BORDETELLA SPECIES: SENSING AND EVADING HOST IMMUNITY

A Dissertation in
Biochemistry, Microbiology and Molecular Biology

by
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ABSTRACT

To evade host immunity and successfully colonize and persist within host, pathogens must sense and appropriately respond to the environment. This dissertation investigates several interactions between the host and the classical bordetellae, which must not only sense their environment, but evade host immunity to persist in the host. Little is known concerning what environmental cues the classical bordetellae sense within the host. Here, we investigated their response to growth in 5% CO$_2$ conditions and identified increased transcription of several known virulence factor genes. Additionally, transcription of two genes, cyaA and fhaB, was found to be increased in 5% CO$_2$ conditions in the absence of a functional BvgS—the master virulence factor gene regulator. These data indicate that there are additional virulence factor gene regulatory mechanisms that respond to growth in 5% CO$_2$ conditions. On the other hand to evade host innate immune response, *Bordetella* species secrete a molecule(s) that inhibits LPS-induced IL-1 secretion in murine macrophages. *B. bronchiseptica* and *B. pertussis* have additional mechanisms to induce secretion of IL-1, but *B. parapertussis* does not. This inhibitory molecule did not affect transcription or downstream inflammasome products, such as caspase-1, suggesting that it blocks release of IL-1 from macrophages. Furthermore, additional host immune factors are important for the control of *Bordetella* species colonization in the respiratory tract, such as complement. *B. parapertussis*$_{ov}$ strains, isolated only from sheep, are rapidly cleared from the lower respiratory tract of mice, unlike the other classical bordetellae. *B. parapertussis*$_{ov}$ strains did not produce an O-antigen, were susceptible to murine complement deposition and killing, and colonized complement deficient mice more efficiently. Unlike mouse serum, sheep serum did not efficiently kill *B. parapertussis*$_{ov}$ strains, indicating a role for complement in host adaptation of
this species. Evasion of cross-protective immune responses through antigenic variation is one strategy employed by pathogens to evade recognition by the immune response. The *Bordetella* species produce antigenically distinct O-antigens that appear, based on SNP density and phylogenetic analysis, to have been horizontally transferred, specifically the genes thought to be responsible for serotype differences. Additionally, poorly immunogenic O-antigens do not appear to generate cross-protective immune responses, suggesting that O-antigen variation allows for evasion of protective immune responses. Combined, the data herein suggest CO₂ sensing by the classical bordetellae, the subversion of IL-1 secretion, a role for complement in host adaptation of a *Bordetella* species, and horizontal transfer of O-antigen serotype-specific genes to evade protective immunity. Finally, this thesis also discusses implications of this research and future directions resulting directly from it.
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<th>Description</th>
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<tbody>
<tr>
<td>ACT</td>
<td>Adenylate Cyclase Toxin</td>
</tr>
<tr>
<td>BG</td>
<td>Bordet Gengou</td>
</tr>
<tr>
<td>BrkA</td>
<td><em>Bordetella</em> resistant to serum killing protein</td>
</tr>
<tr>
<td>Bvg</td>
<td><em>Bordetella</em> virulence genes</td>
</tr>
<tr>
<td>C3</td>
<td>Complement protein 3</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative Genomic Hybridization</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage-associated Molecular Patterns</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle Medium</td>
</tr>
<tr>
<td>dN/dS</td>
<td>change in Non-synonymous mutations over the change in Synonymous mutations</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FHA</td>
<td>filamentous hemagglutinin</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated Molecular Patterns</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PRN</td>
<td>Pertactin</td>
</tr>
<tr>
<td>Ptx</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene diflouride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SAM</td>
<td>Significant Analysis of Microarrays</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>SS</td>
<td>Stainer-Scholte</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence Type</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TTSS</td>
<td>Type III Secretion System</td>
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</table>
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Chapter 1

Introduction
Host-Pathogen Interactions

Sensing the Host Environment:

Host-pathogen interactions are dynamic, ever-changing processes during infection in which invading pathogens encounter diverse host environmental conditions. One strategy that allows for adaptation to the rapidly changing landscape is to sense cues from specific environments, such as temperature, nutrient availability, oxygen, carbon dioxide, reactive oxygen, nitrogen compounds, iron or pH [1-3]. Many of these signals change as bacteria transition from the external environment to the host; for example, temperature is often a cue sensed by mammalian pathogens as host temperature is often elevated as compared to ambient temperature [2]. While these cues can allow for the differentiation between host and non-host, they can also be used by bacteria to discriminate specific within-host niches [3, 4]. For instance, many pathogens have intracellular stages during infection where phagocytic cells engulf bacteria, exposing them to a variety of different environments [4, 5]. To escape from intracellular compartments designed to contain and kill invaders, bacteria must first sense and then coordinately regulate the virulence factors required for escape to the cell cytoplasm or factors that prevent phago-lysosomal fusion [5]. Therefore, sensing the environment is a crucial first step to survival within the host.

Upon host entry, bacteria must express specific sets of genes in order to first colonize and then persist within the new environment by circumventing host defenses [6]. As the infection progresses, the host environment can change. Coping with new environments requires the regulated expression of specific sets of genes, including the silencing of some genes while promoting the expression of others, which is achieved by complex regulatory systems [1, 2, 7, 8]. Aberrant virulence factor expression is not only metabolically costly to the bacteria, but it often
induces host immune responses that can result in the clearance of the pathogen [6, 9]. Virulence factor expression is often regulated by complex and overlapping systems in order to integrate the sensing of multiple signals, such as directly sensing host immunomodulatory compounds, like interferon-γ and antimicrobial peptides [10-12]. Directly sensing products of the immune response can potentially allow regulation of key virulence factors to subvert immunity. The ability of a pathogen to succeed within a host depends not only on the genes encoding specific virulence factors that allow the bacteria to evade the immune response over the course of infection, but an ability to sense and respond to the changing environment.

Some research has focused on determining what specific environmental cues pathogens sense, the regulatory systems they are sensed by, and the downstream virulence factor gene regulation. The known host environmental cues sensed by bacteria are relatively undetermined for some pathogens, and therefore the complex bacterial regulatory systems and genes regulated in response to these cues are also poorly defined. Understanding these environmental cues and systems can lead to the identification of new virulence factor genes and provide further insights into host-pathogen interactions. It is our hope that determining host-induced gene regulation in pathogenic bacteria will lead to improved vaccines and therapeutics for the treatment of infections.

Host Immune Response Evasion:

In order to successfully colonize a host, bacteria must bypass surface barriers to attach to the epithelia and then evade the host immune response [6, 9, 13]. Immune responses can be characterized as either rapid and non-specific, known as innate immunity, or as specific and longer lasting, termed adaptive immunity [6]. Overcoming surface barriers and evading the innate immune response, which is mainly clearance by phagocytic cells, complement and
antimicrobials, are the first steps in bacterial pathogenesis and have often been linked to the restriction of the host range of specific pathogens [13-16]. Additionally, the innate immune response often influences the generation and type of adaptive immune response induced, which is important for not only the clearance of pathogens, but also to provide protective immunity against subsequent infections [17, 18].

Bacterial pathogens have developed many complex strategies to avoid the innate immune response by, for instance, evading immune recognition [6, 13]. Host cells have receptors that recognize highly conserved patterns of some bacterial components, such as lipopolysaccharide (LPS) in Gram-negative bacteria, which are referred to as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) [19, 20]. Pathogens have evolved mechanisms to evade recognition by these receptors by modifying structural components or by preventing the binding of host immune cell receptors to PAMPs via steric hindrance [21]. In contrast to evasion of immune recognition, some microbial pathogens modify their structural components to enhance immune activation and pro-inflammatory responses by expressing molecules that stimulate a maximal immune response [22]. Cell stimulation leading to leukocyte recruitment and disruption of the mucosal layer may allow for bacterial invasion, suggesting that under some circumstances the induction of a pro-inflammatory response may be beneficial to the invading pathogen.

Upon recognition, host molecules termed cytokines and chemokines signal for the recruitment of phagocytic cells to the area of infection to clear the invading pathogen. Bacteria have developed mechanisms to prevent phagocytosis, including the production of certain toxins that mediate cytotoxicity through induction of necrosis or apoptosis, or the production of factors that disorganize the cell cytoskeleton [23-25]. Additionally, some pathogenic bacteria promote
phagocytic uptake, surviving and even replicating inside host cells as means to hide from the immune system and evading contact with antimicrobial agents [26-28].

In addition to evading recognition or using phagocytic cells as a means to avoid the immune response, other strategies include modulating signals following recognition to subvert the downstream innate and adaptive immune responses [16]. One family of receptors that recognizes PAMPs are the toll-like receptors (TLRs)[29]. Once activated TLRs lead to induction of a signal cascades that result in the translocation of transcription factors to the nucleus and the transcription of genes encoding chemokines and cytokines like interleukin-1 (IL-1) and tumor necrosis factor- alpha (TNF-α) [30-32]. Bacterial pathogens, such as 

Salmonella species, produce molecules that disrupt the signaling cascade by preventing the release of the transcription factor NF-κB, thereby inhibiting pro-inflammatory cytokine and

Figure 1.1: Pathogens modulate macrophage signaling. Toll-like receptor (TLR) 4 is a pattern recognition receptor that recognizes bacterial molecules such as lipopolysaccharides (LPS), which induces signaling to degrade the negative regulators of nuclear factor κB (NfκB). Additionally, TRAF6 initiates mitogen activated protein (MAP) kinases that induce Actiavtor Protein-1 (AP-1). NfκB and AP-1 translocate to the nucleus and induce transcription of genes encoding cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Pathogens, such as Pseudomonas aeruginosa modify LPS in order to avoid TLR4 recognition. Downstream of TLR4, bacterial pathogens, like Salmonella spp., inhibit release of NfκB by preventing degradation of IκB. Myleoid Differentiation primary response factor 88 (MyD88), Inhibitor of kinase (Iκκ), Interleukin-1 receptor-associated kinase (IRAK), tumour necrosis factor receptor-associated factor 6 (TRAF6), Jun N-terminal kinases (JNK), extracellular signal-regulated kinases (ERK1/2).
chemokine transcription, and leading to downstream effects on cell recruitment and activation [33]. Additionally, bacterial pathogens have ways of inducing cytokine and chemokine responses that dampen the immune response [16], such as the induction of anti-inflammatory cytokine IL-10 [34, 35]. This strategy is employed by many pathogens, not only to modulate innate cell activation and recruitment to the area of infection [36], but also to influence the adaptive immune response to evade host defenses.

Pathogens that circulate within a population often have mechanisms to circumvent adaptive immunity to facilitate persistence within a host and to evade protective immunity [6, 15]. One mechanism used by bacteria to modulate the adaptive immune response is to interfere with antigen presentation [37, 38]. Some pathogens, like Mycobacterium, decrease cell surface expression of MHC-II molecules while others degrade factors required for antigen processing, thus preventing their presentation [39, 40]. Additionally, other strategies used to evade adaptive immunity include modulation of B- or T-cell effector functions, such as by blocking the signaling cascades downstream of T cell receptor signaling, and therefore inhibiting activation, as demonstrated by the Y. pseudotuberculosis virulence factor YopH [41]. Bacterial pathogens have also evolved to successfully evade protective immunity through antigenic variation, allowing pathogens with similar antigens to circulate within populations of immune hosts due to lack of cross-reactive antibodies [42, 43]. For example, variation in alpha C protein, which confers resistance to opsonophagocytic killing, via repeat region deletions allows Group B streptococci to evade cross protective antibodies [42].

Bacterial pathogen evasion of host immunity is still an active area of investigation, and much remains to be explored. Using a multi-disciplinary approach is key to unraveling host-pathogen interactions and furthering our knowledge of how pathogens successfully evade the
host immune response to facilitate the development of improved prevention strategies and
disease treatments.

**Bordetella species**

The bordetellae are Gram-negative β-proteobacteria consisting of nine species, *Bordetella bronchiseptica*, *B. pertussis*, *B. parapertussis*, *B. holmesii*, *B. hinzii*, *B. ansorpii*, *B. avium*, *B. petrii*, and *B. trematum* [44]. Two clades of *B. parapertussis* strains have been previously identified based on host range, a human adapted and ovine adapted lineage, which appear to be
distinct from each other based on phylogenetic analysis and will be denoted throughout the
following research as *B. parapertussis*<sub>hu</sub> and *B. parapertussis*<sub>ov</sub> [45, 46]. The classical
bordetellae, *B. bronchiseptica*, *B. pertussis* and *B. parapertussis*, which cause respiratory
infections and are the most commonly studied species, will be the focus of the following
research.

**Disease and Prevalence**

*B. pertussis* is a causative agent of whooping cough, a highly contagious respiratory
infection which leads to approximately 50 million cases and 300,000 deaths worldwide each year
[47]. Whooping cough is characterized by severe paroxysmal coughing fits, which are followed
by labored air intake that gives rise to the classic ‘whoop’ sound [44]. The paroxysmal cough can
last for months and can lead to apnea, post-tussive vomiting and hypoxia [48-50]. Whooping
cough was the leading cause of infant mortality until the introduction of a vaccine in the 1940s,
which led to a dramatic decrease in the incidence of whooping cough. Despite high vaccine
coverage in developed countries, whooping cough still persists [49].
B. parapertussis species also cause whooping cough in humans; although there is some evidence to suggest this species causes a milder form of the disease with a shorter duration, reports have varied [50-53]. Symptoms caused by B. parapertussis are clinically indistinguishable from B. pertussis, making it difficult to determine the prevalence of B. parapertussis, which has been reported to be anywhere from 1% to 97% of all whooping cough cases, depending on geographical location [50, 52, 54]. Additionally, B. parapertussis species were long thought to be restricted to the human population until identification of B. parapertussis-like organisms from the lungs of lambs displaying non-progressive pneumonia [45, 46]. Currently, B. parapertussis species are sub-divided into two distinct lineages, human and ovine, that are thought to have diverged from a B.bronchiseptica-like progenitor [55]. B. parapertussis appears to be more genetically diverse than the B.parapertussis clade, based on comparative genomic hybridization, pulse-field gel electrophoresis, and insertion element typing [56-58]. The prevalence of B. parapertussis is currently unknown within sheep populations.

B. bronchiseptica primarily infects the respiratory tract of non-human mammals including, but not limited to, dogs, pigs, cows, rabbits, sheep, monkeys, seals, cats, leopards, horses, and guinea pigs [44, 59]. Additionally, B. bronchiseptica has been isolated from humans, though in many cases immunocompromised individuals [44, 60]. It causes a disease ranging from lethal pneumonias to asymptomatic carriage in the upper respiratory tract in most mammals, and has been shown to predispose animals to secondary infections by other bacterial pathogens, such as Pasteurella multocida [44, 59, 61]. Additionally, B. bronchiseptica is thought to survive outside of the host due to its ability to grow in nutrient poor conditions, which is supported by some limited evidence of growth in lake water [62, 63]. Notably, B. bronchiseptica has not been isolated from an environmental reservoir.
Prevalence of *B. bronchiseptica* in agricultural herds and animal housing units is often greater than 80% [64, 65]. The epidemiology of *B. bronchiseptica* in free-living populations is little understood, but in at least one documented case the seroprevalence was reported for greater than 90% of rabbits, indicating that *B. bronchiseptica* may be highly prevalent in mammalian hosts [66].

**Virulence Factor Gene Regulation**

Survival and success of bacterial pathogens can depend on their ability to adapt to varying environments either outside of or within a host. The classical bordetellae contain a two-component phosphorelay system named BvgAS, which is composed of BvgS, the sensor kinase protein spanning the inner membrane that phosphorylates DNA-binding protein BvgA, the response regulator [67-69]. Phosphorylated BvgA binds to specific motifs upstream of virulence factor genes, recruiting RNA polymerase to initiate transcription [70, 71]. BvgAS activates transcription of virulence factors, such as adhesions, toxins and secretion systems when the bordetellae are grown at 37°C and in the absence of the modulating compounds MgSO₄ or nicotinic acid [67, 72, 73]. In this phase, termed the Bvg⁺ phase, genes associated with survival in the environment, like genes allowing growth in nutrient limiting conditions and motility, are

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**Figure 2.2: BvgAS virulence factor gene regulation.** BvgS and BvgA are comprised of two dimers. In the Bvg⁻ phase BvgS is not activated and BvgA does not bind the promoter region upstream of BvgAS regulated genes. In the Bvg⁺ phase, BvgS is activated by a signal, such as temperature, catalyzes ATP hydrolysis, autophosphorylates, and transfers a phosphate (P) group to BvgA.
repressed [74]. At lower temperatures, associated with a lifestyle outside the host (~25°C) or in the presence of chemical modulators, the opposite occurs, where virulence genes decrease in transcription, while genes encoding motility and nutrient acquisition are increased (Bvg phase) [75, 76]. The exact mechanisms by which MgSO₄ and nicotinic acid modulate BvgAS activation are unknown, nor is it obvious under what conditions outside or within a host the bordetellae would encounter these compounds.

Until recently, temperature was the only known host signal that activated regulation of virulence factor gene expression in the *Bordetella* species. This changed when it was found that iron-limiting conditions induced increased expression of type III secretion system (TTSS) genes in *B. pertussis* and *B. bronchiseptica* [77], although the exact regulatory mechanisms have yet to be determined. BvgAS was required for regulation of the TTSS in response to iron limitation, but additional regulation occurred even when BvgAS was constitutively active [77], suggesting another mechanism may fine-tune regulation of TTSS. In addition, recent work has identified potentially other systems within the bordetellae that regulate virulence factor gene expression [78]. The extracytoplasmic sigma factor, σ⁵, has been implicated in regulation of the TTSS and other genes (Barchinger, S.E. and Zhang, X., unpublished data). Extracytoplasmic sigma factors respond to stress at the outer membrane, and a mutant with high levels of sigma[^E] colonizes at lower levels and clears faster from the murine respiratory tract, indicating that appropriate control of sigma[^E] is necessary for efficient colonization (Barchinger, S.E., unpublished data).

Additional host signals that regulate virulence factor gene expression in the bordetellae have yet to be determined. Despite the large number of transcription factors that are purported to be within the *Bordetella* species genomes [55], few regulatory systems have been described in
detail in the classical bordetellae, and little is understood about transcriptional regulation during infection.

**Virulence Factors and Evading Host Immunity**

The classical bordetellae contain specific repertoires of virulence factors that aid in the evasion of the host immunity and allow for efficient colonization and persistence of the bacteria within the respiratory tract. Some virulence factors are shared between the three species, like adenylate cyclase toxin (ACT), which converts to ATP to cAMP inducing supraphysiological levels that subvert oxidative burst, phagocytosis and eventually leads to apoptosis of macrophages [79-81]. ACT also contributes to pathology and colonization during infection in the lower respiratory tract [81-83]. Also, adhesins are shared between the classical bordetellae as well, like filamentous hemaglutinin (FHA), which is important for adherence to epithelial cells and tracheal colonization [84]. Additionally, some toxins are specific to particular species, such as pertussis toxin (Ptx) in *B. pertussis* [55]. Ptx ADP-ribosylates G proteins, disrupting signaling within cells, and has been shown to delay neutrophil recruitment by inhibiting early chemokine production from

![Figure 3.3: The classical bordetellae virulence factors. *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* share several virulence factors including Filamentous Hemaglutinin (FHA), Dermonecrotic Toxin (DNT), Adenylate Cyclase Toxin (ACT), Fimbriae and Pertactin (PRN). Some virulence factors are shared between only two species or are only expressed in one *Bordetella* species: Pertussis Toxin (Ptx), Type III Secretion System (TTSS), *Bordetella* resistant to serum killing protein (BrkA), and O-antigen. Modified from A.T. Karanikas.](image)
macrophages during infection [85-87]. This toxin is also required for efficient colonization and persistence of *B. pertussis* in the murine model of infection [88].

The classical bordetellae also express secretion systems, the best studied of which is the TTSS of *B. bronchiseptica* [89-92]. *B. parapertussis* is thought to not express a TTSS due to two predicted pseudogenes, one encoding a putative regulatory element and the other a structural element [55]. Conversely, *B. parapertussis* produces a functional TTSS, which is overexpressed in comparison to the TTSS of *B. bronchiseptica* [93]. It was also recently shown that *B. pertussis* isolates produce a functional TTSS, but that laboratory-passaged isolates no longer produce a functional system *in vitro*. The TTSS has been shown in *B. bronchiseptica* to contribute to macrophage cytotoxicity and is known to be important for the induction of IL-10 during infection, which suppresses the pro-inflammatory IFN-γ response allowing the bacteria to persist within the respiratory tract [89, 94].

*B. bronchiseptica* and *B. parapertussis* also have a unique factor that *B. pertussis* has lost, called O-antigen [55, 95]. The O-antigen molecule, which consists of a chain of repeating disaccharide units connected to the inner core of the lipopolysaccharide molecule, protects the bacteria against antimicrobial peptides, complement deposition, and has also been shown to prevent antibody binding [96-98]. Furthermore, it is important for efficient colonization by both *B. bronchiseptica* and *B. parapertussis* strains early during infection [99, 100]. Additionally, there have been three types of O-antigen serotypes identified within the bordetellae that do not induce cross-reactive antibodies against each other, and therefore are thought to allow for evasion of cross-protective antibody responses [101, 102].

Evolution of the bordetellae
The classical *Bordetella* species share a high degree of genome similarity [55, 103]. *B. pertussis* and *B. parapertussis* are predicted to have independently evolved from a *B. bronchiseptica*-like progenitor through genome loss, with little to no evidence for acquisition of new genetic material [55, 104, 105]. Additionally, *B. pertussis* strains have undergone large-scale genome rearrangement, associated with transposases [55]. Genome loss in both human-adapted species correlates with host restriction, which is thought to have occurred relatively recently based on low genetic diversity between both *Bordetella* species [55]. In comparison, *B. bronchiseptica* appears much more diverse, although there is still little evidence for acquisition of novel genes [103, 105-107]. Despite the high clonality of the bordetellae, in comparison to other pathogens, there is evidence of horizontal gene transfer (HGT) within the *Bordetella* species. Evidence suggests HGT of the O-antigen locus, which is thought to provide protection against cross-protective immune responses, and allow bacteria with different serotypes to co-circulate within the host population [101]. Recently, the locus encoding Ptx was identified as being potentially horizontally transferred, although the mechanisms and selective advantage of acquiring different *ptx* loci have not been determined (Park, J, unpublished data). As we sequence additional *Bordetella* genomes we will gain a better insight into the evolution of these unique species.

