to T- and B- lymphocytes to induce adaptive immunity. The
adaptive immune response to *mycoplasma* infection is still poorly understood. While an antibody
response is significant in limiting disease progression, many *mycoplasmas* have mechanisms to
evade or counteract this host defense. Most *mycoplasmas* are extracellular pathogens with
questionable efficacy of antigen presentation. Because of this, a significant mediator of the
adaptive immune response during *mycoplasma* infection is the production of cytokines and
chemokines. Therefore, the ability of the innate response to activate adaptive immunity largely
dictates the whether the host response will be effective in *mycoplasma* clearance, or result in
immunopathology and further exacerbation of disease.

Lesions composed of T and B cells are found in the tracheal mucosa and bronchus-
associated lymphoid tissue (BALT) of the lungs resulting from persistent *mycoplasma* infection
at the mucosal surface (264). As seen in *M. mycoides* ssp. *Mycoides, M. capricolum, M.
agalactiae*, and *M. bovis*, these populations are largely composed of CD4+ and CD8+ T cells,
with CD4+ T cells outnumbering CD8+ T cells 2 to 1 (264, 265). Mice infected with M. pulmonis were found to have and increased T cell populations in the lungs and lymph nodes of the lower respiratory tract, with greater numbers of CD4+ T cells than CD8+. Th1 responses were dominant in the spleen, with a mix of Th1 and Th2 (skewed Th1) in the lungs producing both IL-4 and IFN-γ cytokines. Depletion of CD8+ cells in vivo resulted in disease exacerbation, while CD4+ depletion reduced the severity of disease (266). Neither condition reduced the mycoplasma population, however, it showed that production of IFN-γ by CD8+ T cells can play an important role during infection with an extra-cellular mycoplasma (249). Differences have been observed in the IFN-γ (Th1 response) and IL-4 (Th2 response) cytokine requirements in the upper and lower respiratory tracts. In the upper respiratory tract, loss of either cytokine did not appear to have an impact on mycoplasma numbers or disease pathogenesis. However, it was noted that there was a compensatory immune response dominated by macrophages and CD8+ T cells. In the lower respiratory tract, the loss of IFN-γ resulted in increased mycoplasma numbers and pathogenesis (103). Further analysis of the importance of IFN-γ and IL-4 revealed that the development of adaptive immunity against mycoplasma was impaired when vaccinating IFN-γ-/- mice, whereas vaccinating IL-4-/- mice led to improved ability for the host to clear the mycoplasma (259). These studies show that CD8+ T cells, which may contribute to IFN-γ production, and Th1 IFN-γ, an important macrophage activator, are important in the host response to mycoplasma. While both are pro-inflammatory, they aid in preventing a Th2 skewed response that has shown to result in further chemotaxis of inflammatory cells in the airways as seen in asthma (267).

IL-23 activation of Th17 cells and the subsequent production of IL-17 has also shown to be important during mycoplasma infection. During early M. pneumoniae infection, alveolar
macrophages were shown to be a significant source of IL-23 which was found to induce production of IL-17 by lung CD4+ T cells. Disruption of the IL-23/IL-17 balance resulted in decreased neutrophil recruitment and activity, and reduced *mycoplasma* clearance (260). However, IL-17 has also been shown to be involved with disease exacerbation. BALB/c mice vaccinated with an avirulent *M. pneumoniae* P30 mutant showed increased levels of IL-17 corresponding with eosinophilia upon challenge with a virulent strain (33). It has also been suggested that the breakdown of the immune balance between Th17/IL-17 and Treg/IL-10, as both IL-17 and IL-10 increased in a dose dependent manner upon repeated stimulation with *M. pneumoniae* antigens which may lead to extrapulmonary complications associated with *mycoplasma* infection (262). Recently, it was shown that CD25+ Treg cells help to control the damaging immune response to respiratory *mycoplasma* infection by reducing lymphocyte infiltration into the lungs. This response was not mediated through IL-10 or TGF-β, but instead was mediated by the Treg induced shift away from the Th2 response towards a Th1 response with associated IFN-γ and IL-17 production (268). Therefore, it is has been suggested that the balance between Th1/Th2/Th17 responses to *mycoplasma* may be important to the progression of disease, however further understanding of their roles is still needed.

Antibody response is important in the host defense against *mycoplasma*, and for vaccine development, and may prevent dissemination of disease within a population (269). However, natural infection with *mycoplasma* has shown a dysregulated and sometimes maladaptive response. Additionally, some *mycoplasma* species have developed mechanisms to evade this response by producing chemotactic factors, proteases, and Fc receptors (270). Different species of *mycoplasma* have been found to elicit antibody responses from all classes, with IgM being the first antibody generated and largely involved in the control of *mycoplasma*. IgM is typically
followed by IgG which is able to mediate complement activation and opsonization (271).

Because *mycoplasmas* are predominantly mucosal pathogens, the antibody response is dominated by IgA and IgG, and will occasionally result in IgE isotypes at the mucosal surface (272). *M. pneumonias* resistance has shown better correlation with mucosal IgA than serum IgG or IgM. Similarly, early studies on the adaptive response to *M. gallisepticum* showed that local immunoglobulin titers of IgG and IgA at the mucosal surface positively correlated with immunity *in vivo*, but that serum antibodies titers did not (272). Natural *M. gallisepticum* infection results in infiltration of lymphocytes into the lamina propria including non-specific IgG and IgA secreting B-cells. Challenge after vaccination yields a more organized response with fewer lymphocyte infiltrates, yet contain B-cells secreting anti-mycoplasma IgG which appears to protect chickens from disease. Titers of IgA were far lower than those of IgG, and it is thought that both may play a role in blocking initial cytadhesion (273, 274). While IgG seems to play a significant role in the protection from *M. gallisepticum*, it is important to note that *M. gallisepticum* and *M. synoviae* both have the gene *cysP* that is shown to encode a cysteine protease capable of cleaving chicken IgG into Fab and Fc fragments (275). A recent study on *M. mycoides* ssp. *capri* identified a complex (MIB-MIP) that this species uses to capture and cleave IgG. The MIB component is the *mycoplasma* Ig binding protein that binds the Fv region. The immunoglobulin protease MIP is then recruited to cleave the immunoglobulin (276). These mechanisms likely enable the prolonged survival within the host despite the antibody response (277).

Antibody response is also capable of resulting in further disease exacerbation and complications. Patients with *M. pneumonias* infection of the lower respiratory tract had comparatively higher levels of serum pro-inflammatory molecules, as well as higher Th2
cytokines and IgE within the lungs. This suggested that the exacerbation of disease found with Th2 cytokines and IgE in the lower respiratory tract are precursors for the development of asthma (278). Children infected with *M. pneumoniae* presenting at clinic with wheeze were found to have increased levels of the vasoconstrictor ET-1, IL-5, and serum IgE which was suggestive of the exacerbation of asthma (279). Conversely, patients without wheeze infected with *M. pneumoniae* were shown to have increased IL-5 and vascular epidermal growth factor (VEGF), yet similar levels of IFNγ, IL-4 and IgE as those with wheeze (280). While there is some disagreement, the evidence supporting *M. pneumoniae* contributing to the development or exacerbation of asthma suggests that *M. pneumoniae* induced IgE production plays an important role.

Arthritis mediated by anti-mycoplasma antibodies has also been found in association with *M. arthritidis, M. fermentans, M. hominis, M. synoviae, M. bovis,* and *M. pneumoniae* either by direct infection of the synovial tissues or by antibody mediated disease. The superantigen of *M. arthritidis* (MAM) was shown to be able to both induce arthritis and exacerbate it after immunization with type II collagen in mice (281). Though it has been suggested that MAM is only associated with lethal toxicity and not arthritis in mice (282). In humans, *M. arthritidis* has been found in the synovial fluid of rheumatoid arthritis (RA) patients (283). However, there is inconsistent evidence whether these patients have increased IgG or IgM anti-MAM antibodies (281, 284). *M. fermentens* has been found in the synovial fluid of patients with RA, osteoarthritis, and crystal synovitis (283, 285). In RA patients, IgM and IgG antibodies against *M. fermentens* were found more frequently in the blood than in healthy patients (286). *M. pneumoniae*, also found in the synovial fluid of RA patients, has been associated with specific anti-mycoplasma IgM antibodies. A rapid test is commercially available (ImmunoCard
Mycoplasma; Meridian Bioscience, Cincinatti, OH) to detect IgM anti-
M. pneumoniae antibodies in serum. While a limited number of patients with mycoplasma specific IgM were found to have arthritis (287), a study assessing reactive arthritis in children found a large proportion of patients having anti-M. pneumoniae IgM in their serum (288). Immune complexes (IC) have been found deposited in tissues as well as circulating in the blood of mice experimentally infected with M. arthritidis (289), and upon purification from synovial fluid of RA patients, have been shown to react with proteins from various mycoplasma species (290). These studies have observed the association between mycoplasmas and arthritis, and have offered a potential mechanism (IC) by which mycoplasma may indirectly result in or contribute to arthritic conditions. However, further study is needed to fully appreciate their role in this disease.

**Mechanisms of Immune Evasion**

Two characteristics of mycoplasmas that are suggested to aid in immune evasion are antigenic mimicry and surface variable expression of lipoproteins. Early studies on antigenic mimicry of mycoplasmas observed that rabbit antisera against strains of M. arthritidis reacted with a component of rat muscle tissue (291), and identified a murine monoclonal antibody against M. hyorhinis that reacted with mammalian intermediate filaments. This antibody recognized a protein epitope of M. hyorhinis of which similar epitope components were found in other mycoplasmas including M. pulmonis, M. fermentans, M. hyopneumoniae, and M. pneumoniae, defining an antigenic structure likely important in autoantibody development (292).
Recently, antibodies against *M. penetrans* HF-2 epitopes have been used in efforts to develop a vaccine against melanoma due to the observed cross-reaction with MAGE-A6 expressed on tumor cells (293). Both P1 and P30 adhesion molecules of *M. pneumoniae* have shown antigenic mimicry. Monoclonal antibodies against P1 cross-reacted with human GAPDH and enolase of the glycolytic pathway. It was suggested that these antigenic similarities may be responsible for the non-responsiveness or self-limitation of the immune response to *M. pneumoniae* (294). Further analyses determined that both P1 and P30 adhesins share homology with eukaryotic structural proteins including fibrinogen, keratin, myosin and actin. This was confirmed when P30 antibodies cross-reacted with fibrinogen, and anti-fibrinogen antibodies bound to wild-type P30 (156).

Many *mycoplasma* species are capable of altering the size, structure, or expression of their surface proteins. Possible reasons for the development of these mechanisms may include the need to avoid mechanical clearance by expressing tissue-appropriate adhesins, and avoiding recognition by the host immune response (295). Strategies used to accomplish the variation include phase and antigenic variation and epitope masking. Specifically, on/off switching may be accomplished by genetic events including DNA slippage and site-specific recombination of the genes or their promoters, size variation can occur via DNA slippage, base substitutions, or posttranslational modification, domain shuffling may occur through non-reciprocal, reciprocal recombination or intrachromosomal recombination with deletion (295).

The first described mechanism of *mycoplasmas* gene switching involved the molecular on/off switch of *M. hyorhinis* vlp genes due to DNA slippage in the promoter regions (296, 297). The surface lipoproteins of *M. hyorhinis* are encoded by a genetic system of 3-8 elements in the chromosome (298, 299). Each vlp gene is considered a single transcription unit containing a
poly-A tract within the promoter region between the -35 and -10 sequences. The Vlp is expressed only when the poly-A tract is 17bp long, therefore, a single insertion or deletion prevents transcription (297). Size variation of expressed Vlp products through DNA slippage involving close repeats within coding sequences is advantageous for immune evasion. Isogenic clonal variants of *M. hyorhinis* expressing different Vlp profiles were assessed for their susceptibility to growth inhibition by serum Abs of swine. This study found that the variants expressing longer versions of VlpA, VlpB, or VlpC were resistant to the host serum Abs. Furthermore, it was determined that the Vlp products themselves were not the target of the host Abs, but that the long versions of Vlp were able to mask the antigen targeted by host Abs (300). A similar mechanism is utilized by *M. gallisepticum* for the expression of variable lipoprotein hemagglutinin (*vlhA*) genes. These genes include 38 with signature *vlhA* features and 5 pseudogenes possessing *vlhA* sequence homology, and together comprise approximately 10.4% of their total genome (167). Phase variation is controlled at the transcriptional level, and was once thought to require 12 GAA trinucleotide repeats upstream of the -35 box for expression of a given *vlhA* to enable proper spacing between flanking sequences for accessory factors to bind (301–305). However, recent transcriptional analysis has indicated that 12 GAA repeats were not necessary for expression of a given *vlhA*, and suggested that a different mechanism may be responsible for the regulation of *vlhA* expression (306). Furthermore, a previous assessment found that both chicken serum antibody responses and surface antigens of *M. gallisepticum* underwent rapid change *in vivo*, supporting the hypothesis that the changes in *vlhA* expression may be an adaptive mechanism to evade the host immune response (307). However, while this hypothesis is not directly disputed, it was later found that variation in the expression of surface proteins occurred in nearly 40% of cells within 2 days (302). This was further supported by
transcriptional analysis which also showed changes in \( vlhA \) expression occurring as early as 2 days post infection (306). While interaction with the host may play a role in the changes of \( vlhA \) expression, it remains uncertain whether the changes are mechanisms to evade host defense mechanisms.

