VACCINE-INDUCED-IMMUNITY-MEDIATED COMPETITION
BETWEEN ENDEMIC BORDETELLAE AND HOST IMMUNITY
AGAINST THEM

A Dissertation in
Genetics
by
Xuqing Zhang

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The dissertation of Xuqing Zhang was reviewed and approved* by the following:

Eric T. Harvill  
Associate Professor of Microbiology and Infectious Diseases  
Dissertation Advisor

Robert F. Paulson  
Associate Professor of Veterinary and Biomedical Sciences  
Chair of Committee

Avery August  
Professor of Immunology

Mary J. Kennett  
Professor of Veterinary and Biomedical Sciences

Sarah E. Ades  
Associate Professor of Biochemistry and Molecular Biology

Richard W. Ordway  
Associate Professor of Biology  
Chair, Intercollege Graduate Degree Program in Genetics

*Signatures are on file in the Graduate School
ABSTRACT

Whooping cough is a re-emerging disease in vaccinated populations. *Bordetella pertussis* and *B. parapertussis* are causative agents of this disease. *B. holmesii* is a recently recognized *Bordetella* species that can also cause whooping-cough-like respiratory symptoms. *B. bronchiseptica* infects a wide range of mammals, resulting in diseases of various severities. These bordetellae are endemic in humans and animals, laying burdens on their health. This dissertation investigated several aspects of vaccine-induced-immunity-mediated competition between endemic bordetellae as well as their interactions with host immunity. Current whooping cough vaccines include only *B. pertussis* antigens and are ineffective against *B. parapertussis* and *B. holmesii*. We demonstrated that efficient protection against *B. parapertussis* requires antibodies against O-antigen. Moreover, addition of *B. parapertussis* LPS containing O-antigen to an acellular vaccine conferred protection against this bacterium. O-antigen also enables *B. parapertussis* to avoid *B. pertussis* vaccine-induced immunity by inhibiting binding and functions of cross-reactive antibodies. *B. holmesii* was found to be circulating in Massachusetts in recent years. *B. pertussis* vaccine-induced antibodies did not efficiently bind to *B. holmesii*. Vaccinated mice given *B. holmesii*-specific, but not *B. pertussis*-specific, antibodies quickly controlled *B. holmesii* challenge. These data suggest that future vaccines targeting *B. parapertussis* and *B. holmesii* need to include their own protective antigens. Engagement of *B. pertussis* LPS with TLR4 induces production of several pro-inflammatory cytokines, including IL-1 and IL-6. Their roles in immunity against *B. pertussis* were investigated in this work. Following *B. pertussis* infection, IL-1R⁻/⁻ mice showed uncontrolled bacterial numbers in the respiratory tract, systemic spread, and dysregulated inflammation. These mice survived the challenge with *B. parapertussis* or a *B. pertussis* strain lacking pertussis toxin, indicating a role of IL-1R-mediated effects in overcoming the effects of this toxin. Mice lacking IL-6 generated less *B. pertussis*-specific antibodies, recruited fewer leukocytes to the lungs, produced decreased levels of cytokines, and delayed the clearance of *B. pertussis*. Therefore, IL-1R and IL-6 are required for efficient control of *B. pertussis*. A genetic module, *sigE-BB3751(rseA)-mucB(rseB)*, bearing sequence homology to the *E. coli* *rpoE-rseA-rseB* system, is recently discovered in *B. bronchiseptica* strain RB50. This system is involved in resistance to heat and ethanol stress, as well as cell envelope perturbations by SDS and
some β-lactam antibiotics. Moreover, the mutant lacking SigE failed to cause lethal infection in RAG<sup>−/−</sup> mice. The mutant lacking both RseA and RseA did not efficiently colonize murine hosts. These findings suggest that SigE and its proper regulation are important during infection. Combined, data contained herein demonstrated how <i>B. parapertussis</i> and <i>B. holmesii</i> evade <i>B. pertussis</i> vaccine-induced immunity, how IL-1R and IL-6 signaling contribute to host defenses against <i>B. pertussis</i>, and how SigE system is important for <i>B. bronchiseptica</i> stress responses and pathogenesis. We also discuss the implication of these findings and future avenues of research resulting from this work.
# TABLE OF CONTENTS

**LIST OF FIGURES**..................................................................................................................viii
**LIST OF ABBREVIATION**...........................................................................................................xi
**ACKNOWLEDGEMENTS**................................................................................................................xii

**Chapter 1** Introduction..................................................................................................................1
   The Genus *Bordetella* .....................................................................................................................1
   Evolution of the Endemic bordetellae..............................................................................................1
   Endemic bordetellae; disease and prevalence..................................................................................2
   Current Vaccine Strategies............................................................................................................4
   *Bordetella* Virulence Determinants.............................................................................................5
   Immunity to the endemic bordetellae..............................................................................................7
   Genetic Manipulation Strategies to study host-bordetellae interactions........................................8
   Preface...........................................................................................................................................10
   References.....................................................................................................................................12

**Chapter 2** The O-antigen is a Critical Antigen for the Development of a Protective Immune Response to *Bordetella parapertussis*................................................................17
   Abstract.........................................................................................................................................17
   Introduction....................................................................................................................................18
   Materials and Methods..................................................................................................................20
   Results..........................................................................................................................................24
   Discussion.......................................................................................................................................32
   Authors and Contributions.............................................................................................................35
   References......................................................................................................................................36

**Chapter 3** O-antigen Allows *Bordetella parapertussis* to Evade *B. pertussis* Vaccine-induced Immunity by Blocking Binding and Functions of Cross-reactive Antibodies.........................................................................................39
   Abstract.........................................................................................................................................39
   Introduction.....................................................................................................................................40
   Materials and Methods..................................................................................................................42
   Results..........................................................................................................................................45
   Discussion.......................................................................................................................................55
   Authors and Contributions.............................................................................................................58
   References......................................................................................................................................59
## Chapter 8  Constitutively Active SigE in *B. bronchiseptica* Leads to Decreased Virulence in Murine Hosts

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Results</td>
<td>154</td>
</tr>
<tr>
<td>Abstract</td>
<td>154</td>
</tr>
<tr>
<td>Introduction</td>
<td>155</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>159</td>
</tr>
<tr>
<td>Results</td>
<td>162</td>
</tr>
<tr>
<td>Discussion</td>
<td>168</td>
</tr>
<tr>
<td>Authors and Contributions</td>
<td>172</td>
</tr>
<tr>
<td>References</td>
<td>173</td>
</tr>
</tbody>
</table>

## Chapter 9  Summary and Significance

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implications</td>
<td>176</td>
</tr>
<tr>
<td>Future directions</td>
<td>182</td>
</tr>
<tr>
<td>References</td>
<td>186</td>
</tr>
</tbody>
</table>

## Appendix

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix A: qRT-PCR primers for Chapter 7</td>
<td>190</td>
</tr>
<tr>
<td>Appendix B: Comparison of RB50ΔsigE to RB50 under various stress condistions (Chapter 7)</td>
<td>191</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Chapter 2

Figure 2.1: The O-antigen contributes to the generation of protective immunity to *B. parapertussis* ........24
Figure 2.2: A response against the O-antigen contributes to effective vaccine-induced immunity ............25
Figure 2.3: The O-antigen is not required for the development of splenic IFN-γ or IL-10 responses to *B. parapertussis* .............................................................................................................26
Figure 2.4: The O-antigen contributes to the production of a robust anti-*B. parapertussis* antibody Response .................................................................................................................................26
Figure 2.5: Generation of antibodies that mediate efficient opsonophagocytosis of *B. parapertussis* by PMNs requires the O antigen ........................................................................................................28
Figure 2.6: Antibodies to the O-antigen are required for efficient antibody-mediated clearance of *B. parapertussis* ................................................................................................................29
Figure 2.7: Addition of purified *B. parapertussis* LPS to an acellular *B. pertussis* vaccine confers protection against *B. parapertussis* challenge ..............................................................................30

Chapter 3

Figure 3.1: *B. parapertussis* is more susceptible to wP-induced immunity in the absence of O-antigen ........45
Figure 3.2: aP does not reduce *B. parapertussis* numbers ........................................................................46
Figure 3.3: Splenic production of IFN-γ and IL-10 is cross-reactive ..............................................................47
Figure 3.4: IFN-γ contributes to the protection against *B. parapertussis* by wP ............................................48
Figure 3.5: O-antigen inhibits the binding of *B. pertussis* vaccine-induced antibodies to live, but not denatured, *B. parapertussis* cells .........................................................................................49
Figure 3.6: O-antigen decreases the opsonization of *B. parapertussis* by *B. pertussis* vaccine-induced antibodies .........................................................................................................................50
Figure 3.7: O-antigen blocks *B. pertussis* vaccine-induced antibodies from mediating adherence of *B. parapertussis* to PMNs ....................................................................................................50
Figure 3.8: O-antigen blocks *B. pertussis* vaccine-induced antibody-mediated phagocytosis of *B. parapertussis* ........................................................................................................................51
Figure 3.9: Passive transfer of wP-induced serum antibodies mediates clearance of O-antigen deficient, but not wild-type, *B. parapertussis* from mouse lungs ..................................................................52
Figure 3.10: Passive transfer of *B. parapertussis* specific antibodies rapidly reduces *B. parapertussis* colonization in aP and wP vaccinated animals .................................................................53

Chapter 4
Figure 4.1: *B. holmesii* cases and vaccine coverage in Massachusetts........................................................................................................67

Figure 4.2: Phylogenetic tree of *B. holmesii* isolates......................................................................................................................68

Figure 4.3: Both wP and aP failed to confer protection against *B. holmesii.................................................................69

Figure 4.4: wH/wP/aP splenic IFN-γ and IL-10 responses are cross-reactive.............................................................................70

Figure 4.5: wH/wP/aP antibody responses are not fully cross-reactive..................................................................................71

Figure 4.6: Supplement wP with *B. holmesii*- but not *B. pertussis*-specific antibodies confer protection against *B. holmesii........................................................................................................72

**Chapter 5**

Figure 5.1: IL-1 is induced by *B. pertussis..............................................88

Figure 5.2: Increased mortality and morbidity of *B. pertussis*-infected but not *B. parapertussis*-infected IL-1R⁻/⁻ mice..............................................................................................................89

Figure 5.3: Increased inflammatory pathology and leukocyte recruitment of *B. pertussis*-infected IL-1R⁻/⁻ mice..............................................................................................................92

Figure 5.4: IL-1R deficiency leads to increased pro-inflammatory and decreased anti-inflammatory cytokine production by BMDCs and BMMs.................................................................................................................94

Figure 5.5: IL-1R⁻/⁻ mice BMMs are efficient in killing *B. pertussis.................................................................95

Figure 5.6: Antibody responses are not defective in *B. pertussis*-challenged IL-1R⁻/⁻ mice.................................................................95

Figure 5.7: Increased TNF-α, IFN-γ and decreased IL-10, IL-17 responses in *B. pertussis*-infected IL-1R⁻/⁻ mice..............................................................................................................97

Figure 5.8: Intranasal administration of rmIL-17 restored the pulmonary IL-17 level but did not reduce *B. pertussis* numbers in IL-1R⁻/⁻ mice.................................................................................................................98

Figure 5.9: Pertussis toxin deficient *B. pertussis* failed to cause lethal infection in IL-1R⁻/⁻ mice.............99

**Chapter 6**

Figure 6.1: *B. pertussis* induces IL-6 in vitro and in vivo.........................................................................................................116

Figure 6.2: Delayed clearance of *B. pertussis* in IL-6⁻/⁻ mice.........................................................................................................117

Figure 6.3: IL-6 contributes to the generation of efficient vaccine-induced immunity against *B. pertussis*..............................................................................................................118

Figure 6.4: *B. pertussis*-specific antibody production is decreased in IL-6⁻/⁻ mice.................................................................119

Figure 6.5: Serum antibodies from IL-6⁻/⁻ mice are effective in antibody-mediated clearance of *B. pertussis*..............................................................................................................119

Figure 6.6: Immune functions not corrected by supplementing antibodies are improperly regulated in IL-6⁻/⁻ mice ..............................................................................................................120
Figure 6.7: IL-6 contributes to the recruitment of leukocytes into *B. pertussis*-infected lungs..............121
Figure 6.8: Splenic cytokine production is dampened in *B. pertussis*-inoculated IL-6−/− mice............122

Chapter 7
Figure 7.1: *B. bronchiseptica* SigE is an *E. coli* σE-like sigma factor....................................................138
Figure 7.2: Construction of a SigE-deficient strain of *B. bronchiseptica*.....................................................139
Figure 7.3: Role of SigE in various environmental stress..............................................................................140
Figure 7.4: Colonization of the respiratory tract of C57BL/6 by RB50 and RB50ΔsigE over time.............142
Figure 7.5: Survival and systemic colonization of immunodeficient mice in response to RB50 and RB50ΔsigE.................................................................................................................................143
Figure 7.6: SigE is not required for serum resistance..................................................................................145
Figure 7.7: RB50ΔsigE is less cytotoxic to macrophages..........................................................................145

Chapter 8
Figure 8.1: RB50ΔrseAB does not efficiently colonize the lower respiratory tract of C57BL/6 mice......162
Figure 8.2: RB50ΔrseAB-specific antibodies are efficient in antibody-mediated clearance of RB50ΔrseAB........................................................................................................................................163
Figure 8.3: RB50ΔrseAB fails to cause lethal infection in RAG−/− mice.....................................................164
Figure 8.4: rseAB is not required for serum resistance..............................................................................165
Figure 8.5: RB50ΔrseAB is less cytotoxic to macrophages......................................................................165
Figure 8.6: RB50ΔrseAB does not cause lethal infection in TLR4−/− mice..............................................166
LIST OF ABBREVIATIONS

aP: Adacel, acellular Pertussis vaccine
BG: Bordet-Gengou
BMDC: bone marrow derived dendritic cells
BMM: bone marrow derived macrophages
CDC: the Center for Disease Control and Prevention
cDNA: complementary DNA
CFU: colony forming units
CR3: complement receptor 3
ECF: extracellular function
ELISA: enzyme linked immunosorbent assay
ESR: extracellular stress responses
g: gravity
GM-CSF: granulocyte-macrophage colony stimulating factor
HRP: Horseradish Peroxidase
IACUC: Institutional Animal Care and Use Committee
IFN: interferon
Ig: immunoglobulin
IL: interleukin
i.p.: intraperitoneal
IS: insertion sequence
LD50: Mean Lethal Dose
LDH: lactate dehydrogenase
LPS: lipopolysaccharide
LRT: lower respiratory tract
MDPH: the Massachusetts Department of Public Health
MHC: major histocompatibility complex
MLST: Multi-Locus Sequence Type
PBS: phosphate buffered saline
PBS-T: phosphate buffered saline with tween-20
PCR: polymerase chain reaction
p.i.: post-inoculation
PVDF: polyvinylidene difluoride
Ptx: pertussis toxin
qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TGF: transforming growth factor
TLR: toll-like receptor
Th: T helper
TNF: tumor necrosis factor
TTSS: type three secretion system
UPGMA: Unweighted Pair Group Method with Arithmetic Mean
wP: whole cell B. pertussis vaccine
wH: whole cell B. holmesii vaccine
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Chapter 1

Introduction

The genus Bordetella

The genus *Bordetella* consists of nine species of gram-negative coccobacillus, *Bordetella pertussis*, *B. parapertussis*, *B. parapertussis*<sub>hu</sub> (human adapted), *B. parapertussis*<sub>ov</sub> (ovine adapted), *B. bronchiseptica*, *B. holmesii*, *B. avium*, *B. hinzii*, *B. petrii* and *B. trematum* (62). Since the following research does not involve the ovine-adapted *B. parapertussis*, *B. parapertussis* will refer to the human-adapted organism thereafter. This dissertation will focus on the bordetellae that cause diseases in humans or other mammals. These bordetellae, including *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, and *B. holmesii*, will be referred to “endemic” bordetellae in this work. *B. pertussis* and *B. parapertussis* infect humans, causing the respiratory diseases known as whooping cough (40, 62). In contrast, *B. bronchiseptica* causes diseases in various mammals, including but not be limited to mice, rats, guinea pigs, dogs, cats, pigs, sheep, cows, horses, foxes, monkeys, rabbits, skunks, opossums, raccoons, ferrets, hedgehogs, koalas, leopards, polar bears, seals, bushbabies and occasionally immunocompromised humans (33, 62, 73). This bacterium can cause a wide range of diseases, from asymptomatic colonization in the upper respiratory tract to lethal pneumonia (33, 62). Despite differences in host range and disease severities, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are so closely-related that the three have been suggested to be reclassified as subspecies, also known as the “classical bordetellae” or the “*B. bronchiseptica* cluster” (21, 30, 73, 75, 93). *B. holmesii* is a recently recognized *Bordetella* species that has been isolated from blood or nasopharyngeal specimens of humans (97, 107).

Evolution of the endemic bordetellae

Based on genomic sequences, *B. pertussis* and *B. parapertussis* appear to have evolved independently from *B. bronchiseptica*-like progenitors through large scale gene loss and rearrangement (30, 75, 93). Strains of *B. parapertussis* isolated from various locations over the past 50 years are largely clonal (92, 108), indicating its recent emergence. Together with comparative genomic analysis determining age of divergence (75), this suggests that *B. parapertussis* evolved from a *B. bronchiseptica*-like progenitor more recently than *B. pertussis*. 
Since the complete \textit{B. holmesii} genome sequence is not available, the evolutionary relationship between this species and other bordetellae is still obscure. 16S rRNA sequence and insertion sequence (IS) presence analyses indicate that \textit{B. holmesii} is closely-related to the classical bordetellae (97). However, further research suggests that this might be biased by horizontal transfer between \textit{B. holmesii} and \textit{B. pertussis} (25). Multilocus sequence typing analysis, characterization of the BvgAS and antigen cross-recognition assays (25, 29, 74) also suggest that \textit{B. holmesii} might not be as closely-related to \textit{B. pertussis} as originally assumed. Instead, it might be more closely-related to \textit{B. avium} and \textit{B. hinzii} (25).

\textbf{Endemic bordetellae; disease and prevalence}

The disease and prevalence of the classical bordetellae has been the subject of much research due to their impact on human and animal health. \textit{B. bronchiseptica} is prevalent in companion and agricultural mammals, with more than \(~\sim\)90\% seroprevalence in some swine herds, \(~\sim\)75\% culture-positivity in commercial rabbits, and \(~\sim\)10\% culture-positivity in felines from various sources (7, 24, 83). However, epidemiology of \textit{B. bronchiseptica} in natural settings is still obscure (33). \textit{B. bronchiseptica} is rarely isolated from humans, and typically only from immunocompromised individuals (106). Studies using a murine model of infection suggest that \textit{B. pertussis} immunity may limit the circulation of \textit{B. bronchiseptica} diseases in human populations (32). \textit{B. bronchiseptica} respiratory infections in non-human mammals result in various diseases of veterinary importance, such as kennel cough in dogs, atrophic rhinitis in pigs and snuffles in rabbits, although many \textit{B. bronchiseptica} infections are asymptomatic (62).

\textit{B. pertussis} and \textit{B. parapertussis} are both etiologic agents of whooping cough (40, 62). Whooping cough most commonly presents as a severe paroxysmal cough that may be followed by post-tussive whooping (from the desperate aspiratory effort between fits of coughing) and vomiting, though the disease may also be observed in milder forms, such as a persistent cough or even as a subclinical infection (62). On average, \textit{B. parapertussis} infections are less often associated with the severe symptoms of whooping cough than \textit{B. pertussis}, but the severity or duration of symptoms cannot reliably be used to differentiate between \textit{B. pertussis} and \textit{B. parapertussis} in a clinical setting and both pathogens are capable of causing substantial morbidity and mortality (6, 38, 60, 61).
The first *B. holmesii* isolate was submitted to the Center for Disease Control and Prevention (CDC) in 1983 and was initially designated as “CDC nonoxidizer group 2” (NO-2) (97). Since its establishment as a new *Bordetella* species in 1995 (97), this bacterium has been sporadically isolated from diverse countries, such as Australia, Germany, France, United Kingdom and Switzerland (26, 35, 74, 82, 97). *B. holmesii* infections usually have a relatively mild and uncomplicated course characterized by a nonspecific febrile illness (84), but severe systemic infection has also been reported (82). *B. holmesii* has been isolated from immunocompromised hosts (asplenic or sickle cell diseases patients and transplant recipients), often recovered from blood (49, 64, 71, 74, 84). *B. holmesii* has been isolated from pleural fluid, sputum or lung biopsy specimens of immunocompetent patients (82, 89). *B. holmesii* has also been isolated from nasopharyngeal specimens of otherwise healthy individuals, exhibiting whooping cough like symptoms, such as whoop or post-tussive vomiting (63, 107). Therefore, *B. holmesii* may be an important emerging human pathogen from the *Bordetella* genus, causing whooping cough like symptoms.

It is estimated that whooping cough causes 50 million cases and 300,000 deaths annually worldwide (20). However, a few factors may have confounded more accurate estimation of whooping cough incidence, as well as the proportion of whooping cough diseases caused by individual species. Since differentiating between *B. pertussis* and *B. parapertussis* based on clinical symptoms is difficult and does not affect the course of treatment (62), it is not commonly performed by clinicians. *B. holmesii* is a relatively newly discovered species and may not be detected or distinguished from other bordetellae, leading to low awareness at the clinical level. Even if there is an intention to differentiate among these *Bordetella* species, some technical difficulties interfere with laboratory diagnosis. Culture, a classical method of differential diagnosis, is highly specific but is maximally sensitive only in the initial phases of diseases. Furthermore, culture heavily depends on specimen quality, laboratory expertise, special media and an extended incubation period of 5-10 days (96). Diagnostic serology is highly sensitive and faster than the culture method. However, serology assays for *B. pertussis* or *B. parapertussis* differ among laboratories, and assays specific to *B. holmesii* are not available. Differential diagnosis based on PCR also offers an improvement in sensitivity over that of the culture method. Unfortunately, there is no standardized PCR test available for *B. pertussis* or *B. parapertussis* detection in clinical microbiology laboratories. One commonly used PCR protocol to
differentiate between \textit{B. pertussis} and \textit{B. parapertussis} targets the species-specific insertion sequence (IS) elements. However, \textit{B. holmesii} shares identical sequences within the amplification regions of IS481 and IS1001, otherwise specific to \textit{B. pertussis} and \textit{B. parapertussis}, respectively, which can potentially confound the differential diagnosis (78, 79).

Although whooping cough has been classified as a re-emerging disease by the CDC, diseases caused by \textit{B. parapertussis} or \textit{B. holmesii} are not listed as reportable to this agency, further hindering an accurate estimate of their prevalence. When carefully monitored, \textit{B. parapertussis} infections have been observed at high rates, being reported to cause between 1% and 98% of whooping cough cases in given populations, with most estimates falling between 4% and 40% (9, 54, 95). Random sampling has shown that more than 90% of \textit{B. parapertussis} infections result in mild or subclinical disease (9), suggesting that the vast majority of infections will not be diagnosed as whooping cough. These observations suggest that \textit{B. parapertussis} may be much more prevalent than researchers have previously appreciated. The frequency of \textit{B. holmesii} isolation from nasopharyngeal specimens in two separate studies is similar, being 0.6% (78, 107). Other than that, \textit{B. holmesii} has only been sporadically reported by physicians who encountered patients with underlying conditions. This raises the possibility of a biased and underestimated reporting rate of \textit{B. holmesii} by clinicians only pursuing the identification of a bacterial isolate recovered from immunocompromised patients.

\textbf{Current vaccine strategies}

The common vaccination strategy against \textit{B. bronchiseptica} is intranasal administration of live, attenuated strains (33). These vaccines appear to vary in their efficacies against \textit{B. bronchiseptica} diseases in the swine herd (27, 34, 76).

Early whooping cough vaccines, consisting of whole inactivated \textit{B. pertussis} cells, induce a strong antibody response and a balanced Th1/Th2 response (5, 50, 52). Clinical and experimental studies have shown that whole-cell vaccines are quite effective in reducing the incidence of \textit{B. pertussis} disease (17), but these vaccines confer very low levels of protection against \textit{B. parapertussis} (5, 50). The levels of cross-protection by these vaccines against \textit{B. holmesii} have not been reported.

Most developed countries now use acellular vaccines consisting of some combination of the following \textit{B. pertussis} antigens: pertussis toxin (Ptx), pertactin (PRN), filamentous hemagglutinin (FHA),
fimbriae (FIM) 2, and FIM 3. Acellular vaccines induce robust antibody responses only to this limited set of antigens, and the T cell response is skewed towards a Th2-type response (5, 51, 53). These vaccines are effective against *B. pertussis*, but some data have indicated that they are less effective than the whole cell vaccines against *B. parapertussis* (5, 50, 52). In addition, acellular vaccines may enhance the ability of *B. parapertussis* to colonize hosts (22) (Long G.H., Karanikas A.T., Harvill E.T., Read A.F., and Hudson P.J., unpublished data), and the prevalence of this pathogen may have increased despite, or perhaps due to, the introduction of these vaccines (48). It is unknown whether the acellular vaccines confer cross-protection against *B. holmesii*.

The vast majority of individuals begin to undergo whooping cough vaccinations at 2 months of age in some developed countries, in which vaccine coverage is more than 80% (14, 48, 80). The use of whooping cough vaccines has undoubtedly affected the epidemiology of *B. pertussis*, and has likely affected that of *B. parapertussis* as well. *B. parapertussis* has been found to cause a higher percent of whooping cough cases in vaccinated, compared to unvaccinated, individuals in a certain population (48), suggesting that vaccination may confer a selective advantage to *B. parapertussis*. Differences observed in the age-prevalence of *B. parapertussis* versus *B. pertussis* also support this theory. *B. pertussis* is most common in very young children prior to vaccination and in adolescents in whom vaccine-induced immunity has waned (18, 98). *B. parapertussis*, on the other hand, is most common in young children aging 1 to 10 years old, the age group that has been most recently vaccinated against *B. pertussis* (6, 47, 99) (Lavine J., Han L., Harvill E.T., and Bjornstad O., unpublished data). Overall, these observations suggest that vaccination against *B. pertussis* may confer a selective advantage to *B. parapertussis*. Little is known about the epidemiology of *B. holmesii*, especially in recent years.

