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BORDETELLAE AND THEIR INTERACTIONS WITH THE HOST:
IMPROVING THE FIGHT AGAINST INFECTION

A Dissertation in
Immunology and Infectious Diseases

by
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ABSTRACT

The classical bordetellae are comprised of respiratory pathogens that infect a wide range of hosts. This dissertation focuses on surveying *Bordetella* species and investigating host-pathogen interactions. Chapter 2 identifies 22 different PFGE types of *B. pertussis*, including newly identified PFGE types, which were isolated during outbreaks in California and Washington from 2010-2012. Although the isolates are genetically distinct, they have similarities in susceptibility to antibiotics and *in vivo* growth in the murine respiratory tract. Three (*ptxA, fim2, and fim3*) of 5 genes encoding vaccine antigens were conserved while two others contained mutations (*prn* and *fhaB*) in approximately half of the isolates. Investigation of current vaccine efficacy revealed that the current acellular pertussis vaccine reduced colonization of clinical *B. pertussis* strains in the murine respiratory tract, though not necessarily to the same extent as it reduced that of the laboratory strain. These data provide evidence that the current vaccine is still effective, but the circulating *B. pertussis* isolates have evolved since the generation of the vaccine.

Chapter 3 investigates an avirulent *B. bronchiseptica* strain isolated from human sputum. This *B. bronchiseptica* strain 99R-0433 is beta-hemolytic when grown on a blood agar plate and appears to be negative for O-antigen serotypes O1 and O2. Additionally, in contrast to *B. bronchiseptica* strain RB50, which is an efficient colonizer of the murine respiratory tract, *B. bronchiseptica* strain 99R-0433 cannot colonize the murine respiratory tract of wild type or immunodeficient mice. Based on genome wide SNP analysis, *B. bronchiseptica* strain 99R-0433 is divergent from other *Bordetella*
isolates. Furthermore, *B. bronchiseptica* strain 99R-0433 lacks several important virulence factors including pertactin, tracheal colonization factor, dermonecrotic toxin, adenylate cyclase toxin, Type VI secretion system, and O-antigen. Using this strain as a tool, we can begin to understand which virulence factors are required for different aspects of pathogenesis.

Investigating the host side, Chapter 4 discusses the interactions between *Bordetella* species and microfold (M) cells. The data provide evidence that *B. bronchiseptica* is capable of colocalizing with M cells in vivo and in vitro, and *Bordetella* species associate with and are translocated by M cells in a Bvg-dependent manner. Following infection with *Bordetella* species, induction of tumor necrosis factor α and interleukin-6 by M cells are observed. These preliminary data provide support for determining whether M cells play a role in the initiation of an immune response.

Chapter 5 questions the experimental mouse model of a high dose inoculation system and discusses the capabilities of using *B. bronchiseptica* at a low dose inoculum in order to mimic natural conditions. The low dose inoculum grows in the nasal cavity and results in high bacterial numbers of approximately 100,000 by 7 days post-inoculation, and the bacteria are capable of persisting long term to day 56. Toll like receptor-4 (TLR4) signaling is required for the control of bacterial growth in the nasal cavity in a low dose inoculum, which is in contrast to high dose inoculation systems. In contrast, Tumor necrosis factor-α (TNFα) signaling is not required for control of bacterial numbers following a low dose inoculation. Cell recruitment analysis revealed that while a high dose inoculum induces ~6,500 leukocytes to the nasal cavity 7 days post-inoculation, the low dose inoculum does not induce recruitment of leukocytes or
neutrophils beyond a basal level (< 3,000). Finally, despite the inability of the low dose to induce leukocytosis to the nasal cavity, serum antibody levels are the same in both high and low dose inoculated mice.

Through these studies, we can understand the breadth of *Bordetella* species and their interactions with the host system while beginning to elucidate remaining questions concerning their pathogenesis.
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</tr>
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<tbody>
<tr>
<td>ACIP</td>
<td>Advisory Committee on Immunization Practices</td>
</tr>
<tr>
<td>ACT</td>
<td>Adenylate Cyclase Toxin</td>
</tr>
<tr>
<td>aP</td>
<td>Acellular pertussis vaccine</td>
</tr>
<tr>
<td>Bvg</td>
<td><em>Bordetella</em> virulence gene</td>
</tr>
<tr>
<td>Bvg⁻</td>
<td>Bvg negative phase</td>
</tr>
<tr>
<td>Bvg⁺</td>
<td>Bvg positive phase</td>
</tr>
<tr>
<td>Bvg⁺ⁱ</td>
<td>Bvg intermediate phase</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNT</td>
<td>Dermonecrotic toxin</td>
</tr>
<tr>
<td>DTaP</td>
<td>Diphtheria, Tetanus and acellular Pertussis Vaccine</td>
</tr>
<tr>
<td>DTwP</td>
<td>Diphtheria, Tetanus and whole cell Pertussis Vaccine</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FIM</td>
<td>Fimbriae</td>
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<tr>
<td>FHA</td>
<td>Filamentous hemagglutinin</td>
</tr>
<tr>
<td>g</td>
<td>Gravity</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>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>pAPC</td>
<td>Professional antigen presenting cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field Gel Electrophoresis</td>
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<tr>
<td>PMN</td>
<td>Polymorphonuclear cell</td>
</tr>
<tr>
<td>PRN</td>
<td>Pertactin</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>sIgA</td>
<td>secretory IgA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCT</td>
<td>Tracheal cytotoxin</td>
</tr>
<tr>
<td>TDaP</td>
<td>Tetanus, diphtheria, and acellular pertussis vaccine</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------</td>
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<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type III Secretion System</td>
</tr>
<tr>
<td>T6SS</td>
<td>Type VI Secretion System</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>U.S.</td>
<td>United States</td>
</tr>
<tr>
<td>wP</td>
<td>Whole cell pertussis vaccine</td>
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Chapter 1

Introduction: *Bordetella* species and the host immune responses against them.
The *Bordetella* genus is closely related to the *Achromobacter* genus and contains 9 species\textsuperscript{1,2}. Friedrich von Wintzingerode et al. evaluated the *Bordetella* genus and its most closely related sister genera *Achromobacter* and *Alcaligenes*, and these data provide evolutionary history supporting that *Achromobacter* and *Bordetella* species shared a common ancestor (Figure 1.1)\textsuperscript{2}. Like the bordetellae, *Achromobacter* species are Gram-negative rods. Interestingly, *Achromobacter* species are identified as soil pathogens but can also infect humans as an opportunistic pathogen similar to *B. anisorpii*, which has been isolated from epidermal cyst exudate of immunocompromised patients, *B. avium* and *B. hinzi*, which have been isolated from cystic fibrosis patients, *B. holmesii*, which has been isolated from asplenic and AIDS patients, and *B. tremaatum*, which has been isolated from ear infections and limb wounds\textsuperscript{2}. Although these *Bordetella* species have been isolated from immunocompromised patients, these cases are relatively rare in occurrence, and these *Bordetella* species are typically not seen as human pathogens.

Three *Bordetella* species are identified as common pathogens that affect human health. These most commonly studied species are termed the classical bordetellae – *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. *B. pertussis* and *B. parapertussis* are human-specific pathogens that cause whooping cough, a disease that results in a severe cough, the characteristic inspiratory whoop, post-tussive vomiting, apnea, and sometimes death. According to the World Health Organization, approximately 20-50 million cases occur each year, resulting in approximately 300,000 deaths annually. The number of whooping cough cases has been increasing since the late 1990s in the United States
(U.S.), and in 2010-2012 the nation experienced the largest number of cases observed since the 1970s\textsuperscript{3}.

The closely related species \textit{B. bronchiseptica}, has a much wider host range, and is capable of causing the highly infectious kennel cough in dogs, atropic rhinitis in pigs, snuffles in rabbits, and a wide range of other mammals. These infections cost agriculture producers billions of dollars annually\textsuperscript{4}. All of these classical bordetellae are thought to be derived from a \textit{B. bronchiseptica}-like organism, and research suggests that the host-restricted species, \textit{B. pertussis} and \textit{B. parapertussis}, became more specialized pathogens through gene loss\textsuperscript{5,6}. This chapter will discuss the mechanisms through which these pathogens cause disease in order to colonize and evade immune mechanisms to avoid clearance as well as explore the immune response generated against these pathogens to mediate bacterial clearance.

1.1 Virulence Regulatory System.

Pathogens use virulence factors to cause damage to the host in order to effectively colonize and evade host immune mechanisms that otherwise would remove the infection. To evade the host response, the bordetellae use a battery of virulence factors controlled by a master regulatory system, BvgAS (\textit{Bordetella} virulence genes). The BvgAS system has two components, the sensor kinase – BvgS, and the response regulator – BvgA. BvgS is inactive at low temperatures and in the presence of chemical modulators such as MgSO\textsubscript{4}\textsuperscript{7}. When BvgS is activated by a signal, such as 37°C, it autophosphorylates, followed by a phosphorelay which in turn results in the phosphorylation and activation of
BvgA (Figure 1.2A). Active BvgA is capable of inhibiting virulence-repressed genes and activating virulence-associated genes. This regulatory system allows *Bordetella* to sense the environment and regulate factors that aid in the survival of the pathogen. A variety of virulence genes are regulated under this system, which gives three different phenotypic phases – Bvg⁻, Bvg⁺, and Bvg⁺. For example, genes important for environmental survival, potentially outside the host, are thought to be active in the Bvg⁻ phase as we have seen that the Bvg⁻ phase is required in lower temperatures and in nutrient limiting conditions; conversely, genes important for virulence including those encoding toxins such as pertussis toxin (PTX) and adenylate cyclase toxin (ACT) and adhesins such as fimbriae (FIM) and filamentous hemagglutinin (FHA) are produced in the Bvg⁺ phase, which is required for colonization and survival within the host (Figure 1.2B).

While this regulation is essential in controlling multiple virulence factors, other unknown ways of sensing environmental conditions and regulating virulence factors occur in order to fine-tune virulence factor expression within the host, such as Bvg-independent CO₂ effects on virulence factor expression⁸.

### 1.2 Virulence Factors.

A virulence factor can be defined as a component of a bacterium that aids in the ability of the bacterium to cause damage and disease to its host. The bordetellae have several classes of virulence factors including secretion systems, lipopolysaccharide (LPS) components, toxins, and adhesins. These virulence factors are typically on the outer surface and/or secreted from the bacterium to be able to readily interact with the host.
1.2A Secretion Systems.

The type III secretion system (T3SS) is a needle-like apparatus that punctures the cell membrane of the target host cell (Figure 1.3A). The structure resembles the flagellar hook-basal body complex and is generally conserved across organisms. Features of the T3SS include the absence of a sec-dependent signal sequence on secreted effectors, proteins with chaperone function in the cytosol, and the requirement of environmental signals for full activation. In bordetellae, the T3SS has been shown to be cytotoxic to mammalian cell lines and modulates the immune response via inhibition of IFNγ, which leads to persistence in the lower respiratory tract. T3SS effectors injected can inhibit NF-κB, which results in disruption of several host cell signaling pathways and blocks expression of defensins.

The type VI secretion system (T6SS) is a syringe-like apparatus that also punctures the target cell membrane. This apparatus is structurally analogous to an intracellular membrane-attached contractile T4 phage tail (Figure 1.3B). Interestingly, in some bacteria this apparatus has been shown to mediate injection into both bacteria and eukaryotic cells. When injecting into bacteria, the T6SS has bactericidal activity and gives a competitive edge for growth in mixed culture biofilms. Recently characterized in B. bronchiseptica, the T6SS appears to modulate host cell cytokine expression, inflammasome activity, and antibody generation (Muse, unpublished data). The T6SS also plays a role in nasal cavity persistence in the murine infection model and may be turned off during intracellular survival (Bendor, unpublished data). Although B.
pertussis strains do not appear to have intact T6SS loci, most B. bronchiseptica and B. parapertussis contain the locus\textsuperscript{18}.

1.2B Lipopolysaccharide.

The lipopolysaccharide (LPS) is comprised of 4 components: lipid A, inner core, outer core, and O-antigen encoded by lpx, wlb, waa, and wbm loci respectively (Figure 1.4). The lipid A binds Toll-like receptor (TLR)-4 and is responsible for the endotoxin activity. The structures of the lipid A in the classical bordetellae differ; B. bronchiseptica lipid A contains a hexa-acylated lipid A, while B. pertussis and B. parapertussis have fewer acylated chains, and this structural difference is reflected in the ability of B. bronchiseptica LPS to be more TLR-4 stimulatory than B. pertussis or B. parapertussis\textsuperscript{19,20}. The inner and outer core are not highly variable although expression of band A more pronounced in B. pertussis which lacks the locus encoding O-antigen\textsuperscript{21}. B. bronchiseptica strains have multiple serotypes including O1, O2, and the poorly immunogenic O-antigen recognition defined as serotype O3\textsuperscript{22,23}. Interestingly, B. parapertussis and the O2 serotype of B. bronchiseptica share identical loci encoding the O-antigen, which with other data suggests that horizontal gene transfer within the Bordetella genus may have occurred\textsuperscript{22,24}. Notably, the O-antigen is also an important virulence factor, effective at preventing complement deposition and antibody mediated clearance\textsuperscript{25–27}. 
1.2C Toxins.

Pertussis toxin (PTX) is a well-studied AB$_5$ toxin, belonging to the ADP-ribosylating toxin family secreted by the Type IV secretion system, Ptl$^{28}$. PTX has a catalytic site (subunit A) and five binding domains (subunits B), (shown in Figure 1.5A) that bind sialic acid-containing glycoproteins$^{29,30}$. This binding stimulates endocytosis, and PTX is delivered to the cytoplasm from the late endosome and Golgi apparatus$^{31,32}$. Once inside, the catalytic domain, subunit A, acts by inhibiting G protein activity$^{28}$. Inhibition of G protein activity results in decreased chemokines signaling and thus impaired chemotaxis of macrophages, neutrophils and lymphocytes$^{33}$. The locus encoding Ptl is present in all classical bordetellae and most contain the PTX locus, but only $B$. pertussis expresses the toxin; $B$. parapertussis and $B$. bronchiseptica are not thought to express PTX due to promoter inactivation$^{18,34}$.

Adenylate cyclase toxin (ACT) is a pore-forming toxin with a cytolysin, shown in Figure 1.5B. ACT has an RTX domain, a hydrophobic domain for insertion into host membranes, and an adenylate cyclase enzyme$^{35}$. It enters the host by binding the $\alpha$M$\beta$2 integrin of complement receptor 3 (CR3) found on a multitude of cells including macrophages, polymorphonuclear cells (PMN), dendritic cells (DC), and natural killer cells (NK)$^{5}$. This translocation event causes dysregulation of host signaling pathways by increasing cyclic adenosine monophosphate (cAMP) which reduces the ability of phagocytic cells to phagocytose targets and produce superoxides$^{36}$. ACT leads to a decrease in interleukin (IL)-12 and tumor necrosis factor $\alpha$ (TNF$\alpha$) and an increase in IL-6, IL-10 and IL-1$\beta$ through the NALP3 inflammasome$^{35,37-40}$. Produced by all
classical bordetellae, this toxin is vital for the ability of the bacteria to modulate the immune response.

Tracheal cytotoxin (TCT) is a small 9.2kDa secreted monomer of peptidoglycan$^{41,42}$. TCT has been shown to activate an ancient pathway called the immune deficiency pathway. This pathway, discovered in *Drosophila*, is activated in response to Gram negative bacteria, and is responsible for antimicrobial gene expression$^{43}$. Damaging to epithelial cells and neutrophils, TCT elicits IL-1α, which inhibits DNA synthesis and epithelial cell regeneration$^{42,44}$. The results of this activation include induced cytopathology such as cilia loss, tight junction disruption, and dissociation of ciliated cells from the respiratory tract lining$^{42}$.

1.2D Adhesins.

Adhesins are outer membrane proteins shown to contribute to the adherence of the bordetellae to host cells. Several virulence factors have been identified as contributing to bacterial adherence, including filamentous hemagglutinin (FHA), fimbriae (FIM), and pertactin (PRN).

FHA contains a heparin-binding domain that binds to sulfated polysaccharides, a carbohydrate-recognition domain that aids in adherence to ciliated respiratory epithelial cells and macrophages, and a RGD domain$^{45,46}$. In addition, CR3 is a receptor for FHA. The binding of FHA to these receptors results in activated phagocytes, increasing their phagocytic abilities, and enhancing respiratory burst functions$^{47}$. 
FIM are long, thin protrusions that cover the surface of the microbe. This virulence factor is a large polymer of fimbrial subunits.\textsuperscript{48} The bordetellae are able to adhere to monocytes via a component of the FIM, FimD. FimD is at the tip of FIM and displays molecular mimicry to fibronectin, which is important for host cell adherence.\textsuperscript{49} When FimD binds very late antigen 5, the result is activation of CR3.\textsuperscript{50} This is important because CR3 is an integrin specific to monocytes and macrophages.

PRN is an autotransporter, also known as type V secretion system and has roles as an adhesin through RGD domain binding and as a protective antigen.\textsuperscript{51-55} Because of its role as a protective antigen, pertactin has been included in the current acellular vaccines in several countries including the United States. Some have speculated that \textit{B. pertussis} is losing expression of pertactin due to current vaccine pressures, which are forcing \textit{B. pertussis} to evolve away from the vaccine as a way to avoid immunity, a phrase coined vaccine-driven evolution.\textsuperscript{56}

Although these virulence factors have been characterized as adhesins, their other roles in bacterial pathogenesis continue to be uncovered.

\textbf{1.3 Immune responses to \textit{Bordetella}}

The bordetellae have a host of carefully regulated virulence factors developed to combat host mechanisms that look to prevent colonization and damage and eliminate pathogens capable of overcoming those prevention methods. Both the pathogens and the host have co-evolved to combat each other in an effort to remain status-quo without succumbing to its opposition, a concept termed the Red Queen hypothesis.\textsuperscript{57,58} In the
following section, efforts of the host immune system to combat the bordetellae will be discussed.