**Section 5: Mycoplasma gallisepticum**

**Disease and Significance**

*Mycoplasma gallisepticum* is a significant pathogen of poultry largely affecting the respiratory and reproductive tracts (308). This pathogen spreads both horizontally and vertically (309, 310), resulting in significant economic loss due to poor feed conversion efficiency, reduced egg production and hatchability (311, 312). *M. gallisepticum* is the primary etiologic agent of chronic respiratory disease (CRD), though it is typically found concurrent with other infectious pathogens including Newcastle disease virus, Avian Influenza virus (AIV), infectious bronchitis, pathogenic *E. coli*, *Haemophilus*, and avian rhinotracheitis virus (313–315). Characteristics of *M. gallisepticum* infection include coughing, sneezing, nasal discharge, tracheal rales, increased mucus production and loss of cilia, labored breathing due to airsacculitis, and conjunctivitis (316, 317). Inflammatory lesions are commonly found in the trachea, lungs, and air sacs due to the infiltration of heterophils and macrophages during early
infection, with lymphocytes (predominantly T cells) populating the lesions beginning around 5 days post infection (316, 318).

*M. gallisepticum* was once determined by the United States Federal Government to be one of the top 3 most significant avian pathogens to U. S. poultry producers. As a consequence of the rapid spread, chronicity of infection and significant economic impacts resulting from *M. gallisepticum* infection, it was including in the National Poultry Improvement Plan (NPIP) beginning in the 1960’s (NPIP05). This plan served to provide guidelines to control the spread of the pathogen, and improve poultry and poultry products throughout the U. S. Strategies currently used by commercial farms to reduce the spread and morbidity of this pathogen include strict biosecurity measures, antibiotic therapy, and vaccination programs. However, medications and vaccines have not been able to eradicate the infection, especially in multi-age layer farms. Therefore, in severe cases, culling of entire flocks may be used to control the spread of infection (312, 319).

Further risk of spreading *M. gallisepticum* between backyard flocks is likely due to infection of turkeys and house finches. Infection in turkeys results in sinusitis and conjunctivitis, and house finches present with conjunctivitis (313, 320). Recently, the emergence of a new strain of *M. gallisepticum* was identified in house finch (*Carpodacus mexicanus*) populations in the eastern United States in 1994. This strain causes severe conjunctivitis in house finches; however, it only results in mild disease when used to experimentally infect chickens (321). *M. gallisepticum* has also been isolated from passerine birds including purple finches, blue jays, and American goldfinches which may contribute to the spread and morbidity (322).
Virulence Factors and Pathogenesis

A critical first step in *M. gallisepticum* pathogenesis is cytadhesion to the host’s mucosal epithelium as it prevents clearance by the host mechanisms such as ciliary action, and enables colonization of the respiratory tract (174, 197). Cytadhesion is mediated by the primary adhesin molecule GapA, and the cytadhesion associated molecule CrmA (166). These molecules are thought to be homologs of the P1 and B/C adhesin molecules of *M. pneumoniae* (164, 167). Once attached, pathological changes occur in the host cells including increased mucus production and release from goblet cells, ciliostasis and loss of cilia, and rounding and exfoliation of epithelial cells (323, 324). Genomic sequencing of attenuated *M. gallisepticum* strains indicated that avirulent *R_{high}* lacks several genes compared to virulent *R_{low}* including gapA and *crmA* (325). To restore cytadherence and virulence to *R_{high}* complementation with both gapA and *crmA* was necessary (166). Transposon insertion mutations in *R_{low}* gapA and *crmA* genes has also shown to reduce the cytadherence capabilities by approximately 75% which significantly reduces the virulence of this strain in the host, further indicating their importance as virulence factors (326). Avirulent *R_{high}* was also found to lack two genes responsible for binding fibronectin; hlp3 and plpA (208, 325).

As seen in other *mycoplasma* species including *M. mycoides* ssp. *mycoides*, metabolic proteins have been linked to virulence (86, 87). Dihydrolipoamide dehydrogenase (lpd) is part of the pyruvate dehydrogenase complex pathway that is responsible for ATP production during glycolysis. The *lpd* gene was shown to have an important role in *M. gallisepticum* virulence when disruption of this gene with a transposon insertion resulted in a significantly attenuated strain (327). This strain resulted in minimal to no pathology in the host and was rarely recovered
2 weeks post infection (328, 329). Due to the mutation, the organism was likely unable to produce sufficient ATP to provide energy for survival within the host (328).

A mycoplasma-specific lipoprotein named ‘MslA’, is an immunogenic lipoprotein that exhibits reduced expression in the live attenuated F strain vaccine. Furthermore, R$_{low}$ showed attenuated virulence in experimentally infected chickens when this gene underwent transposon mutagenesis (330). While its association with virulence is not fully understood, it has been hypothesized that MslA may bind TLRs resulting in the activation of NF-$\kappa$B and subsequent production of pro-inflammatory cytokines and chemokines (330).

Sialidase activity has also been shown to play an important role in virulence. Transposon mutagenesis of $M$. gallisepticum gene MGA_0329, a homolog of sialidase in $M$. pneumoniae, resulted in the loss of sialidase activity as well as decreased virulence in experimentally infected chickens. This was characterized by less severe tracheal lesions and mucosal thickening, as well as reduced bacterial recovery (124). Given that sialidase production was shown to positively correlate with virulence, it was surprising that complementation was able to restore sialidase activity but did not restore virulence (125). While the exact role of sialidase in virulence remains unknown, it has been hypothesized that sialylation protects against the hydrolysis of glycosidic bonds on the host cell surface and prevents degradation of the host ECM. Therefore, removal of sialic acid residues may expose host antigens and play a role in autoimmune complications (124). However, given the inability of sialidase complementation to restore virulence, the relationship is likely more complex than originally thought.

As previously discussed, variable expression of vlhA genes may be associated with virulence as they are a significant component of host-pathogen interaction. However, changes in expression of vlhA genes early in the course of infection indicate that they do not play a role in
the evasion of host antibodies (302, 306). It has been suggested, however, that the ability of VlhA products to bind red blood cells may contribute to the dissemination of the organism to other locations within the host (331).

*Mycoplasma gallisepticum* Vaccines

Numerous attempts have been made to develop safe and efficacious vaccines to protect poultry from *M. gallisepticum* infection. The two types of vaccines that are currently available are bacterins, which are inactivated suspensions of whole organisms, and live attenuated vaccines (LAV). Bacterins initially showed promise as vaccinated birds showed reduced clinical signs and air sac lesions, reduced the decline in egg production, and prevented vertical transmission (332–337). However, the reduction in bacterial load was only minimal (338), and the lesions, while less severe, remained present (339). Additionally, bacterins only provided limited protection against virulent R strain challenge (332–337). Adjuvanted LAVs have been used for *M. agalactiae, M. capricolum subsp capricolum, M. mycoides subsp mycoides* large colony type, and *M. hyopneumoniae*. These vaccines have been shown to provide partial protective efficacy and reduction in the spread of disease (340, 341).

The live attenuated F strain vaccine was derived from a strain first identified in 1956 (342). Continued passage of this strain revealed that the F strain had potential efficacy as a vaccine candidate once it had been passaged between 200 and 250 times (343). This vaccine elicits a strong serological response (344), though the antibody response is not as significant as that seen in vaccinated chickens challenged with virulent R_{low} (343). The F strain vaccine is protective without resulting in lesions (345–347), is capable of displacing virulent R_{low} (348)
thereby decreasing the bacterial load, and likely accomplishes this by outcompeting the virulent strain (329). Upon challenge with $R_{\text{low}}$, F strain prevented the development of lesions and disease (264, 349, 350). However, it was also shown that the F strain vaccine was not safe in all conditions. Vaccination in turkeys and young chickens resulted in vertical transmission and severe lesions (343, 351), and was shown to be associated with a delay in the onset of egg production in layer chickens (346, 352). Regardless, chickens vaccinated with F-strain still fared better than non-vaccinated birds.

A temperature sensitive mutant (ts-11) was created through chemical mutagenesis (353). The maximum growth temperature suitable for this mutant is 33ºC, and therefore is only able to colonize the upper respiratory tract. Administration of this vaccine was shown to be safe in both chickens and turkeys and induced a protective response. Egg production remained normal and minimal lesions were only observed in < 10% of animals (336, 337). It has also shown to persist within the vaccinated host for several months with limited horizontal transmission (344, 354). However, further analysis revealed that protection varied likely due to the low antibody response (354–356). Furthermore, while the ts-11 strain was able to displace the F strain, it was unable to displace virulent $R_{\text{low}}$ (357). While this strain did not provide as much protection as the F strain vaccine, it was shown to be a safer option for young chickens and turkeys.

A third live attenuated vaccine, 6/85 was generated through serial passage *in-vitro* (344), and similar to the ts-11 vaccine, was shown to be safe in both chickens and turkeys and resulted in very few mild lesions (358). The immune response upon challenge with a virulent strain was weak and varied between animals, and did not elicit any detectible serum antibodies (344, 358). Additionally, 6/85 was unable to displace or prevent the spread of virulent strains (359, 360).
While it has not been confirmed experimentally, reports of 6/85-like strains have been recovered in un-vaccinated animals (361, 362).

While the three commercially available vaccines have contributed to controlling *M. gallisepticum*, each have limitations. The F strain is protective but is virulent to young chickens and turkeys, both of which are typically in proximity to laying chickens. Ts-11, a temperature sensitive mutant, is not able to displace virulent field strains in multiage facilities (311). 6/85, created through serial passage, only survives for a short period of time and results in very minimal serum antibody response (363). While both are safe, the host response to these vaccines varied and offered limited protection. Therefore, further development of a vaccine that is safe for chickens and turkeys and capable of providing sufficient protection from virulent strains.

Two laboratory vaccine strains have been produced by The Center of Excellence for Vaccine Research (CEVR) at UConn to protect chickens from *M. gallisepticum*. GT5, was created by complementing avirulent R\textsubscript{high} with the cytadhesion gene *gapA*. Complementation did not restore cytadhesion capabilities, but was still shown to prevent lesions due to R\textsubscript{low} infection upon challenge (364). Additionally, the host produced high serum IgG antibodies as well as mycoplasma specific IgG and IgA secreting B cells in the trachea as early as day 4 post challenge. This corresponded with CD4+ and CD8+ T cells and B cells arranged in organized follicles in the lamina propria unlike the disorganized infiltration of lymphocytes seen during natural infection (273).

The second attenuated vaccine strain, Mg 7, was created by transposon mutagenesis with an insertion in the metabolic gene dihydrolipoamide dehydrogenase (*lpd*) (328). The *lpd* gene contributes to the production of ATP during glycolysis. Analysis of this strain indicated limited colonizing capabilities as only 12.5% of samples contained Mg 7 two weeks post infection (328,
Virulence was also limited resulting in minimal lesions in the trachea and air sacs, while the vaccine was still able to evoke antibody levels similar to those induced by virulent $R_{\text{low}}$ infection (329). Analysis of GT5 and Mg7 indicated that they both outperformed commercially available vaccines; however, Mg7 induced higher $M. \text{gallisepticum}$ specific IgG serum antibodies than GT5, and also resulted in lower levels of post-challenge recovery of $R_{\text{low}}$ suggestive of prophylactic immunity (329).

Both laboratory attenuated vaccine strains passed the ‘proof-of-concept’ stage of development and were shown to outperform the F strain, ts-ll and 6-85 vaccine strains under laboratory controlled settings (329). Unfortunately, GT5 and Mg7 were created using transposon insertions that contain a gentamicin selectable marker, making them unsuitable for commercial production. Therefore, there is still a need to develop a safe and efficacious vaccine with fewer limitations than those that are currently available. Better understanding of the pathogenic mechanisms utilized by $M. \text{gallisepticum}$ and the subsequent host response is necessary to guide the development of these future vaccines.

**Immune Response to $M. \text{gallisepticum}$**

Infection of the chicken respiratory tract with $M. \text{gallisepticum}$ at the mucosal epithelium results in the colonization of the trachea and development of lesions due to immune cell infiltrates into the lamina propria (318, 365). Initial host-pathogen interaction is thought to largely occur between the lipoproteins anchored to the mycoplasma membrane with TLR-2 (TLR-2/TLR-1 or TLR-2/TLR-6 heterodimers) on the epithelial cell surface. As seen in *in vivo* analysis, this results in a robust pro-inflammatory response as indicated with an upregulation in
several chemokines and cytokines such as lymphotactin, CXCL-13, CXCL-14, RANTES, IL-6 and MIP-1\(\beta\) between days 1 and 8 post infection (224). *In vitro* exposure of cultured tracheal epithelial cells (TECs) and tracheal *ex vivo* exposure to *M. gallisepticum* revealed a similar yet transient upregulation of gene expression profiles at 1.5, 6, and 24 hours. However, these studies have shown differences regarding the expression levels of CCL-20, IL-1\(\beta\), IL-8, IL-12p40, and IFN-\(\gamma\) (170). Furthermore, it has been suggested that insufficient levels of IL-12p40 and IFN-\(\gamma\) may result in the incomplete activation of macrophages during *M. gallisepticum* infection (366).