Importantly, whooping cough has been increasing in incidence over the past 20 years in countries that have high vaccine coverage (1, 15, 23, 86, 94). It is not clear what the relative roles of *B. pertussis*, *B. parapertussis* and *B. holmesii* are in this resurgence. The modification of vaccine strategies to efficiently protect against all these endemic bordetellae in humans would likely reduce the incidence of whooping cough diseases overall.

*Bordetella* virulence determinants
The colonization and persistence of *B. bronchiseptica* in the murine respiratory tract depend on the expression of an array of virulence determinants regulated by the BvgAS two-component system (19). The BvgAS system is composed of BvgS, the transmembrane sensor kinase (87), and BvgA, the DNA-binding response regulator, present in all endemic bordetellae (4, 10, 29, 69). *bvgAS* expression can be activated by growth at 37ºC, or in the relative absence of MgSO₄ or nicotinic acid (65, 66), although the *in vivo* signals that regulate this system are not fully understood. Bordetellae grown under such conditions are referred to as Bvg⁺-phase. Through a four-step His-Asp-His-Asp phosphor-relay mechanism, BvgA is phosphorated by BvgS, and promotes the transcription of Bvg⁺-phase-specific genes by binding to the BvgA-binding site in their promoter regions (81, 87). Genes that are positively regulated by the BvgAS system encode toxins, such as adenylate cyclase toxin (ACT), Ptx (*B. pertussis* specific), dermonecrotic toxin (DNT), type three secretion system (TTSS), as well as adhesions including FIM, PRN and FHA, and gene products that modify LPS structures (62). Under Bvg⁺ conditions, another set of genes is repressed through the regulation by BvgR, which is activated by BvgA (67). Under “modulating” conditions, such as 25ºC or in the relative presence of MgSO₄ or nicotinic acid, the BvgAS phosphor-transfer system is inactivated, which decreases the production of BvgR, and genes encoding flagellin as well as genes involved in starvation survival are thus expressed (2). Bordetellae growing under sub-modulating conditions can display phenotypes intermediate between the Bvg⁺ and Bvg⁻ phases, designated the Bvg⁰ phase, which has been proposed to play a role in respiratory transmission and biofilm formation (28, 41, 88). Experiments using phase-locked mutants indicate that the Bvg⁺ phase is both necessary and sufficient for respiratory tract infection (19), and suggest the critical roles of virulence determinants expressed in the Bvg⁺ phase during infection.

Although *B. pertussis* and *B. parapertussis* share most of their virulence determinants, each expresses factors not expressed by the other. For example, Ptx is only expressed by *B. pertussis*, whereas O-antigen is only expressed by *B. parapertussis*. The gene encoding Ptx is present in *B. parapertussis* genome, but some mutations in the promoter region prevent its expression (4, 58). Ptx is an AB₅ toxin that can ADP-ribosylate and inactivate a subset of G proteins, which are shared by many chemokine receptor signaling pathways (8, 42). Clinically, Ptx is known to be the cause of some systemic symptoms associated with whooping cough, such as lymphocytosis, histamine sensitivity and insulinemia (72). This toxin has been shown to block the
early migration of neutrophils into the lungs (3, 43). Another study indicates that airway macrophages are the primary target of Ptx in the lower respiratory tract (LRT) (13). Ptx exerts multiple suppressive effects on the immune system other than those observed on innate immune cells; for example, Ptx suppresses the production of anti-\textit{Bordetella} serum antibodies (12, 68), reduces major histocompatibility complex class II on the surface of monocytes (85), and interferes with CD1a expression on dendritic cells (59).

Although Ptx exerts multiple suppressive effects on host immunity, \textit{B. parapertussis} is successful in infecting humans without the expression of this toxin. This may indicate that Ptx is not required for virulence of bordetellae in humans; alternatively, this may also indicate the existence of some alternative \textit{B. parapertussis}-specific virulence mechanisms. O-antigen, the membrane distal repeating chain of disaccharides, is retained by \textit{B. parapertussis}, but not \textit{B. pertussis} (91). O-antigen confers serum resistance by inhibiting C3 deposition onto the surface of \textit{B. parapertussis} (31). O-antigen also enables \textit{B. parapertussis} to avoid \textit{B. pertussis}-infection-induced immunity by preventing cross-reactive antibodies from binding to \textit{B. parapertussis} (101).

Since the complete genome sequence of \textit{B. holmesii} is not available, less is known about its virulence determinants. It has been determined that BvgS is functionally interchangeable between \textit{B. pertussis} and \textit{B. holmesii}, but BvgA of \textit{B. holmesii} cannot substitute for its \textit{B. pertussis} counterpart (99). Njamkepo et al. determined that anti-\textit{B. pertussis} FHA, PRN, Ptx, ACT, FIM2 or FIM3 antibodies do not recognize any protein from five independent \textit{B. holmesii} isolates (74), suggesting that these proteins are either not produced by \textit{B. holmesii} or that the proteins produced do not cross-react with those expressed by \textit{B. pertussis}. \textit{B. pertussis} and \textit{B. holmesii} also produce phenotypically and immunologically distinct LPS, and the LPS profile of \textit{B. holmesii} does not appear to be modulated by temperature (91). One potential virulence determinant of \textit{B. holmesii} may be a putative pathogenicity island containing a functional, iron-regulated locus that appears to have been laterally transferred from \textit{B. pertussis} (25).

\textbf{Immunity to the endemic bordetellae}

Although little is known about the immune responses against \textit{B. holmesii}, clinical studies and experimental studies using animal models have identified key host factors involved in protective immunity against \textit{B. bronchiseptica}, \textit{B. pertussis} and \textit{B. parapertussis}. Toll-like receptor (TLR)4 signaling in response
to *B. bronchiseptica* leads to rapid and robust TNF-α response, which is required for host survival (55-57). Complement is also critical to the control of *B. bronchiseptica* infection as CD11b−/− mice, lacking Complement Receptor 3, succumb to infection between days 3 and 4 post-inoculation (77). Additionally, antibodies are required for efficient clearance of *B. bronchiseptica* (44). TLR4, neutrophils, Fcγ receptors, and complement are required for the antibody-mediated clearance of *B. bronchiseptica* from the lungs (45). Immunoglobulin (Ig) A mediates protection against *B. bronchiseptica* in the upper respiratory tract (104).

TLR4 deficient mice have a protracted course of *B. pertussis* infection, which is associated with elevated inflammatory pathology (39, 57). TNF-α deficient mice harbor more *B. pertussis* in their lungs at the later stages of infection associated with elevated inflammation (105). Both antibodies and T cells are required for efficient control of *B. pertussis* (44, 46). According to clinical studies, antibodies against *B. pertussis* FHA, FIM, PRN and Ptx correlate with protection against disease (16, 62, 70). Ptx inhibits the antibody-mediated clearance of *B. pertussis* until the second week post-infection, when its inhibitory effect is overcome and antibodies clear the infection with the help of neutrophils and Fcγ receptors (43).

Clinically, *B. parapertussis* infection in humans induces antibody responses to FHA, PRN and LPS (6, 90). In contrast to its close relatives, *B. pertussis* and *B. bronchiseptica*, *B. parapertussis* LPS does not efficiently stimulate TLR4-mediated signaling (57). There is very little inflammation or leukocyte recruitment to the lungs during the first few days of a *B. parapertussis* infection (100). During the second week, T cells produce IFN-γ, which contributes to the recruitment of neutrophils to the lungs (102). However, IL-10, produced by macrophages during the first week, skews T cells away from Th1 phenotypes, limiting the IFN-γ responses (102). The combination of limiting pro-inflammatory TLR4 signaling and stimulating anti-inflammatory IL-10 production enables *B. parapertussis* to grow to high numbers and persist longer within its host. Antibodies are produced after about 2 weeks and T cells, IFN-γ, neutrophils, as well as the complement cascade, complement receptor 3, and Fcγ receptors, are critical to the function of antibodies against *B. parapertussis* (103).

**Genetic manipulation strategies to study host-bordetellae interactions**

The complete genome sequence of at least one strain of each classical bordetellae is available (75), and several other strains of classical bordetellae are being sequenced. Through *in silico* analysis using these
genome sequences, genes can be implicated in pathogenesis based on sequence similarity to known virulence determinants in other species. This approach identifies potentially interesting genes, but provides little information regarding specific functions of their encoded products. However, the genetic manipulation of bordetellae allows for the construction of bacterial mutants lacking potential virulence determinants in question. The resultant phenotypes of the strains lacking a specific factor may imply the functions of the encoded products.

Genetic manipulation of the hosts offers another approach to study bacterial pathogenesis on the host side. Murine models have been widely utilized in immunological research due to the availability of mouse strains deficient in specific immune factors. Intranasal inoculation of classical bordetellae in 50µL volume results in the deposition of bacteria throughout the respiratory tract at the time of infection (11, 36). Although transmission and coughing symptoms are not observed in laboratory mice even following high dose inoculation (5×10^5 CFU), the mouse is a natural host of *B. bronchiseptica*, making murine infection of this bacterium a suitable model to study its interaction with the host. Murine infections of the human-adapted bordetellae share many disease characteristics with infected humans, such as the production of *Bordetella*-specific antibodies, neutrophil migration to the lungs and memory T and B cell responses (62, 70, 100, 103). Monitoring the host responses in mice with specific immunodeficiencies distinguishes the indispensable host defense mechanisms from the dispensable ones. Host immune responses are so complex that deficiency in one factor may impact various aspects of immune responses. The interactions between players in host immunity may be better revealed in the context of disease progression or in the host defenses against invading pathogens including bordetellae. Thus studying the immune response following a *Bordetella* infection in immunodeficient mice may also address general immunology questions.

The combination of bacterial mutants and knockout mouse strains permit the dissection of specific interactions between bordetellae and the host. The precise role of a specific virulence determinant in a complicated host-bacterial interaction may sometimes be hindered due to redundant virulence mechanisms or host defense pathways. In this regard, deletion of a bacterial factor may not result in a detectable difference in the course of infection. One way to attack this problem is to combine the tools of bacterial and mammalian genetics to alter both sides of the interaction, more specifically to explore the interactions between bacterial
factors and immune defense functions in vivo by examining bacterial factor mutants in mice with mutations causing specific immunodeficiencies (37). This approach has several advantages. First of all, by creating a defect on the bacteria side and identifying a complementary defect on the host side, bacterial factors can be matched to specific immune functions that they interact with. For instance, if the mutant bacteria lacking a specific factor are defective in wild-type hosts but behave similarly to the wild-type bacteria in hosts lacking immune factor “X”, this would suggest that “X” is the in vivo target for that factor. Secondly, the lack of certain immune functions may disrupt immune regulation, facilitating the identification of differences between mutant and wild-type bacteria. For example, if the mutant bacteria lacking a specific factor are only defective in the host lacking immune function “X”, but not in immunocompetent hosts, this would imply that that factor is required to overcome the defense mechanisms retained by hosts lacking “X”.

Preface

This dissertation will describe the interactions between endemic Bordetella species mediated mainly by vaccination, the contribution of two cytokine pathways to efficient immune responses against B. pertussis, and the role of the B. bronchiseptica SigE system in stress responses and pathogenesis. Chapters 2, 3 and 4 of this dissertation will explore how B. parapertussis and B. holmesii each evade B. pertussis vaccine-induced immunity. In synopsis, the isogenic O-antigen deficient strain of B. parapertussis confers less protection following B. parapertussis vaccination and infection, indicating that the effective adaptive immune responses against B. parapertussis require O-antigen. Furthermore, the addition of LPS, including O-antigen, to B. pertussis vaccines increases the efficacy of current whooping cough vaccines against B. parapertussis (109) (Chapter 2). O-antigen also inhibits the binding of B. pertussis vaccine-induced antibodies to B. parapertussis, antibody-mediated opsonophagocytosis in vitro as well as bacterial clearance in vivo. This lack of binding of cross-reactive antibodies may contribute to the ability of B. parapertussis to circulate in B. pertussis vaccinated populations (110) (Chapter 3). B. holmesii is found to be circulating in Massachusetts in recent years. Experiments using a murine model of infection indicate that B. pertussis vaccine-induced immunity confers little protection against B. holmesii due to the lack of cross-reactive antibodies (Chapter 4).

Chapters 5 and 6 will outline the contributions of IL-1R and IL-6 to immune responses against B. pertussis. Mice lacking IL-1R display increased susceptibility to B. pertussis, which is associated with
uncontrolled inflammatory pathology, atypical disseminated diseases and the lack of IL-17 responses. The IL-1R pathway contributes to the induction of IL-10 responses during *B. pertussis* infection, and also plays a role in overcoming the effects of a *B. pertussis* specific factor, pertussis toxin (Chapter 5). IL-6, which is the focus of chapter 6, contributes to the pulmonary leukocytes recruitment and the generation of *B. pertussis*-specific T cell cytokine responses (Chapter 6). After all, IL-6^−/−_ mice show delayed clearance of *B. pertussis*, as well as decreased anamnestic immunity against this pathogen.

Chapters 7 and 8 will focus on the role of a *B. bronchiseptica* extracytoplasmic function sigma factor, SigE, and its negative regulators in stress responses and pathogenesis. SigE plays a role in the growth of *B. bronchiseptica* under heat or ethanol stress, its thermotolerance, and resistance to cell envelope perturbations posed by detergents or β-lactam antibiotics. In addition, the SigE deficient strain is defective in causing systemic and lethal infection in RAG^−/−_ mice (Chapter 7). The SigE negative regulators deletion strain failed to efficiently colonize immunocompetent mice, which implies the importance of the proper control of SigE activity during infection (Chapter 8).

The last chapter will summarize this dissertation as a whole and discuss the overall significance and implications of the research, as well as some future directions that can be taken to extend this work.
References


Chapter 2

The O-antigen Is a Critical Antigen for the Development of a Protective Immune Response to *Bordetella parapertussis*

Abstract:

Despite excellent vaccine coverage in developed countries, whooping cough is a reemerging disease that can be caused by two closely related pathogens, *Bordetella pertussis* and *B. parapertussis*. The two are antigenically distinct, and current vaccines, containing only *B. pertussis*-derived antigens, confer efficient protection against *B. pertussis* but not against *B. parapertussis*. *B. pertussis* does not express the O-antigen, while *B. parapertussis* retains it as a dominant surface antigen. Since the O-antigen is a protective antigen for many pathogenic bacteria, we examined whether this factor is a potential protective antigen for *B. parapertussis*. In a mouse model of infection, immunization with wild-type *B. parapertussis* elicited a strong antibody response to the O-antigen and conferred efficient protection against a subsequent *B. parapertussis* challenge. However, immunization with an isogenic mutant lacking the O-antigen, *B. parapertussisΔwbm*, induced antibodies that recognized other antigens but did not efficiently mediate opsonophagocytosis of *B. parapertussis*. The passive transfer of sera raised against *B. parapertussis*, but not *B. parapertussisΔwbm*, reduced *B. parapertussis* loads in the lower respiratory tracts of mice. The addition of 10 µg of purified *B. parapertussis* lipopolysaccharide (LPS), which contains the O-antigen, but not *B. parapertussisΔwbm* LPS drastically improved the efficacy of the acellular vaccine Adacel against *B. parapertussis*. These data suggest that the O-antigen is a critical protective antigen of *B. parapertussis* and its inclusion can substantially improve whooping cough vaccine efficacy against this pathogen.
Introduction:

*Bordetella pertussis* and *B. parapertussis* are the causative agents of whooping cough, resulting in approximately 50 million cases and 300,000 deaths annually worldwide (28). While whooping cough is considered by the CDC to be a reemerging disease (5), the relative incidences of *B. pertussis* and *B. parapertussis* are not clear (50). It is known, however, that the resurgence of whooping cough roughly correlates with the introduction of acellular pertussis vaccines (5). These vaccines contain only *B. pertussis*-derived antigens and confer little to no protection against *B. parapertussis* (9, 14, 15, 23, 27, 28). Current acellular pertussis vaccines contain some combination of filamentous hemagglutinin, pertactin, and fimbriae 2 and 3, all of which are expressed by both *B. pertussis* and *B. parapertussis*, and pertussis toxin, which is *B. pertussis* specific (33, 34). Based on genome sequences, the levels of amino acid sequence identity between *B. pertussis* and *B. parapertussis* filamentous hemagglutinin, pertactin, and fimbria 2 and 3 proteins are about 98, 91, 71, and 92% (35), and antibodies raised against these antigens from *B. pertussis* cross-react with *B. parapertussis* (17, 31). However, immunization with purified *B. pertussis* filamentous hemagglutinin or pertactin does not confer protection against *B. parapertussis* (17). *B. pertussis* fimbriae confer some protection against *B. parapertussis*, but at much lower levels than they protect against *B. pertussis* (52). Based on these observations and the fact that *B. parapertussis* infection induces protective immunity to itself (56, 58), we hypothesized that the lack of protective antigens from *B. parapertussis* may be part of the reason why current whooping cough vaccines are ineffective against this bacterium.

Although *B. pertussis* and *B. parapertussis* are very closely related (8, 35, 48), they differ in the structure of their lipopolysaccharides (LPS) (1, 2, 39, 40, 47). *B. pertussis* produces a lipo-oligosaccharide containing lipid A and a branched-chain core oligosaccharide with a complex trisaccharide modification but lacks the O-antigen due to a natural deletion of the *wbm* locus responsible for its synthesis (39, 47). *B. parapertussis* LPS is similar to *B. pertussis* LPS but lacks the trisaccharide modification and includes an O-antigen (39, 40). In addition to conferring serum resistance by inhibiting C3 deposition onto the surfaces of bacteria (11), the O-antigen enables *B. parapertussis* to avoid *B. pertussis*-induced immunity by preventing antibody binding to cross-reactive antigens on the surfaces of *B. parapertussis* cells (56, 59). Since the O-antigen is one dominant surface antigen recognized by *B. parapertussis* immune sera (56) and has been
shown previously to be a protective antigen of various pathogenic bacteria (22, 36), we hypothesized that the O-antigen is a protective antigen of *B. parapertussis*.

To assess the role of the O-antigen in the generation of an adaptive immune response to *B. parapertussis*, the immunity and protection generated by *B. parapertussis* infection or vaccination were compared to those generated by an isogenic mutant of *B. parapertussis* lacking the O-antigen (Δwbm) (39). Animals immunized with *B. parapertussis*, but not *B. parapertussisΔwbm*, were protected against subsequent challenge with *B. parapertussis*. Mice immunized with *B. parapertussisΔwbm* were also deficient in the production of *B. parapertussis*-specific antibodies, and sera collected from these mice were less effective at reducing *B. parapertussis* colonization upon passive transfer than sera raised against *B. parapertussis*. The inclusion of LPS from *B. parapertussis*, but not from *B. parapertussisΔwbm*, rendered the acellular *B. pertussis* vaccine Adacel efficacious against *B. parapertussis* challenge. Together, these data indicate that the O-antigen is an important protective antigen of *B. parapertussis*. 
Materials and Methods:

Bacterial strains and growth. B. pertussis strain 536, B. parapertussis strain CN2591, and the isogenic B. parapertussis mutant strain lacking the O-antigen, CN2591Δwbm, have been described previously (39, 46). For opsonization, attachment, and phagocytosis experiments, these strains were transformed with plasmid pCW505 (kindly supplied by Alison Weiss, Cincinnati, OH), which induces cytoplasmic expression of green fluorescent protein (GFP) without affecting growth or antigen expression (51). Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% sheep blood (Hema Resources) and 20 µg/ml streptomycin (Sigma-Aldrich). Liquid cultures were grown overnight on a roller drum at 37°C to mid-log phase in Stainer-Scholte broth (44, 49).

Cells. Peripheral blood polymorphonuclear leukocytes (PMNs) were isolated from human donors’ heparinized venous blood by using Ficoll-Histopaque (Sigma, St. Louis, MO) gradient centrifugation. PMNs were harvested, and the remaining erythrocytes were removed by hypotonic lysis. Cell viability was >99% as determined by trypan blue exclusion. Prior to functional assays, PMNs were washed twice with Dulbecco’s modified Eagle medium (HyClone) supplemented with 10% fetal calf serum (HyClone) and resuspended, and the cells were used immediately. All experiments were carried out with freshly isolated PMNs lacking FcγRI (CD64) expression, as monitored by fluorescence-activated cell sorter analysis using a FACSScan flow cytometer (Becton Dickinson, San Jose, CA) with anti-FcγRI monoclonal antibody 22 (41).

Opsonization. GFP-expressing strains were opsonized by incubation at 37°C for 30 min in a final volume of 50 µl containing 5% heat-inactivated serum samples from naïve C3−/− mice or convalescent C3−/− mice challenged with CN2591 or CN2591Δwbm. Serum-opsonized bacteria were incubated with Rphycocerythrin (RPE)-labeled goat F(ab’)2 fragments of anti-mouse immunoglobulin G (IgG; Southern Biotechnology, Birmingham, AL) for 30 min at 4°C. The opsonization of each strain was assessed by fluorescence-activated cell sorter analysis (43).

Attachment and phagocytosis. Attachment and phagocytosis of the B. parapertussis strains were evaluated as described previously, with a few modifications (42). Briefly, serum-opsonized, GFP-expressing bacteria were subsequently incubated with PMNs at a multiplicity of infection of 30 for 20 min at 37°C to allow binding. In selected experiments, 200 ng/ml cytochalasin D (Sigma-Aldrich) was added to inhibit
phagocytosis. After extensive washing to remove unattached bacteria, an aliquot was maintained on ice to be used as a bacterial attachment control. Another aliquot was further incubated for 1 h at 37°C to allow internalization. Phagocytosis was stopped by placing PMNs on ice. Cell surface-bound bacteria in both aliquots (obtained before and after 1 h of incubation at 37°C) were detected by incubation with RPE-labeled goat F(ab')2 fragments of antimouse IgG at 4°C for 30 min. To avoid eventual cytophilic binding of antibodies, all incubations were done in the presence of 25% heat-inactivated human serum. After being washed, samples were analyzed by flow cytometry. Ten thousand cells per sample were analyzed. Green fluorescence intensity associated with PMNs maintained at 37°C for 20 min was determined to indicate the level of bacterial attachment. The decrease in red fluorescence after incubation for 1 h at 37°C reflects bacterial phagocytosis. Phagocytosis was calculated from the drop in the mean red fluorescence intensity of green fluorescence-positive cells as described previously (42).

**Animal experiments.** C57BL/6 mice were obtained from Jackson Laboratories. C3−/− mice were kind gifts from Rick Wetsel and have been described elsewhere (7). All mice were bred in our Bordetella-free, specific-pathogen-free breeding rooms at The Pennsylvania State University. Four- to six-week-old mice were sedated with 5% isoflurane (Abbott Laboratory) in oxygen and inoculated by pipetting of 50 µl of phosphate-buffered saline (PBS; Omnipur) containing 5 × 10⁵ CFU of bacteria onto the external nares (18). This method reliably distributes the bacteria throughout the respiratory tract (13). For rechallenge experiments, mice were treated with gentamicin in drinking water (10 mg/ml) for 7 days starting on day 21 postinoculation (57). Mice were challenged with 5 × 10⁵ CFU of bacteria on day 30 postinoculation and dissected 3 days postchallenge (57). For passive transfer of sera, 200 µl serum samples (collected on day 28 postinoculation) from naïve or convalescent C3−/− mice were intraperitoneally (i.p.) injected at the time of inoculation (19, 38). For vaccination, mice were i.p. injected with 10⁸ CFU of heat-killed CN2591 or CN2591Δwbm in 200 µl of PBS with Imject Alum adjuvant (Pierce) on days 0 and 14. For acellular B. pertussis vaccinations, mice were i.p. injected with one-fifth of a human dose of Adacel (Sanofi Pasteur; 0.5 µg of pertussis toxin, 1 µg of filamentous hemagglutinin, 0.6 µg of pertactin, and a 5-µg mixture of fimbriae 2 and 3 per mouse) in a 200-µl volume containing PBS and Imject Alum adjuvant with or without 10 µg of purified CN2591 LPS or CN2591Δwbm LPS (45) on days 0 and 14. Vaccinated mice were challenged with bacteria on day 28. Mice
were sacrificed via CO₂ inhalation, and the lungs, tracheae, and nasal cavities were excised. Tissues were homogenized in PBS, serially diluted, and plated onto Bordet-Gengou agar, and colonies were counted after incubation at 37°C for 3 to 4 days (25). All protocols were reviewed and approved by The Pennsylvania State University Institutional Animal Care and Use Committee, and all animals were handled in accordance with institutional guidelines.

**Splenocyte restimulations.** Spleens were taken from C57BL/6 mice immunized with CN2591 or CN2591Δwmb on day 28 postinoculation. Splenocytes were isolated as described previously (25, 37). In brief, spleens were homogenized and red blood cells were lysed by 0.84% ammonium chloride treatment. Aliquots of cells (2 × 10⁶) were resuspended in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate (HyClone), and 100 µg/ml penicillin-streptomycin (HyClone) and placed into each well of a 96-well tissue culture plate. Splenocytes were stimulated with either medium alone or medium containing 10⁷ CFU of heat-killed CN2591 or CN2591Δwmb (multiplicity of infection of 5) (37). After 3 days, the supernatants were collected and analyzed for gamma interferon (IFN-γ) and interleukin-10 (IL-10) production via enzyme-linked immunosorbent assays (ELISAs) per the instructions of the manufacturer (R&D Systems).