**Preface**

This dissertation will focus on the interactions between host and pathogen: bacterial sensing of and response to host signals, manipulation of pro-inflammatory cytokine secretion in macrophages, role of complement in control of some *Bordetella* species, and the evolution of virulence factor genes important for evading protective immunity.
The first chapter will explore how *B. bronchiseptica* strains respond to growth in 5% CO₂ conditions using transcriptomics analysis of the response of *B. bronchiseptica* strains to determine what genes are regulated in response to these conditions. In brief, we identify genes that are differentially transcribed in response to 5% CO₂ conditions in *B. bronchiseptica* strains, but also in *B. pertussis* and *B. parapertussis*. Additionally, we show that virulence factor genes *cyaA* (encodes ACT) and *fhaB* (encodes FHA) are regulated not only by BvgAS, but also by an additional unknown CO₂ responsive factor.

The next section of this thesis focuses on the subversion of the host immune response by the classical bordetellae. In summary, we show that *B. parapertussis*hu, *B. pertussis* and *B. bronchiseptica* secrete a molecule that inhibits IL-1 secretion by murine macrophages, but also *B. pertussis* and *B. bronchiseptica* have additional mechanisms/factors which overcome inhibition, while *B. parapertussis*hu does not. Furthermore, inhibition of secretion of IL-1 by *B. parapertussis*hu appears to be downstream of transcription and inflammasome activation and likely prevents release of IL-1 from the macrophage.

The following chapter analyzes the clearance of *B. parapertussis*ov strains from the mouse respiratory tract. We show that *B. parapertussis*ov strains produce a unique O-antigen molecule of one length, and we also determined that complement is important for the efficient control of *B. parapertussis*ov colonization in the murine respiratory tract. Additionally, we show that sheep serum does not kill *B. parapertussis*ov strains.

The next chapter focuses on the three different serotypes of O-antigen identified in *B. bronchiseptica* and *B. parapertussis* strains and the evolution of the locus. In short, phylogenetic analysis identifies potential HGT of the serotype-specific genes. Additionally, the prevalence of
the poorly immunogenic O-antigen serotype appears to cluster in *B. bronchiseptica* Complex IV, which is the human-associated lineage.

Finally, the last chapter will summarize the overall findings of each chapter and define the significance of this work in the context of the *Bordetella* field and host-pathogen interactions, as well as discuss the future directions of these studies.
References

Chapter 2

Identification of a CO$_2$ Responsive Regulon in the *Bordetella*
Abstract:

Sensing the environment allows pathogenic bacteria to coordinately regulate gene expression to maximize survival within or outside of a host. Here we show that *Bordetella* species regulate virulence factor expression in response to carbon dioxide levels that mimic *in vivo* conditions within the respiratory tract. We found strains of *Bordetella bronchiseptica* that did not produce adenylate cyclase toxin (ACT) when grown in liquid or solid media with ambient air aeration, but produced ACT and additional antigens when grown in air supplemented to 5% CO$_2$. Transcriptome analysis and quantitative real time-PCR analysis revealed that strain 761, as well as strain RB50, increased transcription of genes encoding ACT, filamentous hemagglutinin (FHA), pertactin, fimbriae and the type III secretion system in 5% CO$_2$ conditions, relative to ambient air. Furthermore, transcription of *cyA* and *fhaB* in response to 5% CO$_2$ was increased even in the absence of BvgS. *In vitro* analysis also revealed increases in cytotoxicity and adherence when strains were grown in 5% CO$_2$. The human pathogens *B. pertussis* and *B. parapertussis* also increased transcription of several virulence factors when grown in 5% CO$_2$, indicating that this response is conserved among the classical bordetellae. Together, our data indicate that *Bordetella* species can sense and respond to physiologically relevant changes in CO$_2$ concentrations by regulating virulence factors important for colonization, persistence and evasion of the host immune response.
Introduction:

Many cues, such as temperature, oxygen (O\textsubscript{2}), iron, pH, osmolarity and bicarbonate, allow bacteria to distinguish between environments within a host and outside of a host, as well as various microenvironments within a host [1]. In sensing multiple cues, bacteria are able to synchronize gene expression to adapt and ultimately thrive [2]. One cue, carbon dioxide (CO\textsubscript{2}), has been shown to affect regulation of virulence factor expression in many bacterial pathogens. *Bacillus anthracis* responds to elevated levels of CO\textsubscript{2} by increasing expression of the genes encoding edema toxin, lethal factor and protective antigen [3-5]. In response to 10% CO\textsubscript{2}, *Streptococcus pyogenes* increases transcription of M protein, an important virulence factor that prevents the deposition of complement onto the bacterial surface [6]. In increased CO\textsubscript{2}, M protein has been shown to be regulated by a trans-acting positive regulatory protein that binds to the promoter of the *emm* gene [6, 7]. CO\textsubscript{2} regulation in *B. anthracis* appears to be more complicated since the transcriptional regulator of the toxins is not increased transcriptionally in response to growth in CO\textsubscript{2} [3]. Additionally, *Staphylococcus aureus, Salmonella enterocolitica* and *Borrelia burgdorferi* are responsive to increased CO\textsubscript{2} concentrations, suggesting this ability is useful to a variety of pathogens [8-11].

*Bordetella bronchiseptica* is a Gram-negative bacterium that infects a wide range of hosts causing respiratory disease varying from asymptomatic persistence in the nasal cavity for the life of the host to lethal pneumonia [12-14]. *B. bronchiseptica* is very closely related to the other two classical bordetellae, *Bordetella pertussis* and *Bordetella parapertussis*, the causative agents of whooping cough in humans [13,15,16]. Several virulence factors are produced by *B. bronchiseptica* such as, pertactin (PRN), filamentous hemaglutinin (FHA), two serotypes of fimbriae, and the two cytotoxic mechanisms, adenylate cyclase toxin (ACT) and the Type III
Secretion System (TTSS) [13,16]. ACT, a member of the repeats-in-toxin (RTX) family, is a bi-functional adenylate cyclase/hemolysin that converts ATP to cAMP, disrupting oxidative burst, phagocytosis, chemotaxis and eventually leads to apoptosis in macrophages and neutrophils [17-19]. ACT has also been shown to contribute to pathology, efficient colonization and persistence of *B. bronchiseptica* and *B. pertussis* species [20-22].

Regulation of virulence factors in bordetellae occurs via the BvgAS two-component system [23]. BvgS, the sensor in the cytoplasmic membrane, is thought to directly sense changes in the environment and, through a phosphorylation-transfer mechanism, activates BvgA, the response regulator [24-26]. Once BvgA is activated (Bvg+ phase), it binds to high and low affinity motifs in the genome, resulting in increased expression of the genes encoding toxins and adhesins, while expression of Bvg− phase genes involved in motility and uptake of certain nutrients are repressed; the opposite occurs in the Bvg− phase [27-32]. The Bvg+ phase has been shown to be necessary and sufficient for host colonization [33]; however an intermediate phase has been described in which a subset of virulence factors are expressed, along with a unique set of factors [34-36]. The ability to respond to multiple signal inputs to differentially regulate transcriptional networks allows for adaptation to different microenvironments within the host. *Bordetella* species have multiple putative transcription factors within their genomes, indicating that gene regulation is likely to be a more complex regulatory system than is currently appreciated [16].

Here we identify, through screening of a collection of *B. bronchiseptica* isolates, strains that only produce ACT in response to growth in elevated CO$_2$ conditions at 37°C. Both 761 and the sequenced laboratory reference strain RB50 increased transcription of *cytA* and production of ACT when grown in 5% CO$_2$ conditions, although only 761 was dependent on 5% CO$_2$ for
efficient expression. Several other virulence factor genes were increased in transcription in response to growth in elevated CO$_2$. BvgAS was required for ACT production, but cyaA and fhaB were transcriptionally increased in response to 5% CO$_2$ conditions in the absence of BvgS. Together these data indicate that an additional regulatory system increases production of ACT and other virulence factors in various *Bordetella* species.
Materials and Methods:

Bacterial strains and growth. *B. bronchiseptica* strain RB50 is an isolate from a rabbit [33]. RB54 and RB50ΔbscNΔcyaA are previously described derivatives of strain RB50 [33, 37]. *B. bronchiseptica* strain 761, 448, and 308 were obtained from the CDC in Atlanta, Georgia and have been previously described [15, 38, 39]. *B. bronchiseptica* strain JC100 has been previously described [15]. *B. parapertussis* strain 12822 was isolated from German clinical trials and has been previously described [40, 41]. *B. pertussis* strain 536 is a streptomycin resistant derivative of Tohama I [42]. Bacteria were maintained on Bordet-Gengou agar (Difco, Franklin Lakes, NJ) containing 10% sheep blood (Hema Resources, Aurora OR) and 20 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO). Liquid cultures were grown at 37°C overnight in a shaker to mid-log phase (O.D. 0.7-1.0) in Stainer-Scholte (SS) broth. Bacteria were grown in either atmospheric concentrations of oxygen and carbon dioxide (atmospheric conditions) or in atmospheric levels of oxygen with the addition of 5% carbon dioxide into a sealed incubator 37°C (5% CO₂ conditions).

cAMP Assay. Murine macrophage-like cell line, J774, was cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone). Cells were grown to approximately 80% confluence, and bacteria were added at a multiplicity of infection (MOI) of 1. After a 5 minute centrifugation at 250 x g, the mixture was incubated for 30 minutes at 37°C. cAMP was measured with a cyclic AMP ELISA system (Tropix, Bedford, MA) according to the manufacturer's instructions. Results were analyzed using analysis of variance with Tukey simultaneous test in Minitab 16 (Minitab Inc., State College, PA), and a *P* value of <0.05 was considered significant.
**Animal Experiments.** C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were bred in our *Bordetella*-free, specific pathogen-free breeding rooms at The Pennsylvania State University. All animal experiments were performed in accordance with institutional guidelines. 4 to 6 week old mice were lightly sedated with 5% isoflurane (IsoFlo, Abbott Laboratories) in oxygen and $5 \times 10^5$ CFU were pipetted in 50ul of phosphate-buffered saline (PBS) (Omnipur, Gibbstown, NJ) onto the external nares. This method reliably distributes the bacteria throughout the respiratory tract [43]. To obtain serum, blood from inoculated or vaccinated mice was obtained 28 days post-inoculation and serum was separated from the blood by centrifugation at 500 x g for 5 minutes.

**Western Immunoblots.** Western blots were performed on whole cell extracts of *B. bronchiseptica, B. pertussis* and *B. parapertussis* grown to mid-log phase in SS broth as described previously [38,39]. Lysates were prepared by resuspending $1 \times 10^9$ CFU in 100 μl of Laemmli sample buffer; total cellular protein content were quantitated using the BCA assay to equalize protein content between samples. Lysates for Figures 2 and 6 are from separate biological samples and resolving gel percentages are 8% and 10%, respectively. $1 \times 10^8$ CFU (10μl) were run on an 8% sodium dodecyl sulfate-polyacrylamide electrophoresis gels in denaturing conditions and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were probed with pooled serum from mice inoculated with *B. bronchiseptica, B. pertussis, B. parapertussis*, or a monoclonal antibody against ACT (anti-ACT) at the following dilutions, 1:1000, 1:500, 1:1000 and 1:1000, respectively. Western blots in Figure 2 and 6 were probed with serum from two different pooled serum samples. A 1:10,000 dilution of goat anti-mouse Ig HRP conjugated antibody (Southern Biotech, Birmingham, AL)
was used as the detector antibody. Membranes were visualized with ECL Western blotting
detection reagents (Amersham Biosciences, Piscataway, NJ).

**RNA Isolation.** RNA was isolated from three independent biological replicates of strains RB50, 761, RB54, 536 and 12822 grown in SS broth overnight. Bacteria were subcultured at a starting
OD$_{600}$ of 0.1 into 5 ml of SS broth and grown at 37°C while shaking in either atmospheric or 5%
CO$_2$ conditions until the OD$_{600}$ reached 0.75. Bacteria were harvested and total RNA was
extracted using a RNAeasy Kit (Qiagen, Valencia, CA) and treated with RNase-free DNase I
(Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

**Preparation of labeled cDNA and microarray analysis.** RNA isolated from strains RB50 and
761 were used in microarray experiments. A 2-color hybridization format was used for the
microarray analysis. For each biological replicate, RNA extracted from cells grown in 5% CO$_2$
conditions was used to generate Cy5-labeled cDNA and RNA extracted from cells grown in
atmospheric conditions was used to generate Cy3-labeled cDNA. Additionally, dye-swap
experiments were performed analogously, in which the fluorescent labels were exchanged to
ensure that uneven incorporation did not confound our results. Fluorescently-labeled cDNA
copies of the total RNA pool were prepared by direct incorporation of fluorescent nucleotide
analogs during a first-strand reverse transcription (RT) reaction [39, 44-46]. The two
differentially labeled reactions were then combined and directly hybridized to a *B. bronchiseptica* strain RB50-specific long-oligonucleotide microarray [45]. Slides were then
scanned using a GenePix 4000B microarray scanner and analyzed with GenePix Pro software
(Axon Instruments, Union City, CA). Spots were assessed visually to identify those of low
quality and arrays were normalized so that the median of ratio across each array was equal to 1.0.
Spots of low quality were identified and were filtered out prior to analysis. Ratio data from the
two biological replicates were compiled and normalized based on the total Cy3% intensity and Cy5% intensity to eliminate slide to slide variation. Gene expression data were then normalized to 16S rRNA. The statistical significance of the gene expression changes observed was assessed by using the significant analysis of microarrays (SAM) program [47]. A one-class unpaired SAM analysis using a false discovery rate of 0.001% was performed. Hierarchical clustering of microarray data using Euclidean Distance metrics and Average Linkage clustering was performed using MeV software from TIGR [48]. All microarray data are available in the supplementary text and have been deposited in MAIMExpress under accession number E-MEXP-2875.

**qRT-PCR.** qRT-PCR was performed using a modified protocol previously described [45, 46]. RNA was extracted as described, and 1 µg of RNA from each biological replicate was reverse transcribed using ImProm-II Reverse transcriptase and 0.5 µg of random oligonucleotide hexamers (Promega, Madison, WI). cDNA was diluted 1:1,000 and 1 µl was used in RT-qPCRs containing 300 nM primers designed with Primer Express software (Applied Biosystems, Foster City, CA, and Integrated DNA Technologies software) [Primer sequences are listed in Appendix A] and SYBR Green PCR master mix (Invitrogen, Carlsbad, CA). Samples without reverse transcriptase were included to confirm lack of DNA contamination and dissociation curve analysis was performed to determine cycle threshold (C_T) for each reaction. Amplification of the *recA* RNA amplicon was used as an internal control and for data normalization. Change in transcript level was determined using the relative quantitative method (ΔΔC_T) [49]. Results were analyzed using analysis of variance with Tukey simultaneous test in Minitab 16 (Minitab Inc., State College, PA), and a *P* value of <0.05 was considered significant.
Cytotoxicity Assay. Cytotoxicity assays were carried out as previously described [44]. J774 cells were cultured in DMEM with 10% FBS. Cells were grown to approximately 80% confluency, and bacteria were added at a MOI of 100, 10 or 1. After a 5 minute centrifugation at 250 x g, the mixture was incubated at 37°C for the indicated times. Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release using the Cytotox96 (Promega) kit according to the manufacturer's protocol. Results were analyzed using analysis of variance with Tukey simultaneous test in Minitab 16 (Minitab Inc., State College, PA), and a P value of <0.05 was considered significant.

Adherence Assay. Adherence assays were modified from a previously described protocol [46]. Rat epithelial cells, L2, were cultured in DMEM/Ham’s F12 50-50 mixture with 10% FBS. Cells were grown to approximately 80% confluency, and bacteria were added at an MOI of 100. After a 5 minute centrifugation at 250 x g, the mixture was incubated for 30 minutes. Cell culture supernatant was removed and cells were washed 4 times with PBS to remove unbound bacteria. Epithelial cells were then trypsinized and resuspended in 1 mL of tissue culture media. The mixture of cells and bacteria were diluted in PBS and plated on BG agar to determine CFU. Results were analyzed using analysis of variance with Tukey simultaneous test in Minitab 16 (Minitab Inc., State College, PA), and a P value of <0.05 was considered significant.
Results:

A *B. bronchiseptica* isolate regulates ACT expression in response to 5% CO$_2$ conditions. *B. bronchiseptica* isolates are generally β-hemolytic when grown in Bvg$^+$ conditions due to the production of ACT, which causes lysis of red blood cells. It was recently discovered that some *B. bronchiseptica* isolates do not have the genes required to produce a functional ACT and therefore are not hemolytic on blood agar plates [39]. However, through screening of 73 isolates based on hemolysis on blood agar plates and PCR amplification of the genes encoding ACT, 4 *B. bronchiseptica* isolates were found to be non-hemolytic, but still retained the genes for production of ACT (data not shown). When one isolate displaying this phenotype, *B. bronchiseptica* strain 761, was grown in a tissue culture incubator where the CO$_2$ concentration

![Figure 2.1: Differential production of ACT in *B. bronchiseptica* strains grown in 5% CO$_2$. Strains RB50 (A, C) and 761 (B, D), grown in normal atmospheric oxygen conditions (A, B) or in elevated 5% CO$_2$ conditions (C, D). (E) RB50 and 761 grown in atmospheric conditions or grown in 5% CO$_2$ conditions, or recombinant ACT (2.5 ng) were probed with a monoclonal antibody to CyaA protein at a dilution of 1:1000. J774 murine macrophage cells were stimulated with media or media containing RB50 (F) or 761 (G) at an MOI of 1 for 30 minutes, and cAMP levels were assessed. * indicates a p-value less than 0.05.](image-url)
is increased to 5%, it was hemolytic (Figure 2.1, compare panels B and D). RB50 was hemolytic even in ambient air (~0.03% CO₂) (Figure 2.1, compare panels A and C), but there appeared to be more hemolysis when it was grown in 5% CO₂ conditions, suggesting both strains produce more ACT in response to growth in 5% CO₂ conditions.

To more directly assess the production of ACT, lysates of RB50 and 761 grown in liquid cultures (mid-log phase, O.D. 0.7-0.9) in normal or 5% CO₂ conditions were probed with a monoclonal antibody to the cyaA protein product, ACT. RB50 produced more ACT when grown in 5% CO₂ conditions than in normal atmospheric conditions (Figure 2.1E). 761 grown in normal atmospheric conditions produced no detectable ACT, while 761 in 5% CO₂ conditions did produce ACT (Figure 2.1E). To determine if strain 761 produces a functional ACT, cyclic-AMP (cAMP) was measured in murine macrophages stimulated with bacteria grown in either normal or 5% CO₂ conditions. Cells were stimulated for 30 minutes with RB50 or 761 to assess their effects on cAMP levels. Both strains grown in 5% CO₂ induced significantly more cAMP than the same strains grown in normal atmospheric conditions (Figure 2.1F, G). Together, these data demonstrate by different measures that the prototypical B. bronchiseptica strain, RB50, and 761 increase production of functional ACT when grown in 5% CO₂ conditions, but only 761 appears to be dependent on 5% CO₂ conditions for production of ACT.

Figure 2.2: Differential recognition of antigens in B. bronchiseptica strains grown in atmospheric or 5% CO₂ conditions. C57BL/6 mice were inoculated with 5 x 10⁵ CFU B. bronchiseptica strain RB50, and serum was collected 28 days later. Strains RB50 and 761 were grown in atmospheric or in elevated CO₂ concentrations, and were probed with serum against RB50. Increased (arrows) or decreased (arrowheads) production of antigens in response to 5% CO₂ conditions is denoted.
Since ACT production was increased in 5% CO$_2$, we hypothesized that other antigens might also be differentially regulated in response to these conditions. To test this, Western blot analysis was performed by probing RB50 and 761 lysates, grown in ambient air or in 5% CO$_2$ conditions, with serum antibodies from animals convalescent from RB50 infection. An antigen approximately 80kDa was produced in greater amounts in RB50 grown in ambient air, while strain 761 produced antigens of approximately 55, 72, 130, and 250kDa in greater amounts compared to growth in 5% CO$_2$ conditions (Figure 2.2, arrowheads). An antigen greater than 250 kDa was produced in greater amounts when RB50 and 761 were grown in 5% CO$_2$ conditions compared to ambient air (Figure 2.2, arrows). Additionally, both 761 and RB50 produced antigens between 36 and 28kDa in greater amounts when grown in elevated CO$_2$ concentrations than in ambient air (Figure 2.2). These data indicate that additional antigens besides ACT are differentially regulated in response to 5% CO$_2$.

**Defining a CO$_2$ responsive regulon in B. bronchiseptica.** To determine which genes are differentially regulated in response to different CO$_2$ concentrations, microarray analyses were performed comparing RB50 grown in atmospheric concentrations of CO$_2$ to growth in elevated CO$_2$ conditions. Expression of 35 genes increased in RB50 in response to 5% CO$_2$, based on SAM analysis (Figure 2.3), including genes encoding cyaA, as well as other genes encoding known virulence factors such as members of the TTSS locus (bscE, bscF, bopD), fhaC, fhaB, fhaD, fhaA, fimA, fim3, and prn, (Figure 2.3). Expression of 452 genes were decreased when RB50 was grown in 5% CO$_2$, many of which are known to be expressed in the Bvg$^-$ phase including, flaA, cheW, cheB, wbmD, flgH, flfS, and cheD (Figure 2.3). A similar trend was observed for strain 761; genes encoding known virulence factors were increased in 5% CO$_2$ growth conditions while genes for flagellar assembly and chemotaxis were decreased in these
Expression of 6 genes, including cyaA, was increased in both strains and expression of 41 genes decreased in both strains when grown in 5% CO₂ conditions (Appendix B). qPCR of 12 genes confirmed the microarray results (Appendix A). Overall, these data indicate there is a CO₂ responsive regulon in B. bronchiseptica that includes several virulence factors, suggesting a role during infection.