Early studies on the adaptive immune response to *M. gallisepticum* showed that chickens were more susceptible to infection upon bursectomy and thymectomy resulting in deficient B and T cell lymphocytes (367), and that IgG antibodies against *M. gallisepticum* inhibited cytadhesion to cultured tracheal rings *ex vivo* (368). Analysis of lymphocyte infiltrates showed consistently increased levels of CD8+ T cells found between the upper and lower respiratory tracts. CD4+ T cell levels outnumbered CD8+ T cells nearly 2 to 1, and were positively correlated with bacterial load (369). As previously discussed, antibodies have been shown to be important mediators of protection in vaccinated chickens. Chickens vaccinated with GT5 were found to have higher serum IgG as well as higher *M. gallisepticum* specific IgG and IgA secreting plasma cells in the trachea mucosa compared to unvaccinated chickens (273). Furthermore, CD8+ T cells were found to be the predominant cell type as early as 1-day post challenge, that were then surrounded by clusters of CD4+ T cells by day 4, with CD8 + T cells scattered throughout the lamina propria (273). However, analysis using the ts-ll vaccine strain indicated an initial response dominated by CD8+ T cells that were gradually replaced by CD4+ T cells and was then predominated by B cells (369). Therefore, these studies suggest that the role of T cells in the adaptive immune response to *M. gallisepticum* is still poorly understood.
Despite the robust host immune response to *M. gallisepticum*, this organism is known for establishing chronicity and the development of significant respiratory disease in chickens. Studies to date have helped to elucidate the role of chemokines and cytokines as key players of the pro-inflammatory host response. However, given the complex nature of the interaction between *M. gallisepticum* and the respiratory mucosa, further analyses are needed to better understand the molecular events that may contribute to the development of CRD.

**Significance, Hypotheses and Specific Aims**

Previous studies have contributed significant data aiding in the understanding of the early pro-inflammatory response to *M. gallisepticum*. However, a broader understanding of the complete tracheal host response is needed to identify key steps in the biological pathways that lead to a maladaptive versus beneficial immune response. The analysis of both pathogenic and attenuated vaccine strains will indicated key differences between the host response and its ability to result in either immunopathogenesis or protective immunity. Elucidation of these initial molecular events will indicate which host cell receptors, immune signaling pathways, cytokines and chemokines play important roles in the response to *M. gallisepticum* that may have previously gone unappreciated. These components could then be targeted and differentially regulated through the development of vaccines to provide protection from wild-type, virulent strains, and induce long-term immunological memory.
Hypotheses:

Hypothesis 1: The host immune response will show broad changes in regulation of genes encoding proteins with immune function in response to pathogenic *M. gallisepticum* strain R_{low}.

Hypothesis 2: On days 1 and 2 post-infection, attenuated *M. gallisepticum* strain Mg 7 will evoke broad differential expression in metabolic and immune related genes comparable to the host response to virulent strain R_{low}, whereas infection with attenuated strain GT5 will result in limited differential gene expression.

Specific Aims:

Specific aim 1: Investigate and characterize the transcriptional profile of the tracheal host response to *M. gallisepticum* strain R_{low} over a seven-day time course.

Specific aim 2: Investigate, characterize and compare the transcriptional profile of the tracheal host response to two attenuated vaccine strains, GT5 and Mg 7, to that of virulent R_{low} on days 1 and 2 post-infection.
References
new species of the class Mollicutes (division Tenericutes). Int J Syst Evol Microbiol
57:2703–2719.


46. Dammann O, Allred EN, Genest DR, Kundsin RB, Leviton A. 2003. Antenatal mycoplasma infection, the fetal inflammatory response and cerebral white matter damage


mycoides subsp. mycoides small colony type to bovine epithelial cells. Infect Immun 76:263–269.


120. Chalker VJ, Owen WMA, Paterson C, Barker E, Brooks H, Rycroft AN, Brownlie J.


199. Roberts DD, Olson LD, Barile MF, Ginsburg V, Krivan HC. 1989. Sialic acid-dependent


CHAPTER 2:

Transcriptional Profiling of the Chicken Tracheal Response to Virulent

*Mycoplasma gallisepticum Strain R_{low}*

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Abstract

*Mycoplasma gallisepticum*, the primary etiologic agent of Chronic Respiratory Disease (CRD) in poultry, leads to prolonged recruitment and activation of inflammatory cells in the respiratory mucosa. This is consistent with the current model of immune dysregulation that ostensibly allows the organism to evade clearance mechanisms and establish chronic infection. To date, studies using qRT-PCR and microarrays have shown a significant transient up-regulation of cytokines and chemokines from tracheal epithelial cells (TECs) *in vitro* and tracheal tissue *ex vivo* in response to virulent strain R<sub>low</sub>, that contributes to the infiltration of inflammatory cells into the tracheal mucosa. To expand upon these experiments, RNA was isolated from tracheas of 20 chickens infected with *M. gallisepticum* R<sub>low</sub> and 20 mock-infected animals at days 1, 3, 5 and 7 post inoculation, and analyzed for differential gene expression using Illumina RNA sequencing. A rapid host response was observed 24 hours post-infection, with over 2,500 significantly differentially expressed genes on day 3, the peak of infection. Many of these genes have immune related functions involved in signaling pathways including the TLR, MAPK, Jak-STAT, and the NOD-Like Receptor. Of interest, was the increased expression of numerous cell-surface receptors including TLR-4 and -15 which may contribute to the production of cytokines. Metabolic pathways were also activated on days 1 and 3 post-infection, ostensibly due to epithelial cell distress that occurs upon infection. Early perturbations in tissue-wide gene expression, as observed here, may underpin a profound immune dysregulation, setting the stage for disease manifestations characteristic of *M. gallisepticum* infection.
Introduction

*Mycoplasma gallisepticum* is the primary etiologic agent of Chronic Respiratory Disease (CRD) in poultry. Infection with this pathogen results in decreased feed efficiency, reduced weight gain, subsequent downgrading of carcasses, and limited egg production and hatchability. Three commercial vaccines are available to control *M. gallisepticum* infection, however, further development of an improved vaccine that is safe and efficacious for susceptible birds of multiple ages and species is needed (1, 2). To ensure eradication, broiler industries cull their entire flocks in the event of a sporadic outbreak. However, with *M. gallisepticum* infection frequently found in US egg-laying facilities, culling is not economically feasible (1-3). These flocks often remain infected for life and provide the opportunity for further spread of infection to other facilities and wild birds (1,4).

The host mounts a vigorous inflammatory response to *M. gallisepticum* resulting in respiratory distress in chickens (1). Lesions of varying severity are found lining the trachea and air sacs due to colonization of the bacteria and the excessive recruitment and activation of inflammatory cells. This is one aspect of the immune dysregulation orchestrated by *M. gallisepticum* that allows it to establish chronic infection (5-10). In addition, virulent strains of *M. gallisepticum*, such as R_{low}, are able to avoid clearance by the host’s natural defense mechanisms of the respiratory tract by cytadhering to tracheal epithelial cells. This is established by the function and co-expression of the primary cytadherence molecules GapA and CrmA that play a critical role in colonization and pathogenesis (11-13).

Previous studies assessing the host tracheal response to *M. gallisepticum* have utilized methods such as exposure of cultured tracheal epithelial cells *in vitro* and whole trachea *ex vivo*
(9, 10, 14, 15). The most relevant to this current study, however, was an in vivo analysis of chemokine and cytokine expression profiles of the tracheal host response to \( R_{\text{low}} \) (14). Over the course of an 8-day infection, increased expression of pro-inflammatory chemokines and cytokines including lymphotactin, CXCL13, -14, RANTES, IL-6 and IL-1\( \beta \) was observed, while decreased expression was seen in CCL20, IL-8, and IL-12p40. While ex vivo and in vitro studies have revealed similar expression profiles, some discrepancies exist regarding the significance and expression kinetics of inflammatory genes. Irrespective of these differences, these studies further support the premise that the overwhelming host response is ineffective at clearing the organism and contributory to the immunopathogenesis observed upon infection; thereby contributing to the development and progression of CRD (9, 10, 14).

Studies to date have provided beneficial but limited insight into the host response to \textit{M. gallisepticum} through methods such as qRT-PCR and microarrays. Given the potential interactions and downstream effects of the pro-inflammatory genes that have been implicated to date, we hypothesized that the tracheal host response will show much broader changes in differential expression than what has currently been identified. To expand on previous studies, we have utilized RNA Sequencing of the host tracheal tissues subsequent to infection with virulent \textit{M. gallisepticum} strain \( R_{\text{low}} \) over a seven-day time course. This will provide a comprehensive, unbiased assessment of the changes in gene expression that comprise the maladaptive host response.
Results

Global Differentially Expressed Genes (DEGs) Between Infected and Control Chickens

Sampling directly from the tracheal lumen yielded sufficient quantities of RNA to assess transcripts from each chicken and mapped to 17,935 *Gallus gallus* genes. Day 1 post-infection contained 1,913 DEGs; 2,523 on day 3; 435 on day 5, and 351 on day 7. This indicated that the host mounts a rapid response to *M. gallisepticum* strain R<sub>low</sub> that peaked at day 3 and decreased towards baseline levels by day 7. Days 1 and 3 post-infection contained the largest number of unique DEGs likely due to the numerous metabolic pathways seen to be involved during early infection as indicated by pathway analysis (Fig. 1a, 2). At each timepoint, the number of DEGs with increased expression values exceeded those with decreased expression (Fig. 1b).

Pathway Analysis

The number of pathways affected by *M. gallisepticum* R<sub>low</sub> as identified by containing a minimum of two DEGs, was 117 on day 1; 126 on day 3; 51 on day 5, and 39 on day 7. Out of 168 total metabolic pathways identified in chickens, days 1 and 3 post-infection indicated that there were 65 and 72 metabolic related pathways, respectively, containing DEGs (Fig. 2). As the course of infection continued, the number of metabolic pathways involved decreased to 18 on day 5 and 9 on day 7 reflecting a return toward homeostasis. There were 46 pathways involving immune related functions categorized within immune signaling, signal transduction, and signaling molecules. Less variability was seen within immune-related pathway categories throughout the time course with 21 pathways identified as affected by infection on days 1 and 3, 15 on day 5, and 18 on day 7.
Functional Gene Ontology (GO)

Functional ontology assessment of the DEGs allowed the genes to be categorized based on enriched biological processes (Fig. 3). On day 1 post-infection, there were 195 genes with a log2 fold change ≥ 2.00. While a large proportion of metabolic pathways were indicated on days 1 and 3 post-infection (Fig. 2), very few differentially expressed metabolic genes had a log2 fold change ≥ 2.00 which resulted in a minimal number of metabolic genes being assessed. The biological process with the highest percentage of genes per GO term was the ‘antimicrobial humoral response’ at nearly 43%. Of interest, was the number of associated DEGs found in biological processes related to the host immune, inflammatory and defense responses as well as ‘immune cell migration’. 176 DEGs were categorized on day 3 post-infection and indicated additional mitogenic and cytokine-related cellular response as shown by the presence of the ‘regulation of ERK1 and ERK2 cascade’, ‘response to interferon-gamma’ and the ‘cytokine-mediated signaling pathway’ that were not seen on day 1. Day 5 post-infection categorized 165 DEGs which included two unique biological processes; ‘phagocytosis’, and ‘antigen processing and presentation of exogenous antigen’ with the highest percentage of genes per GO term at 43%. There were 184 DEGs categorized on day 7 post-infection, and while there was still indication of the continued host response to the bacterial pathogen, there were many more processes related to the regulation of the immune response. In addition, this was the first time that processes related to ‘T-cell selection and differentiation’ were identified, potentially reflecting antigen processing, cellular activation and proliferation/differentiation over time.
Immune Response

Due to the significant enrichment of immune genes further assessment of immune-related pathways was conducted. Pathways including DEGs included, but were not limited to, cytokine-cytokine receptor interaction, the Toll-like receptor (TLR) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the nucleotide oligomerization domain (NOD)-like receptor pathway (Fig. 4). Each of the aforementioned pathways are involved in pathogen recognition and the pro-inflammatory environment initiated by the production of chemokines and cytokines. The number of DEGs per pathway increased from day 1 to day 3 (peak), decreased at day 5, and appeared to level out by day 7. The cytokine-cytokine receptor interaction pathway contained the greatest number of DEGs and these predominantly encoded cytokine receptors (Fig. 5).

As expected, TLR-2 and -1A showed significant differential expression on days 3 and 7 post-infection (Fig. 6). Interestingly, TLR-4, traditionally a pattern recognition receptor (PRR) for lipopolysaccharide, a ligand that is absent from Mycoplasma species, was significantly increased at each time point. TLR-15, unique to avian species, was also found to be significantly increased at all timepoints.