**Titer ELISAs.** Antibody titers were determined as described previously (25, 56). In brief, exponential-phase live CN2591 or CN2591Δwmb bacteria were diluted to 5 × 10⁷ CFU/ml in a 1:1 mix of 0.2 M sodium carbonate and 0.2 M sodium bicarbonate buffers. The wells of 96-well plates were coated with these antigens, and the plates were incubated for 2 h at 37°C in a humidified chamber and then washed and blocked. Serum samples from individual mice were diluted 1:50, added to the first wells of the plates, and serially diluted 1:2 across the plates, and the plates were incubated for 2 h at 37°C. Plates were washed, probed with a 1:4,000 dilution of goat anti-mouse Ig horseradish peroxidase-conjugated antibodies (Southern Biotech) for 1 h, and washed again prior to visualization with 2,2’-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt in phosphate-citrate buffer with hydrogen peroxide at an absorbance of 405 nm. Titers were determined via the end point method based on optical densities in identically treated wells probed with naïve sera.
**Western blot analysis.** Lysates containing 107 CFU of heat-killed CN2591 or CN2591 \( \Delta wbm \) were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels under denaturing conditions. Polyvinylidene difluoride membranes (Millipore) were probed overnight with either naïve sera or sera from CN2591- or CN2591\( \Delta wbm \)-inoculated mice at a 1:500 dilution. Goat anti-mouse Ig horseradish peroxidase-conjugated antibodies (Southern Biotech) were used at a dilution of 1:10,000 as the detector antibody (56, 57). The membrane was visualized with enhanced chemiluminescence Western blotting detection reagent (Pierce Biotechnology).

**Statistical analysis.** The mean ± standard error was determined for all appropriate data. Two-tailed, unpaired Student’s \( t \) tests were used to determine the statistical significance of differences between groups. All experiments were performed at least twice with similar results.
Results:

The O-antigen is required for efficient generation of protective immunity against *B. parapertussis* infection.

To determine whether the O-antigen contributes to the generation of *B. parapertussis* infection-induced protective immunity against secondary challenge, mice were intranasally inoculated with either *B. parapertussis* or *B. parapertussis∆wbm* and challenged with either *B. parapertussis* or *B. parapertussis∆wbm*. Naïve animals challenged with wild-type *B. parapertussis* had mean loads of $10^{6.2}$, $10^{6.1}$, and $10^{6.7}$ CFU in the nasal cavity, trachea, and lungs on day 3 postchallenge (Fig. 2.1, black bars). Mice previously inoculated with *B. parapertussis* were substantially immune to subsequent challenge, harboring approximately $10^3$ CFU in the nasal cavity, and had cleared the bacteria from both the trachea and lungs by 3 days postchallenge (Fig. 2.1, white bars). Prior infection with *B. parapertussis* and *B. parapertussis∆wbm* conferred similar levels of protection in the lower respiratory tract (LRT) against subsequent *B. parapertussis∆wbm* challenge (Fig. 2.1, right). Interestingly, *B. parapertussis* infection induced more protection against the O-antigen-deficient strain in the nasal cavity than *B. parapertussis∆wbm* infection did (Fig. 2.1, top right). However, mice immunized with *B. parapertussis∆wbm* harbored at least 8,000-fold more *B. parapertussis* bacteria in the lungs, $10^{4.9}$ CFU, than *B. parapertussis*- immunized mice ($P < 0.01$) (Fig. 2.1, bottom), indicating that the mutant lacking the O-antigen did not induce effective protective immunity.
Effective vaccine-induced immunity requires a response against the O-antigen.

*B. parapertussisΔwbm* is known to colonize at a lower level than *B. parapertussis* in the presence of complement (11), raising the possibility that its defect in colonization contributes to the decreased protection against subsequent challenge (Fig. 2.1). To deliver equivalent amounts of antigens, mice were vaccinated with heat-killed *B. parapertussis* or *B. parapertussisΔwbm*. Sham-vaccinated control mice challenged with *B. parapertussis* harbored 10^{6.4}, 10^{5.8}, and 10^{6.8} CFU in the nasal cavity, trachea, and lungs 3 days later (Fig. 2.2, black bars). Vaccination with *B. parapertussis* effectively decreased *B. parapertussis* numbers by 99.99% in the LRT and by 80% in the nasal cavity (Fig. 2.2, white bars). Although vaccination with *B. parapertussisΔwbm* reduced *B. parapertussis* numbers in the LRT (Fig. 2.2, striped bars), animals vaccinated with *B. parapertussisΔwbm* had 160-, 16-, and 3-fold more bacteria in the lungs, trachea, and nasal cavity than *B. parapertussis* vaccinated animals (Fig. 2.2). This decreased protection conferred by *B. parapertussisΔwbm* vaccination further strengthens the conclusion that the O-antigen is required for the efficient generation of an adaptive immune response against *B. parapertussis*.

**The O-antigen is not required for the development of splenic IFN-γ and IL-10 responses to B. parapertussis.**

Since the O-antigen contributes to the generation of efficient protective immunity against *B. parapertussis*, we investigated whether the O-antigen is involved in the generation of a T-cell response. Splenocytes from naïve or *B. parapertussis*- or *B. parapertussisΔwbm*-vaccinated mice were stimulated with medium alone or with heat-killed *B. parapertussis* or *B. parapertussisΔwbm*, and 3 days later, IFN-γ and IL-10 concentrations in the culture supernatants were measured. Vaccination with either strain resulted in...
increased IFN-γ and IL-10 production. There was no significant difference in IFN-γ or IL-10 production in response to *B. parapertussis* or *B. parapertussis*Δwbm between mice vaccinated with *B. parapertussis* and those vaccinated with *B. parapertussis*Δwbm (Fig. 2.3). Since the splenic IFN-γ and IL-10 responses are T-cell dependent (D. N. Wolfe, A. K. Karanikas, S. E. Hester, M. J. Kennett, and E. T. Harvill, in press), these data suggest that the O-antigen is not required for the generation of a T-cell response to *B. parapertussis*.

**The O-antigen is required for the generation of an efficient antibody response against *B. parapertussis*.**

As the O-antigen is required for the generation of anamnestic immunity to *B. parapertussis* but not an efficient T-cell response, we assessed whether the O-antigen contributes to efficient antibody generation. In
ELISAs using either strain as the antigen, *B. parapertussis* immune serum had significantly less recognition of the O-antigen mutant than of wild-type bacteria (Fig. 2.4A, left). *B. parapertussisΔwbm* immunization sera had similar Ig titers when probed with the wild-type and O-antigen mutant *B. parapertussis* strains (Fig. 2.4A, right). Sera raised against *B. parapertussisΔwbm* showed a 44% reduction in *B. parapertussis*-specific antibody titers compared to those in sera raised against *B. parapertussis* (Fig. 2.4A, first and third bars). These data suggest that vaccination with *B. parapertussis* induces a robust antibody response against the O-antigen and that vaccination with *B. parapertussisΔwbm* induces an antibody response to other antigens that are shared.

To compare the antigens recognized by sera from different groups, Western blotting analyses were performed with lysates of *B. parapertussis* and *B. parapertussis Δwbm* probed with naïve sera or *B. parapertussis* or *B. parapertussisΔwbm* immune sera (Fig. 2.4B). Naïve sera appeared to minimally bind antigens from either lysate (Fig. 2.4B, lanes 1 and 2). *B. parapertussis*-induced serum antibodies recognized a broad band or smear, band i, present in *B. parapertussis* lysate but not in *B. parapertussisΔwbm* lysate (Fig. 2.4B, lanes 3 and 4), suggesting that it represents LPS containing the O-antigen and that the O-antigen is one of the dominant antigens of *B. parapertussis*. Several higher-molecular-mass antigens shared by the two strains, for example, those represented by bands iii and iv, were also recognized by *B. parapertussis* immune serum antibodies. Interestingly, although *B. parapertussisΔwbm*-induced serum antibodies showed recognition of antigen(s) in band iii, these antibodies lacked recognition of antigen(s) in band iv and had strong recognition of additional antigen(s) in bands ii and v, not recognized by *B. parapertussis*-induced serum antibodies. As expected, the O-antigen (band i) was not recognized by *B. parapertussisΔwbm*-induced serum antibodies. Together, these data indicate that immunization with *B. parapertussis* induces a measurably stronger antibody response, dominated by the O-antigen, than that induced by *B. parapertussisΔwbm* and that immunization with *B. parapertussisΔwbm* induces a different antigen recognition profile from that induced by immunization with the wild-type counterpart.

The O-antigen contributes to the generation of antibodies that mediate opsonophagocytosis of *B. parapertussis* by PMNs.
To determine whether antibodies against the O-antigen are important for some key antibody functions, we assessed the opsonization of bacteria and subsequent attachment to, and phagocytosis by, PMNs mediated by antibodies raised against wild-type or O-antigen-deficient *B. parapertussis*. Because *B. parapertussisΔwbm* is not defective in colonization of mice lacking complement (11), sera were generated in complement deficient mice, thereby removing the difference in bacterial load as a factor affecting antibody production. Compared to the naïve sera, *B. parapertussis* immune sera mediated efficient opsonization of wild-type *B. parapertussis* and subsequent attachment to and phagocytosis by PMNs (Fig. 2.5, middle black bars). *B. parapertussis* immune sera were less effective against O-antigen-deficient *B. parapertussis* in all three assays (Fig. 2.5, middle white bars), suggesting that antibodies recognizing the O-antigen, rather than the shared antigens, are involved. Sera from mice immunized with O-antigen-deficient *B. parapertussis* were similarly effective against the wild-type and O-antigen-deficient strains (Fig. 2.5, right). Control PMNs treated with cytochalasin, a phagocytosis inhibitor, showed no phagocytosis (data not shown).

Figure 2.5: Generation of antibodies that mediate efficient opsonophagocytosis of *B. parapertussis* by PMNs requires the O antigen. GFP-expressing wild-type *B. parapertussis* (*Bpp*) or O-antigen-deficient *B. parapertussis* (*BppΔwbm*) was opsonized with naïve sera or sera from C3-/- mice challenged with *B. parapertussis* (*B. parapertussis* immune sera [B.p.p. IS]) or *B. parapertussisΔwbm* (*B.p.p.Δwbm IS) and stained with RPE-labeled goat F(ab')2 fragments of antimouse IgG. (A) Opsonization levels were measured as mean intensities ± standard errors of red fluorescence from bacteria opsonized with the indicated sera from four individual mice. (B) Opsonized bacteria were incubated with freshly isolated human peripheral blood PMNs for 20 min or 1 h and 20 min. Attachment levels were measured as mean intensities ± standard errors of green fluorescence associated with PMNs incubated for 20 min with bacteria opsonized by the indicated sera from four individual mice. (C) The cell surface-bound bacteria on PMNs were detected by incubation with RPE-labeled goat F(ab')2 fragments of antimouse IgG. Mean phagocytosis levels ± standard errors were calculated from the drop in red fluorescence of green fluorescence positive cells incubated for 1 h and 20 min compared to that of cells incubated for 20 min. Results were obtained from experiments done with four independent serum samples. AU indicates arbitrary units; * indicates a P value of <0.05; ** indicates a P value of <0.01. Maria Eugenia Rodriguez conducted this experiment.
indicating that although indirect, the assay measured phagocytosis. The observed high levels of activity of *B. parapertussis* immune sera against wild-type but not O-antigen-deficient *B. parapertussis* suggest that much of this activity is mediated by antibodies to the O-antigen.

**The O-antigen is required for the generation of antibodies that efficiently clear *B. parapertussis*.**

To determine if the decreased *B. parapertussis*-specific antibody titers of, and opsonophagocytosis mediated by, sera raised against *B. parapertussisΔwbm* result in decreased antibody-mediated clearance in vivo, mice received passively transferred naïve sera or sera raised against wild-type or O-antigen-deficient *B. parapertussis* in C3^−/− mice. Mice were then challenged with *B. parapertussis* and sacrificed on day 14 postchallenge for bacterial enumeration, since *B. parapertussis* poorly stimulates Toll-like receptor 4 (TLR4) and antibodies therefore have no effect until around day 14 after T cells have been generated (19, 55; D. N. Wolfe, unpublished data). Naïve sera had no effect on bacterial loads throughout the respiratory tract on day 14 postchallenge (Fig. 2.6). As seen in previous studies (19, 56), *B. parapertussis* immune sera decreased the bacterial loads in the trachea and lungs by 96 and 99.6% at this time point. However, *B. parapertussisΔwbm* immune sera failed to significantly reduce *B. parapertussis* colonization, indicating that the O-antigen is required for the generation of antibodies that clear *B. parapertussis* from the LRT in vivo upon adoptive transfer. Neither serum treatment affected bacterial numbers in the nasal cavity.

Figure 2.6: **Antibodies to the O antigen are required for efficient antibody-mediated clearance of *B. parapertussis*.** Groups of four C57BL/6 mice were inoculated with *B. parapertussis* and i.p. injected with the indicated serum. Bacterial loads in the nasal cavities, tracheae, and lungs at 14 days postinoculation are expressed as the log10 means ± standard errors. * indicates a *P* value of <0.05 for comparison between results for groups receiving naïve serum (NS) and *B. parapertussis* immune serum. ‡ indicates a *P* value of <0.05 for comparison between results for groups receiving *B. parapertussisΔwbm* immune serum (*B. p.p.Δwbm IS*) and wild-type *B. parapertussis* immune serum (*B. p.p. IS*). The limit of detection is indicated by the y axis. Elizabeth M. Goebel contributed to this experiment.
Supplementing Adacel with *B. parapertussis* LPS containing the O-antigen confers protection against *B. parapertussis* challenge.

Figure 2.7: **Addition of purified *B. parapertussis* LPS to an acellular *B. pertussis* vaccine confers protection against *B. parapertussis* challenge.** Groups of four C57BL/6 mice were vaccinated as indicated and then challenged with *B. parapertussis* (B.p.p.) (A) or *B. pertussis* (B.p.) (B) and dissected at day 3 postchallenge. The numbers of CFU recovered from the nasal cavities, tracheae, and lungs are expressed as the log_{10} means ± the standard errors. ND indicates that CFU were not detectable. * indicates a *P* value of <0.05. The limit of detection is indicated by the y axis.

Since the O-antigen is necessary for the generation of efficient protective immunity to *B. parapertussis* (Fig. 2.1, 2.2, and 2.4), we examined whether *B. parapertussis* LPS alone, containing the O-antigen, is sufficient to induce protective immunity against this pathogen and whether supplementing Adacel with *B. parapertussis* LPS renders this vaccine effective against *B. parapertussis*. Mice were vaccinated with an adjuvant alone, the acellular pertussis vaccine Adacel with an adjuvant, or Adacel with an adjuvant supplemented with purified LPS from *B. parapertussis* or *B. parapertussis*Δwbm. Vaccination with adjuvant alone or Adacel had no effect on *B. parapertussis* loads throughout the respiratory tract 3 days postchallenge (Fig. 2.7A). In contrast, vaccination with *B. parapertussis* LPS, but not *B. parapertussis*Δwbm LPS,
significantly reduced *B. parapertussis* loads in the lungs by 93.8% compared to those in the group vaccinated with adjuvant alone. Moreover, the addition of *B. parapertussis* LPS, but not *B. parapertussisΔwbm* LPS, to Adacel caused significant decreases in bacterial loads, by 70.7, 99.6, and 96.2% in the nasal cavity, trachea, and lungs, respectively, suggesting that the efficacy of an acellular pertussis vaccine against *B. parapertussis* may be increased if *B. parapertussis* LPS containing the O-antigen is included. To ensure that the addition of *B. parapertussis* LPS did not have an impact on the efficacy of Adacel against *B. pertussis*, mice were immunized with this vaccine with or without *B. parapertussis* LPS and challenged with *B. pertussis*. As expected, vaccination with the adjuvant alone did not affect the colonization by *B. pertussis* compared to that of naïve animals (Fig. 2.7B). Vaccination with Adacel reduced the *B. pertussis* load in the lungs by >99.5% (Fig. 2.7B, bottom). This vaccine supplemented with *B. parapertussis* LPS caused a similar reduction of *B. pertussis* numbers (Fig. 2.7B, bottom), suggesting that the inclusion of *B. parapertussis* LPS does not affect the efficacy of the vaccine against *B. pertussis*. All together, our data suggest that the addition of *B. parapertussis* LPS containing the O-antigen to a current acellular vaccine extended its utility to include protective immunity to *B. parapertussis*. 
Discussion:

A clear picture of *B. parapertussis* epidemiology is not available because differential diagnostic methods to distinguish between the two causative agents of whooping cough are rarely performed at the clinical level and diseases caused by *B. parapertussis* are not reportable to the CDC. However, when carefully monitored, *B. parapertussis* has been found to cause a substantial proportion of whooping cough cases and even larger proportions among vaccinated groups (4, 23, 24, 50). Although the mouse model does not replicate coughing symptoms of the disease, mechanisms of immune control and clearance of the bordetellae are consistent with what is known of these mechanisms in humans (19, 29, 30). The data presented here are consistent with the findings of experimental studies using a mouse infection model, as well as those of clinical studies, in which *B. pertussis* immunity failed to induce protective immunity to *B. parapertussis* (Fig. 2.7A) (9, 10, 14, 23, 27, 52, 56, 59). This work extends the findings of those previous studies to examine the role of the O-antigen in the generation of *B. parapertussis*-specific immunity.

We found that although immunization with wild-type *B. parapertussis* induced protective immunity to both the wildtype and the O-antigen-deficient *B. parapertussis* strains, prior infection or vaccination with the O-antigen-deficient strain conferred significantly less protection against the wild type in the lungs (Fig. 2.1 and 2.2). Immunization with *B. parapertussis*Δwbm induced splenic cytokine production similar to that induced by wild-type vaccination (Fig. 2.3), indicating that the decrease in protection conferred by the O-antigen-deficient strain was not due to inefficient T-cell cytokine production. Interestingly, *B. parapertussis*-induced antibodies recognized the O-antigen as a dominant antigen (Fig. 2.4A and B, lanes 3 and 4). Serum antibodies raised against the wild type, but not the O-antigen-deficient strain, mediated efficient opsonophagocytosis and reduced *B. parapertussis* colonization upon passive transfer (Fig. 2.5). Together, these data suggest that the O-antigen is required for the generation of an effective antibody response against *B. parapertussis*.

Antibodies raised against *B. parapertussis*Δwbm lacked recognition of the O-antigen but recognized different antigens from those recognized by antibodies raised against wild-type *B. parapertussis* (Fig. 2.4B) and efficiently cleared *B. parapertussis*Δwbm (Fig. 2.1). These antigens are present in the *B. parapertussis* lysate (Fig. 2.4B), but *B. parapertussis*Δwbm immune serum is much less effective at binding live bacteria,
mediating opsonophagocytosis in vitro, or mediating bacterial clearance in vivo than *B. parapertussis* immune serum (Fig. 2.4 to 6), suggesting that these antigens may not be recognized on the surfaces of live *B. parapertussis* cells expressing the O-antigen. These data further indicate that the O-antigen is a dominant surface antigen of *B. parapertussis* and that antibodies against it are required for efficient clearance of this bacterium.

The O-antigen seems to contribute to the generation of effective protective immunity against *B. parapertussis* in the lungs but not in the trachea or nasal cavity (Fig. 2.1 and 2.2). Wolfe et al. observed that B cells and T cells are required for clearance of *B. parapertussis* from the lungs and that CD4⁺ T cells, complement, and neutrophils are required for antibody mediated clearance in this organ (58). What immune components are required for *B. parapertussis* clearance in the trachea and nasal cavity is less understood. Infection-induced immunity appeared to be more effective than vaccination-induced immunity in the nasal cavity and trachea (Fig. 2.1 and 2.2). This pattern may be due to different clearance mechanisms in infection- and vaccination-induced immunity to bordetellae (12). Vaccination is efficient in controlling disease but may be less effective in preventing subclinical colonization, as observed with *B. pertussis* (28). While the nasal cavity may be a reservoir of asymptomatic carriage of *B. parapertussis*, the protection in the lungs correlates with vaccine efficacy against severe disease and is thus the focus of this study (9).

The incidence of whooping cough has increased over the past 20 years, despite the maintenance of excellent vaccine coverage in developed countries (5). This trend may be due, at least in part, to vaccines’ being ineffective against *B. parapertussis*- induced disease (9, 16, 23). Of note, the switch from whole-cell to acellular vaccines correlates with increased prevalence of *B. parapertussis* (23). Moreover, whooping cough vaccinations have been proposed to shape the age-incidence patterns of the two causative agents. *B. pertussis* is more common in infants prior to vaccination and adolescents in whom vaccine-induced immunity has waned (6, 53), whereas *B. parapertussis* is most common in young children who have been recently vaccinated (3, 21, 54; J. Lavine, L. Han, E. T. Harvill, and O. Bjornstad, unpublished data). All these observations suggest that current whooping cough vaccines confer a selective advantage on *B. parapertussis* in its ongoing competition with *B. pertussis*. 
We have shown that supplementing the acellular pertussis vaccine Adacel with 10 µg of purified *B. parapertussis* LPS containing the O-antigen reduced *B. parapertussis* numbers in the LRT by more than 90% within 3 days compared to the numbers in the group receiving Adacel alone (Fig. 2.7). Thus, the addition of this single antigen increased the efficacy of this vaccine against *B. parapertussis* in the mouse model. These results are not necessarily easily translated to improved human vaccines, since vaccine reactogenicity has been associated with LPS of *B. pertussis*. However, *B. parapertussis* LPS is less stimulatory toward TLR4 than *B. pertussis* LPS, and it is possible to purify the O-antigen portion of the LPS (20, 26, 55), thereby removing the TLR4 agonist, lipid A, to which is attributed most of the proinflammatory stimulation (32). Alternatively, other, as-yet-unidentified antigens of *B. parapertussis* may prove to be protective and could be added to acellular whooping cough vaccines. However, the poor protection conferred by the O-antigen-deficient strain and the ability of the O-antigen to block the effects of antibodies recognizing other antigens (56, 59) suggest that the inclusion of the O-antigen in the whooping cough vaccines should be favored over other, as-yet-unidentified protein antigens.
Authors and Contributions:

Xuqing Zhang\(^1,2,\dagger\), Elizabeth M. Goebel\(^1,3,\dagger\), Maria Eugenia Rodríguez\(^4\), Andrew Preston\(^5\), and Eric T. Harvill\(^1\)

\(^1\)Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, 115 Henning Building, University Park, Pennsylvania 16802

\(^2\)Graduate Program in Genetics, The Pennsylvania State University, University Park

\(^3\)Graduate Program in Immunology and Infectious Diseases, The Pennsylvania State University, University Park

\(^4\)CINDEFI (UNLP, CONICET La Plata), School of Science, La Plata University, La Plata, Argentina

\(^5\)Department of Clinical Veterinary Science, University of Bristol, Bristol, United Kingdom

\(^\dagger\)XZ and EMG contributed equally to this work.

Conceived and designed the experiments: XZ, EMG, MER, AP, ETH.

Performed the experiments: XZ, EMG, MER, AP.

Analyzed the data: XZ, EMG, MER.

Wrote the paper: XZ, EMG, ETH.
References:

Chapter 3

O-antigen Allows *Bordetella parapertussis* to Evade *B. pertussis* Vaccine-induced Immunity by Blocking Binding and Functions of Cross-reactive Antibodies

Abstract

Although the prevalence of *B. parapertussis* varies dramatically between studies in different populations with different vaccination regimens, there is broad agreement that whooping cough vaccines, composed only of *B. pertussis* antigens, provide little if any protection against *B. parapertussis*. In C57BL/6 mice a *B. pertussis* whole cell vaccine (wP) provided modest protection against *B. parapertussis*, that was dependent on IFN-γ. The wP was much more protective against an isogenic *B. parapertussis* strain lacking O-antigen than its wild-type counterpart. O-antigen inhibited binding of wP-induced antibodies to *B. parapertussis* as well as antibody-mediated opsonophagocytosis *in vitro* and clearance *in vivo*. aP-induced antibodies also bound better *in vitro* to the O-antigen mutant than to wild-type *B. parapertussis*, but aP failed to confer protection against wild-type or O-antigen deficient *B. parapertussis* in mice. Interestingly, *B. parapertussis* specific antibodies provided in addition to either wP or aP were sufficient to very rapidly reduce *B. parapertussis* numbers in mouse lungs. This study identifies a mechanism by which one pathogen escapes immunity induced by vaccination against a closely related pathogen, and may explain why *B. parapertussis* prevalence varies substantially between populations with different vaccination strategies.
Introduction

Whooping cough is an acute, highly contagious, paroxysmal coughing illness (25). The first whooping cough vaccines consisting of whole inactivated *B. pertussis* were licensed in the mid-1940s and led to a dramatic decrease of disease incidence (7, 25). However, the potential health risk associated with whole cell vaccines led to the development of acellular vaccines, consisting of some combination of *B. pertussis* antigens including pertussis toxin (PT), pertactin, filamentous hemagglutinin (FHA) and 2 fimbriae serotypes. Despite maintenance of high vaccine coverage, the reported whooping cough incidence has been increasing over the past 20 years in some developed countries (1, 6), although a large portion of whooping cough infections are thought to remain unreported (9). Both *B. pertussis* and *B. parapertussis* are causative agents of whooping cough (17, 25) that appear to have evolved independently from distinct lineages of *B. bronchiseptica* through rearrangements and large scale gene loss, with *B. parapertussis* emerging more recently than *B. pertussis* (10, 32). Although they are closely related, a few striking differences exist between the human-adapted bordetellae. For example, *B. parapertussis* lipopolysaccharide (LPS) includes a repetitive membrane-distal O-antigenic structure, while *B. pertussis* only expresses lipid A and a branched-chain core oligosaccharide with a complex trisaccharide modification, but lacks O-antigen (35, 42). *B. pertussis* expresses PT, but *B. parapertussis* does not due to mutations in the promoter region (3, 23). Since differential diagnosis of *B. pertussis* and *B. parapertussis* does not affect the course of treatment, it is rarely performed in clinical settings (15, 45). The CDC does not list *B. parapertussis* as reportable (1), but a few epidemiological studies have reported the percentage of whooping cough cases caused by *B. parapertussis* to be from 1% to 98%, most commonly 4-40% (45). Although *B. parapertussis* appears to contribute substantially to disease, whooping cough vaccines are solely derived from *B. pertussis* (25).