Among the 35 genes in strain RB50 increased in expression in 5% CO₂ conditions, 19 genes were reported to be positively regulated under Bvg⁺ conditions, 4 genes negatively regulated by BvgAS, and 13 genes not previously known to be regulated by BvgAS, based on previous analysis [45,50]. Similarly, of the 452 genes negatively regulated by 5% CO₂, 252 were known to be negatively regulated under Bvg⁺ conditions, 19 were positively regulated and 181 were not previously known to be regulated by BvgAS [45,50]. The CO₂-responsive regulon appears to contain genes that are Bvg-regulated, as well as genes

![Figure 2.3: Defining the CO₂ responsive regulon in B. bronchiseptica.](image)

(A) Changes in gene expression in B. bronchiseptica in response to 5% CO₂ are analyzed by MeV analysis (Saeed et al. 2003). (B) Genes reported to be regulated by BvgAS in prototypical B. bronchiseptica strain RB50 are shown for strain RB50 (left) and strain 761 (right), with yellow representing increased transcription and blue indicative of decreased transcription in growth in 5% CO₂ conditions, compared to growth in normal atmospheric conditions. This experiment was performed by Tracy Nicholson.
that are not, suggesting a regulatory mechanism that functions independently or cooperatively with BvgAS rather than subordinate to it.

**CO₂ responsiveness is not conferred by differences in the bvgAS loci between strains.** Three additional *B. bronchiseptica* strains, JC100, 308 and 448, were also observed to be hemolytic only when grown in 5% CO₂ but not in normal atmospheric conditions. Based on multi-locus sequence typing of seven conserved housekeeping genes, strain 308 belongs to sequence type 7 and 448 belongs to sequence type 23, while strains 761 and JC100 are sequence type 12 (data not shown, [15]). Other strains of these sequence types did not share this phenotype, suggesting that this phenotype is not lineage-specific. Since some CO₂-responsive genes are Bvg-regulated, the *bvg* locus of these strains was analyzed revealing that strain JC100 carries a 29 amino acid duplication in the region of the *bvgS* gene encoding the periplasmic domain (Figure 2.4). To determine if this duplication is involved in the CO₂/ACT dependent phenotype in JC100, the *bvgAS* locus from JC100 was expressed in a RB50 knockout of *bvgAS* (RB55::pBvgASJC100).

![Figure 2.4: Duplication in the *bvgS* gene in strain JC100.](image)

This strain was hemolytic in the absence of 5% CO₂, indicating that transfer of the *bvgAS* locus does not confer the CO₂-dependence for ACT production (Appendix C). The reverse was also true; when a plasmid carrying the *bvgAS* locus from RB50 was introduced into JC100 (MLJC114::pEG100), ACT production remained dependent on growth in 5% CO₂ (Appendix C).
Furthermore, the \textit{bvgS} gene of 761 did not have this duplication (Figure 2.4). These data indicate that the duplication in \textit{bvgS} in JC100 is neither necessary nor sufficient for the CO$_2$ requirement for hemolysis.

**CO$_2$ responsiveness in the Bvg$^-$ state.** Since some virulence genes known to be Bvg-regulated were responsive to 5% CO$_2$ conditions, we sought to determine if they are differentially regulated in response to 5% CO$_2$ in the absence of BvgAS.

Transcription of six genes responsive to CO$_2$, cya\textit{A}, fha\textit{B}, \textit{bopD}, \textit{bopB}, che\textit{Z} and flg\textit{B}, were analyzed in RB50 and RB54, a Bvg$^-$-phase locked derivative of strain RB50, grown in atmospheric or 5% CO$_2$ conditions (Figure 2.5).

For RB50, addition of 5% CO$_2$ increased transcription of cya\textit{A}, fha\textit{B}, \textit{bopD} and \textit{bopB} (Figure 2.5A-D), but decreased transcription of che\textit{Z} and flg\textit{B} (Figure 2.5 E,F). In RB54, transcription of \textit{bopD} and \textit{bopB} was not increased in response to addition of 5% CO$_2$ (Figure 2.5 C,D), and transcription of che\textit{Z} and flg\textit{B} was not decreased (Figure 2.5 E,F). Therefore, the
differential transcription of *bopD*, *bopB*, *cheZ* and *flgB* in response to 5% CO$_2$ is dependent on BvgS. However, in the *bvgS* mutant RB54, the transcription of genes *cyaA* and *fhaB* was increased in response to addition of 5% CO$_2$ (Figure 2.5 A,B), indicating that some gene regulation in response to 5% CO$_2$ is independent of BvgS.

To determine whether differential transcription results in differential accumulation of antigens in the absence of BvgS, Western blots were performed. Lysates from RB54 grown in ambient air or 5% CO$_2$ conditions were probed with serum antibodies from mice convalescent from RB50 infection (Figure 2.6A). Strain RB54 grown in 5% CO$_2$ also produced antigens >250 kDa and ~60kDa in greater amounts (Figure 2.6A). RB54 grown in ambient air produced an antigen of ~120kDa in greater amounts (Figure 2.6A, arrows) and others in lesser amounts (Figure 2.6A, arrowheads). These data demonstrate that antigen production is differentially regulated in response to 5% CO$_2$ even when a functional BvgS is absent.

**Growth in 5% CO$_2$ affects cytotoxicity and adherence of *B. bronchiseptica* strains.** Since genes, *cyaA* and the TTSS genes, associated with the cytotoxicity of *B. bronchiseptica* were increased when strains RB50 and 761 were grown in 5% CO$_2$ conditions (Figure 2.3), we assessed the relative cytotoxicity of strains grown in atmospheric or 5% CO$_2$ conditions to J774 murine macrophages. Similar to previous findings [51], RB50 killed >90% of cells at an MOI of
10 or 100; however, RB50 only killed ~65% at an MOI of 1 (Figure 2.7A). RB50 grown in 5% CO$_2$ killed >90% at all MOIs, indicating that RB50 grown in 5% CO$_2$ killed more macrophages at a lower MOI than RB50 grown in atmospheric conditions (Figure 2.7A). Strain 761 had detectable (~30%) killing only at high MOIs (10 and 100) but was comparable to background at an MOI of 1, while 761 grown in 5% CO$_2$ was cytotoxic at an MOI of 1 (~45%) and comparable to RB50 at higher MOIs (Figure 2.7A). These data show that growth in 5% CO$_2$ increased killing of murine macrophages by both strains.

ACT and TTSS have been previously shown to account for all cytotoxicity of macrophages when RB50 is grown in ambient air [37, 51]. Since 5% CO$_2$ increased expression of several genes, we examined whether the increased cytotoxicity is due to increases in these known factors or a new cytotoxic mechanism. Cells were stimulated for 4 hours at MOIs of 1, 10 or 100 with wild-type RB50, or a mutant lacking bscN (encoding the ATPase of the TTSS) and cyaA (RB50ΔcyaAΔbscN). RB50ΔcyaAΔbscN caused very low levels of cytotoxicity even after 4 hours at MOI of 100 as seen previously [37] and did not induce increased cytotoxicity when the mutant was pre-grown in 5% CO$_2$ (Figure 2.7B). These data suggest that there are no

Figure 2.7: Cytotoxicity and adherence of strains grown in 5% CO$_2$ conditions. J774 murine macrophage cells were stimulated with media alone or media containing RB50 or 761 (A), RB50 or RB50ΔcyaAΔbscN (B) in atmospheric or 5% CO$_2$ conditions at MOIs of 1, 10 or 100 for 4 hours, and LDH release was assayed. (C) Rat epithelial cells were incubated with RB50 or 761 at a MOI of 100 for 30 minutes. Adherence is expressed as the proportion of adherent bacteria to the amount in the original inoculum. The error bars represent standard deviations. * p-values < 0.05 as compared to the same strain grown in atmospheric conditions. Daryl Nowacki performed the experiment for Figure 2.7A.
other cytotoxic mechanisms and that increased ACT and TTSS function accounts for the increased cytotoxicity when strains are grown in 5% CO₂ conditions.

Since many genes encoding adhesins were increased in transcription in response to growth in 5% CO₂ conditions, we hypothesized that strains grown under these conditions, in comparison to growth in ambient air, would be more adherent to epithelial cells. L2 cells were incubated with RB50 or 761 pre-grown in either atmospheric or 5% CO₂ conditions. Both strains pre-grown in 5% CO₂ conditions adhered to lung epithelial cells more efficiently than bacteria grown in normal conditions (Figure 2.7C).

**B. pertussis and B. parapertussis modulate virulence factor expression in response to 5% CO₂.** Since up-regulation of virulence factors in response to growth in 5% CO₂ conditions is common to multiple *B. bronchiseptica* strains, we hypothesized that *B. pertussis* and *B. parapertussis* may also regulate virulence factor expression in response to growth in 5% CO₂ conditions. Genes shown to be CO₂ responsive (*cyaA, fhaB, fimA*) or non-responsive to CO₂ (*bvgS*) in *B. bronchiseptica* (Figure 2.3), were chosen to be analyzed by qPCR in *B. pertussis* and *B. parapertussis*. *B. pertussis* and *B. parapertussis* had increased expression of *fhaB* and *cyaA*, but not *bvgS* in response to CO₂ (Figure 2.8A, B). *B. pertussis*, unlike *B. parapertussis*, also had increased expression of *fimA*, indicating that the 5% CO₂ responsive regulon may be different among the three classical *Bordetella* species. To further investigate this effect, Western blots with lysates of *B. pertussis* and *B. parapertussis* grown in ambient air or 5% CO₂ were probed with either *B. pertussis*-induced sera or *B. parapertussis*-induced sera (Figure 2.8C, D).
Figure 2.8: Differential expression of virulence factor genes in B. pertussis and B. parapertussis in response to 5% CO2 conditions. qRT-PCR analysis was performed on B. pertussis (A) and B. parapertussis (B) grown in atmospheric or elevated CO2 conditions. Fold-change expression (FCE) in B. pertussis and B. parapertussis grown in 5% CO2 was compared to expression in atmospheric levels of CO2 and expressed as the mean ± standard deviation. Lysates from bacteria grown in either atmospheric conditions or 5% CO2 conditions were probed with serum from mice inoculated with either a B. pertussis (C) or B. parapertussis strain (D). * p-values <0.05 as compared to the same strain grown in atmospheric conditions.

B. pertussis grown in ambient air showed a different antigenic profile from the lysate grown in 5% CO2, with bands from roughly 50 to 250 kDa which were more numerous and intense in 5% CO2 (Figure 2.8C). B. parapertussis grown in ambient air had more intense bands between 95 and 72 kDa as indicated by the arrowheads, while growth in 5% CO2 had more intense bands between 55 and 28 kDa, indicated by the arrows (Figure 2.8D). These data indicate that several antigens in B. pertussis and B. parapertussis are differentially regulated in response to growth in 5% CO2 compared to growth in ambient air.
Discussion:

BvgAS was originally considered an ON/OFF switch, modulating *Bordetella* species between two distinct states, avirulent (Bvg\(^-\)) and virulent (Bvg\(^+\)). The discovery of an intermediate phase has led to a view of BvgAS gene regulation as a rheostat visualized as varying along a one dimensional gradient [10, 58, 62]. In this view of the two-component system few signals, temperature and some chemical cues, are known to affect virulence factor regulation through the BvgAS system. However, the respiratory tract contains many microenvironments, and within each environment there is likely to be great variation. For example, CO\(_2\) levels are thought to vary between air and epithelial cells of the respiratory tract, although these are separated by a fraction of a millimeter of mucous. These sites also change dramatically in the course of the various stages of an infection, and there is likely to be a selective advantage to any strain that can sense these differences and modulate virulence factor expression in response.

Here we show that the classical bordetellae share the ability to sense and respond to physiological changes in CO\(_2\) concentrations at a concentration likely to be encountered in the host. In mammalian tissues and blood, CO\(_2\) concentrations are higher than inhaled ambient air concentrations of CO\(_2\), which are approximately 0.03%. The observed changes in expression of various virulence factors (Figure 2.3), and altered phenotypes (adherence and cytotoxicity, Figure 2.7) provide additional evidence that the ability to respond to changes in CO\(_2\) concentrations allow *Bordetella* species to adjust to microenvironments within the host respiratory tract.

Recently, it has been shown that there is a zone of oxygenation between the anaerobic luminal environment and the host epithelium in the gastrointestinal tract, which likely is sensed
by *Shigella flexneri* [2]. The presence of oxygen alters the expression of TTSS effectors that are important for invasion of host cells, and this ‘aerobic zone’ may enhance secretion of these effectors thereby increasing invasion of host cells [2]. Similarly, the respiratory tract of mammals contains multiple sites where gradients of CO$_2$ or oxygen likely influence virulence factor expression and how respiratory pathogens interact with host cells. *B. bronchiseptica* has been isolated from multiple sites within the respiratory tract (e.g. nasopharynx, trachea, lungs) and the ability to detect these differences could allow this pathogen to respond by expressing the array of factors optimal for success under each condition [2]. As bacteria disseminate from the nasal cavity to the trachea, lung and invade tissues, CO$_2$ concentrations may increase, serving as a signal for increased transcription of factors such as adhesins, which are known to be important for tracheal colonization and toxins that subvert the immune response, which is more robust in these regions [17, 51-53].

*B. bronchiseptica* strains sense and differentially regulate virulence factor gene expression in response to 5% CO$_2$ (Figure 2.3), and differential regulation was observed in multiple strains and species of *Bordetella* demonstrating that sensing and responding to carbon dioxide levels is a shared mechanism among the classical bordetellae. Additionally, the transcription of several Bvg$^+$-phase genes increased in response to 5% CO$_2$, suggestive of BvgAS involvement in the response. Interestingly, regulation of some virulence factor genes (*bopD*, *bopB*) by BvgAS was epistatic to 5% CO$_2$ regulation (Figure 2.5 C,D). However, not all virulence gene expression (*cyA*, *fhaB*) was dependent on *bvgS* (Figure 2.5A,B), demonstrating an independent mechanism for virulence factor gene regulation in response to 5% CO$_2$.

Standard Bvg$^+$ conditions, without additional CO$_2$, are sufficient for production of ACT (Figure 2.1) in RB50, suggesting that additional mechanisms may contribute to increases in
production, but are not required. Of 73 *B. bronchiseptica* strains screened, 4 strains were identified here, 761, 308, 448 and JC100, in which Bvg\(^+\) phase conditions are not sufficient for measurable production of ACT. In these strains both Bvg\(^+\) phase conditions and 5% CO\(_2\) are required for detectable production of ACT. The variation of requirement for 5% CO\(_2\) for the production of virulence factors among strains may reflect evolutionary adaption of *B. bronchiseptica* strains, and suggests that the mechanism of differential sensing of CO\(_2\) may confer a selective advantage.

Collectively these data demonstrate that a CO\(_2\) response mechanism contributes to regulation of virulence factors (Figure 2.5A,B). This is first description of a CO\(_2\) sensing mechanism that regulates virulence factor expression through a mechanism acting cooperatively with or independently of BvgAS. Our data support the idea that virulence factor gene expression can be fine-tuned in response to signals specific to different microenvironments within the respiratory tract.
Authors and Contributions:

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Conceived and designed experiments: SEH, ML, JFM, ETH

Performed experiments: SEH, ML (Figure 2.4), TN (Figure 2.3), DN (Figure 2.7A)

Analyzed the data: SEH, ML, TL, JFM, ETH

Wrote the paper: SEH, JFM, ETH
References:


Chapter 3

Inhibition of Interleukin-1 Secretion in Murine Macrophages by the Classical bordetellae
Abstract:

Bacterial pathogens have evolved to modulate and evade host defenses in order to survive. Interleukin (IL)-1 plays a central role in innate immunity and is part of the first line of defense against invading microorganisms. Here we show that the classical bordetellae secrete a molecule that inhibits IL-1α and IL-1β secretion from murine macrophages. Additionally, we show that bacteria, *B. pertussis* and *B. bronchiseptica*, induce IL-1, while *B. parapertussis* does not. Fractionation methods proved unsuccessful at determining the compound produced by *B. parapertussis* that inhibits IL-1, but *B. parapertussis* O-antigen mutant did not inhibit IL-1 suggesting the molecule is not produced by the mutant. We also found that *B. parapertussis* does not inhibit transcription, maturation of IL-1β by active caspase-1, or alter cell surface-expression of IL-1α. Additionally, *B. parapertussis* did not produce a molecule which degrades IL-1, suggesting that inhibition likely occurs at secretion of IL-1. Visualization of IL-1β throughout the macrophage showed distinct vesicles containing IL-1β indicating that *B. parapertussis* likely inhibits the release of these vesicles from the macrophages. Our findings provide evidence indicating that the classical bordetellae secrete an inhibitory molecule that appears to block the release of IL-1-containing vesicles.
Introduction:

Interleukin (IL)-1 is a family of unconventionally secreted proinflammatory cytokines, such as IL-1α and IL-1β, secreted by a variety of cells including macrophages and monocytes [1-3]. IL-1α and IL-1β are induced by the induction of TLR4 signaling, but a second signal is required for the processing and secretion of biologically active IL-1β while less is known about regulation of IL-1α [4, 5]. Both IL-1α and IL-1β are produced in precursor forms which can be cleaved into mature forms [1]. In the case of IL-1β, pro-IL-1β is cleaved by caspase-1 and is secreted in only the mature form, whereas IL-1α can be cleaved by calpains, but is biologically active in both cleaved and uncleaved forms [1, 6, 7]. Additionally, the molecule required for IL-1β cleavage, caspase-1, is activated upon binding of a complex of molecules termed the inflammasome that recognize LPS, flagellin, ATP and several known toxins [5, 8, 9]. While processing of IL-1β is relatively well understood, several mechanisms have been proposed for secretion of IL-1β, including release via autolysosomes [10], shedding of microvesicles [11], multivesicular body fusion with exosomes [12], and caspase-1 dependent pyroptotic cell death mediated release [13]. Less is understood of IL-1α secretion, but it is known that it is retained within the cell, targeted to the cell surface or secreted, but the mechanisms and signals regulating these processes are poorly understood [14, 15]. Secretion of IL-1α has been shown to be dependent on the presence of IL-1β, as bone-marrow derived macrophages from IL-1β−/− mice do not secrete IL-1α [16]. Recent studies indicate that IL-1α and IL-1β interact and indicate a potential co-secretion of both of these cytokines, which future research will further define [16].

Processing and secretion of IL-1 is a complex process, and misregulation of IL-1 has been implicated in a variety of autoimmune disease including rheumatoid arthritis (RA), Crohn’s disease and multiple sclerosis [2, 17-19]. Additionally, modulation of IL-1 is a mechanism used
by pathogens to evade the immune response. For example, *Francisella tularensis* inhibits IL-1β production in macrophages by preventing the translocation of NF-κB into the nucleus [20]. Other pathogens modulate activation of the inflammasome thereby inhibiting IL-1β secretion.

*Pseudomonas aeruginosa* Type III Secretion System (TTSS) effector ExoU suppresses caspase-1 activation through an unknown mechanism, while *Yersinia* species produce TTSS effectors which block the oligomerization and subsequent activation of caspase-1 [21, 22]. Preventing IL-1β secretion by modulating inflammasome activation is a common strategy employed by several species of bacteria including, *Shigella flexneri, Salmonella typhimurium,* and *Mycobacterium tuberculosis,* but few pathogens are known to directly interfere with IL-1β secretory pathways [23-25].

The bordetellae are Gram-negative respiratory pathogens that are composed of nine species, including the classical bordetellae: *B. bronchiseptica, B. pertussis,* and *B. parapertussis* [26]. *B. pertussis* and *B. parapertussis* are the causative agents of whooping cough in humans, while *B. bronchiseptica* predominantly infects non-human mammalians, causing disease ranging from asymptomatic infection to lethal pneumonia [26, 27]. The three species share a high percentage of genome homology leading to the suggestion that they be re-classified as subspecies [28, 29]. Despite the high degree of similarity the classical bordetellae each have a different repertoire of virulence factors used to evade host immune responses. *B. bronchiseptica* and *B. pertussis* both express a TTSS that is not expressed in *B. parapertussis,* which has been shown in *B. bronchiseptica* to modulate IFN-γ via induction of IL-10 [28, 30, 31]. Additionally, *B. pertussis* expresses Pertussis toxin (Ptx) which ADP-ribosylates G proteins, disrupting G protein coupled signaling pathways [32, 33]. Ptx has been shown to block neutrophil recruitment, inhibit chemokine production from macrophages, induce IL-1β secretion in macrophages, and
prevent antibody-mediated clearance of *B. pertussis* [34-36]. Additionally, adenylate cyclase toxin (ACT) has also been shown to induce IL-1β secretion, a factor shared by all three bordetellae [37]. *B. bronchiseptica* and *B. parapertussis* species also produce an O-antigen, which aids in prevention of complement deposition, antimicrobial peptides and antibody-mediated clearance [38-41]. Variations in virulence factors allow for differential host immune evasion strategies, and therefore specific immune functions are important for control and clearance of each pathogen. Recently, a differential role for IL-1 receptor signaling was shown in control of *B. pertussis* and *B. parapertussis* during infection [36]. While, IL-1 receptor signaling was necessary for the control and clearance from lower respiratory tract and to prevent systemic colonization by *B. pertussis*, there was no evidence for IL-1 receptor-mediated signaling in control of *B. parapertussis* infection [36]. Additionally, it was shown that Ptx was important for induction of IL-1β, though not IL-1α [36].

