**CHARACTERIZATION OF MULTIPLE VIRULENCE FACTORS INVOLVED IN COLONIZATION, PERSISTENCE AND TRANSMISSION OF BOREDETELLA SPP.**

by

LONGHUAN MA

(Under the direction of Eric T. Harvill)

ABSTRACT

The circulating strains of *Bordetella pertussis* have demonstrated a dramatic decline in the expression of pertactin, an outer membrane protein, in the last few decades. Despite substantial interests in using pertactin as a vaccine antigen, surprisingly little is known about its role in the biology and pathogenesis of *B. pertussis*. This dissertation discusses work using *B. bronchiseptica*, a close *evolutionary* progenitor of *B. pertussis* that naturally infects mice, to examine the role of pertactin in the context of the natural processes of colonization, growth, spread within the respiratory tract, shedding and transmission between hosts. The results indicated that pertactin contributes to shedding and transmission of *B. bronchiseptica*. Furthermore, in an attempt to inspect possible reasons behind the loss of pertactin expression from clinical isolates, the dissertation discusses three aspects of pertactin that distinguish it from other vaccine antigens, which may, individually or collectively, explain why only this antigen is being precipitously eliminated. Work on elucidating the functional properties of pertactin in *B. bronchiseptica* is complemented by our previous studies on the evolutionary origins of *B. bronchiseptica* that demonstrate its ability to survive inside amoeba and utilize it as a transmission vector. Here, we aimed to test whether the machinery which allows interactions with predatory amoeba is conserved in some or all *Bordetella* species. The results show that only some *Bordetella* species retain the

ability to successfully interact with amoebae. Complete genomes for all 9 species allowed a Genome Wide Association Study (GWAS) approach that identified a set of genes that are associated with *Bordetella*-amoebal interactions. The final section of this dissertation describes work conducted on a pertussis-toxin-like factor in *B. pseudohinzii* identified via genome comparison between this bacterium and its closely related species. While recent studies have shown that *B. pseudohinzii* colonizes middle ears and persists for an extended time period, this dissertation describes how this ability may be linked to the production of these pertussis toxin-like proteins. Through these studies, we have gained a better understanding of the effects of various virulence factors on pathogenesis, transmission, and interactions with an amoebic transmission vector of *Bordetella* spp.

INDEX WORDS: pertactin, *Bordetella bronchiseptica*, transmission, amoebae, *Bordetella pseudohinzii*

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2021

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May 2021

ACKNOWLEDGEMENTS

Chapter 3 of this dissertation “Pertactin-deficient Bordetella pertussis Strains: Vaccine-driven Evolution and the Reemerging of Pertussis” has been accepted by *Emerging Infectious Diseases*; 2021 June. All permissions have been obtained regarding the reproduction of the text and figures of this manuscript within the dissertation.

First, I would like to thank my mentor, Dr. Harvill, for his time and patience on guiding me throughout my research. I also would like to thank my committee members Dr. Frederick Quinn, Dr. Balázs Rada, Dr. Stephen M Tompkins and Dr. Vincent Joseph Starai for their advice and input. In addition, I would like to thank current members of Harvill lab, Dr. Kalyan K. Dewan, Yang Su, Amanda Danielle Caulfield, Colleen Sedney and Randy Kim, as well as past lab members Dr. Bodo Linz, Dr. Israel Rivera, Dr. Monica Gestal, Dr. Hamidou Illiassou, Dr. Dawn Tylor, Dr. Valerie Ryman, Amanda Lee Skarlupka, Coralis Del Mar Rodriguez-Garcia, Shannon Wagner and Troy King. I would like to give special thanks to Dr. Bodo Linz and Dr. Kalyan Dewan for their guidance on project development. I would also like to thank Dr. Demba Sarr from Dr. Rada’s lab, Uriel Balas-machado from the Diagnostic lab, James P. Barber from the Cytometry core facility and Muthugapatti K. Kandasamy from the Confocal core facility for their training and technical assistance. I would also like to thank the staff from animal facility for their hard work on providing care for our mice. I want to give special thanks to previous CAF facility supervisor, Ilu Castellon who brought joy into every conversation. I would also like to thank Dr. Nancy Manley from the Department of Genetics for her encouragement throughout my Ph.D. study.

Lastly, I would like to thank my family and friends. There are too many to name, but you know who you are. To my parents, brother and sister, thank you for your constant support throughout my Ph.D. study. To my girlfriend, Yanan Zhu, you know I could not have made it without your support, encouragement and LOVE.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Genus *Bordetella*

The genus *Bordetella* is comprised of 10 species, including the closely related "classical Bordetellae," consisting of *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*. In contrast to these classical species, the remaining "non-classical Bordetellae" consist of more distantly related species, including *B. holmesii*, *B. petrii*, *B. avium*, *B. hinzii*, *B. trematum*, *B. pseudohinzii* and *B. ansorpii* (1) (Figure 1.1). The classical Bordetellae are primarily found in mammalian hosts. *B. pertussis* is a human-specific pathogen, *B. parapertussis* includes 2 strains that infect either humans or sheep (2), and *B. bronchiseptica* causes respiratory infections in a broad range of mammals (1, 3). The non-classical *Bordetella* species can cause infections in a diverse range of hosts. *B. holmesii* infects humans (4, 5), *B. avium and B. hinzii* infect birds (6, 7), *B. pseudohinzii* colonizes mice (8), and *B. trematum and B. anosorpi* were isolated from the infected wounds of immunocompromised patients (9, 10) (Table 1.2). In addition to isolation from animal hosts, some *Bordetella* species can also be isolated from the environment. *B. petrii* has been isolated from multiple natural environments, including an anaerobic, dechlorinating bioreactor culture enriched by river sediment, marine sponges and grass root consortia (11-13). Interestingly, in our recent study, *B. bronchiseptica* was shown to interact with the common soil amoeba *Dictyostelium discoideum*, and to use *D. discoideum* as a translocation vector (14, 15) (Figure 1.2). This raises the question whether the mechanisms that facilitate this *Bordetella*-amoebal interaction appear in

other *Bordetella* species and similarly contribute to their life cycles. In Chapter 4, we tested 9 *Bordetella* species on interactions with *D. discoideum*.

To investigate the evolution of *Bordetella* species, our group generated phylogenic trees for 128 genomes from 9 species of the *Bordetella* genus based on genome-wide sequence alignment and gene content (16) (Figure 1.3). Both phylogenic trees revealed the evolution of the genus *Bordetella* into three clades of species: clade A: formed by *B. bronchiseptica*, *B. pertussis* and *B. parapertussis*, clade B: including *B. hinzii*, *B. trematum*, *B. avium* and *B. holmesii*, and the phylogenetically oldest clade C: containing *B. petrii* and *B. ansorpii*. The sequence-based phylogenic tree reveled that the genome of classical *Bordetella* in clade A are closely related to each other, while species in clades B and C are far more diverse. Furthermore, substantial gene loss and gene acquisition occurred in clades A and B species, respectively, which would explain the diverse genome sizes within these two clades. Of the clade A species, pertussis toxin (PT) is expressed by *B. pertussis* but is absent in *B. bronchiseptica* and *B. parapertussis* due to truncated promoters. However, through genome comparison, we have identified a set of genes that have similarity with pertussis toxin genes that appear in the *B. pseudohinzii* genome. To date, there have been no reports of genes encoding a pertussis toxin-like molecule outside the classical Bordetellae and part of this dissertation describes investigations on the putative contribution of this set of toxin genes to the pathogenesis of *B. pseudohinzii*. Furthermore, it has been observed that several virulence factor genes were lost or functionally inactivated among *Bordetella* species, suggesting an ongoing selective evolution of virulence phenotypes within the genus. As a recent example, in the last 20 years, an increasing proportion of *B. pertussis* isolates have lost an immunogenic outer membrane protein, pertactin (16, 17). While it has been shown that *B. pertussis* pertactin-deficient strains demonstrated higher fitness in vaccinated mice (18, 19), the overall biological functions of

pertactin are still unknown. In Chapter 2, we describe the biological functions of pertactin in *B. bronchiseptica* by using a more natural infection model than that which has historically been used in the field.

The following section describes the characteristics of three *Bordetella* species (*B. pertussis*, *B. bronchiseptica* and *B. pseudohinzii*) which are relevant to the work in this dissertation.

***Bordetella pertussis***

*B. pertussis* is the etiological agent of whooping cough, which caused an estimated 24.1 million cases and 160,700 deaths in 2014 around the world according to a survey study conducted by the Centers for Disease Control (CDC) (20). Despite high vaccine coverage, a significant increase in the incidence of pertussis cases has been observed in many countries since the 1990s (21). Potential causes of this resurgence are widely debated, but several hypotheses have been proposed, such as 1) increased surveillance and sensitivity of diagnostic techniques, 2) waning convalescent and vaccine-induced immunities, and 3) the appearance of new variants capable of avoiding vaccine-induced immunity. With respect to the genetic changes in circulating strains, recent *B. pertussis* isolates with elevated expression of pertussis toxin (PT) have been reported (22). In addition, the prevalence of circulating strains that no longer express pertactin, an outer membrane protein and key vaccine antigen, has dramatically increased in the United States and other countries (16, 17). Additionally, a select few variants with limited amino acid changes in Fimbriae subunit 2 (Fim2) or Fimbriae subunit 3 (Fim3) have also been found (23). Genetic modifications in *B. pertussis* antigens included in acellular vaccines (ACV) are suspected to facilitate evasion of vaccine-induced immunity. In an era of pertussis resurgence, research into the possible reasons behind these genetic changes are urgently needed. These findings may help us to

optimize current vaccines or inform new vaccination approaches and/or strategies. In chapter 3, we speculate three possible explanations as to why the prevalence of pertactin-deficient isolates, but no other vaccine antigen-defective strains are rapidly increasing worldwide.

***Bordetella bronchiseptica***

*B. bronchiseptica* causes respiratory infections in a wide range of species, including cats, dogs, swine, mice, rabbits, rats, turkeys, humans, and primates (24). Occasionally, *B. bronchiseptica* has also been isolated from immunocompromised human patients (25-27). Because of the high relevance to *B. pertussis* (98% homology of shared genes) (28) and efficient colonization in mice, *B. bronchiseptica* has been widely used as a model for the study of *Bordetella* infections, including *B. pertussis*. The practical protocol on gene manipulation in *B. bronchiseptica* and availability of accessible mouse models using mice with different genetic backgrounds has enabled research into the pathogenesis and host-pathogen interactions of *Bordetella* species in recent years. However, in the conventional infection model, more than 1 million *B. bronchiseptica* bacilli are delivered into the lungs, which induces a strong immune response and quick bacterial clearance. Though severe symptoms of disease are rapidly observed with this approach, it fails to simulate the more gradually progressing and extended natural infection process. A model that more closely mimics the natural infection is needed, and in chapter 2, we describe the characterization of a more natural infection model for *Bordetella* study.

***Bordetella pseudohinzii***

*B. pseudohinzii* isolated from laboratory-raised mice was earlier mistakenly identified as *B. hinzii*. However, this isolate was later recognized as an independent species and characterized

by Ivanov *et al.* in 2016 (8). Since then, *B. pseudohinzii* has been isolated from additional mouse models, including laboratory mice, by different groups around the world (29-31). Our recent study revealed that *B. pseudohinzii* not only effectively colonizes the respiratory tract, but also causes persistent infection in the middle ears of mice (32), which successfully simulates chronic otitis media. Perniss *et al*. revealed that the colonization of *B. pseudohinzii* induces extensive airway inflammation with epithelial remodeling and causes damage to the structure of ciliated epithelium (33), which may hinder bacterial clearance, potentially explaining why *B. pseudohinzii* persists in the respiratory tract and middle ears for long periods of time. However, the issue of what genes in *B. pseudohinzii* are involved in the interactions between host and bacteria is still unresolved. In chapter 5 we have characterized the functions of *B. pseudohinzii* pertussis toxin-like molecule subunit A in bacterial persistence.

### *Bordetella* Virulence Factors

The expression of virulence factors among the classical Bordetellae is controlled by a two-component regulatory system, BvgAS (*Bordetella* virulence gene), composed of the sensor kinase BvgS and the response regulator BvgA (34). This regulation mechanism allows bacteria to express different sets of genes in response to changing environments, for example via changes in temperature and is often activated when *Bordetella* species detect they are in a host (35). In the Bvg+ phase, virulence factor genes related to colonization and survival inside hosts are highly expressed, including pertussis toxin, filamentous haemagglutinin adhesion, pertactin, Fimbriae, and adenylate cyclase toxin (36). In contrast, the Bvg- phase suppresses virulence related genes (*vrg*), and upregulates genes required for survival in extra-host or nutrient-limited environments, including lipopolysaccharide (LPS), capsular polysaccharide, select genes involved in metabolic

and catabolic pathways, as well as cold shock proteins (37, 38). There is also an intermediate phase called Bvgi in which some but not all Bvg+ regulated genes are expressed at an intermediate level (39, 40). This phase is suggested to be important for the transition between positive and negative phases.

The importance of the functions and regulation of two Bvg+ regulated virulence factors, PT and PRN, will demonstrated in the later chapters.

**Pertussis Toxin**

Pertussis toxin is a classical A-B toxin. The A component consists of subunit 1, which is activated in an unknown step upon entry into host cells and has ADP-ribosyl transferase functions which interfere with G protein-dependent signaling. The B component of pertussis toxin includes four subunits, (S2, S3, S4, and S5), which collectively mediate host-cell membrane binding to facilitate toxin delivery(41) (Figure 1.4). The expression of PT is regulated by the *BvgAS* regulon and it is highly expressed in the Bvg+ phase(42). PT has been shown to inhibit immune cell recruitment (neutrophils and macrophages) in early stages of infection and contributes to colonization in the lungs (43-46). Cyster *et al.* also showed that PT contributes to the recruitment of B cells and T cells into the spleen (47). The PT locus appears in all three classical *Bordetella* species, but *B. parapertussis* and *B. bronchiseptica* do not express this toxin due to promoter inactivation (16). However, genome comparisons between *B. pertussis* and *B. pseudohinzii* revealed that there is a pertussis toxin-like gene locus in *B. pseudohinzii*. The potential functions of this PT-like gene will be characterized in chapter 5.

**Pertactin**

Pertactin (PRN) is an autotransporter protein that appears on the outer membrane of *B. pertussis* (48) (Figure 1.5). Previous studies revealed that PRN may function as an adhesin through its Arg-Gly-Asp (RGD) motif and contributes to adherence and invasion of mammalian cells (49-52). However, in some other studies, the adhesive function of PRN was not verified (53-56). *In vivo* studies using the conventional high dose lung infection mouse model demonstrated that pertactin contributes to immunomodulatory effects in *B. bronchiseptica*, a close *evolutionary* progenitor of *B. pertussis* that naturally infects mice, and that these effects are independent of the RGD motif (57). In our study, we used a more natural infection mouse model to test the role of pertactin in colonization, growth, spread within the respiratory tract, shedding and transmission between hosts. These results and their significance are presented in Chapter 2. Because of its role as a protective antigen, pertactin has been included as a key antigen in acellular vaccines. Problematically, pertactin expression has been disrupted in an increasing proportion of circulating *B. pertussis* strains, now reaching 85% prevalence in the United States (58). Different theories have been proposed to explain the loss of pertactin, including that *B. pertussis* avoids complement-activating anti-pertactin antibodies by losing this antigen. However, there are 5 different components (PT, FHA, PRN, FIM2, and FIM3) included in current acellular vaccines (59), and the reasons behind why only pertactin was lost by *B. pertussis* are likely influenced by a variety of factors and selective pressures. We speculate 3 possible explanations to this question and these arguments are presented in chapter 3.

## Host-pathogen Interactions

### Upper Airway Epithelium

Ciliated epithelial cells and goblet cells form most of the airway's luminal surface(60). Cells are joined by tight junctions to form an integral barrier between self and environment which functions to block the invasion of most pathogens (61). The upper respiratory tract organs are covered by ciliated, pseudostratified, columnar epithelium (60). The cilia located in the anterior region of the nasal cavity beat towards the outside environment and the cilia on the surface of the posterior nasopharyngeal regions beat towards the pharynx, where pathogen and inhaled particles trapped in the mucus are either swallowed or expectorated (62). Various *Bordetella* virulence factors have been reported to disrupt these protective barriers and mechanisms of the respiratory epithelium. For example, Tracheal cytotoxin (TCT) and ACT have been shown to damage the tight junctions of the ciliated epithelium (63, 64). In addition, FHA and PT were reported to circumvent cilia beating-mediated clearance (51, 65, 66). In Chapter 2, we present the functions of pertactin in the induction of inflammation and mucus secretion in the upper respiratory tract, resulting in efficient bacterial shedding and transmission.

**Innate Immune Components**

In a mechanism to confront invading pathogens, epithelial cells secrete antimicrobial short peptides. Two types of antimicrobial peptides, cationic peptides and β-defensins, have been isolated from the upper respiratory tract and play a role in preventing infection (67). They target the plasma membrane, where these antimicrobial peptides form channels to disrupt the membrane's integrity, resulting in bacterial cell lysis (68). Lipopolysaccharide (LPS) and a glycosyltransferase, ArnT, in *B. bronchiseptica* have been reported to aid in the resistance against antimicrobial peptide-induced killing (69).

Another innate immune component combating bacterial infection is the complement system. Complement system components are generated in the liver and circulate throughout the blood system, ready to respond to potential invaders (70). In infections with *Bordetella* species, the activation of the complement system induces inflammation, bacterial killing via the complement membrane attack complex (MAC), and promotes phagocytosis through C3b-C3bR interactions or neutralized antibody-FcR interactions between bacteria and phagocytes (70). Two virulence factors in genus *Bordetella*, O-antigen and *Bordetella* resistance to killing factor A (BrkA), have been reported to resist complement-mediated clearance (71).

Previous studies have shown that infection with *B. pertussis* in mice will induce the recruitment and activation of macrophages and immature dendritic cells (iDCs), followed by an influx of neutrophils and natural killer cells (NKs) (72). Macrophages take up bacteria through phagocytosis, during which proinflammatory cytokines are secreted for the recruitment of other immune cells (73). DCs are the most potent antigen presenting cells and iDCs are matured after recognition of pathogen-associated molecular patterns (PAMPs) from invading pathogens. After maturation, DCs induce T-cell activation and produce numerous pro-inflammatory cytokines (74). *B. pertussis* and *B. bronchiseptica* have been reported to survive inside macrophages and dendritic cells, indicating that *Bordetella* can hijack the phagocytic mechanisms and use macrophages and dendritic cells to evade host immune monitoring and killing (75).

Neutrophils are important cells of the innate immune system and have been shown to have protective functions against various bacterial pathogens in the respiratory tract (76). In infections with *Bordetella* species, neutrophils are recruited to infected areas as early as 2 days post-infection (dpi), and reach peak abundance at 10-14 dpi (77). In previous studies, TCT, ACT, PT and PRN all showed properties which inhibit neutrophil recruitment or/and phagocytosis (45, 57, 77).

Natural Killer (NK) cells were shown to be another critical component in *B. pertussis* clearance (72). In mice lacking NK cells, *B. pertussis* disseminates from the respiratory tract and causes a lethal infection. Allavena *et al.* revealed that PT blocks monocyte chemotactic protein-1(MCP-1) mediated NK cell recruitment (78). In chapter 2, we discuss the functions of PRN in recruitment and activation of various innate immune cells in the upper respiratory tract during *B. bronchiseptica* infection.

**Adaptive Immune Components**

Antibody-mediated humoral immunity and cell-mediated immunity both play roles in protection against pertussis infection (72, 79-81). IgM, IgG and IgA are generated when vertebrate hosts are exposed to *B. pertussis* (72, 82, 83). Antibodies increase host defense by neutralizing toxins, inhibiting attachment of bacteria to host cells, and enhancing phagocytic uptake of various phagocytes by C3b- or FcR-mediated pathways (84). In natural infections with *B. pertussis*, Th1 and Th17 cell responses are both induced (79). A study from Mills *et al.* showed that in T cell-deficient mice, *B. pertussis* caused persistent infection and failed to be cleared from hosts, indicating a key role of T cells in the control and clearance of *B. pertussis*. After transferring CD4+ T cells into nude (athymic) mice, the recipient mice cleared the infection from the lung within 20 days, suggesting an important role of CD4+ cells in protective immunity to *B. pertussis* (85). Accordingly, *Bordetella* species have developed mechanisms to interfere with the functions of adaptive immunity. A study from our group showed that a putative sigma factor, *btrS* in *B. bronchiseptica,* suppresses the recruitment of B and T cells to the lung, disrupting host mechanisms that facilitate bacterial clearance from respiratory tract (86). Also, the *B. bronchiseptica* Type 3 secretion system was shown to induce IL-10 expression and inhibit IFN-γ

production, leading to a decreased antibody-mediated bacterial clearance (87). In chapter 2, we found that pertactin contributes to the recruitment of T and B cells to the nasal cavity. We also demonstrate that pertactin appeared not to contribute to colonization but induced inflammation, and elevated transmission efficiency to naïve hosts.

### *Bordetella* Transmission

*B. pertussis* transmission has been demonstrated to occur via aerosolized respiratory droplets in non-human primate model (88). Due to the prevalence and morbidity of pertussis disease in infants and young children, various vaccination strategies aimed at reducing transmission to young age groups have been utilized. These include different vaccine formulas for initial doses, maternal immunization, and "cocooning" strategies whereby the immediate family in frequent contact with vulnerable neonates are vaccinated (89, 90). However, previous studies showed that while acellular vaccines prevented immunized hosts from developing severe symptoms of whooping cough, they failed to prevent colonization and bacterial transmission among human hosts (91). By using *B. bronchiseptica* transmission models, Smallridge *et al.* proved that a heat-killed whole cellular vaccine was able to reduce bacterial shedding and control bacterial transmission, while acellular vaccine immunized hosts had similar shedding levels to nonimmunized groups, resulting in a relatively high transmission level (92). Therefore, a new or enhanced pertussis vaccine capable of preventing transmission is urgently needed.

A two-component regulon, RisA, has been suggested to play a role in the transmission of *B. pertussis* because of its regulatory function on the expression of motility and extra host survival genes (93). Our group found that an extracellular polysaccharide (tEPS) locus only has modest effect on colonization in murine model but plays critical roles in bacterial transmission (94). In my

study, I found that the outer membrane protein, pertactin, contributes to both shedding and transmission of *B. bronchiseptica*. The results from this study are presented in chapter 2.

**Animal Models for Infection and Transmission**

Different kinds of animal models, including mice, pigs and non-human primates, have been used for studies on the mechanisms of natural and vaccine-induced immunity to *B. pertussis* and in the development of improved pertussis vaccines (91, 92, 94, 95). A recently developed non-human primate model is capable of mimicking pertussis infections with characteristic paroxysmal coughs (95). Warfel *et al.* used baboon models of *B. pertussis* infection and studied pertussis pathogenesis and disease progression(96). Their data showed that after baboons were infected with *B. pertussis*, they developed respiratory colonization and leukocytosis (97). The infected baboon was confirmed to transmit to new hosts via the resultant cough produced by *B. pertussis* infection. In a study comparing the efficacy of acellular pertussis vaccine and whole cellular pertussis vaccine, acellular vaccines protected baboons from severe disease, but failed to prevent transmission, while whole cell vaccines were able to clear infections and prevent transmission better than acellular vaccines(91). In addition, pigs that are infected with *B. bronchiseptica* will develop a wide range of respiratory symptoms, including coughing and sneezing, and antibodies, IgG and IgA, were detected in blood and mucosal surfaces, respectively (98). The well-described disease progress and human-like immune system makes porcine models of pertussis useful for vaccine development. However, the prohibitive costs of establishing and conducting experiments using porcine, non-human primate, and other large animal models is a major limitation.

The efficiency and availability of genetically engineered murine models have been a valuable tool to understand the pathogenesis of pertussis infection, the mechanisms of host immune response

against pertussis, and in the development and testing of improved pertussis vaccines. Even though mice infected with *B. pertussis* do not develop characteristic cough that can manifest in humans, they do have many other features that mimic natural host infections (99). Currently, the widely used (100) mouse model involves a large number of bacteria that are delivered into the lung directly, leading to severe pneumonic disease and a strong immune response (57). However, natural infections begin in the nasopharyngeal region, progressing slowly during a prolonged catarrhal stage, reaching the trachea over a period of 2-3 weeks and rarely involve the lungs. In this study, a more natural experimental system takes advantage of the ability of *B. bronchiseptica*, a closely related species, to naturally colonize mice with inocula as low as 5 CFU. In this system, numerous aspects of pathogenesis can be monitored, including natural colonization, bacterial spread within hosts, progressive immune responses and bacterial shedding and transmission between hosts, offering new tools to understand these important aspects of *Bordetella* infections.

**Preface**

In the following chapters, we will exam the role of various Bordetella virulence factors in the colonization, persistence and transmission of *Bordetella spp*.

In chapter 2, we used a Bordetella natural infection mouse model to exam the role of pertactin on colonization, growth, spread within the respiratory tract, shedding and transmission between hosts. An isogenic pertactin deletion mutant was demonstrated to be similar to its wild-type (WT) parental strain in terms of colonization of nose, trachea and lungs of mice. However, the pertactin-deficient strain was shed from the noses of infected mice in much smaller numbers, resulting in lower transmission between hosts, indicating a role for pertactin in the shedding and transmission of *B. bronchiseptica*. In chapter 3, we speculated the possible reasons on why only pertactin is being lost from the majority of circulating *B. pertussis* strains. in the United States and

other countries. The first is the possible functional redundancy of pertactin. The second is the relatively longer functional persistence of antibodies against pertactin . The third possible reason is its close association with the outer, membrane directing productive complement fixation to that membrane.

*B. bronchiseptica* showed capacity to survive inside the common soil amoeba *Dictyostelium discoideum* and used it as a transmission vector. In chapter 4, we tested if this machinery that allows *B. bronchiseptica* to interaction with predatory amoeba is conserved in some other *Bordetella* species. In this chapter, we tested 9 diverse *Bordetella* species in assays representing three separated aspects of their interactions with *D. discoideum:* intracellular survival, plaque expansion, and translocation to the amoebic sori. The results showed that the species known to have been isolated from various sources generally retain the ability to successfully interact with amoebae. In contrast, these abilities are partly degraded in the two human-specialized species, *B. pertussis* and *B. parapertussis*.

*B. pseudohinzii* infection persists in the respiratory tract and middle ears for a much longer time compared to that of *B. bronchiseptica*. In chapter 5, through a genome wide comparison between *B. bronchiseptica* and *B. pseudohinzii*, a pertussis toxin like locus appears in the genome of *B. pseudohinzii* but not *B. bronchiseptica*. To investigate the role of these toxin genes in the pathogenesis of B. *pseudohinzii*, a toxin subunit A deletion mutant was generated to compare colonization and persistence profiles with *B. pseudohinzii* wild-type (WT) strain. We observed that a mutant strain lacking the subunit A failed to persist in the respiratory tract and middle ears of mice. However, the WT and mutant strain had similar bacterial persistence in mice deficient in B cells and T cells, indicating that pertussis toxin like factor intervenes the function of B cells and T cells.

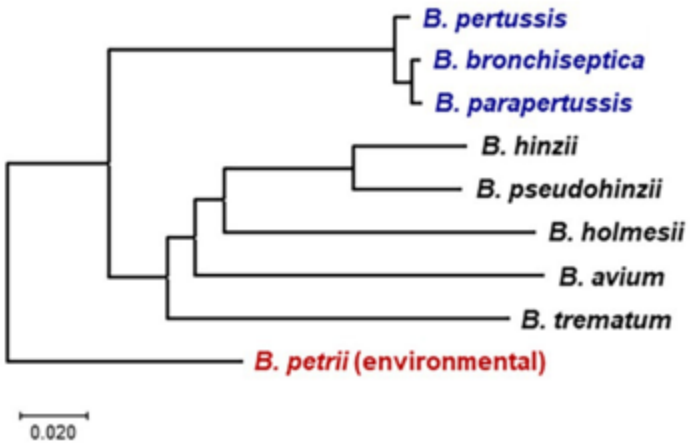


**Figure 1.1 The phylogenic relationships between *Bordetella spp*.** The phylogenic tree was generated based on pairwise average nucleotide identities.

Modification of a figure created by Dr. Bodo Linz.

**Table 1.1 The primary host rang(s) of each *Bordetella* species.**

| *Bordetella* species | Host |
|---|---|
| *B. bronchiseptica* | variety of mammals, including mice, dogs, cats, rabbits, humans etc. |
| *B. parapertussis* | humans and sheep |
| *B. pertussis* | humans |
| *B. hinzii* | turkeys, rabbits, humans |
| *B. pseudohinzii* | mice |
| *B. avium* | birds |
| *B. holmsii* | humans |
| *B. trematum* | humans |
| *B. ansorpii* | humans |
| *B. petrii* | Humans, environmental |

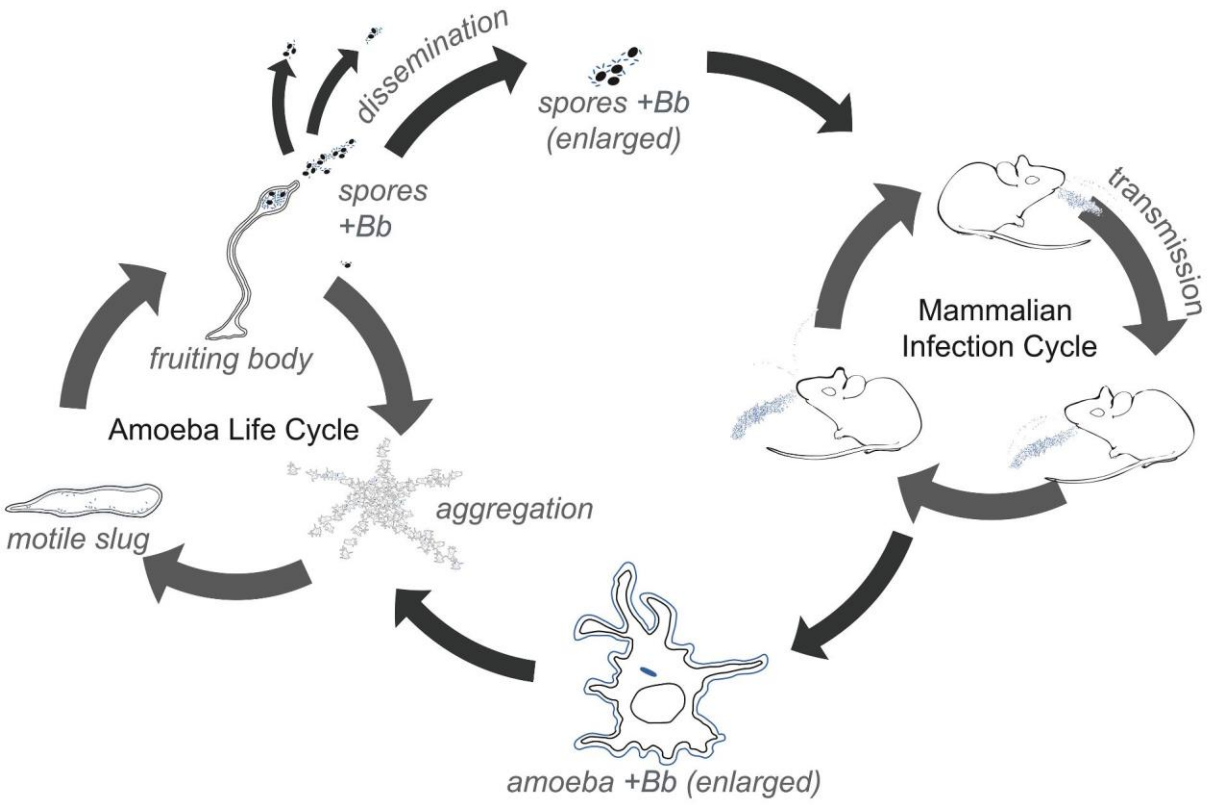


**Figure 1.2 The interconnected life cycles of *Bordetella bronchiseptica* with amoeba and mammals.** The model illustrates the survival and transmission of *B. bronchiseptica* (blue) both in mammalian hosts and along with the amoeba.