Genes with Greatest Differential Expression

Fold change values for the top 25 genes per time point ranged from a log2 fold change value of -5.0 to +9.2 (Table 1). Protein functions encoded by these genes are involved in pathogen recognition, receptor ligation, pro-inflammatory cytokines and chemokines, catalytic activity, negative feedback of inflammation, as well as the maintenance and remodeling of tissue structure. Of the 131 DEGs common to all four time points, many of the genes with the greatest
log2 fold change were involved with the host’s pro-inflammatory response, defense, and repair mechanisms including CCL5, TLR15, TLR4, IL-1β, IL-8, BPI and MMP9 (Table 2). The fold change of differential expression for many of the pro-inflammatory genes was transient and peaked at either day 3 or day 5 post-infection while the fold change of other genes was stable over the course of infection. Importantly, the continued significant differential expression of multiple cell receptors, pro-inflammatory cytokines and chemokines, highlights the sustained signaling of immune-related pathways.

Discussion

This study examined global transcriptomic analysis of differential gene expression in chicken tracheal cells following exposure to *Mycoplasma gallisepticum* Rlow over a seven day time course. In keeping with prior studies, this analysis provided copious data re-affirming the vigorous pro-inflammatory response mounted in the chicken trachea subsequent to *M. gallisepticum* infection. This response is ostensibly due to prolonged signaling through epithelial cell receptors resulting in increased production of cytokines and chemokines paired with tissue infiltration of inflammatory cells (7, 10, 14, 15). Previous *ex vivo* analysis of host tracheal epithelial cells had suggested that the exaggerated influx of inflammatory cells into the respiratory lamina propria is likely dependent upon robust TLR-2 signaling that occurs as early as 1.5 hours post-exposure to *M. gallisepticum* (10). Furthermore, *in vitro* exposure of tracheal epithelial cells to Rlow resulted in a rapid response with increased expression of inflammatory genes CCL20, IL-8, IL-6, IL-12p40, NOS2, CXCL13, -14, MIP-1β and IL-1β within 24 hours of exposure (10). Previous *in vivo* analysis, however, indicated that CCL20, IL-8, IL-12p40 had
decreased expression upon infection with R_{low} while minimal changes were seen with IL-1\beta over the course of 8 days (14). The findings of the current study differed from those of earlier in vitro and in vivo studies; TLR-2 was found to be increased, but not to the extent seen with TLR-4 or -15. CCL20 (MIP-3\(\alpha\)) showed decreased expression however this was only found to be significant on day 5, while differential expression of IL-12p40 (IL-12B) was only observed on day 1. IL-1\beta, IL-8L1 and IL-8L2 showed significantly increased expression at each timepoint, which differed from the findings of the previous in vivo study. Given the important pro-inflammatory function of IL-1\beta and chemotactic properties of IL-8L1 and IL-8L2, significant increased expression was expected. Differences found between these studies is likely a reflection of the difference in experimental methodology and hence the cell types captured in the analysis. It should be pointed out that the sampling methods used here and post-collection analysis was quite different in that the latter study used whole tracheal tissues whereas the current study only sampled cells in the trachea by pipetting TRIzol through the lumen. Based on the gene expression profiles observed in the current study, it appears that heterophils and lymphocytes were likely present in the samples collected, however the extent of tissue penetration is unknown, and certain cell populations may have been excluded (e.g. cells trapped in the connective tissue of the lamina propria). However, this TRIzol method is quite relevant to the study of differential gene expression in that the recovered cells are in close contact with the pathogen.

While increased expression of TLRs may not be a direct reflection of ligand binding and subsequent signaling, it is of interest that the host response increases the sensitivity of the epithelial and immune cells involved through increased expression of receptors. TLR-15, specific to avian and reptilian lineages and largely expressed on the surface of heterophils,
macrophages and fibroblasts, shares approximately 30% identity with TLR-2 (16-18). It has been suggested that TLR-15 is a broad spectrum TLR that recognizes heat-stable components of both gram negative and positive bacteria, CpG oligonucleotides, tripalmitoylated lipopeptide, and lipopolysaccharide (LPS) (17, 18). More recently, it has been shown to be activated in the presence of diacylated lipopeptide from Mycoplasma synoviae (19). In the current study, TLR-15 had an average log₂ fold change of 4.71 throughout the course of the 7-day infection and may reflect the persistent presence of M. gallisepticum (data not shown). The stable differential expression of TLR-15 was greater than that observed for TLR-1A and -2, unlike what was seen in previous in vitro work with M. gallisepticum (10). Its importance with respect to M. gallisepticum pathogenesis, however, remains uncertain. The increase in differential expression of TLR-4, while partly due to increased numbers of immune cells, may also indicate increased cellular response to extrinsic signals. Though M. gallisepticum does not contain LPS, the hallmark TLR-4 ligand, other studies have shown TLR-4 signaling due to the presence of damage-associated molecular pattern molecules (DAMPs) such as heparin sulfate, fibrinogen, fibronectin or heat shock proteins 60 and 70 as a result of damaged extracellular matrix (ECM) or injured cells (20-23). Associated with this may be the significantly increased differential expression of matrix metalloproteases (MMP) 7 and/or 9 found within the top 25 DEGs at every time point. On day 3, MMP7 had the greatest increase in differential expression of all genes with a log₂ fold change value of 9.22. In mucosal epithelium, MMP7 has been shown to play an important role in the injury response with increased expression found in cells surrounding the site of injury (24). Research on Equine chronic obstructive pulmonary disease (COPD), suggested that MMP9 may serve as a marker for ongoing tissue remodeling (25). It has also been proposed that disproportionate expression of MMPs, likely a result of increased cytokine
production, coupled with deficient expression levels of tissue inhibitors of metalloproteinases (TIMPs) may result in excess ECM degradation (20, 26, 27). Components of the ECM may then ligate TLR-4 and result in signaling through MyD88 (16, 20-22). In addition, increased expression of TLR-4 may provide greater accessibility of endogenous gram-negative bacteria to this receptor. Ligation of TLR-4 and -15 and subsequent increase of pro-inflammatory cytokines may contribute to and perpetuate the exaggerated and dysregulated host immune response.

Significant differential expression of many cytokine and chemokine receptors was also observed over the course of infection. For example, on day 3, the peak of infection as indicated by gross and histological analysis (data not shown) and the point at which the highest number of DEGs were observed, the number of cytokine/chemokine receptor genes found to be differentially expressed outnumbered the cytokine genes by a 4 to 1 ratio. This may indicate that *M. gallisepticum* infection results in increased cell sensitivity to inflammatory agonists by virtue of up-regulating receptors for signaling molecules not present on mycoplasmas.

Pathway analysis showed that the greatest change in proportion of pathway categories occurred in those with metabolic function, with the number of immune related pathways remaining constant. However, further analysis for GO revealed that the majority of DEGs with log$_2$ fold change ≥ 2.00 were related to pathogen recognition and the subsequent host immune response. A recent study assessing the changes in miRNA due to *M. gallisepticum* strain HS in the lung, identified numerous miRNAs with gene targets found in immune pathways. These included, but were not limited to, genes found within the MAPK, TLR, and Jak-STAT pathways (28). While the RNA-seq data presented cannot explicitly show gene regulation changes in individual cells, analysis of miRNAs and their targets further supports the differential expression seen in the tracheal host response presented here.
Collectively, these differential gene expression data observed in this study further revealed the complexity of the host response to M. gallisepticum, especially at early time points. It is easy to envisage how the myriad differentially expressed inflammatory, signaling and immune response genes could result in a profoundly dysregulated host response. Numerous genes were found to have significant differential expression over the course of infection, and while there were significantly fewer DEGs were observed on days 5 and 7 post-infection, many pro-inflammatory genes maintained their expression throughout the time course. Both PAMPs and DAMPs likely contributed to the ligation of receptors that may not have been activated based solely on the components of M. gallisepticum and subsequent downstream signaling. The presence of PAMPs and DAMPs coupled with the increased expression of PRRs and cytokine receptors, may underpin the robust host response that results in the recruitment and activation of inflammatory/immunoregulatory cells, thereby establishing an ecological niche favoring pathogen persistence. As previously mentioned, recruitment of various immune cells into the lamina propria during the course of M. gallesipticum infection. While the changing cell populations may also contribute to differences in expression observed here, it does not mitigate the potential importance of these changes in the local host response.

This study has provided global assessment of the chicken tracheal response to M. gallisepticum over the course of early infection and further elucidated the complex response contributing to the early development of CRD. Better understanding of these molecular events is critical in identifying those which lead to a maladaptive rather than beneficial host response and may be important in the development of future therapeutics and vaccines.
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 avian isolators, and allowed to acclimate for one week. Non-medicated feed and water were provided ad libitum throughout the experiment. All components of the study were performed in accordance with approved UConn IACUC protocol number A13-001.

Chicken Infection

Stocks of *M. gallisepticum* strain R\text{low} (passage 17) were grown in Hayflick’s complete medium overnight at 37\degree C until mid-log phase was reached as indicated by a color shift from red to orange. Bacterial concentrations were determined by the OD$_{620}$ 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 minutes and resuspended at 5 x 10$^8$ CFU/ml in Hayflick’s complete medium. Chickens were challenged by pipetting 1 x 10$^8$ CFU/200 µl (‘infected’) or 200 µl of Hayflick’s medium alone (‘control’) into the tracheal lumen as previously described (29).

RNA Extraction

Five infected and five control chickens were humanely sacrificed each day for seven consecutive days. Tracheas were excised and total RNA was collected by pipetting 1 mL TRIzol reagent four times through the lumen of each individual trachea (30) to minimize the changes in
gene expression that may occur during the time between specimen collection and RNA purification (Invitrogen, Carlsbad, CA, USA). Total RNA was then purified using the Zymo-Direct-zol RNA Miniprep Kit according to manufacturer’s instructions (Zymo Research Corporation, Irvine, CA, USA).

**Illumina Sequencing**

cDNA libraries were created from RNA isolated from individual chickens on days 1, 3, 5, and 7 post-infection using the Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the Illumina TruSeq RNA Sample Preparation v2 (HT) protocol. Briefly, PolyA selection was performed using RNA purification beads on 0.1-4 µg of total RNA per sample to obtain mRNA. 10-400ng 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 dNTPs including dUTP, DNA polymerase, and RNase. End repair was performed by adding dATP to all free 3’ ends and adaptor ligation added unique index sequences to the fragments. The products were then amplified by PCR and purified.

The cDNA libraries were quantified using the Qubit 2.0 fluorometer (Invitrogen) and assessed for correct fragment size (~260bp) using the Agilent TapeStation 2200 (Agilent Technologies). Libraries were normalized to 2nM, pooled, denatured and sequenced on the NextSeq500 Sequencing platform (Illumina Inc.) using a 75bp paired-end approach targeting approximately 10 million reads per sample.
**RNA-Seq Analysis**

Fastq data from individual birds were mapped using the TopHat package (version 2.0.1) utilizing the Bowtie2 engine (version 2.2.5) to align reads to the *Gallus gallus* reference genome (WASHUC2) producing BAM alignment files (31). BAM files for each day were processed as experimental condition groups with Cufflinks (version 2.2.1) to assess aligned reads against reference transcripts using Cuffdiff to calculate treatment group-specific expression values and statistical significance of between-group differential expression. Within-condition normalization of data using Cuffdiff was achieved by calculating the fragments per kilobase of exon per million fragments mapped (FPKM) for each gene. The generated p-value was then used to determine the significance of the differential expression based on the Benjamini-Hochberg correction with a false discovery rate (FDR) of <5% to generate the q-value. Between-condition differences in expression values were considered significant when q ≤ 0.05, thus addressing within-condition variation for all biological replicates on each day. Fold change was determined from the log2 transformation of the ratio of FPKM between conditions (31, 32). For this study, all references to differentially expressed genes (DEGs) implies statistical significance. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE101403 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE101403).

**Pathway Analysis**

Identification of biological pathways that significant DEGs were involved in, was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (33). Pathways containing ≥ 2 significant DEGs were categorized and displayed.
**Functional Gene Ontology**

DEGs with a log2 fold change ≥ 2.00, as identified by Cuffdiff, were analyzed to identify enriched biological processes based on Gene Ontology (GO). This was performed using ClueGO v2.3.2, a plugin of the software platform Cytoscape v3.5.0-RC1 (34). Processes that had a p-value ≤ .05 for each time point were considered significant and displayed.

**Acknowledgements**

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References


FIG 1 The number of DEGs between infected and control chickens per time point as identified by Cuffdiff. (a) Venn diagram displaying the total number of DEGs. Gray scale is based on the number and percentage of genes identified. (b) The number of DEGs with increased or decreased expression.
FIG 2 Pathway analysis. Pathways containing ≥ 2 DEGs were categorized based on function using DAVID Bioinformatics software. The number of identified pathways per category is indicated.
Day 1:
Day 7:
FIG 3 Functional Gene Ontology. Significant DEGs with a log₂ fold change ≥ 2.00 were analyzed for enriched biological processes using the ClueGO application for Cytoscape. Bars represent the percentage of associated genes assigned to a unique GO term with the absolute number of associated genes located at the end of the bars. The color of the bars indicates functionally related groups based on the similarity of associated genes. Significance of enriched biological processes are as indicated (* = p ≤ 0.05, ** = p ≤ 0.001).