Here we sought to determine if *B. parapertussis* inhibits the immune response upstream of the IL-1 receptor. We show that *B. parapertussis* secretes a molecule that inhibits LPS-induced IL-1α and IL-1β secretion, but *B. bronchiseptica* and *B. pertussis* also secrete this unknown factor; although, both possess additional virulence factors that appear to induce IL-1 secretion. Additionally, inhibition of IL-1 secretion occurs after transcription of IL-1 precursors and cleavage of IL-1β by activated caspase-1. The secreted inhibitor did not degrade IL-1α or IL-1β, indicating that inhibition occurs at the level of secretion. Overall these data indicate differential mechanisms employed by the three classical *Bordetella* species to modulate IL-1 secretion, and identify a potential mechanism by a bacterial pathogen to modulate secretory pathways involving not only IL-1β, but also IL-1α.
Materials and Methods:

**Bacteria Strains.** *B. bronchiseptica* strain RB50, *B. parapertussis* strains 12822, 2591 and mutant 2591Δwbm, and *B. pertussis* strain 536 have all been previously described [42-45]. Bacteria were maintained on Bordet-Gengou agar (Difco, Franklin Lakes, NJ) containing 20µg/mL of streptomycin and 10% sheep’s blood (Hema Resources, Aurora, OR). Bacteria were grown in liquid culture Stainer-Scholte (SS) broth containing heptakis shaking at 37°C to mid-log phase approximately an OD$_{600nm}$ 0.75, unless otherwise indicated [46, 47].

**LPS purification.** LPS was purified using a modified Westphal method [48]. 500 mL cultures were seeded with mid-log phase *Escherichia coli* (*E. coli*), *B. parapertussis* strain 2591, or *B. parapertussis* strain 2591Δwbm, and grown in a shaking incubator at 37°C. Cultures were grown in Luria Bertani (LB) broth or SS broth to OD$_{600}$ of 1.0. Cells were then pelleted at 500 x g and resuspended in 10 mLs of endotoxin free water. An equal volume of 90% w/v phenol was added and the samples heated to 65 °C for 1 hour with stirring. Chilled samples were then centrifuged at 1,000 x g and the aqueous phase dialyzed against ddH$_2$O for 48 hours. After lyophilization, resulting material was resuspended in Tris buffer (pH 8.0) containing 1mM MgCl$_2$ and 1mM CaCl$_2$, and treated with RNase (25 µg/mL) (Ambion, Austin, TX) and DNase (100 µg/mL) (Mo Bio, Carlsbad, CA). Proteinase K (Ambion, Austin, TX) was then added at a concentration of 100 µg/mL. Following phenol extraction, the aqueous phase was dialyzed for 12 hours against ddH$_2$O and lyophilized. Resulting LPS was suspended in endotoxin free water, examined for purity by silver stain and emerald green strain.

**Cell Stimulations and Cytokine Quantification.** Murine macrophage-like cell line RAW 264.7 was grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (Gemini Bio Products, West Sacramento, CA) and 20ug/mL of penicillin and
streptomycin in 5% CO₂ at 37°C. Cells were grown to approximately 80% confluency and 
bacteria were added in DMEM at a multiplicity of infection (MOI) of 1. Cells were also 
stimulated with 100 ng/10µL of *Escherichia coli* LPS. Additionally, bacterial supernatants were 
collected by removing bacteria using a 0.45µm filter and cells were stimulated with 50µl of 
collected supernatants. Tissue culture supernatants were collected from macrophages at either 0, 
12, 24, 26, 48 or 72 hours post-stimulation and cells were Trypan blue stained. To assess 
intracellular contents, cells were washed three times with PBS before PBS containing 0.01% 
Triton was added for 10 minutes and removed. Cytokines, IL-1α and IL-1β, were analyzed via 
ELISA according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN). Statistical 
significance was calculated using analysis of variance and Tukey simultaneous test in Minitab (v. 
16; Minitab Inc., State College, PA). A *P* value of ≤0.05 was considered significant.

**Lipid Extractions.** 2.5L of *B. parapertussis* strain 12822 was grown to mid-log phase (OD₆₀₀nm 
0.75) and bacteria were spun and filtered (0.45µm) away from the supernatant. Total lipid 
extraction was performed by adding 1N HCl to pH 6 to the supernatant. To separate the aqueous 
from non-aqueous fraction the supernatant was mixed with a 1:1 mixture of hexane and ethyl 
acetate (1:1 v/v) (Sigma Aldrich, St. Louis, MO) in a separating funnel. The aqueous phase was 
removed from the non-aqueous phase, and 10 mLs of either fraction was bubbled with argon to 
remove solvents and resuspended in 500 µl of PBS containing 0.1% DMSO. Cells were 
stimulated with the media containing 0.1% DMSO, 50 µl of the non-aqueous or the aqueous 
fraction, with or without *E. coli* LPS (100 ng). The remaining non-aqueous fraction was further 
separated using a previously described method [49] into fractions containing fatty acids, 
phospholipids, cholesteryl esters, triglycerides, cholesterol, diglycerides or monoglycerides. All
elutants were taken to dryness under argon and resuspended in PBS containing 0.1% DMSO. All solvents were HPLC-grade (J.T. Baker, Phillipsburg, NJ).

**Reverse- and Straight-phase HPLC.** Various fractions were separated by passage through Sep-Pak C18 cartridges. Cartridges were pre-equilibrated with a 0.1% acetic acid:water solution, and Reverse-phase HPLC was performed using a Rainin C18 column with acetonitrile:water:acetic acid (700:300:0.08 v/v/v) as a solvent system. Fractions were eluted at a flow rate of 2 mL/min and the eluant monitored at 220 nm. Straight-phase HPLC was performed using a silica column pre-equilibrated with hexane, hexane:isopropanol:acetic acid (0.5% isopropanol and 0.1% acetic acid) as a solvent system. Fractions were eluted at a flow rate of 200 µl/min and the eluant was monitored at 220nm. All elutants were taken to dryness under argon and resuspended in PBS containing 0.1% DMSO, and further tested by cell stimulation and cytokine ELISA.

**RNA Extraction and qRT-PCR.** RNA was extracted from three biological replicates of cells stimulated with supernatant, *E. coli* LPS or a combination of both for 24 hours using an RNAeasy Kit (Qiagen) and treated with RNase-free DNase I (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA quality was assessed 1µg of RNA was then reverse transcribed in cDNA using Im Prom-II reverse transcriptase and 0.5 µg of random hexamers according to the manufacturer’s instructions (Promega, Madison, WI). cDNA was diluted 1:1000 and 1 µl was used in reactions of Syber Green PCR master mix (Invitrogen, Carlsbad, CA) containing 300nm of the following primers: IL-1β GCCTCGTGCTGTCGGACC (forward) and TGTCGTTGCTTGGTTCTCCTTG (reverse), IL-1α AAAATCTCAGATTCACAACTGTTCGT (forward) and TGGCAACTCCTTCAGCAACAC (reverse), 18S CACGGCCGGTACAGTGAAAC (forward) and CCCGTCGGCATGTATTAGCT (reverse) [50-52]. In order to confirm lack of gDNA samples
without reverse transcriptase were also run and dissociation analysis was used to determine cycle threshold ($C_T$). Samples were normalized to amplification of the 18S gene, which was used as an internal control. The change in transcript levels was determined using the $\Delta\Delta C_T$ method of relative quantification [53].

**Western blots.** Cells were stimulated for the indicated time points with *B. parapertussis* strain 12822 supernatant, *E. coli* LPS, or a combination of both. Following stimulation, cells were trypsinized and spun down at 250 x $g$ for 5 minutes to remove tissue culture supernatant. Cells were then resuspended in 100 μl of Laemmli sample buffer and total protein content was determine using the BCA assay (ThermoScientific, Rockford, IL) in order to equalize amounts between samples. 1μg of protein was loaded on a 12.5% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were probed with rabbit anti-caspase-1 (1:500), anti-IL-1β (1:750) or anti-β-actin (1:1000) (Abcam, Cambridge, MA). Membranes were probed with a 1:10,000 dilution of goat anti-rabbit or goat anti-mouse Ig HRp conjugated antibody (Southern Biotech, Birmingham, AL), and visualized with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

**Flow cytometry.** RAW 264.7 Cells were stimulated with media, *B.parapertussis* supernatant, *E. coli* LPS (100ng/mL), or *B. parapertussis* supernatant with *E. coli* LPS (100 ng/mL) for 12 or 24 hours. Supernatant was removed for IL-1 determination via cytokine ELISA, cells were washed with DMEM containing 10% FBS 3 times, removed and pelleted at 100 x $g$. IL-1alpha surface expression was evaluated as follows, RAW cells were washed and incubated with 1x Monensin (eBioscience, San Diego, CA) for 4 hours. RAW cells were washed, and the single cell suspension was blocked with CD19/32 (1:200)(BD Biosciences, San Diego, CA) and stained for
20 minutes on ice in the dark with α-IL-1α-PE (1:100) (eBioscience, San Diego, CA) and α-CD45-APC-Cy7 (1:400) (BD Biosciences, Sparks, MD). Samples were resuspended in 4% paraformaldehyde until acquisition on a BD LSR Fortessa II. Data were analyzed with FlowJo Software 7.6.1. Results were analyzed using analysis of variance with Tukey simultaneous test in Minitab 16 (Minitab Inc., State college, PA) with a P value of <0.05 considered significant.

**Fluorescent Microscopy.** Monolayers of RAW 264.7 cells were cultured on coverslips, washed three times with PBS, fixed in 4% paraformaldehyde in PBS for 15 minutes, and permeabilized with 0.05% Triton X-100 for 5 min at room temperature, modified from previously described [54]. The cells were again washed three times with PBS and incubated in 3% BSA for 30 min to block. The anti-IL1β primary antibody (Abcam, Cambridge, MA) was diluted 1:200 in 3% BSA and incubated with the cells for 1 hour at room temperature. The cells were washed 3 times in PBS and an Alexa Fluor 488 (1:1000)-conjugated secondary antibody (Invitrogen, Carlsbad, CA) was added to the cells for a 1 hour incubation at room temperature. The cells were washed 3 times with PBS, and Vectashield mounting medium (Vector Laboratories, Inc.) was applied to the coverslips to mount them on the glass slides. The samples were examined with a confocal laser scanning microscope (Olympus Fluoroview FV1000) at the Microscope and Cytometry Facility at Penn State University Park. These experiments were performed in triplicate.
Results:

*B. parapertussis* inhibits LPS-induced IL-1 in murine macrophages. It was recently shown that IL-1 receptor signaling is important for the control of *B. pertussis* colonization in the respiratory tract [36]. Additionally, signaling downstream of this receptor has also been found important for the control of *B. bronchiseptica* (Karanikas, A.T., unpublished data), but conversely, IL-1 receptor signaling was not required for the control of *B. parapertussis* colonization in the respiratory tract, indicating that either events downstream of IL-1 receptor signaling are not important for the control of *B. parapertussis* or this bacteria modulates

![Figure 3.1](image_url)

Figure 3.1: The classical bordetellae secrete a molecule that inhibits LPS-induced IL-1 in murine macrophages. IL-1α (A) and IL-1β (B) levels were determined from cells stimulated with *B. parapertussis* strain 12822 (gray bars) or *B. pertussis* strain 536 (black bars), in the presence or absence of *E. coli* LPS (100ng) (white bars) for 48 hours. Cell were stimulated with or without *E. coli* LPS (100 ng) (white bars) in the presence or absence of *B. parapertussis* strain 12822 bacteria (gray bars) or culture supernatant (black bars), and IL-1α (C) and IL-1β (D) were measured from cell supernatants. Cells stimulated with supernatant from a *B. parapertussis*, *B. bronchiseptica* (strain RB50) or *B. pertussis* (strain 536) culture grown to mid-log phase, in the presence or absence of *E. coli* LPS (100ng) for 48 hours, and IL-1α (E) and IL-1β (F) levels were measured. Bars indicate the average of quadruplicate samples ± the standard error. * indicates a p-value of <0.05 in comparison to *E. coli* LPS-induced IL-1. Andrew Preston purified *E. coli* LPS used in all experiments.
IL-1 upstream of the signaling receptor. To determine if *B. parapertussis* inhibits IL-1α and IL-1β, murine macrophages were stimulated for 48 hours with *B. parapertussis* strain 12822 or *B. pertussis* strain 536 at an MOI of 1 with or without *E. coli* LPS, which induces IL-1. *B. parapertussis* induced IL-1α comparable to media alone levels (Figure 3.1A) and 30 pg/mL of IL-1β (Figure 3.1B), while *B. pertussis* induced more IL-1α and IL-1β, approximately 2,600 and 60 pg/mL respectively (Figure 3.1A,B). *E. coli* LPS induced secretion of approximately 1,700 pg/mL of IL-1α and 117 pg/mL IL-1β (Figure 3.1A,B). When *B. pertussis* was present with *E. coli* LPS, more IL-1α was induced (roughly 2,500 pg/mL) than *E. coli* LPS alone (~1,700 pg/mL) (Figure 3.1A). Unlike *B. pertussis*, *B. parapertussis* inhibited *E. coli* LPS induced IL-1α and IL-1β, reducing it by over 80% in each case (Figure 3.1A,B). These data indicate that *B. pertussis* stimulates production of IL-1, while *B. parapertussis* inhibits it.

To further investigate this inhibitory effect, we sought to determine whether *B. parapertussis* secretes an IL-1 inhibitory factor by stimulating macrophages with supernatant from a mid-log phase culture of *B. parapertussis*. Filtered supernatant alone induced similar levels of IL-1α and IL-1β secretion, compared to the bacteria (Figure 3.1C,D). Compared with *E. coli* LPS stimulated cells, live *B. parapertussis* inhibited LPS-induced IL-1α, approximately 8-fold, as did the supernatant (Figure 3.1C). The supernatant inhibited LPS-induced IL-1α, although not to the same extent as live bacteria, approximately 2-fold (Figure 3.1C). *E. coli* LPS induced IL-1β secretion was also inhibited by supernatant, roughly 450 pg/mL compared to 100 pg/mL (Figure 3.1D), indicating that *B. parapertussis* secretes the IL-1 inhibitory factor. Paradoxically, cells stimulated with *B. pertussis*, or *B. bronchiseptica* culture supernatants also inhibited *E. coli* LPS-induced IL-1 secretion (Figure 3.1E,F), while live *B. bronchiseptica* and *B. pertussis* were unable
to inhibit. These data indicate that although neither *B. pertussis* nor *B. bronchiseptica* bacteria inhibit IL-1, both secrete a factor that inhibits LPS-induced IL-1, which may be the same or different from the molecule produced by *B. parapertussis*.

**Inhibition based fractionation of the bordetellae IL-1 inhibitory factor.** To determine the factor produced by all three classical bordetellae, we used *B. parapertussis* since the live bacteria inhibited *E. coli* LPS-induced IL-1 while the other two species likely produce additional factors, which induce IL-1. *B. parapertussis* supernatant was separated into aqueous and non-aqueous phases using a 1:1 ethyl acetate:hexane mixture. IL-1α and IL-1β levels secreted by cells stimulated with the aqueous phase were higher without (2,400 pg/mL) and with (2,300 pg/mL) *E. coli* LPS, which was higher than cells stimulated with only *E. coli* LPS (1,000 pg/mL).

**Figure 3.2: Fractionation of the bordetellae inhibitory factor.** Supernatant from a *B. parapertussis* culture was separated into aqueous and non-aqueous fractions using a 1:1 mixture of hexane and ethyl acetate. Cells were stimulated with either *B. parapertussis* culture supernatant (light gray), the non-aqueous (dark gray) or aqueous (black) fraction of supernatant, with or without *E. coli* LPS (100 ng) (white bars) for 48 hours, and IL-1α (A) and IL-1β (B) levels were assessed via cytokine ELISA. (C) Cells were stimulated with the non-aqueous fraction after separation via aminoacyl columns into fatty acids (b), phospholipids (c), cholesteryl esters (d), triglycerides (e), cholesterol esters (f), diglycerides (g) or monoglycerides (h) in the presence or absence of *E. coli* LPS (100ng) for 48 hours and IL-1β levels were assessed by ELISA. Bars indicate the average of quadruplicate samples ± the standard error. * indicates a p-value of <0.05 in comparison to *E. coli* LPS-induced IL-1. Ravindra Kodihalli performed fractionation experiments.
pg/mL) (Figure 3.2A); a similar trend was noted with IL-1β secretion (Figure 3.2B). The non-aqueous phase induced low levels comparable to the background of IL-1α and IL-1β (Figure 3.2A,B). The combination of the non-aqueous phase plus *E. coli* LPS reduced LPS-induced IL-1α and IL-1β to approximately 400 pg/mL and 50 pg/mL respectively (Figure 3.2A,B). These data indicate that the inhibitory factor secreted by *B. parapertussis* separates with the non-aqueous fraction.

To further delineate the inhibitory molecule from the non-aqueous fraction we separated the fraction using aminopropyl columns into fractions based on lipid types known to elute, such
as fatty acids, phospholipids, triglycerides, cholesteryl esters, cholesterol, diglycerides and monoglycerides, as previously shown [49]. All fractions stimulated background levels of IL-1β release in comparison to the media alone (Figure 3.2C). Fractions containing triglycerides, cholesteryl esters, cholesterol, diglycerides and monoglycerides did not inhibit *E. coli* LPS-induced IL-1β, while fractions containing fatty acids and phospholipids reduced IL-1β levels to approximately 60 pg/mL and 10 pg/mL respectively (Figure 3.2C). These data indicate that the fractions containing fatty acids and phospholipids contain the inhibitory factor. The fatty acid fraction was then applied to reverse-phase HPLC to further separate out the inhibitory factor from others within the fraction. HPLC resolved 5 peaks, but only molecule(s) within peaks 1 and 2 retained inhibitory activity (Figure 3.3A). Mass spectrometry analysis indicated that fraction 2 contained more than one compound (data not shown), so further separation via straight-phase HPLC was used to further divide the fraction. Fractions 3 and 4 from the straight phase HPLC retained inhibitory activity (Figure 3.3B) and were further characterized by a combination of GC-MS and H-NMR analysis (Appendix D). These spectra indicated that the compound in fraction 4 was most likely 4-(dodecan-6-yl)

![Figure 3.4: 4-(dodecan-6-yl) benzenesulfonic acid induction of IL-1β. Cells were stimulated with the putative compound, 4-(dodecan-6-yl) benzenesulfonic acid at the indicated concentrations in the presence of *E. coli* LPS (100 ng), and IL-1β levels were measured. Cell death was measured via trypan blue staining. Bars indicate the average of quadruplicate samples ± standard error.](image)
benzenesulfonic acid, while fraction 3 appeared to contain a degradation product of this compound (Appendix D). Cells were stimulated with a wide-range of concentrations of 4-(dodecan-6-yl) benzenesulfonic acid, but concentrations of 1µg-100ng caused 100% cell death (Figure 3.4). Additional concentrations down to 1 fg did not inhibit E. coli LPS-induced IL-1β, indicating that this compound is not the inhibitory molecule secreted by B. parapertussis.

**Lack of inhibition by the O-antigen mutant.** Although the molecule identified via the inhibition based fraction did not inhibit E. coli LPS-induced IL-1, we additionally analyzed a B. parapertussis O-antigen mutant.

**Figure 3.5: The B. parapertussis O-antigen mutant does not inhibit LPS-induced IL-1.** Cells were stimulated with wild-type or the O-antigen mutant of B. parapertussis (A,B), supernatant from wild-type or the O-antigen mutant of B. parapertussis cultures (C,D) or purified LPS from wild-type or the O-antigen mutant of B. parapertussis (E,F) with E. coli LPS (100 ng). IL-1α and IL-1β levels were measured by ELISA and the average of quadruplicate replicates ± the standard error was calculated. * indicates a p-value of <0.05 in comparison to E. coli LPS-induced IL-1. Andrew Preston purified LPS from B. parapertussis used in this experiment.
parapertussis mutant lacking O-antigen. Cells incubated with B. parapertussis mutant bacteria lacking O-antigen at an MOI of 1 induced higher levels of secretion of IL-1α and IL-1β than B. parapertussis wild-type (Figure 3.5A,B). Additionally, cells stimulated with E. coli LPS and the B. parapertussis O-antigen mutant secreted similar levels of IL-1α and IL-1β as the E. coli LPS alone (Figure 3.5A,B). Additionally, the secretome from the O-antigen mutant does not inhibit E. coli LPS-induced IL-1α or IL-1β (Figure 3.5C,D). LPS was purified from B. parapertussis or the B. parapertussis O-antigen mutant and cells were stimulated to determine if the LPS inhibited E. coli LPS-induced IL-1.

Cells stimulated with either B. parapertussis LPS or the O-antigen mutant LPS did not inhibit E. coli LPS-induced IL-1 (Figure 3.5E, F). These data suggest that the O-antigen mutant does not produce the inhibitory factor.

**Transcription of IL-1 is not inhibited by B. parapertussis.**

IL-1 secretion is a complex process requiring input from two signaling pathways [55]. To determine at what stage B. parapertussis inhibits IL-1 processing, we analyzed transcript levels of IL-1α and IL-1β in the presence of B. parapertussis supernatant with or without E. coli LPS. B. parapertussis supernatant induced 8-fold more transcripts of IL-1α over the media control, while E. coli LPS induced approximately 4-fold (Figure 3.6A). The combination of both B. parapertussis supernatant and E. coli LPS induced similar transcript levels of IL-1α compared to E. coli LPS alone (Figure 3.6A).
Similar transcript levels of IL-1β were also seen (Figure 3.6B), indicating that the *B. parapertussis* IL-1 inhibitory factor does not affect transcription of either IL-1α or IL-1β, suggesting that inhibition likely occurs post-translationally.