1.3A Innate Immune Components.

Epithelial cells provide a strong border between pathogens breathed into the respiratory tract and the host systems, and they have several mechanisms to promote protection from pathogens by preventing invasion and stimulating immune responses. For example, airway epithelial cells and dendritic cells have tight junctions located at the apical surface to form a sealed barrier, which prevents both solutes and pathogens from paracellular transport. This barrier function acts as a first line of defense by preventing access of pathogen to the remainder of the system. Specialized epithelial cells named microfold (M) cells are a rare cell type, lacking an organized brush border, identified as the gateway of antigen sampling for their unique ability to translocate antigens, solutes, commensals, or pathogens, from the apical side to the basolateral side. M cells are shallow, which in turn leads to increased basolateral surface area, and appears as a pocket for immune cells to more intimately interact. M cells use secretory IgA (sIgA) receptors to transport unbound secretory IgA from the basolateral side to the apical side and bacterial-bound to the basolateral side for uptake by professional antigen presenting cells such as DC. M cells are often studied in the gut but have also been identified in the nasal cavity.

Additionally, epithelial cells and other innate immune cells have been shown to secrete collectins and antimicrobial peptides. Collectins, such as surfactant protein A
and surfactant protein D, have collagen-like domains and bind to bacteria, neutralizing and marking them for phagocytosis. It is thought that the *Bordetella* LPS structure provides steric hindrance resulting in the evasion of this neutralizing molecule\(^75,76\).

Antimicrobial peptides include cationic peptides and \(\beta\)-defensins, both of which are capable of bacterial membrane disruption. One example of pathogen resistance to antimicrobial peptides is lipid A modification in which *B. bronchiseptica* ArnT modifies the glucosamine residues. In the absence of ArnT, *B. bronchiseptica* becomes susceptible to cationic peptides and \(\beta\)-defensins\(^77\). The production of \(\beta\)-defensin 2 has been linked to production of IL-17, a main factor in Th17 immunity found to be crucial to the elimination of extracellular bacteria such as bordetellae, in airway epithelial cells, although this has not yet been investigated in the context of *Bordetella* species\(^78\).

Another innate defense is the complement system. With components produced in the liver and freely circulating throughout the blood stream, activation of the pathway, either through classical, mannose-lectin binding, or alternative pathways, occurs upon proper ligand binding. This elegant system results in inflammatory mediators and either the coating of the target in complement component 3b (C3b) for targeted neutrophil-mediated phagocytosis or bacterial cell death through membrane attack complex assembly, resulting in the creation of lethal channels on the bacterial surface. The bordetellae have evolved to resist complement through the expression of the O-antigen in *B. parapertussis* and *B. bronchiseptica* and the expression of BrkA, a serum resistance protein found in most strains of the classical bordetellae\(^25,79\).

Macrophages and DCs are the first cells to respond to *Bordetella* infection, preceeded only pro-inflammatory cytokines secreted by resident cells (Figure 1.6)\(^80–82\).
Macrophages take up bacteria through phagocytosis, and this activity is enhanced by IFNγ, which induces nitric oxide production, and IL-17. Interestingly, while most bacteria are killed following phagocytosis, B. pertussis has been detected in human and murine pulmonary alveolar macrophages both in vivo and in vitro, indicating it may be capable of living and replicating intracellularly. It has also been suggested that this may be an intracellular niche for B. pertussis in an effort to extend the length of the acute infection. While Bordetella species are capable of intracellular survival within neutrophils, this is not likely the case due to the extremely short neutrophil half-life of only 6-8 hours.

DCs are phagocytic cells, important not only for their ability to take up bacteria, but also to present bacterial peptides to B and T cells as professional antigen presenting cells (pAPC). Using pattern recognition receptors, such as Toll-like receptor (TLR) 4, DCs bind to common pathogen associated molecular patterns like LPS. The binding of DCs to LPS through TLR4 mediates DC maturation, which leads to the presentation of bacterial peptides to B and T cells, and secretion of IL-12 and IFNγ, which promotes the Th1 response. Bordetella ACT stimulates DCs to become activated in a way that the TH17 response is also promoted. This is followed by activation of caspase-1, and the NOD-like receptor family as well as the NLRP3 inflammasome, resulting in production of IL-1β and IL-23, cytokines important for the Th17 response. Interestingly, ACT and FHA stimulation also leads to DC production of anti-inflammatory cytokine IL-10, which in turn promotes T regulatory (Treg) cells.

Another phagocytic cell, the neutrophil (PMN) is important for the early control of Bordetella colonization in the lungs. IL-6 and macrophage inflammatory protein 2, a
chemotactic factor, are required for neutrophil recruitment to the lungs as early as day 5. Although, neutrophils peak 10-14 days post-infection, due to a delay in recruitment because of blocking by Bordetella-secreted PTX\textsuperscript{80,96–98}. While neutrophils have been documented as not required for bacterial clearance during primary infections with B. pertussis, neutrophils may be important in a memory response via antibody-mediated killing\textsuperscript{98}.

Natural killer (NK) cells have been investigated thoroughly for their role in virally-infected cells, although they are also important during extracellular bacterial infections such as Bordetella infection. These cells are first to provide IFNγ, a pro-inflammatory cytokine essential for the containment to the respiratory tract and clearance of Bordetella species as mice lacking IFNγ have detectable levels of Bordetella outside the respiratory tract, specifically in the liver\textsuperscript{81}. Furthermore, the provision of IFNγ helps to promote a Th1 immune response, and in the absence of NK cells, a Th2 immune response is observed\textsuperscript{81}. This is important because pro-inflammatory cytokines, such as IFNγ, are the first of many immune signals released during infection (Figure 1.6).

1.3B Adaptive Immune Components.

Adaptive immunity is the memory response to a pathogen and often takes time to develop in a naïve host. During Bordetella pertussis infection, antibodies appear around 14 days post-infection, while CD4\textsuperscript{+} cells appear in the lungs at approximately 3 weeks post-infection and subsequently in the draining lymph nodes and spleen, as seen in Figure 1.6\textsuperscript{99}. However, upon reinfection, the adaptive immune response is quickly activated and
in a matter of days, a pathogen can be cleared. Adaptive immunity is also important with respect to vaccinations, which will be discussed later.

### 1.3B.1 Humoral Immunity.

Humoral immunity is the protection cells provide through secretions, such as antibodies, in a contact-independent manner. Antibodies can function by 1. neutralizing toxins (i.e. neutralization of PTX), 2. inhibiting extracellular bacteria from binding to host cells, or 3. coating and enhancing phagocytic uptake of extracellular bacteria. *Bordetella* infection induces antibody production important for the passive protection in serum and across the placental barrier *in utero* as IgG and in the form of IgA in colostrum post-natal\(^99,100\). Notably, local antibodies in the lung are thought to have more relevance to protection than in serum\(^101\). Anti-PTX, anti-PRN and anti-O-antigen antibodies have been defined as protective antibodies while anti-FHA antibodies have been deemed less protective\(^22,102\). Interestingly, B cells may also protect in an antibody-independent manner as mucosal vaccination of Ig\(^+\) mice resulted in some protection against *B. pertussis*\(^103\). The B cells could be acting as professional antigen presenting cells, or through organogenesis as B cells have been found to induce M cells in the epithelial lining\(^104\).
1.3B.2 Cellular Immunity.

While antibodies provide protection against *Bordetella*, the importance of cellular immunity has also been recognized and continues to be investigated. Cell-mediated immunity is usually driven by either a Th1 response, responsible for intracellular bacterial elimination and characterized by secretion of pro-inflammatory cytokine IFNγ induced by IL-12, or a Th2 response, responsible for eliminating extracellular pathogens and characterized by secretion of anti-inflammatory cytokines IL-4 and IL-5, induced via IL-4. Additionally, the Th17 response has been identified for elimination of fungal and extracellular pathogens and is characterized by secretion of IL-17. Natural infection with *Bordetella* species induces both Th1 and Th17 responses, which act in synergy to clear the infection from the host lower respiratory tract. IL-17-secreting Th17 cells promote neutrophil recruitment and activate macrophages and PMNs to kill *Bordetella* species. *B. pertussis* infected babies have circulating peripheral T cells that secrete Th1 cytokines IFNγ and IL-2, but not Th2 cytokines IL-4 or IL-5. Furthermore, CD4+ T cells have been observed to be specific for PTX, FHA, and PRN. In the mouse model, restimulation of spleen cells with *B. pertussis* antigens results in IFNγ and IL-17, supporting the Th1/Th17 induced immunity during infection.

T regulatory cells (Treg) are anti-inflammatory cells, induced by IL-10, that may help to control the level of immune-mediated damage to the host. *Bordetella* infection can induce the activation of Tregs in order to persist longer through FHA- and ACT-
mediated IL-10 induction. Additionally, Tregs appear to be induced simultaneously with Th1 cells\textsuperscript{110–114}.

### 1.4 Vaccination against *Bordetella*

Due to the highly contagious nature of *Bordetella* infections, safe and effective vaccines have been of high importance in the field. Currently, vaccines against *B. bronchiseptica*, both as live attenuated nasal vaccines and inactivated whole cell injections, are available and are highly recommended for animals kept in a high density, such as canines staying at dog boarding facilities; however, it is not suggested by veterinarians for animals that are unlikely to come into contact with other animals with the likelihood to be infected, such as indoor cats. Some concerns about the efficacy of the kennel cough vaccines have been raised due to high variability in the efficacy, in addition to the possibility of current vaccines to cause immunosuppression\textsuperscript{115–118}.

Vaccination against whooping cough is of high interest. Currently the vaccination regimen as recommended by the Advisory Committee on Immunization Practices (ACIP) is as follows: 5 doses of Diptheria, Tetanus, and acellular Pertussis (DTaP), one dose at 2 months, 4 months, 6 months, between 15-18 months, and 4-6 years. Because of its highly contagious nature and disease severity to infants (≤ 3 months old) understanding the mechanisms of protection via vaccination is of great importance to the field in order to develop more protective vaccines. Whooping cough presents as a common cold at the beginning of infection, followed by a mild fever and the inspiratory cough (though a cough is not always observed in infants), which is followed by post-tussive vomiting,
apnea and hypoxia. Among hospitalized cases, bacterial pneumonia is common, followed by leukocytosis, which is associated with high mortality, and pulmonary hypertension\textsuperscript{119}.

In order to decrease the overall burden of Pertussis on the community, several vaccination strategies have been implemented. Childhood vaccination with tetanus, diphtheria and acellular pertussis (TDaP) has been in place since the 1990s. Before that, diphtheria, tetanus, and whole cell pertussis (DTwP), containing whole cell pertussis, had been important in the staggering decrease of childhood whooping cough responsible for $>$ 200,000 cases in the United States during the 1950s. The whole cell vaccine (wP) induces a strong Th1/Th17 response that results in both cellular and humoral immunity (Figure 1.7) \textsuperscript{120,121}. Due to high reactogenicity (swelling at site of infection and occasional fever) and public concern of rare neurological effects associated with wP such as convulsions, a new, safer vaccine was developed. In contrast to the wP, the new acellular pertussis (aP) vaccine induces a Th2 or mixed Th1/Th2 response, resulting in high antibody responses including IgG and IgE\textsuperscript{120,122–124}. The updated acellular version of the vaccine showed significantly reduced side effects and was more readily accepted by the public\textsuperscript{125–127}. The current aP includes PTX, FHA, FIM2/3, and PRN, and numerous clinical trials confirm that these multi-component vaccines are most protective as compared to vaccines containing less components\textsuperscript{128–132}. aP induces high levels of antibodies thought to aid in clearance and helps clear \textit{B. pertussis} from the lower respiratory tract, albeit more slowly than with the wP vaccine\textsuperscript{103,133–135}.

Unfortunately, the whooping cough vaccination regimen begins at 2 months and therefore, does not protect infants. In an attempt to shield infants from exposure,
cocooning, the act of exposing infants only to vaccinated individuals, was recommended, but this effort has been deemed ineffective, due to the high cost benefit ratio and the inability to have all encountering the infant vaccinated\textsuperscript{136,137}. Due to the observation of waning immunity from aP vaccination, the ACIP recommends an adolescent vaccine with reduced antigen dose. This booster has effectively reduced the burden on adolescents aged 11-13, but this has had no effect on the number of infant cases\textsuperscript{101}. Therefore, other vaccine strategies such as maternal and neonatal vaccination have been proposed. In baboons, both neonatal and maternal vaccinations reduced leukocytosis and bacterial pneumonia complications\textsuperscript{138}. Interestingly, these vaccinations did not prevent colonization or transmission\textsuperscript{139}.

\section*{1.5 Resurgence}

In the 1950s, whooping cough was a leading cause of childhood deaths, and the introduction of a vaccine reduced the case numbers to a very low level. Since the 1990s, we have begun to observe a substantially greater increase in cases, and whooping cough has been defined as a reemerging infectious disease (Figure 1.8). Many hypotheses have been put forth to explain the recent resurgence of whooping cough cases, with as many as 48,000 cases in the U.S. during 2012 alone, according to the Center for Disease Prevention and Control. Because of the switch from wP to aP in the 1990s, research has focused on the change in memory responses and the length of protection, which has been estimated to be between 5 and 12 years. Others have suggested that vaccine pressures have allowed vaccine escape through evolution of \textit{B. pertussis}, supported by loss of
vaccine-antigen expression\textsuperscript{56}. Other studies show that vaccine avoidance in the population, due to unwarranted and falsified claims on the link between vaccines and autism, resulted in pockets of highly susceptible populations, where whooping cough outbreaks have been observed\textsuperscript{140,141}. Whooping cough resurgence is likely a combination of all these factors and has led to multiple future considerations by the field. These include altering the current vaccine with respects to adjuvants in order to induce a more Th1/Th17 memory response, which should lead to a more protective and longer memory. Additionally, suggestions of changing the vaccine strain to a more recent isolate may result in a more efficacious vaccine. Furthermore, inclusion of other virulence factors such as a more immunogenic pertussis toxoid or ACT, which has been shown in mice to produce a protective response, would provide greater protection. All of these proposed ideas continue to be investigated, but in the meantime, further investigation of \textit{B. pertussis} virulence, evolution, and induction of a protective immune response needs to be pursued.

\textbf{1.6 Mouse Model of Infection}

Because of the impact multiple \textit{Bordetella} species have on human health, studies investigating these pathogens have been performed using animal models. There are many different animals that have been used for the models, but the majority of studies investigating the pathogenesis of \textit{B. pertussis} use the mouse infection model.

The mouse infection model uses either intranasal or aerosol inoculation which reliably distributes bacteria throughout the respiratory tract, and this system can be used
to understand the effects of virulence factors that affect colonization and leukocytosis; however, mice are not physically capable of coughing, and tracheal and lung tissue necrosis and transmission are not observed\textsuperscript{142}.

Studies using newborn piglets have also deemed useful for studying \textit{B. pertussis} infection; infection characteristics including nasal discharge, breathing difficulties, weight retardation, and bronchopneumonia (a common feature of severe pertussis in infants) are observed as in human infections. Notably, piglets are capable of a cough, but this cough is not paroxysmal. Upon necropsy, necrosis is observed in lungs, similar to necrosis in humans, although it is not observed in the trachea\textsuperscript{142,143}. Others have observed transmission between piglets allowing for the studies of vaccination and herd immunity, which is crucial for addressing the current problem of \textit{B. pertussis} resurgence\textsuperscript{144}.

While the piglet model more closely mimics a human infection, it still lacks the paroxysmal cough, which is important for understanding \textit{B. pertussis} infections and whooping cough dynamics. Recently, a non-human primate model has been established and shows all of the infection characteristics of human infant infections. The primates produce mucus and have a paroxysmal cough. Following infection, leukocytosis and lymphocytosis occur. Additionally natural airborne transmission has been observed\textsuperscript{145,146}. These are important features that will allow this new model to help address the problem of resurgence by understanding the mechanism of transmission resulting in its prevention.

Because the primate model is expensive and not feasible for all studies, and the mouse model can lack important features of the infection, the \textit{Bordetella} field has begun to use a closely related species, \textit{B. bronchiseptica}, in the mouse model of infection as a
model for studying pertussis infections. \textit{B. bronchiseptica} shares high similarity with both etiological agents of whooping cough, \textit{B. pertussis} and \textit{B. parapertussis}, with approximately 3,300 genes in common (Figure 1.9)\textsuperscript{147}. The speciation of these classical bordetellae is thought to have taken place between 0.27 and 4.0 million years through gene loss for host adaptation, which gives more credence to considering \textit{B. bronchiseptica} in mice as a model for \textit{B. pertussis} in humans\textsuperscript{18,147}.

1.7 Preface

This dissertation examines the host-pathogen interactions from multiple perspectives: 1. the prevalence and characteristics of \textit{Bordetella} species as pathogens, 2. the host and how it responds to pathogen invasion, and 3. the approach used to study these interactions.

Chapter 2 characterizes recent isolates in the U.S. from 2010-2012. The data show that multiple PFGE types circulate simultaneously and that these strains are genetically similar with greater than 98\% homology. Minor differences in virulence genes lead to the discovery of 9 new \textit{fhaB} allele types and 5 different types of mutations in the \textit{prn} gene. Recent isolates are able to colonize the murine respiratory tract, and the current vaccine is able to reduce colonization by 90\%, although this is reduced from the protection conferred by the vaccine against the laboratory strain (99\% reduction). These data are particularly important in a time when vaccination is a controversial topic.