Modification of a figure created by Dr. Dawn Taylor.

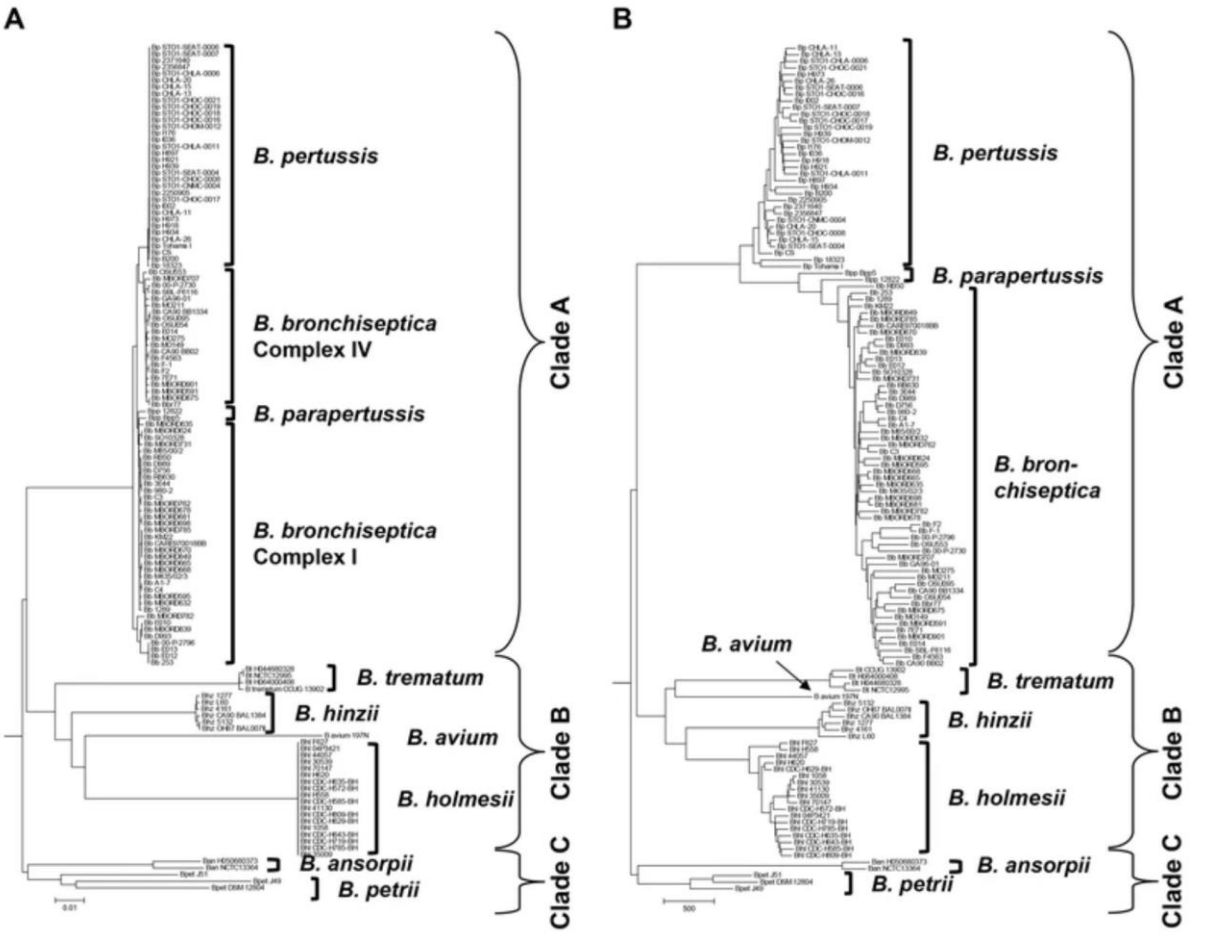


**Figure 1.3 Phylogenetic structure (Neighbor-joining trees) according to A) a genome-wide sequence alignment, and B) presence or absence of genes in 128 genomes from nine species of the genus *Bordetella***. The Bordetellae evolved into 3 clades of species: the phylogenetically oldest clade C formed by *B. petrii* and *B. ansorpii*, clade B formed by *B. trematum*, *B. hinzii*, *B. avium* and *B. holmesii*, and clade A containing the classical bordetellae *B. bronchiseptica*, *B. parapertussis* and *B. pertussis*.

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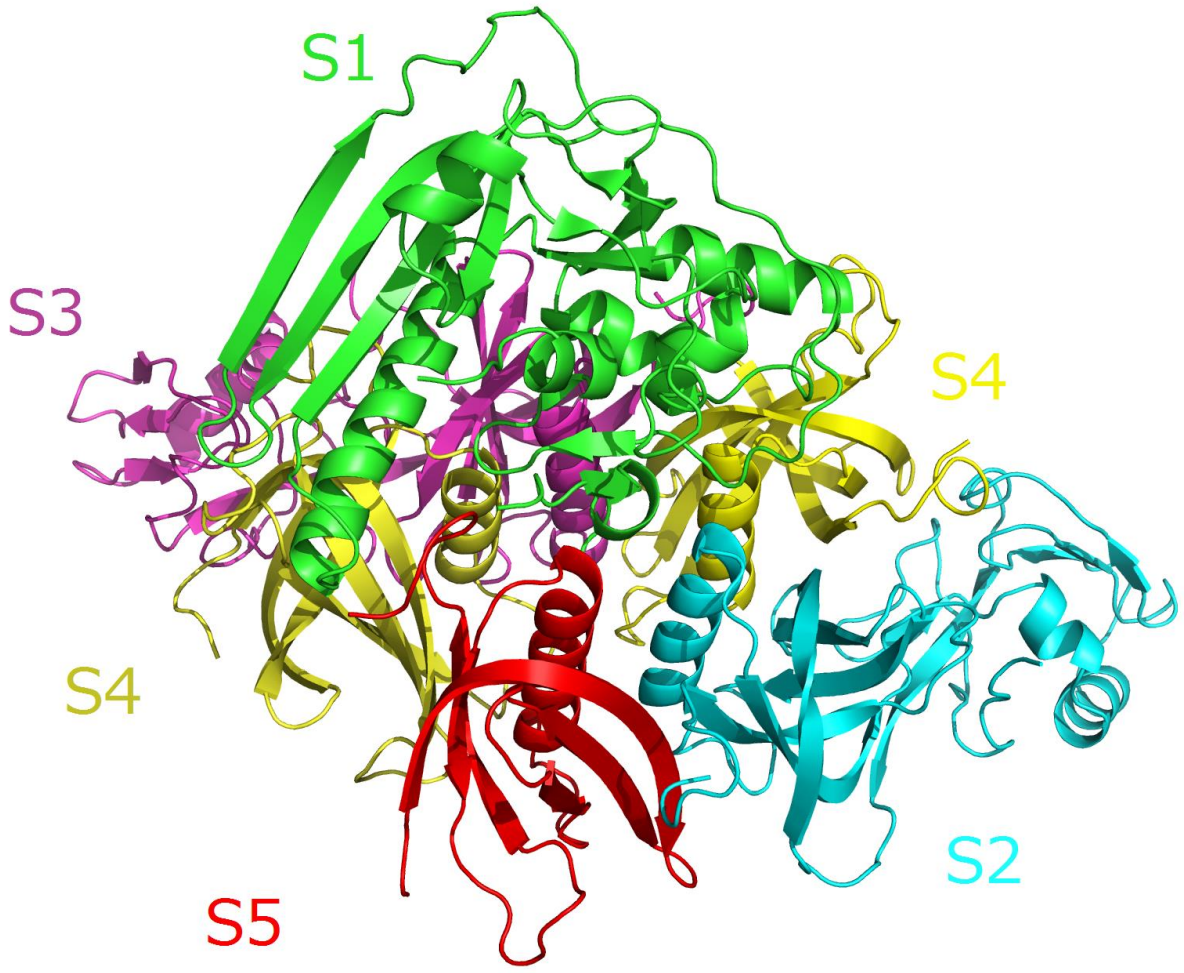


**Figure 1.4 Digital representation of the molecular structure of pertussis toxin.** Pertussis toxin isolated *from B.* pertussis is composed of 5 subunits. S1: green S2: cyan S3: purple S4: yellow (double) S5: red.

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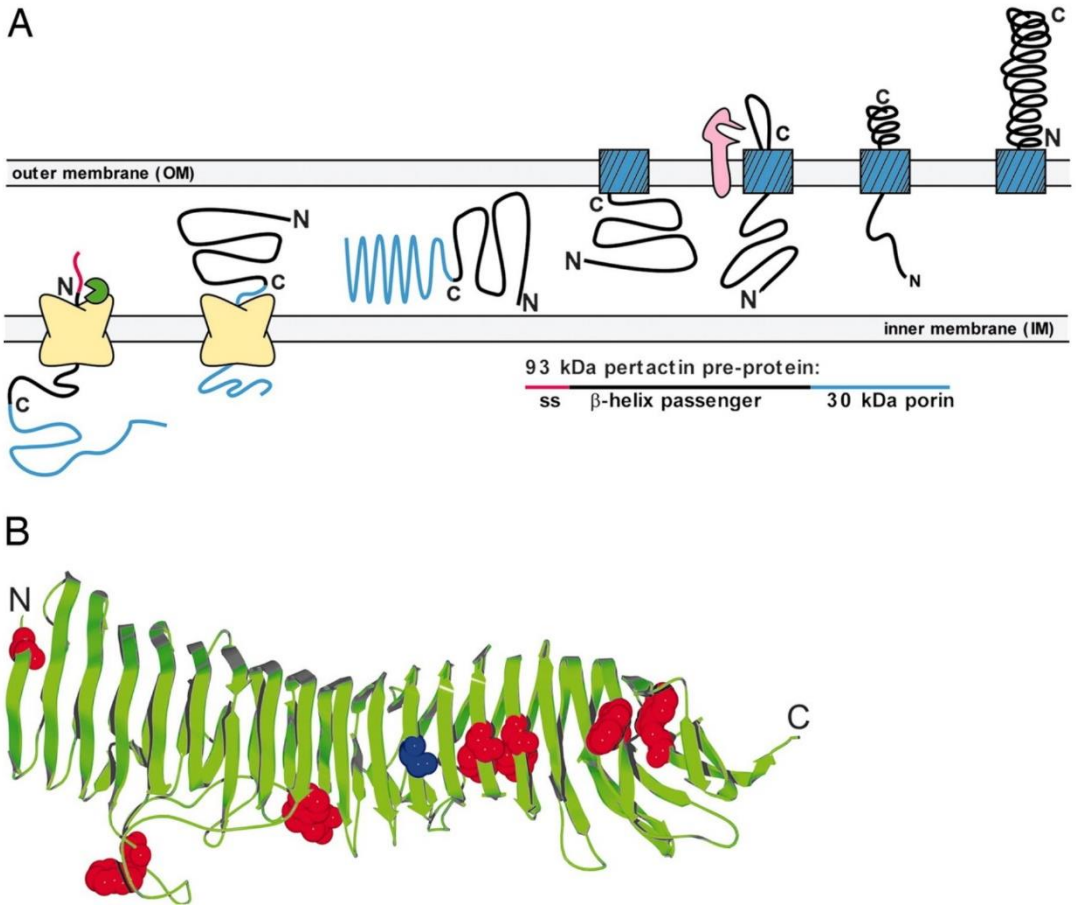


**Figure 1.5 Pertactin secretion mechanism and crystal structure of mature pertactin.** A) The models for pertactin secretion include passage through the IM via an N-terminal signal sequence (pink), followed by insertion of the 30-kDa C-terminal porin sequence (blue) into the OM; the passenger domain (black) then passes through the central pore of the porin before folding to the native structure outside the cell. B) The crystal structure of a mature pertactin protein. Modification of figure created by Junker, *et al. Proceedings of the National Academy of Sciences* 103.13 (2006): 4918-4923 (101).

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# CHAPTER 2

# PERTACTIN CONTRIBUTES TO SHEDDING AND TRANSMISSION OF *BORDETELLA BRONCHISEPTICA*[1]

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**Abstract**

Whooping cough is resurging in the USA despite high vaccine coverage. The rapid rise of *Bordetella pertussis* isolates lacking pertactin, a key vaccine antigen, has led to concerns about vaccine-driven evolution. A recognizable RGD motif motivated studies demonstrating that pertactin can mediate adhesion to host cells *in vitro*. However, *in vivo* studies using the conventional high dose lung infection mouse model demonstrated pertactin contributes to immunomodulatory effects of *B. bronchiseptica,* a close *evolutionary* progenitor of *B. pertussis* that naturally infects mice, and that these effects are independent of the RGD motif. To further investigate *in vivo* roles, we examined the functions of pertactin in the context of newer, more natural, experimental systems that are more sensitive to its effects on colonization, growth, spread within the respiratory tract, shedding, and transmission between hosts. A *B. bronchiseptica* pertactin deletion mutant was found to similar to its isogenic wild-type (WT) parent strain in colonization of nose, trachea and lungs of mice. However, the pertactin-deficient strain was shed from the noses of mice in much lower numbers, resulting in a much lower rate of transmission between hosts. Histological examination of respiratory epithelia revealed that pertactin-deficient bacteria induced substantially less inflammation and mucus accumulation than the WT strain. In addition, only WT *B. bronchiseptica* were recovered from the spleen and observed within isolated splenocytes of inoculated hosts, indicating that pertactin contributes to intracellular survival and systemic dissemination. These results suggest pertactin mediates interactions with immune cells and augments inflammation that contributes to bacterial shedding and transmission between hosts. The frequent loss of pertactin from circulating strains suggests that these functions may be dispensable in *B. pertussis*, perhaps due to alternative strategies and/or redundancy. Understanding

the relative contributions of various factors to inflammation, mucus production, shedding and transmission will guide novel strategies to interfere with the reemergence of pertussis.


## Summary

*B. pertussis* strains lacking pertactin have been rising in prevalence especially in countries using acellular vaccines containing pertactin as a key, membrane-associated surface antigen. Previous *in vivo* studies revealed immune modulatory properties of pertactin in conventional *B. pertussis* infection models in which roughly one million bacteria are delivered into lungs, leading to severe pneumonic disease and a strong immune response. However, natural infections begin in the nasopharyngeal region, progress slowly during a prolonged catarrhal stage, reaching the trachea over a period of 2-3 weeks and rarely involve the lungs. In this study, a more natural experimental system takes advantage of the ability of *B. bronchiseptica*, a closely related species, to naturally colonize mice with inocula as low as 5 colony forming units (CFU). In this system *B. bronchiseptica* can be observed to efficiently colonize, grow, spread within the respiratory tract, shed, and transmit between hosts, allowing each of these steps to be measured and studied. Under these conditions, an isogenic pertactin deletion strain was indistinguishable from its parental strain in its abilities to colonize, grow in numbers and spread within the respiratory tract. However, the pertactin-deficient mutant was shed from these mice in lower numbers than wild type, and it was defective in transmission between mice. These assays reveal novel roles of pertactin in the induction of inflammation, mucus production, shedding and transmission.


## Introduction

Pertussis, or whooping cough, is an acute respiratory disease caused by the gram-negative bacterial pathogen *Bordetella pertussis*. Pertussis had a high morbidity and mortality rate and was the predominant childhood killer before the introduction of whole cell vaccines (WCV) that greatly

reduced its prevalence (1). In highly vaccinated populations, the absence of severe disease led to attention focused on the moderate side effects associated with WCV, including common local reactions (swelling and pain), uncommon systemic reactions (fever, irritability, drowsiness, loss of appetite and vomiting), and very rare neurological reactions (acute encephalopathy in newborns) (2). To address these concerns, less reactive pertussis acellular vaccines (ACV) containing up to five purified, detoxified *B. pertussis* proteins, including pertussis toxoid, pertactin, type 2 and 3 fimbriae, and filamentous hemagglutinin, were introduced in the 1980s and 1990s, and eventually replaced the WCV in most developed countries (3).

Since the switch to ACV, the incidence of whooping cough has been increasing in all age groups, from infants to adults (4). Moreover, in recent years several countries have seen an increasing percentage of clinical isolates that fail to express pertactin (PRN), a prominent outer membrane protein (5, 6). The loss of PRN, which is one of the ACV components used by several countries, has led to concern that pertussis vaccines are driving the evolution of *B. pertussis* (7).However the long-term consequences of this possibility are difficult to predict since we only have limited knowledge of the biological functions of PRN.

PRN is an autotransporter protein that appears on the outer membrane of *B. pertussis* (8). This protein has been included in the currently used acellular vaccine in several countries, due to the critical role of pertactin specific antibodies in *B. pertussis* immunity. Previous studies revealed that PRN exerts cell binding through its RGD motif and contributes to adherence and invasion of mammalian cells (9-12). However, the adhesion functions of pertactin were not detected in some other studies (13-16). *In vivo* studies have shown an immunomodulatory role of PRN (17, 18), in the context of severe pneumonic infection initiated by supernatural challenge with roughly a million CFU of *B. pertussis* or *B. bronchiseptica* delivered deep into the lungs of mice where they

caused severe lung pathology. This experimental system was established to study the most extreme form of disease and has enabled the development and testing of vaccines and therapeutics to prevent and treat severe disease in the lower respiratory tract. Natural *B. pertussis* infections begin with the catarrhal stage, which is limited to the nasopharyngeal region and is a highly contagious period. The very high infectiousness of *B. pertussis* suggests that small numbers of organisms can initiate colonization of the upper respiratory tract. Thus, the weeks of growth within and shedding from the upper respiratory tract are critical aspects of the infectious cycle of *B. pertussis*. However, these aspects are not simulated in the standard inoculation approach that delivers a million bacteria deep into the lungs and measures subsequent pneumonic pathology (19-21).

We have focused on simulating the progression of natural infections in order to study the contributions of individual bacterial factors and host immune functions. We use *B. bronchiseptica* because it naturally and highly efficiently colonizes, grows, causes pathology, is shed, and transmits between mice. Importantly, it can efficiently colonize mice when delivered to the external nares in very small numbers, allowing the entire natural progression of the infection to be studied, including many aspects that are obviated by the conventional approach of washing millions of bacteria deep into the lungs. *B. pertussis* and *B. bronchiseptica* differ in some of their gene complements(22-24) but are very closely related and shared genes are ~ 98% identical at the nucleotide level. Both species infect mammals and, with the notable exception of expression of the pertussis toxin, share nearly all genes implicated in interacting with their hosts, including the vaccine antigens fimbriae (FIM), filamentous hemagglutinin (FHA) and PRN (25). But unlike *B. pertussis*, *B. bronchiseptica* efficiently colonizes mice with an infectious dose less than 5 CFU (26). *B. bronchiseptica* infections progress efficiently, allowing the study of the mechanistic basis for their complex interactions in the context of naturally progressing infection in the natural host.

We also previously described *B. bronchiseptica* efficiently transmitting among TLR4-deficient C3H/HeJ mice (26-29). Although these animals are immunodeficient in their ability to detect and respond to bacterial infections, this experimental model allows all the bacterial components necessary for efficient transmission to be experimentally examined to better understand the transmission process.

In this study, to investigate the biological role(s) of PRN in the infectious process and pathogenicity of *Bordetella* species, we used a mutant of *B. bronchiseptica* with an in-frame deletion in the *prn* gene which was generated by allelic exchange as described by Inatsuka *et al.* (18). *In vivo* experiments showed that *prn* was not necessary for efficient colonization and early growth in the nasopharynx but was required for efficient bacterial shedding and transmission between mice. Furthermore, wild-type *B. bronchiseptica* induced higher levels of inflammation and more mucus secretion than the *prn* mutant, revealing a role for PRN in promoting inflammation and mucus secretion. Moreover, the WT strain, but not the *prn* deletion strain, was recovered from splenocytes of infected mice, indicating that PRN contributes to intracellular survival of *B. bronchiseptica*, leading to a systemic dissemination. Together these data suggest that PRN contributes to the induction of inflammation and mucus production, mediates shedding and transmission to new hosts and, is involved in the intracellular survival and systemic dissemination of the pathogen.

## Results

### PRN is not necessary for *B. bronchiseptica* to efficiently colonize the host and grow.

To evaluate the contribution of pertactin to various aspects of the biology of *B. bronchiseptica*, we used an isogenic *prn* gene deficient mutant (*Bb*Δ*prn*) of *B. bronchiseptica*

strain RB50 (*Bb* WT) (18), which is well-established as being highly efficient in colonizing, persisting, and transmitting among mice. To confirm the deletion was clean and not complicated by other changes, we verified the whole genome by sequencing and the in-frame deletion was verified (S2.1 Fig). The *prn* deletion strain showed no defect in *in vitro* growth, adherence to human alveolar epithelial cells or cytotoxicity to murine RAW 264.7 macrophages (S2.2-2.4 Figs). Using the conventional pneumonic infection model, C57BL/6 mice were inoculated intranasally with 50 µL PBS buffer containing $5x10^5$ CFU of either WT or *Bb*Δ*prn* bacteria. Bacterial numbers in nasal cavities, trachea and lungs harvested at 7-, 14- and 28-days post-inoculation (dpi) did not differ significantly between mice infected with *Bb* WT or *Bb*Δ*prn*, (S2.5 Fig). Interestingly, *Bb*Δ*prn* showed a higher colonization level in the nasal cavity and lunglungslung at 14 dpi, but these differences not observed in later timepoints. Histopathological analysis of nasal cavities at 7 dpi and 14 dpi detected mild suppurative inflammation in both groups with similar incidence and severity. Thus, the standard high dose pneumonic infection experimental system did not reveal a significant role for PRN in the pathogenesis of *Bb*.

**PRN contributes to transmission.**

We recently developed an experimental system to study various aspects of the process of transmission between hosts using C3H/HeJ mice (26-30). Before examining the effects of PRN on transmission, we first assessed the time course of infection in these mice. C3H/HeJ mice were inoculated with 150 CFU of either *Bb* WT or *Bb*Δ*prn* in 5 µL of PBS, a volume that deposits the bacterial inoculum only into the nasal cavity. Respiratory organs were harvested at 7-, 14- and 28-dpi. Both bacteria grew over time, with similar numbers observed in nasal cavity, trachea and lung

(S2.6 Fig), suggesting that PRN has no critical role in colonization, growth, and spread within the respiratory tracts of these mice.

To assess the contribution of PRN to transmission, pairs of C3H/HeJ mice were inoculated with 150 CFU of either *Bb* WT or *Bb*Δ*prn* strain (donor mice) and then co-housed with two naïve C3H/HeJ mice (uninfected recipient mice) in cages of four. To evaluate bacterial transmission, the nasal cavities of 12 such co-housed naïve mice per strain (from 6 cages) were assessed for bacterial colonization after 21 days of co-housing with infected mice. In the *Bb* WT group, all 12 co-housed naïve mice became colonized, while *Bb∆prn* bacteria were only transmitted to 5 out of 12 co-housed naïve mice (Fig 2.1A), suggesting that PRN contributes to bacterial transmission between hosts.

**PRN contributes to bacterial shedding.**

The deficiency of *Bb*Δ*prn* in transmission could be due to either a defect in shedding from infected hosts or colonization of exposed hosts. To test the efficiency of colonization, we inoculated C3H/HeJ mice with decreasing doses of bacteria. Both *Bb* WT and *Bb*Δ*prn* efficiently colonized the respiratory tracts of all mice with a calculated inoculation dose of ~5 CFU (S2.7 Fig) indicating that PRN is not required for efficient colonization of the respiratory tract.

Since PRN contributes to transmission but is not required for efficient colonization, we investigated its effect on shedding from the noses of challenged mice. C3H/HeJ mice (n=10) were inoculated with 150 CFU of either *Bb* WT or *Bb*Δ*prn*, and bacteria shed from the nose were collected by gently swabbing the external nares with Dacron-polyester tipped swab every to two- or three-days post inoculation. Throughout the experiment, WT bacteria were shed from the nares at high numbers, > 1000 CFU at multiple consecutive timepoints, indicating prolonged, high level

shedding. In contrast, the *Bb∆prn* strain was shed at 10 to 100-fold lower numbers from the first day of shedding to the end of the experiment at day 21 (Fig 2.1B), revealing a critical role for PRN in efficient shedding from the nose to the environment.

**PRN contributes to inflammation in the nasal cavity.**

The striking difference in shedding, without a substantially different load of bacteria in the respiratory tract, led us to speculate that PRN might induce shedding by altering the inflammatory state and/or mucus production. To test this hypothesis, C3H/HeJ mice infected with *Bb* WT or *Bb*Δ*prn* were collected at 7 and 14 dpi for histopathological analysis. Histopathology showed that in the WT group, all 10 analyzed mice had inflammation in the nasal cavity ranging from mild to severe (Figs 2.2G-2.2I). In contrast, of the *Bb*Δ*prn* infection group, 2 out of 10 had no apparent inflammation and the other 8 had severity scores which varied from minimal to mild (Figs 2.2D-2.2F). These data indicate that PRN is involved in induction of inflammation in the nasopharynx (Fig 3A; S1 Table). To probe the effect of PRN on immune cell recruitment, flow cytometry was used to analyze immune cell populations in nasal cavities 14 days after inoculation with *Bb* WT or *Bb*Δ*prn* (Fig 2.4). *Bb* WT induced a significant increase in numbers of neutrophils, NK cells, macrophages, and B cells in the nasal cavities (Fig 2.4C-2.4F). In contrast, *Bb*Δ*prn* induced an increase in macrophages, but no significant increase of other cell types (Fig 2.4E). These results indicate that PRN plays a role(s) in activation and recruitment of neutrophils, NK cells, and B cells, leading to higher inflammation levels in the nasal cavity.

**PRN increases mucus secretion in acute inflammation.**

The dramatically higher number of bacteria shed from mice infect with *Bb* WT than those given *Bb⊿prn* led us to examine whether increased mucus production might be involved. To compare mucus secretion in the nasal cavity of *Bb* WT infected and *Bb⊿prn* infected mice, Alcian blue-Periodic acid Schiff (PAS) staining was performed on tissue sections. At 7 dpi all 5 *Bb* WT infected mice displayed moderate mucus secretion. In contrast, 2 out of 5 *Bb*Δ*prn* infected mice displayed only minor mucus accumulation and the other 3 showed no mucus accumulation (Fig 2.3B). The higher mucus secretion in *Bb* WT infected mice compared to *Bb*Δ*prn* infected mice indicates that PRN plays a role in the induction of mucus production during the acute phase of infection.

**PRN contributes to intracellular survival and systemic dissemination.**

*Bb* has been shown to be able to invade and persist in immune cells, which can have profound effects on the local and systemic immune responses (31-33). Since PRN has been implicated in interactions with immune cells, we tested its effects on access to and invasion of immune organs (18, 34). We harvested spleens from mice inoculated with 150 CFU of either *Bb* WT or *Bb*Δ*prn* at 7-, 14- and 28-dpi. *Bb* WT was recovered from 3 of 4 spleens and 5 of 8 spleens of infected mice on days 7 and 14, respectively, while no bacteria were found in the spleens of any *Bb*Δ*prn* inoculated mice (Fig 2.5A). To examine whether *Bb* WT in the spleen were surviving intracellularly, single cell suspension of splenocytes were treated with gentamicin treatment to kill the extracellular bacteria. All mice with *Bb* WT in their spleen had a substantial fraction that survived gentamicin treatment (Fig 2.5B), indicating they were surviving within the splenocytes.

Infectious stimuli can activate the inflammasome complex within immune cells, which is known to trigger the cleavage, activation, and secretion of IL-1β and IL-18 (35, 36). We therefore

investigated inflammasome activation in murine macrophages exposed to *Bb* by quantifying the secretion of IL-1β and IL-18 in the cell culture supernatants. Increased levels of IL-18 were secreted when cells were exposed to *Bb*Δ*prn* than to *Bb* WT (S2.9 Fig). The substantially decreased secretion of IL-18 induced by the wild-type bacteria suggest that PRN can suppress this measure of inflammation in this *in vitro* assay. However, the sudden exposure of cells to extremely high doses of bacteria in such assays might not fully represent in vivo conditions.