FIG 4 Immune-related Pathways. Pathway analysis using DAVID Bioinformatics software identified the number of DEGs involved in immune-related pathways at each time point.
FIG 5 Cytokine-cytokine Receptor Interaction. DEGs identified on day 3 post-infection involved in this pathway are indicated with red stars as identified by DAVID Bioinformatics software. Green shading represents the completeness of the pathway identified within the *Gallus gallus* genome.
**FIG 6** Toll-Like Receptors. TLRs identified by RNA-Seq are displayed. Differential expression of TLRs between infected and control birds is expressed as $\log_2$ fold change. Those with significant differential expression ($q \leq 0.05$) are indicated (*).
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**TABLE 1** Top 25 DEGs for each time point based on log₂ fold change values as indicated by Cuffdiff.
### TABLE 2

Top 30 DEGs Common to All Time Points. Sorted by Day 3 post-infection log\(_2\) fold change values as indicated by Cuffdiff.

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CHAPTER 3:

Transcriptional Comparison of the Chicken Tracheal Response to Virulent and Attenuated Vaccine Strains of Mycoplasma gallisepticum

Beaudet J\textsuperscript{a,b}, Tulman ER\textsuperscript{a}, Pflaum K\textsuperscript{a}, Canter JA\textsuperscript{a}, Silbart LK\textsuperscript{a,b}, and Geary SJ\textsuperscript{a} #

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In Preparation for Submission
Abstract

Host immune responses to *M. gallisepticum* are complex, inducing increased expression of many pro-inflammatory chemokine and cytokine genes, and the influx of leukocytes into the mucosa, resulting in immunopathogenesis. The need for improved vaccines that are both safe and efficacious in birds of different ages and species has driven the development of several laboratory attenuated vaccine strains. As a first step toward understanding the attenuation process, we examined two well characterized strains generated in our laboratory; GT5 and Mg7, each of which were found to be protective upon challenge with virulent R<sub>low</sub>. However, knowledge of the early molecular events that likely differ between the host response to virulent R<sub>low</sub> and attenuated vaccine strains GT5 and Mg7 is limited. To elucidate these differences, we conducted an infection study comparing differential gene expression in tracheal tissues exposed to R<sub>low</sub>, GT5 and Mg7 on days 1 and 2 post-infection. This enabled assessment of the early innate immune response that is critical in the development of disease and subsequent adaptive immunity. Compared to the pronounced response to R<sub>low</sub> infection seen on day 1, GT5 and Mg7 resulted in significantly fewer differentially expressed genes related to metabolic and immune functions. On day 2, similar pathways were activated in R<sub>low</sub> and Mg7 exposed birds, while the genes involved in the response to GT5 exposure were less inflammatory, consisting primarily of genes associated with cellular growth and repair. This study provides a greater understanding of maladaptive vs. beneficial host responses; information critical for the design of future vaccines efficacious in terms of both limiting inflammation and enabling clearance of the pathogen.
Significance

Current vaccines have reduced the incidence and spread of Mycoplasma gallisepticum in poultry, however, a safer and more efficacious vaccine is needed. We conducted host transcriptional analysis to provide comprehensive understanding comparing early responses to virulent R_{low}, and attenuated vaccine strains Mg7 and GT5. Differentially expressed genes (DEGs), both in number and magnitude of response were observed in cytokines and receptors as well as metabolism and other immune-related pathways. Our data indicate several immune-related genes and pathways affected by R_{low} that are likely important in inducing adaptive immunity, but should resemble the response evoked by attenuated strains. Future vaccines should target genes common to virulent and avirulent strains to activate the appropriate host response, promote protective immunity and avoid immunopathogenesis.

Introduction

Mycoplasma gallisepticum, an infectious respiratory pathogen, contributes to the development of Chronic Respiratory Disease (CRD) in poultry. CRD in chickens is frequently associated with clinical signs including coughing, nasal discharge, and tracheal rales (1). Inflammatory lesions are most commonly found in the trachea, air sacs, lungs, and occasionally in the conjunctiva and oviduct (2). Despite efforts to control outbreaks, M. gallisepticum results in significant worldwide economic losses due to decreased feed conversion efficiency, downgrading of carcasses, and reduced egg production and hatchability (1, 3).
Virulent strains of *M. gallisepticum*, such as R\textsubscript{low}, avoid clearance by the host’s respiratory defense mechanisms by initially cytadhering to tracheal epithelial cells and subsequently colonizing the mucosal surface. Tight attachment is mediated by an ‘attachment organelle’ comprising the cytadherence molecules GapA and CrmA, which are required for colonization and pathogenesis (4). Disruption of either of these genes results in limited colonization and a dramatically reduced host response and its associated pathology (4–6).

The tracheal host response to R\textsubscript{low} is characterized by a marked influx of inflammatory cells into the lamina propria (7, 8) and is associated with robust signaling of Toll-like receptors (TLRs) in epithelial cells lining the tracheal lumen (9). This results in increased expression of pro-inflammatory chemokines and cytokines including IL-6, IL-8, CCL20, CXCL-13, -14, and RANTES that are likely sustained due to increased signaling and activation of immune cells associated with R\textsubscript{low} colonization (8–10). Over time, the vigorous host response is associated with a dramatic reduction in bacterial load, however, it is incapable of clearing the infection and the concomitant immunopathogenic sequelae pre-dispose birds to co-infection and the development and progression of CRD (11–13).

Three widely used live attenuated *M. gallisepticum* vaccines are known as ‘F strain’, 6/85, and ts-11. While highly effective, each of these vaccines have limitations that render them unsuitable in certain situations. For example, the F strain vaccine persists for the life of the chicken and is protective against virulent strains, however, it is pathogenic in turkeys and young chickens (14). Vaccine strains ts-II, a temperature sensitive mutant, and 6/85, which was generated by serial passage, are non-pathogenic in chickens and turkeys, and are protective upon challenge, though protection levels and duration of immunity vary. These vaccines are less
effective at displacing virulent strains, and 6/85 elicits little or no humoral antibody response (15–17).

Efforts to develop more efficacious vaccines have resulted in laboratory attenuated vaccine strains including GT5 and Mg7. GT5 was created by complementing avirulent, high passage, strain R_high with wild-type gapA (4). GT5 induces lower levels of pro-inflammatory cytokines and chemokines compared to R_low, and is capable of inducing the local recruitment of CD4^+ and CD8^+ T cells, B cells, and ultimately the production of M. gallisepticum specific IgG, IgA, and IgM antibodies (8, 18-20). Due to the number and magnitude of gene mutations in R_high and the modest immune response, incomplete clearance of organisms and less durable immunity, we consider this vaccine to be highly attenuated. Conversely, Mg7 is an R_low isogenic mutant with a transposon insertional disruption in the dihydrolipoamide dehydrogenase (lpd) gene (21). As such, Mg7 is genetically identical to the fully virulent R_low strain but lacks a single metabolic gene, and hence is considered only mildly attenuated. Virulence assessments of Mg7 indicated limited colonization capabilities and minimal lesions in the trachea and air sacs, while still evoking levels of antibodies consistent with other vaccines (20, 21). Comparing the efficacy of these two live-attenuated vaccines indicated that chickens could rapidly clear the challenge strain (R_low) and had significantly reduced histopathologic lesion scores (20). Both GT5 and Mg7 outperformed the commercially available vaccines in laboratory controlled studies. Mg7 resulted in significantly lower levels of post-challenge recovery of R_low suggestive of, and highly correlated with, prophylactic immunity (20). While GT5 and Mg7 have been shown to be protective without resulting in significant immunopathology in a laboratory setting, further understanding of the host response to these strains is needed to determine which elements
of the host response are required to induce protective immunity versus those which are potentially maladaptive.

Previous transcriptional analysis conducted in our laboratory yielded a global assessment of the chicken tracheal response to \( R_{\text{low}} \) over the course of a 7-day infection. This expanded our knowledge of the immunological pathways involved, and highlighted the changes in cellular metabolism, stress, and cellular injury that occurred during virulent \( M. \text{gallisepticum} \) infection, some of which may contribute to the exaggerated host response (10). Based on the rapid host response that peaked in both gene expression and tracheal thickness during early infection with \( R_{\text{low}} \), the current study aimed to compare the differences between the host response to virulent \( R_{\text{low}} \) and attenuated strains GT5 and Mg7 during these early time points. Comparison of the initial molecular events that occur in the respiratory mucosa upon initial infection is imperative as pathway activation and production of cytokines and chemokines set the trajectory for the magnitude of inflammation and disease related changes, and contribute to the induction of the adaptive immune response resulting in protective immunity. Assessing early infection, prior to significant immune cell infiltrates observed at day 3, will provide a more accurate representation of the epithelial cell response. To accomplish this, we conducted a 2-day infection study followed by RNA sequencing to assess the transcriptional changes occurring in host genes that may help identify key steps in pathways leading to either a maladaptive or beneficial immune response to \( M. \text{gallisepticum} \). This knowledge may provide a framework to guide the development of attenuated vaccines. Future tailored vaccines should retain specific elements of immune activation absent perturbation of genes associated with inflammation or recruitment/activation of cells not contributory to adaptive immunity.
Results

*Differentially Expressed Genes (DEGs) Between Control and Virulent or Attenuated Strains*

RNA collected from cells lining the tracheal lumen of each chicken yielded transcripts that mapped to 17,935 genes within the *Gallus gallus* genome. On day 1 post-infection there were 1824 differentially expressed genes (DEGs) observed in response to \( R_{\text{low}} \) infection, 631 DEGs due to Mg7 exposure, and 257 due to GT5. Approximately 60% of these DEGs showed increased expression in each group. Day 2 post-infection resulted in a greater numbers of DEGs for each infection group; 2967 in \( R_{\text{low}} \) with 57% increased, 1969 in Mg7 with 63% increased, and 425 in GT5 with 74% increased (Fig. 1a, b). Of the total DEGs, only 171 were common to all three infection groups on day 1, with 274 in common on day 2 (Fig. 1c). As expected, the greatest number of unique DEGs were found in the \( R_{\text{low}} \) infected group on both days.

*Pathway Analysis*

Infection with *M. gallisepticum* strain \( R_{\text{low}} \) resulted in the greatest number of affected pathways on both days; with 110 observed on day 1 and 134 on day two. Exposure to Mg7 resulted in 69 pathways affected on day 1 and 115 on day 2, and GT5 exposure affected the fewest pathways with 35 on day 1 and 41 on day 2 (Fig. 2 a, c). The greatest number of activated pathways and DEGs were found within the ‘metabolism’ category on both days for each strain (Fig. 2 a-d). The pathways affected by Mg7 followed a similar trend as the \( R_{\text{low}} \) infected group, however, there were fewer pathways and DEGs observed within these pathway categories. The difference in DEGs between \( R_{\text{low}} \) and Mg7 infection was especially notable within metabolism-related pathways and those with immune related functions including ‘signal transduction’,
‘immune signaling’, and ‘signaling molecules’ (Fig. 2 b, d). GT5 exposure affected the fewest number of pathways in both metabolic and immune related categories, and showed the least variability between time points.

Functional Gene Ontology (GO)

Functional gene ontology assessment of the DEGs with a log2 fold change ≥ 2.00 enabled comparison of enriched biological processes identified in each infection group (Fig. S1). On day 1, there were 245 DEGs with a log2 fold change ≥ 2.00 due to Rlow infection, 79 DEGs due to Mg7, and 24 for GT5. In keeping with our previous study (10), Rlow infection affected numerous biological processes involving host immune, inflammatory, and defense responses involved in pathogen recognition, cytokine and chemokine production, and chemotaxis. The processes identified as a result of Mg7 exposure included similar cell responses such as chemotaxis and leukocyte migration, but appeared to be less broad as reflected by far fewer processes being affected. GT5 exposure resulted in the fewest biological processes being affected. On day 2, the number of DEGs assessed for each infection group were as follows; 194 in the Rlow group, 123 in Mg7 and 125 in GT5. Many of the biological processes affected in the Rlow infected group on day 1 were also affected on day 2. One notable difference however, was the presence of ‘positive regulation of NF-kappaB import into nucleus’. Mg7 exposure affected fewer processes than those observed upon Rlow infection on day 1, however, these differences decreased by day 2. Notable differences included the presence of ‘cellular response to interferon-gamma’ only being indicated in the Rlow infected group, and the presence of ‘positive regulation of interleukin-6 production’, ‘positive regulation of interleukin-8 production’, ‘macrophage activation’, and ‘positive regulation of T-cell activation’ indicated in the Mg7 exposed group. The greatest
difference between days 1 and 2 was seen in the GT5 exposed group with only 5 processes affected on day 1 and 64 processes affected on day 2. The ‘leukocyte chemotaxis’ and ‘neutrophil chemotaxis’ processes were not observed on day 2, nor were there any processes involved in the recognition of a bacterial pathogen or the host immune response. The majority of additional processes that were identified in the GT5 exposed group included those involved in cell growth, repair, development, differentiation, and cell proliferation.