**B. parapertussis does not modulate intracellular levels of IL-1.** Another potential stage at which *B. parapertussis* could modulate IL-1 levels, would be at translation. Since IL-1 is tightly regulated within macrophages, we determined intracellular levels of IL-1α and IL-1β. Cells were stimulated with *B. parapertussis* supernatant with or without *E. coli* LPS, and at the indicated time points intracellular and extracellular levels of IL-1 were assessed. As previously shown

![Graphs showing IL-1 production](image)

**Figure 3.7: Production of IL-1 is not inhibited by *B. parapertussis*.** Cells were stimulated with *B. parapertussis* culture supernatant with or without *E. coli* LPS (100 ng) for the indicated times, and IL-1α or IL-1β extracellular (A,B) and intracellular (C,D) levels were assessed by ELISA. The average of quadruplicate samples ± standard error was calculated.

(Figure 3.1B), *B. parapertussis* supernatant inhibited *E. coli* LPS-induced IL-1α and IL-1β levels (Figure 3.7A,B). Inhibition of *E. coli* LPS-induced extracellular IL-1 was observed as early as 24 hours post-stimulation (Figure 3.7A,B). Intracellular levels of IL-1 peaked at 12 hours and
decreased over time for all stimulations, likely due to a combination of degradation and secretion of IL-1, which has been previously observed (Figure 3.7C,D). *B. parapertussis* supernatant induced high levels of intracellular IL-1α and IL-1β by 12 hours, approximately 12,000 pg/mL and 2,500 pg/mL respectively (Figure 3.7C,D). *E. coli* LPS alone induced 16,000 pg/mL of IL-1α and ~ 8,700 pg/mL of IL-1β, while the combination of *B. parapertussis* supernatant and *E. coli* LPS induced comparable intracellular levels of IL-1 at 12 hours (Figure 3.7C,D). At 24 hours there was significantly more IL-1α and IL-1β when cells were stimulated with both *B. parapertussis* supernatant and *E. coli* LPS then either alone (Figure 3.7C,D). These data indicate that *B. parapertussis* does not inhibit production of IL-1.

**Products of the inflammasome are not inhibited by *B. parapertussis***. Pro-IL-1β requires cleavage by caspase-1 into its mature form in order to be secreted and Pro-caspase-1 is activated through oligomerization of a large internal complex termed the inflammasome [5]. Since IL-1α secretion has been linked to that of IL-1-β [16], we determined if *B. parapertussis* modulates cleavage of IL-1β. Precursor and mature IL-1β were assessed in cells stimulated with *B. parapertussis* decreased over time, but mature IL-1β was higher in cells stimulated with both than with either alone (Figure 3.8A). A similar trend was also observed for caspase-1 (Figure 3.8A), indicating that *B. parapertussis* does not inhibit the cleavage of IL-1β by caspase-1 or activation of caspase-1. IL-1α requires IL-1β for secretion and when IL-1β is absent, IL-1α is retained on the cell surface [16]. To determine if *B. parapertussis* modulates the secretion of IL-1α by preventing association with IL-1β we assessed cell surface expression of IL-1α. Cells stimulated with *B. parapertussis* supernatant alone induced cell surface expression of IL-1α (Figure 3.8B), which was consistent with lack of caspase-1 induction and likely inflammasome activation seen in
Figure 3.8: Mature IL-1β, caspase-1 and cell surface expression of IL-1α were not inhibited by *B. parapertussis*. (A) Cells were stimulated with *B. parapertussis* culture supernatant, *E. coli* LPS or a combination of both for the indicated timepoints and pro-IL-1β, IL-1β, pro-caspase-1 and caspase-1 were determined by Western blot. (B) IL-1α cell surface expression was assessed at 12 and 24 hours post-stimulation by flow cytometry. The average of triplicate technical replicates was graphed ± the standard deviation. Laura L. Goodfield performed the experiment for Figure 3.8B.

Figure 3.8A at 12 and 24 hours post-stimulation. Both *E. coli* LPS alone and with *B. parapertussis* supernatant induced significantly less cell surface expression of IL-1α, which was also consistent with the active IL-1β and caspase-1 (Figure 3.8B), indicating that *B. parapertussis* does not induced high cell surface expression of IL-1α in the presence of *E. coli* LPS suggesting that it likely does not block the association between IL-1α and IL-1β.

**B. parapertussis does not degrade recombinant IL-1.** Some pathogenic bacteria produce proteases that degrade specific host immune components [56]. To determine if *B. parapertussis* produces a factor that degrades IL-1, supernatant from a *B. parapertussis* culture was incubated for 48 hours with recombinant IL-1α (1,500 pg/mL) or IL-1β (2,500 pg/mL). *B. parapertussis* supernatant did not reduce IL-1α or IL-1β levels (Figure 3.9A,B). These data indicate that *B. parapertussis* likely does not produce a factor that either degrades IL-1 or binds IL-1 to prevent antibody recognition.
Localization of IL-1β within the cell. Since *B. parapertussis* does not inhibit *E. coli* LPS-induced IL-1 processing or degrade it, we assessed where intracellular IL-1β was throughout the cell at 12 (Figure 3.10A-D) and 24 (Figure 3.10E-H) hours post-stimulation. *B. parapertussis* supernatant stimulated cells had IL-1β diffuse throughout the cytosol, with some observable discreet localization at 12 and 24 hours (Figure 3.10C,G). Cells stimulated with *E. coli* LPS had IL-1β throughout the cytosol with localized puncta of IL-1β (Figure 3.10B,F). Additionally, IL-1β appeared to be spread throughout the cytosol and localized in vesicles (arrows) when cells were stimulated with either *B. parapertussis* supernatant, *E. coli* LPS, or both (Figure 3.10C-H). These data indicate that *B. parapertussis* induced IL-1β is mainly scattered throughout the cell cytosol, but appears to localize into discrete vesicles.

**Figure 3.9**: *B. parapertussis* does not degrade recombinant IL-1. *B. parapertussis* culture supernatant was incubated with media, recombinant IL-1α (A) or IL-1β (B) for 48 hours and levels measured via ELISA. The average of quadruplicate replicates was graphed ± the standard error.
Figure 3.10: *B. parapertussis* induces diffuse and localized IL-1β within murine macrophages. Cells were stimulated with media (A,B), *E. coli* LPS (100 ng) (C,D), *B. parapertussis* culture supernatant (E,F), or a combination of both *B. parapertussis* supernatant and *E. coli* LPS (G,H) for either 12 (A,C,E,G) or 24 hours (B,D,F,H). Cells were stained with antibody against IL-1β and visualized. Images are representative of three replicates, arrows indicate bodies. Sarah J. Muse performed this experiment.
**Discussion:**

Modulating the innate response is a tactic used by many bacterial pathogens to subvert the host immune response [57, 58]. In this study we demonstrate that three closely related bacterial pathogens secrete a molecule that inhibits IL-1 secretion in murine macrophages. While all three bacterial species secrete an inhibitory factor, *B. bronchiseptica* and *B. pertussis* also have factors to induce the secretion of IL-1 over inhibition, and mice lacking IL-1 receptor-mediated signaling succumb to infection, indicating a role for IL-1 in control of bacterial numbers for both pathogens ([36], Karanikas, A.T., unpublished data). In contrast *B. parapertussis* appears to have adopted a different strategy in that the bacterium does not produce additional factors that abrogate inhibition of IL-1. This correlates with events downstream of IL-1 receptor signaling not being important for the control of *B. parapertussis* numbers in the respiratory tract [36]. Additionally, inhibition of IL-1 secretion in macrophages appears to be downstream of TLR4 and inflammasome signaling, and caspase-1 activation suggesting that the classical bordetellae secrete a factor that blocks the actual secretion of IL-1.

In order to successfully evade the host immune response, bacterial pathogens have strategies to subvert host immunity, such as secretion of molecules into the environment [59]. By secreting factors into the environment, pathogens can dampen or subvert the overall response of the host. The classical bordetellae have previously been shown to subvert immune responses by secretion of molecules, such as ACT or Ptx, both of which have effects on cytokine and chemokine production by antigen presenting cells and downstream recruitment of cells to the area of infection [34, 60, 61]. Paradoxically, *B. bronchiseptica* and *B. pertussis* secrete a molecule(s) that can inhibit IL-1 secretion in macrophages, but when cells are stimulated with bacteria, both pathogens elicit a robust IL-1 response (Figure 3.1). One possible explanation is
that IL-1 inhibition during early infection is beneficial to dampen the immune response to ensure the establishment of both pathogens, but as the infection progresses, induction of IL-1 subverts an efficient immune response from clearing the bacteria. Alternatively, the induction could be the consequence of additional factors that are required for evading the immune response. The production of Ptx by *B. pertussis* has been shown to inhibit several chemokines [34], induced by IL-1 signaling, suggesting that for *B. pertussis* the beneficial effects of having Ptx may outweigh the cost of inducing IL-1. *B. parapertussis*, on the other hand, colonizes IL-1 receptor deficient mice similar to wild-type [36], indicating that either IL-1 receptor signaling is not important or that *B. parapertussis* inhibits IL-1 throughout infection. It has been previously shown that co-infection with *B. bronchiseptica* clears *B. parapertussis* more rapidly from the respiratory tract, and that there is higher induction of pro-inflammatory cytokines, suggesting that the overall strategy *B. parapertussis* employs to modulate the host immune response is one of damping the pro-inflammatory response in order to colonize and persist within the host [62].

Blocking inflammasome activation or modulating downstream products of the inflammasome is the predominant strategy employed by bacterial pathogens to manipulate IL-1 processing and secretion in macrophages and dendritic cells [63]. Few pathogens, such as *Salmonella* spp., modulate secretion of IL-1 downstream of the inflammasome of, although this appears to be the case for *B. parapertussis* [23], (Figure 3.8A). In the case of *B. bronchiseptica* and *B. pertussis*, modulation and induction of IL-1 appears to be a complex process. *B. parapertussis* does not inhibit mature caspase-1 and IL-1β, which were both found intracellularly in macrophages stimulated with either *E. coli* LPS or a combination of *E. coli* LPS and *B. parapertussis*, indicating that inhibition occurs downstream. In assessing the location of IL-1β inside the cell it was diffuse throughout the cytosol, but also localized in discrete puncta
suggesting that *B. parapertussis* does not appear to inhibit IL-1β sequestration into these undefined bodies (Figure 3.10). Previous research indicates that IL-1β co-localizes with lysosomal markers, as well as was shown to be within microvesicles [10, 12]. These data suggest that inhibition is most likely to occur at the final stage of IL-1 secretion, release from the cell. The data suggest that the bordetellae manipulate the secretion of IL-1 from macrophages by a mechanism that remains undefined.

Overall, these data indicate that the strategies used by the three classical bordetellae to evade the host immune response are likely specific to each species. Future work will focus on determining what factor is produced by all three species, which inhibits LPS-induced IL-1 secretion, and the mechanisms behind inhibition. Additionally, defining the mechanism of inhibition of IL-1 secretion by *B. parapertussis* could give rise to alternative therapeutic ways to modulate IL-1 secretion during pro-inflammatory and autoimmune diseases.
Authors and Contributions:

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Performed experiments: SEH, RK (Figure 3.2, 3.3A,B), SJM (Figure 3.10), LLG (Figure 3.8B), AP (Figure 3.1, 3.5)

Analyzed the data: SEH, RK, SJM, LLG, KSP, ETH

Wrote the paper: SEH, ETH
References:


Chapter 4

Host Specificity of ovine *Bordetella parapertussis* and the Role of Complement
Abstract:

Subtle changes in virulence factors can reflect major shifts in the host range of a pathogen and the emergence of new infectious diseases. The classical bordetellae are comprised of three sub-species of varying host specificity. *B. parapertussis* is composed of two clades, one restricted to humans, and the other ovine restricted. Here, we show that *B. parapertussis*<sub>ov</sub> strains are rapidly cleared from the mouse. Using a comparative genomic approach, we show *B. parapertussis*<sub>ov</sub> strain Bpp5 lacks several virulence factors and produces an O-antigen different from that of *B. bronchiseptica* and *B. parapertussis* strains. Additionally, the O-antigen is altered, poorly immunogenic, and does not protect Bpp5 from murine complement-mediated deposition and killing. *B. parapertussis*<sub>ov</sub> strains also colonized the respiratory tract of mice deficient in complement protein 3 more efficiently than wild-type mice. Strikingly, while *B. parapertussis*<sub>ov</sub> strains were more susceptible to mouse complement mediated killing, sheep serum killed *B. bronchiseptica* strain RB50, but failed to kill *B. parapertussis*<sub>ov</sub> strains, indicating a potential mechanism for host restriction of this *B. parapertussis* sheep specific clade.
**Introduction:**

The factors involved in host specificity are poorly defined for most pathogens; yet, understanding host-pathogen co-evolution is crucial to predict and prevent the emergence and adaptation of new infectious disease. Initial interactions between host and pathogen often determine host specificity, such as attachment and evasion/induction of the innate immune response [1]. Adherence factors can mediate bacterial attachment to specific cellular receptors that may be host- or even tissue-specific [2-6]. For example, the human pathogen *Listeria monocytogenes* enters host cells via the binding of the outer membrane surface protein internalin to E-cadherin. Internalin binds only human E-cadherin and not mouse E-cadherin due to a single amino acid difference [4, 5]. Other bacteria, such as *Neisseria meningitidis*, use molecular mimicry to bind a negative regulator of complement, complement factor H (fH), which prevents bacterial killing. The meningococcal protein only binds human fH, suggesting host range is determined by a host-specific mechanism of complement modulation [2, 3]. Furthermore, Group A streptococci secrete a streptokinase that specifically cleaves only human plasminogen, which is critical for pathogenicity [6].

The classical bordetellae are Gram-negative closely related respiratory pathogens that offer a unique model to study host specificity [7-9]. *Bordetella bronchiseptica* infects the widest range of mammalian hosts, and causes disease ranging from asymptomatic infection to lethal pneumonia, while *B. pertussis* is host restricted and causes whooping cough in humans [10, 11]. *B. parapertussis* is divided into two genetically distinct clades; *B. parapertussis*_hu is specific to humans and another causative agent of whooping cough, while the other clade is isolated specifically from the respiratory tracts of sheep (*B. parapertussis*_ov)[12]. *B. parapertussis*_ov was first isolated from the lungs of lambs in two separate locations (Scotland and New Zealand).
displaying chronic non-progressive pneumonia, but has also been isolated from the lungs of healthy sheep [12, 14]. Additionally, infection with *B. parapertussis*<sub>ov</sub> strains has been shown to facilitate the colonization of other known sheep pathogens, such as *Pasteurella haemolytica* [15]. *B. parapertussis*<sub>ov</sub> infection is marked by an increase in pathology associated with neutrophil recruitment [13].

Despite the differences in host specificity, about 65% of genes are shared between the classical bordetellae ([13], Park, J., unpublished data), but the mechanisms behind host specificity remain poorly understood. While closely related to *B. parapertussis*<sub>hu</sub> strains, *B. parapertussis*<sub>ov</sub> strains differ in virulence factor repertoire, including over-expression of the Type III Secretion System (TTSS) and differential transcription of dermonecrotic toxin and adenylate cyclase toxin [13, 16]. Additionally, the *B. parapertussis*<sub>ov</sub> O-antigen locus is missing genes, such as *wbmE* and *wbmK*, and several genes predicted to modify the polysaccharide shared lower sequence similarity with *B. bronchiseptica* and *B. parapertussis*<sub>hu</sub> [13]. Also, *B. parapertussis*<sub>ov</sub> strains have been shown to induce cytoxocity in murine macrophages to the same levels as *B. bronchiseptica* strains [16, 17]; however, *B. parapertussis*<sub>ov</sub> isolates are rapidly cleared from the mouse respiratory tract [17], suggesting that differences in virulence factor repertoire may reflect niche adaptation of *B. parapertussis*<sub>ov</sub> strains to sheep.

In this study we determine mechanisms contributing to efficient clearance of *B. parapertussis*<sub>ov</sub> strains from the mouse respiratory tract using a combination of genomic analysis and mouse molecular models. Here, we show that *B. parapertussis*<sub>ov</sub> strains do not produce an O-antigen and are susceptible to mouse complement activation, C3b deposition onto the bacterial cell surface and killing. Furthermore, complement was determined to contribute to the efficient clearance of *B. parapertussis*<sub>ov</sub> strains from the mouse respiratory tract. Strikingly, sheep serum
killed *B. bronchiseptica* strain RB50, but did not kill *B. parapertussis* strains, indicating that complement mediated killing may contribute to the host specificity of the *B. parapertussis* clade.
Materials and Methods:

Bacterial Strains and growth. *B. bronchiseptica* strain RB50 is a rabbit isolate[18], RB50Δwbm is a derivative of RB50 lacking O-antigen[19, 20], *B. parapertussis* strain 12822 was isolated from German clinical trials, and *B. parapertussis* strains 2591 and its derivative, 2591Δwbm have been previously described [20, 21]. *B. parapertussis* ov strains Bpp5 and HI were isolated from sheep in Scotland and New Zealand, respectively, and have been previously described [17]. Bacteria were maintained on Bordet-Gengou agar (Difco, Franklin Lakes, NJ) containing 10% sheep blood (Hema Resources, Aurora OR) and 20 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO). Liquid cultures were grown at 37°C overnight in a shaker to mid-log phase in Stainer-Scholte (SS) broth with heptakis[22, 23].

Animal Experiments. C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). C3 knockout (C3−/−) mice were a kind gift from Rick Wetsel and have previously been described [24]. Mice were bred in our *Bordetella*-free, specific pathogen-free breeding rooms at The Pennsylvania State University. All animal experiments were performed in accordance to institutional guidelines. 4 to 6 week old mice were lightly sedated with 5% isoflurane (IsoFlo, Abbott Laboratories) in oxygen and 5 x 10⁵ CFU were pipetted in 50ul of phosphate-buffered saline (PBS) (Omnipur, Gibbstown, NJ) onto the external nares. This method reliably distributes the bacteria throughout the respiratory tract [25]. To quantify bacterial numbers mice were sacrificed on the indicated time points and the lungs, trachea and nasal cavities were excised. Organs were then homogenized in PBS, the appropriate dilution plated on BG agar, and CFU determined by counting colonies. For all appropriate data the average +/- the standard error (error bars) were determined. Results were analyzed using analysis of covariance in Minitab 16 (Minitab Inc., State college, PA). The explanatory variable used was either the bacterial or
mouse strain, and a covariate for day was fitted to control for the change in bacterial load over time. A \( P \) value of <0.05 was considered significant.

**Virulence factor comparison.** Genes and the loci that encode the known virulence factors, filamentous haemagglutinin (FHA), fimbriae (Fims), pertactin (PRN), tracheal colonization factor (TcfA), invasive adenylate cyclase/haemolysin (ACT), dermonecrotic toxin (Dnt), *Bordetella* resistance to killing (BrkA) [28], O-antigen [29], and Type III secretion system (TTSS) [30], were compared among the three genomes via ACT [66]. Percent sequence similarity was calculated based on RB50 sequences with BLASTN [58], and genes that either contain a frame-shift mutation or an in-frame stop codon, or that are absent were highlighted with different colors in the heatmap that was generated by R [68].

**Comparative protein sequence analysis.** The protein sequences corresponding to all the genes present in O antigen locus of *B. bronchiseptica* strain RB50 and *B. parapertussis* human strain 12822 were obtained online (http://www.ncbi.nlm.nih.gov), while those in *B. parapertussis* ovine strain Bpp5 O antigen locus were obtained from newly sequenced and annotated Bpp5 genome at Sanger and Penn State ([8], Park et al., unpublished). The amino acid sequence similarity was determined by comparing 12822 genes to orthologous genes in RB50 and Bpp5 using the online NCBI protein BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

**Western Immunoblots.** Lysates were prepared by resuspending 1mg/mL of purified LPS in 100 μl of Laemmli sample buffer [26, 27]. 10 μgs were run on an 8% sodium dodecyl sulfate-polyacrylamide electrophoresis gels in denaturing conditions and transferred to a polyvinylidene diflouride membrane (Millipore, Bedford, MA). Membranes were probed with serum from *B. bronchiseptica* (RB50 or 1289)-, *B. parapertussis* hu-inoculated, or *B. parapertussis* ov-heat-killed vaccinated mice at the following dilutions, 1:1,000, 1:500 and 1:1,000, respectively. A 1:10,000
dilution of goat anti-mouse Ig HRP conjugated antibody (Southern Biotech, Birmingham, AL) was used as the detector antibody. Membranes were visualized with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

**LPS Purification.** LPS was purified by a modified Westphal method [28]. 500 mL cultures were seeded with mid-log phase bordetellae and grown in a shaking incubator at 37°C. Cultures were grown in Stainer-Scholte broth with heptakis to an OD₆₀₀ of 0.75. Cells were then pelleted at 500 x g and resuspended in 10 mLs of endotoxin free water. An equal volume of 90% w/v phenol was added and the samples were heated to 65 °C for 1 hour with stirring. Chilled samples were then centrifuged at 1,000 x g and the aqueous phase dialyzed against ddH₂O for 48 hours. After lyophilization, resulting material was resuspended in Tris buffer (pH 7.5) and treated with RNase (Ambion, Austin, TX) and DNase (Mo Bio, Carlsbad, CA) to concentrations of 25 and 100 μg/mL, respectively. Proteinase K (Ambion, Austin, TX) was then added to 100 μg/mL. Following phenol extraction, the aqueous phase was dialyzed for 12 hours against ddH₂O and lyophilized. Resulting LPS was suspended in endotoxin free water.

**Emerald Green Stain.** Purified LPS was resuspended to a final concentration of 250 μg/mL in Laemelli sample buffer, and separated by SDS-PAGE on a Mini-PROTEAN® TGX™ 4-20% gradient pre-cast gel (Bio-Rad, Hercules, CA). The gel was run at 70 volts and stained using the Pro-Q® Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen, Carlsbad, CA). Briefly, the gel was fixed with two 45 minute washings in a 50% methanol, 5% acetic acid solution. The gel was then washed with gentle agitation for two 20 minute cycles in 100 mL of a 3% acetic acid solution. Carbohydrates were then selectively oxidized with a periodic acid solution for 30 minutes. After three washes, the gel was stained with Pro-Q® Emerald 300 stain (Invitrogen, Carlsbad, CA) for 110 minutes. Following two more washes, the gel was visualized with a
The image was taken with Quantity One® imaging software.