## Discussion

Previous studies have used different and unrelated clinical isolates of *B. pertussis* with or without PRN, for example the utilization of an isolate lacking PRN sustained a longer infection in mice immunized with an acellular pertussis vaccine (37). Such differences cannot be specifically attributed to the loss of PRN, since the genomes of these two separate isolates differ in many other genes. Moreover, because *B. pertussis* poorly colonizes, sheds, and transmits between mice, it has been difficult to assess the role of PRN in these critical aspects of the infectious cycle. To address these problems, we and Inatsuka *et al*. used an in-frame deletion of *prn* in *B. bronchiseptica*, a close *evolutionary* progenitor of *B. pertussis* that naturally infects mice, to test the roles of PRN in infections of a natural host. In the conventional *Bordetella* experimental system in which a very high inoculation dose ($5 \times 10^5$ CFU) delivered in a large volume (50 µL) to deposit bacteria deep in the lungs, PRN had a modest effect in BALB/c mice in the prior study. In our high dose pneumonic infection in C57BL/6 mice assessed at different time points, we did not observe measurable effects of *prn* deletion on bacterial growth or induction of respiratory tract pathology. Since BALB/c and C57BL/6 mice have different immune responses upon *B. pertussis* infection or vaccination (38-40), the different genetic backgrounds of two mouse strains may explain the different observations in these two studies. Alternatively, we have recently demonstrated that in

this conventional pneumonic infection experimental system, the very large numbers of bacteria delivered deep into the lung bypass many aspects of the early stages of natural infection, instead rapidly inducing massive lung damage and triggering an artificially robust and rapid immune response that results in relatively rapid clearance of bacteria (33, 41-48). To better simulate a more gradual and natural *Bordetella* species infection, we used a low dose inoculation model in which an experimental dose as low as 5 CFU is delivered into the external nares of mice in a 5µl droplet that deposits bacteria only in the nose. In this more natural experimental system, PRN was not required for efficient colonization and spread within inoculated hosts, but *Bb* WT-inoculated mice shed significantly more bacteria and transmitted between mice much more efficiently than *Bb*Δ*prn*-inoculated mice (Fig 2.1). *Bb* WT also induced significantly more inflammation and mucus production than RB50Δ*prn*. Inflammation and mucus production contribute to the rhinorrhea that facilitates shedding and transmission, as noted previously with *Streptococcus pneumonia* transmission (49).

PRN, as an outer surface protein with a demonstrated ability to affect adhesion to mammalian cells *in vitro* (9-11), has been considered an "adhesin". However, it is not clear that PRN contributes to adhesion to either bronchial or laryngeal cells *in vivo*. In this study, deleting PRN did not affect the ability of *B. bronchiseptica* to efficiently colonize mice with a remarkably small inoculum, indicating that PRN is not required for the efficient adherence involved in initial colonization. However, *Bb* WT showed significantly higher recovery from spleens of infected hosts, with substantial numbers intracellular within splenocyte (Fig 2.5), suggesting that PRN may affect invasion of immune cells and/or phagocytosis Upon encountering invading bacteria, immune cells trigger cascades of inflammatory responses by secreting cytokines, chemokines, small lipid mediators (SLM), and antimicrobial peptides (AMPs) that can contribute to increased

phagocytic capacity and bacterial clearance (50), amongst other potential effects. After phagocytosis, peripheral immune cells can carry engulfed bacteria to deeper tissues including draining lymph nodes for T cell priming (51). Thus, systemic dissemination may result in a stronger immune response against *Bb* WT than *Bb*Δ*prn*, consistent with the histopathology analysis and IgG antibody titers observed (Figs 2.2; S2.8).

The increasing prevalence of PRN-deficient *B. pertussis* strains raise questions about both positive and negative (purifying) selection. It appears increasingly likely that PRN-containing ACV's select for PRN-deficient *B. pertussis*. It is also likely that the epidemiology of pertussis has changed as host behavior, population density, and worldwide travel affect the network of connected hosts. Even though PRN-deficient *B. pertussis* isolates may be shed less, a dense and well-connected population of vaccinated hosts in countries like the USA may be sufficient for a successful chain of transmission. Alternatively, the loss of PRN may have less cost in *B. pertussis* due to compensation by other genes. There are 15 other autotransporter genes in the genome of *B. pertussis* (5, 25), one or more of which may compensate for the loss of PRN. Since PRN plays roles in systemic dissemination and induction of inflammation murine murine transmission models (Figs 2.2-2.5), other factors facilitating systemic dissemination and promoting inflammation may compensate for its loss. Alternatively, since *B. pertussis* is different from *B. bronchiseptica* in various aspects, the functions of PRN in these two species may vary to some extent. To identify the role of pertactin in the transmission of *B. pertussis*, an efficient *B. pertussis* transmission model is urgently needed. In addition, further studies are needed to identify factors that might compensate for the loss of PRN in current circulating strains. This might lead to the identification of alternative antigens to include in next generation vaccines against *B. pertussis*.

## Material and methods

### Bacterial strains and growth

*B. bronchiseptica* WT strain RB50 and isogenic pertactin knockout strain *Bb*Δ*prn* (SP5) have been previously described(11, 18). To allow mutant and WT to be distinguished in "competition assays", SM10λpir cells carrying allelic exchange vector pEH10 was used to generate gentamicin-resistant Δ*prn* mutant strain. The generation of pEH10 was described previously(52). Liquid cultures were prepared using Stainer Scholte (SS) medium supplemented with 0.5% (w/v) Heptakis (2,6-di-O-methyl)-β-cyclodextrin) (Sigma H0513). Plate cultures were grown on Bordet Gengou (BG) agar supplemented with 10% (v/v) defibrinated sheep blood (Hemstat, Hemostat Laboratories) and streptomycin (20 μg/mL). Comparative growth curves were generated from triplicate cultures of bacteria grown 48 hours in SS medium at 37°C and shaking at 200 rpm.

### Adherence assay

Adherence assays were conducted following protocols described earlier (12, 53). In brief, human epithelial lung A459 cells were seeded in triplicate in 24-well plates at a density of $2.5 \times 10^5$ cellcell/well in Dulbecco's modified Eagle's medium (DMEM) (supplemented with 10% fetal bovine serum, 10 mM glutamine, 25 mM sodium pyruvate, 10 mM HEPES). Log-phase bacteria were suspended in warm DMEM medium and added to each well at a multiplicity of infection of 10:1 (bacteria: eukaryotic cells). The plate was centrifuged at 300X*g* for 10 minutes to synchronize infection and the assay plates were incubated for 5 minutes at 37°C. Unattached bacteria were then removed by washing the cells 4 times with 1 mL phosphate-buffered saline (PBS). A459 cells were lysed with 100 μL of 0.1% sodium deoxycholate for 5 minutes and released bacteria suspended in 900 μL of PBS. The bacteria were enumerated by dilution plating on BG agar plates.

RB50 Δ*fha*B (12), a mutant strain deleted of the gene encoding the filamentous hemagglutinin and known to be defective in adherence, was used as the negative control.

**Cytotoxicity Assay**

Cytotoxicity assays were conducted on RAW 264.7 cells, using the CytoTox 96 Nonradioactive Cytotoxicity Assay Kit (Promega) following manufacturer's protocols. In brief, 100 μL of $2.5 \times 10^4$ macrophages were seeded in triplicate in a 96-well plate followed by adding bacteria at a multiplicity of infection of 10:1. The assay plate was centrifuged at 300X*g* for 10 minutes. Bacteria were incubated with the macrophages for 4 hours, following which the plate was centrifuged for 5 minutes (300X*g*). 50 μL of the supernatant was placed into a fresh flat-bottomed 96-well plate and was calorimetrically assayed for lactate dehydrogenase. A noninfected group was used as a negative control (S 2.4 Fig).

**Cytokine Test**

To assess inflammasome activation in macrophages, the supernatant of infected cells (MOI=1 or MOI=10) was exposed to either WT or mutant strain for 4 hours or exposed to 40nM (54) and 120 nM purified PRN or BSA to quantify levels of two cytokines, IL-1β and IL-18, via commercially available ELISA kits (R&D Systems Mouse IL-1 beta DuoSet ELISA and RayBiotech Mouse IL-18 ELISA).

**Mouse Infections**

All work with mice was conducted following institutional guidelines. Six-week-old female C57 BL/6 or C3H/HeJ mice (Jackson Laboratories, Bar Harbor, Maine) were used for assessing the colonization and the progress of infection of the respiratory tract. As required, 5–150 CFU of bacteria was delivered in 5 μL of PBS to the nares of mice anesthetized with isoflurane/oxygen. For the colonization profiles, groups of 4 mice were inoculated with WT or mutant bacteria, and

at the indicated days 4 mice of each group were euthanized with carbon dioxide ($CO_2$) and the nasal cavity, trachea, and lungs were collected in PBS and homogenized using a bead tissue disruptor. Bacterial load was enumerated by dilution plating.

**Transmission and Shedding Assay**

Transmission assays were conducted using the transmission permissive C3H/HeJ (TLR4 deficient) strain of mouse (Jackson Laboratory) whereby infected (index) mice were co-housed with uninfected (naive) mice (28). In brief, mice were lightly anaesthetized with isoflurane/oxygen and inoculated with 150 CFU of bacteria delivered in 5 μL of PBS onto the nares. Inoculated (index) mice were then placed in cages with 2 uninfected (naive) mice. Transmission of *B. bronchiseptica* was assessed after 3 weeks of co-housing by enumerating the bacterial load in the nasal cavities of the naive mice. To monitor shedding, the external nares of the index mice were swabbed (32 swipes) with a dry Dacron polyester tipped swab at the indicated times. The swab was vortexed vigorously in 1 mL PBS for 30 seconds and bacteria enumerated on BG agar plates.

**Histopathology**

Following fixation in neutral-buffered, 10% formalin solution and subsequent decalcification in Kristensen's solution, coronal sections were made through the nose. Tissues were subsequently processed, embedded in paraffin, sectioned at approximately 5μm, and stained with hematoxylin and eosin. Histopathological examination consisted of evaluation of the nose for the incidence (presence or absence), severity, and distribution of inflammation. Histopathologic severity scores were assigned as grades 0 (no significant histopathological alterations); 1 (minimal); 2 (mild); 3 (moderate); or 4 (severe) based on an increasing extent and/or complexity of change, unless otherwise specified. Lesion distribution was recorded as focal, multifocal, or diffuse, with distribution scores of 1, 2, or 3, respectively (55).

**Flow cytometry**

Five mice per experimental group were euthanized by $CO_2$ inhalation. Nasal cavities were harvested, placed in 1mL of RPMI 1640 medium and homogenized via a syringe plunger against a 40µm cell strainer. Cell suspensions were centrifuged at 1,500rpm for 10 minutes and remaining red blood cells were lysed with ACK lysing buffer. After washing with PBS, the cells were incubated with 1µl Zombie aqua (Biolegend) for 20 minutes, washed again, and incubated with 1µl Fc Block (Biolegend) for 30 minutes. Surface marker staining was added to each sample from a master mix of antibodies. Cells were fixed, washed, and resuspended in 250µl FACS buffer. Flow cytometry (Acea Novocyte Quanteon) was performed was used to sort neutrophils (CD11b-Pe-Cy7, CD115-APC, Ly6G-AF488) and macrophages (CD11b-PE-Cy7 CD115-APC, Ly6G-AF488, F4/80-PE). In a separate panel, T cells (CD45-AF700, CD3-APC), B cells (B220-PE-Cy7), and NK cells (NK1.1-PE) were sorted via a separate gating strategy. Viable cells were gated as Zombie aqua-negative cells. The data were analysed with FlowJo 10.0. (S 2.10 Fig)

**Cell isolation for intracellular survival test**

The spleen organs harvested from WT or mutant infected Hej mice was cut into 3-4 pieces using sterile scissors. Spleen pieces were mashed with the rough surfaces of two sterile frosted microscope slides and slide surfaces were rinsed with 1 mL (FBS-free) DMEM medium. The samples were then passed through a 70 µm cell strainer to obtain a single-cell suspension. A 100µl sample was plated on BG agar containing 20 µg/ml streptomycin to estimate the number of total bacteria. Cells were washed twice with PBS and then incubated for 1 h with 300 µg/ml gentamicin to eliminate extracellular bacteria. Cells were washed twice with PBS and then lysed with 0.1 % triton X-100 for 15 minutes at room temperature. CFU counting was performed on cell lysates by plating 10-fold serial dilutions onto BG agar plates containing 20 µg/ml streptomycin to estimate

the number of intracellular bacteria. Control experiments to assess the efficacy of gentamicin were performed in parallel (56). Briefly, samples of $10^3$, $10^4$ or $10^5$ bacteria were incubated with 300 µg/ml gentamicin for 1 hour at 37°C and plated on BG agar. There were 3 replicates for each group. The results showed that more than 99.9% bacteria were killed.

**Ethics Statement**

Mouse experiments used in this study were performed in strict accordance to recommendations outlined in the Guide for Care and Use of Laboratory Animals of the National Institute of Health. Protocols were approved by the Institutional Animal Care and Use Committee at University of Georgia (A2016 02-010-Y2-A3, *Bordetella*-Host Interactions). Mice were closely monitored during experiments and any mouse found moribund was euthanized using $CO_2$ inhalation to prevent unnecessary suffering.

**Statistical Analysis**

Statistical analysis of differences between the WT and mutant groups was performed using the Unpaired Student 2-tailed *t* test, One-way ANOVA and Two-way ANOVA test, as appropriate. P value less than 0.05 shown as *; P value less than 0.01 shown as **; P value less than 0.001 shown as ***.

**Acknowledgements**

We acknowledge the staff of the mouse facilities at University research animal resources (URAR), University of Georgia for help in facilitating the work. We also thank members of the Harvill laboratory for helpful discussions and editing of the manuscript.

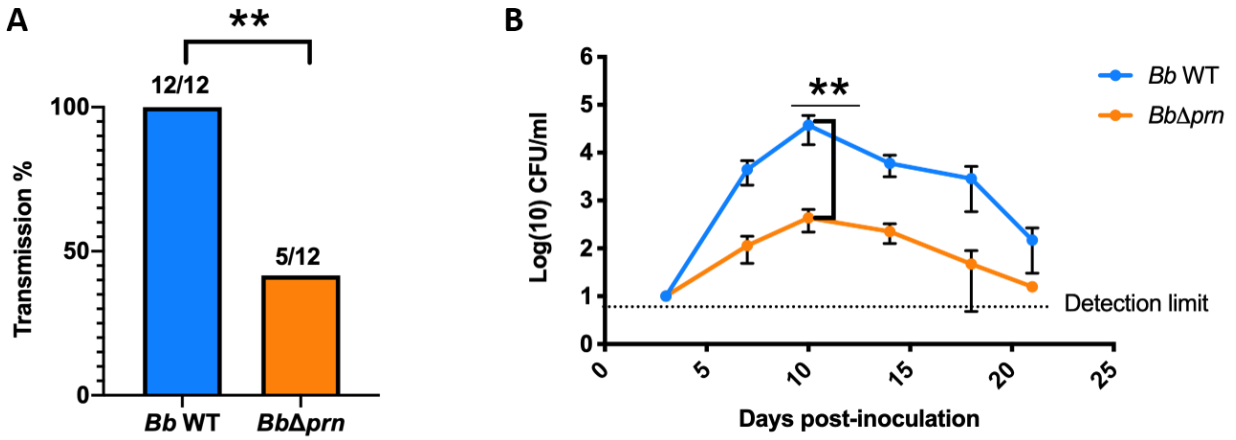



**Fig 2.1 *B. bronchiseptica* PRN contributes to bacterial transmission and shedding. A**) Transmission rates among *Bb* WT (blue) or *BbΔprn* (orange) inoculated mice. **B**) Number of bacteria shed from the external nares of *Bb* WT infected mice (blue) and *BbΔprn* infected (orange) mice. Twelve mice were utilized in each time point per group. Error bars show the standard error of mean. Statistical significance was calculated by using chi-square test in panel A and two-way ANOVA in panel B. **$p < 0.01$.

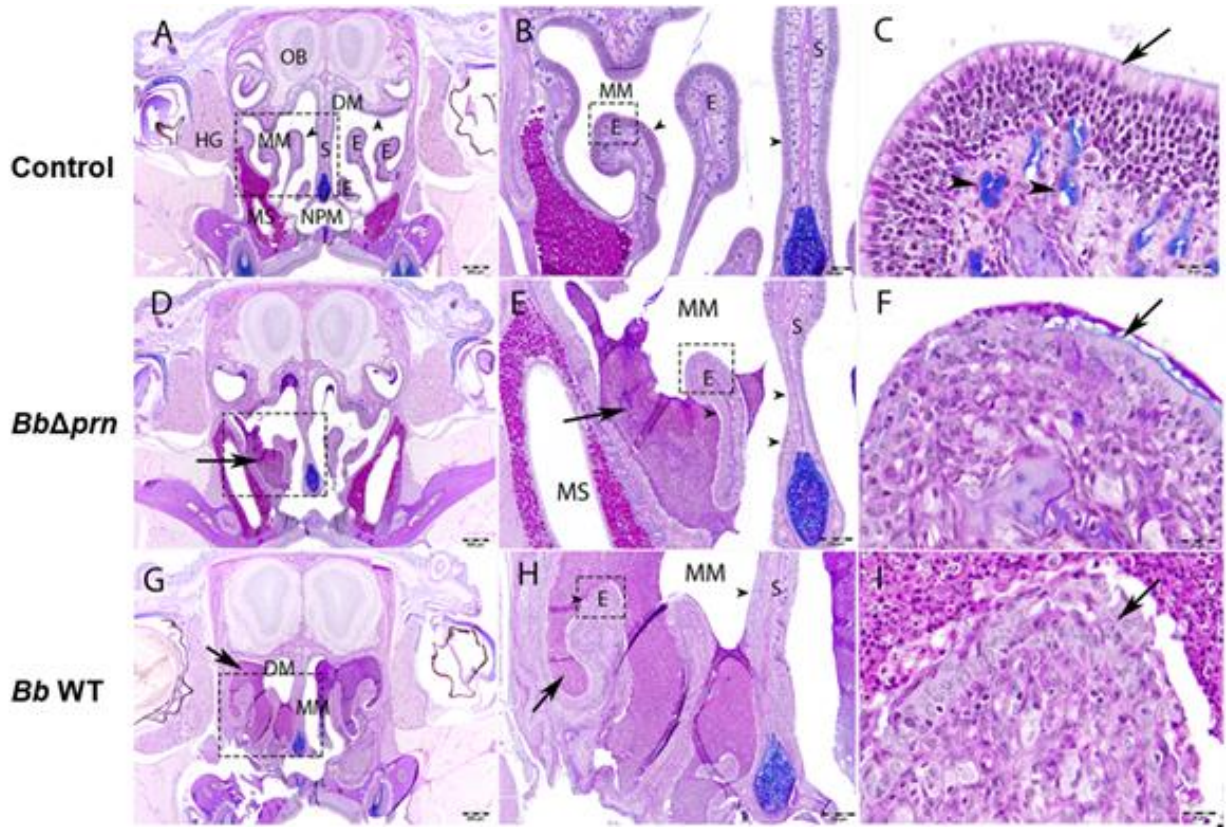



**Fig 2.2 *B. bronchiseptica* PRN induces inflammation and mucus secretion.** (A-I) Coronal sections of the nasal cavity (level 4) of control or *B. bronchiseptica (BbΔprn* or *Bb* WT strains*)* infected C57BL/6J mice at 14 dpi with stained with Alcian blue – Periodic acid-Schiff hematoxylin (AB-PASH) stain. A) Image of normal nasal cavity, level 4, from a PBS-inoculated control mouse. At this level, a layer of olfactory epithelium (arrowheads) lines the nasal mucosa within the dorsal and middle nasal meatus. Scale bar = 500μm. B) Higher magnification of Fig A (dashed rectangle) showing ethmoturbinates (E) and septum (S) lined by a layer of olfactory epithelium (arrowhead) within the middle meatus (MM). Scale bar = 200 μm. C) Higher magnification of Fig B (dashed rectangle) showing an ethmoturbinates lined by olfactory epithelium (arrow). Arrowhead points to a mucus producing (alcian blue-positive) gland within the lamina propria. Scale bar = 20 μm. D) Image of the nasal cavity, level 4, from a mouse inoculated with *BbΔprn*. The arrow points to a moderate accumulation of mucopurulent within the middle meatus. Scale bar = 500 μm. E)

Higher magnification of Fig D (dashed rectangle) showing deposition of mucopurulent exudate (arrow) covering the olfactory epithelium (arrowhead) within the middle meatus (MM). There is thinning of the olfactory epithelium (arrowheads) along the septum (S) and ethmoturbinate (E). Scale bar = 200 μm. F) Higher magnification of Fig E (dashed rectangle) showing an ethmoturbinate. There is loss of olfactory epithelium and replacement by ciliated epithelium covered by a thin layer of mucus (arrow). There is loss of mucus producing glands within the lamina propria. Scale bar = 20 μm. G) Image of the nasal cavity, level 4, from a *B. bronchiseptica (RB50)*-inoculated mouse. The arrow points to a severe accumulation of mucopurulent within the dorsal (DM) and middle meatus (MM). Scale bar = 500 μm. H) Higher magnification of Fig G (dashed rectangle) showing deposition of mucopurulent exudate (arrow) covering the olfactory epithelium within the middle meatus (MM). There is thinning and loss of the olfactory epithelium (arrowheads) along the septum (S) and ethmoturbinate (E). Scale bar = 200 μm. I) Higher magnification of Fig H (dashed rectangle) showing an ethmoturbinate. Large numbers of PAS-positive neutrophils cover the ethmoturbinate. There is total loss of olfactory epithelium and replacement by non-ciliated epithelium (arrow). There is loss of mucus producing glands within the lamina propria. Scale bar = 20 μm. Abbreviations: Dorsal Meatus (DM); Ethmoturbinate (E); Harderian Gland (HG); Maxillary Sinus (MS); Middle Meatus (MM); Nasopharyngeal Meatus (NPM); Olfactory Bulb (OB); Septum (S). There were 5 mice in each time point per group.

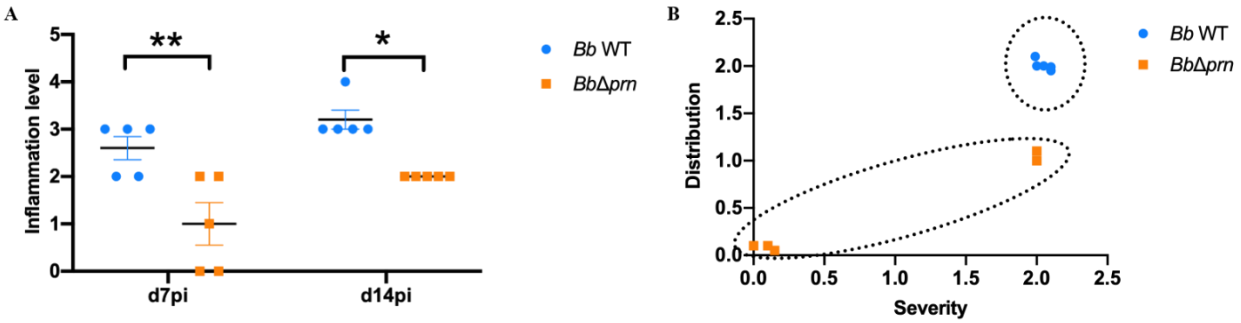



**Fig 2.3 *B. bronchiseptica* PRN induces inflammation in the nasal cavity.** A) Inflammation level in the nasal cavities of mice inoculated with either *Bb* WT (blue) or *Bb* Δ*prn* (orange) at 7-and 14-dpi. B) Mucus accumulation in the nasal cavity of *Bb* WT (blue) or *Bb* Δ*prn* (orange) inoculated mice at day 7 pi. Individual samples from either group are highlighted with dashed circles. The veterinary pathologist conducting the inflammation level analysis (UBM) was blinded to the sample source. There were 5 mice in each time point per group. Error bar shows the standard error of mean. Statistical significance was calculated using Two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

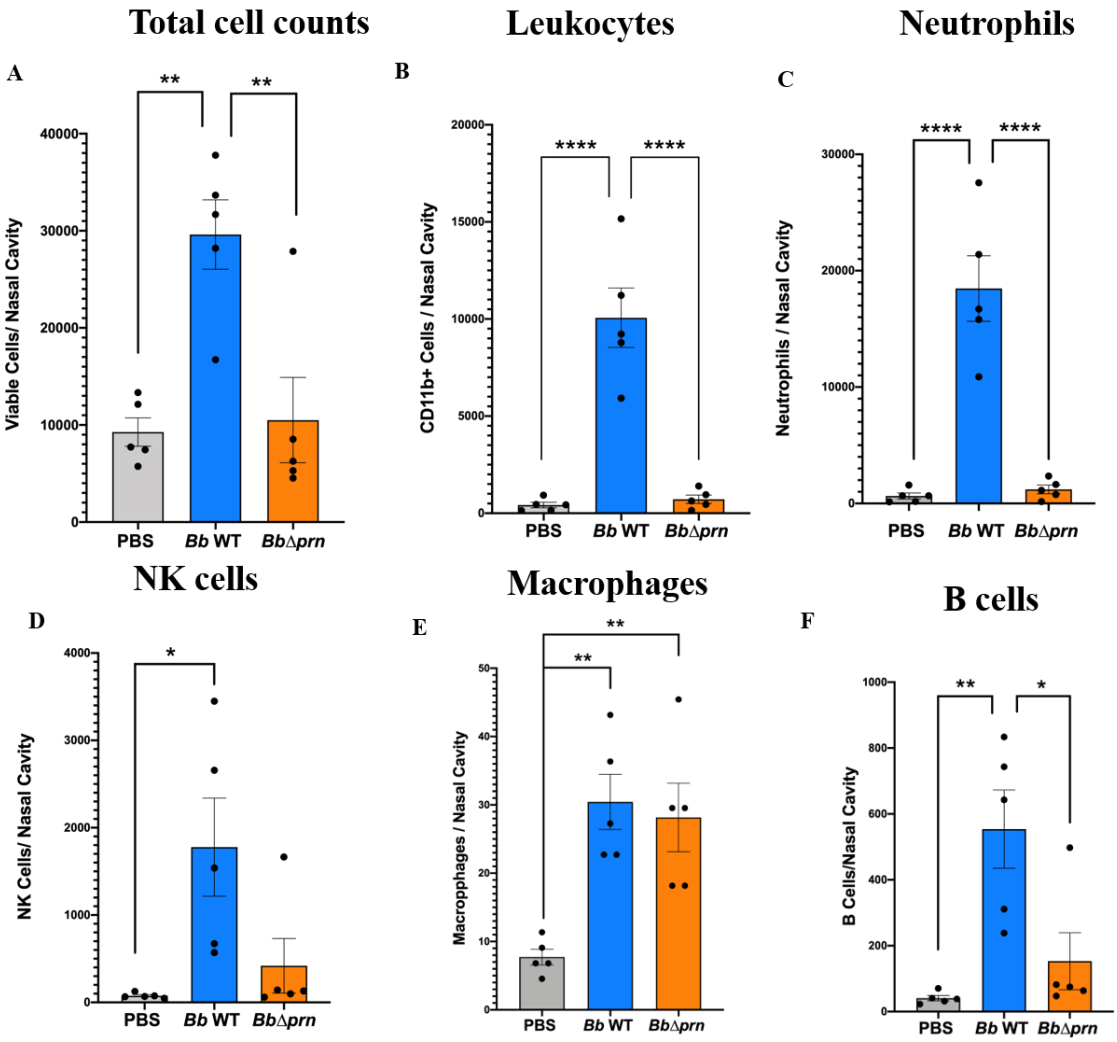



**Fig 2.4 *B. bronchiseptica* PRN contributes to the recruitment of leukocytes, neutrophils and B cells in the nasal cavity.** A) Total cells recruited in nasal cavities. B) Comparison of CD11b+ cells recruited in nasal cavities. C) Neutrophils recruited in nasal cavities. D) NK cells recruited in nasal cavities. E) Macrophages recruited in nasal cavities. F) B cells recruited in nasal cavities. There were 5 mice in each time point per group. Error bar shows the standard error of mean. Statistical significance was calculated using One-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

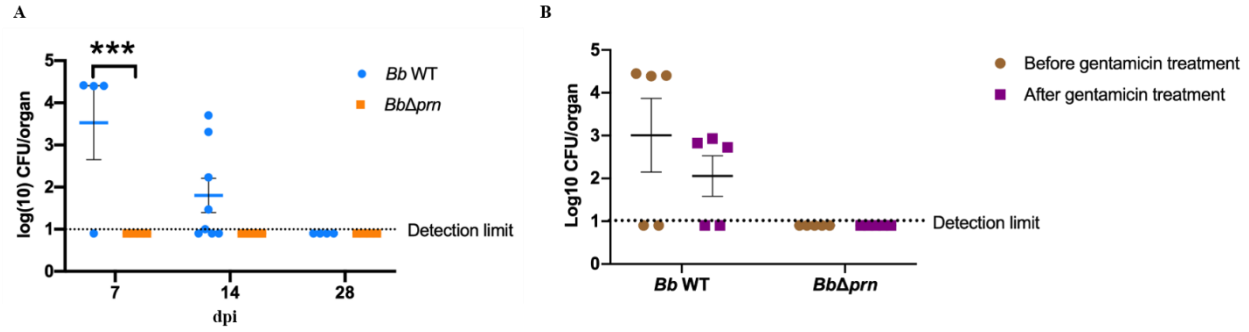
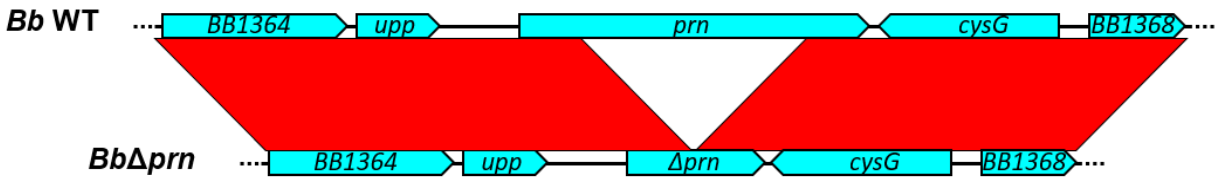
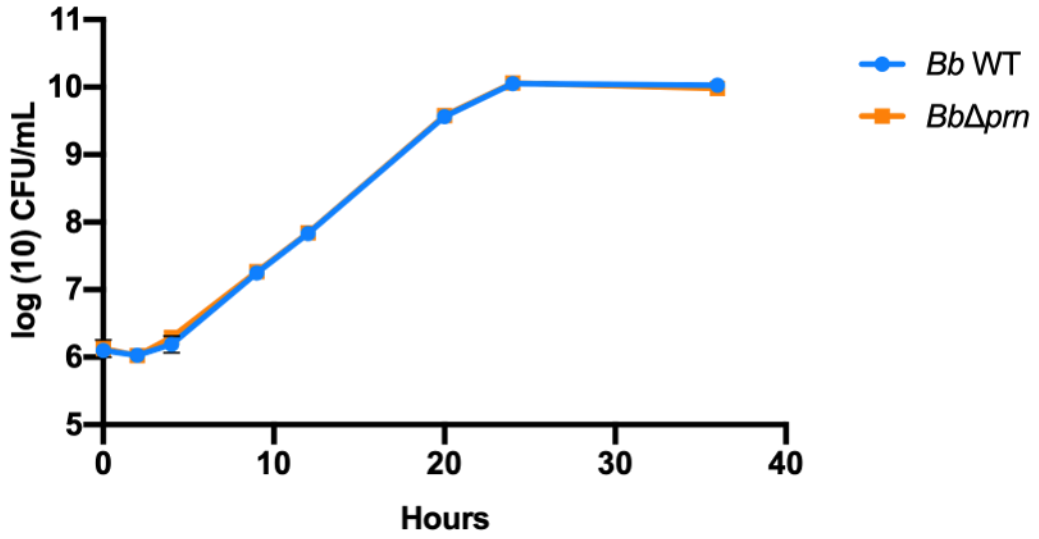