Immune Pathways

Pathways of interest based on the differences in the host immune response to virulent and attenuated strains, included cytokine-cytokine receptor interaction, the mitogen-activated protein kinase (MAPK) signaling pathway, the nucleotide oligomerization domain (NOD)-like receptor pathway, the Janus kinase- signal transducers and activators of transcription (Jak-STAT) pathway, and the Toll-like receptor (TLR) pathway. As expected based on the total number of DEGs found in each infection group (Fig. 1), the R\text{low} infected group contained the most DEGs in each of these pathways at both time points, and GT5 exposure resulted in a limited number of DEGs. On day 1, the NOD-like receptor pathway indicated that R\text{low} infection uniquely affected pro-inflammatory genes including IL-6, RANTES and CASP1, while GT5 exposure did not affect any genes in this pathway. On day 2, the cytokine-cytokine receptor interaction pathway indicated that R\text{low} infection affected IL-6, IL-12, TNFSF11, and approximately 24% more receptors than Mg7 (Fig. 3). The differences in the MAPK pathway (not shown) included DEGs such as PKC, FAS, HSP72 and CASP that were only differentially expressed in the R\text{low} infection group, and TGF-β was only affected as a result of GT5 exposure. Few differences were observed in the Jak-STAT pathway (Fig. 3); however, only R\text{low} infection affected the signal
transduction adapter molecule (STAM). These molecules become activated upon stimulation of the Jak-STAT pathway due to cytokines and growth factors, and are involved in intracellular signal transduction (22, 23). In the TLR pathway, the most prominent differences between R_{low} infection and Mg7 exposure were seen in the toll-like receptors and the down-stream products of the pathway including both inflammatory cytokines and costimulatory molecules. IL-6, IL-12 and CD40 were only affected in R_{low} infection; IL-1β, RANTES, and CD80 were affected in R_{low} infection and Mg7 exposure; and IL-8 was affected in all three groups (Fig. 3). The greatest fold changes were seen in TLR-4 and -15 upon R_{low} infection and were also significant in Mg7 exposed chickens (Fig. 4). While there were more TLRs found to be significantly altered due to R_{low} infection and Mg7 exposure on day 1, Mg7 exposure only resulted in significant differential expression of TLR-4 and TLR-15 on day 2. The only TLR significantly affected by GT5 was TLR-21 on day 2.

**Genes with Greatest Differential Expression**

To compare the host response to each strain, the top 30 common DEGs were determined and ranked based on the log2 fold change values of the R_{low} infected group (Table 1). Many of the common DEGs encode proteins with immune-related functions such as IL-13RA2, IL-1β, LYG2, IL-4I1, IL-20RA, IL-8L2, and BPI. However, a greater proportion of these genes are related to functions including injury response, metabolism, cell adhesion and remodeling, extracellular matrix (ECM) degradation, and membrane transport. Assessing the top DEGs due to GT5 exposure revealed that by day 2, most of the genes were involved in cell growth/proliferation, wound repair, and maintenance of the ECM (Fig. S2). Therefore, to provide a more focused examination of specific differences in the host immune response, the top
30 DEGs unique to $R_{\text{low}}$ infection and Mg7 exposure (not found in GT5) were determined (Fig. S3). Several DEGs potentially involved in the host’s pro-inflammatory response include TLR-4, -15, IL-1β, IL-1R2, IL-8L1, IL-22RA2, CXCL1, CCLI10, IRG1. On day 1, $R_{\text{low}}$ infection resulted in greater differential expression than Mg7 exposure, whereas on day 2 the extent of differential expression observed in these two groups were less pronounced. Lastly, to deduce differences in the host response to $R_{\text{low}}$, the DEGs unique to $R_{\text{low}}$ infection were assessed (Fig. S4). DEGs with immune related functions included IL-6, IL-12B, IL-22, CCL4, CD80, CSF2RA, TNFSF11, and NCF2.

**Discussion**

The development of attenuated vaccine strains derived from pathogenic bacteria has traditionally been performed randomly (e.g. serial passage, chemical/UV mutagenesis or by generating transposon mutant libraries), with subsequent assessments consisting of immune response, histopathology and protection studies. These time-honored yet inefficient approaches may give way to ‘precision’ vaccine design in which host responses are specifically tailored to match correlates of immune protection on a gene/pathway-specific basis, especially as we enter the age of CRISPR/Cas9 gene editing of synthetic bacterial genomes.

As a first step, we must learn what current attenuated vaccines have in common, and more importantly, how they differ with respect to host responses to pathogenic strains. In this regard, the current study examined the global transcriptional differences in the host response to virulent versus attenuated vaccine strains of *M. gallisepticum* during early stages of infection. Previous studies have largely assessed the efficacy and adaptive host immune response to GT5
and Mg7 (8, 20, 21, 24), with only one study assessing the innate immune response to GT5 (19). These studies concluded that GT5 and Mg7 are non-pathogenic as they result in minimal inflammatory cell infiltration into the lamina propria and nominal increases in tracheal thickness. Both attenuated strains provide protection against virulent R\textsubscript{low}; interfollicular zones of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were found in GT5 vaccinated birds upon challenge (8), and both GT5 and Mg7 resulted in IgG- and IgA- secreting plasma cells in tracheal tissue, and anti-\textit{M. gallisepticum} IgG in serum (8, 20). Comparatively, Mg7 resulted in greater tracheal thickness than GT5 likely due to a more robust cellular infiltration, however, it outperformed GT5 in protection as indicated by less pathology and lower rates of R\textsubscript{low} recovery 4 weeks after challenge (20).

Differential expression of chemokines and cytokines were previously compared between R\textsubscript{low} infected and GT5 exposed chickens on days 1, 4 and 8 post-infection (19). Notable findings of that study included decreased expression of CCL20, IL-8 and IL-12p40 in both GT5 and R\textsubscript{low} infected chickens 1 day post-infection. Day 4 post-infection showed decreased expression of CCL20 and IL-12p40 in both GT5 exposure and R\textsubscript{low} infection, and GT5 exposure continued to show decreased IL-8 expression while R\textsubscript{low} infection was comparable to controls. In the current study, expression of CCL20 was also found to be decreased in both R\textsubscript{low} infected and GT5 exposed chickens on days 1 and 2. Conversely, expression of IL-12B (IL-12p40) and IL-8 was increased, though not significant, on day 1 due to GT5 infection and was significantly increased as a result of R\textsubscript{low} infection. Day 2 yielded significant expression of IL-8 as a result of both Mg7 exposure and R\textsubscript{low} infection, and IL-12B as a result of R\textsubscript{low} infection. It is important to note that our previous transcriptomic 7-day R\textsubscript{low} infection study utilizing RNA sequencing showed comparable results for these genes on days 1 and 3 post infection except for IL-12B which was
only significantly increased on day 1 (10). The current study used a different study design; performing a single inoculation with a dosage equal to or less than those in the previous studies (8, 19–21, 24), and sampling cells from the trachea by pipetting TRIzol through the lumen. In comparison, this approach may have resulted in a limited host response that peaked at an earlier time point, and may have only captured epithelial cells and cells loosely associated with the lamina propria. However, these cells are most likely to be in close contact with the organism and hence more likely to differentially express genes in response to infection.

The significant number of DEGs found within metabolic pathways is ostensibly due to the epithelial cell distress that occurs upon infection (25); suggesting that *R. low* infection results in greater cell stress and subsequent injury. Cell stress is also known to trigger an immune response through activation of the inflammasome within the NOD-like receptor pathway (26, 27). While activation of inflammasomes during host defense typically occurs in macrophages (26), the Nlrp3-inflammasome can be activated in murine respiratory epithelium during induced airway inflammation (28), and in human lung epithelial cells in the presence of silica (29). Additionally, recent studies have shown that other *Mycoplasmas* including *M. salivarium* (30), *M. hominis* (31), and the CARDS toxin of *M. pneumoniae* (32), are involved in the activation of inflammasomes. Caspase-1, a regulator of the processing and secretion of interleukins IL-1β, IL-18, and IL-22, a component of the Nlrp3-inflammasome, requires the presence and binding of extracellular ATP to the P2X7 purinergic receptor to become activated. Cellular efflux of ATP is known to occur during cellular stress and as a consequence of bacterial infections including *Mycoplasma pneumoniae* and *M. hominis* (33–36). Additionally, it has been shown that the presence of TLR ligands, such as bacterial lipoproteins, are required to prime the cells to activate caspase-1 in response to ATP (26, 37). In the current study, we note that many of these genes
were indeed differentially expressed. For example, expression of the P2X7 receptor gene was significantly increased on Days 1 and 2 in both R<sub>low</sub> and Mg7 exposed chickens, with higher expression seen in the R<sub>low</sub> infected group (Fig. S5). TLR-2, a well-characterized receptor for *M. gallisepticum* lipoproteins (9), was differentially expressed in all three infection groups on both days, however it was only statistically significant in the R<sub>low</sub> infected group (Fig. 4). CASP1 showed increased expression in R<sub>low</sub> and Mg7 on both days, with higher levels in R<sub>low</sub> infected chickens which was significant on day 1. In the GT5 exposed group, CASP1 expression was minimally increased on day one, and actually decrease on day 2 in comparison to controls (Fig. S5). Significantly increased expression of IL-1β was found on both days in response to R<sub>low</sub> infection and Mg7 exposure, with the greatest differences found in the R<sub>low</sub> infected group. In GT5 exposed chickens, IL-1β was only significantly expressed on day 1 (Table 1). In the current study, we were unable to determine which cell type(s) the observed differential expression was attributed to, and it is unknown whether sufficient extracellular ATP was present. However, given the capabilities of other *Mycoplasmas* to activate inflammasomes and the expression profiles seen here, further study on the potential role of Nlrp3-inflammasomes in the pathogenesis of R<sub>low</sub> is warranted.

Comparing gene ontologies between infection groups further supported the differences between virulent and attenuated vaccine strains. The most prominent observation was the number of functional processes that are affected by R<sub>low</sub> on day 1 compared to those of the attenuated strains. While it has been well established that the host response to R<sub>low</sub> is rapid (8, 9), its magnitude was further highlighted when comparing it to the limited number of mitogenic and cytokine related host response processes activated upon Mg7 and GT5 exposures. However, it is important to note that host cells responded to the presence of GT5 as indicated by the affected
chemotactic processes despite the limited cytadherence capabilities of this attenuated strain. On day 2, the presence of ‘positive regulation of NF-kappaB import into the nucleus’ likely represents the expression and possible ligation of TLRs acting as ‘master switches’ are responsible for transcription of many downstream immune related genes. The processes affected by Mg7 more closely resembled those seen in R_{low}, indicating a more gradual activation of the host response. GT5 exposure on day 2 affected processes primarily involved in repair and regrowth with only 2 processes involved in the pro-inflammatory host response. The difference in the processes observed in the R_{low} infected, and Mg7 exposed groups compared to the GT5 exposed group may be due to its diminished cytadhesion capabilities, thereby reducing its attachment and residence time in association with the host mucosal epithelium.

An interesting difference seen in the gene ontology of Mg7 exposed chickens on day 2 was the ‘macrophage activation’ process. The ability of R_{low} to recruit macrophages has been established in previous in vivo and ex vivo studies, however, the extent to which they become activated is unknown based on conflicting data regarding IL-12p40 (IL-12B) and IFN-γ typically produced by activated macrophages, and may be yet another indicator of the dysregulated host response (19, 38). It should be noted that the expression of IL-12B does not necessarily indicate the presence of activated macrophages; this cytokine can also be produced by dendritic cells and respiratory epithelial cells (9, 39). In the current study, expression of IL-12B was significantly increased on both days in the R_{low} infected group, but varied greatly within Mg7 and GT5 exposed groups and was not found to be significant. Additionally, IFN-γ gene expression was not found to be significantly differentially regulated in any of the infection groups. Whether the ‘macrophage activation’ process indicated by gene ontology would result in activated macrophages, or whether the sampling method used in the study prevented accurate measures of
macrophage produced cytokines is unknown. However, it would be beneficial to determine whether these attenuated vaccine strains are capable of activating macrophages that enable successful antigen presentation, as this would be a desirable trait in future vaccines.

In addition to IL-12B, genes encoding proteins with immune related functions including IL-6, IL-22, and CCL4 (MIP-1β), were uniquely upregulated in R\textsubscript{low} infected chickens. IL-6 is a multifunctional cytokine that has been shown to play a role in both host protection and disease exacerbation depending on which other cytokines are present (40–42). Therefore, whether this cytokine is a contributing factor in the pathogenesis that results from R\textsubscript{low} infection remains unclear. IL-22 is a pro-inflammatory cytokine produced by immune cells including Th17 lymphocytes, acts on epithelial cells and requires tight regulation (reviewed in 37). Similar to IL-6, this cytokine can contribute to maladaptive host immune responses depending on the presence of other pro-inflammatory cytokines, and may also result in increased levels of IL-6 and IL-8 (reviewed in 37). This may explain the unique expression of IL-6 and the greater expression levels of IL-8 in R\textsubscript{low} infected chickens compared to Mg7 and GT5 exposures. CCL4 is a chemoattractant for immune cells such as NK cells and monocytes (44), and may further contribute to the immune cell infiltrates and immunopathology of R\textsubscript{low}.