Chapter 3 describes an avirulent strain of \textit{B. bronchiseptica}, isolated from a human. Using the mouse model of infection, we have identified the inability of this
isolate, strain 99R-0433, to colonize wild type mice following delivery of $5 \times 10^5$ CFU to the respiratory tract despite its ability to cause beta-hemolysis on Blood Agar. Interestingly, this strain cannot establish colonization in immunodeficient mice either. Based on SNP analysis, \textit{B. bronchiseptica} strain 99R-0433 varies significantly from other bordetellae, with 176,602 SNPs different from prototypical \textit{B. bronchiseptica} strain RB50 and 142,027 SNPs different from avirulent \textit{B. bronchiseptica} strain 253. Immunoblot analysis also reveals that this isolate does not have an O1-type or O2-type O-antigen. Interestingly, this isolate lacks many of the common virulence factors identified in \textit{Bordetella} pathogenesis.

In Chapter 4, we investigate the interactions between M cells and \textit{Bordetella} species at the cellular level in an effort to understand the first interactions and subsequent recruitment of immune cells that occur during infection. Our data show that both \textit{B. bronchiseptica} and \textit{B. pertussis} are capable of associating with M cells. To evaluate the ability of M cells to translocate bacteria from the apical side to the basolateral side, we used a co-cultures system and found that \textit{B. bronchiseptica} strain RB50 is readily translocated while Bvg$^-$ \textit{B. bronchiseptica} strain RB54 was translocated to a lesser degree, suggesting that Bvg$^+$ factors are important for M cell interactions. Because M cells are seen as part of the initial immune response, we evaluated the mRNA response of M cells to \textit{B. bronchiseptica} and \textit{B. pertussis}. We found that stimulation with \textit{Bordetella} species resulted in induction of pro-inflammatory cytokines TNF$\alpha$ and IL-6, and these signals may be responsible for the initiation of cell recruitment to the site of infection.

Chapter 5 discusses the effect of different doses in inducing immune responses for control of bacterial burden. Using a more natural dose, we determined that high levels of
leukocytes, more specifically neutrophils, are only recruited to the site of infection during a high dose inoculation. Interestingly, in the absence of high immune cell recruitment, the low dose was still capable of inducing serum antibodies. This chapter takes into consideration the immune requirements for efficient control of bacterial growth and how those immune requirements may differ or remain consistent based on starting inoculating dose.

The dissertation ends with Chapter 6, which discusses the summary of the above findings by chapter. Additionally, the significance of these results on the field of pertussis surveillance, host-pathogen interactions, and mouse models are discussed as well as potential future directions.
Figure 1.1 Phylogenetic tree based on 16S rRNA for *Bordetella*, *Achromobacter*, and *Alcaligenes* genera.

The dendrogram was generated using the neighbor-joining method with 100 bootstrap resamplings. Phylogenetic trees generated by the parsimony and the compatibility criterion methods showed identical overall tree topologies. Branching patterns within the *Bordetella* and the *Achromobacter* clusters changed among different algorithms. Numbers at branching points indicate bootstrap proportions of confidence in percentages. (N, neighbor-joining; P, parsimony; C, compatibility). Only values ≥ 70% are shown. *Zoogloea ramigera* ATCC 19544 T (EMBL accession no. D14254) was used as an outgroup. Bar, 10% estimated sequence divergence. Republished with permission of Society for General Microbiology, from *Bordetella petrii* sp. nov., isolated from anaerobic bioreactor, and emended description of the genus *Bordetella*, Friedrich von Wintzingerode, Antje Schattke, Roman A. Siddiqui, Ullrich Rosick, Ulf B. Gobel and Roy Gross, 51, 2001; permission conveyed through Copyright Clearance Center, Inc.
BvgS is a polydomain histidine sensor kinase that contains (from the amino to the carboxyl terminus) two periplasmically located venus flytrap domains (VFT1 and VFT2), a transmembrane domain, a PAS domain, a histidine kinase domain (HK), a receiver domain (Rec) and a histidine phosphoryl transfer domain (Hpt). BvgA is a response regulator protein that has an N-terminal Rec and a C-terminal helix–turn–helix domain (HTH). BvgS is active at 37 °C and becomes autophosphorylated at a conserved histidine (H) in the HK domain. The phosphoryl group is then transferred to the Rec domain, followed by the Hpt and finally to the Rec domain of BvgA. Phosphorylated BvgA (BvgA-P) dimerizes and activates the expression of virulence-associated genes (vag loci; which are subdivided into class 1 and class 2 genes) and represses the expression of virulence-repressed genes (vrg loci; which are class 4 genes) (B) BvgAS controls four classes of genes and three distinct phenotypic phases. The Bvg⁺ phase occurs when BvgAS is fully active and is characterized by maximal expression of genes that encode adhesins (class 2 genes, such as fhaB and fim; expression levels are indicated by the green line) and toxins (class 1 genes, such as cyaA–E, ptx–ptl and bsc genes; expression levels are indicated by the red line) and minimal expression of class 3 and class 4 genes (expression levels are indicated by purple and blue lines, respectively. The Bvg⁻ phase occurs when BvgAS is inactive and is characterized by maximal expression of class 4 genes and minimal expression of class 1, class 2 and class 3 genes. The Bvg⁻ phase is required for growth under nutrient-limiting conditions, such as those that may be encountered in the ex vivo environment. The Bvg⁻ phase occurs when BvgAS is partially active and is characterized by the maximal expression of class 2 and class 3 genes and minimal expression of class 1 and class 4 genes. The only class 3 gene that has been characterized so far is bipA, which is activated by BvgA under Bvg⁻ phase conditions and repressed by BvgA under Bvg⁺ phase conditions. The Bvg⁻ phase may be important for transmission between hosts, but this has not been fully elucidated. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, Melvin, et al., Vol. 12, Issue 4, pp. 274-288, © 2014.
Figure 1.3 Schematic of T3SS and T6SS structures.

Figure 1.4 Schematic of LPS structure.

LPS is made up of a lipid A (red), inner (pink) and outer core (blue and orange), and an O-chain (green). *B. bronchiseptica* and *B. parapertussis* contain an O-antigen while *B. pertussis* lacks an O-antigen and therefore contains LOS instead of LPS. Adapted from: Elder Pupo & Eugenio Hardy. (2009) Complexity and solutions to the isolation problem of Gram negative lipopolysaccharides’ bacteria molecular species. Biotecnología Aplicada. 26:9-15
Figure 1.5 Toxin-mediated virulence from *Bordetella* spp.

Figure 1.6 Kinetics of cell recruitment during a *B. pertussis* infection.

Relative levels of inflammatory cytokines (red), macrophages and dendritic cells (Mac/DC) (royal blue), γδ T cells (sky blue), neutrophils (green), natural killer (NK) cells (pink), *B. pertussis* colony forming units (Bp CFU) (black dashed line), IgA (olive green)CD4+ T cells (royal blue) and IgG (peach) during infection. *Reprinted by permission from Nature Publishing Group: Mucosal Immunology, Higgs, et al., Vol. 5, Issue 5, pp. 485-500, © 2014.*
Figure 1.7 Comparison of immune response associated with wP and aP.

Prevalence of *B. pertussis* in the U.S.

Figure 1.8 Prevalence of *B. pertussis* in the U.S. Reported Cases.

Data adapted from CDC.
Figure 1.9 Venn diagram showing gene complements of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*.

Numbers in parentheses indicate numbers of unique genes, excluding those from ISEs. Figures outside the circles indicate the average synonymous substitution rates (number of synonymous substitutions per potential substitution site) for the set of core genes between each pair of organisms and the estimated age of divergence in million years (My) calculated from these rates. Reprinted by permission from Macmillan Publishers Ltd: Nature Genetics, Parkhill, et al., Vol. 35, Issue 1, pp. 32-42, © 2014.
1.8 References


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38. Boyd, A. P. \textit{et al.} \textit{Bordetella pertussis} adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in


Chapter 2

Understanding the Resurgence of Pertussis: Measuring Genomic Diversity and Acellular Vaccine Efficacy against Clinical *Bordetella pertussis* Isolates from 2010-2012.
2.1 Abstract.

**Background:** *Bordetella pertussis* is a reemerging pathogen that causes whooping cough, which can lead to fatal infections, particularly in infants. To understand the recent resurgence, we evaluated strains circulating during the 2010-2012 U.S. outbreaks.

**Methods:** Phylogeny of 35 strains was assessed using pulsed-field gel electrophoresis (PFGE) analysis and sequence of vaccine-associated genes and entire genomes. Recent isolates and reference strains were compared in their ability to grow, both *in vitro* and *in vivo* in murine lungs, and the protection conferred by the current acellular vaccine, Adacel.

**Results:** 22 different PFGE types were identified, with multiple types isolated from the same location. Although genetically distinct, the isolates are similar in their resistance to erythromycin and azithromycin and in their *in vivo* growth within the murine respiratory tract. Two genes encoding vaccine antigens contained mutations (*prn* and *fhaB*) while three others (*ptxA*, *fim2*, and *fim3*) were conserved in all strains. Irrespective of strain, the current acellular pertussis vaccine reduced colonization of the murine respiratory tract, though not necessarily to the same extent as it reduced that of the laboratory strain.

**Conclusions:** This characterization of multiple strains circulating simultaneously revealed near uniform antibiotic sensitivity, suggesting that recent antibiotic use has not selected for resistance. The efficacy of the acellular vaccine against these diverse strains is consistent with clinical evidence that current vaccines remain effective. The data are inconsistent with the hypothesis that *B. pertussis* is rapidly evolving in response to
pressures created by current vaccines or antibiotics use, but leave important questions unanswered about the recent resurgence of pertussis.

2.2 Introduction.

*B. pertussis* is the etiological agent of the reemerging respiratory disease, whooping cough. According to the World Health Organization, approximately 20-50 million cases occur each year, resulting in approximately 300,000 deaths annually. The number of whooping cough cases has been increasing since the late 1990s, and in 2010-2012 the United States (U.S.) experienced the largest number of cases observed since the 1970s. In 2010, a major outbreak occurred in California, resulting in 1,337 cases by July. In 2012, outbreaks occurred in Washington and elsewhere, resulting in 48,277 cases nationwide compared to only 18,719 in 2011. This recent resurgence of whooping cough in the U.S. and other countries raises important and pressing public health concerns.

The vaccine coverage in the U.S. has remained high; coverage ranged between 93 and 96.1% for 3+ doses of DTwP or DTaP between 1994 and 2012 for children aged 19-35 months and has already reached 84.6% for 13-17 year olds since the recent licensure of adolescent vaccine. Some have proposed that genotypic and phenotypic variation of currently circulating strains could explain the resurgence of whooping cough cases over the past few years. For example, the anecdotal observations of increased antibiotic resistance or enhanced growth in vivo could allow for greater survival and transmission of *B. pertussis*, although these characteristics have not yet been attributed to a growing
proportion of currently circulating strains. Alternative explanations involve mutations in protective antigens that allow for escape from vaccine-induced immunity and variation in pertussis toxin (PTX) expression\textsuperscript{56}. Loss of expression of the vaccine component and surface protein antigen pertactin (PRN) have also been observed and related to vaccine-escape\textsuperscript{154,155}. Changes in vaccine antigens in response to vaccine pressure fit the paradigm of other infectious diseases like influenza and supports our expectations of a pathogen causing new outbreaks by rapidly evolving in response to new anthropogenic forces. However, evidence to support this view is still quite limited.

We examined strains from the recent outbreaks in California (2010) and Washington (2012) and others for evidence of changing phenotypes or genotypes that might explain the resurgence of pertussis. The prevailing explanations for this resurgence, involving recent evolution of strains that are more virulent or have escapes vaccine-induced immunity, predict that the newly adapted strain would expand rapidly in prevalence. However, pulsed field gel electrophoresis (PFGE) typing and genome-wide single nucleotide polymorphism (SNP) -based analysis showed that many diverse strains circulated simultaneously during outbreaks, including PFGE types first observed years ago. Both PFGE and genome wide SNP-based analyses indicate that recent isolates are genetically diverse, inconsistent with the idea of the outgrowth of a small number of newly dominant strains. Recent isolates also are highly similar in various \textit{in vitro} and \textit{in vivo} phenotypes. For example, we observe similar antibiotic sensitivities, growth rates \textit{in vitro} and virulence characteristics in the host respiratory tract. Most importantly, vaccination with the current acellular \textit{pertussis} (aP) vaccine was protective against recent isolates. Together, these data do not fit the hypothesis that the emergence of newly
adapted strains is causing the resurgence of whooping cough cases. Instead, they suggest that understanding the recent resurgence of whooping cough may require additional studies exploring new insights.

2.3 Materials and Methods.

Bacterial Strains.

*B. pertussis* strains were a kind gift from the Collaborative Pediatric Critical Care Research Network (CPCCRN), Dr. Christopher Newth, and the Centers for Disease Control and Prevention (CDC). Bacteria were maintained on Bordet-Gengou agar (Difco) containing 10% sheep blood (Hema Resources). Bacteria were grown in Stainer-Schölte broth containing heptakis shaking at 37°C. The laboratory strain was represented by Tohama I, which was isolated from Japan in the 1950s or 536, its streptomycin-resistant variant.

Animal Experiments.

C57BL/6 mice were obtained from Jackson Laboratories. All animal experiments were performed in accordance with institutional animal care and use committee (IACUC protocol # 40029) guidelines. C57BL/6 mice were vaccinated with Adacel or alum and challenged with 5x10^5 CFU *B. pertussis* isolates as previously described. The lower respiratory tract was excised on day 3 post-challenge to evaluate the colonization numbers by serial plating on Regan-Lowe agar containing 40µg/mL cephalexin. For antigen-specific antibodies, C57BL/6 mice were vaccinated on days 0, 14, and 21 and euthanized on day 25 for serum collection. The mice were vaccinated with 15.2µg FHA
or PRN (Sanofi Pasteur) in 200µL PBS supplemented with 20µL alum (Fisher Scientific). Serum was isolated as previously described\(^8\).

**PFGE Typing.**

PFGE typing was performed as previously described\(^{160}\). Briefly, isolates were suspended in agarose plugs and DNA was digested with restriction enzyme XbaI. Electrophoresis was conducted in 1% agarose gels using 0.5X Tris-Borate-EDTA buffer at 14°C. The voltage used was 6V/cm for 18 hours with a ramped switch time of 2.2-35 seconds. After staining with ethidium bromide, the DNA bands were visualized using UV light. A TIFF image was captured for each gel, which was subsequently analyzed using BioNumerics software, version 5.01 (Applied Maths, Austin, TX).

**SNP-based Tree Generation.**

*B. pertussis* clinical and reference strains as well as *B. bronchiseptica* strain RB50 genomic sequences were randomly shredded into 54 base pair-long reads and mapped onto the reference genome (Tohama I), using Ssaha v2.2.1\(^{161}\). High-quality candidate SNPs were identified using ssaha_pileup, and 1,496 SNP sites were identified in at least one strain based on Tohama I. Phylogenetic trees were constructed with RAxML v7.0.4 for all SNP sites in the reference genome, using a General Time Reversible model with a gamma correction for among site rate variation and ten random starting trees\(^{162,163}\).

**Allele typing.**

Bacterial genomes were assembled as previously reported\(^{164}\). Using Basic Local Alignment Search Tool (BLAST), each virulence factor gene and the *ptxP* promoter were queried against each *B. pertussis* genome. Returned hits were manually curated for gene allele typing in Artemis genome browser\(^{165}\). To identify alleles, *B. pertussis* strain
Tohama I’s genome (RefSeq ID: NC_002929.2) was used as the reference for pairwise comparison.

**Immunoblots.**

Bacterial lysates were made as previously described, and $10^8$ CFU (10 µl) were run on 7% sodium dodecyl sulfate-polyacrylamide electrophoresis gels as previously described. Membranes were probed overnight at 4°C with pooled serum from mice vaccinated with PRN or FHA at 1:500 or 1:1,000 dilutions respectively.

**Statistics.**

The mean ± the standard error was determined for each treatment group in the individual experiments. Statistical significance was calculated by using a paired Student’s $t$ test, with a significance level set at $P$ values of <0.05

### 2.4 Results.

Currently circulating isolates have diverse, known PFGE types.

Major outbreaks in the U.S., including the 2010 California outbreak, resulted in several infant deaths. To study this and other outbreaks and cases throughout the United States, we obtained 32 isolates from 8 pediatric hospitals (Figure 2.1A). Twenty-two PFGE types were identified, indicating that many different strains are currently circulating (Figure 2.1B). Multiple PFGE types were isolated from each of the California hospitals, indicating that in none of these local areas was there an outbreak associated with a single strain. While there were some cases of reoccurring PFGE types, such as CDC253 and CDC082, most isolates were of different types, indicating substantial
variety. These observations are not consistent with the concept of an outbreak being caused by a single strain that enters a susceptible population, or that exhibits increased virulence or antigenic variation. In contrast, the large number of PFGE types identified is more consistent with a diverse set of strains that are endemic in this population.

Among a diverse set of strains present within a population, it is possible that there can be substantial variation in virulence characteristics. Emergence of a particularly virulent strain could explain increased observation of disease. We therefore sought to identify and type isolates from the most severe cases, patients admitted to the pediatric intensive care unit (PICU). Isolates from PICUs across the country were determined to be of different PFGE types, indicating that these severe and often-deadly cases are not caused by a single virulent strain, but rather by a seemingly random assortment of strains. These data indicate that it is not a single virulent strain, or a small set of high-pathology strains, that cause the most deadly form of the disease.

PFGE typing is limited in that it only reveals changes that substantially affect the location of restriction sites. To create a more robust and detailed tree relating these strains, we used genome sequence data to generate a phylogenetic tree based on every single nucleotide polymorphism (SNP) site that distinguishes them (Figure 2.1C). This tree shows that all of these isolates were derived from the Tohama I lineage, with ~3,500 SNPs separating them from the more distantly related *B. pertussis* strain 18323 (Figure 2.1C). Isolates from 2010 and 2012 and from different states were widely distributed across the tree, suggesting that a single strain did not evolve and emerge to dominate a region. Additionally, strains STO1-CHOC-0008 and STO1-CNMC-0004, isolated from severe and fatal pertussis cases, are on separate branches suggesting that no single lineage
of isolates caused the most severe disease. Importantly, several strains of *B. pertussis* were circulating throughout the U.S., and most can be distinguished from all others by at least 50 SNPs.