**Fig 2.5 PRN contribute to systemic dissemination and intracellular survival of *B. bronchiseptica*.** A) *Bb* WT were recovered from spleen of infected mice at 7- and 14 dpi, while there was no bacterial recovery from spleen in *Bb*Δ*prn*-infected mice in both time points. There were 4 mice for assays performed 7- and 28 dpi, and 8 mice for assays performed at 28 dpi per group. B) *Bb* WT were recovered from isolated splenocytes of infected mice at 7 dpi. Error bar shows the standard error of mean. Statistical significance was calculated by using Two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

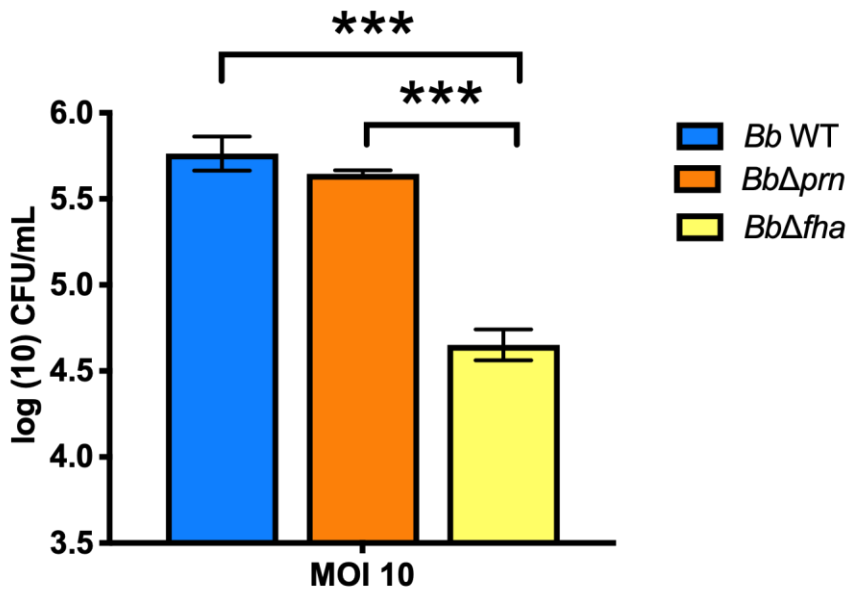



**S2.1 Fig. Schematic of in-frame deletion of pertactin gene.**

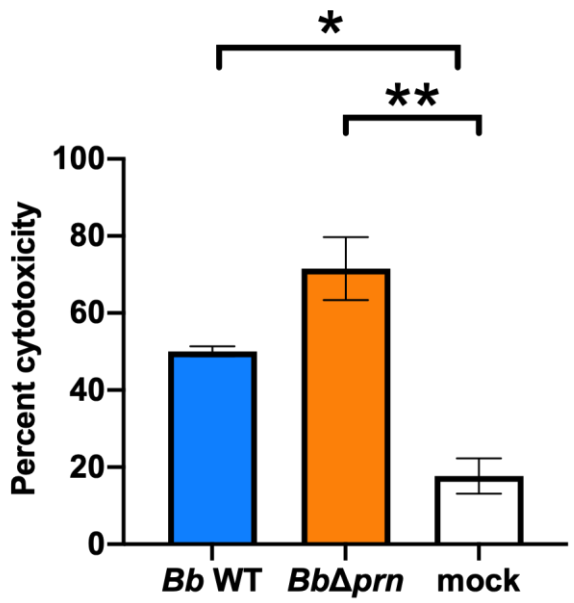



**S2.2 Fig. Similar laboratory growth *in vitro* of *Bb* WT (blue) and *BbΔprn* (orange) bacteria.**

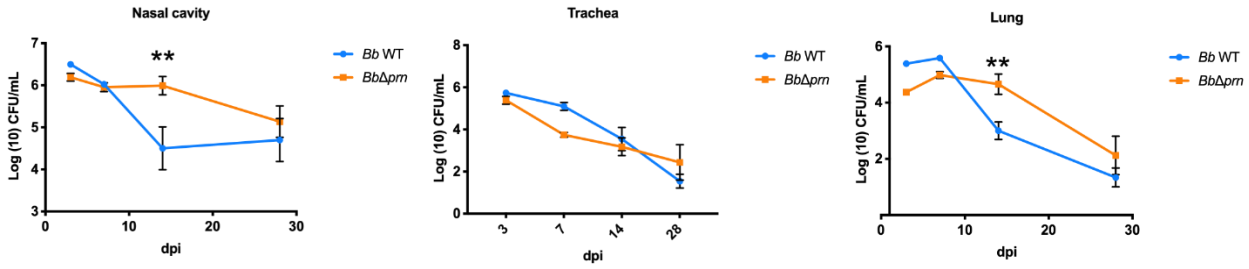
There were 3 replicates in each time point per group. Error bar shows the standard error of mean. Statistical significance was calculated using Two-way ANOVA.




**S2.3 Fig. No effect of pertactin on bacterial adhesion to human alveolar epithelial cells.** Adherence to A549 lung epithelial cells of *Bb* WT (blue), *Bb*Δ*prn* (orange) and *Bb*Δ*fha* (yellow) that was previously shown to be impaired in its ability to adhere to epithelial cells. There were 3 replicates in each group. Error bar shows the standard error of mean. Statistical significance was calculated using One-way ANOVA. ***$p < 0.001$.

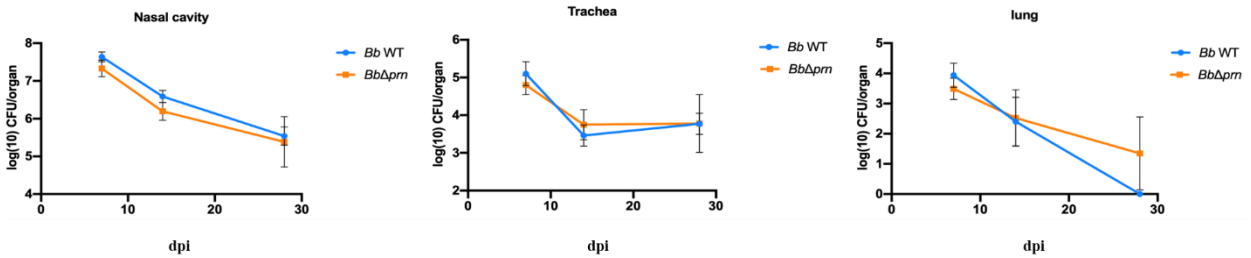



**S2.4 Fig. No significant difference in cell cytotoxicity of *Bb* WT and *Bb*Δ*prn* to RAW 264.7 macrophages.** There were 3 replicates in each group. Error bar shows the standard error of mean. Statistical significance was calculated using One-way ANOVA.

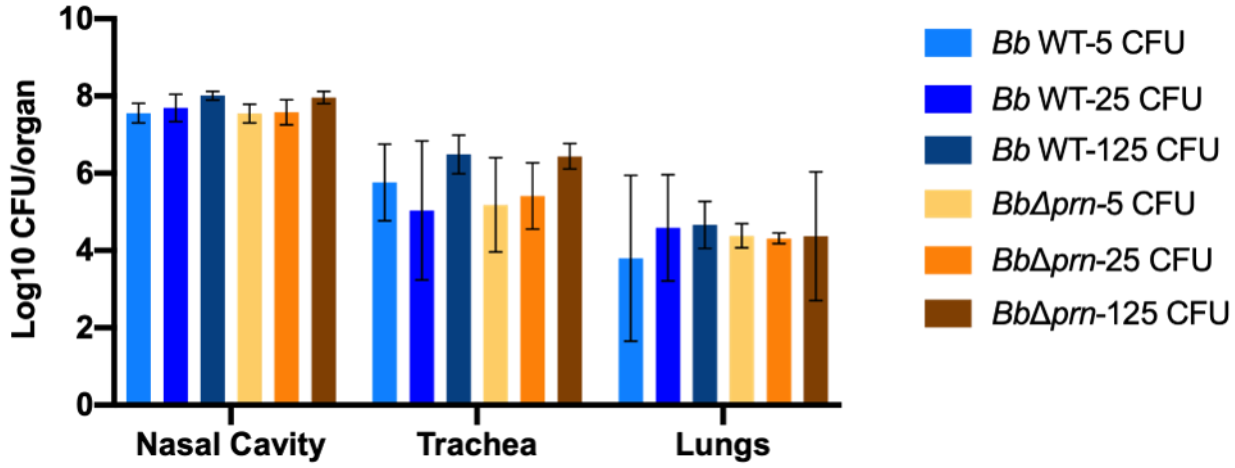



**S2.5 Fig. Comparative colonization profiles of *Bb* WT and *Bb*Δ*prn* in C57 BL/6 mice.** Bacterial CFU recovered on days 3-, 7-, 14-, and 28 dpi from the nasal cavities, trachea, and lungs of mice inoculated with either wild-type (blue) or mutant (orange) bacteria. There were 4 mice in

each time point per group. Error bar shows the standard error of mean. Statistical significance was calculated by Two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.




**S2.6 Fig. The role of PRN in colonization of *B. bronchiseptica* in C3H/HeJ mice.** Comparative colonization profiles of *Bb* WT and *Bb*Δ*prn* in C3H/HeJ mice. Number of colony-forming units (CFU) recovered on days 3-, 7-, 14-, and 28 pi from the nasal cavities, trachea, and lungs of mice infected with either wild-type (blue) or mutant (orange) bacteria. There were 4 mice in each time point per group. Error bar shows the standard error of mean. Statistical significance was calculated by using Two-way ANOVA.

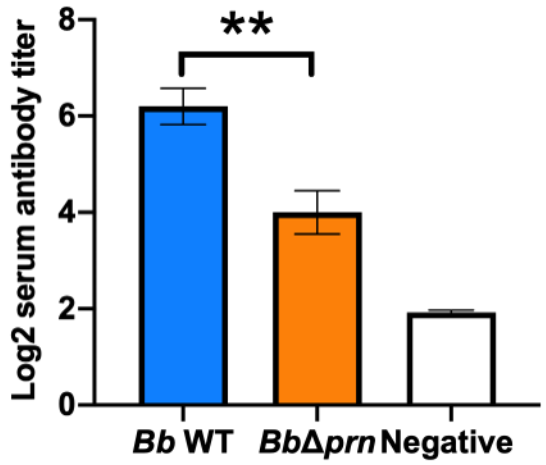



**S2.7 Fig. *B. bronchiseptica* PRN is not required for efficient colonization.** $ID_{50}$ test of *Bb* WT and *Bb*Δ*prn* showing bacterial numbers at 7 dpi in respiratory organs of C3H/HeJ mice inoculated

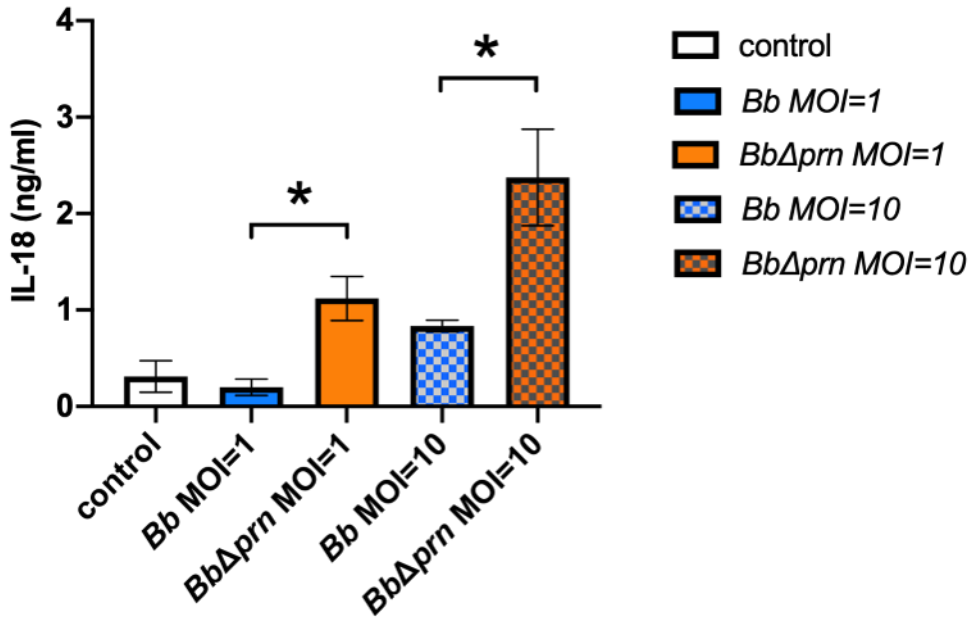
with incrementally increasing doses of 5 CFU, 25 CFU and 125 CFU. Number of CFU recovered from nasal cavities, trachea, and lungs revealed no difference in colonization between wild-type and mutant. There were 4 mice in each time point per group. Error bar shows the standard error of mean. Statistical significance was calculated by using Unpaired t-test.

## S2.1 Table. Scoring standard for histopathology analysis.

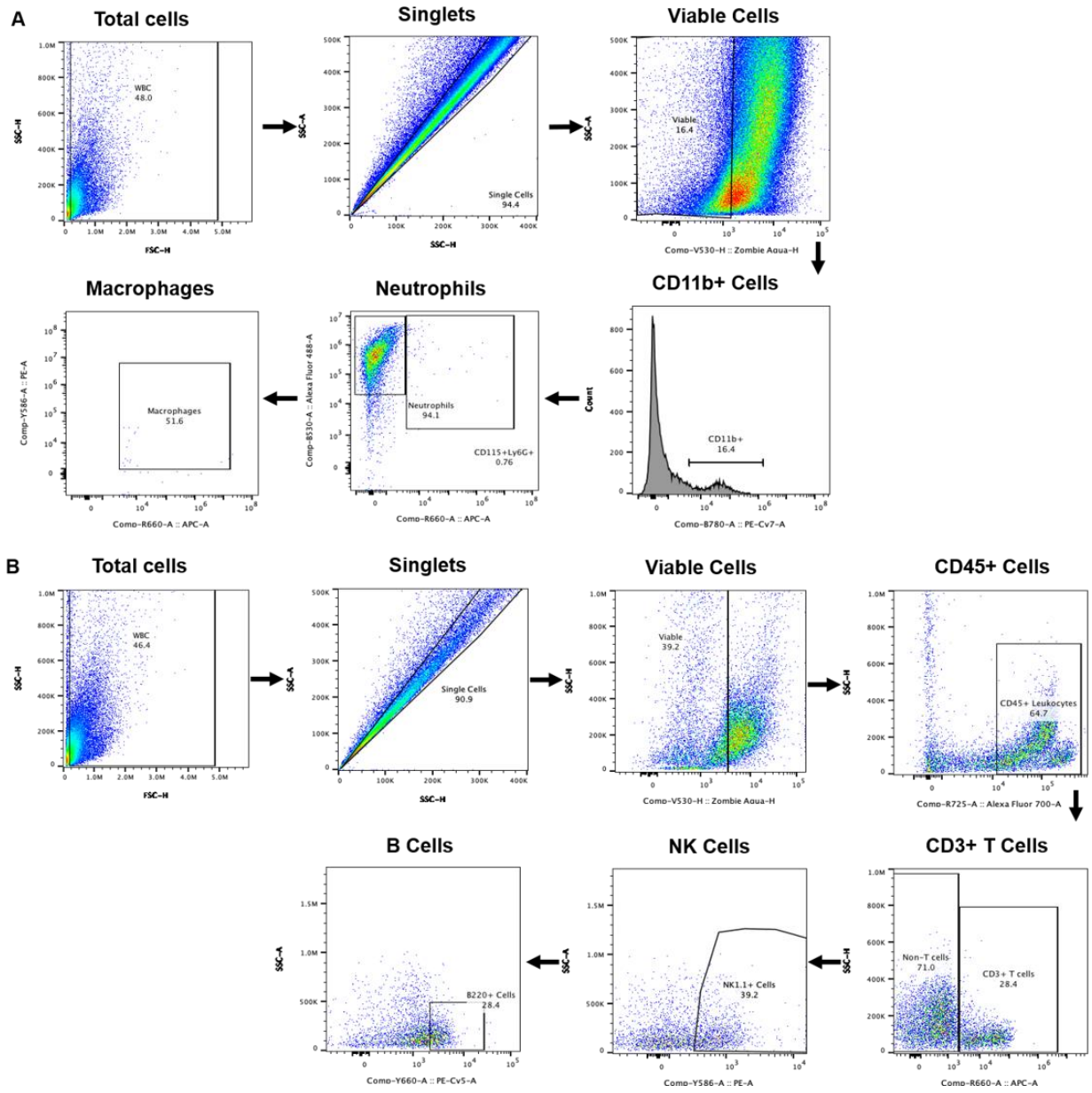
| Organ | | | Nose | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Lesion | | | Inflammation | | | Cartilage Degeneration | | | Mucus | |
| | DPI | Mouse ID | Sev | Dist | | Sev | Dist | | Sev | Dist |
| Naive | - | 1 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| Naive | - | 2 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| Naive | - | 3 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| Naive | - | 4 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| | | | | | | | | | | |
| PBS | 7 | 5 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| PBS | 7 | 6 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| PBS | 7 | 7 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| PBS | 7 | 8 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| PBS | 7 | 9 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| | | | | | | | | | | |
| *BbΔprn* | 7 | 10 | 1 | 1 | | 0 | 0 | | 0 | 0 |
| *BbΔprn* | 7 | 11 | 2 | 2 | | 0 | 0 | | 2 | 1 |
| *BbΔprn* | 7 | 12 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| *BbΔprn* | 7 | 13 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| *BbΔprn* | 7 | 14 | 2 | 1 | | 0 | 0 | | 2 | 1 |
| | | | | | | | | | | |
| *Bb* WT | 7 | 15 | 3 | 3 | | 1 | 1 | | 2 | 2 |
| *Bb* WT | 7 | 16 | 3 | 3 | | 1 | 1 | | 2 | 2 |
| *Bb* WT | 7 | 17 | 2 | 3 | | 0 | 0 | | 2 | 2 |
| *Bb* WT | 7 | 18 | 3 | 2 | | 0 | 0 | | 2 | 2 |
| *Bb* WT | 7 | 19 | 2 | 2 | | 0 | 0 | | 2 | 2 |
| | | | | | | | | | | |
| PBS | 14 | 20 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| PBS | 14 | 21 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| PBS | 14 | 22 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| PBS | 14 | 23 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| PBS | 14 | 24 | 0 | 0 | | 0 | 0 | | 0 | 0 |
| | | | | | | | | | | |
| *BbΔprn* | 14 | 25 | 2 | 2 | | 0 | 0 | | 2 | 2 |
| *BbΔprn* | 14 | 26 | 2 | 2 | | 1 | 1 | | 2 | 2 |
| *BbΔprn* | 14 | 27 | 2 | 2 | | 0 | 0 | | 2 | 2 |
| *BbΔprn* | 14 | 28 | 2 | 2 | | 1 | 1 | | 0 | 0 |
| *BbΔprn* | 14 | 29 | 2 | 2 | | 1 | 1 | | 2 | 2 |
| | | | | | | | | | | |
| *Bb* WT | 14 | 30 | 4 | 2 | | 2 | 2 | | 2 | 2 |
| *Bb* WT | 14 | 31 | 3 | 3 | | 2 | 2 | | 2 | 2 |
| *Bb* WT | 14 | 32 | 3 | 2 | | 1 | 2 | | 2 | 2 |
| *Bb* WT | 14 | 33 | 3 | 2 | | 2 | 2 | | 2 | 2 |
| *Bb* WT | 14 | 34 | 3 | 2 | | 2 | 2 | | 2 | 2 |
| | | | | | | | | | | |
| DPI = DAYS POST INFECTION | | | | | | | | | | |
| HISTOPATHOLOGIC SCORES WERE ASSIGNED AS GRADES 0 (NO SIGNIFICANT HISTOPATHOLOGICAL ALTERATIONS); 1 (MINIMAL); 2 (MILD); 3 (MODERATE); OR 4 (SEVERE) BASED ON AN INCREASING EXTENT AND/OR COMPLEXITY OF CHANGE, UNLESS OTHERWISE SPECIFIED | | | | | | | | | | |
| SCORED BY/DATE: Uriel Blas-Machado DVM, PhD, DACVP | | | | | | | | | | |




**S2.8 Fig. PRN contributed to the generation of anti- *B. bronchiseptica* IgG antibodies.** IgG antibody titers against *B. bronchiseptica* were determined in sera of mice infected with either *Bb* WT (blue) or *Bb*Δ*prn* (orange) at 28 dpi. There were 4 mice per group. Error bars show the standard error of mean. Statistical significance was calculated by using One-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.




**S2.9 Fig. *B. bronchiseptica* PRN suppresses inflammasome activation in murine RAW 264.7 macrophages.** There were 3 replicates in each group. Error bar shows the standard error of mean. Statistical significance was calculated using Unpaired t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.




**S2.10 Fig. Gating strategy for flow cytometry. A**) Gating strategy for myeloid cell types. **B**) Gating strategy for lymphoid cell types.

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# CHAPTER 3

# PERTACTIN-DEFICIENT BORDETELLA PERTUSSIS STRAINS: VACCINE-DRIVEN EVOLUTION AND THE RE-EMERGENCE OF PERTUSSIS[2]

[2] Longhuan Ma[1], Amanda Caulfield[1], Kalyan Dewan*, Eric T. Harvill. Accepted by Emerging Infectious Diseases, 3/3/2021. Reprinted here with permission of the publisher.

## Abstract

The recent re-emergence of pertussis (whooping cough) in highly vaccinated populations and the rapid expansion of *Bordetella pertussis* strains lacking pertactin, a common acellular vaccine antigen, have raised the specter of vaccine-driven evolution and the potential return of what was once the major killer of children. Indeed, the discovery that most circulating *B. pertussis* strains in the USA have acquired new and independent disruptive mutations in pertactin is compelling evidence of strong selective pressure. Interestingly, the other four antigens included in acellular vaccines do not appear to be selected against so rapidly. In this perspective, we consider three aspects of pertactin that distinguish it from other vaccine antigens, which may, individually or collectively, explain why only this antigen is being precipitously eliminated. An understanding of the rise of pertactin-deficient strains should inform the current search for new protective antigens and provide broader lessons for the design of improved subunit vaccines.


## Introduction

*Bordetella pertussis*, the causative agent of pertussis (whooping cough), continues to reemerge in countries that have high vaccine coverage, such as the United States, and has accelerated since the switch during the mid-1990s from whole cell formulations comprising many partially characterized bacterial proteins to the less reactogenic 1–5 component acellular pertussis (aP) vaccines (*1,2*). These aP vaccines, including DTaP and Tdap, protect against disease, but this protection wanes rapidly, and does not prevent colonization or transmission of the pathogen (*3–5*). In this background of suboptimally performing aP vaccines, many countries have noted the emergence and expansion of strains specifically lacking pertactin (PRN), a membrane bound autotransporter, and 1 of up to 5 *B. pertussis* protein antigens included in the vaccines (*6–11*).

PRN-deficient *B. pertussis* strains have recently been reported in countries using aP vaccines, including the United States, Australia, Sweden, Italy, Norway, the United Kingdom, France, Belgium, Finland, the Netherlands, and Japan. The frequency of PRN-deficient strains has been variable but has risen to dominance in the United States (85%), Australia (>80%), Sweden (69%), and Italy (55%) (*7–11*). Lower frequencies were reported from Japan, which showed a major decrease from a prevalence of 41% during 2008–2010 to 8% during 2014–2016 and correlated with a change to aP vaccine formulations that exclude pertactin (*6,11–12*). Denmark, which uses the monocomponent pertussis toxin (PT) vaccine, had no reports of pertactin-deficient isolates before 2012, and the 4 PRN-deficient strains detected since have been associated with human migration from countries with PRN in their vaccines (*6*). There is limited data available from the predominantly developing countries that use whole-cell vaccines to enable a robust comparison between the effects that aP and wP have on the selection of PRN. A sequencing study of the only 2 clinical isolates reported from India, which still uses wP, showed that the isolates still retained the broadly encountered PRN gene allele *prn-1* (*13*). Considered together, these observations provide a strong correlation between the use of aP vaccines containing PRN and the appearance and increase to prominence of PRN-deficient strains.

Lineages of all bacteria are constantly evolving, but increasing to dominance alone is not conclusive evidence of a causal relationship between use of PRN-containing aP vaccines and loss of PRN. A PRN mutation could be carried along with a strain that is increasing in dominance because of 1 or many other mutations. However, the appearance of a wide variety of PRN mutations, each arising from a diversity of *B. pertussis* lineages over time, provides additional strong evidence in favor of vaccine-driven selection on PRN in particular. Although insertions of IS481 at multiple genomic locations are the most common PRN mutation, there is a large diversity

of disruptions to PRN expression, including deletions within the signal sequence, promoter inversion, transversions resulting in a stop codon, deletions resulting in a stop codon, and full-gene deletion (*2,6,12*). The variety of genomic lesions that have led to loss of PRN indicates that numerous independent selection and expansion events have occurred in most of the lineages now circulating in many countries using aP vaccines. Providing more direct experimental data, such as murine models aimed at investigating pertactin-deficient *B. pertussis* infection, have demonstrated an overall defect in colonization in unvaccinated mice, but advantages in both colonization and competition in assays using aP-vaccinated mice (*14–16*).

Together, these observations strongly support the hypothesis that loss of PRN confers a fitness advantage over wildtype *B. pertussis* particular to the aP vaccinated populations in which they are arising. However, there are 4 other antigens included in aP vaccines that are not being disrupted or lost. Why are the other vaccine antigens not being mutated at similar rates? What are the characteristics of PRN that might lead to the loss of this antigen in particular? Understanding multiple possible explanations, and distinguishing between them where possible, will be useful for ongoing efforts to improve vaccines to control *B. pertussis* spread and disease.

## Results

### Role of Pertactin

Pertactin is an autotransporter protein located on the surface of *B. pertussis* (*17*). Similar to all autotransporters found in gram-negative pathogens, PRN has 3 functional domains: the N terminal signal sequence, the passenger domain, and a C-terminal autotransporter domain. The signal sequence guides the passenger and transporter domains into the periplasm, enabling the transporter domain to form a pore in the outer membrane for translocation of the passenger domain

to the cell surface. The protein is then cleaved by an outer membrane protease, with the passenger domain remaining in contact with the surface by noncovalent interactions (*18,19*).

The function of PRN is only partially understood. PRN is considered one of several virulence factors found in *B. pertussis* and has been shown to serve as an adhesin, facilitating attachment to various mammalian epithelial cells (*20,21*). The 3-dimensional structure of pertactin (PDB no. 1DAB) shows 16 right-handed parallel β-helixes, the largest β-helix structure recorded to date. Two Arg–Gly–Asp tripeptide motifs within the helical structure appear to be potential attachment sites to many mammalian adhesion proteins (*22–24*). PRN is reported to be essential for resisting neutrophil-mediated clearance and possesses additional immunomodulatory abilities that aid *B. pertussis* in suppressing the production of proinflammatory cytokines (*25,26*). The benefits of functional expression of PRN in pathogenesis are consistent with its conservation in *B. pertussis* and other pathogenic *Bordetella* species. Loss of such a factor would be expected to be costly to the organism, yet pertactin-deficient strains appear to be rapidly expanding in aP vaccinated populations, suggesting a recent rebalancing of fitness costs and benefits.

**Functional Redundancy of Pertactin**

PRN is conserved in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* under the same *Bordetella* virulence gene regulatory system, suggesting that PRN has a more general role in pathogenesis that is not restricted to the human-specific *B. pertussis*. However, there are 16 other autotransporter genes identified in the genome of *B. pertussis*, 5 of which are disrupted by frameshift mutations (*27*). Park et al. (*28*) observed that autotransporters, as a group, are much more highly mutated or lost than other virulence factors. There appears to be no major loss in in vitro growth or in vivo fitness that prevents the expansion of *B. pertussis* strains lacking PRN, agreeing with the notion that the functional contributions of PRN in pathogenesis might be

redundant or that complementary functions, potentially mediated by other autotransporters, might be compensating for any deficiencies caused its loss. This finding can be contrasted with pertussis toxin, which requires a complex operon to assemble and another to export and has a central and nonredundant role in the pathogenesis of *B. pertussis* and has no paralogs in the genome that can replace it. This finding is consistent with the rarity of clinical isolates lacking PT, compared with the abundance of PRN-deficient strains (*29–31*). For these and potentially other reasons, it might be that loss of PRN can be tolerated, but loss of PT would result in a more serious fitness defect.

Examination of the location and conformation of the different acellular vaccine components showed that most antibody functions directed against these antigens occur away from the bacterial surface. Both PT and the mature surface-associated filamentous hemagglutinin (FHA) (*32*) are secreted and diffuse away into the surrounding host environment. Therefore, antibodies directed against released FHA and PT would primarily neutralize toxin function by binding to the secreted molecules in the surrounding milieu, or to molecules in the process of being shed, mitigating potential surface-directed antibody effects. These antibodies do not effectively localize to the bacterial surface, and thus do not facilitate complement activation or FcR-mediated phagocytosis that would result in direct bacterial killing (Figure 3.1, panel A) (*33,34*). Fimbriae (FIM), although generally anchored to the bacteria, might extend more than a cell length away from the cell surface. Because FIM are composed of repeated structures with many copies of the same antigenic molecule, most FIM antibodies would be bound to parts of the structure extended >1 micron from the bacterial membrane, which is an enormous distance for highly reactive complement components to travel. Antibodies bound to fimbria would not be arrayed on a 2-dimensional surface required to optimally bind and activate complement or FcRs (Figure 3.1, panel B) (*35*).