Given the importance that TLRs have shown in *Mycoplasma* infections (9, 45, 46), it is important to consider the differences in expression of TLRs between virulent and attenuated strains. Based on ex vivo analysis, it has been suggested that TLR-2 signaling in tracheal epithelial cells contributes to the notable influx of inflammatory cells into the lamina propria during R\textsubscript{low} infection (9). In the current study, TLR-2 expression was only significantly increased in the R\textsubscript{low} infected chickens. As seen in our previous R\textsubscript{low} infection study (10), TLR-4 and TLR-15 were significantly increased on both days in R\textsubscript{low} infected chickens. Mg7 exposure
resulted in increased expression of these TLRs, but was still significantly lower than the expression seen in response to R_{low}; GT5 exposure resulted in an equivocal increase in the expression of TLR-4 and -15. As previously discussed, increased expression of TLR-4 may indicate increased cellular response to extrinsic factors including the presence of damage-associated molecular pattern molecules (DAMPs) such as heat shock proteins, heparin, fibrinogen and fibronectin as a result of damaged extracellular matrix (ECM) or cellular injury (10, 47–52). Aside from cell stress related-injury, damage to cells and the ECM may be due to a disproportionate increase in the expression of matrix metalloproteases (MMPs) 7 and/or 9. These MMPs have been shown to play important roles during cellular injury and repair (47, 48), but also result in excess degradation of the ECM when increased cytokine production is coupled with deficient expression levels of tissue inhibitors of metalloproteinases (TIMPs) (49, 50, 53). Our studies have confirmed the observed perturbations in MMP7 and/or -9 expression, with significant increases in response to R_{low} infection at all time points (10); in the current study an increase in expression of these MMPs was observed in response to both Mg7 and GT5 exposure. The greatest difference was seen in response to R_{low} infection, but these genes were also expressed at higher levels in response to Mg7 infection, with the lowest levels seen in GT5 (10). TLR-15, which is unique to avian and reptilian lineages, is a broad spectrum TLR that recognizes lipopolysaccharide (LPS), CpG oligonucleotides, gram-negative and gram-positive bacteria, tripalmitoylated lipopeptide as well as diacylated lipopeptide from Mycoplasma synoviae (54–57). The expression patterns of TLR-15 seen in each infection group was similar to that of TLR-4, and was significantly increased in R_{low} infection throughout our previous 7-day time course (10). Therefore, it is possible that the interaction of lipoproteins and the greater levels of cellular stress and damage due to R_{low} infection, collectively contributes to the
increased expression of TLRs 2, 4, and 15, which in turn promotes disease exacerbation through the subsequent increased expression of pro-inflammatory cytokines and chemokines that are not expressed at the same magnitude in attenuated vaccine strains.

Lastly, \textit{R}_{\text{low}}\textsuperscript{infection} resulted in significant differential expression of many cytokine and chemokine receptors (Fig. 4). On day 1, the number of differentially expressed receptors was approximately 60\% greater than that observed upon \textit{Mg7} exposure, and approximately 24\% greater on day 2. \textit{GT5} exposure resulted in only one differentially expressed receptor on day 1 and 2 receptors on day 2. Taken together, wide-spread increases in expression of cytokine, chemokine, and TLR receptors, may indicate that \textit{R}_{\text{low}}\textsuperscript{infection} results in increased cell sensitivity to signaling molecules including host factors (e.g. cytokines/chemokines and their receptors) and those present on mycoplasmas, DAMPs, and endogenous gram-negative bacteria. The fact that \textit{GT5} exposure does not appear to induce increased sensitivity of host cells is significant and is consistent with the lack of immunopathology observed in response to this strain.

Overall, based on the gene expression profiles observed in this study, it appears that the differences in host response to virulent \textit{M. gallisepticum} strain \textit{R}_{\text{low}}\textsuperscript{infection} compared to avirulent strains \textit{Mg7} and \textit{GT5} is partly due to the complex interplay between metabolism, cellular stress/injury and the subsequent pro-inflammatory response, and a possible increase in cellular sensitivity due to increases in gene expression of immune recognition receptors. The host response to \textit{Mg7} involves far fewer metabolic genes which suggests reduced cellular stress and damage, and evokes a proinflammatory response that is less robust and slower to evolve than that observed in response to \textit{R}_{\text{low}}\textsuperscript{infection}. \textit{GT5} evokes a response that is markedly dampened compared to both \textit{R}_{\text{low}}\textsuperscript{infection} and \textit{Mg7} exposure; not only was the response on day 1 minimal in comparison, it
was the only strain that did not result in a significant increase in metabolic and immune related functions. This may explain why previous studies showed that Mg7 out-performed GT5 upon R<sub>low</sub> challenge (20). As previously mentioned, recruitment of various immune cells into the lamina propria due to <i>M. gallisepticum</i> infection varies between virulent strain R<sub>low</sub> and attenuated vaccine strains Mg7 and GT5. Examining the differences of the host response to these strains during early time points has allowed comparison of the host’s initial interaction with <i>M. gallisepticum</i>, and was intended to assess whole tissue gene expression prior to the influx of immune cells into the lamina propria due to prolonged chemotaxis and activation of leukocytes observed in response to R<sub>low</sub> infection. It is possible that the decreased response seen in Mg7 is partly due to its reduced metabolic abilities and limited viability, however it was shown that recovery of Mg7, though limited, occurred 2 weeks post-infection (21). It is also assumed that differences seen in GT5 may in part be due to its limited cytadherence capabilities, resulting in reduced contact/residence in association with the host’s mucosal epithelium.

Global transcriptional comparison of the early chicken tracheal response to virulent and attenuated vaccine strains of <i>M. gallisepticum</i> has illustrated the varied molecular events in metabolic and immune related pathways of potentially maladaptive vs. protective host responses. Development of future vaccines should aim to promote the expression of those immune-related genes expressed as a result of both Mg7 and GT5 infection, limit the magnitude of expression of those genes common to R<sub>low</sub> and Mg7, while avoiding proinflammatory and metabolism-related genes uniquely associated with R<sub>low</sub>. Targeting those components of attenuated vaccine strains Mg7 and GT5 that have stimulated a host response lacking immune-related pathogenesis while promoting the successful connection between the innate and adaptive responses, will result in a safe vaccine capable of providing long-term protection from virulent strains of <i>M. gallisepticum</i>.
Materials and Methods

Animals

Four-week-old female specific-pathogen-free White Leghorn chickens (SPAFAS, North Franklin, CT, USA) were divided randomly into four groups of 10, placed in HEPA-filtered avian isolators, and allowed to acclimate for one week. Non-medicated feed and water were provided ad libitum throughout the experiment. All components of the study were performed in accordance with approved UConn IACUC protocol number A13-001.

Chicken Infection

Stocks of *M. gallisepticum* strain R<sub>low</sub> (passage 17) were grown in Hayflick’s complete medium overnight at 37°C until mid-log phase was reached as indicated by a color shift from red to orange. Vaccine strains Mg7 (passage 6) and GT5 (passage 5) were grown in Hayflick’s complete medium with gentamicin selection (150 µg/ml) as previously described (20). Bacterial concentrations were determined by the OD<sub>620</sub> 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 minutes and resuspended at 5 x 10<sup>8</sup> CFU/ml in Hayflick’s complete medium. Chickens were infected by pipetting 1 x 10<sup>8</sup> CFU/200 µl of R<sub>low</sub>, Mg7, or GT5 (‘infection groups’), or 200 µl of Hayflick’s medium alone (‘control group’) into the tracheal lumen as previously described (52).

RNA Extraction

Fifteen infected (5 per ‘infection group’) and five ‘control’ chickens were humanely sacrificed at 24 and 48 hours (‘days 1 and 2’) post-infection. Tracheas were excised and total
RNA was immediately stabilized by pipetting 1 mL TRIzol reagent four times through the lumen of each individual trachea (52) to minimize the changes in gene expression that may occur during the time between specimen collection and RNA purification (Invitrogen, Carlsbad, CA, USA), and stored at -80º C. Total RNA was then purified using the Zymo-Direct-zol RNA Miniprep Kit according to manufacturer’s instructions (Zymo Research Corporation, Irvine, CA, USA).

**Illumina Sequencing**

cDNA libraries were created from RNA isolated from each individual chicken on days 1 and 2 post-infection using the Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the Illumina TruSeq RNA Sample Preparation v2 (HT) protocol. Briefly, PolyA selection was performed using RNA purification beads on 0.1-4 µg of total RNA per sample to obtain mRNA. 10-400ng 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 dNTPs including dUTP, DNA polymerase, and RNase. End repair was performed by adding dATP to all free 3’ ends and adaptor ligation added unique index sequences to the fragments. The products were then amplified by PCR and purified.

The cDNA libraries were quantified using the Qubit 2.0 fluorometer (Invitrogen) and assessed for correct fragment size (~260bp) using the Agilent TapeStation 2200 (Agilent Technologies). Libraries were normalized to 2nM, pooled, denatured and sequenced on the NextSeq500 Sequencing platform (Illumina Inc.) using a 75bp paired-end approach targeting approximately 10 million reads per sample.
**RNA-Seq Analysis**

Fastq data from individual birds were mapped using the TopHat package (version 2.0.1) utilizing the Bowtie2 engine (version 2.2.5) to align reads to the *Gallus gallus* reference genome (WASHUC2) producing BAM alignment files (53,11). BAM files for each day were processed as experimental condition groups with Cufflinks (version 2.2.1) as previously discussed (10). Within-condition normalization of data using Cuffdiff was achieved by calculating the fragments per kilobase of exon per million fragments mapped (FPKM) for each gene. The generated p-value was then used to determine the significance of the differential expression based on the Benjamini-Hochberg correction with a false discovery rate (FDR) of <5% to generate the q-value. Between-condition differences in expression values were considered significant when q ≤ 0.05, thus addressing within-condition variation for all biological replicates on each day. Fold change was determined from the log2 transformation of the ratio of FPKM between conditions (53, 54). For this study, all references to differentially expressed genes (DEGs) implies statistical significance.

**Pathway Analysis**

Identification of biological pathways that significant DEGs were involved in, was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.7 (55). Pathways containing ≥ 2 significant DEGs were categorized and displayed.
**Functional Gene Ontology**

DEGs with a log$_2$ fold change $\geq 2.00$, as identified by Cuffdiff, were analyzed to identify enriched biological processes based on Gene Ontology (GO). This was performed using ClueGO v2.3.3, a plugin of the software platform Cytoscape v3.5.1 (56). ‘GO term fusion’ was applied to reduce redundancy by comparing ‘parent-child’ relation sharing similar genes and displaying the more representative ‘parent’ or ‘child’ term (56). Processes that had a p-value $\leq .05$ for each time point were considered significant and displayed.

**Acknowledgements**

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References


48. Raulo SM, Sorsa T, Tervahartiala T, Piril?? E, Maisi P (2001) MMP-9 as a marker of inflammation in tracheal epithelial lining fluid (TELF) and in bronchoalveolar fluid


FIG 1 The number of DEGs determined by comparing each infection group (Rlow, Mg 7 and GT5) to the respective control group as identified by Cuffdiff. (a) The overall number of DEGs identified in each group on days 1 and 2 post-infection. (b) The number of DEGs with increased or decreased expression. (c) Venn diagram displaying the DEGs per infection group. The number of unique and common DEGs are displayed.
Day 1

a)

![Bar graph showing pathway categories on the x-axis and the number of pathways/category on the y-axis. The graph compares Rlow, Mg7, and GT5 conditions.]

b)

![Bar graph showing DEGs/pathway category on the y-axis and pathway categories on the x-axis. The graph compares Rlow, Mg7, and GT5 conditions.]
Day 2

c)

![Graph showing the number of pathways/Category across different conditions Rlow, MG7, GT5.](image)


d)

![Graph showing the number of DEGs/Pathway across different conditions Rlow, MG7, GT5.](image)
**FIG 2** Pathway analysis. Pathways containing $\geq 2$ DEGs were categorized based on function using DAVID Bioinformatics software. (a & c) The number of identified pathways per category is indicated for each infected group on day 1 (a) and day 2 (c) post-infection. (b & d) The number of DEGs per pathway category is indicated for each infected group on day 1 (b) and day 2 (d) post-infection.

**Day 1:**

![Pathway Diagram](image-url)
Day 2:

**FIG 3** Immune Pathways. DEGs involved in these pathways are indicated for each infection group; red stars (R_{low}), blue stars (Mg 7), and yellow stars (GT5) as identified by DAVID Bioinformatics software. Green shading represents the completeness of the pathway identified within the *Gallus gallus* genome.
FIG 4 Toll-like receptors. TLRs identified by RNA-Seq analysis are displayed. Differential expression is expressed as log$_2$ fold change. Individual bars indicating $R_{low}$, Mg 7, and GT5
represent differential expression seen between each infection group and the control group.

Significant differential expression of TLRs between infected and control birds are indicated with an (*) above its respected bar. Significant differential expression compared directly between infection groups (R\textsubscript{low} vs. Mg 7) and (R\textsubscript{low} vs. GT5) is indicated across the top of the bars with *. Differential expression is considered significant at q ≤ 0.05.