*B. pertussis* outbreak isolates have similar phenotypes.

The number of whooping cough cases has been rising significantly since the early 1990s. One hypothesis to explain this rise is that the bacteria have evolved to become more resistant due to overuse and/or incorrect use of antibiotics. To determine whether any of our isolates are more resistant to antibiotics, we investigated whether SNP changes were observed in the 23S rRNA gene that might confer resistance to erythromycin. No changes in the 23S rRNA gene were observed, and no strains appeared to be resistant to erythromycin or azithromycin with all strains having a MIC of ≤ 1µg/mL (Table 2.1). These results indicate that although there may be genetic differences, these differences do not correlate with changes in antibiotic resistance, suggesting that the speculated overuse of antibiotics has not contributed to the reemergence of *B. pertussis*.

An *in vitro* growth assay showed that none of the collected isolates grew at a faster rate than *B. pertussis* laboratory strain 536 (Tohama I derivative) (data not shown). To determine whether these results correlate with *in vivo* persistence in murine lungs, we inoculated wild type mice with 5x10^5 CFU of one of five geographically-diverse isolates. We observed no significant differences in bacterial burden on days 3 or 7 compared to *B. pertussis* laboratory strain 536 (Figure 2.2). These data indicate that there are no changes in growth patterns, suggesting that genetic changes have not allowed for greater growth.
Genetic variation in vaccine antigens.

Because bacteria rapidly evolve in response to environmental pressures, it is possible that vaccine pressures could have driven the evolution of *B. pertussis* to change the small number of antigens present in the current vaccines. Therefore, we investigated variation among the genes encoding the 5 vaccine antigens *ptxA*, *fim2*, *fim3*, *prn*, and *fhaB* as well as the *ptx* promoter. With the exception of strain 2250905 that contains the *ptxP1* allele, all of our isolates have the *ptxP3* allele, which has been previously been observed ([Table 2.S1](#)). In order to evaluate the homogeneity of PTX, we analyzed *ptxA* and found that all recent strains contain the *ptxA1* allele. Likewise, *fim2* and *fim3* genes are highly conserved. All strains contained the *fim2-1* allele with the exception of strain H897, which has this allele truncated by the insertion of *cyoA*, resulting in 144 base pairs instead of the 630 base pair full length gene ([Table 2.S1](#)). Two very similar alleles, *fim3-1* and *fim3-2*, which differ by 1 SNP, were also observed in 29 isolates.

In contrast to the high level of conservation in *ptxA*, *fim2*, and *fim3*, there is substantial variation in *prn* and *fhaB*. In our collection, 13 out of 29 isolates did not produce PRN (PRN⁻) that could be recognized by anti-PRN antibodies ([Figure 2.3A, B](#)). Consistent with prior observations, we observed substantial variation in the *prn* gene among our strains ([Figure 2.3A, Figure 2.S1](#)). One strain, H918, contains the *prn9* allele. The remaining isolates contain most or all of the *prn2* allele and have been categorized into groups based on gene structure ([Figure 2.S1](#)). Fifteen isolates in group 1 have an intact signal peptide, outer membrane protein, and translocator and appear to be wild type. However, 14 strains contained mutations or insertions resulting in 5 different forms of the gene and major structural changes ([Figure 2.3A, Figure 2.S1](#)). One isolate in group 2 has a signal peptide
deletion while another isolate in group 3 has a portion of the translocator deleted. An isolate in group 4 has an amber codon change resulting in a stop codon in the outer membrane portion. Isolates from groups 5 and 6 (8 and 3 isolates respectively) contain an IS481 element disrupting the gene within the outer membrane protein domain. These results suggest that there is a high level of variation in prn of almost half of our isolates that distinguishes it from the highly conserved genes ptxA, fim2, and fim3. Importantly, most of these changes are not simply accumulations of SNPs that typically distinguish alleles.

Although several reports have focused on the appearance of PRN- B. pertussis strains, not much is known about fhaB variants. High variation in fhaB alleles was observed, resulting in the discovery of 9 new fhaB alleles (Figure 2.3C). The fhaB3 allele was present in the majority of isolates, whereas other allele types are only represented by a single isolate with the exception of fhaB9 and strains with truncations, which could not be determined (Figure 2.3C, Table 2.S2). Regardless of allele type, all strains expressed FHA by western blot, suggesting that these SNPs did not disrupt the expression and maturation of this complex exported protein (Figure 2.3B). These data, taken together, suggest that some of the vaccine antigen-encoding genes (fim2, fim3, and ptxA) are highly conserved, while others (prn and fhaB) are much more variable.

Acellular vaccination is more protective against the laboratory strain than recent isolates.

Because there is substantial variation in prn and fhaB in spite of high conservation in ptxA, fim2, and fim3, we sought to assess the effects of these changes on the efficacy of the current aP vaccine. Mice were sham- or aP-vaccinated and challenged with 4
geographically-diverse isolates. aP vaccination decreased the bacterial numbers of strain 536 (streptomycin-resistant derivative of Tohama I) recovered from the lungs three days later by greater than 90%, relative to sham-vaccinated mice (Figure 2.4). The vaccine was similarly effective against 3 currently circulating strains (STO1-CHOC-0008, STO1-CNMC-0004, and STO1-CHLA-0011), and slightly less protective against STO1-SEAT-0004. These data suggest that the aP vaccine is effective but not as protective against all recent isolates as it is against the laboratory strain 536, which will be important for continuing to improve the vaccine.

2.5 Discussion.

*Bordetella pertussis* is an imminent threat for children younger than six months, for whom the risk of hospitalization is 60% higher than other age groups and accounts for more than 90% of pertussis-related deaths\(^\text{170}\). Because the number of whooping cough cases has escalated in the last decade, we sought to evaluate circulating strains for genetic or phenotypic evidence of evolution, for example in response to antibiotics or vaccination. On a side note, our sampling of circulating isolates was bias towards area where outbreaks were observed, specifically the 2010 outbreak in California and the 2012 outbreak in Washington. In our collection, isolates from individual hospitals were genetically distinct suggesting that local outbreaks are not clonal expansions, and demonstrating that many distinct lineages were circulating in the U.S. from 2010-2012 (Figure 2.1).
Although there have previously been reported cases of erythromycin resistance, recent outbreaks were not caused by *B. pertussis* isolates with high antibiotic resistance (Table 2.1). This suggests that, unlike *Staphylococcus aureus* and other pathogens, recent antibiotic use has not selected for *B. pertussis* strains that are more resistant. Likewise, we observed no evidence of greater virulence or accelerated growth in the murine respiratory tract (Figure 2.2)\(^1\). This crude method of determining ability to colonize the murine lungs would alert us to any gross changes in colonization ability; however, it is possible that, due to the limited ability of the mouse model, there are subtle changes that we cannot detect.

While we observed high conservation among genes encoding fimbriae and PTX, the *prn* sequences showed substantial variation with insertions, deletions, and amber codon changes, and these mutations are associated with loss of expression of PRN (Figure 2.3). In contrast, several mutations noted in *fhaB* were all SNPs and none prevented expression of FHA. In order to evaluate whether these changes were due to vaccine-driven evolution, a comparison of non-synonymous to synonymous SNPs between vaccine-associated antigens and other virulence factors would need to be evaluated. While there is a sampling bias toward the 2010 California and 2012 Washington outbreaks, the genotypic differences we observe are similar to results of other groups, both in the U.S. and abroad in other countries\(^{155,169,172}\). The high rate of PRN\(^{-}\) strains could be explained by increased fitness in a vaccinated host, but the current aP vaccine conferred substantial protection against PRN\(^{-}\) strains as compared to PRN\(^{+}\) strains (90% versus 99% bacterial reduction) (Figure 2.4). One caveat to this experiment is that the laboratory adapted strain *B. pertussis* strain 536 was used to determine changes
in vaccine efficacy. However, even though the current aP vaccine is able to reduce colonization by recent isolates, it is detectably less effective against these strains than against the laboratory isolate.

In conclusion, we provided evidence that multiple *B. pertussis* strains were circulating simultaneously from 2010-2012. These data are consistent with the genetic changes observed globally. We observed circulation of diverse *B. pertussis* strains, as revealed by PFGE and genome-wide SNP analysis. In contrast to expectations, most vaccine antigen encoding genes do not appear to have changed since the introduction of vaccines. However, *fhaB* and *prn* have disproportionate numbers of SNPs and other changes, respectively. These changes may have provided escape from the effects of the aP vaccine, which reduced bacterial numbers by 90%. To determine this, mutations in *prn* could be made to evaluate whether the absence of a functional pertactin would result in reduced colonization following vaccination.

This work provides vital information on current outbreak strains and informs the development of hypotheses about *B. pertussis* resurgence. Furthermore, by using current outbreak strains, these data indicate that the current aP vaccine is still effective but also provides information important for the development of future vaccination strategies.
A geographically diverse set of strains is genotypically distinct.

(A) A United States map indicates the sample size at each isolation location. (B) PFGE analysis was performed on isolates to determine their PFGE type. Black bars indicate strains that were isolated from PICUs. Hatch-marked bars indicate strains that were isolated from the Children’s Hospital of Los Angeles during the 2010 outbreak. White bars indicate strains isolated during 2012 from the CDC. Asterisks (*) indicate new PFGE types that have not been previously identified. (C) Genome wide SNP analysis is presented. Blocks next to the isolate represent the location and year of isolation (Michigan, - New York, - Washington D.C., - California, - Washington, - 2010, - 2011, - 2012).
Figure 2.2 Murine lung bacterial burden is similar among newly circulating isolates.

Bacterial numbers in the lung were determined by serial plating 3 days after inoculation with $5 \times 10^5$ CFU in 50µL PBS. The mean ± the standard error was determined for each treatment group in the individual experiments. Statistical significance was calculated by using a paired Student’s t test, with a significance level set at $P$ values of $<0.05$ and was not achieved in this experiment.
Figure 2.3 *prn* and *fhaB* have SNP differences in new isolates.

(A.) proportions of *prn* mutations are shown. (B.) Table describing the *prn* and *fhaB* alleles, mutation and antibody recognition status of each individual isolate. (C.) proportions of *fhaB* SNPs and mutations. *ND* denotes alleles we could not determine due to truncation or frameshifts.
Figure 2.4 aP vaccination is not as effective at reducing bacterial colonization of circulating isolates.

(A) Bacterial counts from sham (white bar) and aP (black bar) vaccinated mice. The mean ± the standard error was determined for each treatment group in the individual experiments. ** denotes p≤0.002 between sham and vaccinated groups. (B) Percent reduction in murine lung colonization following acellular vaccination. * denotes p≤0.05 as compared to reference B. pertussis strain 536 group based on a Student’s T test.
Figure 2.S1 prn mutations found in circulating isolates.

A schematic describes the different mutations observed in prn, categorized by group. The signal peptide (SP) in red, the outer membrane portion in blue, and the translocator region in yellow. Δ denotes deletion, Φ denotes an amber codon change resulting in a premature stop codon, and rectangles denote insertion of an IS481 element in three separate locations.
Table 2.1 Minimum Inhibitory Concentration 50 (MIC50) values for erythromycin and azithromycin inhibition of strain growth.

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* Bacterial isolates were incubated with varying concentrations of antibiotics in a 96 well plate. Plates were incubated at 37°C while shaking at 210 rpm and OD_{600} values were measured at 0, 24, 48, and 72 hours. MIC_{50} value was determined based upon the OD_{600} value recorded at the 48-hour time point for each strain, and was calculated as the antibiotic concentration at which the growth of the strain was decreased to at least half that of the strain’s growth in PBS-only.

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2.6 Author Contributions.

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Conceived and designed experiments: LLG, JAM, ATK

Performed experiments: LLG, JAM, YVI, SJM, JP, WES, PKC, MRM, LB

Analyzed data: LLG, JAM, YVI, SJM, JP, WES, PKC, MRM, ETH

Wrote the paper: LLG, JAM, YVI, JP, ETH
2.7 References

1. CDC - Pertussis: Surveillance – Cases by Year. at <http://www.cdc.gov/pertussis/surv-reporting/cases-by-year.html>


Chapter 3

Identification of an avirulent and divergent human isolate of *Bordetella bronchiseptica*. 
3.1 Abstract.

Background: *Bordetella bronchiseptica* is a respiratory pathogen that has a wide host-range, which typically consists of non-human mammals. Occasionally, *B. bronchiseptica* is isolated from immunocompromised or immunosuppressed humans.

Methods: *B. bronchiseptica* strain 99R-0433 was grown on a blood agar plate to evaluate hemolytic abilities. This strain was evaluated for O-antigen based on immunoblot using convalescent serum to determine whether it expresses an immunogenic O-antigen. Using the mouse model of infection, we determined the ability of *B. bronchiseptica* strain 99R-0433 to colonize wild type and immunodeficient mice. To determine strain relatedness and virulence content, we generated a SNP-based tree and a virulence table showing the presence or absence of virulence factor genes.

Results: *B. bronchiseptica* strain 99R-0433 is beta-hemolytic when grown on a plate and is negative for O-antigen serotypes O1 and O2. *B. bronchiseptica* strain 99R-0433 cannot colonize the murine respiratory tract of wild type or immunodeficient mice, and is divergent from other *Bordetella* isolates. *B. bronchiseptica* strain 99R-0433 lacks several important virulence factors including pertactin, tracheal colonization factor, dermonecrotic toxin, adenylate cyclase toxin, Type VI secretion system, and O-antigen.

Conclusions: *B. bronchiseptica* strain 99R-0433 has undergone significant gene loss and SNP acquisition compared to other *B. bronchiseptica* isolates and appears avirulent in the murine model of infection, despite being isolated from a human case. Further investigations will inform us on the pathogenesis of *Bordetella* species.
3.2 Introduction.

Bacteria are constantly evolving to adapt to the ever changing environmental pressures they encounter throughout their lifecycles. When the energy required to maintain a gene outweighs the use of that gene product, those genes will acquire single nucleotide polymorphisms (SNPs) until the gene degrades and no longer leads to a functional protein. Alternatively, gene degradation can occur when a gene or its product no longer confers a selective advantage to that bacteria and subsequently can result in clearance from the host. Gene loss is a common form of evolution, and the *Bordetella* genus contains many examples. *B. pertussis* is said to have evolved to be more host-restrictive from a *B. bronchiseptica*-like isolate through gene loss\(^1,2\). Human isolates of *B. bronchiseptica* have been observed predominantly in multi-locus sequence type complex IV, a complex more evolutionarily related to *B. pertussis*.

*Bordetella bronchiseptica* are Gram negative coccobacili respiratory pathogens. *B. bronchiseptica* has a wide host-range, and infection in animals results in kennel cough in dogs, snuffles in rabbits, atrophic rhinitis is swine, and bordetellosis in other animals\(^3\). *B. bronchiseptica* species use an arsenal of virulence factors including toxin systems, adhesins, secretion systems and lipopolysaccharide (LPS) to colonize, evade host immune responses, mediate damage, and are seen as efficient colonizer of the respiratory tract of mammals\(^3\).

In this study, we characterized the growth and O-antigen serotype of *B. bronchiseptica* human isolate 99R-0433. We further evaluated the ability of this strain to colonize the murine respiratory tract. Because of its inability to colonize both wild type
and immunodeficient mice, the genome was sequenced and virulence factor genes were investigated. We observe that *B. bronchiseptica* strain 99R-0433 has accumulated 142,027 SNPs compared to its most closely related *B. bronchiseptica* strain 253. Further evaluation reveals that *B. bronchiseptica* strain 99R-0433 lacks the genes encoding several virulence factors including pertactin, tracheal colonization factor, and dermonecrotic toxin, as well as adenylate cyclase toxin (ACT), the Type VI secretion system (T6SS), and the O-antigen. By studying strains that have lost the ability to colonize, we can learn about the evolution of *B. bronchiseptica*.

### 3.3 Materials and Methods.

**Bacterial Strains.**

*B. bronchiseptica* strain 99R-0433, also known as D754, is a human sputum isolate identified by multi-locus sequence type as 60⁴. Bacteria were maintained on Bordet-Gengou agar (Difco) containing 10% sheep blood (Hema Resources) and 20µg/mL streptomycin (Sigma). Liquid cultures of bacteria were grown in Stainer-Schölte broth containing heptakis shaking at 37°C to mid-log phase⁵,⁶.

**Immunoblots.**

Bacterial lysates were made as previously described, and 10⁸ CFU (10µl) were run on 7% sodium dodecyl sulfate-polyacrylamide electrophoresis gels as previously described⁷. Membranes were probed overnight at 4°C with pooled convalescent serum of mice infected with 5x10⁴ CFU/50µL of either RB50 (representing O1-type), 1289 (representing O2-type), or MO149 (representing O3-type) at 1:1,000 dilution.
Animal Experiments.

C57BL/6, IFNγ−/−, and TNFα−/− mice were obtained from Jackson Laboratories. C3−/− mice were a kind gift from Rick Wetsel and have previously been described. All animal experiments were performed in accordance with institutional animal care and use committee (IACUC protocol # 40029) guidelines. Mice were anesthetized with 5% isofluorane in oxygen and intranasally inoculated with 5x10^5 CFU in 50µL. This method reliably distributes bacteria throughout the respiratory tract. Mice were euthanized by 5% CO₂ inhalation according to IACUC guidelines. The respiratory tract was excised on the appropriate time point, bacterial counts were enumerated by serial plating.

SNP-based Tree Generation.

Publically available B. bronchiseptica, B. pertussis, and B. parapertussis strain genomic sequences were randomly shredded into 54 base pair-long reads and mapped onto the reference genome (RB50), using Ssaha v2.2.1. High-quality candidate SNPs were identified using ssaha_pileup. Phylogenetic trees were constructed with RAxML v7.0.4 for all SNP sites in the reference genome, using a General Time Reversible model with a gamma correction for among site rate variation and ten random starting trees.