However, pertactin is the only aP vaccine antigen that remains closely associated with the outer membrane. When cognate antibodies bind PRN, they become arrayed on the bacterial surface in a conformation that is particularly effective in binding and activating complement component C1q. The subsequent complement cascade that is activated rapidly deposits component in the adjacent membrane that opsonizes the cell and assembles into a membrane attack complex to lyse the bacterial membrane (*33–36*). The combination of the array of antibodies, bolstered by the complement components cleaved and activated in the immediate vicinity, would effectively opsonize the bacteria for efficient phagocytic killing (Figure 3.1, panel C).

The model (Figure 3.1) we provide, although largely hypothetical, is consistent with evidence that aP vaccination is effective in preventing severe disease (by binding and neutralizing a key factor that mediate aspects of disease), but is much less effective in preventing nasopharyngeal colonization. Most antibodies, similar to those directed against secreted PT/FHA or distal FIM, do not effectively target and kill the bacteria. This model would also explain why loss of PRN might enable partial evasion of aP-induced immunity, and is also consistent with human surveys that showed that 3-component aP vaccines containing PT, FHA, and PRN were more efficacious than 2-component vaccines lacking PRN (*37,38*).

**Persistence of Pertactin Antibodies**

Despite the shortcomings of aP vaccines in generating a strong memory response, and the resulting waning immunity of these vaccines against disease (*39,40*), aP vaccines induce robust IgG titers against most of its component antigens. However, this strong humoral response, although protecting against symptoms of pertussis, does not prevent the pathogen from colonizing the upper respiratory tract or from transmitting between hosts (*3*). Furthermore, although initially induced at high levels, circulating antibodies decay relatively rapidly across all age groups (*4,40–*

*43*). Studies evaluating dynamic levels of specific antibodies across time have consistently show differential rates of decay for the various antigen-specific antibodies, with PTX antibody titers decaying more rapidly than antibodies to FHA and PRN (Figure 3.2). Similarly, FIM2/3 have been reported to poorly stimulate generation of protective antibodies postinfection and postvaccinations (*4,43*), although these data conflict with those of other reports that show higher levels of FIM antibodies, even at 10 years postvaccination. However, these reports, similar to PTX, show a sharp decrease in antibody titers that might decrease below protective levels over time. This rapid waning immunity against PTX and FIM would be expected to narrow the window of selective pressure against these antigens.

In contrast to antibodies against PTX, those against PRN and FHA are relatively more persistent (Figure 3.2) (*38,44,45*), suggesting that there is a longer period after vaccination when there are effective titers of antibodies against FHA and PRN. This finding would be expected to result in strong pressure against PRN and FHA. However, in a search for serologic correlates of immunity to pertussis, Le et al. (*4*) noted that FHA provides relatively little contribution to protection but pertactin had a higher protective role.

These observations have recently been validated in studies by Lesne et al., who used human serum bactericidal assays to determine that antibodies to PRN, but no other aP component, are bactericidal in in vitro complement killing assays (*46*). These findings somewhat conflict with those of previous studies and testing methods, which often prioritize PT IgG as an indicator for protection against pertussis (*4,45,47*). However, the short period during which levels of neutralizing antibodies against PT remain elevated, in contrast to bactericidal antibodies against PRN, suggests that PRN antibodies might be a more appropriate measure for pertussis immunity.

The short period during which antibodies to PTX remain at elevated levels indicates that there is a longer period when antibodies to PRN remain at high levels, but levels of antibodies to PTX have decreased. Le et al. also noted that a much larger proportion of enrolled patients tested before aP vaccination already had high titers of antibodies to PRN, and quite few had antibodies to PTX (*4*). Therefore, in addition to its surface localization and strong opsonizing potential, the persistence of the PRN antibodies is likely to contribute to prolonged selection against this antigen in particular.

## Discussion

The rapid reemergence of pertussis noted in the early 2000s brought much initial speculation. Factors such as human migration, increased sensitivity of testing, increased volumes of testing, and reporting through heightened surveillance have been proposed to contribute to the observed resurgence (*1–3*). However, the collective experience in countries switching from whole cell to acellular vaccines strongly suggests that these safer, but less effective vaccines have contributed to the resurgence of pertussis. In addition, the way multiple pertactin-deficient strains have swept across countries that use acellular vaccines presents a strong case in favor of vaccine-driven selection against pertactin (*6–12*). What has remained unclear is what will be the consequences of these changes. Will PRN-deficient strains continue to evolve, loosing other vaccine antigens until they escape vaccine effects completely? Or will the loss of these factors result in strains attenuated in virulence such that they become more like commensals than pathogens? Or is PRN really the only antigen that can be lost without serious fitness costs?

The 3 aspects of PRN that have been highlighted in this article for selective loss of PRN (i.e., its functional redundancy, the relatively longer functional persistence of antibodies against it, and its close location to the surface membrane for productive complement fixation) are not an

exhaustive list of all possibilities and are not mutually exclusive. Sufficient sustained selective pressure against any particular antigen is likely to lead to its loss or change. Efforts to improve the current vaccine should be informed by a careful consideration of the lessons learned from this instance. If PRN is being lost because it is the only surface antigen, then should we replace it with 1 or more new surface antigens? If it is lost because its function is at least partly redundant then should we select some molecule(s) that is less likely to be redundant? In designing new vaccines, it would be prudent to carefully consider the issues that appear to be enabling potential vaccine escape mutants, such as PRN-deficient strains, to rapidly expand and rise to prominence.

This study was supported by the National Science Foundation (grant no. DGE-1545433) and NIH (grant nos. 1R21AI156293-01 and R56 A1149787)

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Longhuan Ma and Amanda Caulfield equally contributed to this paper on conceptualization and writing. Kalyan Dewan and Eric Harvill contributed to paper writing and review.

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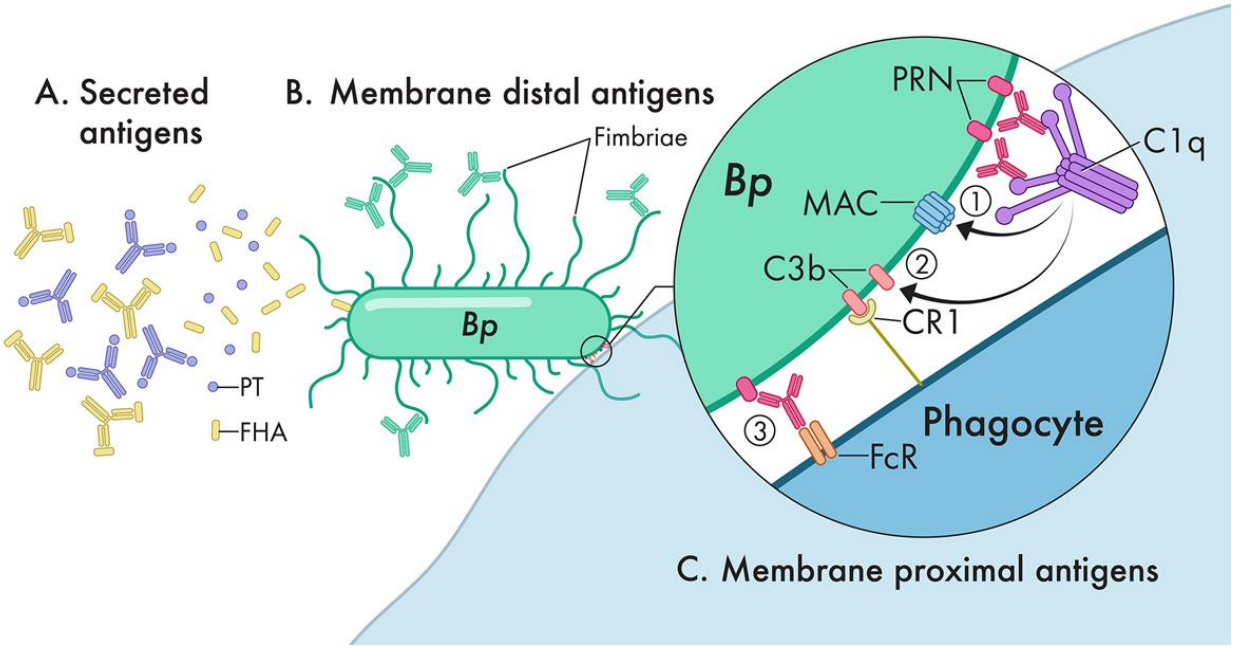


**Figure 3.1 The various roles of antibodies against antigens in acellular pertussis vaccine.** (A) Antibodies against PT and FHA neutralize secreted virulence factors and mitigate disease progression but are not targeted to bacterial surface. (B) Antibodies attaching to fimbriae poorly activate the complement system far from the bacterial membrane. (C) Antibody-pertactin complexes induce strong bactericidal activity via multiple synergistic functions: 1. Activate complement to form a membrane attack complex. 2. Activate complement to deposit components like C3b that opsonize the bacterial surface. 3. Bind FcRs on phagocytes to activate phagocytosis.

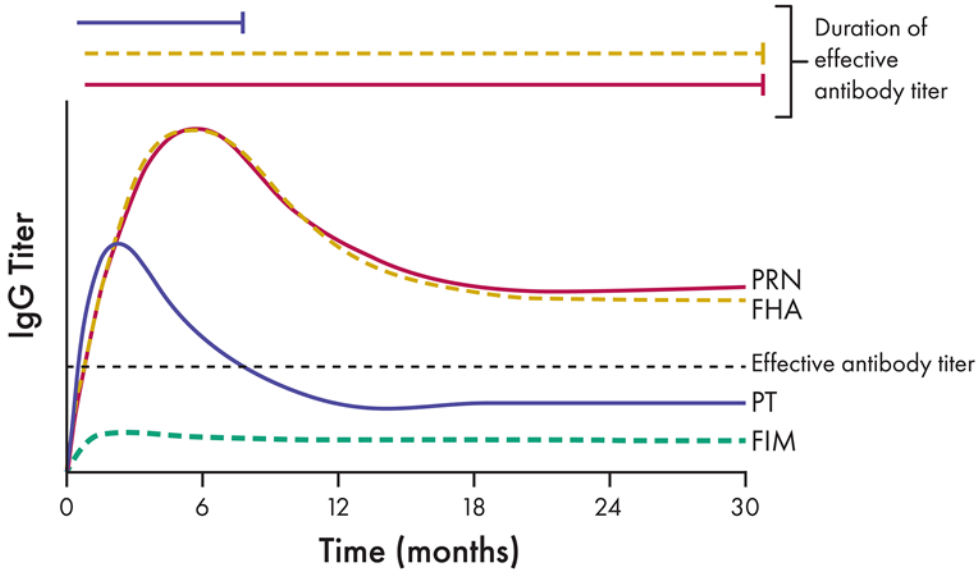


**Figure 3.2 The differential decay of antibodies against acellular vaccine antigens and their effective capacity for protection.** Antibodies against PRN and FHA remain at relatively higher titers for a longer period. However, PT specific antibodies decrease to very low titers rapidly. A consistently low-level of antibodies against FIM are induced. Solid lines indicate antibodies with high protective capacity, while dotted lines indicate antibodies with low protective capacity. Only anti-PRN antibodies are both highly protective and persist at high titers for years.

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# CHAPTER 4

# NATURAL HISTORY AND ECOLOGY OF INTERACTIONS BETWEEN *BORDETELLA* SPP. AND AMOEBAE[3]

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**Abstract**

The ability to evade diverse environmental phagocytic predators such as amoebae allows some environmental bacteria to invade and survive within mammalian host phagocytes, where they are protected from soluble antimicrobials and can cause persistent infections. Our recent study found that *Bordetella bronchiseptica*, the animal pathogen and apparent progenitor of important human pathogens, can evade predation by the common soil amoebae *Dictyostelium discoideum*, survive within and hijack its complex life cycle as a propagation and dissemination vector. This interaction may have served as an evolutionary training ground for *Bordetella* species to develop mechanisms that allow intracellular survival within mammalian phagocytes. Following the divergence, evolution, and adaptation of *Bordetella* species to different hosts and ecological niches, accompanied by loss and gain of multiple genes, it remains unclear whether the mechanisms allowing interactions with predatory amoebae are conserved in some or all *Bordetella* species. In this study, we tested 9 diverse *Bordetella* species in three assays representing separated aspects of their interactions with *D. discoideum:* intracellular survival, plaque expansion and translocation to the amoebic sori. The results show that the species known to have been generally isolated from various sources retain the ability to successfully interact with amoebae. In contrast, these abilities are partly degraded in the two human-specialized species, *B. pertussis* and *B. parapertussis*. Interestingly, a different lineage of *B. parapertussis* that is only known to infect sheep retains the ability to interact with *D. discoideum*, suggesting this lineage may not have fully committed to a closed life cycle in sheep. Complete genomes for all 9 species allowed a genome-wide association study (GWAS) approach that identified a set of genes that are associated with the ability to interact with *D. discoideum*. These observations suggest that *Bordetella* species that have specialized to a closed life cycle in humans have lost some ability to interact with amoebae,

but those observed in humans as well as other environments and niches (*B. bronchiseptica*) have retained some or all of these abilities, suggesting they may translate to useful functions within mammalian hosts.

## Introduction

The genus *Bordetella* comprises 9 species, including the "classical *Bordetella*" consisting of *B. bronchiseptica, B. pertussis* and *B. parapertussis* and the seven other more distantly related species, including *B. holmesii*, *B. petrii*, *B. avium*, *B. hinzii*, *B. pseudohinzii*, *B. trematum* and *B. ansorpii* (1, 2). Our group generated phylogenic trees for 128 genomes from 9 species of genus *Bordetella* based on genome-wide sequence alignment and presence or absence of genes. Both phylogenic trees revealed the evolution of genus *Bordetella* into three clades of species: clade A formed by *B. bronchiseptica*, *B. pertussis* and *B. parapertussis*, clade B including *B.hinzii, B. trematum, B. avium* and *B. holmesii* and clade C containing *B. petrii* and *B. ansorpii*. The sequence-based phylogenic tree reveled that the genome of classical *Bordetella* in clade A are closely related to each other, while species in clade B and C are far more diverse. Furthermore, substantial gene loss and gene acquisition occurred in clade A species and clade B species, which would explain the diverse gene content within these two clades (3).

The classical *Bordetella* are mostly found in mammalian hosts, as *B. pertussis* infects humans, the two lineages of *B. parapertussis* infect either humans (*B. parapertussis* $_{hu}$) or sheep (*B. parapertussis* $_{ov}$), and *B. bronchiseptica* is a pathogen of various mammals (1, 4). Meanwhile, the non-classical *Bordetella* species can cause infections in a variety of hosts, *B. holmesii* in humans (5, 6), *B. avium and B. hinzii* in birds (7, 8), *B. pseudohinzii* in mice and other rodents (9) and *B. trematum and B. anosorpi* in the infected wounds of immunocompromised patients (2, 10).

In addition to isolation from animal hosts, some *Bordetella* species can even be isolated from the environment. *B. petrii* has been isolated from multiple natural environments, including an anaerobic, dechlorinating bioreactor culture enriched by river sediment, marine sponges and grass root consortia (11). In 2015, Tazato and his colleagues isolated three new *Bordetella* species, designated as *B. muralis, B. tumulicola*, and *B. tumbae*, from a plaster wall surface of 1,300-year-old mural painting in Japan (12). Through a recent study from our laboratory, numerous 16S rRNA sequences of bacteria isolated from various environmental sources, including soil, water, plants and sediment were subjected to a phylogenetic analysis to determine the relatedness between *Bordetella* species and those isolated from environmental samples. The phylogenetic tree provided evidence for an environmental origin of the genus *Bordetella*, showing environmental *Bordetella* species possess a significantly higher genetic diversity than those from human- and animal-associated samples (13).

*B. pertussis*, *B. parapertussis* and *B. bronchiseptica* have been found surviving inside various mammalian cell types *in vivo*, including macrophages, neutrophils, dendritic cells, and epithelial cells (14-18). Different virulence factors, such as adenylate cyclase toxin, filamentous hemagglutinin and pertussis toxin, have been reported to contribute to the ability to survive within mammalian cells (19-21). An in vitro assay showed that *B. bronchiseptica* appeared in the cytoplasm of infected epithelial cells while *B. pertussis* did not, indicating that different invasion and persistence strategies may be used by the two species (22). In an intracellular survival study in RAW 264.7 macrophages, the results showed that bacterial survival and persistence is not restricted to three classical *Bordetella* species, but *B. hinzii*, *B. pseudohinzii*, *B. trematum* and *B. petrii* also survived at an equally high ratio, suggesting that the machinery of intracellular survival is conserved in some *Bordetella* sub-species (14).

Various infectious pathogens have been found in free living amoeba (23). These amoeba resistant microorganisms (ARMs) show higher gene numbers and DNA contents compared to their relatives, suggesting that the interactions of pathogens with amoebae may pose a selective pressure on ARMs for a well-developed intracellular survival machinery. In other words, amoebae may work as a training ground for bacterial intracellular survival. Our previous study reported that *B. bronchiseptica* could survive inside *D. discoideum* and use it as a dissemination vector for bacterial transmission (24). Considering the similarities between amoebae and mammalian phagocytes, interactions between *B. bronchiseptica* and amoebae may represent the starting point for bacterial adaption to mammalian cells. Since *Bordetella* species have diverged, evolved, and adapted to different hosts and ecological niches along with loss and gain of multiple genes, whether the machinery allowing interaction with predatory amoebae is conserved in some or all *Bordetella* species is still unknown.

In this study, we tested 9 diverse *Bordetella* species in assays representing three separate aspects of their interactions with *D. discoideum:* intracellular survival, plaque expansion, and translocation to the amoebic sori. The results demonstrate that the species known to have been isolated from environmental sources generally retain the ability to successfully interact with amoebae. In contrast, these abilities are partly degraded in the two human-specialized species, *B. pertussis* and *B. parapertussis*. Interestingly, a different lineage of *B. parapertussis* that is only known to infect sheep retains the ability to interact with *D. discoideum*, suggesting this lineage may not have fully committed to a closed life cycle in sheep. Complete genomes for all 9 species allowed a GWAS approach that identified a set of genes that are associated with the ability to interact with *D. discoideum*. These observations suggest that *Bordetella* species that have specialized to a closed life cycle in humans have lost the capacity to utilize amoebae as

environmental reservoir and transmission vectors, but those observed in humans as well as other environments (like *B. bronchiseptica*) have retained this capacity.

## Materials and methods

### Bacterial strains and growth

*B. bronchiseptica* strain RB50, *B. pertussis* 536, *B. parapertussis* ovine strain (Bpp5), *B. parapertussis* human strain (12822), *B. pseudohinzii* 8-296-03, *B. hinzii* L60, *B. petrii* DSM12804, *B. avium* 197N, *B. holmesii* P3421 and *B. trematum* H044680328 were grown and maintained on BG agar (Difco) supplemented with 10% defibrinated sheep's blood (Hema Resources). Liquid cultures were grown overnight at 37°C to mid-log phase (OD ~0.6) in Stainer Scholte (SS) liquid broth. *Klebsiella pneumonia* was grown and maintained on Luria-Bertani (LB) agar (Difco) and liquid cultures were grown at 37°C to mid-log phase in LB broth (Difco).

### Amoeba strains and growth

*D. discoideum* strain AX4 has been used in this study. The trophozoite amoebal cells were cultured in HL5 medium at 21°C, and subcultured twice a week in fresh medium to prevent the cultures from reaching confluence. They were also grown on bacterial lawns as described below.

### Intracellular survival assay in *D. discoideum*

*D. discoideum* cells were grown to 80% confluency (~1 x $10^5$ CFU/well) in HL/5 medium in 96-well tissue culture treated plates (Greiner Bio-One) at 21°C. 10 µl of *bacteria* were added to wells at a multiplicity of infection (MOI) of 10 or 1. Plates were centrifuged at 300 Xg for 10 minutes at room temperature and then incubated at 21°C. After 45 minutes, supernatant was removed from remaining wells and replaced with 100 µl of 300 µg/ml gentamicin solution (Sigma-Aldrich) in HL/5. Plates were incubated at 21°C, and then at 1 or 2 hours post gentamicin treatment,

the appropriate wells were washed three times with PBS and then 100 µl of 0.1% Triton-X solution in PBS was administered to a subset of wells, followed by a 5-minute incubation at 21°C and vigorous pipetting to lyse open cells. 10 µl from each were serially diluted and plated on BG or LB to quantify total *intracellular survived bacteria.*

**Intracellular survival inside macrophage**

RAW 264.7 macrophages cells were grown to 80% confluency (~$1 \times 10^5$ CFU/well) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, glucose and glutamine in 48-well tissue-culture plates at 37°C. Bacteria were added in 10 µl PBS containing $10^5$ CFU (MOI of 1) as indicated. Plates were centrifuged at 300 X*g* for 10 minutes at room temperature and incubated at 37°C for 1 hour, after which gentamicin solution (Sigma-Aldrich) was added to a final concentration of 300 µg/ml. Plates were incubated at 37°C for an additional 2 hours and subsequently washed with PBS. 0.1% Triton-X solution was administered, followed by a 5-minute incubation and vigorous pipetting to lyse the macrophages. The samples were serially diluted and plated on BG agar plates to quantify total bacteria numbers.

**Predation resistance assay**

Bacterial isolates grown on BG agar plates or LB agar plates were resuspended in 1 mL of PBS. The resuspended bacteria were plated on sm5 medium agar plates. The plates were allowed to dry under sterile conditions to obtain bacterial lawns. The *D. discoideum* cells were resuspended in fresh HL5 with no antibiotic before counting them in a hemacytometer chamber. Serial dilutions were prepared in HL5 medium to obtain the *D. discoideum* cell concentrations at 50 cells per 10 µl. The bacterial lawns were spotted with 10 µl of the serial *D. discoideum* dilutions. The plates were allowed to dry and were incubated at 21°C for 16 days. The area of plaques formed on bacterial lawns are measured on days 4, 7 and 16.

**Gentamicin protection assay of Bordetella spp. in *D. discoideum* sori**

To determine the number of intracellular *B. bronchiseptica* in amoebal spores, sori grown on lawns of *B. bronchiseptica* RB50 were collected and treated with gentamicin. For each sample, one sorus was collected into 500 μL PBS and vortexed. The sample was then split into two aliquots of 250 μL. One aliquot was treated with 50 μL gentamicin (20 mg/ml), while the other aliquot was treated with 50 μL PBS. The aliquots were incubated 4 hours at 21°C, washed three times in PBS, and then plated on BG agar plates.

**Genome comparisons and protein similarity analysis.**

For comparison between "classical *Bordetellae*", we compared the genes and predicted proteins of *B. bronchiseptica* strain RB50 (NC_002927.3), *B. parapertussis*$_{ov}$ BPP5 (NC_018828.1), *B. parapertussis*$_{hu}$ 12822 (NC_002928.3), and *B. pertussis* Tohama I (NC_002928.2) using mGenomeSubtractor (25) and using the Artemis Comparison Tool (ACT) (26). Pseudogenes containing mutations that would prevent complete translation (premature stop codon, frame shift, truncation) were identified by direct pairwise comparisons between *B. bronchiseptica* RB50 and either of the other three genomes. Protein similarities of *B. bronchiseptica* genes and their homologs in the "non-classical *Bordetellae*" were determined as pairwise BLASTp comparisons as previously described (14). Total protein sequences were extracted from NCBI for *B. bronchiseptica* RB50 (RefSeq assembly accession: GCF_000195675.1), *B. hinzii* L60 (GCF_000657715.1), *B. pseudohinzii* 8-296-03 (GCF_000657795.2), *B. avium* 197N (GCF_000070465.1), *B. petrii* DSM12804 (GCF_000067205.1), *B. trematum* H044680328 (GCF_900078695.1), and *B. holmesii* 04P3421 (GCF_000662215.1). Similarities between *B. bronchiseptica* proteins and their corresponding

homologs in the "non-classical *Bordetellae*" were calculated in mGenomeSubtractor as the H value for each protein, defined as the product of the highest BLASTp identity score 'i' and the length of the matching sequence length 'lm', divided by the query length 'lq' (H = i x lm / lq). Based on our previous work (Rivera et al., 2019), homologs of *B. bronchiseptica* genes with a protein similarity value of $H \geq 0.5$ were considered to be present. Proteins with values of $H \geq 0.5$ were validated as true orthologs by pairwise tBLASTx genome comparisons in ACT.

**Statistical Analysis**

The mean ± standard error (error bars in figures) was determined for all appropriate data. Two-tailed, unpaired student's *t*-tests were used to determine the statistical significance between two normally distributed populations. GraphPad Prism version 6.04 was used to conduct these statistical tests and to generate figures.

## Results

**Human-specific classical *Bordetella* species can survive within *D. discoideum* but fail to persist.**

Our group has previously reported the ability of *B. bronchiseptica* to survive inside amoebal cells. However, whether this capacity is conserved in other classical *Bordetella* species is still unknown. To compare the intracellular survival of classical *Bordetella* species inside *D. discoideum*, a gentamicin protection assay was performed after *D. discoideum* was exposed to *B. bronchiseptica*, *B. pertussis*, *B. parapertussis* $_{hu}$ or *B. parapertussis* $_{ov}$. After 2 hours of gentamicin treatment, viable intracellular bacteria were enumerated by plating the lysed amoebal cells on BG agar plates. High numbers of *B. bronchiseptica* and *B. parapertussis* $_{ov}$ were recovered. In contrast,

*B. pertussis* and *B. parapertussis* $_{hu}$ failed to survive inside the amoebal cells (Fig 4.1). The results suggest that within classical *Bordetella* species, the strains that are restricted to human have lost machinery that allows them to survive inside predatory amoebae.

**_D. discoideum_ efficiently feed on human-restricted Bordetella spp.**

*B. bronchiseptica* has been shown to resist predation by *D. discoideum*. To investigate whether other classical *Bordetella* species can defy amoebal predation, *D. discoideum* was inoculated onto the bacterial lawns of classical *Bordetella* species. Plaques were formed in areas where *D. discoideum* consumed the bacteria. To test the predation of amoebae on classical *Bordetella* species, the area of plaque formation on different bacterial lawns was measured and compared at different time points. Plaques formed on the bacterial lawn of *B. pertussis* and *B. parapertussis* $_{hu}$ at early time points and the size increased along the time course, however, the plaques formed on the bacterial lawn of *B. bronchiseptica* and *B. parapertussis* $_{ov}$ were not obvious at early timepoints and were much smaller compared to that of the plaques formed on bacterial lawns of *B. pertussis* and *B. parapertussis* $_{hu}$ (Fig 4.2). The results indicate that human specific *Bordetella* species have lost the ability to resist the predation from amoebae.

**Human-specific _Bordetella_ species fail to survive in amoeba sori.**

In previous studies, *B. bronchiseptica* has been proven to survive through the amoebic life cycle and was carried to amoebal sori. To test the intra-sori survival of other classical *Bordetella* species, the sori formed on lawns of different *Bordetella* species were harvested at various time points, and bacteria surviving inside sori were enumerated. A high number of *B. bronchiseptica* and *B. parapertussis* $_{ov}$ were consistently recovered from sori across different time points, while

*B. pertussis* and *B. parapertussis* $_{hu}$ were not recovered from sori at any timepoint (Fig 4.3). The absence of *B. pertussis* and *B. parapertussis* $_{hu}$ in sori indicates that human specific *Bordetella* species have lost mechanisms involved in translocation to or survival within amoebic sori.

**Different intracellular survival rates observed among non-classical *Bordetella* species.**

We expanded the intracellular amoebal survival test to include the remaining 6 non-classical Bordetella species. In gentamicin protection assay, 5-10 percent of *B. trematum* and *B. hinzii* survived inside amoebal cells after 2 hours treatment, while only around 0.1 percent of *B. avium* and *B. petrii* were recovered from amoebal cells. Between these two groups, *B. parapertussis* $_{ov}$, *B. bronchiseptica*, *B. pseudohinzii* and *B. holmesii* had an intermediate recovery percentage between 0.5 to 5%. (Fig 4.4). The results showed that species isolated from mammals have a relatively high intracellular survival rate in amoebae, suggesting that the ability to survive inside amoebal cells remains important for these species with mammalian hosts.

**Some non-classical *Bordetella* species resist predation by amoebae.**

To investigate the abilities of non-classical *Bordetella* species to resist amoebal predation, we carried out plaque formation assays by using bacterial lawns of classical and non-classical *Bordetella* species. Results showed that *B. pertussis*, *B. parapertussis* $_{hu}$, *B. avium* and *B. petrii* failed to resist the predation of *D. discoideum*. The plaques formed on the bacterial lawns of these species had similar sizes of that on the lawn of *Klebsiella. Pneumoniae*, food resource for soil amoebae. In contrast, *B. hinzii*, *B. pseudohinzii*, *B. trematum* and *B. holmesii* successfully resisted the predation from *D. discoideum*, resulting in the small plaques formed on the bacterial lawn of these species. Besides these two groups, *B. bronchiseptica and B. parapertussis* $_{ov}$ showed an

intermediate ability to resist the predation from *D. discoideum*. The plaques formed on the bacterial lawns of *B. bronchiseptica* and *B. parapertussis* $_{ov}$ are larger than those of *B. hinzii*, *B. pseudohinzii*, *B. trematum* and *B. holmesii*, but far smaller than that of *B. pertussis*, *B. parapertussis* $_{hu}$, *B. avium* and *B. petrii* (Fig 4.5). The results indicate that the ability to survive within cells is correlated to the resistance to amoebal predation. However, *B. bronchiseptica* and *B. parapertussis* $_{ov}$ had relatively high intracellular survival in *D. discoideum* but failed to efficiently resist the predation from amoebae, indicating that extra machinery is needed besides intracellular survival to resist amoebal predation.

**Some non-classical *Bordetella* species translocate to and grow within amoeba sori.**

*B. bronchiseptica* and *B. parapertussis* $_{ov}$ were able to translocate into amoebal sori, while *B. pertussis* and *B. parapertussis* $_{hu}$ failed to do so. To test if other *Bordetella* species can enter amoebal sori for possible vector transmission, the sori formed on bacterial lawns were collected at various timepoints. The results showed that within the tested non-classical *Bordetella* species, *B. avium* and *B. petrii* failed to be recovered from collected sori, indicating that the machinery used for translocation to and/or survival within sori is not expressed (Fig 4.6). The species that failed to survive inside amoebae and resist amoebal predation also lost the ability to translocate into the amoebal sori, suggesting that the three aspects of ameoba interaction we tested may positively correlate to each other.