**TABLE 1** Top 30 common DEGs as determined by Cuffdiff and ranked based on the log\textsubscript{2} fold change values in the R\textsubscript{low} infection group.
Day 1 $R_{low}$ Infected
Day 2 GT5 Infected
FIG S1 Functional Gene Ontology. Significant DEGs with a log$_2$ fold change $\geq$ 2.00 were analyzed for enriched biological processes using the ClueGO application for Cytoscape. ‘GO term fusion’ was applied to reduce redundancy by comparing ‘parent-child’ relation sharing similar genes and displaying the more representative ‘parent’ or ‘child’ term. Bars represent the percentage of associated genes assigned to a unique GO term with the absolute number of associated genes located at the end of the bars. The color of the bars indicates functionally related groups based on the similarity of associated genes. Significance of enriched biological processes are as indicated (* = $p \leq 0.05$, ** = $p \leq 0.001$).

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FIG S2 Top 30 DEGs identified in the GT5 infection group as determined by Cuffdiff. Genes are ranked based on the log$_2$ fold change. Fold change is considered significant at a q-value of $\leq 0.05$. 

149
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<th>Gene:</th>
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<th>Log FC Mg7:</th>
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**FIG S3** Top 30 DEGs common only to the R$_{$low} and Mg 7 infection groups as determined by Cuffdiff. Genes are ranked based on the log$_2$ fold change of the R$_{$low} infection group.
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**FIG S4** Top 30 DEGs unique to the R<sub>low</sub> infection group as determined by Cuffdiff. Genes are ranked based on the log<sub>2</sub> fold change values.

**FIG S5** Log<sub>2</sub> fold change of CASP1 and P2RX7 expression in each infection group as determined by Cuffdiff. Fold change is considered significant at a q-value of ≤0.05.
CHAPTER 4:

Conclusions and Future Directions

Conclusions

The overarching goal of this research was to evaluate the host tracheal response using an unbiased, global approach. To do this, we sampled host cell RNA by pipetting TRIzol through the tracheal lumen, enabling the recovery of RNA from cells that were in the closest proximity to *M. gallisepticum*. Collectively, these transcriptomic studies have expanded our understanding of the magnitude of the chicken tracheal response to virulent *M. gallisepticum* strain R<sub>low</sub> over a 7-day time course, and the comparatively dampened host response elicited by laboratory attenuated vaccine strains Mg 7 and GT5 during early infection. Furthermore, these data enabled the functional analysis of biologically relevant pathways and how their varied involvement may contribute to the maladaptive or beneficial host response to virulent and attenuated strains of *M. gallisepticum*, thereby providing critical information with regards to pathways and gene products to either be included or excluded in the response to future vaccine preparations.

In the time course analysis of the tracheal host response to virulent R<sub>low</sub>, we observed a significant pro-inflammatory response involving several immune related pathways involving many DEGs that peaked at day 3, and trended towards baseline expression values by day 7. The peak of infection, as indicated by the greatest number and fold change seen in DEGs, gross pathology, and bacterial recovery, was approximately 10-12 days earlier than the peak of infection and subsequent lesion scores that have been observed in other studies (1–3). However,
it is important to note that previous studies inoculated chickens twice, whereas our study used a single inoculation.

The significant pro-inflammatory response was not only reaffirmed through transcriptional analysis, it identified additional genes involved in this response that were not previously appreciated in the host response to *M. gallisepticum*. Previous studies have shown the importance of predominant TLR-2 signaling and the subsequent production of pro-inflammatory cytokines in response to R<sub>low</sub> infection, while other TLRs including TLR-15 and TLR-4 were observed, they were expressed at a far lesser degree than TLR-2 (4, 5). Our data further support the importance of TLR-2, however the increased expression of TLR-15 and TLR-4 were considerably greater than TLR-2. TLR-15 was recently shown to be activated in the presence of *M. synoviae* lipoproteins (6), and therefore, it is not surprising to find increased expression of this TLR given the interaction between *M. gallisepticum* lipoproteins and host tracheal mucosa.

It is however, important to consider the role that TLR-15 may play in the pro-inflammatory response. TLR-4 expression has also been associated with other *Mycoplasmas* including *M. pneumoniae* (7), *M. arthritidis* (8), and *M. hyorhinis* (9), though the direct role it may play in the pathogenesis of disease remains uncertain. The potential role of TLR-4 as a receptor for DAMPs (10–12) suggests a possible scenario in which the damage to host cells caused by *M. gallisepticum* results in the activation of TLR-4. The increased expression of TLR-4 may indicate another pathway capable of resulting in pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 and IL-12. Furthermore, expression of TLR-15 and TLR-4 in Mg 7 and GT5 infected chickens were significantly less than R<sub>low</sub> infected chickens, suggesting an association of the host response to the virulence status of the bacteria. While the changes seen in TLR expression may in part be due to changes in the inflammatory cells infiltrating the lamina propria, these findings
have identified additional potential mediators of the host inflammatory response that warrant further study.

In addition to TLRs, there were numerous cytokine and chemokine receptors that showed significant differential expression as a result of R<sub>low</sub> infection. In comparison, Mg7 infection resulted in 60% fewer differentially expressed receptors on day 1 post-infection and 24% on day 2, and GT5 infection only resulted in three differentially expressed receptors. This suggests that R<sub>low</sub> infection may increase host cell sensitivity to signaling molecules present on mycoplasma, DAMPs, endogenous gram-negative bacteria, and host factors such as chemokines and cytokines. With the increased expression of cytokines and chemokines and probable cell stress present upon R<sub>low</sub> infection, the increased expression of host cell receptors may contribute to the exaggerated and dysregulated host response to virulent <i>M. gallisepticum</i>.

The extent of the host’s metabolic activity upon <i>M. gallisepticum</i> R<sub>low</sub> infection was also striking; affecting the greatest number of genes on days 1, 2, and 3 post infection. The number of metabolic genes activated in Mg 7 was considerably less, while GT5 infection activated the least number of metabolic genes. This finding is likely indicative of reduced cellular stress during infection with attenuated strains, and correlates with the levels of TLR-4 expression seen in each infection group and the likely presence of DAMPs. It is also possible that the increased cell stress and metabolic activity seen during R<sub>low</sub> infection yields greater levels of extracellular ATP which subsequently binds purinergic receptors, and potentially activating the Nlrp-3 inflammasome. This was reflected in the gene expression profiles as the P2X<sub>7</sub> purinergic receptor for extracellular ATP, and CASP1, a regulator of the Nlrp-3 inflammasome, were significantly expressed in both R<sub>low</sub> and Mg 7 infected chickens, with greater values observed in the R<sub>low</sub> infected chickens. Tthe end-product of inflammasome activation, IL-1β, was
significantly expressed in each infection group, with the greatest levels were seen in $R_{\text{low}}$ infected chickens. By day 2, expression was no longer significant in the GT5 infected chickens. As these genes are components of the NOD-like receptor pathway, these data suggest that this pathway may play an important role in pathogenesis during infection with virulent mycoplasma. This offers insight as an immune related pathway to avoid activating by using strains with limited cytadhesion capabilities to limit the contact with host cells when designing future vaccines.

Comparison of the host response to virulent of $R_{\text{low}}$ to the attenuated immune responses elicited by Mg 7 (13, 14) and GT5 (1, 14, 15), has identified host genes that may serve as safe and effective targets for future vaccines. These potential gene targets include those that are uniquely expressed in Mg 7, and genes expressed in either $R_{\text{low}}$ and/or Mg 7 that are common with GT5 (Fig. 1c, p. 139). It may be necessary to include some genes whose expression is induced in $R_{\text{low}}$ and Mg 7, however, those that are unique to $R_{\text{low}}$ likely contribute to the immunopathology observed during $R_{\text{low}}$ infection. For example, as seen in the cytokine-cytokine receptor pathway, there are several host cell receptors significantly expressed only during infection with $R_{\text{low}}$, several others that are expressed during Rlow and Mg 7 infection, while GT5 infection only resulted in a select few. This suggests that ligands of the receptors activated during $R_{\text{low}}$ infection should be avoided; thereby decreasing the sensitization of the host cell and reducing the downstream products resulting from receptor activation. In contrast, while activated by cytokines, expression of genes within the Jak-STAT pathway were similar between the three strains, suggesting that this pathway does not contribute to a maladaptive host response.
**Future Directions**

A caveat to the studies presented here, is the uncertainty as to which cells the mRNA transcripts were produced from. Therefore, some of the changes seen in expression values may well be in part due to the changing abundance of immune cells. Nonetheless, this data provides us with important insights into the host’s overall response to infection with *M. gallisepticum*. Although beyond the scope of this project, future studies should focus on the identification of the specific host cells which are responsible for the increase in transcripts thought to be important in the host immune response. This could be accomplished by in situ hybridization, laser capture microdissection, or single-cell sequencing analyses. This would further elucidate the host response and the critical roles that some cells likely play in either immunopathology or a protective immune response.

It was recently shown that interaction of *M. bovis* with neutrophils prevents the release NETs, however, it also induces increased elastase production in neutrophils which degrades the ECM (16). Early infection with *M. gallisepticum* results in the infiltration of heterophils into the lamina propria which are thought to play a significant role in host immune response (17, 3). It would be of interest to determine whether interaction of *M. gallisepticum* with chicken heterophils also prevents the release of HETs and whether elastase production is increased (18). While elastase is intended to aid in the killing of pathogens by degrading the membrane, release of elastase and the subsequent degradation of the host ECM may only contribute to tissue damage during acute inflammation. Additionally, the degradation of the ECM may play a role in the pathogenesis of disease as *M. gallisepticum* contains surface molecules OsmC and PlpA that
bind ECM components heparin and fibronectin respectively, and may therefore contribute to an environmental niche aiding in the chronicity of *M. gallisepticum* (19, 20).

Transcriptional analysis has provided a global assessment of the host response; however, transcription does not necessarily mean translation. To better understand which gene transcripts are actually being translated and therefore affecting the host response, it would be beneficial to utilize ribosomal profiling to then perform transcriptional analysis only on those transcripts that were undergoing active translation (21).

Creation of the Mg 7 vaccine strain through transposon mutagenesis of the *lpd* gene of *R*low did not affect its capability of cytadhesion, however, GT5 cytadhesion capabilities remained equivalent to that of *R*high at approximately 24%. By two weeks post infection, GT5 is not recovered from infected chickens (1, 14, 15), and Mg 7 was only recovered in approximately 12% of infected chickens. While this may be partly due to clearance of the organism through host mechanisms and/or the immune response, it is likely that the inadequate ATP production of Mg7 and limited cytadhesion of GT5 play roles in this clearance. While the growth curve of Mg7 has shown to be comparable to that of *R*low over a 54-hour growth period, it is uncertain how long Mg7 stays in contact with the tracheal mucosa (13). Therefore, transcriptional analysis of the F strain that is capable of providing protection from virulent strains and known to persist in the host over a long period of time (22), may provide useful insights regarding a host response that develops during constant contact with the organism. Additionally, because the host response to Mg 7 more closely resembled the host response to *R*low by day 2 post-infection, it may be advantageous to understand the changes that occur in the host response to Mg 7 as the course of infection continues. This would enable comparisons to be made at the height of infection/peak host response, and may assist in understanding the differences between *R*low and Mg 7 in the
transition from the innate to adaptive host immune response to virulent and attenuated strains. This would be important especially given the significance that the Th1 and Th2 balance play in mycoplasma pathogenesis and the cytokines and chemokines that may be involved in this process.

In conjunction with these studies, Dr. Katherine Pflaum of our lab, sequenced *M. gallisepticum* RNA obtained from the tracheas of the same infected chickens to assess the changes in *vlhA* expression (23). This was a comprehensive host/pathogen analysis over the course of early infection. Interestingly, changes in *vlhA* gene expression were seen as early as 1-2 days post-infection. And while these changes are occurring before the development of specific antibodies (1), it is likely that interaction between the host and pathogen contribute to *vlhA* expression changes. Further understanding of the changes in *vlhA* expression and their interaction with host cell receptors such as TLRs 1, 2, 6, 15 and NOD-like receptors may identify important mechanisms in cellular activation and signaling that occur in the presence of *M. gallisepticum*.

The global transcriptional analysis of the host response to virulent *M. gallisepticum* *R*<sub>low</sub> and the differences seen in the response to attenuated vaccine strains Mg 7 and GT5, has provided further understanding of how complex the response to this pathogen is. The studies presented here emphasized significant changes in genes with metabolic and immune functions during *M. gallisepticum* infection, and have implied the importance of host cell receptors and the production of chemokines and cytokines that likely ensues. While many genes active in the host response to *R*<sub>low</sub> are likely important in the activation of the adaptive immune response, the magnitude of expression should resemble that seen in response to Mg7. Overall, future vaccine development should focus on targeting those genes that were differentially expressed in both
virulent $R_{\text{low}}$ and attenuated vaccine strains Mg7 and GT5. Specifically, limiting the expression of host cell receptors and proinflammatory cytokines while ensuring the production of chemokines and cytokines including, but not limited to IL-8, IL-12B, CCL4, and IFN-$\gamma$, will enable the innate response to activate protective adaptive immunity. These data, coupled with future analyses aimed at identifying the specific cells, gene products, and their direct implications on the host response will aid in the development of future therapeutics and vaccines.
References