Virulence factor comparison.

Genes and the loci that encode the known virulence factors pertactin (PRN), tracheal colonization factor (TcfA), invasive adenylate cyclase/hemolysin (ACT), dermonecrotic toxin (Dnt), O-antigen, and Type III secretion system (T3SS), were compared between B. bronchiseptica strains RB50 (Accession: NC_002927) and 99R-0433 (GenBankID: JGWN00000000.1) via the Artemis Comparison Tool. Presence or absence of each gene or system was identified.
Statistics.

The mean ± the standard error was determined for each treatment group in the individual experiments. Statistical significance was calculated by using a paired Student's t test, with a significance level set at $P$ values of <0.05

3.4 Results.

*B. bronchiseptica strain 99R-0433 causes hemolysis on a blood agar plate.*

Previous studies have identified avirulent lineage of strains of *B. bronchiseptica*, which have the adenylate cyclase toxin (ACT) locus replaced by a Ptl locus, resulting in the inability of this lineage to be hemolytic\(^{13}\). To determine whether *B. bronchiseptica* strain 99R-0433 was hemolytic, bacteria were grown on agar containing blood. We observed that *B. bronchiseptica* strain 99R-0433 is beta-hemolytic on a blood agar plate like our prototypical *B. bronchiseptica* strain RB50 (Figure 3.1).

*B. bronchiseptica strain 99R-0433 is not seropositive for O1- or O2-type O-antigen.*

The O-antigen has been identified as an important virulence factor that prevents bacterial clearance by complement deposition or antibody recognition\(^{14–17}\). Using immunoblot analysis, we determined that *B. bronchiseptica* strain does not produce an O-antigen recognized by O1- or O2 type specific antibodies (Figure 3.2A, B). Interestingly, *B. bronchiseptica* strain 99R-0433 produces band A and band B (between 10 and 17kDa) as part of its LPS as identified by MO149-specific antibodies (Figure 3.2C).
B. bronchiseptica strain 99R-0433 cannot colonize the murine respiratory tract.

B. bronchiseptica has been shown to efficiently colonize the respiratory tract of mammals, including mice. To determine virulence of this strain, we inoculated wild type mice with $5 \times 10^5$ CFU and enumerated bacterial counts in the lungs and nasal cavity. Despite having indistinguishable bacterial counts 30 minutes post-inoculation on day 0, B. bronchiseptica strain 99R-0433 is rapidly cleared from the respiratory tract as compared to B. bronchiseptica strain RB50, which colonizes the entire respiratory tract at high numbers (Figure 3.3). No detectable levels of B. bronchiseptica strain 99R-0433 were observed by 3 days post-infection in either the lungs or the nasal cavity.

The immune system has developed a series of defenses against invading pathogens. For example, complement component 3 (C3) deficient mice are colonized to the same extent with the O-antigen deficient B. parapertussis as with wild type B. parapertussis\(^\text{17}\). Other immune components, such as tumor necrosis factor α (TNFα) are required for control of bacterial growth using this high dose\(^\text{18}\). To evaluate whether the murine immune response was quickly eliminating B. bronchiseptica strain 99R-0433 and clearance is likely attributed to innate immune mechanisms of bacterial clearance, we inoculated mice deficient in C3, which renders the complement system inactive and should recover any deficiency observed by O-antigen negative B. bronchiseptica isolates. Additionally, we challenged mice deficient in pro-inflammatory cytokines TNFα or IFNγ as each of these have been shown to be important for host control of bacterial growth in the respiratory tract\(^\text{18,19}\). On day 7, the mice were euthanized, and bacterial counts in the lungs were enumerated. We observed no detectable bacteria in the lungs of C3\(^{-/-}\).
TNFα⁺, or IFNγ⁺. These data suggest that even in a weakened host, *B. bronchiseptica* strain 99R-0433 is not capable of colonizing the murine respiratory tract. These data suggest that *B. bronchiseptica* strain 99R-0433 may not have the necessary virulence factors required for establishing colonization, whether those tools are used to compete with microbiota or evade the immune response.

*B. bronchiseptica* strain 99R-0433 is divergent from other classical bordetellae and lacks common virulence factors.

Since a weakened immune response is capable of clearing *B. bronchiseptica* strain 99R-0433, we investigated how similar this isolate is based on genome wide SNPs compared to avirulent *B. bronchiseptica* strain 253 and hypervirulent *B. bronchiseptica* strain 1289. Based on SNP analysis, we see that *B. bronchiseptica* strain 99R-0433 is highly divergent from other bordetellae, including avirulent *B. bronchiseptica* strain 253 (Figure 3.5A). There are 142,027 SNPs separating *B. bronchiseptica* strains 99R-0433 and avirulent 253 and 250,161 SNPs from the most closely related human *B. bronchiseptica* isolate, MO149 (Figure 3.5B). These data suggest that *B. bronchiseptica* strain 99R-0433 has undergone major changes and may not contain many of the virulence factors the bordetellae have in their arsenal to compete with a host immune system.

Since *B. bronchiseptica* strain 99R-0433 has so many SNPs differentiating it from other *Bordetella* isolates, we investigated the presence of outer membrane and adhesin proteins pertactin (prn), tracheal colonization factor (tcfA), dermonecrotic tocin (dnt) and virulence systems such as adenylate cyclase toxin (ACT), the outer-most component of the LPS – O-antigen, and the type VI secretion system (T6SS) locus. While the sequencing results will need to be confirmed by PCR, we observe that *B. bronchiseptica*
strain 99R-0433 lacks all of these components (Table 3.1). Interestingly, although *B. bronchiseptica* strain 99R-0433 lacks the locus for ACT, it still appears beta-hemolytic on a blood agar plate (Figure 3.1), and may have developed other mechanisms for hemolysis independent of ACT.

### 3.5 Discussion.

*B. bronchiseptica* commonly infects non-human mammals but has also shown to have the propensity to infect humans that have become immunocompromised. One such human isolate is *B. bronchiseptica* isolate 99R-0433. Full genome sequencing of this isolate and subsequent SNP-based tree analysis revealed this isolate has acquired over $10^5$ SNPs compared to its next closest relative (Figure 3.5). Accompanying this high number of SNPs is the observation that this strain has also suffered degenerate loss of some of the genes responsible for a number of different virulence factors important for *B. bronchiseptica* infection, colonization, survival and induction of a disease state (Table 3.1). One of these genes is the immunogenic O-antigen, which is absent based on the no recognition of O-antigen serotype via immunoblotting for O1- or O2-type O-antigen (Figure 3.2) and absence of the *wbm* locus (Table 3.1). Because the O-antigen protects *B. bronchiseptica* strain RB50 from complement deposition and killing, we determined whether complement deficient (C3−) mice could rescue the deficiency of *B. bronchiseptica* strain 99R-0433 to colonize the respiratory tract, and found that the absence of complement did not restore the colonization phenotype (Figure 3.4).
Because the phenotype is not rescued, we can conclude that the complement alone is not responsible for clearance of *B. bronchiseptica* strain 99R-0433.

The high number of SNPs and gene acquisitions could also be due to a number of different causes. The most common manner in which this much differentiation occurs genetically between isolates of the same species is due to a hypermutable phenotype. This generally occurs when there have been mutations that occur in the genes that are responsible for DNA maintenance and repair\(^{20}\). Looking at the genome assembly and assessing loss or mutations in DNA maintenance genes is included in future work on *B. bronchiseptica* strain 99R-0433. Another explanation for the observation of a high number of SNPs is that selective pressure and host adaptation have shaped the *B. bronchiseptica* strain 99R-0433 genome. *B. bronchiseptica* is the only species in the *Bordetella* genus that has a broad host range, and it likely has mechanisms in place to maintain a selective advantage in all its hosts. The significant acquisition of SNPs and gene loss may be the direct effect of host specificity attempted by *B. bronchiseptica* strain 99R-0433.

While the genome reveals that *B. bronchiseptica* strain 99R-0433 lacks many virulence factors identified as important in *Bordetella* virulence, *B. bronchiseptica* strain 99R-0433 was still able to survive in the human host despite its inability to survive in the mouse model. While the mouse is a natural host of *B. bronchiseptica*, *B. bronchiseptica* strain 99R-0433 may have found a niche in the environment of a human respiratory tract for a long period of time. This may have allowed *B. bronchiseptica* strain 99R-0433 to adapt through genetic change to maintain colonization within its host.
Despite lacking many of its known virulence factors including ACT, *B. bronchiseptica* strain 99R-0433 retained the ability to cause hemolysis, suggesting it has either acquired a new system to induce hemolysis or may have retained an unidentified gene with redundant function to ACT. Hemolytic activity has been observed previously via autotransporters such as that encoded by *Bartonella henselae’s cfa*\(^{21}\). Further analysis of autotransporters could reveal homology to this autotransporter responsible for hemolysis\(^{21}\). The ability to be hemolytic is critical for pathogenic bacteria despite host specificity as it allows the bacteria acquire essential nutrients such as iron. Many bacteria have evolved systems to acquire iron from their surrounding by activities such as the secretion of siderophores or heme transporters like the *chu* system\(^{22}\). Genome analysis may reveal the presence of one or more genes homologous to the *chu* system, and that may explain how *B. bronchiseptica* strain 99R-0433 could survive in the human as long as it was able to sequester iron. However, it is also possible that *B. bronchiseptica* strain 99R-0433 was accidentally isolated during sputum collection of a patient with a respiratory condition such as cystic fibrosis. Interestingly, several other *Bordetella* species such as *B. avium* and *B. hinzii*, which typically infect birds, have been isolated from the respiratory tract of cystic fibrosis patients\(^{23}\).

The inability to colonize the mouse requires further investigation, especially in the absence of immune signaling since missing these components usually allows *Bordetella* species to colonize to a much higher degree\(^{18,19,24}\). The data collected here suggests that a factor important for initial adherence may have been lost through SNP or through gene loss. Another explanation as to why *B. bronchiseptica* strain 99R-0433 is rapidly cleared is that it is not able to evade the immune response, and any of the many immune
signaling pathways is capable of identifying and preventing *B. bronchiseptica* strain 99R-0433 from colonization.

Most *B. bronchiseptica* isolates use these virulence factors to evade the host immune system and, in fact, require them to be successful in the host. Investigating strains that have lost important virulence factors, much like *B. bronchiseptica* strain 99R-0433, will inform our understanding of *Bordetella* evolution and pathogenesis.
Figure 3.1 *B. bronchiseptica* strain 99R-0433 is beta-hemolytic on Bordet-Gengou Agar containing blood.

(A) RB50, (B) 99R-0433 on Bordet-Gengou plates containing 10% sheep’s blood and incubated for 3 days.
Figure 3.2 *B. bronchiseptica* strain 99R-0433 is not seropositive for O1- or O2-type O-antigen.

7% SDS-PAGE gels were run at 150Volts and transferred to PVDF membranes at 100 Volts. Following blocking with 5% milk in TBS-T, membranes were probed with convalescent antiserum against (A) RB50 (O1), (B) 1289 (O2), or (C) MO149 (O3). In (B) 99R-0433 was run on the same gel but had several wells separating it from MO149, and this space was cropped. The red bracket indicates the location of O-antigen.
Figure 3.3 *B. bronchiseptica* strain 99R-0433 does not colonize the respiratory tract of wild type mice.

Mice were inoculated with 5x10⁵ CFU of *B. bronchiseptica* strains 99R-0433 or RB50 in 50µL and bacterial counts were enumerated at days 0, 1, 3, and 7 post-inoculation. The mean ± the standard error was determined for each treatment group in the individual experiments. Except for day 0, all time points are statistically significantly different with p<0.05 based on a Student’s T-test. The lower limit of detection is 10 bacteria as indicated by the dashed black line at log 1.
Figure 3.4 *B. bronchiseptica* does not efficiently colonize immunodeficient mice.

Immunodeficient mice were inoculated with $5 \times 10^5$ CFU *B. bronchiseptica* strain RB50 or 99R-0433 in 50µL, and CFU in the murine lungs were enumerated on day 7 post-inoculation. The mean ± the standard error was determined for each treatment group in the individual experiments. * indicates p<0.05 based on a Student’s T-test. The lower limit of detection is 10 bacteria as indicated by the dashed black line at log 1.
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Figure 3.5 SNP analysis of *B. bronchiseptica* strain 99R-0433 compared to previously sequenced bordetellae.

Bb indicates *B. bronchiseptica* strain, Bpp indicates *B. parapertussis* strain, and Bp indicates *B. pertussis* strains.
Table 3.1 Virulence factor genes absent in *B. bronchiseptica* strain 99R-0433.

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3.6 Author Contributions.

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Conceived and designed experiments: LLG, SEH

Performed experiments: LLG, JP, MP

Analyzed data: LLG, JP, SEH, ETH

Wrote the paper: LLG, ETH
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Chapter 4

Examining the local response in the mucosa: How microfold cells act as first responders during a *Bordetella* infection.
4.1 Abstract.

**Background:** M cells are a specialized and rare population of epithelial cells, which have been identified as antigen sampling sites in the mucosa. Often studied in the gut, these cells have also been observed to sample antigens in the respiratory tract.

**Methods:** Using the mouse model of respiratory infection, we investigated the ability of *B. bronchiseptica* to colocalize 30 minutes following inoculation. Using an established co-culture system, we assayed for bacterial association with and translocation by Caco-2 cells that have been induced to become M cells (C2-M cells) as well as the C2-M cell transcriptional response.

**Results:** The data provide evidence that *B. bronchiseptica* is capable of colocalizing with M cells *in vivo*. *In vitro*, *Bordetella* species associate with and are efficiently translocated by C2-M cells in a Bvg-dependent manner. Following infection with *Bordetella* species, induction of tumor necrosis factor α and interleukin-6 are observed.

**Conclusions:** Our data provide the new evidence that *Bordetella* species could interact with nasal M cells *in vivo*. In addition, *Bordetella* species associate with and are translocated by C2-M cells in a Bvg-dependent manner *in vitro* and can induce M cell cytokine responses, which may lead to activation and/or recruitment of immune cells.
4.2 Introduction.

During a respiratory tract infection, the first interaction between the pathogen and host occurs in the mucosa. Mucosal regions are found in several different parts of the body including the gastrointestinal tract, urogenital tract, and the respiratory tract. A recent study reveals that the respiratory tract mucosa, much like that in the gut, contains specialized antigen sampling cells, called microfold (M) cells, that are important for the generation of an immune response.

M cells are responsible for picking up antigens passing through the lumen, a process termed antigen sampling. While the precise mechanisms of action used by M cells to initiate an immune response are not completely understood, studies have shown that M cells make up approximately 8-10% of the gut epithelium during a steady state and are morphologically and functionally distinct compared to the surrounding epithelial cells. Like epithelial cells, M cells express tight junction proteins that aid in maintaining the epithelial barrier. M cells express different carbohydrate structures on their apical surface compared to epithelial cells based on α-1-fucose residue expression. Additionally, M cells have a much thinner glycocalyx layer, making them substantially more likely to encounter antigens in the lumen. Furthermore, studies have shown that murine M cells are able to bind IgA-opsonized commensals but not serum IgM nor IgG. This ability to bind only sIgA suggests direct interactions with M cells, which express an IgA receptor on their apical surface.
M cells are known to closely interact with immune cells using pockets, which appear as sites where the depth of the M cell is substantially shorter than surrounding epithelial cells\(^2,5,10,11\). This pocket allows M cells to increase their interactions with “pocket lymphocytes”, which leads to immune cell recruitment and activation and is predicted to be crucial for the generation of an appropriate immune response\(^4-6,11,12\). By studying this relatively rare cell population, we can improve our understanding of the primary cell interactions that shape an appropriate immune response, resulting in the development of more protective mucosal vaccines.

M cells are considered the gateway of antigen sampling because they are constantly binding and taking up both soluble and inert antigens, as well as commensals and pathogens present in the environment. Dendritic cells (DCs) also aid in the process of clearing bacteria by extending their dendrites beyond the epithelial surface in order to catch and phagocytose free antigens before trafficking to a draining lymph node\(^2\). However, DCs only spend approximately two days in the respiratory tract, suggesting that this role may not be their most important\(^2\).

When the host generates an immune response, there is a series of processes that the pathogen and surrounding cells will undergo: recognition of antigen, processing of M cell surface expression molecules, uptake of antigen, signaling to peripheral lymphocytes, vesicular transport of antigen to basolateral surface, and finally recruitment and activation of lymphocytes. If the pathogen evades the mucus, it will likely interact with the epithelium, leading to the release of cytokines such as IL-8 and TNF-\(\alpha\) by these cells which intercellularly communicate with M cells\(^13-15\).
Since M cells are important for antigen sampling and the initiation of an appropriate immune response, we sought to investigate villous M cells in the nasal cavity using a murine infection system in combination with an established M cell-like cell line, in which \textit{B. bronchiseptica} is the invading pathogen. \textit{Bordetella bronchiseptica} has been known to infect a variety of mammals, including rabbits, dogs, cats, leopards, cows, and otters\textsuperscript{16}. Infection with this Gram-negative pathogen causes symptoms ranging from asymptomatic carriage to fatal pneumonia disease which results in substantial agricultural losses every year\textsuperscript{16}. After disease resolves, animals are thought to remain colonized for the remainder of their lives. During infection, \textit{B. bronchiseptica} adheres to the respiratory epithelium, with new data suggesting that a small proportion of bacteria can survive intracellularly\textsuperscript{16,17}. Additionally, \textit{B. bronchiseptica} expresses numerous virulence factors, including adenylate cyclase toxin (ACT), a Type III secretion system, a Type VI secretion system, filamentous hemagglutinin (FHA), pertactin, O-antigen, and fimbriae (FIM)\textsuperscript{16}.