**The possible genes that contribute to interactions with amoeba.**

Of the *Bordetella* species, *B. pertussis*, *B. parapertussis* $_{hu}$, *B. petrii* and *B. avium* failed to interact with amoebal cells, while other species were able to survival inside amoebal trophozoites,

resist amoebal predation, and use amoebal sori as potential translocation vector. To probe the genes that might be involved in interactions with amoebal cells, GWAS was used to filter target genes. Genes that were present in *B. bronchiseptica*, *B. parapertussis* $_{ov}$, *B. holmesii*, *B. hinzii*, *B. pseudohinzii* and *B. trematum* but absent or inactive in any species that failed to interact with amoebae were considered in this study. Following these criteria, we identified 83 genes which relate to metabolism, regulation, stress response or unknown functions. (Fig 4.7; S4.1).

**The correlation between *Bordetella*-amoebal interaction and mammalian cell intracellular survival**

*Bordetella*-amoebal interactions are proposed to be a training ground for *Bordetella* species in adaptation to the mammalian host. To test the correlation between *Bordetella*-amoebal interactions and mammalian cell intracellular survival of *Bordetella* species, we carried out a gentamicin resistance assay on murine macrophages. To determine the number and proportion of bacteria that survive inside macrophages, these cells were infected with *Bordetella* bacilli at a multiplicity of infection (MOI) of 1 for 45 minutes followed by 2 hours gentamicin treatment. Around 15% of *B. holmesii* and *B. parapertussis* $_{ov}$ and 5-10% of *B. bronchiseptica*, *B. pseudohinzii*, *B. hinzii* and *B. trematum* were recovered from antibiotic treated macrophages. In contrast, much smaller numbers of *B. parapertussis* $_{hu}$, *B. petrii* and *B. avium* and undetectable levels of *B. pertussis* were recovered from macrophages in this experiment (Fig 4.8). To probe the possible correlation between intracellular survival inside amoebae and that in murine macrophages, we plotted all *Bordetella* species with survival percentage inside amoebae on the X axis and survival percentage inside macrophages on Y axis. The distribution of *Bordetella* species tells us that species that survive inside amoebae could also survive and persist inside macrophages.

The correlation coefficient value between these two parameters is 0.889035 among all tested *Bordetella* spp., excepting *B. parapertussis* $_{hu}$ and *B. holmesii*. Only *B. parapertussis* $_{ov}$ and *B. holmesii* have intermediate capacity to survive inside amoebae but can evade and survive inside murine macrophages with a much higher efficiency compared to other species (Fig 4.9), suggesting that these two mammal-specialized species may have gained other machinery enabling mammalian cell survival compared the other *Bordetella* species.

## Discussion

*Bordetella* species have been shown to survive inside various mammalian cell types (15-18, 27). The capacity of *B. bronchiseptica* to survive inside soil amoebal cells implies that the interaction of *Bordetella* species and soil amoebae may have served as an evolutionary training ground for Bordetella to adapt to intracellular survival inside mammalian cells. However, throughout the evolution of genus *Bordetella*, many genes have been lost and acquired (3), and whether the machinery that allows *Bordetella* to interact with soil amoebae is conserved in some or all *Bordetella* species is unknown. Herein, we tested 9 *Bordetella* species and their interactions with *D. discoideum*. *B. pertussis*, *B parapertussis* $_{hu}$ and *B. avium*, which have a closed life cycle circulating within a specific host population, have lost the capacity to interact with *D. discoideum* as an environmental shelter. In addition, *B. petrii*, a *Bordetella* species that was isolated from natural environment, was unable to interact with *D. discoideum* trophozoites, suggesting a different life cycle for this species. In contrast, *B. bronchiseptica*, *B. hinzii*, *B. pseudohinzii*, *B. trematum*, *B. holmesii* and *B. parapertussis* $_{ov}$ can survive inside amoebal trophozoite cells, resist predation by amoebae and translocate into the sori. These interactions with amoebae are likely to facilitate an interconnected life cycle for these species, as previously described for *B. bronchiseptica*.

The "classical" *Bordetella* species, *B. bronchiseptica*, *B. pertussis*, *B. parapertussis* $_{hu}$ and *B. parapertussis* $_{ov}$, are closely related and share many virulence factors (3). However, only *B. bronchiseptica* and *B. parapertussis* $_{ov}$ share successful interactions with *D. discoideum* in all three assays. Furthermore, within "non-classical" Bordetella species, *B. trematum*, *B. pseudohinzii*, *B. trematum* and *B. holmesii* interact with *D. discoideum*, while the other two species, *B. petrii and B. avium*, do not. These studies reveal that while human adapted species have lost their ability to survive intracellularly, many of the environmental species of *Bordetellae* continue to possess this ability. There remains a need to remain vigilant since it is possible that, as in the human adapted species, these environmental species possess the biological machinery to infect and opportunistically adapt to humans. To investigate the genes that contribute to interactions with amoebae, we used GWAS to probe target genes. The genes that are present and apparently functional in *Bordetella* species that interact with amoebae but are absent or inactive in the other species were collected and analyzed. *Bordetella* species that have a high survival level inside amoebal trophozoites survived better inside mammalian phagocytes, suggesting that the interactions between *Bordetella* species and amoebal cells may have served as training ground for *Bordetella* species to adapt to survival in their mammalian hosts. The genes we identified that contribute to amoebal intracellular survival and amoebal predation resistance may also have functions in interactions with mammalian host cells, suggesting new approaches to the study of host-pathogen interactions.

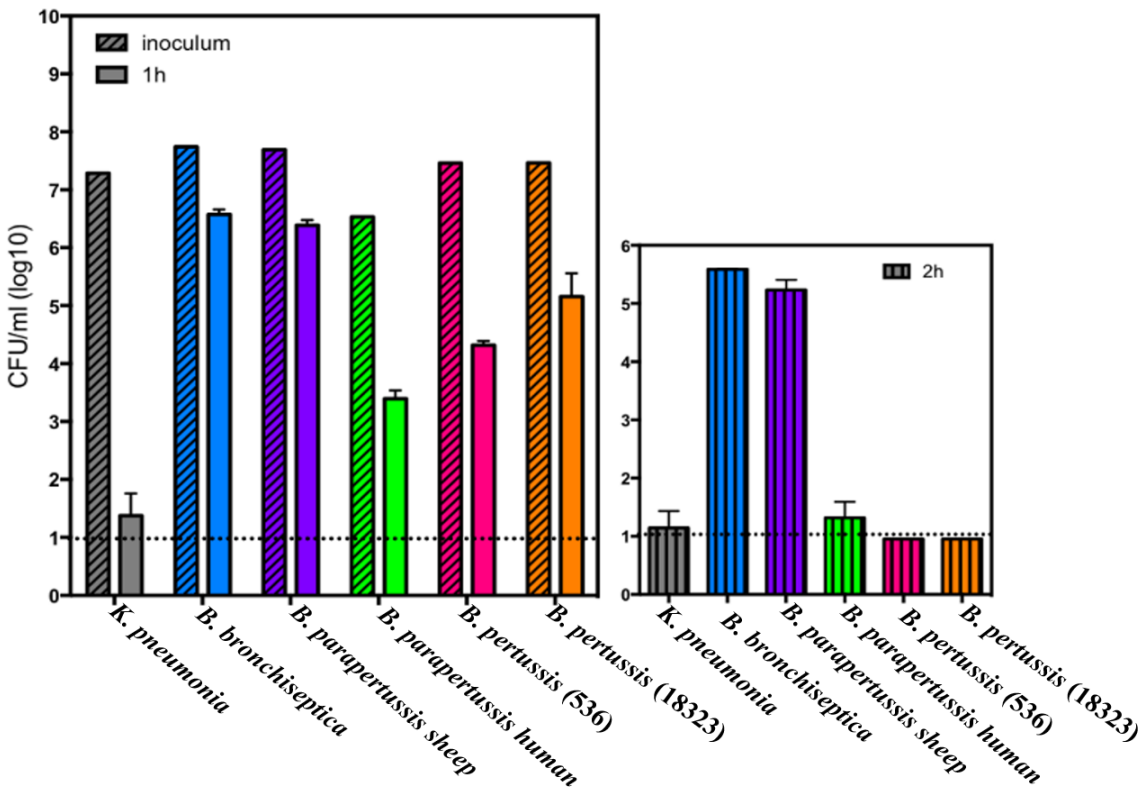


**Figure 4.1 Human-restricted classical Bordetella species can survive within *D. discoideum* but fails to persist.** Intracellular survival of three "classical" *Bordetella* species inside amoebal trophozoites was assessed in a 1-hour and 2-hour gentamicin protection assays. There were 3 replicates in each group. Error bars show the standard error of mean.

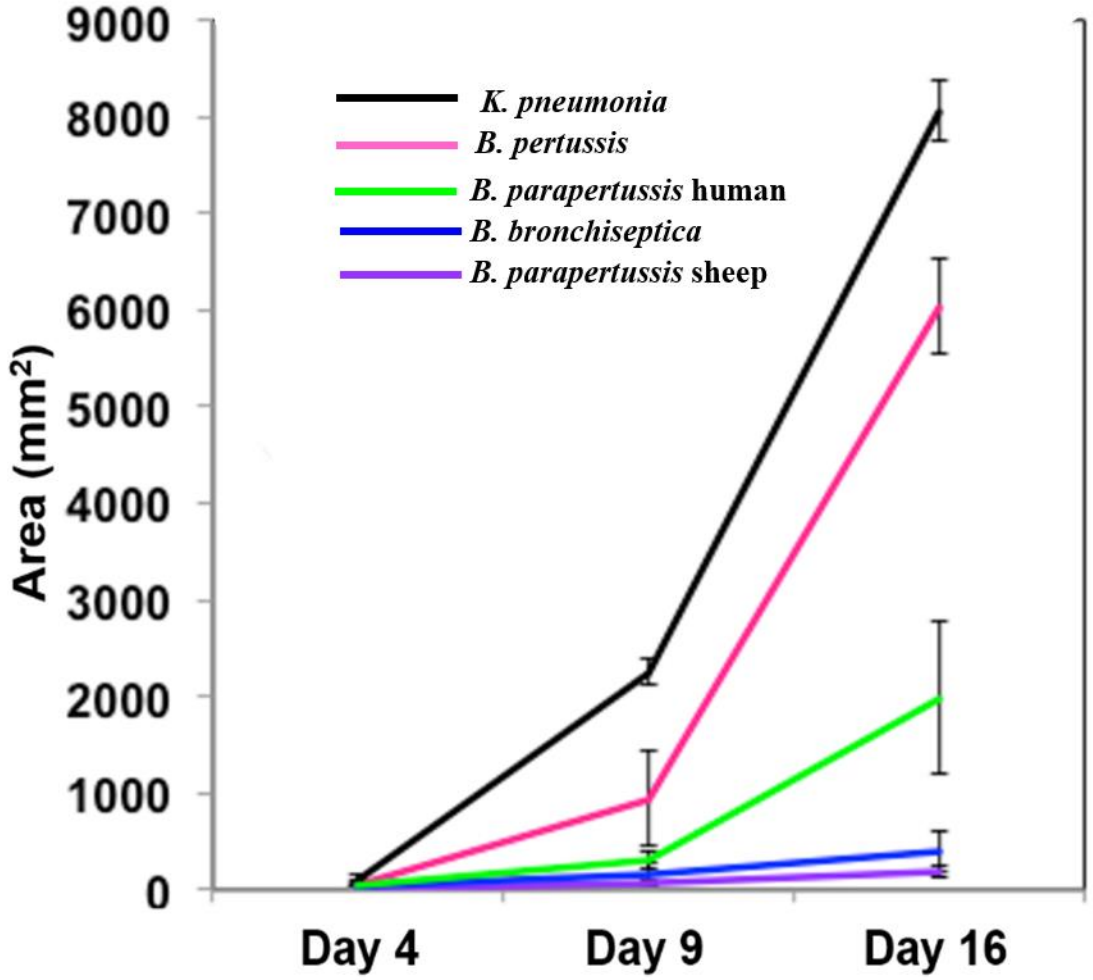


**Figure 4.2 *D. discoideum* efficiently feed on human-restricted *Bordetella* species.** The plaque area formed on the lawn of *Bordetella* species were measured at various time points. There were 3 replicates in each group. Error bars show the standard error of mean.

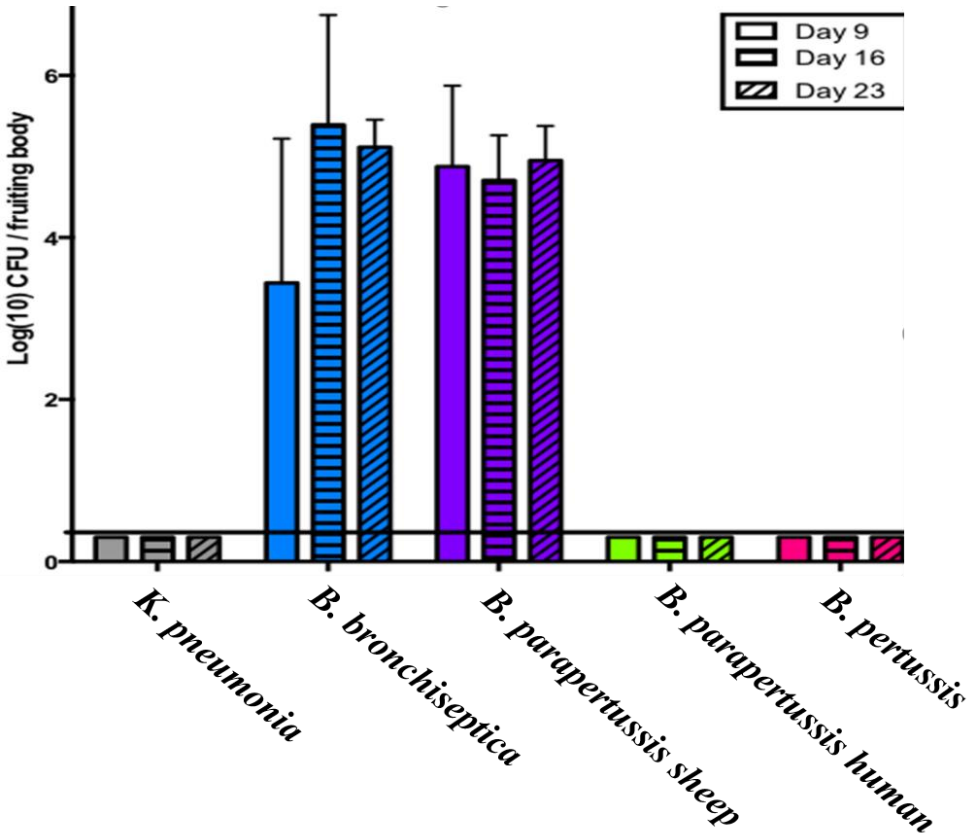


**Figure 4.3 Human-specific *Bordetella* species fail to colonize sori.** The sori formed on the lawn of *Bordetella* species were collected at days 9, 16 and 23 post inoculation of amoebae. The bacteria that survived inside sori were enumerated by plating samples on BG agar plates. There were 3 replicates in each group. Error bars show the standard error of mean.

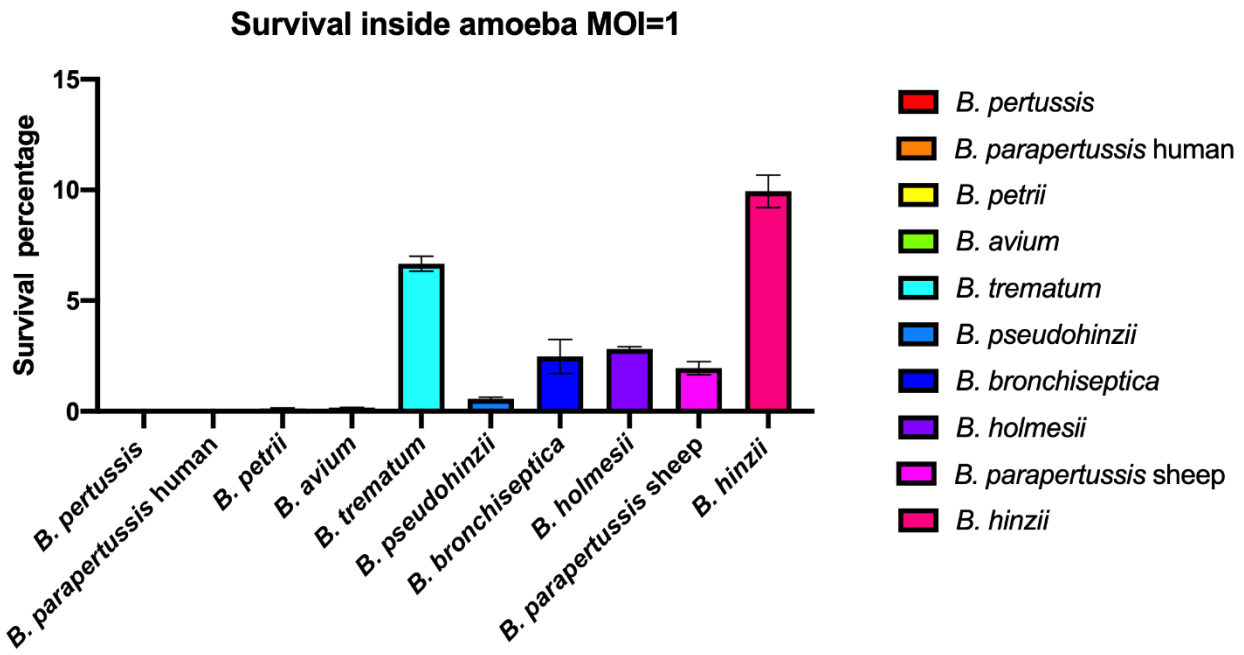


**Figure 4.4 Different intracellular survival ratios observed within non-classical Bordetella species.** Intracellular survival of three *Bordetella* species inside amoebal trophozoites was assessed in a 2-hour gentamicin protection assay using an MOI at 1. There were 3 replicates in each group. Error bars show the standard error of mean.

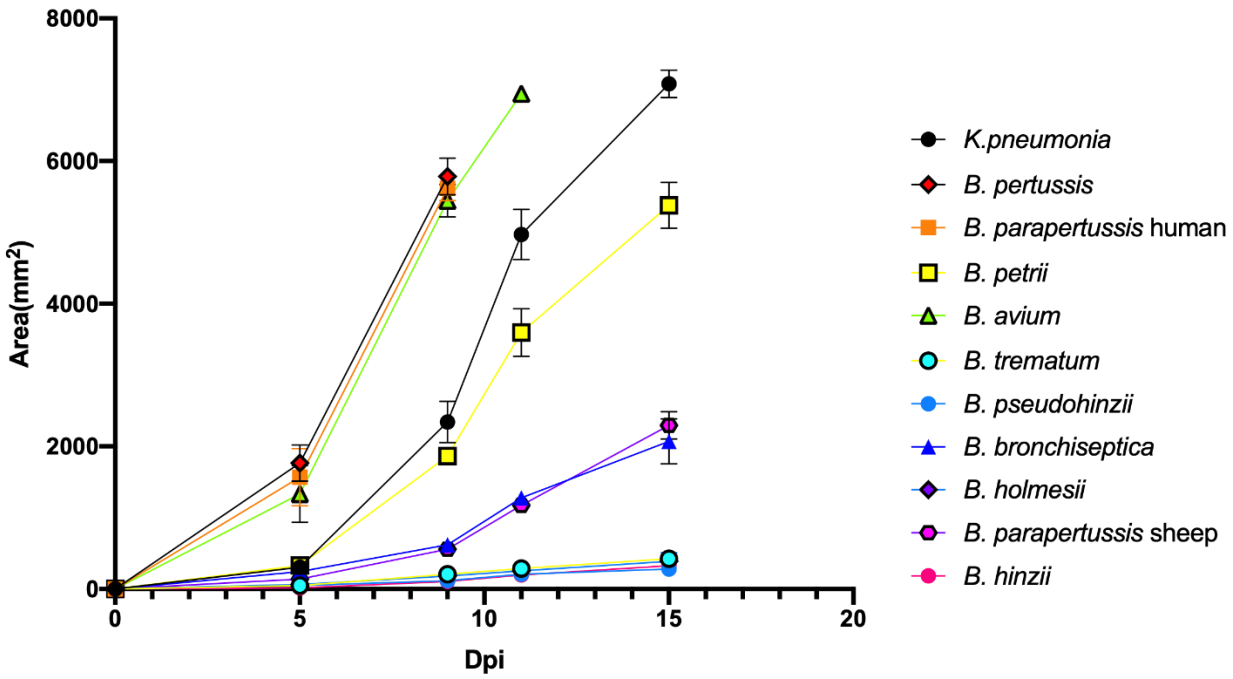


**Figure 4.5 Non-classical *Bordetella* species showed different capacities on resisting the predation of amoebae.** The plaques formed on the lawn of *Bordetella* species were measured at various time points post inoculation of amoebae. There were 3 replicates in each group. Error bars show the standard error of mean.

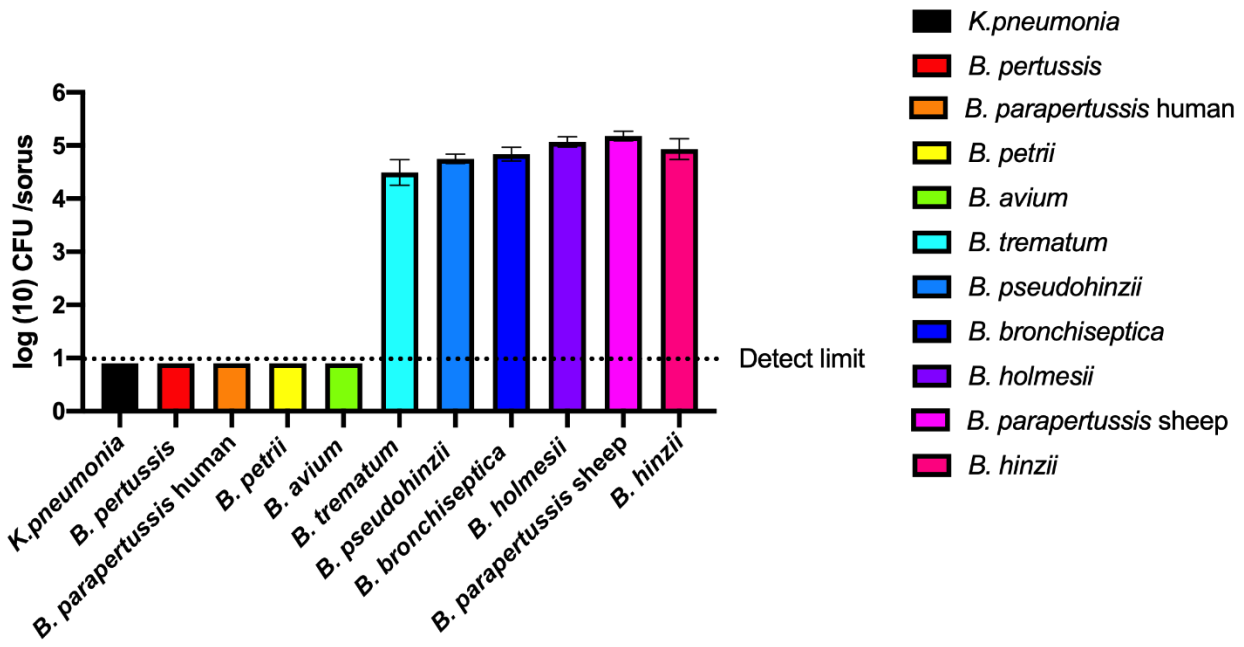


**Figure 4.6 Some *Bordetella* species can survive inside amoebal sori.** The sori formed on the lawn of *Bordetella* species were collected at day 16 post inoculation of amoebae. The bacteria that survived inside sori were enumerated by plating samples on BG agar plates. There were 3 replicates in each group. Error bars show the standard error of mean.

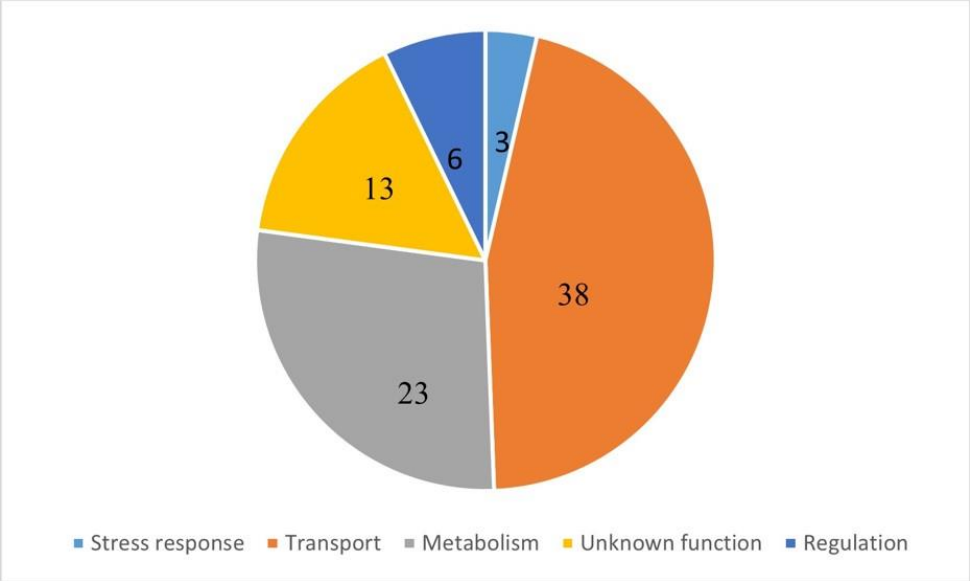


**Figure 4.7 Potential genes that contribute to *Bordetella*-amoebal interactions.** By using GWAS approach, 83 genes which may contribute to the interaction between *Bordetella* spp. and amoebae were identified. The genes are related to different functions, including transport, metabolism, regulation, stress response, and unknown functions.

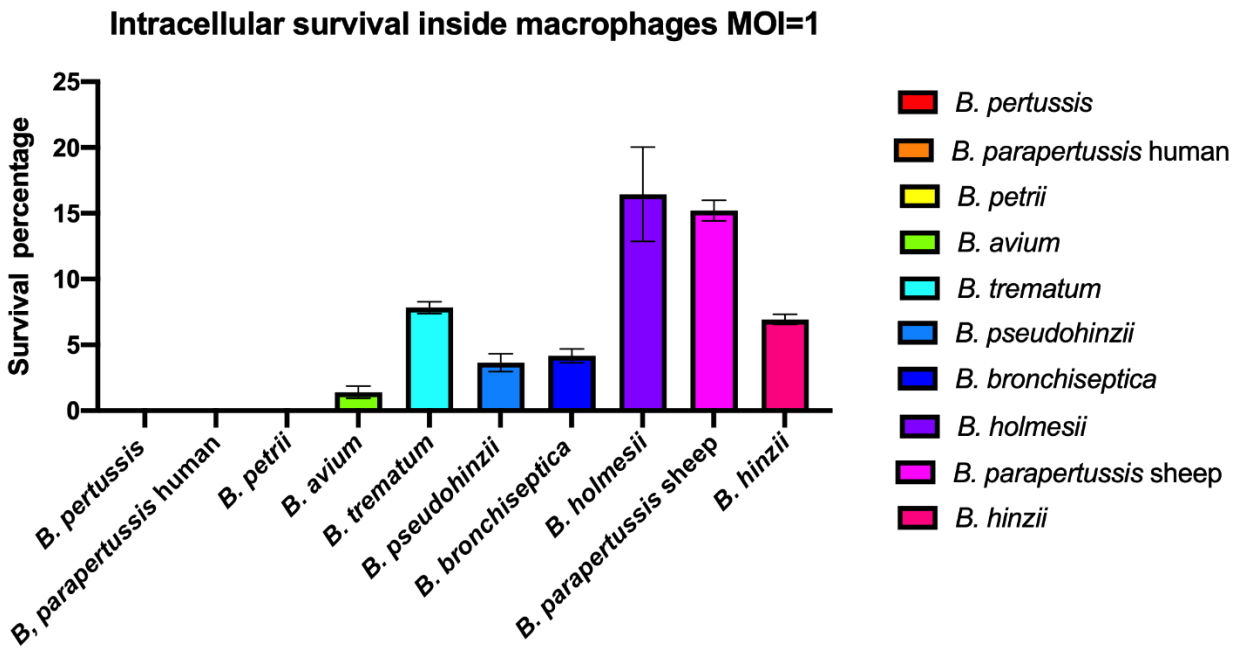


**Figure 4.8 Different intracellular survival rates of non-classical *Bordetella* species in RAW 264.7 murine macrophages.** Intracellular survival of *Bordetella* species inside murine RAW macrophages was assessed in a 2-hour gentamicin protection assay using a MOI at 1. There were 3 replicates in each group. Error bars show the standard error of mean.