**Serum Killing Assays.** Complement killing assays were performed as previously described [21]. Briefly, blood collected from C57BL/6 mice, C3<sup>-/-</sup> mice, or sheep was pooled, incubated at 4 °C for 1 hour and centrifuged at 250 x g for 10 min. Approximately 1,000 CFU of RB50, Bpp5, HI and RB50Δ<sub>wbm</sub> from mid-log-phase cultures were incubated with the indicated concentration of serum or PBS for 1 hour at 37 °C. Bacterial numbers before and after incubation were determined by plating and CFU counts. For all appropriate data the average +/- the standard error (error bars) were determined. Results were analyzed using analysis of variance in Minitab 16 (Minitab Inc., State college, PA) with a P value of <0.05 considered significant.

**Complement Deposition.** Approximately 10<sup>8</sup> CFU were taken from a mid-log phase culture and incubated in the absence or presence of 20% complement sufficient or deficient mouse serum for 30 minutes. After two washes with cold PBS, bacteria were resuspended in the absence or presence of FITC conjugated anti-mouse C3b antibodies (1:1,000) (eBioscience, San Diego, CA) for 15 minutes on ice in the dark. Bacteria were washed twice with cold PBS and resuspended in 4% paraformaldehyde until acquisition with a Becton Dickinson FC500. Data analysis was performed using FlowJo 7.6.1 software. For all appropriate data the average +/- the standard error (error bars) were determined. Results were analyzed using analysis of variance in Minitab 16 (Minitab Inc., State college, PA) with a P value of <0.05 considered significant.
Results:

*B. parapertussis*<sub>ov</sub> strains are rapidly cleared from the mouse respiratory tract.

Although the various *Bordetella* species and lineages efficiently infect their natural hosts, the majority of the classical bordetellae have been shown to efficiently infect mice. However, *B. parapertussis*<sub>ov</sub> strain Bpp5 poorly colonizes the respiratory tract of mice [17]. To determine if this defect is common amongst *B. parapertussis*<sub>ov</sub> strains, we inoculated mice with strains Bpp5 and HI. In comparison, *B. bronchiseptica* RB50 persists at $10^4$ CFU, as does *B. parapertussis*<sub>hu</sub> strain 12822 in the mouse nasal cavity (Figure 4.1A). *B. parapertussis*<sub>ov</sub> strains Bpp5 and HI are present at low numbers throughout the time course in the nasal cavity in comparison to RB50 (Figure 4.1A) ($F_{1,72} = 4.25, p = 0.05; F_{1,72} = 4.28, p = 0.05$).

Within the trachea, *B. bronchiseptica* RB50 and *B. parapertussis*<sub>hu</sub> 12822 are still present on day 28 at approximately $10^{3.5}$ and $10^2$ CFU, while *B. parapertussis*<sub>ov</sub> Bpp5 and HI are cleared significantly faster ($F_{1,72} = 5.63, p = 0.026; F_{1,72} = 16.55, p = 0.001$) (Figure 4.1B). *B. bronchiseptica* strain RB50 and *B. parapertussis* strain 12822 are able to efficiently colonize lower respiratory tract, despite the mouse not being a natural host for...
**B. parapertussis** (Figure 4.1C). Unlike the other classical bordetellae, **B. parapertussis** strains Bpp5 and HI are cleared much faster from the lungs of mice compare to **B. bronchiseptica** strain RB50 ($F_{1,72}=10.06, p=0.004$; $F_{1,72}=10.44, p=0.003$) reaching the limit of detection by 14 days post-inoculation (Figure 4.1C). The relatively rapid clearance of **B. parapertussis** strains suggests this lineage may lack some factor required for efficient colonization of mice, but presumably not colonization in sheep.

**Virulence factor genes of **B. parapertussis** strains.** Several virulence factors of the classical bordetellae are known to be important for attachment or innate immune response subversion, and are required for efficient colonization of the respiratory tract [21, 29-32]. To identify the molecular basis for the apparent colonization limitation of the **B. parapertussis** strains, we used comparative genomic analysis to assess the virulence factor in both **B. bronchiseptica** and **B. parapertussis** strains [8]. Since defects in adherence can lead to rapid clearance and host specificity of bacterial strains, we determined whether **B. parapertussis** strain Bpp5 has genes encoding known adherence factors, such as filamentous hemaglutinin (**fhaB, fhaL, fhaC, fhaS**), pertactin (**prn**) and fimbriae (**fimD, fimC, fimB, fimA, fim2, fim3, fimN, fimX**) ([13], Park, J. unpublished data). **B. parapertussis** strains Bpp5 had intact genes for all known adherence factors and the sequence similarity was greater than 90% in comparison to **B. bronchiseptica** strain RB50, suggesting that lack of adherence factors does not explain rapid respiratory tract clearance (Figure 4.2). Evading the innate immune response is also important for initial colonization by pathogens. Virulence factors, such as the TTSS and ACT, have been shown to be important for overcoming aspects of the innate immune response and crucial for efficient initial colonization over the first seven days of infection [31, 32]. In comparison to **B. bronchiseptica** strain RB50 and **B. parapertussis** strain 12822, **B. parapertussis** strain Bpp5 has intact and
apparently functional copies of all the genes for ACT and the TTSS [13] (Figure 4.2). In comparing virulence factors genes, we found that several O-antigen genes were missing in Bpp5 compared to B. bronchiseptica strain RB50 and B. parapertussis strain 12822 (Figure 4.2), while most other virulence factor genes were present. This analysis indicates that differences in the B. parapertussis O-antigen locus may play a role in the rapid clearance from the mouse lower respiratory tract.
**Bpp5 has a unique O-antigen locus and does not produce an O-antigen.** Since the O-antigen locus has previously been shown to be important for colonization of both *B. bronchiseptica* and *B. parapertussis* strains, we further determined whether or not Bpp5 produced a unique O-antigen. O-antigen loci in both *B. bronchiseptica* and *B. parapertussis* contain 24 genes (BB0121 to BB0144/ BPP0121 to BPP0144)[8, 33]. We observed that genes likely to encode modifications to the polysaccharide backbone are less conserved in comparison to *B. parapertussis* strain 12822 and *B. bronchiseptica* strain RB50 (wbmP, wbmN, wbmM, wbmL, wbmJ, and wbmD), similar to previous CGH analysis (Figure 4.3A)[13]. Also, wbmO and wbmI are predicted to be pseudogenes via a frame shift mutation, whereas only wbmI had been previously predicted to be a pseudogene ([13], Figure 4.3A). Additionally, we also observed that...
is completely missing in Bpp5 (Figure 4.3A) and \textit{wbmK} is replaced by a unique gene, which shows low similarity to a gene that encodes a methyltransferase type 11 in other bacteria, such as \textit{Wolinella succinogenes} ([13], Figure 4.3A). Overall, this comparative analysis of the Bpp5 O-antigen locus indicates that Bpp5 potentially has a novel or defective O-antigen.

The differential gene content, together with prior evidence of differential gene expression[13], prompted us to examine whether antibodies against either \textit{B. parapertussis}_{hu} strain 2591 (O2 serotype) or \textit{B. bronchiseptica} strain RB50 (O1 serotype) O-antigens cross-reacted with an Bpp5 O-antigen. Serum obtained from \textit{B. bronchiseptica} strain RB50 infection recognized purified LPS from RB50, but not RB50\textit{Δwbm} (O-antigen mutant), \textit{B. parapertussis}_{hu} strain 2591, 2591\textit{Δwbm} (O-antigen mutant), or \textit{B. parapertussis}_{ov} Bpp5 strain, indicating that the RB50 O-antigen is antigenically distinct from that of both \textit{B. parapertussis} strains (Figure 4.3B). Antibodies raised against \textit{B. parapertussis}_{hu} strain 2591 O-antigen did not cross react with RB50, RB50\textit{Δwbm}, 2591\textit{Δwbm}, or the \textit{B. parapertussis}_{ov} strain Bpp5, indicating that the \textit{B. parapertussis}_{ov} strain Bpp5 does not produce an O-antigen or that it produces one that is antigenically distinct from \textit{B. bronchiseptica} strain RB50 (O1) and \textit{B. parapertussis}_{hu} strain 12822 (O2) serotypes (Figure 4.3C). Furthermore, \textit{B. parapertussis}_{ov} strains probed with serum antibodies from mice vaccinated with heat-killed Bpp5 did not produce any discernible bands from 17-32kDa, suggesting that Bpp5 does not produce an O-antigen molecule or that it produces a molecule that is poorly immunogenic (Figure 4.3D).

To determine if \textit{B. parapertussis}_{ov} strain Bpp5 produces an O-antigen, LPS was purified from \textit{B. bronchiseptica} strain RB50, RB50\textit{Δwbm}, or \textit{B. parapertussis}_{ov} strain Bpp5 and stained for the presence of O-antigen. \textit{B. bronchiseptica} strain RB50 at either 10µg or 1µg produced an O-antigen, while LPS from RB50\textit{Δwbm} at either concentration only contains Band A, the inner
core trisaccharide, and Band B, the outer core branched-chain oligosaccharide attaching Lipid A to the O-antigen (Figure 4.3E). LPS purified from *B. parapertussis*<sub>ov</sub> strain Bpp5 produced bands that correlate with Band A and B, but there was no discernible O-antigen produced at any of the indicated concentrations of LPS (Figure 4.3E). Together these data overall indicate that Bpp5 does not produce an O-antigen.

**Complement deposition and killing of *B. parapertussis*<sub>ov</sub> strains.** *B. bronchiseptica* and *B. parapertussis*<sub>hu</sub> strains are protected from complement mediated killing in the mouse model of infection by O-antigen, which blocks complement from depositing onto the bacterial cell surface [19, 21]. Since *B. parapertussis*<sub>ov</sub> strains do not appear to have an O-antigen, we determined if complement can efficiently deposit on and kill *B. parapertussis*<sub>ov</sub> strains. In serum killing assays, 100% of RB50 survived in 80% mouse serum concentrations, while serum at concentrations as low as 20% efficiently killed RB50<sub>Δwbm</sub>, as previously observed [19] (Figure 4.4A). In comparison, only 30% of Bpp5 survived in 20% serum and all were killed in 80% serum (Figure 4.4A). To determine if complement was responsible for the killing of Bpp5, *B. parapertussis*<sub>ov</sub> strains were incubated with complement sufficient (solid lines) or complement deficient serum (dashed lines) at the indicated concentrations for 1 hour (A). The average percent survival of three independent experiments is shown +/- standard error. (B-D) Flow cytometry analysis of C3b deposition onto RB50, RB50<sub>Δwbm</sub> or Bpp5 incubated with complement sufficient (solid line) or deficient (dashed line) serum. Samples were unstained or stained with FITC-anti-mouse C3 antibodies and analyzed. (E) The average percentage of FITC-positive cells of three replicates of RB50 (white bar), RB50<sub>Δwbm</sub> (gray bar) or Bpp5 (black bar) are indicated +/- standard error. * indicates p value <0.05 in comparison to RB50. Laura L. Goodfield performed the experiment for Figure 4.4B.

![Figure 4.4: Complement efficiently deposits onto the cell surface and kills Bpp5.](image-url)
Becksteinia bronchiseptica strain RB50, RB50Δwbm, and Bpp5 were incubated in complement deficient serum as well. As previously shown, 100% of RB50 survived in the presence and absence of complement and in fact, it grew over the course of 1 hour within 20% and 80% serum [19] (Figure 4.4A). RB50Δwbm was efficiently killed in the presence of complement, but survived (>100%) in the absence of complement (20% and 80% serum), as previously shown [19] (Figure 4.4A). However, Bpp5 was efficiently killed in complement sufficient serum, but survived (>95%) in complement deficient serum at either 20 or 80% serum (Figure 4.4A). Together, these data indicate that complement mediates killing of Bpp5.

Previous studies have shown that the O-antigen protects bacteria against complement mediated killing by preventing complement deposition and subsequent complement membrane attack complex formation [21]. To determine if complement deposits onto the bacterial cell surface of Bpp5, B. bronchiseptica strain RB50, RB50Δwbm, and B. parapertussis ov strain Bpp5 were incubated in mouse complement sufficient and deficient serum, and then analyzed via flow cytometry for complement protein 3(C3)b deposition (Figure 4.4B). In 20% complement sufficient mouse serum, there was approximately 7% of B. bronchiseptica strain RB50 stained FITC-positive for C3b (Figure 4.4B), while over 80% of RB50Δwbm stained positive, indicating that O-antigen blocks C3b deposition onto the bacterial surface (Figure 4.4B). However, 56% of B. parapertussis ov strain Bpp5 stained FITC-positive for C3b (Figure 4.4B), which was significantly more C3b-positive bacteria than B. bronchiseptica strain RB50. These data suggest that the Bpp5 does not prevent complement deposition onto the bacterial cell surface and that killing is likely mediated by deposition.

**Complement contributes to the efficient control of B. parapertussis ov strains.** Since B. parapertussis ov strains were reduced in numbers over the first week post-inoculation, do not
produce an O-antigen, and are efficiently killed by mouse serum complement, we hypothesized that complement contributes to the efficient control of \( B. parapertussis_{\text{ov}} \) strains in the lower respiratory tract. Wild-type or C3 deficient mice were inoculated with \( 5 \times 10^5 \) CFU of \( B. bronchiseptica \) strain RB50 or \( B. parapertussis_{\text{ov}} \) strain Bpp5.

Numbers of RB50 within the respiratory tracts colonization of wild-type mice and mice deficient in complement were not significantly different, as previously seen ([19], Figure 4.5A). However, Bpp5 colonized the nasal cavity of C3 knockout mice at higher levels compared to wild-type mice on days 7 and 14 post-inoculation \( (F_{1.27}=4.93, p=0.004) \) (Figure 4.5B). Also, Bpp5 was able to colonize C3\(^{-}\) tracheas and lungs of mice on days 7, 14 and 28 post-inoculation at higher levels than wild-type mice \( (F_{1.27}=12.23, p=0.002; F_{1.27}=18.51, p=0.0009) \) (Figure 4.5B).
4.5B), indicating that complement contributes to the efficient control of *B. parapertussis* in the respiratory tracts of mice.

**Sheep serum does not kill Bpp5.** Complement is an evolutionarily conserved innate immune defense conserved amongst vertebrates and even invertebrates [1]. Sensitivity to complement would be disadvantageous to bacterial pathogens, yet *B. parapertussis* is successful in sheep leading us to hypothesize that sheep complement may differ from that of mice; we therefore hypothesized that *B. parapertussis* would be able to survive in sheep complement. To determine if Bpp5 survived, we incubated *B. parapertussis* strain Bpp5 in sheep serum. Approximately 90% of RB50 incubated with either 20% or 80% sheep serum was killed, while nearly 100% of RB50Δwbm was killed in 20% serum and 0% in 80% sheep serum, indicating that *B. bronchiseptica* strain RB50 survives in mouse serum, but not sheep serum (Figure 4.6). Surprisingly, Bpp5 and HI survived in 20% and 80% sheep serum and even grew in 80% serum (Figure 4.6), showing that although efficiently killed in mouse serum, *B. parapertussis* strains survive in sheep serum. Overall, this suggests a potential mechanism that contributes to host specificity of the classical bordetellae.

![Figure 4.6: Sheep serum does not kill Bpp5.](image)

*Figure 4.6: Sheep serum does not kill Bpp5. B. bronchiseptica* RB50 (○), RB50Δwbm (□), or *B. parapertussis* Bpp5 (△) or HI (●) were incubated with sheep serum for 1 hour at the indicated concentrations. The average percent survival of four replicates and two independent experiments is shown +/- standard error. * indicates a *p* value <0.05 between RB50 and Bpp5, or RB50 and HI.
**Discussion:**

What is the basis for host specificity of closely related bacterial species with markedly different host ranges? *B. parapertussis*<sub>ov</sub> strains are a distinct clade within the classical bordetellae that appear to be restricted to circulation within sheep populations, but are closely related, based on whole-genome wide SNP analysis, to human restricted *B. parapertussis*<sub>hu</sub> and generalist *B. bronchiseptica* ([12, 34], Park, J., unpublished data). Unlike the rest of the classical bordetellae species, *B. parapertussis*<sub>ov</sub> poorly infects the mouse lower respiratory tract even at high doses ([17], Figure 4.1). Paradoxically, despite the apparent host specificity of *B. parapertussis*<sub>ov</sub> strains and the rapid clearance from the mouse respiratory tract, human host restricted pathogens *B.parapertussis*<sub>hu</sub> and *B. pertussis* efficiently colonize the mouse respiratory. However, rapid clearance from the lower respiratory tract provides a basis to understand and study the mechanisms behind the host specificity of *B. parapertussis*<sub>ov</sub> strains, in comparison to the other classical bordetellae.

Here we show that *B. parapertussis*<sub>ov</sub> strain Bpp5 has a different repertoire of virulence factors in comparison to *B. bronchiseptica* strain RB50 and *B. parapertussis*<sub>hu</sub> strain 12822 (Figure 4.2). Since *B. parapertussis*<sub>ov</sub> strains were cleared efficiently from the lower respiratory tract we investigated interactions between the bacteria and complement. Mouse serum killed *B. parapertussis*<sub>ov</sub> strains similar to a *B. bronchiseptica* mutant lacking O-antigen, but serum lacking C3 did not kill *B. parapertussis*<sub>ov</sub> (Figure 4.4A,B). Additionally, mice lacking complement did not efficiently control the coloniztion of *B. parapertussis*<sub>ov</sub> in the lungs, trachea or nasal cavity (Figure 4.5B). These data suggest that complement is a factor controlling host specificity of this *B. parapertussis*<sub>ov</sub> clade.
Complement has been previously implicated in mediating the host restriction of pathogens such as *N. meningitidis* and *Borrelia* species [2, 3, 35]. Our data indicate that Bpp5 likely does not produce a O-antigen molecule (Figure 4.3) and does not prevent deposition of mouse complement onto the bacterial surface (Figure 4.4C), as compared to the other *Bordetella* species. This suggests that lack of O-antigen in *B. parapertussis* strain Bpp5 leads to greater susceptibility to complement deposition, and therefore, mouse complement-mediated killing. Despite efficient complement-mediated killing of *B. parapertussis* strains by mouse complement, sheep serum did not kill either *B. parapertussis* strain Bpp5 or *B. bronchiseptica* strain RB50. One possible explanation is that the structure of sheep complement may differ from that of mouse, so that additional serum resistance components of *B. parapertussis* strains retain the ability to bind complement in sheep, thus blocking complement-mediated killing. It would additionally indicate that *B. bronchiseptica* strain RB50 does not share this factor (Figure 4.6). Alternatively, differences in activation of complement pathways may change the susceptibility of *B. parapertussis* strains to complement from different animal species; for example, in mouse serum, the classical complement pathway may become activated, whereas in sheep serum either the alternative or mannose-binding lectin pathway may be activated by the bacteria. Determining which pathways are activated and where within pathways inhibition of complement occurs during *B. parapertussis* infection in differing animal models will give greater insight into the factors behind complement resistance to sheep serum and mechanisms that restrict the host range of the *B. parapertussis* clade.

While the efficient killing of *B. parapertussis* strains by mouse complement may potentially lead to a greater understanding of *B. parapertussis* host restriction, the potential loss of O-antigen within this clade could lead to greater insights into the evolution and adaption of the
bordetellae sub-species to specific host populations. The O-antigen locus is missing from \textit{B. pertussis}, and yet this bacterium circulates efficiently throughout the human population, as do O-antigen containing \textit{B. parapertussis}_{\textit{hu}} strains [16, 36, 37]. Since O-antigen is a dominant antigen, the loss of O-antigen or loss of immunogenicity may be beneficial in evading cross protective immune responses [38]. While \textit{B. parapertussis}_{\textit{ov}} strains are only found in sheep populations, \textit{B. bronchiseptica} strains have also been isolated from sheep [12]. It is therefore possible that the change in O-antigen allows \textit{B. parapertussis}_{\textit{ov}} strains to circulate in a \textit{B. bronchiseptica} immune population.

Previous work has shown that the classical bordetellae possess several complement resistance factors, including BrkA, O-antigen, FHA and Vag8 [29, 39-41]. While Bpp5 does not appear to produce an O-antigen, it possesses an intact \textit{brkA} (Park, J., unpublished data). Additionally, Bpp5 \textit{vag8} is a predicted pseudogene, and although FHA has been shown to bind complement 4 binding protein there is no evidence that this protects against \textit{in vitro} complement-killing [42]. Since the only overlapping factors between \textit{B. bronchiseptica} strain RB50, \textit{B. parapertussis}_{\textit{hu}} strain 12822 and \textit{B. parapertussis}_{\textit{ov}} strain Bpp5 is O-antigen, it is likely that the altered O-antigen confers susceptibility to murine complement. In sheep serum, survival of Bpp5 may be due to additional factors, such as BrkA preventing sheep complement-mediated killing. This possibility could be likely considering that in \textit{B. bronchiseptica} strain RB50 \textit{brkA} is a predicted pseudogene, and RB50 does not survive in sheep serum [37]. Additionally, the differing repertoires of complement resistance factors suggest that specific sets may confer differing susceptibilities to complement of various hosts and may play a role in the host specificity of some \textit{Bordetella} sub-species.
Authors and contributions:

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Analyzed data: SEH, JP, LLG, ETH

Wrote the paper: SEH, ETH
References:


Chapter 5

Horizontal Gene Transfer and Selective Advantage of O-antigen Diversity among the \textit{Bordetella} species
Abstract:

Horizontal Gene Transfer (HGT) allows for the acquisition and rapid spread of novel genetic material between species of bacteria. HGT is very common amongst many bacteria, but rare or absent from others. Within the classical bordetellae there are no published examples of horizontally transferred genes. Alignment of the O-antigen loci from eight classical bordetellae genomes showed high conservation of genes at the ends of the locus, but amongst genes within the locus there was greater variability, as well as lower GC content than the average. Additionally, dN/dS ratios indicated purifying selection within the locus despite the high SNP density. A comparison between phylogenetic trees of each gene within the O-antigen locus and the genome-wide tree indicated that portions of the locus did not follow the evolutionary relationship of the strains, specifically the serotype-specific genes and the highly variable middle portion of the locus. This suggests that HGT of portions of the O-antigen locus has occurred. Antibodies generated against O1 and O2 O-antigens allowed for some cross-evasion of immunity, but antibodies against OX-type offered no protection, suggesting a potential selective advantage for each different O-antigen serotype. The results of this study demonstrate evidence of HGT of portions of the *Bordetella* O-antigen locus as a mechanism to evade cross-protective immunity.
Introduction:

Bacterial genetic variation can be caused by mutations, recombination events, or horizontal gene transfer (HGT)[1, 2]. HGT can rapidly and dynamically change genetic material between closely and distantly related organisms, which can change the ecological niche and pathogenicity of bacterial species [2-4]. HGT, within the closely related species, has been observed in the emergence of new disease, such as transfer of the *Shigella flexineri* toxin to Shiga-toxin producing *Escherichia coli* (STEC) strain O157:H7 by bacteriophages [5]. Additionally, interspecies HGT of plasmids and pathogenicity islands between bacteria often allows for the spread of antimicrobial resistance factors, as well as novel secretion systems [1, 6]. However, HGT is rare or undocumented in many bacterial species.