Fimbriae are adhesion factors expressed on the surface of many bacteria that can directly bind to M cell receptors, leading to antigen uptake\textsuperscript{18}. For instance, bacteria expressing FimH, such as \textit{Escherichia coli} and \textit{Salmonella typhimurium}, can bind directly to glycoprotein 2 (GP2), which is a receptor expressed on M cells\textsuperscript{19,20}. The bordetellae are able to bind to monocytes via another component of the FIM, FimD\textsuperscript{21}. FimD displays molecular mimicry of fibronectin, which is important for cell adhesion. When FimD binds very late antigen 5, the result is activation of complement receptor 3 (CR3)\textsuperscript{21}. This is important because CR3 is an integrin specific to monocytes and macrophages and may also be expressed on M cells. In addition, CR3 is a receptor for
FHA, another adhesion factor important to the bordetellae. By expressing the integrin CR3, an M cell will better bind to antigens expressing both FimD and FHA. FHA binds carbohydrates as well as to the leukocyte response integrin LR1, which activates phagocytes, increasing their phagocytic abilities, and enhancing respiratory burst functions\textsuperscript{22}. On the other hand, the binding of bacteria may be based on pattern recognition receptors (PRR) or Toll-like receptors (TLR) expressed on epithelial cells recognizing pathogen associated molecular patterns (PAMP) present in \textit{Bordetella}\textsuperscript{10,23}. Interestingly, another study showed that \textit{Vibrio} binding to and transcytosis by the M cell could be an active process, since dead \textit{Vibrio} cannot be bound and be transcytosed\textsuperscript{24,25}.

M cells are seen as the gateway between the mucosa and the immune system due to their antigen capture and transepithelial transport capabilities. Because of their role in antigen sampling, we investigated the interactions between M cells and \textit{B. bronchiseptica}. While M cells are able to bind and take up both soluble and insoluble materials, research shows that M cells are 50 times more likely to take up adherent macromolecules\textsuperscript{26}. Some studies suggest that pathogens may selectively target M cells to more efficiently invade, while others suggest that the M cells lack of a thick glycocalyx, leaving them more susceptible to pathogen invasion\textsuperscript{11,26}. A previous study by Kim, \textit{et al.} showed that nasal cavity M cells colocalized and interacted with both \textit{Salmonella typhimurium} and Group A \textit{Streptococcus}, resulting in phagocytosis\textsuperscript{1}. \textit{Y. enterocolitica} expresses an outermembrane protein, invasin, which binds an integrin expressed on the surface of M cells called $\beta 1$\textsuperscript{27}. Therefore, we investigated the effects of the interplay of \textit{B. bronchiseptica} and \textit{B. pertussis} with this specialized immune cell type.
This study is the first, to our knowledge, to identify respiratory M cells as capable of interacting with *Bordetella* species. Using an M cell co-culture system, we found that *Bordetella* species were capable of associating with and being translocated by C2-M (M-like cells differentiated from Caco-2 epithelial cells) cells *in vitro* and increased mRNA levels of pro-inflammatory cytokines tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6). These novel findings are the first steps in characterizing the responses of M cells with the bordetellae and delineating important immune responses elicited by early events in infection.

### 4.3 Materials and Methods.

**Bacterial Strains.**

*B. bronchiseptica* strain RB50 is an isolate from a rabbit\(^2^8\). RB54 is a previously described Bvg\(^-\) phase-locked derivative of strain RB50\(^2^8\). *B. pertussis* strain 536 is a streptomycin-resistant derivative of Tohama I\(^2^9\). Bacteria were maintained on Bordet-Gengou agar (Difco) containing 10% sheep blood (Hema Resources). Liquid cultures of bacteria were grown to mid-log phase in Stainer-Schölte broth containing heptakis shaking at 37°C\(^3^0,3^1\).

**Animal Experiments.**

C57BL/6, mice were obtained from Jackson Laboratories. All animal experiments were performed in accordance with institutional animal care and use committee (IACUC protocol # 40029) guidelines. Mice were anesthetized with 5% isoflurane in oxygen and
intranasally inoculated with $5 \times 10^4$ CFU in 50µL, and euthanized 30 minutes post-inoculation by CO$_2$ inhalation according to IACUC guidelines.

*Immunofluorescence Microscopy.*

Murine nasal cavities were extracted using a fresh frozen protocol and 10µm sections were stained with DAPI, anti-M cell (Miltenyi Biotech), anti-RB50 (rabbit serum generated against *B. bronchiseptica* strain RB50, and images overlaid and imaged with confocal microscopy at 40x magnification.

*Cell Culture.*

C2Bbe1 Caco-2 cells are a human colon cell line (ATCC Reference # CRL-2102) and were maintained in DMEM supplemented with 10% FBS, human transferrin (Sigma), non-essential amino-acids, and penicillin/streptomycin (Gemini Bio-products). Raji cells are a human B lymphocyte cell line (ATCC Reference # CCL-86) and were maintained in RPMI supplemented with 10% FBS and non-essential amino acids until they were co-cultured with Caco-2 cells, when the Caco-2 media was used.

*Co-culture system.*

Approximately $2 \times 10^5$ Caco-2 cells were split onto 1µm transwell inserts, and the media was changed every 2 days for 21-30 days. Then, $10^6$ Raji cells per well were co-cultured with Caco-2 cells to encourage the enterocytes to differentiate into C2-M cells (Figure 4.S1). C2-M cells were stimulated with bacteria at an MOI of 1 for 30 minutes or 2 hours. For translocation experiments, the BIOCOAT HTS Caco-2 Assay System (Corning) was used to terminally differentiate the Caco-2 cells for 3 days. Caco-2 and $5 \times 10^5$-$10^6$ Raji cells were co-cultured for 3-4 days to fully differentiate the Caco-2 cells.
into C2-M cells. Caco-2 cells (C2) that were not incubated with Raji cells were used as controls.

**Adherence and Translocation Assays.**

Following stimulation with $2 \times 10^5$ bacteria for 30 minutes, flow-through was collected from the wells, and the membranes were washed 4x with PBS and trypsinized for 5 minutes at 37°C. Serial plating was used to calculate numbers of both bacteria that adhered to C2-M cells and bacteria that were translocated to the basolateral side.

**RNA Extraction and RT-qPCR Analysis.**

C2 and C2-M cell lysates and media were harvested from the Transwell filters in 1mL Trizol (Life Technologies) for RNA extraction. Trizol extraction was performed according to previously established methods. The extracted nucleic acid was treated with RNase free DNase I (Ambion) for 20 minutes at 37°C to remove DNA contamination, and then the concentration of RNA was measured by Nanodrop (Thermo Scientific).

1μg of RNA from each sample was used to synthesize cDNA using the Superscript III First Strand Synthesis System (Invitrogen) according to manufacturer’s instructions. The resulting cDNA was used as a template for qPCR. SYBR GreenER (Invitrogen) Master Mix was used with primers previously described in the literature (Table 4.1). ROX was used as a passive reference dye. 2μL cDNA and 23μL Master Mix were added to each well of a 96-well optical reaction plate (Denville), and each plate was run using an Applied Biosystems StepOne Real-Time PCR System. All reactions were performed with 3 biological replicates in technical duplicate with no-template and no-reverse transcriptase controls. The data were analyzed using the 2-ΔΔCt method, and
expression of all genes was normalized to the ACTB reference gene\textsuperscript{33}. The data are mean increases relative to samples treated with only medium. To confirm that the epithelial cells were differentiated into M cells (C2-M cells), control gene expression of Tnfrsf9 was measured and found to be upregulated (Figure 4.S2).

**Statistics.**

The mean ± the standard error was determined for each treatment group in the individual experiments. Statistical significance was calculated by using a paired Student's \( t \) test, with a significance level set at \( P \) values of <0.05.

### 4.4 Results.

*B. bronchiseptica co-localizes with M cells in the murine nasal cavity.*

Previous studies have discovered intestinal M cells as important for antigen sampling. However, only one study has identified respiratory M cells in the nasal cavity, and no other work to examine the interactions of M cells with respiratory pathogens has been performed\textsuperscript{1}. Using the natural host-pathogen model of the respiratory pathogen *B. bronchiseptica* infection in mice, we sought to determine whether *B. bronchiseptica* was capable of interacting with nasal M cells. Using immunofluorescence microscopy, we determined that *B. bronchiseptica* strain RB50 colocalized with M cells in nasal cavity tissue sections 30 minutes post-inoculation (Figure 4.1). These results support the *in vivo* relevance of further studying *Bordetella/M* cell interactions.
B. bronchiseptica and B. pertussis associate with C2-M cells.

Because M cells are a rare population in the mucosa, to characterize the responses of M cells to Bordetella we used an established in vitro co-culture system capable of differentiating gut epithelial cells (C2) into terminally differentiated M cells (C2-M). To determine the ability of Bordetella species to associate with M cells, we inoculated M cells with either B. bronchiseptica strain RB50 or B. pertussis strain 536. We observed that both B. bronchiseptica and B. pertussis strains were able to associate with M cells (Figure 4.2). B. pertussis associates at a higher level than B. bronchiseptica, which may be indicative of the host specificity of B. pertussis or may be due to variation in virulence factor repertoires.

B. bronchiseptica is efficiently translocated in a Bvg-dependent manner.

To determine whether M cells act as antigen sampling sites, we investigated the ability of M cells to translocate B. bronchiseptica. We observed that a greater number of wild type B. bronchiseptica strain RB50 was translocated than Bvg phase-locked mutant B. bronchiseptica strain RB54 (Figure 4.3), suggesting that translocation occurs in part through a Bvg+ factor. As a control, C2 cells were infected, and extremely low levels of bacteria were observed in the basolateral chamber suggesting that translocation is an M cell specific effect (Figure 4.3).

Bordetella species induce expression of IL6 and TNF but not CLDN4.

To determine the immune response initiated by M cells, we investigated RNA changes following inoculation with B. pertussis or B. bronchiseptica. Differentiated C2-M cells show 2.4-fold upregulation of CLDN4, a gene encoding tight junction protein claudin 4 compared to undifferentiated Caco-2 (C2) cells (Figure 4.S3). This suggests
that C2-M cells enhance tight junctions in order to ensure bacteria are not transported paracellularly. However, in the presence of *B. bronchiseptica* and *B. pertussis*, no significant differences in gene expression are observed when compared to media alone (Figure 4.S3). This suggests that bacteria do not affect the expression of the tight junction protein.

Because M cells are specialized epithelial cells important for the translocation of pathogens from the apical side to the basolateral side in order to pass the pathogen off to immune cells, we investigated whether M cells were capable of changing their transcriptional programming in an effort to recruit immune cells. We observe upregulation of inflammatory cytokine gene *TNF* in response to *B. bronchiseptica* strain RB50 and *B. pertussis* strain 536, albeit to a statistically significantly higher degree with *B. bronchiseptica* strain RB50 (Figure 4.5A). We also observe significant upregulation *IL6* in response to *B. bronchiseptica* strain RB50 and *B. pertussis* strain 536 (Figure 4.5B). Similarly, *B. bronchiseptica* strain RB50 induces a higher level of mRNA expression by C2-M cells than *B. pertussis* strain 536. These data indicate that M cells signal via pro-inflammatory cytokines, and this may potentially result in the recruitment of immune cells in response to infections.

### 4.5 Discussion.

This study presents a novel interaction between *Bordetella* species and M cells. Here we show that *B. bronchiseptica* colocalizes with nasal M cells *in vivo* and *Bordetella* species associate with M cells *in vitro*. While these findings are not surprising
due to previous research indicating that *Bordetella* species bind to ciliated epithelial cells, the data suggest that bacterial interaction with specialized M cells could affect early initiation of an immune response\textsuperscript{16,18,34}. Notably, *B. pertussis* strain 536 had significantly increased numbers of associated bacteria with M cells than *B. bronchiseptica* strain RB50. This differential association may be due to variation in virulence factors such as lipopolysaccharide composition and expression of secretion systems or it may be due to host specificity of *B. pertussis* for human cells\textsuperscript{16,35,36}. Because we observe wild type *B. bronchiseptica* strain RB50 is translocated to a higher degree than Bvg\textsuperscript{-} *B. bronchiseptica* strain RB50 (Figure 4.3), one or more virulence factors expressed in the Bvg\textsuperscript{i} or Bvg\textsuperscript{+} phase is the ligand responsible for binding to M cells.

Conflicting reports in the field have discussed whether M cells could act as antigen-processing cells or simply translocate bacteria from the apical to the basolateral side. Because we observe live bacteria are translocated through to the basolateral size, our data suggest that M cells are responsible for antigen sampling, although they may also be involved in antigen processing (Figure 4.3).

Because M cells interact with *B. bronchiseptica* and *B. pertussis*, we investigated transcriptional responses and observed that expression of *CLDN4*, which encodes a tight junction protein, by C2-M cells is increased when compared to C2 cells, but stimulation of C2-M cells with either *B. bronchiseptica* or *B. pertussis* does not significantly alter *CLDN4* expression further (Figure 4.S3). In contrast, induction of genes encoding pro-inflammatory cytokines TNF\(\alpha\) and IL-6 is observed. Previously, intestinal M cells have been shown to produce TNF\(\alpha\)\textsuperscript{3}. Notably, upregulation of *TNF* is greater when C2-M cells are stimulated with *B. bronchiseptica* as compared to when stimulated with *B.
pertussis. These data may suggest that M cells signal to the surrounding epithelial cells as well as to immune cells in order to alert them of invading pathogens. These preliminary investigations of the host response have been informative, but future studies to investigate the bacterial transcriptional response to M cells is also of interest.

Future studies should aim at the association between secretory IgA (sIgA) and M cell uptake of Bordetella species since previous studies have shown that sIgA is important for M cell uptake of pathogens as shown with V. cholerae and others. While we observe Bordetella species association and translocation, not all bacteria are translocated, and we may observe greater numbers of bacteria sampled and translocated in the presence of sIgA.

These are the beginning of a series of studies aimed at understanding the role of M cells in initiating an immune response. Most likely, professional antigen presenting cells such as dendritic cells are recruited for antigen processing. Future directions include studying the resulting immune cell recruitment following M cell Bordetella association. Better understanding the role of M cell interactions with pathogens can lead to the development of more effective mucosal vaccines that will more efficiently target the antigen to the adaptive immune system resulting in a more protective memory response. Based on the recent resurgence of whooping cough, a mucosal vaccine targeted to M cells might better stimulate the location response and provide protection for infants who cannot yet be vaccinated and mount a systemic immune response.
Figure 4.1 M cells and *B. bronchiseptica* strain RB50-expressing GFP colocalize with M cells in the nasal cavity 30 minutes post-inoculation.

Wild type mice were inoculated with $5\times10^4$ CFU of *B. bronchiseptica* strain RB50-pCC5, expressing green fluorescence protein (GFP) on a plasmid. Murine nasal cavities were extracted and 10µm sections were stained with DAPI (A), anti-M cell (B), anti-RB50 (C), and images overlaid and imaged with confocal microscopy at 40x magnification. This image is a representative figure.
Figure 4.2 *Bordetella* species associate with C2-M cells.

30 minutes post-inoculation with 2x10^5 CFU, 2x10^5 C2-M cells were washed 4x with PBS, and Cellstripper was used to remove them from the inserts. Bacterial counts were enumerated via serial plating. Bars are representative of the mean with error bars as ±standard error. * indicates p<0.05 based on a Student’s T test.
Figure 4.3 Bvg-dependent factors are required for efficient translocation of *B. bronchiseptica* by C2-M cells.

C2 (undifferentiated) and C2-M cells (differentiated) cells were inoculated with 2x10^5 CFU of *B. bronchiseptica* strain RB50 or an isogenic Bvg^- mutant (RB54) for 30 minutes. Bacteria translocated to the basolateral side were enumerated. * indicates p<0.05 based on a Student’s T test.
Figure 4.4 *Bordetella* species induce C2-M cell expression of *TNF* and *IL6*.

C2-M cells were inoculated with $2 \times 10^5$ CFU, and cell lysates were collected for RNA extraction by the Trizol method. Relative expression levels ($2^{-\Delta \Delta C_t}$) with *ACTB* as a reference gene is shown. Error bars represent standard error of the mean. * indicates p<0.05 based on a Student’s T test.
Figure 4.S1 Set up of co-culture system to differentiate Caco-2 epithelial cells (C2) into M cells (C2-M cells).

Caco-2 cells are seeded onto a transwell filter at a $2 \times 10^5$ cells per insert density. Cells are allowed to terminally differentiate into mature enterocytes for 21 days. Alternatively, using the HTS Caco-2 differentiation kit (Corning), terminal differentiation lasts 3 days. Subsequently $5 \times 10^5$-$10^6$ Rajis are added to the basolateral compartment for 3-4 days. This process results in fully differentiated epithelial cells exhibiting M cell phenotypes such as decreased brush border and ability to translocate molecules and pathogens$^{22,23}$. 
Figure 4.S2 Confirming the differentiation of Caco-2 cells (C2 cells) to an M cell-like cell line (C2-M cells).

C2 and C2-M cell lysates were collected for RNA extraction by the Trizol method. Relative expression of *TNFSF9* was determined to evaluate whether C2-M cells were properly differentiated. Error bars represent ± standard error of the mean. * indicates p<0.05 based on a Student’s T test. Relative expression (2^-ΔΔCt) with ACTB as a reference gene is shown.
Figure 4.S3 *Bordetella* species have no measurable effect on *CLDN4* expression by C2-M cells following 30 minutes post-infection.

C2 and C2-M cells were inoculated with 2x10⁵ CFU, and cell lysates were collected for RNA extraction by the Trizol method. Relative expression levels (2⁻ΔΔCt) with *ACTB* as a reference gene is shown. Error bars represent standard error of the mean. * indicates p<0.05 based on a Student’s T test.
Table 4.1 qPCR Primers

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4.6 Author Contributions.