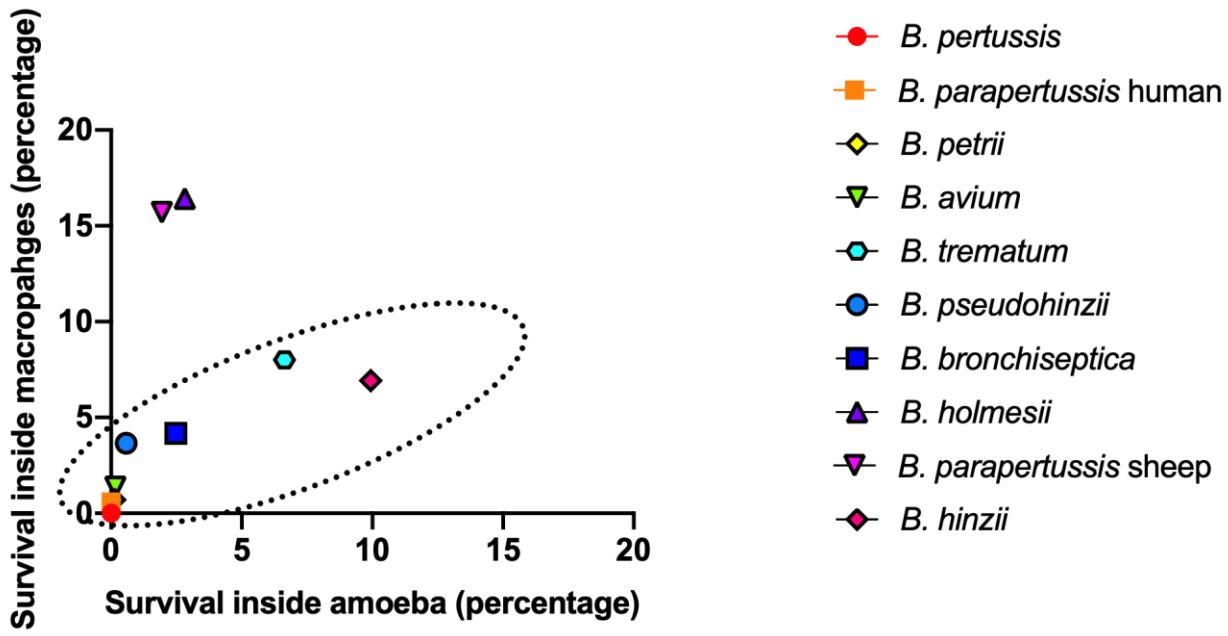


**Figure 4.9 The correlation between Bordetella-amoebal interaction and mammalian cell intracellular survival.** The survival percentage inside sori is located on the X axis and the survival percentage inside murine RAW 264.7 macrophages located on Y axis. The *Bordetella* species highlighted with dash line has a correlation coefficient value at 0.889035 for two variables.

## Table S4.1 The identified genes that may contribute to *Bordetella*-amoebal interactions.

| *B. bronchiseptica* | | Gene presence in | | | | *B. bronchiseptica* protein similarity with | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus_tag | **product** | ***B. bronchiseptica*** | ***B. parapertussis* ovine** | ***B. parapertussis* human** | ***B. pertusssi*** | ***B. hinzii*** | ***B. pseudohinzii*** | ***B. avium*** | ***B. petrii*** | ***B. trematum*** | ***B. holmsii*** |
| BB0039 | hydrolase | + | + | **Stop** | + | **0.683** | **0.676** | 0.355 | 0.375 | **0.721** | **0.669** |
| BB0163 | ABC transporter permease MlaE | + | + | + | + | **0.759** | **0.767** | **0.767** | 0.222 | **0.77** | **0.749** |
| BB0164 | ABC transporter ATPase MlaF | + | + | + | + | **0.778** | **0.774** | **0.778** | 0.344 | **0.771** | **0.789** |
| BB0165 | ABC transporter periplasmic MlaD | + | + | + | **FS** | **0.629** | **0.622** | **0.641** | 0 | **0.644** | **0.619** |
| BB0215 | ferric uptake regulator | + | + | + | - | **0.63** | **0.603** | 0.452 | 0.445 | **0.623** | **0.569** |
| BB0216 | hypothetical protein | + | + | + | - | **0.515** | **0.508** | 0 | 0.439 | **0.508** | **0.508** |
| BB0224 | ArsR family regulator | + | + | + | - | **0.73** | **0.739** | 0.324 | 0.315 | **0.667** | **0.685** |
| BB0388 | Periplasmic aromatic solutes transporter | + | + | + | + | **0.533** | **0.539** | **0.621** | 0.4 | **0.521** | **0.536** |
| BB0886 | Periplasmic aromatic solutes transporter | + | + | + | + | **0.591** | **0.591** | **0.604** | 0 | **0.557** | **0.615** |
| BB0898 | hypothetical protein | + | + | + | + | **0.793** | **0.797** | **0.747** | 0 | **0.731** | **0.782** |
| BB0945 | Phosphonate ABC transport permease | + | + | + | + | **0.858** | **0.853** | 0 | **0.85** | **0.842** | **0.824** |
| BB0946 | Phosphonate ABC transport permease | + | + | + | + | **0.791** | **0.747** | 0 | **0.801** | **0.777** | **0.723** |
| BB0947 | Phosphonate ABC transport ATP-binding | + | **FS** | + | **Trunc** | **0.875** | **0.887** | 0.375 | **0.848** | **0.871** | **0.867** |
| BB0948 | Phosphonate ABC transport periplasmic | + | + | + | + | **0.863** | **0.866** | 0 | **0.863** | **0.896** | **0.907** |
| BB0950 | haloacid dehalogenase-like hydrolase | + | + | + | + | **0.792** | **0.781** | 0 | **0.849** | **0.774** | **0.811** |
| BB1143 | CoA-transferase | + | + | + | - | **0.869** | **0.543** | 0.415 | 0.421 | **0.741** | **0.867** |
| BB1352 | aldo/keto reductase | + | + | + | + | **0.684** | **0.678** | **0.657** | 0.313 | **0.636** | **0.651** |
| BB1384 | acetate kinase | + | + | + | + | **0.758** | **0.755** | **0.765** | 0 | **0.768** | **0.742** |
| BB1385 | phosphate acetyl/butaryl transferase | + | + | + | + | **0.83** | **0.84** | **0.811** | 0.176 | **0.811** | **0.805** |
| BB1442 | ABC transport periplasmic protein | + | + | + | - | **0.87** | **0.876** | 0.237 | **0.873** | **0.806** | **0.855** |
| BB1443 | ABC transporter ATPase | + | + | + | - | **0.896** | **0.892** | 0.401 | **0.903** | **0.874** | **0.874** |
| BB1444 | ABC transporter permease | + | + | + | - | **0.928** | **0.924** | 0.364 | **0.92** | **0.902** | **0.898** |
| BB1737 | acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha | + | + | + | + | **0.928** | **0.928** | **0.931** | 0.112 | **0.925** | **0.922** |
| BB1897 | phosphohydrolase | + | + | + | + | **0.783** | **0.767** | **0.762** | 0 | **0.735** | **0.772** |
| BB1947 | N-formylglutamate amidohydrolase | + | + | + | + | **0.832** | **0.832** | 0.241 | **0.804** | **0.77** | **0.818** |
| BB1948 | glutamate transport periplasmic receptor | + | + | + | + | **0.836** | **0.833** | 0.457 | **0.827** | **0.823** | **0.845** |
| BB1949 | LysR-family transcriptional regulator | + | + | + | + | **0.879** | **0.879** | 0.422 | **0.869** | **0.827** | **0.869** |
| BB1999 | Periplasmic aromatic solutes transporter | + | + | + | **Trunc** | **0.818** | **0.787** | 0.441 | 0.468 | **0.732** | **0.772** |
| BB2000 | aldolase | + | + | + | + | **0.81** | **0.806** | 0.488 | **0.845** | **0.841** | **0.798** |
| BB2157 | osmotically inducible protein C | + | + | + | **FS** | **0.766** | **0.759** | 0.177 | 0.22 | **0.674** | **0.752** |
| BB2160 | ectoine/hydroxyectoine ABC transporter substrate-binding protein EhuB | + | + | + | + | **0.663** | **0.677** | 0.19 | **0.764** | **0.621** | **0.647** |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BB2161 | ectoine/hydroxyectoine ABC transporter permease subunit EhuC | + | + | + | + | **0.765** | **0.751** | 0.381 | **0.657** | **0.714** | **0.751** |
| BB2162 | ectoine/hydroxyectoine ABC transporter permease subunit EhuD | + | + | + | + | **0.83** | **0.848** | 0.296 | **0.848** | **0.786** | **0.798** |
| BB2163 | ATP-binding component of ABC transporter | + | + | + | + | **0.863** | **0.867** | 0.496 | **0.871** | **0.836** | **0.816** |
| BB2252 | hypothetical protein | + | + | + | + | **0.833** | **0.838** | **0.812** | 0 | **0.812** | **0.833** |
| BB2391 | DUF445 protein | + | + | + | + | **0.701** | **0.724** | 0 | 0 | **0.712** | **0.701** |
| BB2501 | amino-acid ABC transporter, APT-binding | + | + | + | + | **0.793** | **0.785** | 0.469 | **0.742** | **0.695** | **0.738** |
| BB2502 | amino-acid ABC transporter, permease | + | + | + | + | **0.907** | **0.894** | 0.327 | **0.916** | **0.832** | **0.872** |
| BB2503 | amino-acid ABC transporter, periplasmic | + | + | + | + | **0.752** | **0.756** | 0.317 | **0.786** | **0.762** | **0.798** |
| BB2885 | DUF1800 protein | + | + | + | **FS** | **0.532** | **0.56** | 0 | 0 | **0.513** | **0.517** |
| BB2886 | DUF1501 protein | + | + | + | + | **0.684** | **0.674** | 0 | 0 | **0.643** | **0.674** |
| BB3121 | DUF4377 protein | + | + | + | - | **0.617** | **0.609** | 0 | 0.399 | **0.56** | **0.584** |
| BB3211 | aspartate 1-decarboxylase precursor | + | + | + | + | **0.943** | **0.943** | **0.975** | 0 | **0.943** | **0.943** |
| BB3226 | Uncharacterized membrane protein YeiB | + | + | + | + | **0.558** | **0.568** | 0 | 0 | **0.533** | **0.516** |
| BB3279 | PEPSY-like protein | + | + | + | + | **0.776** | **0.781** | 0 | **0.694** | **0.78** | **0.757** |
| BB3280 | DUF2270 protein | + | + | + | + | **0.801** | **0.813** | 0 | **0.854** | **0.731** | **0.813** |
| BB3281 | DUF4198 protein | + | + | + | + | **0.718** | **0.722** | 0 | **0.748** | **0.673** | **0.699** |
| BB3282 | thiamine biosynthesis lipoprotein ApbE | + | + | **FS** | + | **0.589** | **0.609** | 0 | **0.586** | **0.565** | **0.586** |
| BB3283 | oxidoreductase | + | + | + | + | **0.54** | **0.538** | 0.083 | **0.632** | **0.538** | **0.558** |
| BB3358 | Periplasmic aromatic solutes transporter | + | + | + | + | **0.799** | **0.796** | 0.437 | 0.433 | **0.693** | **0.78** |
| BB3359 | malonyl-CoA synthetase | + | + | + | + | **0.844** | **0.836** | 0.33 | **0.85** | **0.811** | **0.803** |
| BB3360 | methylmalonyl-CoA decarboxylase | + | + | + | + | **0.649** | **0.631** | 0.295 | **0.683** | **0.679** | **0.627** |
| BB3361 | malonyl-CoA decarboxylase | + | + | + | + | **0.749** | **0.741** | 0 | **0.862** | **0.732** | **0.73** |
| BB3362 | gntR-family transcriptional regulator | + | + | + | + | **0.701** | **0.709** | 0.276 | **0.72** | **0.622** | **0.673** |
| BB3409 | acyl-CoA dehydrogenase | + | + | + | + | **0.712** | **0.712** | 0.163 | **0.744** | **0.662** | **0.662** |
| BB3850 | betaine/carnitine/choline transporter | + | + | + | + | **0.717** | **0.716** | **0.709** | 0 | **0.708** | **0.697** |
| BB4017 | osmotically inducible lipoprotein B | + | + | + | + | **0.877** | **0.877** | **0.822** | 0.342 | **0.808** | **0.863** |
| BB4028 | periplasmic transport protein | + | + | + | + | **0.889** | **0.891** | **0.889** | 0.432 | **0.882** | **0.891** |
| BB4187 | Periplasmic aromatic solutes transporter | + | + | + | + | **0.758** | **0.768** | 0.379 | 0.404 | **0.722** | **0.743** |
| BB4236 | TolA translocation protein | + | + | + | + | **0.691** | **0.668** | 0.306 | 0.288 | **0.675** | **0.539** |
| BB4284 | DUF533 protein | + | + | + | + | **0.788** | **0.765** | **0.695** | 0 | **0.73** | **0.637** |
| BB4285 | Periplasmic aromatic solutes transporter | + | + | + | + | **0.699** | **0.684** | **0.636** | 0.425 | **0.693** | **0.678** |
| BB4523 | Anion permease ArsB/NhaD | + | + | + | + | **0.849** | **0.847** | **0.821** | 0 | **0.851** | **0.855** |
| BB4524 | L-lactate dehydrogenase | + | + | + | + | **0.786** | **0.772** | **0.772** | 0.336 | **0.766** | **0.766** |
| BB4537 | universal stress protein | + | + | + | + | **0.771** | **0.774** | **0.733** | 0.149 | **0.767** | **0.729** |
| BB4556 | membrane efflux protein | + | + | + | + | **0.781** | **0.778** | 0 | 0 | **0.731** | **0.751** |
| BB4559 | Serine aminopeptidase | + | + | + | **Trunc** | **0.588** | **0.567** | 0 | 0.141 | **0.534** | **0.563** |
| BB4593 | Succinylglutamate desuccinylase | + | + | + | + | **0.718** | **0.683** | 0.308 | **0.7** | **0.683** | **0.671** |
| BB4637 | fatty acid hydroxylase | + | + | + | - | **0.761** | **0.764** | **0.737** | 0 | **0.767** | **0.752** |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BB4638 | polysaccharide deacetylase | + | + | + | - | **0.752** | **0.744** | **0.733** | 0 | **0.74** | **0.713** |
| BB4639 | alcohol dehydrogenase YncE | + | + | + | + | **0.775** | **0.769** | **0.792** | 0 | **0.745** | **0.757** |
| BB4660 | DUF523 protein | + | + | + | + | **0.669** | **0.651** | **0.62** | 0 | **0.663** | **0.602** |
| BB4950 | DUF1653 protein | + | + | + | + | **0.753** | **0.753** | **0.753** | 0 | **0.765** | **0.728** |
| BB4954 | periplasmic binding protein | + | + | + | + | **0.897** | **0.884** | 0.091 | **0.909** | **0.85** | **0.853** |
| BB4955 | periplasmic transporter DctM | + | + | + | + | **0.902** | **0.899** | 0.159 | **0.895** | **0.862** | **0.884** |
| BB4961 | ABC transporter, periplasmic binding protein | + | + | + | + | **0.794** | **0.791** | **0.771** | 0.201 | **0.768** | **0.772** |
| BB4962 | ABC transporter, permease | + | + | + | + | **0.833** | **0.841** | **0.825** | 0.416 | **0.805** | **0.802** |
| BB5008 | TCS sensor kinase | + | + | + | + | **0.769** | **0.766** | 0.354 | **0.798** | **0.752** | **0.752** |
| BB5009 | Tripartite-type tricarboxylate receptor TctC | + | + | + | + | **0.88** | **0.883** | 0.281 | **0.886** | **0.856** | **0.859** |
| BB5010 | Tripartite tricarboxylate transporter TctB | + | + | + | + | **0.828** | **0.814** | 0 | **0.828** | **0.793** | **0.779** |
| BB5011 | Tripartite tricarboxylate transport receptor TctA | + | + | + | + | **0.905** | **0.909** | 0.381 | **0.905** | **0.907** | **0.909** |

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# CHAPTER 5

# THE ROLE OF PSXA IN THE PERSISTENCE OF *BORDETELLA PSEUDOHINZII* IN THE MIDDLE EAR[4]

[4] Longhuan Ma[1], Kalyan Dewan, Bodo Linz, Colleen Sedney, Eric T Harvill. To be submitted to *Otitis Media Issue of Frontiers*.

## Abstract

Otitis media has a high prevalence among young children. The natural infection model developed in our laboratory has shown that *Bordetella pseudohinzii* can persist for an extended time, over 100 days, in the middle ears of mice. In contrast, a closely related species, *Bordetella bronchiseptica,* can cause colonization in middle ears but fails to persist. Genome comparison between these species showed that a pertussis toxin-like gene is present only in *B. pseudohinzii*, which may explain the difference in persistence between the two species. To investigate the role of this pertussis like-toxin, which we have named pseudohinzii toxin (Psx), in pathogenesis of *B. pseudohinzii*, a deletion mutation was generated in the gene encoding pseudohinzii toxin subunit A (*psxA*) and used for characterizing the functions of pertussis like toxin. We observed that *psxA* deleted in the *B. pseudohinzii* strain failed to persist in the respiratory tract and middle ears in the late stage of infection in mice. However, WT and mutant strains persisted similarly in mice without B cells and T cells indicating that Psx is only required when B and T cells are mediating bacterial clearance and suggesting that Psx interferes with important antimicrobial functions of B cells and T cells. These findings shed light on mechanisms of immune clearance of bacteria from the middle ear and may guide new approaches to the prevention and treatment of otitis media.


## Introduction

The genus *Bordetella* contains 10 species that include 3 closely related "classical" *Bordetella* species consisting of *B. pertussis, B. parapertussis* and *B. bronchiseptica* and 7 other more distantly related species, including *B. holmesii, B. petrii, B. avium, B. hinzii, B. pseudohinzii, B. trematum* and *B. ansorpii*, which are called "non-classical" *Bordetella* species (1). *B. pertussis* is the causative agent of whooping cough in humans (2), while *B. bronchiseptica* induces

respiratory infections in a wide range of mammals (2, 3). Within "non-classical" *Bordetella* species, *B. hinzii* and *B. pseudohinzii* are closely related to each other (4), which made it difficult to distinguish these two species in early studies.

*B. pseudohinzii* was isolated from laboratory-raised mice and characterized as a distinct species by Ivanov *et al*. in 2016 (4). Since then, *B. pseudohinzii* has been found in various animals, including laboratory mice and other rodents by different groups around the world (5-8). Our recent study revealed that *B. pseudohinzii* not only colonizes respiratory tract organs but can also cause infection of the middle ears where it persists for an extended period, and simulates chronic otitis media; a very important human disease that is poorly modeled in mice (9). Perniss *et al*. revealed that the colonization by *B. pseudohinzii* induces extensive airway inflammation with epithelial remodeling and causes damage to the cilia structure of epithelium, which may contribute to bacterial persistence (7). It has also been shown that a Th17-dominant immune response was activated in *B. pseudohinzii* infection suggesting *B. pseudohinzii* skews T cell differentiation (10). Ma *et al*. has shown that *B. pseudohinzii* replicates in both respiratory tract organs and the digestive tract via the observation of this bacteria in feces-facilitated transmission among mice (8). These characteristics of *B. pseudohinzii* likely contribute to various aspects of pathogenesis and may help explain why this bacterium can persist within hosts for long periods of time.

*B. bronchiseptica* efficiently colonizes and persists in the nasopharynx of mice for life. It also efficiently ascends the Eustachian tube and colonizes the middle ears of mice efficiently but is controlled within about one-month, while *B. pseudohinzii* bacilli persists in both the nasopharynx and middle ears at a high level for at least 3 months. To investigate the possible mechanisms underlying the persistence of *B. pseudohinzii*, we compared the genomes of *B. bronchiseptica* and *B. pseudohinzii* to screen for possible involved genes. A set of *B. pseudohinzii*

specialized virulence factor genes were identified, in which a set of pertussis toxin-like genes were targeted for further study. After we deleted the pertussis toxin-like subunit 1(*psxA*) in *B. pseudohinzii*, the mutant strain showed decreased persistence in the trachea, lungs, and middle ears. However, the mutant strain was indistinguishable from the wildtype strain in $Rag1^{-/-}$ mice, indicating that PsxA was involved in compromising the functions of B cells and/or T cells. The role of PsxA revealed in this study offers a new target for therapeutic intervention in *B. pseudohinzii* infection and presents Psx as a novel immunomodulating agent.

## Material and Methods

### Bacterial strains and growth

*B. pseudohinzii* strain 8-296-03 and *B. bronchiseptica* RB50 have been previously described (4, 11). *Both* were grown and maintained on Bordet-Gengou (BG) agar (Difco) supplemented with 10% defibrinated sheep's blood (Hema Resources). For mouse inoculations, *bacteria* were grown at 37°C, with shaking at 200 rpm, to mid-log phase in Stainer Scholte liquid broth (SS). Numbers of bacterial colony forming units (CFU) were estimated by measuring the optical density at 600 nm, validated by dilution in phosphate buffered saline (PBS), plating on BG agar and counting viable colonies after incubation for 2 days at 37°C.

### Mutant generation

The allelic exchange vector pSS4245 was used for the generation of deletion mutants. Briefly, ~1 kb of DNA flanking each end of gene *psxA* was PCR amplified (Upstream forward: 5'-AGGGCGGCCGCACTAGGGGTTGAGTTCGCGGGCGAAACCAG-3'; Upstream reverse: 5'-TGGCTGCCAGTTATTGACGCATACCCACGCCATTCCTGCTATG-3'; Downstream forward: 5'-TGGCGTGGGTATGCGTCAATAACTGGCAGCCACGATATGGTG-3';

Downstream reverse: 5’-GATCTGTACACCTAGGGGACGATGAGTACACGCGAATAC-3’), joined and inserted into the allelic exchange vector, pSS4245, by PIPE cloning (Plasmid vector amplification primers: Forward primer: 5’-CTAGTGCGGCCGCCCTAGCATAGG-3’; Reverse primer: 5’-CCTAGGTGTACAGATCCGGACCTGC-3’ ) (12). The construct was verified by sequencing, transformed into *E. coli* SM10λ*pir*, and transferred into the parental *B. pseudohinzii* 8-296-03 by mating. Colonies containing the integrated plasmid were selected and incubated on BG agar to stimulate allelic exchange by homologous recombination. Emerging colonies were screened by PCR for replacement of the wildtype by the mutant allele and confirmed by Sanger sequencing.

**Mouse experiments**

Four- to six-week-old C57BL/6J (000664) andC57BL/6J-Rag-/- (002216) mice were procured from The Jackson Laboratory (Bar Harbour, ME) and bred in the Harvill laboratory mouse colony (University of Georgia, GA). All mice were maintained in specific pathogen-free facilities, and all experiments were conducted following institutional guidelines. Mice were lightly sedated with 5% isoflurane (IsoFlo, Abbott Laboratories) and inoculated (150 CFU in 5 μL PBS) by pipetting the inoculum as droplets onto their external nares to be inhaled. To quantify bacterial numbers colonizing the respiratory tract and peripheral auditory system, mice were euthanized via $CO_2$ inhalation and the organs were excised. Tissues were homogenized in 1 ml PBS, serially diluted, and plated on BG agar. Colonies were counted (n = 3 or 4 per group) following incubation for two days at 37°C.

**Statistics**

For experiments determining differences in bacterial loads in the organs of mice, the following statistical analysis were performed using GraphPad PRISM (GraphPad Software, Inc):

Two-tailed unpaired Student t-tests, One-way ANOVA and Two-way ANOVA were used to determine statistical differences between two groups. The specific test used is indicated in the Figure Legends.

**Ethics statement**

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committees at The University of Georgia at Athens, GA (A2016 02-010-A13 Host-Pathogen Interactions, A2016 07-006-A5 Breeding Protocol). Mice were consistently monitored for signs of distress over the course of the experiments to be removed from the experiment and euthanized using carbon dioxide inhalation to prevent unnecessary suffering.

## Results

**Differential persistence of *B. bronchiseptica* and *B. pseudohinzii* in mice.**

To compare the colonization and persistence of *B. bronchiseptica* and *B. pseudohinzii*, groups of C57BL/6J mice were inoculated with 150 CFU bacteria in 5 µl PBS touched to their external nares. At day 3, 7, 14, 28, and 56, four mice from each group were euthanized and the bacteria in the nasal cavity, trachea, lung, and middle ears were enumerated. *B. pseudohinzii* colonized the respiratory tract and middle ears and persisted at consistently high numbers through day 56. *B. bronchiseptica* also efficiently and rapidly colonized these organs but numbers declined steadily after 28 days post inoculation (Fig 5.1), after the generation of a robust adaptive immune response. The prolonged persistence of *B. pseudohinzii* suggests that it has some mechanism(s) to avoid adaptive immune-mediated clearance that *B. bronchiseptica* is lacking.

***B. pseudohinzii* contains genes not shared by *B. bronchiseptica*.**

To probe genes that may contribute to the persistence of *B. pseudohinzii*, we compared the genomes of *B. pseudohinzii* and *B. bronchiseptica*. The comparison showed 1,319 unique genes present in the genome of *B. pseudohinzii* that relate to metabolism, transport systems, regulation systems, virulence, and insertion sequences (Fig 5.2A). We hypothesized that the genes unique to *B. pseudohinzii* and with homology to known virulence factors may contribute to its persistence. Within these *B. pseudohinzii*-specialized genes, there were 114 virulence factor genes which relate to serum resistance factors, Type IV secretion systems, adhesion factors and toxins (Fig 5.2B). Based on the known functions of pertussis toxin, we focused on an intriguing set of genes that appear to encode a related but distinct AB toxin.

**Generation of *B. pseudohinzii*Δ*psxA* mutant strain**

We identified 4 genes in *B. pseudohinzii* that have high similarity with subunit genes of pertussis toxin from *B. pertussis*. We previously named this putative toxin pseudohinzii toxin (Psx) and annotated these four genes as pseudohinzii toxin subunit A through D (psxA-D). Like pertussis toxin, Psx is also comprised of one enzymatically active subunit A, PsxA, and various transport subunits, PsxB-D, that comprise the B subunit of the toxin. When we compared the similarity between *B. pseudohinzii* toxin and their apparently orthologous proteins in *B. bronchiseptica*, no more than 50 percent of similarity appeared in any comparisons (Table 5.1), whereas the general similarity of proteins between these two species is 66.3 percent. To investigate the function of the apparently horizontally acquired Psx in the persistence of *B. pseudohinzii*, an isogenic *B. pseudohinzii* 8-296-03 derivative with a deletion of *psxA* was generated by allelic exchange (Fig S5.1-5.2).

**PsxA contributes to persistence of *B. pseudohinzii* in the respiratory tract and middle ears.**

To determine the role of PsxA in the colonization and persistence of *B. pseudohinzii*, C57BL/6 mice were inoculated intranasally with 5 µL PBS buffer containing 150 CFU of either WT or *B. pseudohinzii*Δ*psxA* bacteria. Four mice from each group were euthanized at 7-, 14-, 28-, 42-, 56- and 100-days post inoculation (dpi) to harvest nasal cavities, trachea, lungs and middle ears. The bacteria were enumerated by dilution plating on BG agar plates. WT *B. pseudohinzii* colonized the respiratory tract and middle ears and remained at a constantly high number. The *psxA* deletion mutant strain efficiently colonized all organs, albeit initially at lower numbers in the nasal cavity, but began to decrease in numbers in all organs but the nose, although the defect was only statistically significant in the trachea on days 56 and 100 post-inoculation (Fig 5.3). To examine the difference in persistence that appeared on day 56 more closely, this time point was repeated using 8 mice per group. These larger numbers revealed that the *B. pseudohinzii*Δ*psxA* mutant was significantly defective in persistence in the tracheas, lungs, and middle ears (Fig 5.4), being present at 90 to 99% fewer numbers than the wild-type strain. These data indicate that Psx plays a key role in the high-level persistence of *B. pseudohinzii*.

**PsxA inhibits lymphocyte-derived immunity.**

We and others have reported the role of adaptive immunity in control and clearance of *Bordetella* species (13-23). To determine whether PsxA is involved in manipulating host adaptive immunity in *B. pseudohinzii* infection, we used mature B/T cell (Rag1-/-) deficient mice to test persistence of WT and *psxA* deletion mutant bacteria. Rag1-/- mice were used to test the bacterial numbers in the nasal cavity, trachea, lung and middle ears at day 56 post inoculation. The results showed that there was no significant difference between the two strains on the persistence in

respiratory tract and middle ears (Fig 5.5). The observation that the persistence defect observed in C57BL/6J mice disappeared in mature T cell and B cell deficient mice, suggests that PsxA interferes with the anti-bacterial clearance functions of B cells and/or T cells, allowing *B. pseudohinzii* to persist.

## Discussion

*B. pseudohinzii* has shown the capability to persist in the respiratory tract and ears for at least three months (9). However, the closely related species, *B. bronchiseptica,* was controlled by the host one month after infection (11, 24, 25). To probe the genes that contribute to the persistence of *B. pseudohinzii*, in this study we compared the genomes of *B. pseudohinzii* and *B. bronchiseptica* and identified 1,817 genes that uniquely appeared in *B. pseudohinzii.* Within these genes, a set of pertussis toxin-like genes, named *B. pseudohinzii* toxin genes, were identified. Considering the critical functions of pertussis toxin in the pathogenesis of *B. pertussis* (26-43), we hypothesize that the *B. pseudohinziii* toxin may play a role in the persistence of *B. pseudohinzii*. To investigate the role of *B. pseudohinzii* toxin genes, we generated an isogenic mutant with an in-frame deletion of *B. pseudohinzii* toxin subunit A. Compared to the parental strain, *B. pseudohinzii*Δ*psxA* was defective in persistence in tracheas, lungs, and middle ears, indicating a key role of Psx in the remarkable persistence of *B. pseudohinzii*.

Since we previously showed *B. bronchiseptica* is cleared via B and T cell functions (14-16, 44, 45), we hypothesized that Psx may mediate persistence by disrupting these functions. To determine whether Psx activities are focused primarily on disrupting B cell and T cell functions, we used mice lacking both cell types (Rag1-/-) to examine the effect of Psx in their absence. We observed no defect for the Psx mutant in $Rag1^{-/-}$ mice, indicating that in the absence of B and T

cells Psx plays no critical role. PsxA is therefore required to limit adaptive immune mechanisms that are functional in C57BL/6J mice but absent in Rag1-/- mice and that more dramatically reduce numbers of the mutant.

Dewan *et al*. have shown that persistent infection of *B. pseudohinzii* in middle ears mimics chronic otitis media in humans and offers a natural infection model for studying otitis media (9). In our study, we found that the *B. pseudohinzii* toxin gene, *psxA*, contributes to persistence of bacteria in respiratory tract and middle ears, offering a potential therapeutic target for the treatment of otitis media. It should be noted that *B. pseudohinzii* is not the leading cause of human otitis media, so the findings in this study may not be directly applicable to common pathogens that induce human otitis media. However, organisms that cause persistent ear infections in humans likely have mechanisms to accomplish a similar disruption of B and T cell mediated bacterial clearance. Understanding the critical role of adaptive immunity in suppressing persistent infection, and how pathogens can disrupt these effects, may offer some insights on prevention and treatment of chronic otitis media.

Due to *B. pseudohinzii* being a newly identified *Bordetella* species (4), few studies have been carried out to analyze its pathogenesis. In our study, we generated the first *B. pseudohinzii* isogenic mutant strain by generating a deletion of *B. pseudohinzii* toxin gene, *psxA*, and determined that this gene plays a critical role in the ability of *B. pseudohinizii* to persist in the respiratory tract and middle ear. The *psxA* deletion in *B. pseudohinzii* paves the way for future genetic manipulation in this bacterium for other studies.