Clinical and experimental data indicate that whooping cough vaccines are very efficacious against *B. pertussis* but not against *B. parapertussis* (8, 15, 16, 21, 24), however, a mechanistic understanding of this phenomenon has not been described. While whooping cough vaccines may fail to generate efficient cross-immunity against *B. parapertussis*, it is also possible that cross-reacting adaptive immunity is generated but is evaded by *B. parapertussis*. Recently, our lab showed that the O-antigen of *B. parapertussis* shields it from *B. pertussis*-infection-induced antibodies (50), relevant to the natural immune-mediated competition between
B. pertussis and B. parapertussis in unvaccinated population. However, nearly all people in industrialized countries are vaccinated, changing the immune landscape of the host population and the immune-mediated competition between these two human pathogens.

To examine the mechanisms used by B. parapertussis to evade B. pertussis vaccines-induced immunity, we showed that a B. pertussis whole cell vaccine (wP) had some effect, but a commercial acellular vaccine (aP) had no effect against B. parapertussis growth in mouse lungs. IFN-γ contributes to the protection against B. parapertussis by wP. O-antigen shielded B. parapertussis from the binding of vaccine-induced antibodies, interfered with opsonophagocytosis of B. parapertussis mediated by aP and wP-induced antibodies and blocked antibody-mediated clearance in vivo. wP conferred more protection against an isogenic B. parapertussis strain lacking O-antigen, indicating that O-antigen contributed to the evasion of wP-induced immunity. aP, however, failed to induce cross-protection against B. parapertussis with or without the hindrance of O-antigen. In B. pertussis vaccinated hosts, supplement of B. parapertussis-specific, but not B. pertussis-specific, antibodies conferred protection against B. parapertussis, indicating that the lack of proper antibody responses causes the failure of these vaccines against B. parapertussis. Together these results explain the clinical finding that B. parapertussis avoids clearance by the current vaccines, and provides a mechanistic understanding that will guide new approaches to overcoming this problem.
Materials and Methods

Bacterial strains and growth. B. pertussis strain 536, B. parapertussis strain CN2591 and its isogenic mutant lacking O-antigen, CN2591Δwbm, have been described previously (35, 40). For opsonization, attachment and phagocytosis experiments, these strains were transformed with plasmid pCW505 (kindly supplied by Dr. Alison Weiss, Cincinnati, Ohio) which induces cytoplasmic expression of GFP without affecting growth, or antigen expression (46). Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% sheep’s blood (Hema Resources) and 20µg/mL streptomycin (Sigma-Aldrich). Liquid cultures were grown overnight in Stainer-Scholte broth at 37°C to mid-log phase (39, 44).

Cells. Peripheral blood polymorphonuclear leukocytes (PMNs) were isolated from heparinized venous blood using Ficoll-Histopaque (Sigma, St Louis, MO) gradient centrifugation. PMNs were harvested and the remaining erythrocytes were removed by hypotonic lysis. Cell viability was >99% as determined by Trypan Blue exclusion. Prior to functional assays, PMNs were washed twice with Dulbecco’s modified Eagle medium (DMEM) (Hyclone) supplemented with 10% of fetal calf serum (FCS) (Hyclone), resuspended, and used immediately. All experiments were carried out with freshly isolated PMNs lacking FcγRI (CD64) expression, as monitored by FACS analysis using a fluorescence-activated cell sorter FACScan (Becton Dickinson, San Jose, CA) with anti-FcγRI mAb 22 (36).

Opsonization. GFP-expressing strains were opsonized by incubation at 37°C with 5% heat-inactivated wP-induced/naive or aP/adjuvant-induced serum samples for 30 min in a final volume of 50 µL. Serum opsonized bacteria were incubated with R-phycoerythrin (RPE)–labeled goat F(ab')2 fragments of anti-mouse IgG (Southern Biotechnology, Birmingham, AL) for 30 min at 4°C. Opsonization of each Bordetella strain was determined by FACS analysis (38).

Attachment and phagocytosis. Attachment and phagocytosis of the Bordetella strains were evaluated as previously described with a few modifications (37). Briefly, serum opsonized GFP-expressing bacteria were incubated with PMNs at multiplicity of infection (MOI) of 30 for 20 min at 37°C to allow binding. After extensive washing to remove non-attached bacteria, an aliquot was maintained on ice to be used for bacterial attachment control. Another aliquot was further incubated for 1h at 37°C to allow internalization. Phagocytosis was stopped by placing PMNs on ice. Cell surface bound bacteria in both aliquots (before and
after 1 hour incubation at 37°C) were detected by incubation with RPE–labeled goat F(ab')2 fragments of anti-
mouse IgG at 4°C for 30 min. To avoid eventual nonspecific binding of antibodies, all incubations were done
in the presence of 25% heat-inactivated human serum. After washing, samples were analyzed by flow
cytometry. Ten thousand cells were analyzed per sample. Green fluorescence intensity associated with
PMNs maintained at 37°C for 20 min has previously been shown to represent bacterial attachment (38).
Phagocytosis was calculated from the drop in mean red fluorescence intensity of green-positive cells after
incubation for additional 1 h at 37°C as described (38).

Animal experiments. C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor) and bred in our
Bordetella-free, specific pathogen-free breeding rooms at The Pennsylvania State University. 4-6 week old
mice were sedated with 5% isoflurane (Abbott Laboratory) in oxygen and vaccinated by intraperitoneally
(i.p.) injection of 1×10^8 CFU of heat-inactivated bacteria in 1 mL of phosphate balanced saline (PBS,
Omnipur) (wP), 1/5 human dose of Adacel (Sanofi Pasteur) (0.5µg PT, 1µg FHA, 0.6 µg pertactin, 5 µg
fimbriae 2 and 3 per mouse) with Imject Alum (Thermo Scientific) (aP) or only Imject Alum in 200µL PBS
on day 14 and 28 prior to challenge (13). For challenge, mice were sedated and inoculated by pipetting 50 µL
PBS containing 5×10^5 CFU of the indicated bacteria onto the external nares (19). This method reliably
distributes the bacteria throughout the respiratory tract (14). For adoptive transfer of immune serum, mice
were vaccinated with the indicated bacteria on day 0 and 14 and sera were collected on day 28 or sera were
collected from naïve animals. 200 µL of sera were i.p. injected at the time of inoculation (20, 34). For
quantification of bacteria numbers, mice were sacrificed via CO2 inhalation and the lung, trachea, and nasal
cavity were excised. Tissues were homogenized in PBS, serial diluted and plated onto Bordet-Gengou agar
plates with 20 µg/mL streptomycin, and colonies were counted after incubation at 37°C for 3-5 days (20).
Gamma interferon (IFN-γ) was depleted by i.p. injections of 5 mg of the antibody from hybridoma XMG1.2
one day prior to challenge (31). The lower limit of detection was 10 CFU. For all experiments, protocols
were reviewed and approved by the Pennsylvania State University IACUC and all animal were handled in
accordance with institutional guidelines.

Splenocyte re-stimulations. Spleens were excised from groups of 3-4 C57BL/6 mice after vaccination.
Splenocytes were isolated as previously described (13, 33). In brief, spleens were homogenized and red blood
cells were lysed with 0.84% ammonium chloride. 2×10⁶ cells were re-suspended in DMEM supplemented with 10% FCS, 1mM sodium pyruvate (HyClone), and 100 µg/mL penicillin and streptocycin (HyClone) and placed into each well of a 96-well tissue culture plate. Splenocytes were stimulated with either media alone or media containing 10⁷ CFU (MOI of 5) of the indicated bacteria that had been heat-killed (13, 33). After three days, the supernatants were collected and analyzed for IFN-γ and interleukin-10 (IL-10) production via Enzyme-linked immunosorbent assays (ELISA) as per the manufacturers’ instructions (R&D Systems).

**Titer ELISAs.** Antibody titers were determined as previously described (29, 50, 51). Briefly, heat-inactivated or exponential phase live bacteria were diluted to 5×10⁷ CFU/mL in a 1:1 mix of 0.2 M sodium carbonate and 0.2 M sodium bicarbonate buffers. These antigens were coated onto 96-well plates, incubated for 2h at 37°C in a humidified chamber, washed and blocked. A1:50 or 1:10 dilution of wP-induced/naïve or aP/adjuvant-induced serum samples from an individual mouse was added to the first well of each row and serially diluted 1:2 across the plates. Plates were incubated for 2h at 37°C, washed and probed with 1: 4000 dilution of goat anti-mouse Ig horseradish peroxidase (HRP)-conjugated antibodies (Southern Biotech) for 1h and visualized with 2,2’-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diaminonium salt in phosphate-citrate buffer with hydrogen peroxide at an absorbance of 405 nm. Titers were determined via the endpoint method based on optimal density of identically treated wells probed with naïve or adjuvant-induced sera.

**Western Blot analysis.** Lysates containing 5×10⁵ CFU of indicated heat-killed bacteria were run on 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels in denaturing conditions. Polyvinylidene Fluoride (PVDF) membranes (Millipore) were probed overnight with either naïve serum or serum from vaccinated mice at a 1:10 or 1: 100 dilution for aP and wP-induced serum respectively. 1:10,000 dilution of goat anti-mouse Ig HRP-conjugated antibodies (Southern Biotech) was used as the detector antibody (50, 52). The membrane was visualized with ECL Western Blotting Detection Reagent (Pierce Biotechnology).

**Statistical analysis.** The means +/- standard error (error bars) were determined for all appropriate data. Two-tailed, unpaired Student’s T-tests were used to determine statistical significance between groups. Results were also analyzed by ANOVA and Tukey simultaneous test in Minitab with similar significance. All experiments were performed at least twice with similar results.
Results:

*B. parapertussis* O-antigen contributes to the evasion of wP-induced immunity.

To examine whether wP is cross-protective against *B. parapertussis* and whether O-antigen interferes with its cross-protection, naïve or wP vaccinated C57BL/6 mice were challenged with $5 \times 10^5$ CFU of *B. pertussis*, *B. parapertussis* or an isogenic *B. parapertussis* strain lacking O-antigen (BppΔwbm). In comparison to naïve mice, wP treatment reduced *B. pertussis* numbers by 91.9%, 97.8% and >99.9% in the nasal cavity, trachea and lungs by day 3 post challenge; naïve mice having about 7000 fold more bacteria in the lungs than vaccinated mice (Fig. 3.1A). wP vaccination reduced *B. parapertussis* loads by 76.6%, 83.0% and 97.6% in the nasal cavity, trachea and lung; naïve mice having about 40 fold more bacteria in the lungs than vaccinated mice (Fig. 3.1A). These results are consistent with multiple clinical studies showing whole cell vaccines confer good protection against *B. pertussis* but relatively little protection against *B.

![Figure 3.1: B. parapertussis is more susceptible to wP-induced immunity in the absence of O-antigen.](image)

Groups of four naïve (black) or wP vaccinated (white) C57BL/6 mice were challenged with the indicated bacteria. (A) The number of CFUs recovered from the respiratory tract on day 3 post-challenge is expressed as the Log 10 mean ± the standard error. Decreases in Log 10 CFU in vaccinated mice compared to naïve mice on day 3 post-challenge are indicated underneath the x axes. (B) The change in CFU number over the first 3 days after challenge is expressed as change in Log 10 mean ± the standard error. * indicates $P \leq 0.05$. ** indicates $P \leq 0.01$. The limit of detection is indicated as the lower limit of the y axes.
Interestingly, compared to naïve mice, wP reduced numbers of an O-antigen deficient *B. parapertussis* strain by 89.4%, 99% and >99.9% in the nasal cavity, trachea and lung; naïve mice having around 2000 fold more bacteria in the lungs than vaccinated mice (Fig. 3.1A). The fold protection (reduction of bacterial number in each individual vaccinated mouse compared to mean number of bacteria in naïve mice on day 3 post-challenge) of wP against O-antigen deficient *B. parapertussis* was significantly higher than against wild-type *B. parapertussis* in both trachea and lung (lung: P=0.0006, trachea: P=0.018), indicating that wP is more efficacious against O-antigen deficient *B. parapertussis*.

To understand how vaccination affects the infection, it is important to examine these effects in the context of the dynamic infectious process. *B. pertussis* and *B. parapertussis* increased in numbers throughout the respiratory tract of naïve mice over 3 days, reflecting effective colonization and bacterial growth (Fig. 3.1B). O-antigen deficient *B. parapertussis* grew in the nasal cavity and trachea but not in the lungs of naïve mice due to its increased susceptibility to complement (12) (Fig. 3.1B). wP decreased the numbers of *B. pertussis* in all respiratory organs over 3 days, resulting in a net decline in numbers, particularly in the lower respiratory tract (LRT). Vaccination did not decrease *B. parapertussis* numbers as efficiently in the lung and *B. parapertussis* actually grew in numbers in the trachea and nasal cavity, reflecting successful colonization and expansion despite vaccination (Fig. 3.1B). Interestingly, wP vaccination decreased O-antigen deficient *B. parapertussis* numbers as efficiently as it did *B. pertussis* numbers in the trachea and lung (Fig. 3.1B). Together these results are consistent with clinical studies showing that wP vaccination confers relatively little protection against *B. parapertussis* and show that O-antigen is required for *B. parapertussis* to avoid efficient wP vaccine-induced immunity.
Many developed countries have switched to acellular vaccines, although these provide even less protection against *B. parapertussis* (8, 21, 47). We therefore determined if the O-antigen of *B. parapertussis* contributes to the evasion of aP-induced immunity. While aP reduced *B. pertussis* colonization in both lung and trachea of vaccinated mice by 99.7% and 96.8% compared to the mice given just adjuvant, aP had no effects on either *B. parapertussis* or O-antigen deficient *B. parapertussis* colonization (Fig. 3.2). These data indicate that aP does not induce protective immunity against *B. parapertussis*.

**wP induces T cells that cross react with *B. parapertussis***.

To investigate why aP is less effective than wP against *B. parapertussis* and why wP confers different levels of protection against *B. pertussis*, O-antigen deficient and wild-type *B. parapertussis*, we compared their induction of T cell responses known to be important for control and clearance of *B. parapertussis* (51).

Splenocytes from mice that were naïve, vaccinated with wP, aP or adjuvant were stimulated with heat-killed *B. pertussis* or wild-type or O-antigen deficient *B. parapertussis* (horizontally hatched) for 3 days. The concentration of IFN-γ and IL-10 in the supernatant is expressed as the mean ± the standard error. ND indicates none detected.

![Figure 3.3: Splenic production of IFN-γ and IL-10 is cross-reactive.](image)

Splenocytes from groups of four naïve C57BL/6 mice or mice vaccinated with the indicated vaccine were stimulated with media only (vertically hatched), heat-killed *B. pertussis* (black), *B. parapertussis* (white) or O-antigen deficient *B. parapertussis* for 3 days. The concentration of IFN-γ and IL-10 in the supernatant is expressed as the mean ± the standard error. ND indicates none detected.

wP-vaccinated mice responded similarly to heat-killed *B. parapertussis* or *B. pertussis*, producing significantly higher IFN-γ and IL-10 than splenocytes from naïve or adjuvant-treated mice (Fig. 3.3). IFN-γ and IL-10 production was abolished in TCRβ\(^{-/-}\) mice (data not shown), indicating that vaccine-induced IFN-γ and IL-10 production is dependent on T cells. These data indicate that the T-cell response to wP is cross-reactive to *B. parapertussis* and that O-antigen did not affect the T cell response.

**IFN-γ contributes to wP-induced protection against *B. parapertussis***.
In the vaccination studies above (Fig. 3.1, 3.2), protection against *B. parapertussis* correlates with the high IFN-γ responses of wP but not aP vaccinated animals (Fig. 3.3). IFN-γ has been shown to contribute to leukocyte recruitment and the reduction of bacterial numbers during *B. parapertussis* infection (D.N. Wolfe, A.T. Karanikas, S.E. Hester, M.J. Kennett, E.T. Harvill, in press). To determine how the cross-reactive IFN-γ response after wP vaccination might contribute to its protection against *B. parapertussis*, naïve or wP vaccinated mice were left untreated or depleted of IFN-γ, challenged with *B. pertussis* or *B. parapertussis* and sacrificed 3 days later for bacterial enumeration. Vaccination or IFN-γ depletion had no effects on colonization of the nasal cavity (Fig. 3.4). However, *B. pertussis* numbers were reduced by >99.9% and >99.5% in the lung and trachea of vaccinated mice compared to naïve mice regardless of the presence or absence of IFN-γ (Fig. 3.4). Although wP reduced *B. parapertussis* numbers in the lung and trachea by about 98.6% and 99.6%, this effect was abolished in mice given IFN-γ neutralizing antibodies (Fig. 3.4), indicating that IFN-γ contributes to the protection conferred by wP against *B. parapertussis*.

**Serum antibody responses to aP and wP are cross-reactive to denatured but not live *B. parapertussis*.**

We have previously shown that antibodies are required for anamnestic immunity to *B. parapertussis* (51). To determine whether *B. pertussis* vaccines induce antibodies that recognize and/or protect against *B. parapertussis*, we first tested whether antibody responses are cross-reactive between strains and if O-antigen affects the responsiveness of antibodies. In a western blot, whole cell extracts of *B. pertussis*, wild-type and
O-antigen deficient *B. parapertussis* were probed with serum antibodies from aP, adjuvant only, wP vaccinated or naïve mice. Compared to the control, aP vaccination induced serum antibodies that recognized distinct antigens present in all three bacteria, although the size and intensity of bands appeared to differ between *B. pertussis* and *B. parapertussis*. While wP-induced serum antibodies recognized four major bands in denatured *B. parapertussis*, they recognize more antigens in *B. pertussis*, especially those of high molecular weights (Fig. 3.5A).

To quantify the cross-reactivity of antibodies, we determined the titers of aP or wP-induced serum antibodies by ELISA using heat-inactivated bacteria as antigens. wP-induced sera had much higher titers than aP-induced sera (Fig. 3.5B). For both aP and wP-induced serum antibodies, *B. pertussis*-specific antibody titers were much higher than *B. parapertussis*-specific antibody titers regardless of the presence or absence of O-antigen (Fig. 3.5B). Since heat killing releases many antigens that are not exposed on the surface of live bacteria, similar experiments were performed with live bacteria as antigens. Interestingly, both wP and aP-induced serum antibodies bound to live *B. parapertussis* more efficiently in the absence of O-antigen (Fig. 3.5C). These data suggest that O-antigen interferes with the binding of *B. pertussis* vaccine-induced antibodies to live *B. parapertussis*

**O-antigen inhibits aP and wP-induced-antibody-mediated opsonization and subsequent attachment to, and phagocytosis by, PMNs.**
To determine whether O-antigen interferes with key functions of antibodies, we assessed its effects on opsonization and subsequent attachment to and phagocytosis by PMNs mediated by aP or wP induced antibodies. Consistent with the ELISA results (Fig. 3.5C), *B. pertussis* was efficiently opsonized by both aP and wP-induced serum antibodies (Fig. 3.6A, B, D). Although *B. parapertussis* was efficiently opsonized by whole cell *B. parapertussis* (wPP)-induced serum antibodies (Fig. 3.6C, D), aP or wP-induced antibodies failed to opsonize this bacterium (Fig. 3.6A, B, D). In contrast, O-antigen deficient *B. parapertussis* was efficiently opsonized by aP or wP-induced antibodies (Fig. 3.6A, B, D), indicating that O-antigen hinders the opsonization of *B. parapertussis* by both aP and wP-induced serum antibodies.
To examine if O-antigen blocking of opsonization results in inhibitory effects on attachment of *B. parapertussis* to phagocytes, bacteria opsonized with vaccine-induced antibodies were further incubated with polymorphonuclear leukocytes (PMN) for 20 mins and cell surface bound bacteria numbers were identified by flow cytometry. aP or wP-induced-antibody-opsonized *B. pertussis* attached to PMNs efficiently (Fig. 3.7A, B, D). *B. parapertussis* attached to PMN after opsonization with wPP-induced serum antibodies (Fig. 3.7C, D), but neither aP nor wP-induced antibodies mediated attachment of this bacterium to PMNs (Fig. 3.7A, B, D). *B. parapertussis* lacking O-antigen, opsonized with aP or wP-induced antibodies, efficiently attached to phagocytes (Fig. 3.7A, B, D), indicating that O-antigen also inhibits this process.

To determine if O-antigen interferes with aP or wP-induced antibodies ability to mediate phagocytosis of *B. parapertussis* by PMNs, an aliquot of cells from the attachment experiment was incubated for one extra hour and phagocytosis was measured. No significant internalization of naïve serum-opsonized bacteria was observed. *B. pertussis* opsonized with aP or wP-induced antibodies was efficiently phagocytosed, but similarly treated *B. parapertussis* was not (Fig. 3.8). In contrast, wPP-induced antibody-opsonized *B. parapertussis* was efficiently internalized by PMNs (Fig. 3.8). *B. parapertussis* lacking O-antigen opsonized by aP or wP-induced antibodies was efficiently phagocytosed by PMNs. Together these results indicate that O-antigen protects *B. parapertussis* from aP or wP-induced serum antibody mediated opsonization, attachment to phagocytes and subsequent internalization by these cells.

**O-antigen blocks antibody-mediated clearance of *B. parapertussis* from mouse lungs.**

Since O-antigen interferes with the binding of *B. pertussis* vaccine-induced antibodies to live *B. parapertussis* and phagocytosis dependent on these antibodies *in vitro*, we tested if O-antigen inhibits...
antibody-mediated clearance \textit{in vivo}. Serum from naïve (NS), wP- or wPP-vaccinated mice were transferred to naïve animals. Bacterial loads in the lungs were determined on day 14 post \textit{B. parapertussis} challenge since antibodies have no effect until \textit{B. parapertussis} specific T cell responses are generated around day 14 (20, 49) (D.N. Wolfe unpublished data). Serum from wPP vaccinated animals significantly lowered numbers of both wild-type and O-antigen deficient \textit{B. parapertussis} compared to naïve serum treated animals and modestly reduced \textit{B. pertussis} numbers (Fig. 3.9). This indicates that although lower level of phagocytosis of O-antigen deficient \textit{B. parapertussis} mediated by wPP-induced serum antibodies were observed compared to wild-type strain (Fig. 3.8), this low level of phagocytosis and/or other biological activities of passively transferred antibodies might be sufficient to reduce O-antigen deficient strain \textit{in vivo} (Fig. 3.9). wP-induced serum completely cleared \textit{B. pertussis} from the lung by day 14 post-challenge but did not reduce \textit{B. parapertussis} numbers. These serum antibodies did, however, reduce the numbers of O-antigen deficient \textit{B. parapertussis} by more than 90% (Fig. 3.9), indicating that O-antigen prevents the wP-induced serum antibody mediated reduction of \textit{B. parapertussis} numbers \textit{in vivo}.

\textbf{\textit{B. parapertussis} specific antibodies augment \textit{B. pertussis} vaccine-induced protective immunity against \textit{B. parapertussis}.}

We have previously shown that both antibodies and T cells are required for anamnestic protective immunity against \textit{B. parapertussis} (51). Although wP vaccination induced T cell responses that were cross-reactive (Fig. 3.4) and antibodies that bound antigens from heat-killed \textit{B. parapertussis} (Fig. 3.5B), O-antigen decreased the binding of these antibodies to live \textit{B. parapertussis} (Fig. 3.5), the opsonophagocytosis mediated by these antibodies \textit{in vitro} (Fig. 3.6-8) and their antibody-mediated clearance \textit{in vivo} (Fig. 3.9). Based on these observations, we hypothesized that wP induces sufficient T cell response but the antibody response is
not sufficient to clear *B. parapertussis* because O-antigen protects *B. parapertussis* from wP-induced antibodies. If this were the case, then adding antibodies that bind live *B. parapertussis* should render wP induced immunity sufficient to rapidly clear *B. parapertussis*. To test this, mice were vaccinated with aP (Fig. 3.10A) or wP (Fig. 3.10B), these vaccinated mice were left untreated or given naïve, wP or wPP-induced serum antibodies at the time of *B. parapertussis* challenge and sacrificed three days after challenge. To compare the protective immunity to that generated by *B. parapertussis*, a group of mice were vaccinated with wPP and adoptively transferred with wPP-induced antibodies. Less than 100 CFUs of *B. parapertussis* were recovered in the LRT of this group of mice by day 3 post-challenge (Fig. 3.10B). The very high titers of this wPP-induced serum antibodies alone had modest effect on reducing *B. parapertussis* numbers (Fig. 3.10B), consistent with prior results with convalescent serum (20, 49). In both aP and wP vaccinated mice, naïve sera had no effect, indicating that components in the serum other than those induced by vaccination do
not affect bacteria numbers. Transfer of wP-induced sera had no effect on *B. parapertussis* colonization throughout the respiratory tract. However, *B. parapertussis* numbers were significantly lower in the LRT of both aP and wP vaccinated animals given wPP-induced sera than in those given naïve sera (Fig. 3.10). These data suggest that the addition of *B. parapertussis* specific antibodies is sufficient to render wP and aP effective against *B. parapertussis*. 
Discussion:

The increasing incidence of whooping cough in highly vaccinated developed countries includes an unknown proportion of *B. parapertussis* infections (1, 6). Multiple clinical and experimental studies have shown that current whooping cough vaccines have little efficacy against this bacterium (8, 15, 16, 21, 24), but have not revealed why. In this study, we determined that O-antigen shields vaccine-induced antibodies from binding to *B. parapertussis* and prevents antibody-mediated opsonophagocytosis *in vitro* and antibody-mediated clearance *in vivo*. Although O-antigen requires a large multigenic *wbm* locus to produce and *B. pertussis* is still successful in the human population without this surface antigen (4), it is retained in *B. parapertussis* (32). Apart from its role in inhibiting complement deposition and complement-mediated killing (12), our study suggests that O-antigen, by decreasing *B. pertussis*-vaccine-induced antibody binding, may confer a selective advantage to *B. parapertussis* in human populations in which there is high prevalence of detectable immunity to *B. pertussis*.