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Conceived and designed experiments: LLG, SJM, ETH

Performed experiments: LLG, SJM

Analyzed data: LLG, SJM, ETH

Wrote the paper: LLG, SJM, ETH
4.7 References.


Chapter 5

Measuring the feasibility of a low dose inoculation: a more natural infection model.
5.1 Abstract.

Background: High dose infection models have been used for decades, but naturally acquired infection likely occurs with very small numbers of bacteria.

Methods: To identify potential artifacts of the high dose inocula commonly used, we inoculated mice with a very low dose (~50 colony forming units (CFU)) and examined the ability of bacteria to colonize the murine respiratory tract, contribute to immune signaling in a mouse model, and induce immune cell recruitment using flow cytometry and systemic antibody responses via ELISA.

Results: We found that a low dose inoculum grows in the nasal cavity and results in high bacterial numbers of approximately 100,000 by 7 days post-inoculation, and that these bacteria are capable of persisting long term to day 56. We determined that Toll-like receptor-4 (TLR4) signaling is required for the control of bacterial growth in the nasal cavity in a low dose inoculum. Although previous findings suggested TLR4 acted primarily via tumor necrosis factor (TNF) α, which is required to control high dose inoculation, TNFα was not required to control this more natural low dose inoculum. While high dose inoculum induced recruitment of ~6,500 leukocytes to the nasal cavity 7 days post-inoculation, the low dose inoculum did not induce recruitment of leukocytes or neutrophils above the basal level (< 3,000). Low dose inoculation did induce serum antibody levels that are similar to high dose.

Conclusions: The high dose inoculation system has revealed much about interactions with host immunity and the effects of various bacterial factors on it. Understanding how
these findings relate to the process following a low dose inoculation will improve our ability to apply knowledge about the effects of each factor to their roles during natural infection. For example, determining the differential roles of TLR4 and TNFα during high and low dose inoculation might predict susceptibility of hosts lacking TLR4, but not TNFα, but predict a role for both in an effective response to some vaccines.

5.2 Introduction.

For decades, the infectious disease field has used high dose inoculations ranging from $10^4$-$10^9$ bacteria/mL in mouse models of infection to study the capabilities of the pathogen to cause disease as well as the immune response to control pathogen-growth and limit tissue damage$^{1-3}$. These high dose models have several benefits, such as consistent colonization throughout the organ being studied, resulting in a robust phenotype with lower variability in colonization and survival rate$^{1,4}$. The concept behind this model is that a robust effect will be observed quickly with the assumption that infection kinetics are not likely to change, only to be accelerated. This approach has given us a great deal of information concerning the immune components necessary to limit infection. However, fine tuning of the infection model is now needed to investigate mechanisms that may be overlooked or only result in overwhelming the immune system in a high dose model. For example, the ability of *Bordetella pertussis* and *B. bronchiseptica* to colonize the murine respiratory tract has been studied extensively, but using lower doses, researchers were able to discriminate between the abilities of these pathogens to displace murine microbiota$^5$. This may lead to further understanding of
host-restricted features of some pathogens and wide host ranges of others as well as the requirement for large numbers of bacteria to overcome the inabilities of bacteria to colonize in non-natural hosts.

The low dose inoculum is relevant because aerosolized droplets in a cough are thought to expel 50 bacteria of a diameter no larger than 2µm or 290 bacteria ranging from 2-4µm in diameter. Because *Bordetella* species are observed to be 0.5x2.0µm, a reduction in dose from the common 5x10⁵ CFU in 50µL inoculum size to 150 or less CFU in 5-10µL will more accurately reflect a natural acquisition of infection and therefore inform us on the initiation and capabilities of the immune response during a natural infection via requirements of the immune system for bacterial colonization and control.

*B. bronchiseptica* is a Gram-negative respiratory pathogen with a wide host range, including the rodent as a natural host. With an ID₅₀ of 5 colony forming units (CFU) and the rodent as a natural host (Rolin and Goodfield, unpublished data), *B. bronchiseptica* is an ideal pathogen to study whether bacterial infection and host immune system requirements are different than those identified using the high dose model. Using *B. bronchiseptica* as the model invading pathogen of the murine respiratory tract, we investigated the importance of Toll-like receptor (TLR) 4 and tumor necrosis factor α (TNFα), immune components previously identified as essential for control of *Bordetella* growth. Additionally, we investigated the capabilities of a lower, more natural dose in generating an immune response in the nasal cavity using flow cytometry and a systemic memory antibody response as detected by ELISA in serum and found that low
dose inoculation results in little cell recruitment to the nasal cavity, but serum antibody titers are equivalent to those in a high dose-inoculated mouse.

5.3. Materials and Methods.

**Bacterial Strains.**

*B. bronchiseptica* strain RB50 is an isolate from a rabbit. Bacteria were maintained on Bordet-Gengou agar (Difco) containing 10% sheep blood (Hema Resources). Bacteria were grown in Stainer-Schölte broth containing heptakis shaking at 37°C overnight to mid-log phase.

**Animal Experiments.**

C57BL/6, TNFα−/−, HEN, and HEJ mice were obtained from Jackson Laboratories. All animal experiments were performed in accordance with institutional animal care and use committee (IACUC protocol # 40029) guidelines. Mice were anesthetized with 5% isoflurane in oxygen and intranasally inoculated with 500 or 5x10⁵ CFU in 5μL. Mice were euthanized by 5% CO₂ inhalation according to IACUC guidelines. The respiratory tract was excised on the appropriate days post-challenge to evaluate the colonization numbers by serial plating on Bordet-Gengou agar containing 10% sheep blood and 20μg/mL streptomycin. Serum was isolated as previously described.

**Flow cytometry.**

Mice were inoculated with a high or low dose as indicated above, and the nasal cavity was excised on the appropriate time point. Samples were incubated with collagenase D (Sigma) and homogenized through a 70μm cell strainer to obtain a single cell suspension.
Samples were resuspended in FC blocking buffer containing anti-CD16/32 (BD Biosciences) and incubated on ice for 20 minutes. Following wash, cell surface markers were labeled with the following antibodies in PBS + 2% FBS: anti-CD45 APC-cy7 400:1 (BD Biosciences), anti-Ly6G APC (E Bioscience). Samples were resuspended in 4% paraformaldehyde until acquisition on a BD LSR Fortessa II. Data were analyzed with FlowJo Software 7.6.1.

Statistics.

The mean ± the standard error was determined for each treatment group in the individual experiments. Statistical significance was calculated by using a paired Student's t test, with a significance level set at P values of <0.05.

5.4 Results.

Low doses of bacteria can establish colonization early and persist long term in the nasal cavity.

To evaluate the ability of the low dose to establish colonization and persist in the nasal cavity as is characteristic of *B. bronchiseptica* strain RB50, we inoculated wild type mice with approximately 50 or 50,000 CFU in 5µL. Throughout the first week, bacterial burden in the nasal cavity, trachea and lungs was assessed. We observed that although the starting inocula were vastly different, bacterial growth kinetics in the nasal cavity appeared similar during the first three days post-infection (Figure 5.1). Notably, bacterial counts in the nasal cavity on day seven are the same, regardless of dose with approximately $10^5$ bacteria in the nasal cavity (Figure 5.1A). The ability of the low dose
to reach the same colonization level may suggest a carrying capacity in the nasal cavity, which is maintained persistently, that, beyond a certain threshold, sends out danger signals to remove the pathogen. The low dose is crucial in observing this phenomena because administering a dose higher than the carrying capacity, as the high dose inoculation system dose, conceals these capacity limitations. Consistent bacterial colonization in the trachea is not achieved regardless of dose (Figure 5.1B). When a high dose is administered, higher bacterial numbers in the lungs are observed on day 3, up to approximately 1,000 CFU, but bacteria are cleared by day 7 (Figure 5.1C). In contrast, while a few bacteria may reach the lungs during a low dose infection, the opportunity to colonize and multiply likely never presents itself due to efficient immune surveillance and the presence of microbiota inhibiting establishment of infection. Surveying immune cells such as macrophages, neutrophils, and dendritic cells may clear low numbers without inducing a detectable immune response. In order to push the system and have an immediate immune response that results in some damage, a large number of invading bacteria are likely required.

*B. bronchiseptica* has long been characterized as a naturally persistent infection in the upper respiratory tract\(^8\). To determine whether a low dose inoculum infection with *B. bronchiseptica* could achieve persistence, mice were inoculated with a low dose, and bacteria were enumerated 56 days later. All four mice were colonized by \(\sim10^4\) bacteria, indicating that a low, more natural dose is capable of persisting long term in the upper respiratory tract of mice at a consistent load.
**TLR4, but not TNFα, is required for the control of bacterial growth in the murine nasal cavity.**

Due to the ability of low dose inoculum to colonize at levels equivalent to the high dose in the nasal cavity and persist long term, the low dose inoculum has proven to be a reasonable tool for investigating host-pathogen interactions to determine whether previous observations could be artifacts. Previous studies have investigated the early immune response detailing the importance of TNFα-dependent TLR4 signaling\(^9\)–\(^{11}\). Therefore, we sought to determine whether TLR4 and TNFα are required for host control of bacterial growth using a low dose. As shown previously using a high dose model, TLR4 is required for the control of *B. bronchiseptica* in the nasal cavity (Figure 5.3A)\(^{10}\). Using TNFα\(^{−}\) mice, Wolfe, et al. determined that TLR-4-dependent TNFα signaling was a primary source of the control of *B. bronchiseptica* in the nasal cavity, because pro-inflammatory cytokines such as TNFα are important in recruiting and activating immune cells\(^{16}\). In contrast to these data, TNFα, which is required for control of bacterial growth in a high dose model, is not required in a low dose inoculation model (Figure 5.3B)\(^{16}\). These data suggest that a low dose model may be better able to delineate which immune components are required for the control of bacterial growth in a natural setting because we can identify features that are artifacts of a high dose inoculation system.

*Leukocyte recruitment to the nasal cavity.*

Because TLR4 signaling is critical for controlling *B. bronchiseptica* growth, we next investigated leukocyte recruitment to the nasal cavity following low or high dose inoculation using flow cytometry. By day 1, a high dose inoculum yields a higher trend of leukocytes and neutrophils recruited to the nasal cavity with \(>6,500\) leukocytes of
which >4,500 are neutrophils (Figure 5.4). In contrast, low dose-inoculated mice have lower numbers of leukocytes and neutrophils, not reaching beyond 3,000 leukocytes of which ~1,500 are neutrophils. These immune cell counts are indistinguishable from naïve mice. Notably, on day 7 post-inoculation, both doses yielded the same bacterial numbers in the nasal cavity (Figure 5.1A). These data suggest that a low dose allows the immune response to maintain invading pathogens at a low level in the mouse. This may occur through immune surveillance which readily removes low numbers of pathogens, while heightened danger signals from substantially more bacteria early on would rapidly recruit immune cells.

*Antibody levels are indistinguishable on day 28 regardless of dose.*

Because the high dose inoculum induces about 6,500 leukocytes and the low dose inoculum induces less than half the number, we wanted to determine the level of humoral response by measuring antibodies in the serum via ELISA. We observed that induced antibodies (total Ig) were at similar levels on both days 7 and 28 (Figure 5.5). These data suggest that the low dose inoculum is as capable of inducing a systemic antibody response as a high dose inoculum.

5.5 Discussion.

By using a low dose in the initial colonization model, we can begin to investigate the subtleties of host-pathogen interactions in the nasal cavity. Notably, because *B. bronchiseptica* does not appear to disseminate to the lower respiratory tract, studies understanding the closely related species *B. pertussis* may not be a good fit for this model
(Figure 5.1). Additionally, the infectious dose at which 50% of mice are infected (ID$_{50}$) is much higher for *B. pertussis* likely due to microbiota competition, and perhaps *B. bronchiseptica* is better armed to compete with and displace microbiota to establish the infection$^5$. Because we do not yet have the ability to sensitively detect transmission of a few bacteria and subsequent dissemination of *B. bronchiseptica* to the lower respiratory tract, we do not know if this actually occurs naturally in rodents. *B. bronchiseptica* can migrate to the lower respiratory tract of larger mammals such as cats and dogs (causing ‘kennel cough’), and this suggests that the bacteria either need to be transmitted in a high inoculum or have strategies at their disposal to evade these initial host responses to allow for dissemination to the lower respiratory tract.

The data show that the low dose increases in bacterial numbers until bacterial numbers plateau, and the high dose inoculation shows a population that increases then decreases to meet capacity of persistent infection. We observe that much like infection with a high dose inoculum, bacteria persist in the nasal cavity at about 10,000 CFU, which suggests stabilization in the population and the potential to become commensal-like (Figure 5.1). The bacterial burden in the nasal cavity cannot exceed this limit of $10^4$ CFU as suggested by the decrease in the high dose growth kinetics. There are many different factors that could play a role in defining this boundary, including the host immune system, host microbiota, and phenotypic switch of the *B. bronchiseptica* that could cause the down-regulation of important virulence factors involved in tissue damage and immune evasion. Other instances of pathogens using virulence factors to become less pathogenic to hosts have previously been observed, and this needs to be further
investigated in the field\textsuperscript{17}. Elucidating the intricacies of these interactions can help us further understand the multi-factorial nature of infectious kinetics of pathogens.

In this work, we have uncovered a difference in immune requirements between the high and low dose inoculation models, showing that there is no requirement of TNF\(\alpha\) to control bacterial growth in the low dose system as compared to previously published information using a high dose high volume system (Figure 5.3)\textsuperscript{11}. In contrast, the data indicate that TLR4 is required for control of bacterial growth regardless of starting dose (Figure 5.3). Other pro-inflammatory cytokines downstream of NF-\(\kappa B\), such as interferon gamma and chemokines such as KC may be responsible for the control of bacterial numbers, and in the absence of all of these downstream signals, the immune system cannot control bacterial growth.

These data suggest that low, more natural doses, capable of establishing an infection as early as day 7 post-inoculation and long term as far as at least day 56, inducing an immune response at the local site of infection, and generating a systemic antibody response, are robust enough to measure changes and should be considered when designing experiments. In fact, a recent study by Rolin et al. shows that low dose inoculation of an index mouse can lead to the shedding and transmission of \textit{B. bronchiseptica} to susceptible mice\textsuperscript{9}. The robust antibody response and local immune responses may play a significant role in keeping these bacteria in check and could give us insights into mechanisms of pathogens that are commensal bacteria that can become opportunistic infections by studying this dynamic. Using a lower, more natural inoculum dose to study host-pathogen interactions will lead us to better evaluate abilities of
pathogens to induce immune responses and immune systems to limit the invasion and control of pathogens.
Figure 5.1 Bacterial growth kinetics of *B. bronchiseptica* strain RB50 during the first week of infection.

The bacterial burden in the nasal cavity (A), trachea (B), and lungs (C) was assessed throughout a one week period in wild type mice following infection with ~500 or 5x10^5 CFU in 5µL PBS.
Nasal cavity persistence at a low dose ranges from Log10 CFU 3.8-4.1.

C57BL/6 mice were inoculated with 50 CFU *B. bronchiseptica* strain RB50. On day 56, the mice were euthanized, and the bacteria in the nasal cavity were enumerated.
Figure 5.3 TLR4, but not TNFα, is required for control of bacterial growth during a low dose inoculum.

HEJ (TLR4 Deficient), HEN (Wild type control), TNFα−/−, or C57BL/6 mice were inoculated with 50 CFU B. bronchiseptica strain RB50. On day 7 post-inoculation, mice were euthanized, and the bacterial numbers in the nasal cavity were enumerated. * indicates p<0.05, NS indicates no significant difference.
Wild type mice were inoculated with 50 CFU *B. bronchiseptica* strain RB50. On day 7 post-inoculation, the nasal cavities were removed, incubated in Collagenase D for 45 minutes, and homogenized over a 70µm filter. The single cell suspension was stained, and the samples were analyzed on the BD LSRII Fortessa. * indicates p<0.05, NS indicates no significant difference.
C57BL/6 mice were inoculated with 500 or 5x10⁵ CFU *B. bronchiseptica* strain RB50, and at the 7 and 28 days post-inoculation, serum was isolated and total antibody titers were determined via the endpoint method. NS indicates no significant difference based on a student’s t-test.
5.6 Author Contributions.

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5.7 References.


Chapter 6

Summary and Significance
6.1 Synopsis.

The classical bordetellae are comprised of respiratory pathogens successful at surviving within the host, including humans and a wide variety of mammals, and evading the immune response. For decades we have studied the host-pathogen interactions between the bordetellae and their hosts, but their pathogenicity is still poorly understood. This work aims to assess current outbreaks and understand factors that contribute to the resurgence of *B. pertussis* infection and severe whooping cough cases, to understand the evolution of *Bordetella* species by studying isolates that have lost the ability to be virulent, to better understand host-pathogen interactions by investigating the role of early defenses in the mucosa, and to reevaluate the way we investigate these questions using the mouse model of infection.

6.2 Understanding the resurgence of pertussis: Measuring genomic diversity and acellular vaccine efficacy against clinical *Bordetella pertussis* isolates from 2010-2012.

6.2A Summary and Implications.

The alarming resurgence of whooping cough has gained a great deal of attention in the past 5 years. To narrow down the factors responsible for increased numbers of pertussis cases, we evaluated multiple isolates collected from children’s hospitals across the United States from 2010-2012 (Figure 2.1A). Using pulsed-field gel electrophoresis (PFGE) and single nucleotide polymorphism (SNP) trees, we determined that multiple closely related isolates were circulating simultaneously during outbreaks and within
hospitals. Specific PFGE types were not associated with hypervirulent cases that resulted in death, suggesting that host factors and not hypervirulence of new isolates contributed to the fatalities. Furthermore, isolates from different geographical locations did not group on the SNP tree branches, suggesting that the changes observed among these closely related strains are not due to the evolution of a couple strains (Figure 2.1B).