The bordetellae consist of nine species of Gram-negative bacteria, including *B. bronchiseptica*, *B. parapertussis*, and *B. pertussis*, which are referred to as the classical *Bordetella* species. *B. parapertussis* and *B. pertussis* are the causative agents of whooping cough in humans and appear to have independently evolved from a *B. bronchiseptica*-like progenitor [7, 8]. *B. bronchiseptica* infects a wide-range of non-human mammals and has been occasionally isolated from immunodeficient humans[8, 9]; disease severities can range from asymptomatic carriage to lethal pneumonia [10]. Each of the classical bordetellae produces a distinct repertoire of virulence factors to evade the host immune response [7]. For example, all three of the classical bordetellae species produce a LPS, but it varies between the species. In *B. bronchiseptica* the LPS is comprised of Lipid A, an inner core (Band B), outer core trisaccharide (Band A) and O-antigen encoded by *lpx*, *waa*, *wlb*, and *wbm* loci, respectively [11]. The architecture of the LPS amongst the species is similar in production of an acylated Lipid A and branched-chain core oligosaccharide, although there are marked differences in acylation patterns
of the Lipid A between all three species [12, 13]. In addition, *B. parapertussis* does not produce the trisaccharide, likely due to a mutation in the *wlb* locus, while *B. pertussis* does not produce an O-antigen due to the lack of the *wbm* locus [12, 14, 15].

The O-antigen loci in *B. bronchiseptica* and *B. parapertussis* species contain 24 genes, while the recently characterized *wbm* locus of one *B. bronchiseptica* strain only contains 15 genes, most of which are divergent from the previously characterized loci [7, 9, 11, 16]. Genes within this locus are thought to be responsible for the biosynthesis of the pentasaccharide linker region connecting the O-polysaccharide to the inner core, synthesis of the repeated polymer, and the capping sugar [11, 12, 14]. Genes within the middle of the O-antigen locus are predicted to be responsible for the modifications of the terminal sugar residue [9, 12]. Two modifications have been noted that correlate with O1 and O2-serotypes, suggesting that antibodies against the O-antigen are directed against these terminal modifications [14, 17, 18]. Additionally, it has been shown that O1-specific immune serum does not recognize O2-specific O-antigen molecules and vice versa, suggesting that varying antigenicity allows for evasion of existing immunity within hosts [18].

This study seeks to further define genomic differences in the O-antigen locus in several newly sequenced *Bordetella* strains that were selected based on diverse phenotypes. We analyzed the currently known O-antigen types and observed high SNP density but low dN/dS ratios within the variable region of the O-antigen locus, suggesting that HGT may have occurred. Phylogenetic analyses produced different branching patterns for highly conserved and variable genes. Additionally, there appears to be extensive recombination within the middle portion of the locus in some strains. Lastly, the OX-type does not confer any protective immunity against any type of O-antigen. Overall, this work identifies HGT as a mechanism of *Bordetella* species
evolution and adaptation, and provides evidence of selective pressure favoring the acquisition of
new functional sets of genes.
Materials and Methods:

Bacterial strains and growth. All strains used in this study have been previously described [7, 9]. Bacteria were maintained on Bordet-Gengou agar (Difco, Franklin Lakes, NJ) containing 10% sheep blood (Hema Resources, Aurora OR) and 20 µg/mL streptomycin (Sigma Aldrich, St. Louis, MO). Liquid cultures were grown at 37°C overnight in a shaker to mid-log phase in Stainer-Scholte (SS) broth and heptakis [19, 20].

Lipopolysaccharide purification. LPS was purified by a modified Westphal method [21]. 500 mL cultures were seeded with mid-log phase (0.5 OD<sub>600nm</sub>) bordetellae and grown in a shaking incubator at 37°C. Cultures were grown in Stainer-Scholte broth with heptakis to an OD<sub>600nm</sub> of 1.0. Bacterial cells were then pelleted at 500 x g and resuspended in 10 mLs of endotoxin free water. An equal volume of 90% w/v phenol was added and the samples were heated to 65 °C for 1 hour with stirring. Samples were then chilled followed by centrifugation at 1,000 x g. The aqueous phase dialyzed against ddH<sub>2</sub>O for 48 hours. After lyophilization, resulting material was resuspended in Tris buffer (pH 7.5) and treated with 25 µg/mL of RNase (Ambion, Austin, TX) and 100 µg/mL of DNase (Mo Bio, Carlsbad, CA). 100µg/mL of Proteinase K (Ambion, Austin, TX) was then added. Following the phenol extraction, the aqueous phase was dialyzed for 12 hours against ddH<sub>2</sub>O and lyophilized. Resulting LPS was suspended in endotoxin free water.

Western blots. Purified LPS from the indicated bacterial strains were separated via sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to polyvinylidene difluoride membrane (Millipore, Bedford, MA) as previously described [16, 18, 22]. Membranes were probed with day 28 convalescent serum from mice inoculated with <i>B. bronchiseptica</i> strains RB50, 1289 or MO149 at a 1:1000 dilution. Membranes were then probed with goat anti-mouse (immunoglobulin H+L) horseradish
peroxidase-conjugated (1:10,000) antibody (Southern Biotech, Birmingham, AL). All membranes were visualized with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

**Animal Experiments.** C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were bred in our *Bordetella*- and pathogen-free breeding rooms at The Pennsylvania State University, and all animal experiments were performed in accordance to institutional guidelines. 4 to 6 week old mice were vaccinated intraperitoneally with 200µl of LPS (100ng) and Imject Alum (Thermo Scientific, Waltham, MA) on days 28 and 14 prior to challenge as previously defined [23]. Mice were lightly sedated with 5% isoflurane (IsoFlo, Abbott Laboratories) in oxygen, and $10^4$ CFU were pipetted in 50ul of phosphate-buffered saline (PBS) (Omnipur, Gibbstown, NJ) onto the external nares. This method reliably distributes the bacteria throughout the respiratory tract [24, 25]. To quantify bacterial numbers, mice were sacrificed on the indicated time points and the lungs, trachea and nasal cavities were excised. Organs were then homogenized in PBS, the appropriate dilution plated on BG agar with antibiotics, and CFU determined by counting colonies. For collection of convalescent or vaccination-induced serum from mice, inoculated animals were sacrificed 28 days post-inoculation or vaccination with 100ng of purified LPS and bleed orbitally as previously described [26]. To obtain the serum, blood was incubated at room temperature for 30 minutes and then spun for 5 minutes at 250 x g. Serum was collected and stored at -80°C. For all appropriate data the average +/- the standard deviation (error bars) were determined. Results were analyzed using analysis of variance in Minitab 16 (Minitab Inc., State College, PA) with a $P$ value of <0.05 considered significant.

**Maximum Likelihood Trees and dN/dS ratios.** Multiple alignments of individual genes in the O-antigen locus were generated by the MEGA5 software, and maximum likelihood trees were
constructed with a Tamura-Nei model and 1,000 bootstrap replicates[27]. dN and dS values were computed using PAML package [28] with the Nei-Gojobori method[29].

**Sequence Analysis and GC content.** Sequence percent similarity of the O-antigen locus and flanking regions based on 12822 was plotted between 0% and 100% using zPicture [30]. GC content was calculated with sliding window method (window size 1,000 base pairs) across the genome or the O-antigen locus using R [31]. Average and standard deviation for the genome-wide GC content were also calculated by R.

**Complement-killing Assay.** As previously described [32], bacteria were grown to mid-log phase, and $10^3$ CFU were incubated in serum containing antibodies generated against either O1-, O2- or OX-type LPS at the indicated concentrations, with naïve serum and PBS for 1 hour. Following incubation at 37°C bacteria were serially diluted and plates on BG containing 25µg/mL of streptomycin. CFU were counted and compared to the initial inoculums in order to determine percent survival of bacteria. For all appropriate data the average +/- the standard error (error bars) were determined. Results were analyzed using analysis of variance in Minitab 16 (Minitab Inc., State college, PA) with a $P$ value of <0.05 considered significant.
Results:

Diversity in the O-antigen locus and antigenicity of the *Bordetella* species. Two *Bordetella* O-antigen types were previously identified based on cross-reactive antigenicity and defined as O1- or O2 serotype [18]. Recently a *B. bronchiseptica* isolate was shown to produce a poorly immunogenic O-antigen [16]. The O-antigen was not recognized by antibodies raised against any *Bordetella* O1- or O2- serotypes [14, 17, 16]. Therefore we propose to classify currently known O-antigen types among the bordetellae as O1, O2, and OX (poorly immunogenic O-antigens which were previously referred to as O3, [16]).

![Figure 5.1: The O-antigen locus among the classical bordetellae.](image)

Our previous analysis has indentified genetic diversity in the genes that correlate with O1- and O2- serotypes, BB0124-*wbtO* and *wbuU-wbtO*, respectively [7, 11, 14, 17, 18]. Additionally, previous Comparative Genomic Hybridization (CGH) analysis revealed variability...
across most of the locus [9]. To precisely define the genetic diversity within the classical bordetellae O-antigen locus of 10 strains, we compared the sequence similarity of the entire locus and flanking genes to *B. parapertussis* strain 12822, including both *B. pertussis* isolates 18323 and Tohama I, which lack the entire locus (Figure 5.1). As previously indicated, the O1- and O2-serotypes correlate with a 7 gene cluster [18], although the rest of the O-antigen locus appears to be highly conserved in the *Bordetella* species, except *B. parapertussis* strain Bpp5 and *B. bronchiseptica* strain MO149. In all *B. bronchiseptica* and *B. parapertussis* strains, genes *wbmA*, *wbmB* and *wbmC* shared between 90 and 100% sequence similarity (Figure 5.1), and are consistent with annotation that designates these genes involved in the assembly of the polysaccharide. In comparison to the variable middle region of the O-antigen locus, nearly all the strains analyzed had the O2-serotype specific genes, with the exceptions of strains RB50 and D445, which had the O1-serotype specific genes, and strain MO149 which carried neither set (Figure 5.1). The serotype specific genes were highly conserved amongst strains with greater than 95% sequence similarity between O2-serotype strains, while D445 and RB50 O1 serotype specific genes were greater than 95% sequence similarity in comparison (Figure 5.1).

Additionally, *B. bronchiseptica* strain D445 had a more divergent *wbmE* gene (Figure 5.1). These data indicate that the genes at the beginning and end of the locus are highly conserved amongst *B. bronchiseptica* and *B. parapertussis* strains, but the middle of the O-antigen locus varies between strains suggesting that portions of the locus may be more likely to have been horizontally transferred.

**The O-antigen Locus has low GC content.** The O-antigen locus has been previously predicted to be horizontally transferred; this study further investigates the role of HGT in the O-antigen locus diversity among the classical bordetellae using multiple newly available *Bordetella*
genomes. Newly developed horizontal gene transfer search software, Alien-Hunter, previously identified the O-antigen locus as a HGT candidate (Park, J. unpublished data). The O-antigen locus GC content in previous analysis of 4 loci indicated variations between the variable middle region and the highly conserved genes on either side [16]. Here, we observed that the O-antigen locus contained low GC content in comparison to the entire genome in B. bronchiseptica strain RB50, suggesting that the locus may be transferred from another species that had different GC content (Figure 5.2A). To determine if portions of the O-antigen locus had lower GC content or if the entire locus did, we analyzed GC content in each gene, including two of flanking genes on each side, using a 1,000 base pair sliding window for B. bronchiseptica strains RB50 and D445. This analysis revealed that the flanking genes had GC content within the standard deviation of the genome-wide average, as did genes wbmC wbmB, and wbmA (Figure 5.2B). Genes within the middle of the locus appeared to have GC content lower than the average of the genome,
suggesting origination from another bacterium (Figure 5.2B). Similar GC content trends were observed in all *Bordetella* strains (Appendix F) over several genes within the variable middle region, indicating that genes within the locus are likely to have been horizontally transferred from another species.

**High SNP Density Despite Evidence of Purifying Selection suggest HGT.** To further investigate horizontally transferred O-antigen loci within the *Bordetella* species, sequence similarity across each locus was calculated. This analysis showed that several genes had high density SNP accumulations (Figure 5.3), within the variable middle region and not in highly conserved genes. In *B. bronchiseptica* strains RB50 and D445, which have the same O-antigen serotype genes, O1-serotype specific gene SNPs are nearly identical (Figure 5.3). Strikingly, *B.*

![Figure 5.3: Sequence similarity across the classical bordetellae O-antigen locus.](image)

Percent similarity of the O-antigen locus and flanking genes based on *B. parapertussis* strain 12822 was plotted between 0% and 100% using zPicture [30]. Intergenic regions and coding regions were highlighted with red and blue, respectively. Jihye Park performed this experiment.
*parapertussis* strain Bpp5 contains several genes in the middle of the locus that have high SNP accumulations, suggesting horizontal gene transfer or recombination events, which have changed the content of this region (Figure 5.3). *wbmF, wbmE* and *wbmD* in strains D445, MO149 and Bpp5 additionally have high SNP density, suggesting that these genes were obtained from an alternative species likely through HGT. This also suggests that recombination of the the O-antigen loci have resulted in gene changes in perhaps MO149 and Bpp5 (Figure 5.3). Overall, these data suggest HGT within the variable middle region of this locus.

**Figure 5.4: dN/dS ratios of the genes within the O-antigen loci.** dN/dS ratios for O2-type (A), O1-type (B), and all O-antigen loci (C) were plotted. Jihye Park performed this experiment.

One potential explanation for high SNP density within a region is positive selection. To determine whether selective pressure played a role in transforming the O1 or O2-type O-antigen loci, we calculated the dN/dS ratio for each gene across the entire locus for all strains (Figure
5.4). For all genes within the O1-, O2-, and OX-type loci, dN/dS ratios were below 1, indicating that positive selection is not the driving evolutionary force behind the SNP density (Figure 5.4A, B, and data not shown). Also, the overall dN/dS ratios were calculated for all the O-antigen loci (Figure 5.4C), and there was no indication of positive selection within any O-antigen locus. Overall, these data indicate the locus is not under positive selection and therefore suggests that portions of the locus are being horizontally transferred, either from within the bordetellae or from different sources.

**Evidence for Serotype-specific Horizontal Gene Transfer.** To further assess the likelihood of HGT within the variable middle region of the O-antigen locus, we constructed maximum-likelihood trees based on individual O-antigen genes and compared them with the genome-wide SNP tree (Figure 5.5A-F). The branch patterns of the flanking genes, BB0120 and blpL were roughly comparable to the genome SNP tree (data not shown). Genes at the beginning of the locus, BB0121 and BB0122, showed similar clustering patterns with the genome SNP tree, because *B. bronchiseptica* Complex I and IV strains were phylogenetically separate, although *B. parapertussis* strains were no longer clustered together (Figure 5.5A). Genes specific to the O1 and O2 serotypes (BB0123, *wbmR*, *wbmS*, *wbmO*) showed different branch patterns that correlated with O-serotype, but not with the overall genome tree, indicating that these genes are likely to be laterally transferred (Figure 5.5B,C). Additionally, other genes (*wbmL*, *wbmJ*, *wbmE*, *wbmD*) within the locus showed an isolated branch with either one or two strains (Bpp5, MO149 or D445) distinct from the rest of the *Bordetella* strains, but the other strains appeared to follow a phylogeny similar to that of the genome-wide SNP tree (Figure 5.5D,E), suggesting
HGT within a separate portion of the locus or recombination. The highly conserved genes, \( \text{wbmB}, \text{wbmC} \), within the O-antigen locus showed comparable clustering patterns to the genome SNP tree. These data suggest that portions of the O-antigen locus may have been horizontally transferred.

**O-antigen-specific antibodies mediate-serum killing.** We have previously shown that O-antigens in the bordetellae block antibody and complement deposition onto the bacterial cell surface [32-34]. To determine if different O-antigen serotypes offer a selective advantage in evasion of cross-protective immune responses, we incubated \( B. \) bronchiseptica strain RB50 (O1-type), 1289 (O2-type) or MO149 (OX-type) with serum containing antibodies against either RB50, 1289 or MO149. 10% serum-containing RB50-specific antibodies and complement killed

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**Figure 5.5: Phylogenetic analysis of genes within the O-antigen locus.** All the trees were reconstructed by maximum likelihood methods with 1,000 bootstrap replicates based on the O-antigen locus sequences (A-F). Jihye Park performed this experiment.
up to >90% of RB50 in a dose-dependent manner, but did not kill 1289 or MO149 (Figure 5.6A). Antibodies specific to 1289 contributed to >90% killing of 1289 in 10% serum and the response was also dose-dependent (Figure 5.6B). 1289-specific antibodies did not kill either RB50 or MO149 (Figure 5.6B). Surprisingly, MO149-specific antibodies did not kill RB50, 1289 or MO149, indicating antibodies against MO149 are not effective at assisting in complement mediated killing of any of the three B. bronchiseptica strains (Figure 5.6C). These data suggest that O1 and O2-type antibodies mediate killing only against a specific O-antigen type, and OX-type antibodies do not mediate any killing.

**Figure 5.6: Antibody-specific mediated serum-killing of RB50, 1289 or MO149.** *B. bronchiseptica* strains RB50 (green), 1289 (red) or MO140 (blue) were incubated with convalescent immune serum from C57/BL6 mice inoculated with $10^8$ of RB50 (A), 1289 (B) or MO149 (C) at the indicated serum percentages in the presence of naïve serum. Dashed line indicates 100% survival of bacteria. Laura L. Goodfield performed experiment 5.6C. Heather Feaga performed experiment Figure 5.6B.

**Lack of O-antigen cross-reactivity mediates a selective advantage.** In order to determine if having multiple O-antigen serotypes circulating within a population could confer the ability of *Bordetella* species to evade cross-protective immunity, mice
were vaccinated with O-type specific LPS and challenged with O1-, O2-, or OX-type bacteria. Nasal cavity and tracheal colonization was not affected by any LPS vacation (Figure 5.7A,B). However, O1-type LPS vaccination reduced RB50 (O1-type) colonization in the lungs by approximately 80%, but colonization of 1289 (O2-type) and MO149 (OX-type) was unaffected (Figure 5.7C). Similarly, 1289 (O2-type) lung colonization was reduced 99% by vaccination with O2-type LPS, but RB50 and MO149 colonization was not changed (Figure 5.7C). Strikingly, OX-type LPS vaccination did not reduce colonization of any B. bronchiseptica strain (Figure 5.7C), suggesting that lack of an antigenic O-antigen may allow these strains to circulate in an immune population.

Overall, these results indicate different O-antigen structures may allow bacterial strains to evade cross-protective immunity.

Figure 5.7: OX-type O-antigens do not protect against colonization. C57/BL6 mice were vaccinated with purified LPS (100 ng/per mouse) from RB50 (green), 1289 (red), MO149 (blue), or were sham vaccinated with PBS (white) on days 0 and 14. On day 28, mice were inoculated with $10^4$ CFU of RB50, 1289 or MO149, and nasal cavity (A), trachea (B) and lung (C) colonization were determined 3 days post-inoculation. The error bars represent standard deviation of 4 mice per group. * indicates a p value <0.05. Andrew Preston purified LPS for Figure 3.7.
Discussion:

O-antigens are known to be horizontally transferred between different bacterial species [35-37]. Recent studies suggest that HGT occurs between closely related bacteria species more frequently than between distantly related bacteria, although it is harder to detect [1, 38]. This could be explained by preferential exposure to similar species that share overlapping ecological niches or have similar genomic characteristics making it less likely the new genetic material will be rejected [38]. Interestingly, our analysis revealed that the *Bordetella* O-antigen loci have GC content well below the genome average, indicating either a functional requirement for lower GC content or that the locus was initially acquired from another bacterial species. Based on low GC content, high SNP densities with low dN/dS ratios, and dissimilar phylogenetic trees across the locus, it appears that the genes specific for O1 and O2-types have been acquired via HGT although we have not yet identified a specific mechanism behind the transfer, i.e, regions flanked by a tRNA[39], or insertion sequence elements[40].