It is interesting that wP, but not aP, induced IFN-γ, which contributed to protection against *B. parapertussis* but not *B. pertussis* (Fig. 3.4). IFN-γ plays a role in the recruitment and activation of phagocytic cells (11, 41). In *B. parapertussis* infected mice, the peak of neutrophil numbers in the lung on day 7 and subsequent control of *B. parapertussis* numbers are dependent on IFN-γ (D.N. Wolfe, A.T. Karanikas, S.E. Hester, M.J. Kennett, E.T. Harvill, in press). *B. pertussis* challenged IFN-γ R-/- mice, however, have an indistinguishable course of infection in the respiratory tract and recruit similar numbers of leukocytes to the site of infection as compared to wild-type mice (22) (Wolfe unpublished data), indicating that IFN-γ is not required for *B. pertussis* clearance and leukocyte recruitment. In vaccinated IFN-γ R-/- mice, no impaired reduction of *B. pertussis* numbers is observed during the first week after challenge, although a rebound of bacteria number was observed on day 10 (28). This is consistent with our observation that IFN-γ is not required for vaccine-mediated control of *B. pertussis* numbers on day 3 post-challenge (Fig. 3.4).

Consistent with multiple clinical and experimental studies, we found that while wP confers some level of protection against *B. parapertussis*, aP does not (8, 21). The decrease of pertussis acellular vaccines efficacy against *B. parapertussis* compared to whole cell vaccines has been suggested to be attributable to the failure of antibodies induced by pertussis acellular vaccines to block the adherence of *B. parapertussis* to
epithelial cells (43) or the immune suppressive effects of the FHA included in those vaccines (26). Among
the antigens included in the aP, only fimbriae, but not FHA and pertactin, was shown to confer cross-
protection against \textit{B. parapertussis} in a mouse model (18, 47). wP contains more antigens than aP, among
which there may be cross-protective antigens. Alternatively, the differences in Th1/Th2 skewing of wP and
aP may affect vaccine efficacy. wP induces a relatively balanced Th1/Th2 response whereas aP induces a
Th2-type response (27). Our data showed that wP, but not aP, induced high splenic IFN-\(\gamma\) production and wP
was no longer protective against \textit{B. parapertussis} when IFN-\(\gamma\) was depleted, suggesting that IFN-\(\gamma\) contributes
to the protection conferred by wP (Fig. 3.3, 3.4). These data are therefore consistent with the idea that
inducing a Th1 response enhances immunity against \textit{B. parapertussis}. The different Th1/Th2 skewing,
quantity and quality of cross-reactive antibodies and possible immune suppression factors in aP may also
explain why aP vaccination was not sufficient to induce protection against \textit{B. parapertussis} even without the
hindrance by O-antigen.

Our data reveal an interesting paradox. wP-mediated clearance of \textit{B. parapertussis} by day 3 requires
IFN-\(\gamma\), which aP does not induce (Fig. 3.3, 3.4). Yet when wPP-induced antibodies, which had limited effect
in naïve animals in the first week of infection (Fig. 3.10B) (20), were given to aP-vaccinated animals, \textit{B. parapertussis}
was effectively cleared from mouse lungs within 3 days (Fig. 3.10A). IFN-\(\gamma\) appears to
contribute to the protection conferred by wP, but aP-vaccination contributes to rapid clearance despite the
lack of detectable IFN-\(\gamma\) induction in our splenocyte re-stimulation assay (Fig. 3.3). It is possible that some
low level of IFN-\(\gamma\) was induced by aP vaccination, which we failed to detect, or some other cross-reactive
protective T cell cytokine responses aid in the rapid clearance mediated by \textit{B. parapertussis}-specific
antibodies in aP-vaccinated animals.

Although it is not clear how much \textit{B. parapertussis} contributes to the resurgence of whooping cough,
the low efficacy of current vaccines against this bacterium might confer a selective advantage to \textit{B. parapertussis}, relative to \textit{B. pertussis}. Moreover, the lower protection against \textit{B. parapertussis} conferred by
acellular vaccines than whole cell vaccine could affect the relative prevalence, a possibility of greater
significance since the recent switch from whole cell to acellular vaccines and the even more recent
introduction of acellular vaccines, including the one used here, for adolescents and adults (2, 5, 8, 21).
Considering adults to be a possible reservoir for transmission to infants (30, 48), the lack of cross-protection of Adacel, determined in this study, may open a niche for *B. parapertussis* not only in adolescents/adults but also in infants. This study shows that *B. parapertussis* evades *B. pertussis* vaccine induced immunity by blocking cross-reactive antibodies binding via O-antigen. Our current data provide strong evidence that including more *B. pertussis* proteins that induce antibodies that recognize orthologs in *B. parapertusis* is unlikely to improve vaccines so that they protect against *B. parapertusis*. Since our study indicates that supplementing wP or aP with *B. parapertusis*-specific antibodies rendered them effective against *B. parapertussis* (Fig. 3.10), addition of protective antigens of *B. parapertussis* to the vaccine may substantially improve its efficacy against this pathogen.
Authors and Contributions:

Xuqing Zhang\textsuperscript{1,2}, Maria Eugenia Rodríguez\textsuperscript{3}, and Eric T. Harvill\textsuperscript{1}

\textsuperscript{1}Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, 115 Henning Building, University Park, Pennsylvania 16802
\textsuperscript{2}Graduate Program in Genetics, The Pennsylvania State University, University Park
\textsuperscript{3}CINDEFI (UNLP, CONICET La Plata), School of Science, La Plata University, La Plata, Argentina

Conceived and designed the experiments: XZ, MER, ETH.

Performed the experiments: XZ, MER.

Analyzed the data: XZ, MER.

Wrote the paper: XZ, ETH.
References:


Chapter 4

Emergence of *Bordetella holmesii* in Massachusetts and Its Evasion of *B. pertussis*

Vaccine-induced Immunity

Abstract:

*Bordetella holmesii* is closely-related to *B. pertussis*, but only rarely observed. Since 2005, culture-confirmed *B. holmesii* cases have been identified in Massachusetts every year. Using a murine model of infection, we determined that immunity induced by a *B. holmesii* isolate from Massachusetts resulted in protection against itself. However, a whole-cell (wP) or an acellular (aP) *B. pertussis* vaccine failed to confer protection against this *B. holmesii* isolate. Although T cell responses induced by wP or aP cross-reacted with *B. holmesii*, vaccine-induced antibodies failed to efficiently bind *B. holmesii*. *B. holmesii*–specific antibodies provided in addition to wP were sufficient to rapidly reduce *B. holmesii* numbers in mouse lungs. This study demonstrates the established presence of *B. holmesii* as a respiratory pathogen in a highly vaccinated population and suggests that failure to induce cross-reacting antibodies may explain the poor cross-protection conferred by whooping cough vaccines against *B. holmesii*. 

Introduction:

Whooping cough is a highly contagious and acute coughing illness in humans (17). Both *Bordetella pertussis* and *B. parapertussis* are causative agents of whooping cough (13, 17). The first whooping cough vaccines, consisting of whole inactivated *B. pertussis*, were licensed in the mid-1940s and led to a dramatic decrease of disease incidence by the mid-1960s (4, 17). However, the potential health risks associated with these vaccines led to the development of acellular vaccines, consisting of some combination of pertussis toxin, pertactin, filamentous hemagglutinin and two fimbriae serotypes, all solely derived from *B. pertussis*. Despite high vaccine coverage, reported whooping cough incidence in some developed countries has been increasing over the past 20 years (1, 3). Furthermore, a large portion of whooping cough infections are thought to remain unreported (5).

In November 1983, the Centers for Disease Control and Prevention (CDC) received an isolate of a Gram-negative bacterium recovered from an asplenic patient (39). During the decade that followed, additional clinical isolates with the same microbiological characteristics (slow-growing, gram-negative, small coccoid, asaccharolytic, oxidase negative, nonmotile and brown-soluble-pigment-producing) were submitted to the CDC for identification (39). This previously unidentified bacterium was initially designated as “CDC nonoxidizer group 2” (NO-2). Subsequent biochemical analysis, 16S rRNA sequencing, and DNA relatedness studies revealed that the NO-2 strains were a single new species belonging to the genus *Bordetella*. In 1995, the NO-2 group was named “*Bordetella holmesii*” in honor of Barry Holmes (39). Since then, this bacterium has been isolated from diverse countries, including Australia, Germany, France, the United Kingdom, the United States and Switzerland (7, 10, 26, 31, 39), indicating that *B. holmesii* is a widespread pathogen.

*B. holmesii* infections usually have a relatively milder infectious course (32), though severe systemic infection of a healthy adolescent has also been reported (31). *B. holmesii* has been isolated from immunocompromised hosts (asplenic or sickle cell disease patients and transplant recipients) (16, 19, 23, 26, 32). The association of *B. holmesii* with underlying conditions may be due to immunodeficiencies in these patients that increase risk for *B. holmesii* infection, but it may also reflect the reporting bias of clinicians who are more likely to pursue the identification of isolates associated with severe disease. Although *B. holmesii*
was often isolated from blood samples, it has also been found to cause respiratory diseases (18, 35, 43). *B. holmesii* has been isolated from the pleural fluid and lung biopsy specimens of an immunocompetent adolescent presenting with fever and pulmonary fibrosis (31) and from sputum of patients with respiratory failure (35). Moreover, *B. holmesii* was isolated from nasopharyngeal specimens of otherwise healthy individuals presenting with whooping cough-like symptoms such as paroxysms, whoop or post-tussive vomiting (18, 43). Therefore, *B. holmesii* appears able to infect the respiratory tract like other *Bordetella* species.

In this study, we demonstrated the increased presence of *B. holmesii* in a highly vaccinated population in Massachusetts since 2005. Sequence-based phylogenetic analyses on 30 independent isolates identified limited genetic variation among *B. holmesii* isolates. Using a murine model of infection, we found that both wP and aP failed to confer protection against a *B. holmesii* nasopharyngeal isolate from Massachusetts. Although these vaccines induced cross-reactive T cell responses, vaccine-induced antibodies did not efficiently bind *B. holmesii*. The administration of *B. holmesii*-specific antibodies to wP-vaccinated mice quickly reduced *B. holmesii* numbers in the lungs, indicating that the poor cross-protection might be attributable to the lack of *B. holmesii* protective antigens in the vaccine.
Materials and Methods:

Identification of *B. holmesii* culture-positive cases in Massachusetts. A serum test was performed if the patient was ≥ 11 years old and had a cough for more than 14 days. In all other cases (< 11 years old or ≤ 14 days of cough) a nasopharyngeal swab was cultured. Details on culturing methods and tests performed for identification of *Bordetella* spp. have been previously described (18).

Bacterial strains and growth. *B. pertussis* strain 536 (34) and *B. parapertussis* strain CN2591 (29) have been previously described. Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% sheep’s blood (Hema Resources) without antibiotics (*B. holmesii*) or with 20 µg/mL streptomycin (*B. pertussis* or *B. parapertussis*) (Sigma-Aldrich). Liquid cultures were grown overnight in Stainer-Scholte broth at 37°C to mid-log phase (33, 37).

Phylogenetic analysis. Phylogenetic analyses based on *atpD, rpoB, tuf,* and *rnpB* sequences were performed as previously described (6). Information on the *B. holmesii* isolates is listed in Figure 2. Concatenated sequences were aligned for the construction of unweighted-pair group method using average linkages (UPGMA) trees using MEGA 4.0 software.

Animal experiments. 4-6 week old C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbor) and bred in *Bordetella*-free, specific pathogen-free breeding rooms. Mice were vaccinated on days 14 and 28 prior to challenge as previously described (9, 45). For challenge, mice were sedated and inoculated by pipetting 50 µL PBS containing 5×10⁵ CFU of *B. pertussis* or *B. parapertussis* or 10⁷ CFU of *B. holmesii* onto the external nares (14). For adoptive transfer of serum antibodies, mice were vaccinated with the indicated bacteria on days 0 and 14, and sera were collected on day 28 from vaccinated mice or from naïve animals. 200 µL of sera were i.p. injected immediately before inoculation (15, 28). To quantify bacterial numbers, mice were sacrificed via CO₂ inhalation, and the lungs were excised, homogenized in PBS, serially diluted and plated onto Bordet-Gengou agar plates. Bacterial colonies were counted after incubation at 37°C for 3-6 days (15). The lower limit of detection was 10 CFU. All protocols were approved by Institutional Animal Care and Use Committee (IACUC), and all animal were handled in accordance with institutional guidelines.
**Splenocyte re-stimulations.** Spleens were excised from vaccinated mice. Splenocytes were isolated as previously described (27, 45) and stimulated with either media alone or media containing $10^7$ CFU (multiplicities of infection of 5) of the indicated heat-killed bacteria (9, 27). After three days, the supernatants were collected and analyzed for Interferon (IFN)-γ and interleukin-10 (IL-10) production via enzyme-linked immunosorbent assays (ELISA) as per the manufacturer’s instructions (R&D Systems).

**Titer ELISAs.** Antibody titers were determined as previously described (25, 40, 41) with the following modifications. wP or wH-induced/naive serum samples (1:200 dilution) or aP/adjuvant-induced serum samples (1:50 dilution) from each mouse was added to the first well of each row of 96-well plates.

**Western Blot analysis.** Lysates containing $10^7$ CFU of indicated heat-killed bacteria were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels in denaturing conditions. Polyvinylidene Fluoride (PVDF) membranes (Millipore) were probed overnight with either naïve serum (1:100 dilution) or wH- (1:500 dilution), wP- (1:500 dilution), aP- (1:100 dilution) induced serum. A 1:10,000 dilution of goat anti-mouse Ig HRP-conjugated antibodies (Southern Biotech) was used as the detector antibody (40, 42). The membrane was visualized with ECL Western Blotting Detection Reagent (Pierce Biotechnology).

**Statistical analysis.** The means +/- standard error (error bars) were determined for all appropriate data. Two-tailed, unpaired Student’s T-tests were used to determine statistical significance between groups. Results were also analyzed by ANOVA and Tukey simultaneous test in Minitab with similar significance.
Results:

*B. holmesii* is endemic in a highly vaccinated population.

Probert et al. have described detection of *B. holmesii* from nasopharyngeal specimens collected from 2003 to 2007 by PCR assays, but these isolates were not validated by culture (30). Other than this study, few publications have reported the prevalence of *B. holmesii* in recent years. The MDPH provides a robust pertussis surveillance program and pertussis diagnostic services. It has been estimated that vaccine coverage for children among 19-35 months of age in Massachusetts with four or more doses of any pertussis vaccines was more than 85% between 1995 and 2008, ranking 5th amongst the 50 states in the U.S. on average (Fig. 4.1) (2). Here, we reported the numbers of *B. holmesii*-culture-positive nasopharyngeal specimens submitted to the MDPH from 1990 to 2008. *B. holmesii* was sporadically isolated from 1990 to 2004; however, since 2005 this bacterium has been isolated from nasopharyngeal swabs of 40 patients with respiratory symptoms in 2005, 2006, 2007, 2008 and 2009, respectively (Fig. 4.1). Together, these data indicate that *B. holmesii* is consistently present in the nasopharynx of a small number of patients suffering respiratory infections in Massachusetts where the vaccine coverage is high. This suggests that *B. holmesii* has established a successful chain of transmission despite the high level of immunity to *B. pertussis*.

**Phylogenic relationship among *B. holmesii* isolates.**

To evaluate the phylogenic relationship among *B. holmesii* isolates, an UPGMA tree was constructed based on concatenated sequences amplified from regions of *atpD*, *rpoB*, *tuf*, and *rnpB* genes (6). Consistent with previous findings (6), all the *B. holmesii* isolates tested are more closely-related to *B. hinzii* and *B. avium* than to the classical bordetellae (Fig. 4.2). Although single-nucleotide polymorphisms do exist among *B.
holmesii isolates, all the nasopharyngeal isolates from Massachusetts belong to branches with bootstrapping value less than 50%, indicating their close-relatedness. B. holmesii isolates from blood and nasopharyngeal specimens do not cluster separately, suggesting that evolutionary relationship among B. holmesii isolates are not associated with differences in anatomic isolation sites.

B. holmesii is not susceptible to B. pertussis vaccine-induced immunity. Since B. holmesii is endemic in a highly-vaccinated population, we hypothesize that this bacterium may evade B. pertussis vaccine-induced immunity. We first examined whether B. holmesii immunization induces protection against itself and cross-protection against B. pertussis and/or B. parapertussis. C57BL/6 mice were vaccinated with heat-killed B. holmesii (wH). These mice or naïve mice were challenged with B. pertussis, B. parapertussis or B. holmesii and sacrificed on day 3 post-inoculation (p.i.) for bacterial number quantification. Since colonization efficiency of B. holmesii in the murine respiratory tract is low compared to
the classical bordetellae, possibly due to the decreased attachment to mouse respiratory epithelium (Karanikas A.T., Weyrich L.S., Goebel E.M., and Harvill E.T., unpublished data), a high challenge dose was used. Similar numbers of *B. pertussis* were recovered from the lungs of naïve and wH-vaccinated mice (Fig. 4.3A), indicating that *B. holmesii* vaccination failed to reduce *B. pertussis* numbers within 3 days. *B. holmesii* vaccination reduced the *B. parapertussis* load by ~70% in the lungs, indicating a modest cross-protection provided by *B. holmesii* against *B. parapertussis*. Compared to naïve mice, *B. holmesii* vaccination reduced *B. holmesii* numbers by ~97% in the lungs (Fig. 4.3A), indicating that *B. holmesii* induces an effective anamnestic responses against itself.

To test whether *B. pertussis* vaccines provide cross-protection against *B. holmesii*, C57BL/6 mice were vaccinated with wP or aP. These mice or naïve mice were challenged with *B. holmesii* or *B. pertussis* and euthanized 3 days later. wP reduced *B. pertussis* numbers in the lungs by > 99.99% compared to naïve mice; however, wP failed to reduce *B. holmesii* numbers (Fig. 4.3B). Compared to the adjuvant-only-vaccinated mice, aP-vaccinated mice reduced *B. pertussis* numbers in their lungs by ~98% (Fig. 4.3C), but this vaccine did not reduce *B. holmesii* numbers either. Together, these data indicate that *B. holmesii* evades wP- and aP-induced immunity.

T cell responses following wH and wP/aP are cross-reactive.
To determine whether T cell responses following vaccination are cross-reactive, splenocytes from naïve or wH-, wP-, or aP-vaccinated mice were stimulated with media alone or media containing heat-killed *B. pertussis*, *B. holmesii*, or *B. parapertussis* for 72h. Concentrations of IFN-γ and IL-10, representing Th1 and Treg cytokines, respectively, in the cell culture supernatants were determined. Splenocytes from wH-vaccinated mice produced low levels of IFN-γ and IL-10 upon stimulation by *B. pertussis*, *B. holmesii*, or *B. parapertussis*, and the levels were comparable to those produced by naïve splenocytes (Fig. 4.4). However, splenocytes from wP-vaccinated mice, produced high levels of IFN-γ and IL-10 upon stimulation with *B. pertussis*, *B. holmesii*, or *B. parapertussis*, indicating that wP induces a strong cross-reactive T cell response. Although splenocytes from aP-vaccinated mice produced little IFN-γ, they produced similar level of IL-10 upon stimulation with heat-killed *B. pertussis*, *B. holmesii*, or *B. parapertussis*, indicating a low but cross-reactive T cell response following aP vaccination. These data indicate that *B. pertussis* vaccines induce cross-reactive T cell responses to *B. holmesii*.

**wP/aP-induced antibodies do not efficiently recognize *B. holmesii***.

To examine whether *B. holmesii* and *B. pertussis*-induced antibodies are cross-reactive, Ig titers of wH-, wP-, or aP- induced sera were determined by ELISA using heat-inactivated bacteria as antigens. The *B. holmesii*-specific Ig titer of wH-induced serum was >450,000, which is around 10-fold and 25-fold higher than *B. parapertussis*- and *B. pertussis*-specific titer of this serum, respectively (Fig. 4.5A). This indicates that *B. holmesii*-induced antibodies bind less efficiently to *B. parapertussis* or *B. pertussis* than to *B. holmesii*. 

![Figure 4.4](image-url)
B. pertussis-specific Ig titer of wP- and aP-induced serum antibodies were 290,000 and 2,500, respectively. wP- and aP-induced antibodies bind less well to B. parapertussis (Fig. 4.5A) and confer little protection against B. parapertussis in vivo (45). wP- or aP-induced sera bound even less well to B. holmesii. A similar trend was observed when live bacteria were used to coat the ELISA plates (data not shown), which rules out the possibility that heat-inactivation destroys cross-reactive antigens.

To compare the antigens recognized by sera from different groups, Western blot analyses were performed on B. pertussis, B. parapertussis or B. holmesii lysates probed with naïve serum or wH-, wP-, aP-induced sera. wH-induced serum antibodies recognized B. holmesii antigens of various molecular masses but lacked cross-recognition of some higher-molecular-mass (> 60kDa) antigens of B. holmesii. aP-induced antibodies only poorly recognized a single B. holmesii antigen.

B. holmesii-specific antibodies augment wP-induced immunity against B. holmesii.

Based on our data, we hypothesized that wP induces sufficient T cell responses but the antibody responses are not sufficient to confer cross-protection. If this were the case, adding B. holmesii-specific antibodies would render wP effective against B. holmesii. To test this, C57BL/6 mice were left untreated or vaccinated with wP, and later treated with either naïve serum or serum from wP- or wH-vaccinated mice and

Figure 4.5: wH/wP/aP antibody responses are not fully cross-reactive. (A) B. pertussis (B.p.) (black bars), B. parapertussis (B.p.p.) (tiltedly dashed bars) or B. holmesii (B.h.) (white bars) specific Ig titers of serum antibodies form wH-, wP- or aP- vaccinated mice are expressed as mean Log_{10} Ig Titer ± the standard error. ** indicates P ≤ 0.01 determined by ANOVA and Tukey simultaneous test. (B) Western blots were performed on B. pertussis, B. parapertussis or B. holmesii lysates probed with naïve serum or wH-, wP-, aP-induced serum (IS). wP: B. pertussis whole cell vaccine; wH: B. holmesii whole cell vaccine; aP: B. pertussis acellular vaccine.
challenged with *B. holmesii*. These mice were euthanized on day 3 post-challenge to determine *B. holmesii* numbers in the lungs. Neither wP-induced serum nor vaccination alone reduced *B. holmesii* numbers, but vaccinated mice given *B. holmesii*-immune serum had substantially lowered *B. holmesii* numbers in the lungs (Fig. 4.6), indicating that the addition of *B. holmesii*-specific antibodies to wP increases its efficacy against *B. holmesii*.

Figure 4.6: **Supplement wP with *B. holmesii*- but not *B. pertussis*- specific antibodies confer protection against *B. holmesii***. Groups of four wP-vaccinated C57BL/6 mice were left untreated (none) or adoptively transferred naïve serum (NS), or wP-, wH- induced serum, and challenged with *B. holmesii*. Bacterial numbers in the lungs on day 3 post-inoculation are expressed as mean Log$_{10}$CFU ± the standard error. * indicates $P \leq 0.05$ determined by ANOVA and Tukey simultaneous test. wP: *B. pertussis* whole cell vaccine; wH: *B. holmesii* whole cell vaccine; aP: *B. pertussis* acellular vaccine.
Discussion:

Our epidemiology data indicate that *B. holmesii* is endemic in a highly vaccinated population (Fig. 4.1). Since its establishment as a species in 1995 (39), sporadic reports of *B. holmesii* cases have been reported worldwide (10, 16, 23, 24, 26, 31, 32, 35). However, few reports on its routine isolation have been described. Here we report that *B. holmesii* was cultured from nasopharyngeal specimens submitted to the MDPH each year from 2005 to 2009. These observed cases are likely to be a small fraction of the total number of *B. holmesii* infections. One major obstacle to the identification of *B. holmesii* infection is the lack of awareness, since *B. holmesii* is not generally considered as an inhabitant of the respiratory tract and is excluded from commercially available identification systems. Current estimation of *B. holmesii* prevalence is further confounded by difficulties in the identification of *Bordetella* spp.. Culture-based laboratory diagnosis is highly specific but is sensitive only in the initial phases of disease and requires special media with an extended incubation period of 5-10 days (38). Moreover, cephalexin, the antibiotics commonly incorporated in both charcoal and Bordet-Gengou agar for *Bordetella* spp. recovery, has been reported to inhibit *B. holmesii* growth (18), likely lowering *B. holmesii* recovery rate. Diagnostic serology and PCR can be highly sensitive and quicker than culture, but no serology or PCR assay specific for *B. holmesii* is widely accepted.

2,628 *B. pertussis* cases were identified by culture between 1992 and 2008 in Massachusetts, while a total of 11,577 *B. pertussis* cases were identified by culture, serology and PCR combined. 8,949, or 77%, of *B. pertussis* cases were identified by serology or PCR, neither of which would detect *B. holmesii*. It is likely that the 44 culture-positive *B. holmesii* cases reported here only comprise a small proportion of all the *B. holmesii* infections in Massachusetts. Overall, due to the low awareness and the identification difficulties, *B. holmesii* infection might occur more frequently than the limited reported cases would suggest.