To investigate the ability of current isolates to infect and colonize the murine respiratory tract, five geographically and temporally distinct isolates were intranasally inoculated into mice. No isolate showed the ability to grow to a higher extent than others in the murine lung, suggesting that the resurgence is not due to the outgrowth of a hypervirulent *B. pertussis* strain (Figure 2.2). Since other studies in the field have suggested whooping cough resurgence may be due to vaccine escape, we investigated whether isolates had changes in genes encoding vaccine antigens. We found that *ptxA* and the promoter *ptxP*, both important for the expression of PTX, *fim2*, and *fim 3*, genes encoding fimbriae, were highly conserved (Table 2.S1). In contrast, *prn*, encoding pertactin (PRN), and *fhaB*, encoding filamentous hemagglutinin (FHA), were highly divergent among examined isolates. Changes in *prn*, such as signal peptide deletions, presence of insertion elements, and amber codons, were observed in approximately half of the strains (Figure 2.3A). These mutations all resulted in loss of anti-PRN antibody recognition (Figure 2.3B). Changes in *fhaB*, such as truncations, frameshift mutations, and SNPs, were observed in approximately half of the strains, resulting in new allele types identified (Figure 2.3C, Table 2.S2). These mutations did not affect anti-FHA antibody recognition (Figure 2.3B).
Since variability was found in two genes encoding vaccine-associated antigens, we investigated whether the current acellular vaccine, Adacel, was capable of reducing colonization in the murine lungs following inoculation of recent isolates. We observed that the vaccine provided protection resulting in a decrease of 1-2 logs of bacteria in the murine lungs (Figure 2.4A). Importantly, we investigated whether the level of protection against these isolates was comparable to the laboratory strain. We found that although the vaccine was protective, it is slightly less protective at reducing colonization of isolates than the laboratory strain during murine infection (Figure 2.4B). This difference is small (90% vs. 99%), but it is detectable and reproducible and may offer some insight as to how we can further improve the current vaccine, because small changes (i.e. SNPs) may have affected the recognition of anti-pertussis antibodies against newer isolates.

6.2B Future Directions.

While the evaluation of these outbreak strains is important, further investigation is needed to determine whether vaccine escape is becoming a concern. To investigate the hypothesis that acellular vaccines are driving evolution, both vaccine- and non-vaccine associated gene evolution should be investigated now that hundreds of clinical B. pertussis strains from pre- and post-vaccine eras are publicly available. A similar mutation rate in vaccine- and non-vaccine associated genes would suggest that while evolution of B. pertussis is taking place, this evolution is not due to vaccine pressures specifically. Additionally, since the mouse model does not perfectly mimic the disease state of pertussis due to lack of cough, evaluation using the baboon model would allow
characterization of these strains. Since the vaccine has played such an important role in initially reducing the number of cases, research focusing on updating the current vaccine continues to be a focus. Further investigation into the mechanism behind maternal vaccination, whether induction of protective antibodies transferred transplacentally or via breast milk, remains a question. These data would contribute to a better understanding of how to protect the most vulnerable of all cases, infants. Other studies could focus on the modeling of non-medical exemption cases and how people who choose not to vaccinate their children might be driving the resurgence of whooping cough. Until whooping cough becomes a vaccine-preventable disease, ongoing studies on treatment, such as anti-pertussis toxin treatment, need to be tested in pre-clinical and clinical studies (Goodfield and Smallridge, unpublished data).

6.3 Identification of an avirulent *B. bronchiseptica* isolate.

6.3A Summary and Implications.

Biological fitness among bacterial isolates is created by selective pressure, but sometimes, this pressure is ineffective and results in bacterial lineages that are not adapted to all environments they must encounter. In Chapter 3, we identified *B. bronchiseptica* strain 99R-0433 as a human isolate. This isolate may have been isolated as a secondary infection. Importantly, this strain does not show similar features of avirulence to *B. bronchiseptica* strain 253, which has been previously identified as a naturally-occurring mutant of adenylate cyclase toxin (ACT)\(^1\). *B. bronchiseptica* strain
99R-0433 is beta-hemolytic on Bordet-Gengou Agar containing blood, suggesting that expression of ACT is intact (Figure 3.1). Additionally, the O-antigen type of *B. bronchiseptica* strain 99R-0433 is not identified by O1- or O2-type antibodies, suggesting that its O-antigen is either divergent, immunogenic, or not expressed. This feature is important because the O-antigen is a highly immunogenic antigen in *B. bronchiseptica* immunity, and *B. bronchiseptica* isolates have been shown to express O-antigens; however, other isolates have been identified which express non-immunogenic O-antigens\textsuperscript{2,3}. *B. bronchiseptica* as a species has been identified as an efficient colonizer of the respiratory tract, an important feature of host-pathogen interaction studies in mice; however, *B. bronchiseptica* strain 99R-0433 is incapable of establishing colonization in wild type or immunodeficient mice (Figure 3.3, Figure 3.4)\textsuperscript{4}. Because of these very different phenotypic features of *B. bronchiseptica* strain 99R-0433, the genome was sequenced and a SNP-based tree was created to see how divergent this unique strain is from other known *B. bronchiseptica*, *B. parapertussis*, and *B. pertussis* isolates. Figure 3.5 shows that *B. bronchiseptica* strain 99R-0433 is separated by 176,602 SNPs from the prototypical strain RB50, 142,027 SNPS from avirulent strain 253, and 230,071 SNPs from human isolate *B. bronchiseptica* strain D445. Understanding how this strain is different from other *B. bronchiseptica* strains in phenotype but is still effective at surviving in hosts such as humans can give the field novel insights to the field of how adaptions in *B. bronchiseptica* can affect host specificity and host-pathogen dynamics. However, it is also possible that this strain did not use humans as a reservoir but rather was an accidental isolation during sputum collection.
6.3B Future Directions.

Since *B. bronchiseptica* strain 99R-0433 is incapable of colonizing mice, future studies aimed at the ability of the strain to adhere and cause cytotoxicity would give a better understanding of its poor survival *in vivo*. Interestingly, in the absence of the well known adenylate cyclase toxin/hemolysis system, *B. bronchiseptica* strain 99R-0433 still retains the ability to cause beta-hemolysis. Determining the novel toxin responsible for beta-hemolysis would greatly enhance the understanding of how this *Bordetella* strain might cause pathogenesis. Using sodium dodecyl sulfate gel electrophoresis would allow us to evaluate whether the protein and LPS content is markedly different from other *B. bronchiseptica* strains. Additionally, we could also determine the mechanism of innate immune clearance. Furthermore, infection of TLR-4 deficient mice would give us a better idea of whether the clearance of *B. bronchiseptica* strain 99R-0433 is TLR-4-dependent. Finally, determining the gene(s) responsible for virulence can be better investigated through avirulent isolates such as *B. bronchiseptica* strain 99R-0433.

6.4 Examining the local response in the mucosa: How microfold cells act as first responders during a *Bordetella* infection.

6.4A Summary and Implications.

The mucosal barrier is the first of many physical host barriers that are in place to prevent the invasion of pathogens into the host. Chapter 3 investigates the initial response of the specialized epithelial cells called microfold (M) cells. Our data show that *B. bronchiseptica* is able to colocalize with M cells in 30 minutes, suggesting that this
may be an important part of the host-pathogen interaction (Figure 3.1). Using an established *in vitro* co-culture system that differentiates enterocytes in M cells, we evaluated the ability of *B. bronchiseptica* and *B. pertussis* to associate with M cells and found that both are capable of associating within 30 minutes (Figure 3.2)\(^5\)–\(^7\). Since the main role of M cells is to translocate potential antigens from the apical side to the basolateral side in order to hand off pathogens to other immune cells such as professional antigen presenting cells, we assayed for the ability of *B. bronchiseptica* to be translocated. *B. bronchiseptica* strain RB50 is translocated, and that this translocation is Bvg-phase-dependent, as a phase locked Bvg\(^{-}\) mutant was less readily translocated (Figure 3.3). Importantly, live bacteria are translocated through to the basolateral size, suggesting that M cells are at least in part, responsible for translocation, and not necessarily for antigenic processing of *Bordetella* species. However, future studies need to be carried out to determine whether *Bordetella* peptides are presented on major histocompatibility complexes on the basolateral side to determine this.

M cells have been found to associate with commensals and respond by changing their transcriptional program\(^5\)–\(^8\). Because we know that M cells interact with and translocate *Bordetella* species, we investigated whether these interactions result in M cell mRNA changes. Compared to undifferentiated Caco-2 cells, expression of the tight junction gene *Cldn4* is upregulated 2.8-fold (Figure 4.4A). In contrast, the transcript levels of *Cldn4* trend lower with stimulation by *B. bronchiseptica* and *B. pertussis*. M cells are also capable of initiating an immune response when stimulated by bacteria as indicated by upregulation of *Tnf* and *Il6*, genes encoding pro-inflammatory cytokines (Figure 4.5). These data are important because they show a novel role for respiratory M
cells in recruiting immune cells as well as maintaining the epithelial barrier and decreasing paracellular transport in response to interactions with *Bordetella* species. To date, these are novel findings in that no evidence has been provided that the *Bordetella* species interact with M cells. Additionally, these findings may aid in uncovering new aspects of the immune response initiation that has not yet been identified in both the M cell and *Bordetella* field.

### 6.4B Future Directions.

Future studies aimed at the mechanism of *Bordetella* binding, by delineating which bacterial ligand and M cell receptor, will inform the field on how M cells take up bacteria of different species\(^5\). Furthermore, ascertaining knowledge of the resulting immune cell recruitment in response in M cell signaling may uncover more about how the host responds to different types of infection. Understanding how or if these interactions change in cases where *Bordetella* become commensal-like, as in persistence of *B. bronchiseptica*, may also give novel insights into the cell to cell mechanisms involved in desensitization of previous pathogens.

Other areas of interest include whether sIgA contributes to efficient association and translocation of *Bordetella* species. Additionally, investigation into the mRNA response *in vivo* is underway. Furthermore, experiments investigating whether *Bordetella* species are able to use virulence factors to permeabilize membranes and/or cause cytotoxicity in epithelial cells would educate the field on these host-pathogen interactions. This may also help us gain a new perspective on the ability of M cells to
differentiate commensals from pathogens as there is a clear enhancement of transcytosis when *Bordetella* are in the Bvg$^+$ phase.

6.5 Measuring the feasibility of a low dose inoculation system: a more natural infection model.

6.5A Summary and Implications.

For decades, host-pathogen interactions have been examined in high dose infection model systems, and many immune components have been identified using this system$^{9-15}$. In this study, we investigated the ability of a lower, more natural dose to study host-pathogen interactions using a natural infection system with *B. bronchiseptica* as the invading pathogen. We found that a low dose inoculum of 50 colony forming units (CFU) was capable of establishing colonization to the same extent as a high dose inoculum in the nasal cavity by day 7 post-inoculation (Figure 4.1). This dose did not reach the lower respiratory tract. Additionally, a low dose inoculum resulted in a long term persistent nasal infection, which is characteristic of *B. bronchiseptica* (Figure 4.2). To determine the immune components required for control of bacterial growth following a low dose, we inoculated Toll-like receptor 4 (TLR4) and tumor necrosis factor α (TNFα) and determined nasal cavity colonization on day 7 post-inoculation. There were 100x more bacteria in the nasal cavity of mice deficient in TLR4 compared to wild type (Figure 4.3A). In contrast, bacterial counts in TNFα-deficient mice were indistinguishable from wild type infected mice (Figure 4.3B). These data suggest that, contrary to requirements in a high dose high volume inoculation system, TNFα-
independent TLR4 signaling is required for the control of bacterial growth in the murine nasal cavity. To determine the effects of dose on immune cell recruitment to the nasal cavity, we inoculated wild type mice with 50 or $5 \times 10^5$ CFU in 5µL and found that, while the high dose inoculation resulted in ~7,000 leukocytes, the low dose inoculation resulted in numbers similar to basal levels (Figure 4.4). Due to the stark difference in cell recruitment, we wanted to determine whether antibody responses were affected. We found that serum antibody titers were the same by day 28, regardless of dose, which suggests that inoculation with a low dose still retains the ability to cause systemic immunity (Figure 4.5).

### 6.5B Future Directions.

These data suggest that a low, more natural dose is feasible when investigating the host-pathogen interactions between *B. bronchiseptica* and the host immune response. Future studies will evaluate the use of a low dose model in the context of a commonly used high dose high volume system. This comparison will allow researchers to evaluate whether the immune components identified as important in a high dose are also critical in a low dose. Additionally, we will determine whether the antibody response induced by day 28 post-inoculation is protective to secondary infection. Finally, this model may help the field better understand the strategies employed by different pathogens to overcome barriers in order to initiate infection.
6.6 References.


Appendices
Appendix A

*B. bronchiseptica* strain 1289 lacking O-antigen expression is more susceptible to clearance.

Figure A.1 O-antigen is important for nasal persistence of *B. bronchiseptica* strain 1289.

C57BL/6 mice were inoculated with 5x10^4 CFU *B. bronchiseptica* strain 1289 or 1289Δwbm. On the appropriate day, mice were euthanized, the respiratory tract was excised, and bacterial counts were enumerated. The mean ± the standard error was determined for each treatment group in the individual experiments. Statistical significance was calculated by using a paired Student’s *t* test, with a significance level set at *P* values of <0.05 for a single comparison as denoted by *. 
The O-antigen of *B. bronchiseptica* strain 1289 contributes to protection against complement deposition.

Figure A.2 O-antigen prevents complement deposition on *B. bronchiseptica* strain 1289.

Mid-log phase bacteria were incubated with mouse serum for 2 hours, and then washed and stained for C3b. Flow cytometry was used to determine complement deposition. * denotes P values <0.05 as determined by a Student’s T Test.
Appendix B

*sigE* is important for *B. bronchiseptica* survival in the blood.

A.

![Graph showing bacterial survival in different organs.]

B.

![Graph showing bacterial survival in different organs.]

Figure B.1 *sigE* allows *B. bronchiseptica* to adapt and survive in the blood.

C57BL/6 mice were intravenously injected with $10^5$ CFU in 200µL and euthanized at (A) 8 hours or (B) 1 day post-injection. Bacteria in the blood, liver, kidney, spleen and lungs were enumerated. The mean ± the standard error was determined for each treatment group in the individual experiments.
Appendix C

LPS from different *Bordetella* species differentially affect pro-inflammatory signaling.

Figure C.1 TNFα is induced similarly in RAW 264.1 macrophages by LPS stimulation from multiple *B. pertussis* and *B. parapertussis* strains.

RAW 264.1 murine macrophages were stimulated with varying amounts of LPS or LOS for 24 hours, and ELISA was used to determine levels of TNFα in the supernatant. MC denotes the media control. The mean ± the standard error was determined for each treatment group in the individual experiments.
Figure C.2 Greater IL-6 production in RAW 264.1 macrophages is induced by LPS of *B. parapertussis* strains than LOS of *B. pertussis* strains.

RAW 264.1 murine macrophages were stimulated with varying amounts of LPS or LOS for 24 hours, and ELISA was used to determine levels of IL-6 in the supernatant. MC denotes the media control.
Appendix D

*B. bronchiseptica* strain RB50 does not kill IL-1R−/− mice by induction of leukocytosis.

Figure D.1 Immune cell recruitment to the lungs following *B. bronchiseptica* infection of IL-1R−/− mice.

IL-1R−/− mice were inoculated with 5x10⁵ CFU *B. bronchiseptica* strain RB50. After 2 days post-inoculation, mice were euthanized and lungs were excised. Lungs were homogenized over a 100µm filter, leukocytes were isolated with Histopaque 1119, and a single cell suspension was made. Cells were incubated with blocking buffer (CD16/32) before staining with appropriate antibodies and acquisition on a BD LSR II Fortessa. Data were analyzed using FlowJo software. * denotes P values <0.05 as determined by a Student’s T Test.
Appendix E

FoxP3+ Tregs do not affect vaccine induced immunity.

Figure E.1 FoxP3+ Tregs are not required for vaccine-induced immunity.

FoxP3-DTR mice were vaccinated with alum (Sham) or acellular pertussis (aP) on days 0 and 14 and challenged on day 28 with $5 \times 10^5$ CFU *B. pertussis* strain 536. Some mice were treated with diphtheria toxin (DT) 1 day prior to inoculation in order to deplete Tregs as indicated by the dashed line. On day 3 or 8 the respiratory tract was excised, and bacterial counts were enumerated.
Appendix F

Adacel aP vaccination reduces colonization of complex IV \( B. \) bronchiseptica isolates.

Figure F.1 Colonization by Complex IV isolates Followed by aP vaccination.

C57BL/6 mice were vaccinated on days 0 and 14 and inoculated with \( 5 \times 10^5 \) CFU/50\( \mu \)L on day 28. Three days post-inoculation, mice were euthanized and bacterial counts in the lung were enumerated. Acellular pertussis vaccination significantly reduces bacterial colonization. The mean ± the standard error was determined for each treatment group in the individual experiments. * denotes P values <0.05 as determined by a Student’s T Test.
VITA

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EDUCATION
The Pennsylvania State University  2009-2014  Ph.D. Immunology and Infectious Disease
Cabrini College  2005-2009  B.S. Biology/Biotechnology, B.A. Spanish

PROFESSIONAL POSITIONS
2013  Student Representative, Search Committee for Chair of the Veterinary and Biomedical Sciences Department, Pennsylvania State University
2013  Founder, Huck Student Seminar, The Pennsylvania State University
2010  Instructor, Introduction to LSR II Fortessa, The Pennsylvania State University
2010  BD Bioscience LSR II Fortessa Operator Course
2007-2009  Certified Title III Advanced Tutor/Classroom Coach, Cabrini College
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GRANTS AND UNRESTRICTED GIFTS
2014  ASM Student Travel Grant - $500
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SELECT PUBLICATIONS AND PRESENTATIONS