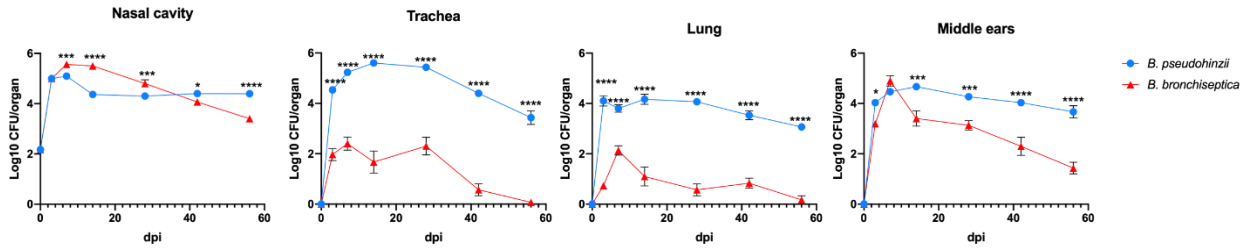


**Fig 5.1 *B. pseudohinzii* persists in the respiratory tract and ears, while *B. bronchiseptica* will be controlled in these organs.** Number of colony-forming units (CFU) recovered on days 3, 7, 14, 28, 42 and 56 from the nasal cavities, trachea, and lungs of mice inoculated with either *B. bronchiseptica* (green) or *B. pseudohinzii* (red) bacteria. There were 4 mice in each time point per group. Error bar shows the standard error of mean. Statistical significance was calculated by using Two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001**,** ****p < 0.0001.

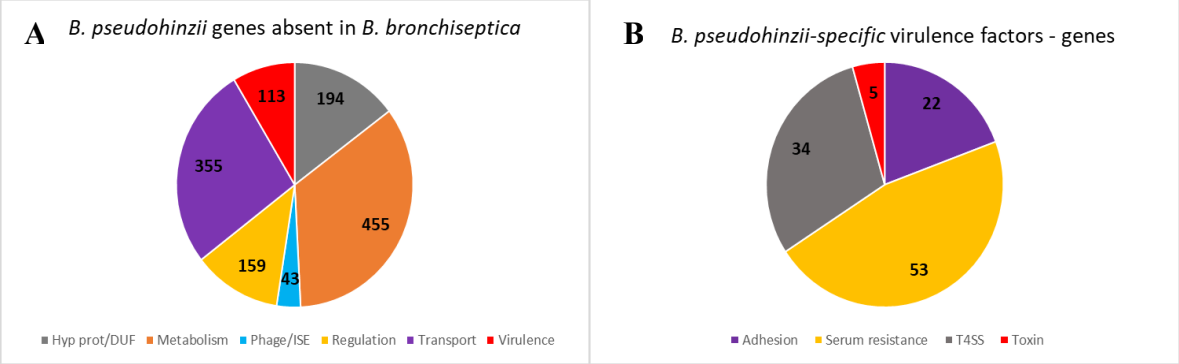


**Fig 5.2 Unique genes expressed in *B. pseudohinzii* but not in *B. bronchiseptica*.** A) Genes only appeared in the genome of *B. pseudohinzii*. B) Virulence factor genes that only exist in the genome of *B. pseudohinzii*.

**Table 5.1 The similarity between *B. pseudohinzii* toxin subunits and its correlated protein in *B. bronchiseptica*.**

| ***B. pseudohinzii* toxin subunit** | **Gene ID of Psx subunits** | **Correlated protein in *B. bronchiseptica* (gene ID)** | **Similarity (H-value)** |
|---|---|---|---|
| PsxA | BBN53_RS01715 | AYT36_RS24835 | 0.5 |
| PsxB | BBN53_RS01720 | AYT36_RS24855 | 0.388 |
| PsxC | BBN53_RS01725 | AYT36_RS24845 | 0.224 |
| PsxD | BBN53_RS01730 | AYT36_RS24850 | 0.252 |

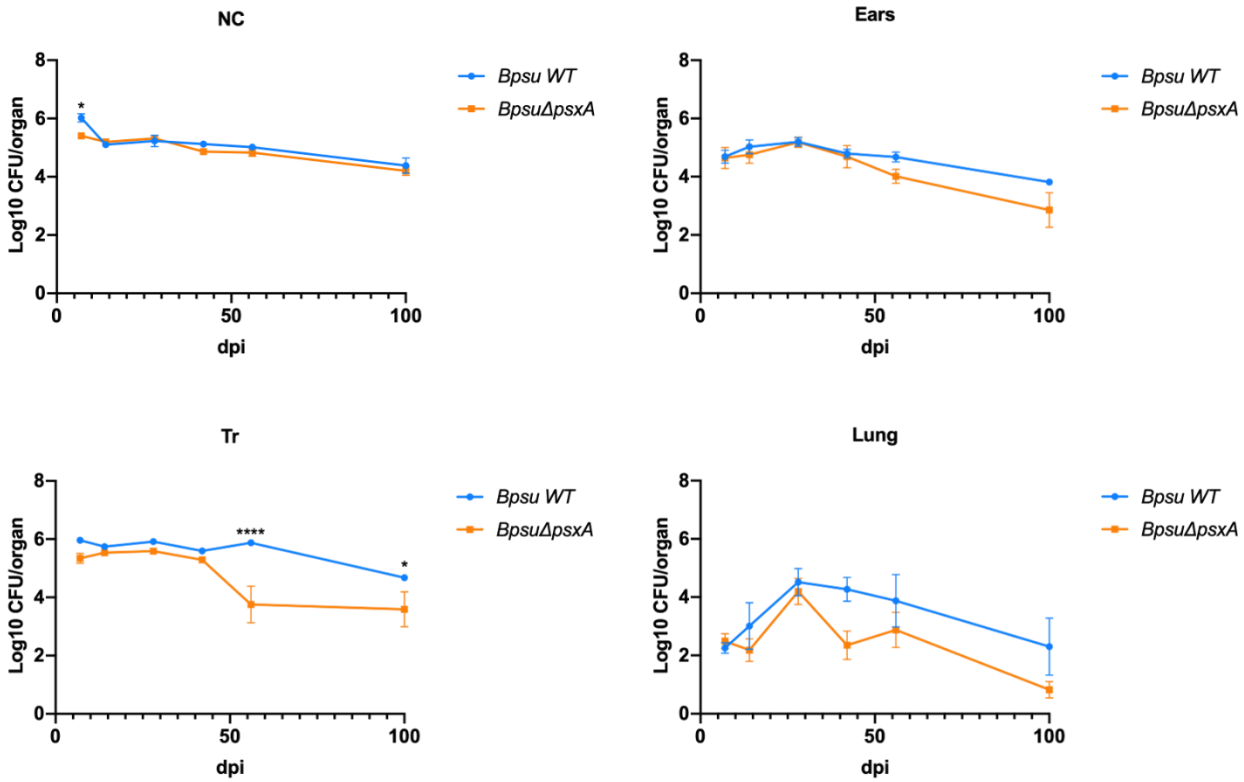


**Fig 5.3 PsxA contributes to persistence of *B. pseudohinzii* in trachea.** Number of colony-forming units (CFU) recovered on days 3, 14, 28, 42, 56 and 100 from the nasal cavities, trachea, and lungs of mice inoculated with either WT (blue) or mutant (orange) bacteria. There were 4 mice in each time point per group. Error bar shows the standard error of mean. Statistical significance was calculated by using Two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$**,** ****$p < 0.0001$.

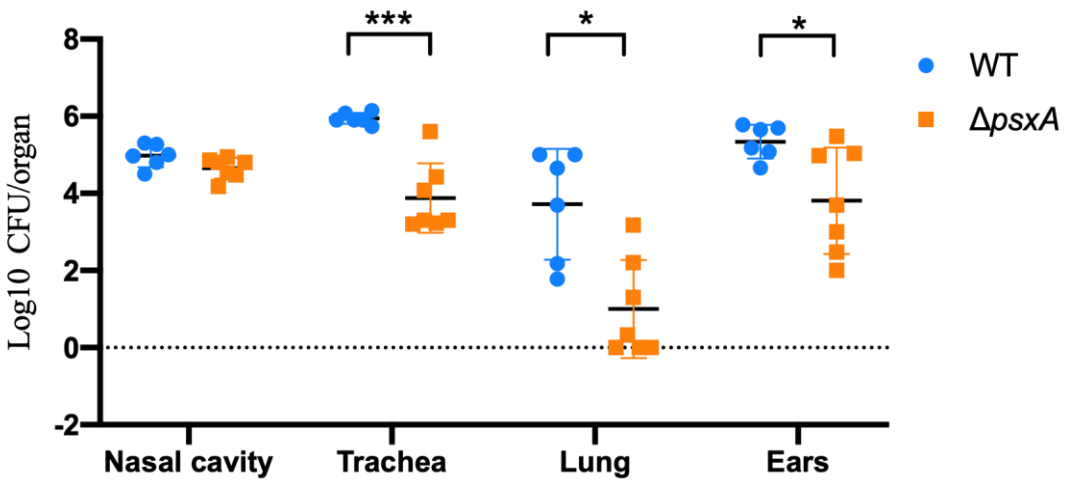


**Fig 5.4 PsxA contributes to persistence of *B. pseudohinzii* in trachea, lung and middle ears.** Number of CFU recovered on days 56 from the nasal cavities, trachea, and lungs of mice inoculated with either WT (green) or mutant (red) bacteria. There were 8 mice in each time point per group. Error bars show the standard error of mean. Statistical significance was calculated by using multiple T test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

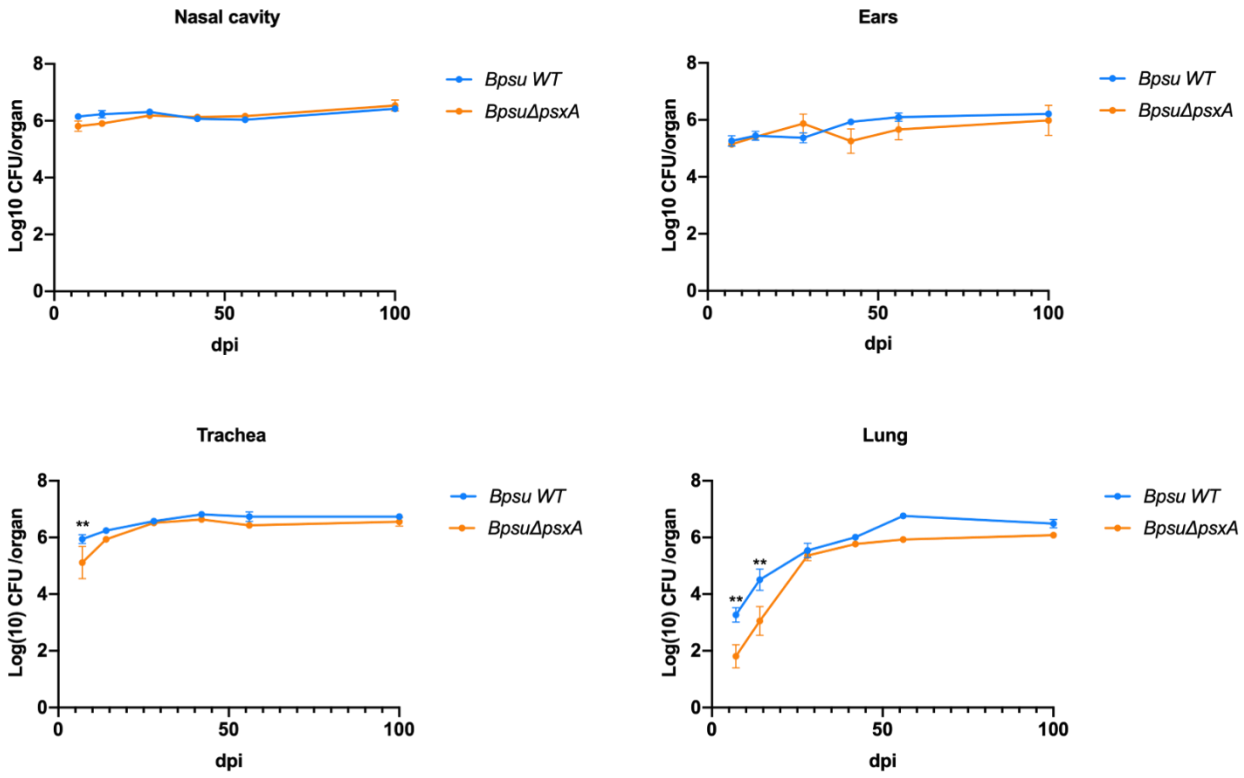


**Fig 5.5 PsxA does not affect persistence in Rag1$^{-/-}$ mice.** Number of colony-forming units (CFU) recovered on days 3, 14, 28, 42, 56 and 100 from the nasal cavities, trachea, and lungs of Rag1-/- mice inoculated with either WT (blue) or mutant (orange) bacteria. There were 4 mice in each time point per group. Error bars show the standard error of mean. Statistical significance was calculated by using Two-way ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

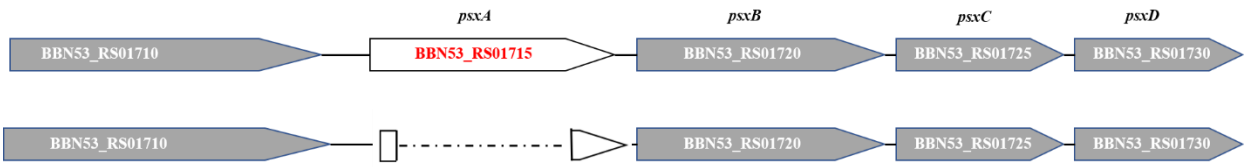


**Fig S5.1 Genetic context of *B. pseudohinzii*Δ*psxA* mutant strain.**

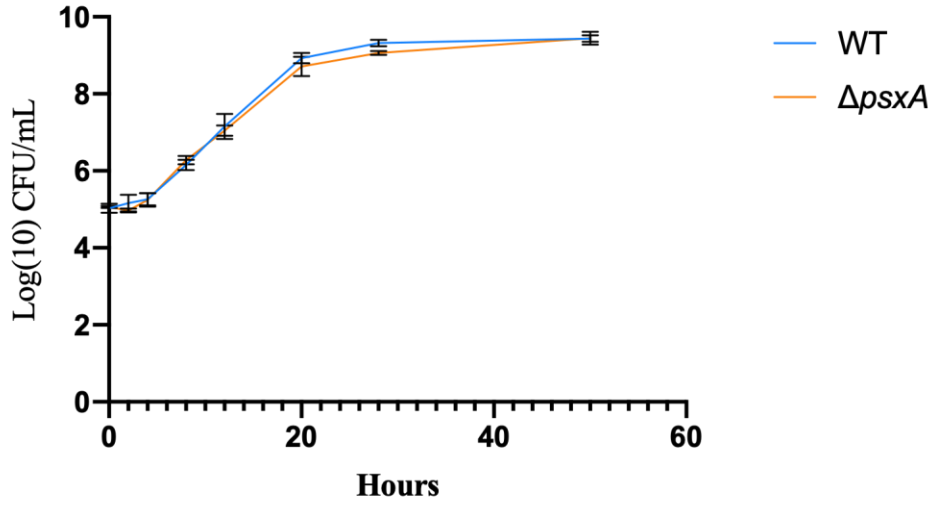


**Fig S5.2 Similar laboratory growth *in vitro* of *B. pseudohinzii* WT (blue) and Δ*psxA* (orange) bacteria.** There were 3 replicates in each time point per group. Error bars show the standard error of mean. Statistical significance was calculated using Unpaired t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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# CHAPTER 6

# SUMMARY AND SIGNIFICANCE

## Overall Summary and Significance

In these studies, we have characterized multiple *Bordetella* virulence factors that are involved in colonization of the host early in the disease process; in persistence, allowing the pathogen to cause prolonged infection, and in transmission which allows microorganism to transmit from one host to another. Pertactin, an outer membrane protein and one of the antigen components in the currently used acellular vaccines, has been lost from most circulating strains of *B. pertussis* in the United States, and pertactin deficient strains of *B. pertussis* are increasing in the prevalence in other countries worldwide. To speculate why pertactin is the primary antigen being lost from *B. pertussis* circulating strains, but not other vaccine antigens, we proposed three possible reasons: (1) The potential functional redundancy of pertactin, (2) the long-lasting, high-titer anti-pertactin antibodies, and (3) the selective pressure derived from the bactericidal function of pertactin-specific antibodies. These three reasons may individually or collectively explain the increased prevalence of *B. pertussis* pertactin negative strains. However, while most attention within the field has been drawn to explain why pertactin is being lost, the precise function of this protein still unknown. To investigate the biological functions of pertactin, we used a mouse infection model to assess the role of pertactin in the colonization, persistence, shedding, and transmission of *B. bronchiseptica,* a species that is a natural pathogen of mice and closely related to *B. pertussis.* Our data showed that pertactin contributes to shedding and transmission of *B. bronchiseptica*.

Chapter 3 of the thesis relates to the evolutionary aspects of how the genus *Bordetella* has acquired the ability to become a highly successful mammalian pathogen. In addition to transmission between mammalian hosts, *B. bronchiseptica* was found to use the sori of amoeba as a transmission vector in the environment. To examine whether the bacterial mechanisms that facilitate interactions with predatory amoebae are conserved in some or all *Bordetella* species, we tested 9 diverse *Bordetella* species in three assays representing separated aspects of their interactions with *D. discoideum:* intracellular survival, plaque expansion and translocation to the amoebic sori. We observed that only some *Bordetella* species retain the ability to successfully interact with amoebae and using GWAS (Genome wide association studies) identified a set of genes as probable genetic determinants of *Bordetella*-amoeba interactions.

In the final chapter of this dissertation, we characterized a pertussis-toxin-like factor that is uniquely identified in *B. pseudohinzii*. To investigate its role in the pathogenesis of *B. pseudohinzii*, an enzymatic domain deletion mutant was generated followed by a time course study in mice. The results showed that this toxin contributes to bacterial persistence by circumventing B and T cell-dependent immunity. Overall, these studies help us to better understand how *Bordetella* species utilize their molecular weapons to fight against cellular or systemic bacterial killing mechanisms.

**Pertactin Contributes to Shedding and Transmission of *Bordetella bronchiseptica***

**Implications**

Whooping cough is reemerging in western countries despite a high level of vaccination coverage. Increasing proportions of clinical *B. pertussis* isolates have lost the outer membrane protein pertactin, which is included in the current acellular vaccine due to its high antigenicity.

Researchers have proposed various reasons that may explain the loss of pertactin, including the functional redundancy of pertactin and vaccine-induced selective pressure. Much attention has been placed on this issue, however research into the detailed biological functions of pertactin *in vivo* have not fully determined how it contributes to many aspects of disease. In previous studies, pertactin has been shown to resist neutrophil-mediated clearance and suppress pro-inflammatory cytokine expression in the lungs of infected mice. However, in these studies, a very high number of *B. bronchiseptica* or *B. pertussis* bacilli were directly delivered into the lungs of mice, which does not accurately simulate some aspects of the natural infection progress. In our study, we developed and utilized a more natural infection mouse model to study the role of pertactin in the pathogenesis of *B. bronchiseptica*, a progenitor-like species of *B. pertussis.* In this model, a very low number of bacteria, as low as 5 CFU, were inoculated into Toll-like receptor 4 deficient mice (C3H/HeJ mice) through the external nares, resulting in a successful colonization in nose and a following spread into the lower respiratory tract. By using this model, we can study the natural colonization, bacterial spread within hosts, the host's progressive immune response, bacterial shedding, and transmission. To investigate the role of PRN in the pathogenesis of *B. bronchiseptica*, we compared the colonization and persistence of a wildtype *B. bronchiseptica* strain and its isogenic *prn* deletion mutant in HeJ mice. Our results showed that PRN does not contribute to the colonization and persistence of *B. bronchiseptica* in the respiratory tract. We then investigated the bacterial shedding and transmission of these two strains. We observed that mice infected with the WT strain had a significantly higher level of bacterial shedding from the nares and resultant transmission, indicating a role of PRN on the shedding and transmission of *B. bronchiseptica*. When we looked at the nasal cavities of two groups of mice, histopathological results indicated that there was a higher inflammation level and more mucus accumulation in the

WT infected group. To analyze the immune cell types recruited in the nasal cavities during this infection, flow cytometry was used, and the results showed that more neutrophils, NK cells and B cells were recruited into the nasal cavity of WT infected mice, suggesting a role of PRN on the recruitment of these cell types. At the same time, WT *B. bronchiseptica* was recovered from the spleens of infected mice while the *prn* mutant failed to reach the spleen, suggesting a contributing role of PRN on systemic dissemination.

To identify whether WT *B. bronchiseptica* was able to survive inside host cells, splenocytes were isolated and treated with antibiotics before harvesting surviving intracellular bacteria. We observed that all mice with WT *B. bronchiseptica* in the spleen had a substantial fraction that survived antibiotic treatment, suggesting that PRN contributes to intracellular survival, resulting in a systemic dissemination which may be partially if not entirely responsible for the high inflammation level we observed in WT infected mice. In this study, PRN has shown its critical role in the transmission of *B. bronchiseptica*. However, PRN is being lost from its closely related species, *B. pertussis*. Since *B. pertussis* induces acute disease in humans while *B. bronchiseptica* causes life-long chronic infection in its hosts, different genes and mechanisms may be used by these two species for their shedding and transmission. But, as *B. pertussis* and *B. bronchiseptica* share 90% similarity on their pertactin protein, the functions of PRN in *B. bronchiseptica* are likely to reflect its effects in *B. pertussis* to some extent.

**Future Directions**

In this study, PRN showed a critical role in intracellular survival, contributing to systemic dissemination. However, the type of cells that house bacteria are still unknown. In the next step, we will identify cell types within which bacteria survive using a combination of microscopy and

flow cytometry. Interaction of pertactin and these cell types will be investigated through *in vitro* systems and various mouse models. The ability of *B. pertussis* to persist inside host cells has important connotations for the modes of treatment that need to be administered in eradicating *B. pertussis* from infected hosts.

Pertactin has been lost from primarily *B. pertussis* isolates. In this study, it is shown that pertactin played an important role in bacterial shedding and transmission, suggesting that some alternative genes may complement the functions of pertactin in circulating *B. pertussis* strains. Beside pertactin, there are another 15 autotransporter proteins that appear in the genome of *B. pertussis*. In the next step, we will test the functions of other autotransporter proteins in shedding and transmission of *B. bronchiseptica* and *B. pertussis*. Autotransporter proteins that contribute to transmission of *B. bronchiseptica*, would be important targets for improved acellular vaccines required to address the increase in transmission of the pathogen.

**Pertactin-deficient Bordetella pertussis Strains: Vaccine-driven Evolution and The Re-emergence of Pertussis**

The incidence of pertussis began to increase since the 1980s in the USA and other countries using aP vaccines. The resurgence has been linked to many factors, like more sensitive diagnostic technologies, waning immunity of current acellular vaccines, and the appearance of *B. pertussis* variants lacking vaccine antigens. In the last 20 years, more and more *B. pertussis* isolates have lost PRN, an outer membrane protein. Since pertactin is 1 out of 5 components in the current acellular vaccines for several counties, the rise of pertactin deficient *B. pertussis* strains has been correlated with the reemergence of pertussis. It was proposed that vaccine-induced immunity placed selective pressures on circulating strains, resulting in an increase of pertactin deficient isolates. However, there are 5 components in the acellular vaccine, why only pertactin-deficient

strains were selected but not others is still unknown. In this section, we propose three possible reasons behind the loss of pertactin. The first one is the possible functional redundancy of pertactin in *B. pertussis*. Because there are another 15 autotransporter protein genes in the genome of *B. pertussis*, the functions of pertactin may be compensated by other autotransporter proteins after its loss. The second possible reason is that antibodies specific for pertactin stay at an effective high level and last for a longer time compared to some other components. Based on previous studies, it has been shown that after acellular vaccine immunization, the titers of IgG against PT and Fimbriae increase slightly, but decrease to a very low level in a matter of months. In contrast, IgG specific for FHA and pertactin increased to a very high level and stay at a relatively high level. The high and long-lasting level of antibodies against PRN and FHA poses the selection pressure on the mutant strains that lack FHA or/and PRN. The third possible reason is that only antibodies against PRN will induce bacterial killing outcomes. Because most of PT and FHA will be shed into the environment, the antibodies will neutralize the shed antigens first without encountering the bacteria. Fimbriae is bound on the surface of bacteria but being thin and filamentous extend away beyond the bacterial membrane surface. It is more than likely that antibodies against fimbriae will interact with the most distal part of the fimbria first which will keep the recruitment of complement system components away from the membrane of bacteria, failing to induce bactericidal mechanisms. Combining three reasons together, we can suggest that maybe only pertactin specific antibodies induce bacterial killing and pose selection pressure on circulating strains.

**Future Directions**

Because of the possible functional redundancy of pertactin, the identification of the potential alternative genes that compensate for the loss of pertactin will be our primary task. There

are additional 15 autotransporter proteins appearing in the genome of *B. pertussis*, we will start investigating the functions of these autotransporter proteins in the pathogenesis of *B. pertussis*.

In our speculated scenario only pertactin specific antibodies could induce bacterial killing mechanisms, so vaccine-induced selective pressure might favor the pertactin-deficient *B. pertussis* strains. While the antibody titers induced by any candidate vaccine antigen is an important aspect of vaccine design, future development should consider the cell localization and presentation of the antigen in its natural state on the bacterial surface. Our results suggest that future experiments should consider membrane proximal antigens which are likely to more directly kill bacteria, rather than simply reduce disease symptoms, to address the ongoing problem of *B. pertussis* colonization and transmission.

**Natural History and Ecology of Interactions Between *Bordetella* spp. and Amoebae**

In our recent study, *B. bronchiseptica* showed capability to interact with *D. discoideum* in these three aspects: survive inside amoebal *D. discoideum* trophozoites, resist amoebic predation, and use amoebal sori as a translocation vector. To investigate other classical *Bordetella* species on their interactions with amoebae, we tested *B. pertussis*, *B. parapertussis* human strain, and *B parapertussis* ovine strain on these three aspects. The results showed that *B. pertussis* and *B. parapertussis* human strain failed to interact with amoebae. In contrast, *B. parapertussis* ovine strain survived inside amoebal trophozoites, resisted amoebic predation, and translocated inside the amoebal sori, suggesting that within classical *Bordetella* species, human restricted species have lost capability to interact with amoebae and to use amoebal sori as a translocation vector.

To expand our investigation to non-classical *Bordetella* species, we tested another 6 *Bordetella* species. We observed that *B. pseudohinzii*, *B. hinzii*, *B. trematum* and *B. holmesii* could

interact with amoebae well in all three aspects, while *B. avium* and *B. petrii* failed to do so. The results suggest that *Bordetella* species with a closed life cycle, circulating between hosts without an environmental niche, fail to interact with amoebae. In contrast, the species that can be detected in multiple sources retain this capability, indicating a role of amoebae in the multiple life cycles of these later species. The positive correlation between intracellular survival of *Bordetella* species inside amoebal trophozoites and inside macrophages suggests that *Bordetella*-amoebal interaction may have been used as a training ground for *Bordetella* species to evolve and develop mechanisms for surviving inside mammalian hosts.

**Future Directions**

The correlation between intracellular survival of *Bordetella* species inside amoebae and mammalian phagocytes suggests that genes involved in *Bordetella*-amoebal interactions may also contribute to evading killing by host phagocytes. Through GWAS comparison, we have identified genes that may contribute to the ability to survive *Bordetella*-amoebal interactions. Future experiments will test the contributions of the identified genes to both surviving amoebal encounter and to the pathogenesis of *Bordetella* species in animal hosts. We will combine bacterial genome manipulation and gene knockout mouse models to investigate the roles of relevant genes in host-pathogen interactions.

**The Role of PsxA in The Persistence of *Bordetella pseudohinzii* in The Middle Ear**

In our recent report, *B. pseudohinzii* has been shown to colonize and persist in the respiratory tract and middle ears of mice. In comparison, *B. bronchiseptica* also colonizes the respiratory tract and middle ears of mice but is controlled after about 1 month infection. Through genome comparison between *B. pseudohinzii* and *B. bronchiseptica*, 114 *B. pseudohinzii*

specialized virulence factor genes were identified, within which a set of pertussis toxin-like genes we have named pseudohinzii toxin genes (*psx*), were targeted for further study. To investigate the role of Psx in the pathogenesis of *B. pseudohinzii*, we generated a *B. pseudohinzii* deletion mutant lacking the gene encoding Psx subunit A (Δ*psxA*). After being inoculated in mice, both the WT and mutant strains successfully colonized, but the mutant was cleared more rapidly from the respiratory tract and middle ears, by day 56 post inoculation, suggesting a defect of the *psxA* deletion mutant in persistence. Based on the observation that PsxA contributes to bacterial persistence in later stages of infection, we hypothesize that PsxA may interfere with the adaptive immune response, facilitating long-time persistence. To test whether the functions of B cells and T cells are affected by PsxA, Rag1-/- mice, which are deficient with B cells and T cells, were inoculated with either the WT or mutant strain for a colonization time course comparison. The results showed that WT and mutant colonized and persisted in Rag1-/- mice with similar bacterial numbers along the whole-time course, confirming the role of PsxA in disrupting the functions of T cells and B cells.

**Future Directions**

Our data suggest that PsxA interferes with the functions of B cells and T cells, allowing for the long-term persistence of *B. pseudohinzii*. To determine whether B cells or/and T cells are affected by PsxA, in the next step, we plan to use B cell and T cell deficient mice to examine their effect on bacterial numbers over the course of infection while comparing WT and mutant bacteria. We will then examine how PsxA directly affects the functions of these cells by comparing WT and mutant bacteria *in vitro* effects on cell's gene expression, cytokines/chemokines secretion and cell apoptosis.

It is possible that PsxA has an indirect influence on the functions of B cells and/or T cells. If no significant differences are observed with the above *in vitro* experiments, upstream signaling pathways could be considered. The *B. pseudohinzii*-mouse infection model represents the first natural mouse middle ear infection model and occupies a unique position in the study of chronic otitis media. The elucidation of Psx as a virulence factors that contribute to the persistence of this pathogen in mice establishes the first of the genes that the pathogen uses to persist in the host.