*B. holmesii* isolates seem to show little genetic variance. Several previous reports have demonstrated limited numbers of pulsed-field gel electrophoresis (PFGE) banding pattern (18) and little 16S rRNA heterogeneity (32, 39) among *B. holmesii* isolates. Diavatopoulos et al. also analyzed seven independent *B. holmesii* isolates by multilocus-sequence-typing and observed only one non- synonymous polymorphism among 3,666 bases (6). Using a similar method, we further analyzed 20 isolates from the CDC and 10 isolates from the MDPH. These isolates, although isolated from diverse geographic locations and dates, seem
to be a nearly uniform group (Fig. 4.2). Taken together, *B. holmesii* seems to be clonal, which indicates its recent emergence and may suggest a chain of transmission from an isolated source.

The murine model of human-adapted bordetellae infection has been well-established. *B. pertussis* and *B. parapertussis* infections in mice mimic the course of infection and most of the immune responses in humans with the exception of the coughing symptom (11, 12, 15, 20). Moreover, bacterial clearance from lungs following *B. pertussis* challenge in vaccinated adult mice correlates with vaccine efficacies in children (21, 22). An animal model of *B. holmesii* infection has not been previously described. *B. holmesii* efficiently colonizes the murine respiratory tract only when large intranasal inoculums are delivered, a phenomenon currently under investigation in our laboratory.

We determined, for the first time, that *B. pertussis* vaccines confer little, if any, protection against *B. holmesii*. Comparative analysis of *B. holmesii* and *B. pertussis* 16S rRNA sequences suggested that *B. holmesii* is closely-related to *B. pertussis* (39). However, subsequent comparative genomic hybridization analysis suggested that this might be a result from lateral gene transfer of *B. pertussis* 16S rRNA to *B. holmesii* (6). Analysis of cellular fatty acid composition, sequencing of housekeeping genes and characterization of the BvgAS locus also suggested that *B. holmesii* might not be as closely-related to *B. pertussis* as originally assumed (6, 8, 39). Furthermore, anti-*B. pertussis* pertactin, pertussis toxin, fimbriae 2 and 3, adenylate cyclase toxin and filamentous hemagglutinin failed to recognize any protein from multiple *B. holmesii* isolates (26), suggesting that these proteins are either not produced by *B. holmesii* or that the proteins produced are antigenically distinct from those in *B. pertussis*. Using the murine model of infection, we determined that both wP and aP do not confer efficient protection against *B. holmesii* (Fig. 4.3B, 4.3C). The vaccine-induced T cell responses are cross-reactive to *B. holmesii* (Fig. 4.4); however, vaccine-induced antibodies bind poorly to *B. holmesii* (Fig. 4.5). Furthermore, *B. holmesii*-specific, but not *B. pertussis*-specific antibody administration, efficiently decreased *B. holmesii* numbers in the lungs of vaccinated mice (Fig. 4.6), suggesting that the lack of cross-reactive antibody responses might result in the poor cross-protection of *B. pertussis* vaccines against *B. holmesii*.

A similar lack of cross-protection against *B. parapertussis* was attributed to the lack of a *B. parapertussis*-specific protective antigen, O-antigen, in the *B. pertussis* vaccines (44), and the blockage of
cross-reactive antibodies binding by O-antigen (45). *B. holmesii* may avoid *B. pertussis* vaccine-induced immunity via similar mechanisms. *B. holmesii* induced anamnestic immunity against itself (Fig. 4.3A), indicating that this bacterium produces protective antigens. The antigens recognized by wH could be the potential protective antigens of *B. holmesii*, but are yet to be identified. *B. holmesii* LPS is phenotypically and immunologically distinct from *B. pertussis* LPS (36), but the detailed structure of *B. holmesii* LPS and its ability to block the binding of cross-reactive antibodies require further investigation.

Our data indicate that *B. holmesii* is present in a highly vaccinated U.S. population. *B. pertussis* vaccines confer little protection against *B. holmesii*, which may potentially favor the emergence of *B. holmesii* in the human population. Careful surveillance of *B. holmesii* infections is needed to better evaluate its prevalence and potential mode of transmission. Genome sequencing of this organism will help address the presence of novel virulence determinants of *B. holmesii*, and guide the design of effective vaccines against this human pathogen.
Authors and Contributions

Xuqing Zhang\textsuperscript{1,2}, Laura S. Weyrich\textsuperscript{1,3}, Alexia T. Karanikas\textsuperscript{1,4}, Jennie S. Lavine\textsuperscript{5} and Eric T. Harvill\textsuperscript{1}

\textsuperscript{1}Department of Veterinary and Biomedical Sciences, the Pennsylvania State University, 115 Henning Building, University Park, PA 16802

\textsuperscript{2}Graduate Program in Genetics

\textsuperscript{3}Graduate Program in Biochemistry, Microbiology, and Molecular Biology

\textsuperscript{4}Graduate Program in Cell and Developmental Biology

\textsuperscript{5}Center for Infectious Disease Dynamics, the Pennsylvania State University, 501 Agriculture and Sciences Industry Building, University Park, PA 16802

Conceived and designed the experiments: XZ, ETH.

Performed the experiments: XZ, LSW (Fig. 4.2), ATK (Fig. 4.2).

Analyzed the data: XZ, LSW, JSL.

Wrote the paper: XZ, ETH.
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78


Chapter 5

IL-1R Signaling Contributes to Controlling Inflammatory Pathology and Overcoming the Effects of Pertussis Toxin during *B. pertussis* Infection.

Abstract:

Interleukin (IL)-1R −/− mice are healthy despite being colonized by commensal microbes, but are defective in defenses against specific pathogens, suggesting that IL-1R-mediated effects contribute to immune responses against specific pathogenic mechanisms. To better define the critical role of IL-1R in immunity to respiratory infections, we challenged IL-1R −/− mice with *Bordetella pertussis* and *B. parapertussis*, the causative agents of whooping cough. Following infection by *B. pertussis*, but not *B. parapertussis*, IL-1R −/− mice showed increased mortality associated with elevated bacterial numbers, atypical systemic spread, and higher inflammatory pathology compared to wild-type mice. IL-17 responses were nearly abolished, but restoring the pulmonary IL-17 levels did not decrease *B. pertussis* burdens in IL-1R −/− mice. *B. pertussis*-stimulated dendritic cells and macrophages from IL-1R −/− mice produced higher levels of tumor necrosis factor (TNF)-α and IL-6 but lower levels of IL-10 than wild-type cells. Moreover, elevated levels of gamma-interferon (IFN-γ) and TNF-α were detected locally and systemically following *B. pertussis* infection in IL-1R −/− mice. These data demonstrate a role for IL-1R signaling in protecting against *B. pertussis* infection by restricting infection within the respiratory tract and controlling inflammatory pathology. Since *B. parapertussis* did not cause severe disease in IL-1R −/− mice, we hypothesized that the extreme requirement for IL-1R involves pertussis toxin (Ptx), which is expressed only by *B. pertussis*. An isogenic Ptx deficient strain of *B. pertussis* was completely defective in causing lethal disease in IL-1R −/− mice, indicating that the virulence of *B. pertussis* in these mice involves Ptx and that IL-1R protects wild-type mice from aspects of Ptx-mediated virulence.
**Introduction:**

Inflammatory responses effectively combat infection and, when properly controlled, ensure restoration of normal tissue architecture. A complex array of cytokines can contribute to the regulation of inflammation under different conditions. By discovering the specific conditions under which key cytokines are required for regulation, we can better define their individual roles. Interleukin (IL)-1, a pleiotropic pro-inflammatory cytokine presented either as IL-1α or IL-1β, is a key player in this regulation. IL-1α is active in both 31 kDa precursor polypeptide form (pro-IL-1α) and calpain-cleaved “mature” 17 kDa form (19, 22). Pro-IL-1β is inactive and requires cleavage by caspase-1 to be active and secreted (11, 19, 22, 75). There are two membrane receptors for IL-1, type I IL-1 receptor, which mediates signal transduction, and type II IL-1 receptor, which lacks the cytosolic domains and acts as a decoy receptor (73). Both IL-1α and IL-1β bind to the same receptors and induce indistinguishable responses including endothelial activation, leukocyte recruitment, T helper cytokines production, and alterations of the hypothalamic thermoregulatory set point (19).

IL-1 has been implicated in various inflammatory diseases as well as during microbial infections. IL-1 signaling plays pathogenic roles in some autoimmune diseases, such as rheumatoid arthritis and Crohn’s disease (19). It is also involved in inducing pathogenic damage by bacterial pathogens, for example *Yersinia enterocolitica* and *Shigella flexneri* (21, 66). On the other hand, IL-1 signaling also plays beneficial roles in combating microbial infections. For example, exogenous administration of rmIL-1α provided protection against an intracellular pathogen, *Listeria monocytogenes*, infection (16). IL-1β deficient mice challenged with *Staphylococcus aureus*, a gram-positive bacterium, developed larger lesions associated with decreased neutrophil recruitment (55). IL-1R−/− mice showed increased intestinal damage and lethality following challenge by *Citrobacter rodentium*, a gram-negative pathogen (41).

IL-1R−/− mice are also defective in host defenses against some respiratory pathogens, suggesting that IL-1 signaling is particularly important in the control of respiratory infection. For example, mice deficient in IL-1R had higher *Pseudomonas aeruginosa* loads in the lungs (65), and suffered lethal necrotic pneumonia following *Mycobacterium tuberculosis* infection (24). Although IL-1 signaling is required for the control of several pathogens, the critical aspects of host-pathogen interactions that require this pathway for effective
immune responses have not yet been determined. Since IL-1α−/−, IL-1β−/− and IL-1R−/− mice are viable and healthy despite a complex flora resident in them, IL-1 signaling is apparently not required for healthy homeostasis or the containment of non-pathogens. There appear to be specific virulence mechanisms of certain pathogens that stress the host responses in ways that reveal important roles of the IL-1 signaling. Here, we examine the role of IL-1R in the immune responses to *Bordetella pertussis* and *B. parapertussis*, identifying a key role in mitigating the virulence associated with pertussis toxin (Ptx) secreted only by *B. pertussis*.

*B. pertussis* and *B. parapertussis* are gram-negative coccobacillus, causing whooping cough in humans, an acute and severe respiratory disease (51). Despite high vaccine coverage in developed countries, whooping cough causes approximately 50 million cases and 300,000 deaths annually worldwide (14). Even though a large portion of infections remain unreported (18), whooping cough incidence is on the rise (25, 35, 70, 76). *B. pertussis* utilizes complex strategies to modulate and evade host immune responses by producing various virulence determinants, such as Ptx, adenylate cyclase toxin, tracheal cytotoxin, filamentous hemagglutinin, fimbriae, pertactin and lipopolysaccharide (51, 56). *B. parapertussis* shares most of the virulence determinants with *B. pertussis* (51), but does not express Ptx (4, 49). Although *B. pertussis* and *B. parapertussis* are closely-related (62), they use distinct strategies to modulate host immune responses, revealing details of the different sophisticated immune regulations necessary to combat each.

Ptx is a multi-subunit toxin with an AB5 configuration. The B-oligomer binds to glycoproteins or glycolipids on the surface of target cells (77, 80). The enzymatic activity of Ptx resides in the A subunit, also known as S1. Once in the cell cytosol, S1 mediates ADP-ribosylation of the α-subunit of a subset of Gi proteins in mammalian cells (6, 36). The modification results in the inhibition of Gi protein-coupled signaling pathways, causing a variety of downstream effects. Ptx is known to be the cause of some systemic symptoms associated with whooping cough, such as lymphocytosis, histamine sensitivity and insulinemia (58). Ptx also exerts multiple modulating effects on the immune system, including targeting airway macrophages to promote infection (10), blocking the early migration of neutrophils into the lungs (2, 38), suppressing the production of anti-*Bordetella* serum antibodies (9, 54), reducing major histocompatibility complex class II on the surface of monocytes (68), and interfering with CD1a expression on dendritic cells.
A recent study indicates that Ptx contributes to the induction of pro-inflammatory cytokine production at the peak of infection (3), but whether these cytokines are important for host defenses against B. pertussis are not fully understood.

A murine model of B. pertussis infection in specific gene-deficient mice has established the non-redundant contribution of several innate-immune system components or effectors, such as toll-like-receptor (TLR)-4, tumor necrosis factor (TNF)-α and IL-6 in host defenses against B. pertussis (34, 48, 56, 79) (Goel T., Zhang X. and Harvill E.T., unpublished data). TLR4 deficient mice have a protracted infectious course of B. pertussis, which is associated with elevated inflammatory pathology (34, 48). TNF-α deficient mice are more susceptible to B. pertussis infection due to overwhelming pathology (79). Downstream signaling of IL-1R overlaps with that of TLR4 and TNFR, and IL-1β is produced in response to B. pertussis activation of TLR4 (34). It is not known if IL-1, a pro-inflammatory cytokine downstream of TLR4, is dispensable or is required to combat B. pertussis infection.

We demonstrated here that although mice lacking type I IL-1 receptor (IL-1R−/−) showed comparable macrophage bactericidal efficiency, normal neutrophil recruitment in the early stages of infection and sufficient antibody responses against B. pertussis, they suffered increased mortality from B. pertussis infection. This was associated with increased bacterial burdens throughout the respiratory tract, atypical disseminated diseases, increased histopathology, increased leukocyte recruitment, elevated pro-inflammatory cytokine production and decreased anti-inflammatory cytokine production. IL-1R is not required for the control of B. parapertussis, which lacks the expression of Ptx. IL-1R−/− mice did not succumb to infection by a B. pertussis strain lacking Ptx. Overall, our study suggests an indispensable protective role of IL-1R signaling during B. pertussis infection in limiting inflammatory pathology and overcoming the effects of Ptx.
Materials and Methods:

**Bacterial strains and growth.** The *B. pertussis* strain 536, a streptomycin resistant derivative of Tohama I, and the *B. parapertussis* strain 12822, an isolate from German clinical trials, have been previously described (32, 72). BPH101 (*B. p. Δptx*), a pertussis toxin-deficient derivative of strain 536, is a gift from Dr. Drusilla Burns (US Food and Drug Administration) (31). Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% defibrinated sheep blood (Hema Resources) and 20 μg/mL streptomycin (Sigma-Aldrich). Liquid cultures were grown overnight in Stainer-Scholte broth at 37°C to mid-log phase (71).

**Stimulation of macrophage cell lines.** The murine macrophage cell line, RAW 264.7, was obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone) supplemented with 10% fetal calf serum (FCS) (HyClone). Approximately 10⁵ cells were placed in each well of 96 well tissue culture plates (Falcon) and incubated with media or media containing live *B. pertussis* at multiplicities of infection (MOI) of 5 or 25. Cell culture supernatant was collected after 48h incubation and IL-1α or IL-1β concentration was measured via enzyme-linked immunosorbent assays (ELISA) as per the manufacturers’ instructions (R&D Systems).

**Animal experiments.** C57BL/6, B6.129S7-*Il1r1tm1Imx/J* (IL-1R-/-) and B6.129S2-*Igh-6tm1Cgn/J* (μMT) mice were obtained from Jackson Laboratories (Bar Harbor) and bred in *Bordetella*-free, specific pathogen-free breeding rooms at The Pennsylvania State University. For inoculation, 4-6 week-old mice were lightly sedated with 5% isoflurane (Abbott Laboratories) in oxygen and inoculated by pipetting 50μL phosphate buffered saline (PBS) containing 5×10⁵ CFU (unless otherwise specified) of bacteria onto the external nares (39). This method reliably distributes the bacteria throughout the respiratory tract (30). For survival curves or mean lethal dose (LD₅₀) determination, mice were inoculated with the indicated dose and the percent survival was monitored over a 100-day period. Mice with lethal bordetellosis, indicated by ruffled fur, labored breathing, and diminished responsiveness, were euthanized to alleviate unnecessary suffering (29, 79). For adoptive transfer of serum antibodies, sera were collected from naïve animals or on days 21 or 28 post *B. pertussis* inoculation and 200μL pooled serum was i.p. injected at the time of inoculation (39). These mice were euthanized on day 14 p.i. for bacterial number quantification in their lungs, by which time the inhibition by pertussis toxin in antibody-mediated clearance of *B. pertussis* is overcome with T cell help in
wild-type animals (38) (D.N. Wolfe unpublished data). For intranasal administration of rmIL-17, C57BL/6 or IL-1R−/− mice were lightly sedated and intranasally inoculated 50µL PBS or PBS containing rmIL-17 (R&D systems) on days 3 (1.25µg/mouse) and 6 (1µg/mouse) post-inoculation (p.i.) and sacrificed on day 7 p.i.. For quantification of bacterial numbers, mice were sacrificed via CO2 inhalation, lung, trachea, nasal cavity, spleen and liver were excised and around 750µL blood was collected from each mouse by cardiac puncture into tubes with 50µL 0.5M pH8 EDTA. Tissues were homogenized in 1 mL PBS, serially diluted and plated onto Bordet-Gengou agar plates with 20µg/mL streptomycin, and colonies were counted after 4-5 days incubation at 37°C (39). The lower limit of detection was 10 CFU. All protocols were reviewed and approved by The Pennsylvania State University Institutional Animal Care and Use Committee (IACUC) and all animals were handled in accordance with institutional guidelines.

**Lung pathology.** For analysis of lung pathology, mice were intranasally inoculated as described above and euthanized at the indicated days p.i.. The tracheas and lungs were excised and inflated with approximately 2 mL of 10% formaldehyde. The tissues were then sectioned and stained with haemolysin and eosin at the Animal Diagnostic Laboratories Facility of The Pennsylvania State University. Sections were examined and scored by a veterinarian with training and experience in rodent pathology (M.J.K.) who was blinded to experimental treatment (46, 47, 63). A score of 0 indicates no noticeable inflammation or lesions; a score of 1 indicates few or scattered foci affecting less than 10% of the tissue, typically with a few mild perivascular and/or peribronchial lymphoid aggregates; a score of 2 indicates frequent mild perivascular and/or peribronchial lymphoid aggregates, with or without occasional small foci of pneumonia, with overall inflammation affecting no more than 10 to 20% of the tissue; a score of 3 indicates moderate lesions, typically with abundant perivascular and peribronchial lymphoid infiltrates and multiple mild to moderate foci of pneumonia, with inflammation affecting approximately 20 to 30% of the tissue; and a score of 4 indicates extensive pneumonia and marked inflammation affecting more than 30% of the tissue; a score of 5 indicates extensive lesions with >50% of the tissue affected. If a severity falls between categories, 0.5 was added to the pathology score of the lower category.

**Bone-marrow derived cell assays.** Bone marrow derived macrophages (BMM) and dendritic cells (BMDC) were prepared as previously published with modifications (43, 69). In brief, bone marrow was isolated from
femurs of C57BL/6 or IL-1R−/− mice and cultured for 10 days in DMEM supplemented with 10% FCS, 100µg/mL penicillin-streptomycin (HyClone), 20 ng/mL macrophage-colony-stimulating-factor (M-CSF) (PeproTech) for BMM differentiation or in RPMI supplemented with 2mM L-glutamine (HyClone), 10% FCS, 100µg/mL penicillin-streptomycin, 40 ng/mL granulocyte-macrophage-colony-stimulating-factor (GM-CSF) (PeproTech) for BMDC differentiation. For cytokine responses of the BMDCs, 2×10⁵ cells were seeded into each well of 96 well tissue culture plates in media without antibiotics and stimulated with media or media containing *B. pertussis* at a MOI of 10. After 2h, 12h, 24h or 36h, the culture supernatant was removed and assayed for TNF-α, IL-6 or IL-10 by ELISA (R&D Systems). For BMM cytokine secretion assays, 10⁵ cells were seeded into each well of 96 well tissue culture plates in media without antibiotics and stimulated with media or media containing *B. pertussis* lipopolysaccharide (LPS) (final concentration 1µg/mL) or *E. coli* LPS (final concentration 100ng/mL). After 12h, the culture supernatant was removed and assayed for TNF-α, IL-6 or IL-10 by ELISA (R&D Systems). For BMM bactericidal assay, 10⁵ cells were seeded into each well of 96 well tissue culture plates and primed with media without antibiotics and containing 25ng/mL rm gamma-interferon (IFN-γ) (R&D systems) or 1µg/mL *B. pertussis* LPS for 2h. Early exponential-phase *B. pertussis* were opsonized with 5% convalescent-phase complement depleted (incubated at 56°C for 30 min) immune serum from C57BL/6 mice for 30 min in a shaking 37°C incubator. Primed BMMs were inoculated with opsonized *B. pertussis* at a MOI of 10. After 2h or 4h, 1% ice-cold Triton X-100 in PBS was added to lyse the macrophages. 1/10 serial dilutions of the lysed macrophages from each well were plated for bacterial enumeration. Bacterial numbers in similarly treated wells without cells were enumerated as total bacterial numbers.

**Splenocyte re-stimulation.** Spleens were excised on the indicated days p.i. from groups of *B. pertussis*-inoculated C57BL/6 or IL-1R−/− mice. Splenocytes were isolated as previously described (64, 78). In brief, spleens were homogenized and red blood cells were lysed with 0.84% ammonium chloride treatment. 2×10⁶ cells were re-suspended in 200µL DMEM supplemented with 10% FCS and 100µg/mL penicillin-streptomycin, and placed into each well of 96 well tissue culture plates. Splenocytes were stimulated with 10µL media alone or media containing 10⁷ CFU (MOI of 5) of heat-inactivated *B. pertussis* (64, 78). After 3
days, the supernatants were collected, and TNF-α, IFN-γ, IL-10 and IL-17 concentrations were determined by ELISA (R&D Systems).

**Titer ELISAs.** Antibody titers were determined as previously described (46). Briefly, heat-inactivated exponential–phase *B. pertussis*, diluted to $5 \times 10^7$ CFU/mL in a 1:1 mix of 0.2M sodium carbonate and 0.2M sodium bicarbonate buffers, was used to coat 96 well plates (Greiner Bio-one) by incubating for 4h at 37°C in a humidified chamber and the plates were washed and blocked. A 1:50 [for immunoglobulin (Ig)] or 1:20 (for IgG1 and IgG2a) dilution of serum samples collected from individual C57BL/6 or IL-1R−/− mouse on days 7, 14 or 21 p.i. were added to the first well and serially diluted 1:2 across the plates. Plates were incubated for 2h at 37°C, washed and probed with 1:4000 dilution of goat anti-mouse Ig, or 1:2000 dilution of goat-anti-mouse IgG1 or IgG2a horseradish peroxidase (HRP)-conjugated antibodies (Southern Biotechnology Associates and Pharmingen) for 1h and visualized with 2,2’-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt in phosphate-citrate buffer with hydrogen peroxide at an absorbance of 405 nm. Titers were determined via the end-point method using a cut off that is 0.1 higher than the optical density of similarly treated wells probed with naïve serum (46).

**Quantification of leukocyte and cytokine in the lungs.** To quantify leukocytes, lungs were perfused with 2-3mL sterile PBS, excised, and placed in 4 mL of RPMI 1640 (HyClone). Lungs were homogenized, laid over Histopaque 1119 (Sigma Aldrich) and centrifuged for 30 min at 700g at 20°C. The leukocyte layer was collected, spun at 300g for 5 min and re-suspended in PBS supplemented with 2% FCS. The total number of leukocytes was determined by counting at 40× magnification on a hemocytometer. Aliquots of cells were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences Pharmingen) and stained with FITC-labeled anti-mouse-Ly6G, F4/80 or CD4 antibodies (BD Biosciences Pharmingen), and the percentages of Ly6G+, F4/80+ or CD4+ cells were determined by flow cytometry. Percentages were multiplied by the total number of leukocytes to calculate the number of Ly6G+, F4/80+ or CD4+ cells respectively. To measure the cytokine concentration in the lungs, lungs were homogenized in 1mL PBS, tissues were spun down at 8,000g for 10 min at 4°C, and IL-1α, IL-1β, TNF-α, IFN-γ, IL-10 and IL-17 concentrations in the aliquots of the supernatants were examined via ELISAs (R&D Systems).
**Statistical analysis.** The means ± the standard error (error bars) were determined for all appropriate data. Two-tailed, unpaired Student’s T-tests were used to determine statistical significance between groups. Results were also analyzed by ANOVA and Tukey simultaneous test in Minitab with similar significance.
Results:

*B. pertussis* induces IL-1 production *in vitro* and *in vivo*.

To determine if *B. pertussis* stimulates the production of IL-1, we stimulated RAW cells, a murine macrophage cell line, with media or media containing *B. pertussis* and the concentrations of IL-1α or IL-1β in the cell culture supernatants were measured after 48h. Macrophages incubated with media alone produced little IL-1 (Fig. 5.1A). Macrophages incubated with *B. pertussis* for 48h had produced ~40ng/mL or ~600pg/mL IL-1α, and ~800pg/mL or ~2000pg/mL IL-1β at MOI 5 or 25, respectively (Fig. 5.1A).

To examine if IL-1 is produced at the site of *B. pertussis* infection *in vivo*, C57BL/6 mice were inoculated with 5×10⁵ CFU of *B. pertussis* in 50µL PBS and IL-1α and IL-1β concentrations in the lungs were examined on days 0, 1, 3, 7, 10 and 26 p.i.. Compared to the level in naïve mice (day 0), IL-1α levels were elevated within 24h post *B. pertussis* infection, peaked at 2,400pg/mL by day 7 p.i. and declined thereafter (Fig. 5.1B white circle). IL-1β levels showed a similar trend, peaking at ~3,800pg/mL by day 7 p.i. (Fig. 5.1B black circle). Together these data indicate that *B. pertussis* induces IL-1α and IL-1β production *in vitro* by macrophage and *in vivo* in the lungs.