O1- and O2-serotypes have been defined by amplification of a unique gene within the O-specific clusters and cross-reactivity with antibodies generated against either O1- or O2-type O-antigens [18], but the OX-type O-antigen can only be identified by the presence of an O-antigen molecule that lacks antibody recognition [16]. The genes, or lack of genes, contributing to this phenotype have yet to be determined. One gene within the locus, *wbmE*, was lost in strain MO149, suggesting this gene may be involved in the poor immunogenicity phenotype of this strain (Figure 5.1). Additionally, *wbmE* had low sequence similarity in *B. bronchiseptica* Complex IV strain D445 (Figure 5.1), and this strain also produces an O-antigen molecule that poorly cross-reacts with antibodies specific to the O1-serotype (data not shown). Also, serum from a D445 inoculated mouse did not recognize any O-antigen molecule (data not shown),
indicating that D445 likely produces a poorly immunogenic O-antigen. *wbmE* has previously been shown to convert uronamides to uronic acids along the polysaccharide [41]. This suggests that the patterns of uronamides, in additional to the terminal sugar previously implicated in determining O-serotype[14], may influence the binding of antibodies to O-antigen molecules or the ability of the immune system to recognize them.

Cross-immunity can select for antigenic variation and genetic polymorphisms within bacterial populations, which is predicted to occur with O-antigens [42, 43]. In *Salmonella enterica* and *Escherichia coli*, multiple O-antigen serotypes have been identified circulating throughout populations, likely due to evasion of cross-immunity [44-46]. Loss of the O-antigen in *B. pertussis* had been suggested to be a result of competition between *B. pertussis* and a *B. bronchiseptica*-like ancestor, which may have circulated within the human population prior to the emergence of *B. pertussis* [47]. Additionally, *B. parapertussis* strains, which do not produce O-antigen molecules are only found to infect sheep. The loss of antigenicity in some *B. bronchiseptica* Complex IV strains, which have been suggested to be more frequently associated with human infections than Complex I strains [9], may in part be due to immune-mediated competition with host restricted *B. parapertussis* in the human population. The apparent divergent virulence factors of *B. bronchiseptica* Complex IV strains, especially the presence of the OX-type O-antigen, may reflect ongoing adaptation of these isolates to a human host range.

Our study suggests HGT of O1- and O2-serotype specific genes may have occurred throughout the *Bordetella* species as a mechanism to avoid cross-protective immunity (Figure 5.8). We show O-antigen differences lead to the evasion of immunity, providing selective pressure to drive propagation of HGT events in *Bordetella* genomes that are otherwise relatively stable and closed.
Authors and Contributions:

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Conceived and designed experiments: SEH, JP, ETH

Performed experiments: SEH, JP (Figures 3.1-3.5), LLG (Figure 3.6C), HAF (Figure 3.6B), AP (Figure 3.8)

Analyzed data: SEH, JP, ETH

Wrote the paper: SEH, JP, ETH
References:

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Chapter 6
Summary and Significance
Synopsis

Sensing and responding to the host environment via the regulation of virulence factors to evade immunity are critical for the survival of pathogens within the host. However, the complex strategies the classical bordetellae use to subvert the immune response are still being investigated in order to understand the respiratory disease caused by this pathogen. This dissertation analyzes several aspects of host-pathogen interactions in order to better understand how the bordetellae evade host immune responses.

Summary and Implications

Bordetella species Virulence Factor Gene Regulation

Sensing and responding to changes within the host are crucial abilities that enable pathogenic bacteria to properly regulate virulence factor expression and efficiently evade the host immune response [1-3]. In Bordetella species, the most defined regulator of virulence factor gene expression is BvgAS, a two-component system shown to respond to temperature and chemical cues [4]. The classical bordetellae also appear to have a disproportionate number of putative transcription factors relative to genome size (B. pertussis-481, B. parapertussis-370, B. bronchiseptica-435) [5], indicating that the bordetellae have complex regulatory networks that control virulence factor expression within the host.

In Chapter 2, we focused on the response of B. bronchiseptica to growth in 5% CO₂ conditions, which the bordetellae may encounter within the host. While this growth condition caused differential hemolysis amongst various B. bronchiseptica isolates (Figure 2.1), it also increased transcription of several virulence factors, including cyaA, fhaB, and some TTSS genes (Figure 2.3). Previous analysis has shown differential regulation of TTSS genes in response to iron limiting conditions independent of constitutive expression of BvgAS [6]. In addition,
mutants lacking *E. coli* $\sigma^E$-like sigma factor, SigE have been shown to have differential TTSS expression (Barchinger, S.E., Zhang, X, unpublished data), though regulation of *cyaA* and *fhaB* has been attributed to BvgAS. Here, we show differential regulation of *cyaA* and *fhaB* in response to 5% CO$_2$ conditions in a mutant lacking a functional BvgS, indicating that there may be additional factors besides BvgAS that contribute to regulation of these two virulence factors (Figure 2.5). BvgS activates BvgA through a two-step phosphorylation cascade [4]. It has long been hypothesized that there are additional regulatory inputs into this system that differentially regulate the phosphorylation of BvgA, and additional regulatory elements may modulate BvgA in response to 5% CO$_2$ conditions by directly affecting phosphorylation. Another possible mechanistic explanation for the differential regulation of *cyaA* and *fhaB* is that there is another transcription factor that binds upstream of *cyaA*, *fhaB*, and potentially other virulence factor genes. These data suggest that while this additional regulatory system may contribute to fine-tuning of virulence factor gene regulation, BvgAS is required for production of these virulence factors. Therefore, regulatory systems in the classical bordetellae may fine-tune regulate virulence factor gene expression in specific microenvironments within the host.

**Determining IL-1 Inhibition Mechanism**

A key mechanism for subverting the immune response used by bacterial pathogens is to modulate cytokine production [7-10]. IL-1$\alpha$ and IL-1$\beta$ are pro-inflammatory cytokines important for the control of many bacterial pathogens, such as *Pseudomonas aeruginosa*, *Yersinia pestis*, and *Mycobacterium tuberculosis* [11-14]. Additionally, several pathogens have been shown to modulate IL-1 secretion from macrophages either by subverting the TLR signaling pathways or via inflammasome activation [9, 15-17]. Less is understood about the release of IL-1$\alpha$ and IL-1$\beta$ from macrophages, though several different pathways have been suggested to be involved in
release IL-1 from the cell [18-20]. This would be a first description of a bacterial pathogen modulating both IL-1α and IL-1β release via manipulation of secretory pathways, although the mechanism of inhibition remains yet to be determine.

Since the classical bordetellae produce a molecule that inhibits IL-1α and IL-1β secretion downstream of both TLR signaling and inflammasome activation (Figure 3.1, 3.7, 3.8), this molecule may have potential therapeutic uses. IL-1 has been implicated in several autoimmune and inflammatory diseases, including but not limited to, rheumatoid arthritis (RA), osteoarthritis, chronic obstructive pulmonary disease (COPD), asthma, irritable bowel syndrome (IBD), Crohn’s disease, Muckle-Wells syndrome, multiple sclerosis, and Alzheimer’s disease [21-27].

Compounds have previously been identified to target inflammasome activation and inhibit caspase-1 [28]. One compound approved for use in treating RA is IL-1 receptor antagonist (IL-Ra), anakinra, an IL-1 receptor-like molecule that does not have the adaptors to induce signaling once bound to IL-1 [18, 28, 29]. There are only a few compounds identified that are known to specifically target release of IL-1α and IL-1β, but the inhibitory mechanism of these compounds remains unknown [30]. Identifying and understanding the mechanism by which this molecule produced by the classical bordetellae inhibits secretion of IL-1 could provide alternative therapies to modulate these cytokines during autoimmune and inflammatory diseases.

**Host Specificity and Complement**

The classical bordetellae have long been defined as being clonal [31, 32], and it has even been suggested that they should be reclassified as sub-species. However, the host specificity provides a unique opportunity to analyze host adaptation of these pathogens. *B. bronchiseptica* colonizes a wide-range of mammals, while *B. pertussis* is human adapted [33, 34]. *B. parapertussis* is separated into two specific lineages that are each host adapted: *B.*
parapertussis\textsubscript{hu} (human) and \textit{B. parapertussis}\textsubscript{ov} (ovine) [35, 36]. In Chapter 4, we investigated the rapid clearance and control of \textit{B. parapertussis}\textsubscript{ov} strains from the mouse respiratory tract, and determined that a host component, complement, contributes to the control of \textit{B. parapertussis}\textsubscript{ov} strains (Figure 4.1, 4.5). We also determined that sheep serum does not efficiently kill \textit{B. parapertussis}\textsubscript{ov} strains (Figure 4.6), suggesting that \textit{B. parapertussis}\textsubscript{ov} has other mechanisms to avoid complement-mediated killing within its natural host.

\textit{B. parapertussis}\textsubscript{ov} strains are not the only \textit{Bordetella} species that have been isolated from sheep; \textit{B. bronchiseptica} strains have also been isolated from this host [33, 34]. While \textit{B. bronchiseptica} strains have specific O-antigen serotypes, \textit{B. parapertussis}\textsubscript{ov} strain Bpp5 appears to have a variable middle region of the O-antigen locus and does not appear to produce an O-antigen at all (Figure 4.2, 4.3). Similar to the adaptation of \textit{B. pertussis} and \textit{B.parapertussis}\textsubscript{hu} to the human population, \textit{B. parapertussis}\textsubscript{ov} isolates may have adapted to co-circulate with \textit{B. bronchiseptica} strains in the sheep population by losing this immunodominant molecule.

Overall, while complement may define the host specificity of \textit{B. parapertussis}\textsubscript{ov} strains, complement does not solely contribute to the host restriction of human pathogens \textit{B. pertussis} and \textit{B. parapertussis} as both are not efficiently killed by mouse serum. Bacterial pathogens must overcome several host barriers, such as attaching to the epithelia, competing with the host microflora, and evading the host immune response in order to successfully colonize. Likely, an inability to overcome any one of these barriers is likely to result in the clearance of the pathogen from a host. Although evading complement is thought to contribute to host restriction of several bacterial pathogens, it is one factor out of many likely to define the host specificity of bacterial pathogens.

\textbf{Serotype-specific HGT of O-antigen}
Previous research has suggested that the *Bordetella* species O-antigen locus was horizontally transferred from another unidentified bacterial species [37]. To further analyze HGT within the classical bordetellae, we investigated the O-antigen loci of several newly sequenced strains in addition to the previously three published strains [32]. We determined that the highly variable middle region of the O-antigen loci appears to have been horizontally transferred by using sequence and phylogenetic analysis of each gene within the locus, which contains the genes thought to confer differences in serotype (Figure 5.3, 5.5). Furthermore, the dN/dS ratio indicates that the locus in all the strains analyzed is under purifying selection (Figure 5.4), suggesting that there is selective pressure to maintain the locus. The O1-and O2-serotypes do not cluster within lineages or even within particular sequence types, suggesting that there has been HGT of the O-serotype specific genes in the *Bordetella* species (Figure 5.6). This antigenic variation could provide a mechanism by which *B. bronchiseptica* strains evade protective immunity and co-circulate within populations of animals.

**Future Directions**

**CO₂ response mechanism**

The finding that there is regulation of virulence factor genes, *cyA* and *fhaB* (Figure 2.5), indicates there are additional complex levels of regulation of virulence factor genes in the classical bordetellae. While BvgAS has been identified as a master regulator of virulence factor gene expression in the bordetellae [38], our data indicate that there may be fine-tuning of regulation by additional regulatory systems. CO₂ may be a signal sensed by the bacteria that differentiates locations within the host to the bacteria, as has previously been proposed for several other pathogens [39-42]. Also, determining what factors increase transcription of some virulence factor genes in response to 5% CO₂ conditions and the mechanisms behind this gene
regulation will help determine the role of CO$_2$ sensing during infection. One approach to determining the unknown mechanism would be to analyze genes differentially transcribed in a Bvg$^-$ phase-locked mutant in response to 5% CO$_2$ conditions since there is increased transcription of virulence factor genes independent of BvgS. Putative regulatory genes could then be knocked out using an allelic exchange system. Alternatively, since $B$. pertussis and $B$. parapertussis respond to growth in 5% CO$_2$ conditions, comparing putative two-component systems across all three species (20 putative two-component systems with conserved sensor kinase or response regulator domains) and using a systematic knockout approach could potentially identify the mechanism.

In addition, finding other host signals that the bordetellae sense and respond to will provide more insights into bordetellae signal sensing mechanisms and virulence factor gene regulation. With the advent of next generation sequencing technology, understanding bacterial gene expression during infection will allow for a better view into global bacterial gene expression and hopefully identify new virulence factors, potentially resulting in new targets for vaccines and prevention therapies.

**Identifying the IL-1 Inhibitory Factor**

$B$. parapertussis secretes an unknown molecule that inhibits LPS-induced IL-1 in macrophages. Conversely, stimulation with $B$. pertussis and $B$. bronchiseptica does not inhibit IL-1 secretion (Figure 3.1). Unexpectedly, $B$. pertussis and $B$. bronchiseptica appear to secrete an inhibitory molecule(s) as supernatant from both bacteria inhibit LPS-induced IL-1. Often the immune system first encounters secreted bacterial products during infection [43], which influences the downstream response to the invading pathogen. One potential explanation for inhibition of IL-1 secretion by $B$. bronchiseptica and $B$. pertussis could be that during initial
invasion the bacteria modulate the pro-inflammatory response in order to evade activating an immune response. When the immune response subsequently becomes activated and \( B. \) *bronchiseptica* and \( B. \) *pertussis* come into contact with macrophages they may produce factors, such as TTSS and Ptx, which are known to subvert cell signaling and induce aberrant pro-inflammatory cytokine secretion, as a potential mechanism to avoid clearance by the host response [44-48]. Another possible explanation is that the IL-1 inhibitory factor is a metabolic by-product, and since \( B. \) *parapertussis* does not have the TTSS and Ptx that subsequently induce IL-1, inhibition of LPS-induced IL-1 still occurs ([5, 49], Weyrich, L.S., unpublished data).

Fractionation attempts to identify the molecule that inhibits LPS-induced IL-1 secretion from murine macrophages yielded a likely contaminant, future studies will determine what compound all three classical bordetellae produce in order to determine the contributions of IL-1 inhibition to colonization and persistence of each *Bordetella* species.

**Inhibition of IL-1\( \alpha \) and IL-1\( \beta \) Secretion**

Inhibition of pro-inflammatory cytokine secretion often occurs at the level of transcription. Several pathogens have been shown to produce factors that modulate NF-κB release and translocation into the nucleosome, where it binds upstream of several pro-inflammatory cytokine genes and directs transcription [50]. Other pathogens, such as *Yersinia*, *Pseudomonas*, and *Streptococcus* species, have been shown to subvert the inflammsome, the second signaling cascade complex required for the cleavage and secretion of mature IL-1\( \beta \) [9, 51]. Inhibition of IL-1 secretion by \( B. \) *parapertussis\( _{hu} \) appears to occur downstream of both transcription and cleavage of IL-1 (Figure 3.7 and 3.8). IL-1 has been shown to co-localize with lysosomes, endosomes, microvesicles, and exosomes [19], and preliminary studies of IL-1\( \beta \) localization within macrophages indicates an association with putative vesicles. Future studies
using markers for specific vesicles, such as lysosomes or endosomes, as well as electron microscopy will define the vesicles and give insight into the inhibitory mechanism.

**B. parapertussis**<sub>ov</sub> O-antigen and serum resistance factors

*B. parapertussis*<sub>ov</sub> strains do not produce an O-antigen molecule, but *B. bronchiseptica* strain RB50 does based on emerald green stain analysis of purified LPS (Figure 4.4). The loss of O-antigen in *B. parapertussis*<sub>ov</sub> strains likely influences the binding of mouse complement to the bacterial cell surface (Figure 4.5); therefore swapping sections of the O-antigen locus between *B. bronchiseptica* strain RB50 or *B. parapertussis* strain 12822, and *B. parapertussis*<sub>ov</sub> would determine if changes in O-antigen alone make *B. parapertussis*<sub>ov</sub> strains susceptible to mouse complement. Additionally, determining what prevents sheep complement or other serum components from killing *B. parapertussis*<sub>ov</sub> will give greater insight into the restriction of these pathogens to the sheep population.

Each of the classical bordetellae have a unique set of complement resistance factors that may potentially reflect host adaption. For example, *B. bronchiseptica* strain RB50 produces O-antigen, Vag-8 and FHA [52-54]. Although O-antigen appears to play the dominant role in preventing complement-mediated killing [53], the roles of Vag-8 and FHA are less understood for the *Bordetella* species (Table 4.1). *B. parapertussis*<sub>ov</sub> strain Bpp5 appears to have a different set of serum resistance molecules, including BrkA, but not Vag-8 (Table 4.1), which may provide resistance to sheep complement or other antimicrobial components of sheep serum, or alternatively, *B. parapertussis*<sub>ov</sub> strains may produce a unique factor not shared by the other *Bordetella* species. Understanding the role of these factors in complement resistance can lead us to determine a potential mechanism defining host specificity of *B. parapertussis*<sub>ov</sub> strains.

**Defining Poorly Immunogenic O-antigens**
Previously research has shown that *B. bronchiseptica* strain MO149 produces a poorly immunogenic O-antigen (OX-setotype) [55]. The O-antigen locus of *B. bronchiseptica* strain MO149 contains several genes that have low sequence similarity in comparison to most of the other bordetellae O-antigen orthologous genes (Figure 5.2)[55], suggesting that multiple recombination events have occurred within this locus and the loss of genes likely contributes to the poor immunogenic phenotype (Figure 5.2). Interestingly, *B. bronchiseptica* strain D445 produces an O-antigen that was partially recognized by antibodies against the O1-serotype O-antigen, but antibodies generated against D445 did not cross-react with the D445 O-antigen molecule or *B. bronchiseptica* strain RB50 O-antigen (O1-serotype) (Hester, S.E., unpublished data). *B. bronchiseptica* strain D445 has an O-antigen locus with high sequence similarity to that of *B. bronchiseptica* strain RB50, with the exception of the *wbmE* gene, which shows 58% sequence similarity (Figure 5.2). *B. bronchiseptica* strain MO149 lacks this gene, as does *B. parapertussis*, strain Bpp5, which does not produce an O-antigen (Figure 4.3 and 5.2). These data indicate that *wbmE* may play a role in the poorly immunogenic phenotype observed within the Complex IV strains. The enzyme that *wbmE* encodes has been previously shown to convert uronamides to uronic acid along the polysaccharide backbone of the O-antigen, but it is unknown if changes in the ratio of uronamides to uronic acid would alter the host response towards the O-antigen [56]. Additionally, determining the genes that contribute to this poorly immunogenic phenotype could lead to a greater understanding of how the O-antigen locus is evolving in *B. bronchiseptica* Complex IV strains and the prevalence of the poorly immunogenic O-antigen type.

**Inefficient Cross-protective Immunity Generated by O-antigen**
Previous analysis suggested that there was HGT of the O-antigen genes, particularly the exchange of the genes thought to encode the O1- and O2-serotypes [37]. Additionally, previous research suggests that the two O-antigen serotypes provide incomplete immune cross-protection [37] (Figure 5.7). Similarly, Salmonella species have different O-antigen types that have been shown to be poorly cross-reactive and in some cases provide no cross-protection [57, 58]. O-antigen serotypes correlate with the circulation of particular Salmonella species within distinct host populations, and are thought to competitively exclude other species of Salmonella from the host population that have cross-protective antibodies against specific O-antigens [54]. The competitive exclusion theory predicts that, within a host population more than one circulating O-antigen type can exist dependent on the ability to evade cross-protective immune responses [59]. However, there is no evidence of B. bronchiseptica strains with O1-or O2-serotype circulating throughout the same host populations. There are currently few population based studies assessing the circulation of B. bronchiseptica in natural populations, but determining whether B. bronchiseptica strains with different O-antigen serotypes circulate in the same host population could shed light on why there are two distinct O-antigen serotypes in the classical bordetellae.

HGT is a means of rapidly acquiring new genetic material [60], which can often change virulence factors and induce antigenic variation (as suggested in the case of O-antigen) [57,58]. As new infectious diseases emerge, our understanding of how they evolve to evade host immune responses will be important in determining how best to prevent disease and the emergence of new infectious disease within populations.
References:


## Appendix A. Chapter 2, qRT-PCR Primers and Data.

### qRT-PCR Primers

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### qRT-PCR Data

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Appendix B. Chapter 2, Genes Increased and Decreased in Transcription in Response to 5% CO$_2$ conditions in *B. bronchiseptica* strains RB50 and 761.

### Genes Increased in Transcription in response to 5% CO$_2$ conditions in *B. bronchiseptica* strains RB50 and 761.

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### Genes Decreased in Transcription in response to 5% CO$_2$ conditions in *B. bronchiseptica* strains RB50 and 761.

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Appendix C. Chapter 2, Colony Characteristics of RB50 and JC100 Derivatives under Different Conditions.

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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RB55::pBvgASJC100</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>RB53 (RB50 bvgs-C3)</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>JC100</td>
<td>J</td>
<td>+</td>
<td>-</td>
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<tr>
<td>MLJC114 (JC100DbvgAS)</td>
<td>-</td>
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</tr>
<tr>
<td>MLJC114::pEG100</td>
<td>J</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MLJC106 (JC100 bvgs-C3)</td>
<td>J</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Colony characteristics:**
+ = Bvg+ phase (hemolytic, small, domed)
- = Bvg- phase (non-hemolytic, large, flat)
J = JC100 at 37°C in normal atmospheric condition (non-hemolytic, flattish, intermediate size)
Appendix D. Chapter 3, GC-MS analysis of compounds B3 and B4 eluted from straight-phase-HPLC.
Appendix E. Chapter 5, *Bordetella* O-antigen loci GC content.

GC content of O2-serotype *B. bronchiseptica* strains R77 (pink), 253 (green), 1289 (purple) and *B. parapertussis* strain 12822 (blue) (A), Bpp5 (B) or MO149 (C) is plotted in 1,000 base pair increments. The overall average GC content of the entire genome is indicated by the red line, and the blue lines represent the standard deviation across the genome.
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Publications:

2. Wolfe, DN, Karanikas, AK, Hester, SE, Kennett, MJ, Harvill, ET. Interleukin-10 Induction by Bordetella parapertussis Limits a Protective Interferon-γ Response. J. Immunology 2009 Feb 1;184(3):1392-400