**Increased mortality, and elevated respiratory tract and systemic colonization in B. pertussis-infected IL-1R−/− mice.**

To determine whether IL-1R signaling is an important aspect of the immune response to *B. parapertussis* or *B. pertussis* infection, we monitored survival after challenging wild-type C57BL/6 or IL-1R−/− mice with 10⁶ CFU of *B. parapertussis* or *B. pertussis*. Challenged C57BL/6 mice showed no signs of disease
and were euthanized at the end of the experiment (100 days p.i.) (data not shown). Similarly, IL-1R<sup>−/−</sup> mice challenged with <i>B. parapertussis</i> did not show any sign of disease throughout the 100-days period (Fig. 5.2A square). However, IL-1R<sup>−/−</sup> mice challenged with <i>B. pertussis</i> showed signs of disease during the second week p.i., including ruffled fur, hunched posture, decreased activity, labored breathing, and were euthanized when the morbidity became apparent. All IL-1R<sup>−/−</sup> mice succumbed to lethal bordetellosis by day 17 p.i. (Fig. 5.2A triangle). We also performed similar survival curve studies at different challenge doses and determined that LD<sub>50</sub> of <i>B. pertussis</i> in C57BL/6 was greater than 5×10<sup>7</sup> CFU, whereas in IL-1R<sup>−/−</sup> mice the LD<sub>50</sub> was approximately 10<sup>3</sup> CFU, indicating that IL-1R deficiency greatly increases sensitivity to lethal <i>B. pertussis</i> infection.

Groups of C57BL/6 or IL-1R<sup>−/−</sup> mice were challenged with 5×10<sup>5</sup> CFU of <i>B. parapertussis</i> and euthanized on days 0, 3, 7, 14 or 28 p.i. to determine whether IL-1R contributes to the control of bacterial
numbers following *B. parapertussis* infection. Other than small and transient differences in the tracheas, *B. parapertussis* had similar colonization kinetics throughout the respiratory tracts in C57BL/6 and IL-1R−/− mice (Fig. 5.2B), indicating that IL-1R signaling is not required for the control and clearance of *B. parapertussis*.

To test if the increased mortality of IL-1R−/− mice following *B. pertussis* infection is associated with higher bacterial loads in the respiratory tract, mice were inoculated with $5 \times 10^5$ CFU of *B. pertussis*, and sacrificed on days 0, 1, 3, 7, 10, 14 and 26 p.i.. In wild-type mice, *B. pertussis* grew rapidly throughout the respiratory tract during the first week p.i., and decreased thereafter (Fig. 5.2C solid lines). In IL-1R−/− mice, *B. pertussis* similarly grew to high numbers during the first week p.i., but these mice failed to reduce bacterial numbers throughout the respiratory tract thereafter. We originally planned a day 28 p.i. time point, but two out of five IL-1R−/− mice showed signs of severe disease on day 24 p.i. and were euthanized. The surviving IL-1R−/− mice became very morbid on day 26 p.i. and were sacrificed along with the C57BL/6 controls for bacterial numbers determination. The IL-1R−/− mice harbored two orders of magnitude more *B. pertussis* in the nasal cavity and three orders of magnitude more *B. pertussis* in the lower respiratory tract (LRT) compared to C57BL/6 mice (Fig. 5.2C). In a separate experiment, groups of C57BL/6 and IL-1R−/− mice were challenged with $5 \times 10^5$ CFU *B. pertussis* and sacrificed on day 21 p.i. to avoid the loss of sick animals. On this time point, C57BL/6 mice harbored $10^{2.7\pm0.2}$ CFU of *B. pertussis* in their lungs, whereas $10^{7\pm0.3}$ CFU of *B. pertussis* were recovered from the lungs of IL-1R−/− mice (data not shown). Together, these data indicate that IL-1R is required for the clearance of *B. pertussis*, but not *B. parapertussis*, from the respiratory tract.

To test if IL-1R is required for the control of relatively low numbers of *B. pertussis*, separate groups of mice were challenged with $10^3$ CFU of *B. pertussis*. 28 days following this low dose inoculation, *B. pertussis* burdens were more than 4 orders of magnitude higher in the lungs, and approximately 2 orders of magnitude higher in the tracheas and nasal cavities, in IL-1R−/− mice than wild-type mice (data not shown). By day 56, although *B. pertussis* was completely cleared throughout the respiratory tract of wild-type mice, half of IL-1R−/− mice became morbid. The surviving IL-1R−/− mice still harbored ~300 CFU of *B. pertussis* in their lungs on this time point (data not shown).

*B. pertussis* does not colonize systemic organs of wild-type mice but causes atypical disseminated diseases in mice lacking certain immune functions (8, 44). To test if *B. pertussis* colonizes systemic organs of
IL-1R−/− mice, in a separate experiment, C57BL/6 and IL-1R−/− mice were challenged with 5×10⁵ CFU B. pertussis and the bacterial loads in their blood, spleen and liver were enumerated on days 3, 7, 14 and 21 p.i. B. pertussis was not recovered from blood or spleen of C57BL/6 mice on any time point. However, B. pertussis was recovered from blood and spleen of IL-1R−/− mice on days 14 and 21 p.i. (Fig. 5.2D). In the liver, B. pertussis colonized at a low level in one out of three of both C57BL/6 and IL-1R−/− mice on day 3 p.i., but was not recovered from the livers of C57BL/6 mice on any of the later time points. Interestingly, B. pertussis consistently colonized the livers of IL-1R−/− mice on days 7, 14 and 21 p.i. (Fig. 5.2D). These data indicate that in the absence of IL-1R signaling, B. pertussis causes severe systemic infection.

IL-1R−/− mice showed elevated inflammatory pathology post B. pertussis infection.

Since IL-1R−/− mice have been reported to show increased inflammation during infection by other bacteria (24, 41), and we visually observed that the lungs of B. pertussis-inoculated IL-1R−/− mice were consistently more inflamed than C57BL/6 lungs in multiple experiments, we examined the role of IL-1R signaling in the control of inflammatory pathology post B. pertussis infection. Sham (PBS) inoculated C57BL/6 or IL-1R−/− mice showed little sign of inflammation (Fig. 5.3A). On day 3 p.i. with 5×10⁵ CFU of B. pertussis, C57BL/6 mice showed pulmonary edema and scattered mild neutrophil infiltration in the parenchyma and bronchi, whereas IL-1R−/− mice showed severe diffuse suppurative pneumonia with fibrin, areas of consolidation (microabcesses), and some hemorrhage. On day 7 p.i., lung tissues from both C57BL/6 and IL-1R−/− mice showed severe diffuse suppurative pneumonia with fibrin and areas of consolidation, necrosis and hemorrhage, and the overall affected area of IL-1R−/− mice lungs (average pathology score 4.4) was significantly larger than that of C57BL/6 mice lungs (average pathology score 3.8) (Fig. 5.3B). On day 21 p.i., lungs of C57BL/6 mice showed marked peribronchiolar, perivascular, lymphoid aggregates, and patchy to diffuse pneumonia with areas of consolidation and lymphocytic infiltrates. IL-1R−/− lungs, however, showed severe diffuse pneumonia with mixed inflammatory cell infiltrates (primarily neutrophils), fibrin and large areas of consolidation, and no lymphocytic cuffing (Fig. 5.3A), indicating poor resolution.

To quantify the numbers of different cell types infiltrated into the lungs, C57BL/6 or IL-1R−/− mice were inoculated with B. pertussis, and total leukocytes were separated from lung homogenates and evaluated by flow cytometry after cell surface staining. 1 and 3 days later, wild-type and IL-1R−/− mice contained
similar numbers of total leukocytes, Ly6G+ neutrophils, F4/80+ macrophages and CD4+ T cells in their lungs (Fig. 5.3C), suggesting that IL-1R−/− mice are not defective in cell recruitment during the early stages of *B. pertussis* infection. Consistent with results from histopathology examination (Fig. 5.3A and 5.3B), more total leukocytes, neutrophils, macrophages and CD4+ T cells were recovered from IL-1R−/− mice lungs on day 7 p.i. than from the wild-type mice lungs (Fig. 5.3C). By day 14 p.i., total leukocytes, neutrophils, macrophages and CD4+ T cells numbers had declined in C57BL/6 mice but these numbers were ~20, 150, 50 and 30 fold higher, respectively, in IL-1R−/− mice. On day 21 p.i., total leukocytes, neutrophils, macrophages and CD4+ T cells numbers in the lungs of IL-1R−/− mice were ~20, 90, 80 and 60 fold higher, respectively, than those in wild-type mice (Fig. 5.3C). Together, these data indicate that IL-1R signaling deficiency results in
uncontrolled *B. pertussis*-induced inflammation, marked by increased cell recruitment and tissue architecture destruction.

**Bone-marrow derived cells from IL-1R<sup>−/−</sup> mice produce higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines in response to *B. pertussis.***

Since *B. pertussis*-infected IL-1R<sup>−/−</sup> mice harbored more bacteria by the first week p.i. (Fig. 5.2B), we hypothesized that innate immune functions might be improperly regulated in these mice. We first examined the production of pro-inflammatory cytokines, TNF-α and IL-6, and the anti-inflammatory cytokine, IL-10, by bone-marrow derived dendritic cells (BMDC) from C57BL/6 or IL-1R<sup>−/−</sup> mice after stimulation with live *B. pertussis* at a MOI of 10. Incubation in media alone resulted in little cytokine production from either type of BMDCs (Fig. 5.4A-C, white symbols). *B. pertussis*-stimulated BMDCs from C57BL/6 mice had produced ~350pg/mL TNF-α by 2h, but these levels decreased to ~250pg/mL by 12h and plateaued at this level. TNF-α production by *B. pertussis*-stimulated IL-1R<sup>−/−</sup> mice BMDCs was similar to that of wild-type cells by 2h post-stimulation, but increased thereafter and was significantly higher than that of wild-type cells at all later time points (Fig. 5.4A). IL-6 production by *B. pertussis*-stimulated wild-type and IL-1R deficient BMDCs increased to high levels by 12h and remained at similar levels thereafter (Fig. 5.4B). IL-6 production by IL-1R deficient BMDCs was somewhat higher than that of wild-type cells; with statistical significance only reached at an earlier time point (2h) (Fig. 5.4B). IL-10 production by wild-type BMDCs increased to ~16,000pg/mL by 24h post *B. pertussis* stimulation and stayed at this level by 36h post-stimulation (Fig. 5.4C). Interestingly, IL-1R deficient BMDCs produced ~50% less IL-10, compared to wild-type cells, at 12, 24 and 36h post-stimulation (Fig. 5.4C). Thus BMDCs lacking IL-1R produced higher levels of the pro-inflammatory cytokines TNF-α and IL-6 and lower levels of the anti-inflammatory cytokine IL-10 in response to *B. pertussis*, suggesting substantial contribution of IL-1R signaling to regulation of cytokine responses to *B. pertussis*.

To test if the cytokine production profile of cells from IL-1R<sup>−/−</sup> mice is specific to *B. pertussis*, bone-marrow derived macrophages (BMM) from wild-type or IL-1R<sup>−/−</sup> mice were stimulated with media alone or media containing *B. pertussis* or *E. coli* LPS, and TNF-α, IL-6 and IL-10 levels in the supernatants were measured after 12h incubation. Cells incubated with media alone produced little cytokine (Fig. 5.4D-F).
IL-1R deficiency leads to increased pro-inflammatory and decreased anti-inflammatory cytokine production by BMDCs and BMMs. (A-C) BMDCs from groups of 4 C57BL/6 (square) or IL-1R-/- (triangle) mice were stimulated with media alone (white) or media containing live *B. pertussis* at a MOI of 10 (black). (A) TNF-α, (B) IL-6 or (C) IL-10 concentrations after 0, 2, 12, 24 or 36h incubation were expressed as mean ± standard error. (D-F) BMMs from groups of 4 C57BL/6 (black bars) or IL-1R-/- (white bars) mice were incubated with media alone or media containing *B. pertussis* (Bp) or *E. coli* LPS. (D) TNF-α, (E) IL-6 or (F) IL-10 concentration after 12h incubation was expressed as mean ± standard error. * indicates P ≤ 0.05. ** indicates P ≤ 0.01.

Similar to the BMDC cytokine production profile, IL-1R deficient BMMs produced significantly more TNF-α, more IL-6 and less IL-10 than wild-type BMMs by 12h post-stimulation (Fig. 5.4D-F). *E. coli* LPS-stimulated IL-1R deficient BMMs also produced significantly more TNF-α, more IL-6 but similar levels of IL-10 compared to similarly treated wild-type BMMs (Fig. 5.4D-F). Thus the higher pro-inflammatory cytokine production by IL-1R deficient BMMs appears to be a general response to microbial stimulus, but the decreased IL-10 production may be *B. pertussis*-specific.

**IL-1R-/- mice BMMs are efficient in killing *B. pertussis***.

Since large amounts of phagocytes infiltrated into the lungs of IL-1R-/- mice but failed to control bacterial numbers, we hypothesized that bactericidal efficiencies of phagocytes from these mice might be decreased. To test this, BMMs from C57BL/6 or IL-1R-/- mice were primed with either rmIFN-γ or *B.*
Pertussis LPS, inoculated with opsonized *B. pertussis*, and the viable bacteria were enumerated after 2h or 4h incubation. Around 90% of *B. pertussis* was killed by a 2h incubation with BMMs from either C57BL/6 or IL-1R-/- mice (Fig. 5.5). Similar percent killing was observed by 4h incubation with BMMs from C57BL/6 or IL-1R-/- mice (Fig. 5.5). These data indicate that BMMs from IL-1R-/- mice are similarly efficient in killing *B. pertussis* compared to wild-type BMMs.

**IL-1R signaling is not required for efficient antibody responses against *B. pertussis***

To test if the failure of IL-1R^-/- mice to control large numbers of *B. pertussis* is associated with decreased antibody generation, *B. pertussis*-specific titers of serum antibodies from C57BL/6 or IL-1R^-/- mice collected on days 7, 14 and 21 p.i. were measured. The titers of sera collected on day 7 p.i. from both strains of mice were under the limit of detection (Fig. 5.6A). On day 14 p.i., Ig titers of sera from wild-type and IL-1R^-/- mice were comparable. On day 21 p.i. the *B. pertussis*-specific Ig titers of sera from IL-1R^-/- mice were significantly higher than that of wild-type mice. These data indicate that IL-1R signaling is not required for the generation of *B. pertussis*-specific antibodies. The higher titer of IL-1R^-/- mouse serum is likely due to higher bacterial loads later during infection. The ratio of *B. pertussis*-specific
IgG2a/IgG1 titers of serum collected on day 21 p.i., an indication of T helper (Th) 1/Th2 skewing, was higher in serum from IL-1R−/− mice than C57BL/6 mice (Fig. 5.6B), indicating that T cell responses in IL-1R−/− mice may be more polarized towards Th1 responses.

To test the efficiency of serum antibodies collected from wild-type versus IL-1R−/− mice in antibody-mediated clearance of *B. pertussis in vivo*, B cell deficient mice (µMT) were adoptively transferred naïve serum or immune serum from C57BL/6 or IL-1R−/− mice collected on day 21 p.i. and sacrificed to enumerate bacterial numbers in the lungs 14 days later. Compared to the naïve serum treated mice, C57BL/6 immune serum treatment reduced *B. pertussis* numbers from µMT mice lungs by 75% (Fig. 5.6C). IL-1R−/− mouse immune serum treated µMT mice completely cleared *B. pertussis* from their lungs (Fig. 5.6C), indicating that the higher titer and Th1-skewed immune serum from IL-1R−/− mice is more effective in antibody-mediated clearance *in vivo*.

Although antibodies from IL-1R−/− mice efficiently cleared *B. pertussis* from B cell deficient mice (Fig. 5.6C), these antibodies did not protect the IL-1R−/− mice themselves from disease (Fig. 5.2A, C, D), suggesting a defect in antibody function in the absence of IL-1R. To test this, naïve or immune serum from wild-type mice, collected on day 28 p.i., was adoptively transferred to C57BL/6 or IL-1R−/− mice and *B. pertussis* numbers in the lungs were determined on day 14 p.i. Naïve serum treatment had no effect on *B. pertussis* numbers in the lungs of either mouse strain (compare Fig. 5.6D and 5.2B). Immune serum treatment decreased *B. pertussis* numbers in both C57BL/6 and IL-1R−/− mice by more than 95% (Fig. 5.6D). However, adoptively transferred serum antibodies failed to reduce *B. pertussis* numbers below 10^7 CFU in IL-1R−/− mice, indicating that some immune functions not ameliorated by the addition of antibodies are improperly regulated in these mice.

**Higher Th1 cytokine and lower Th2 and Th17 cytokine responses in *B. pertussis*-infected IL-1R−/−s.**

Since *B. pertussis*-stimulated BMDCs from IL-1R−/− mice produced higher levels of TNF-α (Fig. 5.4A), we further tested if TNF-α levels are elevated at the site of infection in IL-1R−/− mice. TNF-α levels in the lungs of *B. pertussis*-inoculated C57BL/6 mice peaked on day 7 p.i. and declined thereafter, whereas in the lungs of IL-1R−/− mice, TNF-α levels continued to increase throughout the infectious course and were about 7 fold higher than the levels in the wild-type mice lungs on day 21 p.i. (Fig. 5.7A). Since Th1 skewing
was indicated (Fig. 5.6B), we also examined IFN-γ, IL-10 and IL-17 concentrations, as representative of Th1, Th2 and Th17 cytokines, in the lungs during *B. pertussis* infection to test if the increased inflammatory responses in IL-1R−/− mice correlates with altered T cell cytokine production. The IFN-γ concentrations were higher in the lungs of IL-1R−/− mice than in the lungs of C57BL/6 mice by day 7 p.i. and at later time points, although they followed a similar pattern, peaking on day 7 p.i. and declining thereafter (Fig. 5.7B). IL-10 levels in the lungs of both C57BL/6 and IL-1R−/− mice increased modestly, ~2 fold compared to the basal level, on day 3 p.i. and plateaued thereafter (Fig. 5.7C). A dramatic peak in IL-17 concentrations at ~2,000 pg/mL on day 7 p.i. was observed in wild-type mice lungs but not in IL-1R−/− mice (Fig. 5.7D).

Together, these data indicate that pro-inflammatory cytokine levels were higher in the lungs of *B. pertussis*-infected IL-1R−/− mice than in wild-type mice, consistent with their higher bacterial loads (Fig. 5.2B), higher inflammatory pathology (Fig. 5.3) and higher pro-inflammatory cytokine production by innate immune system cells (Fig. 5.4).

To measure systemic cytokine responses, which might not be affected as much as lung cytokine concentrations by differential bacterial loads in the lungs, splenocytes from the same groups of mice described above were stimulated *in vitro* with media or media containing heat-killed *B. pertussis*, and TNF-α, IFN-γ, IL-10 and IL-17 concentrations in the cell culture supernatants were determined. Incubation in media resulted in little production of any tested cytokine (Fig. 5.7 E-H white symbols). Consistent with the findings of lung TNF-α concentrations (Fig. 5.7A), splenic TNF-α responses from both C57BL/6 and IL-1R−/− mice
increased to over 3,000 pg/mL by day 7 p.i. (Fig. 5.7E). Interestingly, splenic TNF-α response declined thereafter in C57BL/6 mice but remained high in the IL-1R<sup>−/−</sup> mice (Fig. 5.7E). IFN-γ concentration in the culture supernatants of splenocytes from both types of mice increased to high levels by day 7 p.i. and stayed high thereafter (Fig. 5.7F). Interestingly, production of IL-10 by C57BL/6 splenocytes kept increasing throughout the time course (Fig. 5.7G square), whereas IL-1R<sup>−/−</sup> splenocytes failed to increase IL-10 production after an early peak on day 3 p.i. and had produced significantly less IL-10 by day 21 p.i. (Fig. 5.7G triangle) than wild-type splenocytes. Splenic IL-17 production by C57BL/6 mice cells was high (~2,000pg/mL) by day 3 p.i., kept increasing thereafter and reached ~10,000pg/mL on day 21 p.i., whereas IL-1R<sup>−/−</sup> mice splenocytes barely produced any IL-17 (Fig. 5.7H). Together, these data indicate that IL-1R<sup>−/−</sup> mice induce an increased TNF-α and IFN-γ response, a decreased IL-10 responses and virtually no IL-17 response.

**Intranasal administration of rmIL-17 did not reduce B. pertussis numbers in IL-1R<sup>−/−</sup> mice.**

![Figure 5.8: Intranasal administration of rmIL-17 restored the pulmonary IL-17 level but did not reduce B. pertussis numbers in IL-1R<sup>−/−</sup> mice. Groups of 4 C57BL/6 or IL-1R<sup>−/−</sup> mice were inoculated with 5×10<sup>5</sup> CFUs of B. pertussis, intranasally administrated PBS (dashed bars) or PBS containing rmIL-17 (black bars) on days 3 and 6 p.i., and euthanized on day 7 p.i.. (A) IL-17 concentration in the lungs was expressed as mean ± the standard error. (B) B. pertussis numbers in the lungs were expressed as mean of Log<sub>10</sub>CFU ± the standard error.

Since very little IL-17 response was detected in the lungs of IL-1R<sup>−/−</sup> mice or was produced by splenocytes from IL-1R<sup>−/−</sup> mice, and since IL-17 has been implicated in the control of B. pertussis colonization (3), we hypothesized that the lack of IL-17 responses in IL-1R<sup>−/−</sup> mice might partially account for the failure of these mice to control B. pertussis numbers. To test this, C57BL/6 and IL-1R<sup>−/−</sup> mice were inoculated with B. pertussis, intranasally administrated PBS or PBS containing rmIL-17 on days 3 and 6 p.i. and sacrificed on day 7 p.i.. Consistent with previous finding (Fig. 5.7D), little IL-17 was detected in the lungs of PBS-treated IL-1R<sup>−/−</sup> mice (Fig.
5.8A white bars). Although rmIL-17 treatment restored IL-17 level in the lungs of IL-1R−/− mice to that in wild-type mice (Fig. 5.8A black bars), PBS- or rmIL-17-treated IL-1R−/− mice harbored similar numbers of *B. pertussis* in their lungs (Fig. 5.8B), tracheas and nasal cavities (data not shown), and the bacterial loads were ~30-fold higher than those in C57BL/6 mice, indicating that restoration of IL-17 level in the lungs on day 7 p.i. is not sufficient to decrease *B. pertussis* burdens of IL-1R−/− mice.

**IL-1R signaling contributes to overcoming the effects of pertussis toxin.**

IL-1R is required for the control of bacterial numbers following *B. pertussis* but not *B. parapertussis* infection (Fig. 5.2A, B, C), suggesting that IL-1R may be important in the host response to some *B. pertussis*-specific virulence mechanisms. Since Ptx is not expressed by *B. parapertussis* (4, 49), we hypothesized that IL-1R might contribute to overcoming the effects of Ptx. To test if Ptx is required for *B. pertussis* to cause lethal diseases in IL-1R−/− mice, we challenged C57BL/6 or IL-1R−/− mice with 2×10⁶ CFU of *B. pertussis* or *B. pertussis* Δptx and monitored survival.

C57BL/6 showed no signs of disease following challenge with either strain and were euthanized on day 100 p.i. (Fig. 5.9). High dose wild-type *B. pertussis*-challenged IL-1R−/− mice showed signs of severe disease as early as day 5 p.i. and all of them were euthanized by day 13 p.i. to avoid suffering. Interestingly, *B. pertussis*Δptx challenged IL-1R−/− mice did not show any sign of disease by day 100 p.i. (Fig. 5.9), indicating that IL-1R signaling is only required to prevent lethal disease caused by *B. pertussis* when Ptx is expressed.

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**Figure 5.9: Pertussis toxin deficient *B. pertussis* failed to cause lethal infection in IL-1R−/− mice.** Groups of 4 C57BL/6 (square) or IL-1R−/− (triangle) mice were inoculated with 2×10⁶ CFUs of *B. pertussis* (black) or *B. pertussis*Δptx (white) and monitored for survival.
Discussion:

IL-1R, TLR or TNFR engagement of their distinct ligands results in activation of overlapping signal transduction cascades that share signaling intermediates. The Toll-like receptor /IL-1 domain recruits the myeloid differentiation primary response gene (88) (MyD88) adaptor protein upon activation, leading to further activation of signal transduction events that induce cellular responses known to regulate the innate immune responses. Elucidating the specific and/or redundant functions of these receptor mediated signaling pathways is critical to understanding the innate immune response. TLR4 deficient mice are colonized to higher numbers after the first week of \textit{B. pertussis} infection, and have higher pathology and cellular infiltration than wild-type mice (34). TNF-α deficient mice also have increased \textit{B. pertussis} loads and elevated inflammation in their lungs (79). We showed here that mice lacking IL-1R are more susceptible to \textit{B. pertussis} infection, likely due to uncontrolled inflammation and atypical disseminated diseases. Thus IL-1 is not a dispensable cytokine downstream of TLR4, but rather its signaling pathway is an essential component of immune response to \textit{B. pertussis} infection. Blocking IL-1 secretion or IL-1R mediated signaling has been a proposed treatment of chronic inflammatory diseases, such as rheumatoid arthritis and Crohn’s disease (7, 26). Some respiratory infections have been identified in patients using drugs blocking IL-1R signaling (23). This, together with our data, indicates that therapeutics strategies involving IL-1 blockage may lead to increased susceptibility of \textit{B. pertussis} infection.

IL-1R signaling has been shown to play detrimental or beneficial roles during inflammatory responses on mucosal surfaces (19). In the digestive tract, for example, blockage of components of IL-1R signaling during \textit{Y. enterocolitica} or \textit{S. flexneri} infection controls inflammation (21, 66, 67), whereas IL-1 signaling deficiency leads to increased inflammation caused by \textit{C. rodentium} (41). Similar to the digestive tract, the respiratory tract has a large mucosal area where mounting the proper inflammatory response is critical. However, in comparison, which innate immune signaling pathways are important for inducing and resolving inflammatory responses during respiratory pathogen invasions are less defined. Here we showed that during infection by a respiratory pathogen, \textit{B. pertussis}, IL-1R\(^{-/-}\) mice lungs had extensive infiltration of neutrophils and lymphocytes (Fig. 5.3). In contrast, the wild-type mice showed only mild lymphoid peribronchiolar cuffing. The increased inflammation and lung pathology were associated with decreased anti-inflammatory
cytokine and increased pro-inflammatory cytokine responses by BMDCs and BMMs from IL-1R−/− mice (Fig. 5.4), and both at the site of infection and systemically during *B. pertussis* infection (Fig. 5.7). Thus, consistent with the observation during *C. rodentium* infection, we defined a beneficial role of IL-1R signaling in controlling inflammatory responses by *B. pertussis*.

The contributions of IL-1R signaling pathway to the control of *B. pertussis* infection can be multifold, including direct and/or indirect effects. BMMs lacking IL-1R are as effective as wild-type cells in killing *B. pertussis* in vitro (Fig. 5.5), suggesting that IL-1R−/− mice might not be defective in direct bactericidal efficiency. Instead, the effects of losing IL-1R signaling might be indirect. IL-1R−/− mice showed extensive lung pathology with elevated cellular infiltration (Fig. 5.3), as well as increased pro-inflammatory cytokine production by dendritic cells/macrophages (Fig. 5.4), in the lungs and systemically during *B. pertussis* infection (Fig. 5.7). In addition, antibody (Fig. 5.6A, C) and *B. pertussis*-specific IFN-γ responses (Fig. 5.6B) were increased in these mice, which may reflect a higher bacterial burden. However, since IFN-γ and antibody responses are normally suppressed at the acute stage of *B. pertussis* infection (38, 52, 57), the high bacterial load might not be the only reason for the elevated responses. Alternatively, the enhanced adaptive immune responses in IL-1R−/− mice may reflect the relative lack of regulatory cytokine production. Compared to the cells from wild-type mice, BMDCs and BMMs from IL-1R−/− mice decreased the production of IL-10 (Fig. 5.4C, F), a prototypic regulatory cytokine. Moreover, these mice failed to increase Ag-specific systemic IL-10 responses in the later stages of infection (Fig. 5.7G). The involvement of IL-1R in inducing *B. pertussis*-specific IL-10 response is in line with the finding that TLR4-mediated innate IL-10 response inhibits Th1 responses and inflammatory pathology in the lungs during *B. pertussis* infection (34), since IL-1R and TLR4 share downstream signaling events. IL-10 has been shown to dampen inflammatory cytokine responses to *B. pertussis* (53). Dirix et al. recently reported that peripheral blood mononuclear cell-derived IL-10 depresses *B. pertussis*-specific IFN-γ production in vaccinated infants, further supporting the role of IL-10 in controlling *B. pertussis*-specific inflammatory cytokine responses (20). These data suggest that following *B. pertussis* infection IL-1R contributes to the induction of IL-10, which is important for controlling inflammatory responses.
Our data indicated that IL-1R signaling is required for the IL-17 responses during *B. pertussis* infection (Fig. 5.7D, H). IL-17 producing Th17 cells are distinct from Th1 or Th2 cells and have been shown to play pathogenic roles in autoimmune diseases (28, 40, 42, 61). Additionally, this cytokine has been shown to contribute to host defenses against bacterial infections, including *Klebsiella pneumonia* (27), *Bacteroides fragilis* (13), *Streptococcus pneumonia* (81) and *M. tuberculosis* (37). IL-17 has been shown to promote macrophage killing of *B. pertussis* (33). Depletion of IL-17 reduces the efficacy of a *B. pertussis* whole cell vaccine (33), and affects *B. pertussis* numbers in murine lungs (3). These data suggest a role for IL-17 in immune responses to *B. pertussis*.

IL-23 (28, 61), transforming growth factor (TGF)-β and IL-6 (5, 45) have been shown to influence the production of IL-17. Besides IL-6, pro-inflammatory cytokines, such as TNF-α and IL-1β, can increase the efficiency of Th17 differentiation in mice (12). IL-1β has been found to be essential for the differentiation of IL-17 producing human Th cells (1). Mouse models of arthritis and encephalomyelitis have also revealed the involvement of IL-1 in the generation of IL-17 producing cells (59, 74). The role of IL-1R signaling in induction of local and systemic IL-17 production during bacterial infection has not been previously described. We showed here a severe lack of IL-17 induction in *B. pertussis*-challenged IL-1R−/− mice, which might be attributable to the role of IL-1 in the generation of IL-17 producing cells. Alternatively, since Th17 cells are negatively regulated by IFN-γ (15, 28, 61), the high IFN-γ responses observed in *B. pertussis*-challenged IL-1R−/− mice (Fig. 5.7B, F) might have inhibitory effect on IL-17 production in these mice. Although we have established that IL-1R signaling is required for the generation of IL-17 responses during microbial infection, restoring the pulmonary IL-17 peak did not decrease *B. pertussis* burdens in the lung (Fig. 5.8). This could be due to the limitations of our delivery method or could indicate that the defect of IL-1R−/− mice in controlling *B. pertussis* infection is not simply their failure to induce IL-17.

IL-1R signaling deficiency does not lead to lethal infection by *B. parapertussis* or *B. pertussisΔptx* (Fig. 5.2A, 5.2B, 5.9), both lacking Ptx expression. This highlights an interesting interaction between IL-1R signaling and a specific bacterial virulence factor, Ptx. Nasso, M. et al. recently showed that human Ptx-treated dendritic cells promote Th1 cytokine secretion by T cells, which are down-regulated by IL-10 (60). Although whether IL-1R is involved in the production of IL-10 by Ptx-treated dendritic cells has not been
directed tested, mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), required for this effect (60), are important signaling components downstream of or interacting with IL-1R (17). Moreover, our data showed that *B. pertussis*-challenged IL-1R-/- mice BMDCs and splenocytes produce lower IL-10 (Fig. 5.4, 5.7). Murine macrophages produce lower levels of IL-1β following stimulation with *B. pertussis*ΔPtx than with wild-type *B. pertussis* (data not shown), indicating that Ptx contributes to the induction of IL-1β. It is likely that Ptx induces IL-1β, which activates IL-1R mediated signaling via MAPK and PI3K, leading to the production of IL-10 and thus the control of inflammatory responses. In the absence of IL-1R, lower levels of IL-10 are produced, resulting in overwhelming inflammation, tissue damage, uncontrolled bacterial growth and ultimately lethality of the animal. The *B. pertussis* strain lacking Ptx induces lower inflammatory responses (3, 60), thus eliminating the requirement for IL-1R-mediated regulation.
Authors and Contributions:

Xuqing Zhang\textsuperscript{1,2}, Sara E. Hester\textsuperscript{1,3,4}, Mary J. Kennett\textsuperscript{1}, and Eric T. Harvill\textsuperscript{1}

\textsuperscript{1}Department of Veterinary and Biomedical Sciences, the Pennsylvania State University, 115 Henning Building, University Park, Pennsylvania 16802

\textsuperscript{2}Graduate Program in Genetics, the Pennsylvania State University, University Park

\textsuperscript{3}Department of Biochemistry, Microbiology, and Molecular Biology, The Pennsylvania State University, University Park

\textsuperscript{4}Graduate Program in Biochemistry, Microbiology and Molecular Biology, The Pennsylvania State University, University Park

Conceived and designed the experiments: XZ, ETH.

Performed the experiments: XZ, SEH, MJK.

Analyzed the data: XZ.

Wrote the paper: XZ, ETH.
References:


Chapter 6

Decreased Leukocyte Accumulation and Delayed *Bordetella pertussis* Clearance in IL-6−/− Mice.

Abstract

IL-6, a pleiotropic cytokine primarily produced by the innate immune system, has been implicated in the development of acquired immune responses, though its roles are largely undefined and may vary in the context of different diseases. Using a murine model of infection, we established that IL-6 influences the adaptive immune responses against the endemic human respiratory pathogen, *Bordetella pertussis*. IL-6 was induced in the lungs of C57BL/6 mice by *B. pertussis*. IL-6−/− mice showed a protracted infectious course and were less efficiently protected by *B. pertussis* vaccination than wild-type mice. Antibodies from IL-6−/− mice, though lower in titer, efficiently reduced *B. pertussis* numbers in IL-6 sufficient mice. IL-6−/− mice treated with antibodies from wild-type mice harbored significantly more *B. pertussis* than similarly treated wild-type mice, suggesting additional defects not ameliorated by supplementing antibodies. Pulmonary leukocyte recruitment and splenic T cell cytokine responses to *B. pertussis*, including T\(_{\text{H}1}\), T\(_{\text{H}2}\) and T\(_{\text{H}17}\) cytokine production, were lower in IL-6−/− mice than in wild-type mice. Together, these results reveal the dysregulation of multiple aspects of adaptive immune responses in *B. pertussis*-infected IL-6−/− mice and suggest that IL-6 is involved in regulating antibody generation, pulmonary leukocyte accumulation and T cell cytokine production in response to *B. pertussis* as well as the generation of effective vaccine-induced immunity against this pathogen.
Introduction

IL-6, though primarily produced as a part of the innate immune response, has recently been recognized as a crucial regulator of the generation of adaptive immune responses (23). For example, IL-6 plays a critical role in the differentiation of B cells and promotes the proliferation of plasmablasts during their final stages of maturation into immunoglobulin producing plasma cells (1, 34, 57, 61). Additionally, by binding to its soluble receptor, IL-6 can promote the production of some chemokines, such as MCP-1 and IL-8, by endothelial cells, which increase expression of adhesion molecules and contribute to the recruitment of leukocytes to the site of inflammation (51). IL-6 is also a regulator of T cell proliferation, differentiation and survival (13, 23, 46). Recent reports have implicated this cytokine in the differentiation of naïve T cells to T\(_{H}17\) cells, a lineage shown to be involved in the development of autoimmune diseases and host defenses against invading pathogens (5, 18, 47).

The role of IL-6 in regulating protective immune responses has been revealed by challenging IL-6 deficient (IL-6\(^{-/-}\)) mice with various pathogens. For instance, IL-6\(^{-/-}\) mice fail to induce a protective T\(_{H}1\) response against the fungal pathogen, *Candida albicans* (50). These mice also have a decreased T\(_{H}1\) response and an increased mortality rate following infection with the intracellular pathogen *Chlamydia trachomatis* (65). Additionally, IL-6\(^{-/-}\) mice have decreased T\(_{H}2\) cytokine responses, decreased IgG2b production, and increased lyme arthritis incidence in response to *Borrelia burgdorferi* infection (3).

Several lines of evidence support the importance of IL-6 in host defenses against certain respiratory pathogens. For example, *Mycobacterium tuberculosis* induces decreased IFN-\(\gamma\) responses and a lethal infection in IL-6\(^{-/-}\) mice (30). These mice also show elevated pulmonary pro-inflammatory cytokine levels and an increased susceptibility to *Streptococcus pneumoniae* (60). Furthermore, IL-6 has been shown to protect against *Klebsiella pneumoniae* infection by augmenting neutrophil-mediated killing of bacteria (59). Notably, IL-6\(^{-/-}\) mice are not diseased by the normal flora of the respiratory tract, suggesting that IL-6-mediated immune responses are important for host defenses against specific virulence mechanisms of certain pathogens.

The complex interactions between various adaptive immune factors following *Bordetella pertussis* infection make the murine model of *B. pertussis* infection suitable to further explore how IL-6 impacts the
adaptive immune responses in the respiratory tract during infection. B. pertussis is one of the etiologic agents of whooping cough (41), an acute and severe respiratory disease, causing approximately 50 million cases and 300,000 deaths worldwide annually (9). The incidence of whooping cough is on the rise in regions of high vaccine coverage in developed countries (16, 21, 52, 62). Notably, a large portion of whooping cough infections are thought to remain unreported (12). B. pertussis produces various toxins and adhesins, such as pertussis toxin, adenylate cyclase toxin, filamentous hemagglutinin, fimbriae, pertactin and lipopolysaccharide, many of which are known to contribute to pathogenesis and immune subversion (41).

Unlike many other bacterial and viral diseases in which antibodies against a single surface antigen or toxin mediate protection, immunity against B. pertussis is much more complex, in that no single arm of the immune response alone can confer effective protection (43). Both antibodies and T cells are required to clear the infection, but neither alone is sufficient (28, 38, 44, 67). Although previous clinical and experimental studies have established the roles of various host immune factors, such as B cells, antibodies, neutrophils, CD4+ T cells, TNF-α and IFN-γ, in immunity against B. pertussis (27, 32, 36, 37, 41-43, 45, 68), many other contributing host factors to immunity against this bacterium are still being identified (X. Zhang, S.E. Hester, M.J. Kennett, E.T. Harvill, submitted for publication; A.T. Karanikas, E.T. Harvill, unpublished observations). Since IL-6 is crucial for host defenses against various respiratory pathogens (8, 25, 29, 60) and is induced by B. pertussis LPS in vitro (40), we sought to determine the role of this cytokine in the immune response against B. pertussis. The complexity of immune responses against B. pertussis provides us the opportunity to dissect the involvement of IL-6 in regulating various arms of the immune responses.

IL-6−/− mice were delayed in their clearance of B. pertussis and were less efficient in generating vaccine-induced immunity against B. pertussis compared to wild-type mice. Compared to serum antibodies from wild-type mice, antibodies from B. pertussis-infected IL-6−/− mice had decreased B. pertussis-specific titer, despite higher bacterial numbers in these mice. Both sera efficiently reduced bacterial numbers in IL-6 sufficient mice. Additionally, wild-type-serum-treated IL-6−/− mice still harbored more bacteria than similarly treated wild-type mice, suggesting that the inefficient clearance of B. pertussis in IL-6−/− mice is not due solely to decreased antibody generation. IL-6−/− mice had decreased leukocyte accumulation in the lungs during the later stages of B. pertussis infection and lower levels of splenic T_{H1}, T_{H2} and T_{H17} cytokine production as
compared to wild-type mice. Overall, this study establishes roles for IL-6 in antibody generation, leukocyte recruitment and T cell cytokine production in response to *B. pertussis* infection.
**Material and Methods**

**Bacterial strains and growth.** The *B. pertussis* strain 536, a streptomycin resistant derivative of Tohama I, has been previously described (56). Bacteria were maintained on Bordet-Gengou agar (Difco) supplemented with 10% defibrinated sheep blood (Hema Resources) and 20 µg/mL streptomycin (Sigma-Aldrich). Liquid cultures were grown overnight in Stainer-Scholte broth at 37°C to mid-log phase (54).

**Stimulation of macrophage cell line.** The murine macrophage cell line, RAW 264.7, was obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (HyClone) supplemented with 10% fetal bovine serum (FBS) (HyClone). Approximately 10⁵ cells were placed in each well of 96-well plates (Falcon) and stimulated with live *B. pertussis* at multiplicities of infection (MOI) of 1 or 10. Cell culture supernatants were collected at the indicated time points and IL-6 concentration was measured via Enzyme-linked immunosorbent assays (ELISA) as per the manufacturers’ instructions (R&D Systems).

**Animal experiments.** C57BL/6J, B6.129S6-Il6tm1Kopf/J (IL-6−/−) and B6.129S2-Igh-6tm1Cgn/J (μMT) mice were obtained from Jackson Laboratories (Bar Harbor) and bred in *Bordetella*-free, specific pathogen-free breeding rooms at The Pennsylvania State University. For inoculation, 4-6 week-old mice were lightly sedated with 5% Isoflurane (Abbott Laboratories) in oxygen and inoculated by pipetting 50 µL phosphate-buffered saline (PBS) containing 5×10⁵ CFU of bacteria onto the external nares (28). This method reliably distributes bacteria throughout the respiratory tract (19). For vaccination, mice were i.p. injected with 10⁸ CFU of heat-inactivated bacteria in 200 µL PBS on days 14 and 28 prior to challenge (70). For adoptive transfer of serum antibodies, sera were collected on day 28 post *B. pertussis* inoculation or from naïve animals, and 200 µL of pooled serum was i.p. injected before the bacterial inoculation (28). Bacterial numbers in the lungs were quantified on day 14 post-inoculation since pertussis toxin delays the antibody-mediated clearance of *B. pertussis* until T cell responses are generated (27) (D.N. Wolfe, E.T. Harvill, unpublished observations). For quantification of bacteria numbers, mice were sacrificed via CO₂ inhalation and lungs, tracheas, and nasal cavities were excised. Tissues were homogenized in 1 mL PBS, serially diluted and plated onto Bordet-Gengou agar plates with 20 µg/mL streptomycin, and colonies were counted after incubation at 37°C for 4-5 days (28). The lower limit of detection was 10 CFU. All protocols were reviewed and approved by The
Pennsylvania State University Institutional Animal Care and Use Committee (IACUC) and all animals were handled in accordance with institutional guidelines.

**Splenocyte re-stimulation.** Spleens were excised on the indicated days post-inoculation from groups of 3-4 *B. pertussis*-inoculated C57BL/6 and IL-6−/− mice. Splenocytes were isolated as previously described (48, 66). In brief, spleens were homogenized and red blood cells were lysed with 0.84% ammonium chloride treatment. 2×10⁶ cells were re-suspended in 200 µL DMEM supplemented with 10% FBS, and 100 µg/mL penicillin and streptomycin (HyClone) and placed into each well of 96-well tissue culture plates. Splenocytes were stimulated with 10 µL media alone or media containing 10⁷ CFU (MOI of 5) of heat-inactivated *B. pertussis* (48, 66). After 3 days, the supernatants were collected and analyzed for TNF-α, IL-2, IL-4, IL-5, IL-12, GM-CSF, IL-10 and IFN-γ concentrations by Bio-plex™ T H1/ TH2 Panel (Bio-Rad). IL-17 concentrations were quantified via ELISA (R&D Systems) as per the manufacturers’ instructions.

**Titer ELISAs.** Antibody titers were determined as previously described (39). Briefly, heat-inactivated exponential–phase *B. pertussis* diluted to 5×10⁷ CFU/mL in a 1:1 mix of 0.2 M sodium carbonate and 0.2 M sodium bicarbonate buffers was used to coat 96-well plates (Greiner Bio-one) by incubating for 2 h in a humidified chamber at 37°C. The plates were washed and blocked, and a 1:50 dilution of serum samples collected from individual C57BL/6 or IL-6−/− mouse on days 28, 49, 77 or 108 post-inoculation were added to the first well and serially diluted 1:2 across the plates. Plates were incubated for 2 h at 37°C, washed and probed with 1:4,000 dilution of goat anti-mouse Ig, IgG1, IgG2a or IgG2b horseradish peroxidase (HRP)-conjugated antibodies (Southern Biotechnology Associates and Pharmingen) for 1 h and visualized with 2,2’-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt in phosphate-citrate buffer with hydrogen peroxide at an absorbance of 405 nm. Titers were determined via the end-point method using a cut off that is 0.1 higher than the optical density of similarly treated wells probed with naïve serum (39).

**Quantification of leukocytes and cytokine in the lungs.** To quantify leukocytes, lungs were perfused with sterile PBS, excised, and placed in 4 mL of RPMI 1640 medium (HyClone). Lungs were homogenized and laid over Histopaque 1119 (Sigma Aldrich) and centrifuged for 30 min at 700 g at room temperature. The leukocyte layer was collected, spun down at 300 g for 5 min and re-suspended in PBS supplemented with 2% FBS. The total number of cells was determined by counting at 40× magnification on a hemocytometer.
Aliquots of cells were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences Pharmingen) and stained with FITC-labeled anti-mouse-CD4 antibodies, or FITC-labeled anti-mouse-Ly6G (BD Biosciences Pharmingen). The percentages of CD4⁺ and Ly6G⁺ cells were determined by flow cytometry. Percentages were multiplied by the total number of leukocytes to calculate the total number of CD4⁺ or Ly6G⁺ cells, respectively. To measure the cytokine concentrations in the lungs, lungs were homogenized in 1 mL PBS, tissues were spun down at 8,000 g for 10 min at 4°C, and IL-6 concentrations were determined via cytokine ELISAs in accordance with the suppliers’ protocols (R&D Systems).

**Statistical analysis.** The means ± standard error (error bars) were determined for all appropriate data. Two-tailed, unpaired Student’s T-tests were used to determine statistical significance between groups. Results were also analyzed by ANOVA and Tukey simultaneous test in Minitab with similar significance.
Results

IL-6 is induced in response to *B. pertussis* in vitro and in vivo.

As IL-6 has been shown to be elicited in response to various respiratory pathogens and by *B. pertussis* LPS (8, 25, 29, 40, 60), we examined whether macrophages produce IL-6 in response to live *B. pertussis* in vitro. Murine macrophages were incubated with media alone or media containing *B. pertussis* and IL-6 concentrations in the supernatants were measured via ELISA at various time points thereafter. Macrophages incubated with media alone produced little IL-6 (less than 5 pg/mL) by 24h. However, macrophages incubated with *B. pertussis* at a MOI of 1 produced increasing levels of IL-6 over time and the concentration of IL-6 in the culture supernatant reached 2,000 pg/mL by 24 h. Furthermore, in culture supernatants of macrophages incubated with *B. pertussis* at a MOI of 10, the IL-6 concentration reached 1,700 pg/mL by 6 h and plateaued at ~3,500 pg/mL by 8 h (Fig. 6.1A).

To determine if *B. pertussis* induces IL-6 production in vivo, C57BL/6 mice were inoculated with 5×10^5 CFU *B. pertussis* and their lungs were excised on days 0, 3, 7, 14, 28, 49, 77, and 108 post-inoculation to measure the concentrations of IL-6 in the lungs. IL-6 levels increased from levels in naïve mice (160 pg/mL) by day 3 (~1,100 pg/mL) and peaked on day 7 (~2,000 pg/mL) post *B. pertussis* inoculation. IL-6 levels thereafter were not significantly different between infected and naïve mice (Fig. 6.1B). Combined, these data suggest that *B. pertussis* induces IL-6 production by murine macrophages in vitro and in mouse lungs during the first week of infection.
IL-6 contributes to the clearance of *B. pertussis*.

To determine whether IL-6 is required for the control of *B. pertussis in vivo*, C57BL/6 and IL-6−/− mice were inoculated with *B. pertussis* and sacrificed on days 0, 3, 7, 14, 28, 49, 77 and 108 post-inoculation for bacterial enumeration throughout the respiratory tract.

Consistent with previous findings, *B. pertussis* loads throughout the respiratory tract of C57BL/6 mice peaked on day 3 post-inoculation and declined thereafter. The bacteria were cleared from nasal cavities and tracheas by day 28 post-inoculation and from lungs by day 49 post-inoculation (Fig. 6.2) (28).

Similar numbers of *B. pertussis* were recovered from the nasal cavities of IL-6−/− and C57BL/6 mice until day 14 post-inoculation, but the IL-6 deficient mice had elevated burdens thereafter, with clearance observed about 80 days later than that in C57BL/6 mice (Fig. 6.2). Clearance of *B. pertussis* from the tracheas of IL-6−/− mice was also delayed relative to that of C57BL/6 mice. In the lungs of IL-6−/− mice, *B. pertussis* loads were similar to those in C57BL/6 mice until day 7 post-inoculation, but were about 40-fold and 13,000-fold higher on days 14 and 28 post-inoculation, respectively. Approximately 1,700 and 80 CFU *B. pertussis* were recovered from the lungs of IL-6−/− mice on days 49 and 77 post-inoculation, respectively, when no detectable *B. pertussis* was recovered from the lungs of wild-type mice. Notably, IL-6−/− mice did not clear *B. pertussis* until day 108 post-inoculation, which was a delay of approximately 9 weeks as compared to C57BL/6 mice (Fig. 6.2). Overall, these data indicate that IL-6 is critical to the efficient clearance of *B. pertussis* from the respiratory tract of mice.

**IL-6 is required for the generation of efficient vaccine-induced immunity against *B. pertussis***.
Since IL-6−/− mice are defective in controlling B. pertussis numbers 2 weeks or more post-inoculation (Fig. 6.2), we hypothesized that IL-6 contributes to anamnestic immunity against B. pertussis. To deliver equivalent amounts of antigens, C57BL/6 and IL-6−/− mice were vaccinated with heat-killed B. pertussis. Naïve or vaccinated mice were challenged with B. pertussis and sacrificed 3 days later for bacterial number quantification. B. pertussis vaccination did not have significant effect on B. pertussis colonization in the nasal cavities of C57BL/6 mice, but completely cleared B. pertussis from the tracheas of these mice (Fig. 6.3). Naïve C57BL/6 mice harbored > 800-fold more B. pertussis in their lungs as compared to vaccinated C57BL/6 mice, indicating that vaccination protects the lungs of these mice. In IL-6−/− mice, B. pertussis vaccination had no effect in bacterial numbers in the nasal cavities but was able to reduce B. pertussis numbers in the tracheas and lungs relative to naïve IL-6−/− mice. Notably, vaccinated IL-6−/− mice harbored significantly more B. pertussis in their lungs compared to vaccinated wild-type mice (Fig. 6.3), indicating that IL-6 contributes to the generation of efficient vaccine-induced immunity against B. pertussis in the lungs.

Despite lower titers, serum antibodies from IL-6−/− mice are sufficient for antibody-mediated clearance of B. pertussis.
To examine the role of IL-6 in the generation of an efficient antibody response, we compared *B. pertussis*-specific serum antibody titers on days 28, 49, 77 and 108 post *B. pertussis* inoculation in wild-type and IL-6−/− mice via ELISA. *B. pertussis*-specific total Ig titers of serum antibodies collected from C57BL/6 mice were high (titer>5,000) by day 28 post-inoculation and stayed at high levels on days 49, 77 and 108 post-inoculation (Fig. 6.4). In comparison, *B. pertussis*-specific Ig titers of serum antibodies from IL-6−/− mice were below 1,000 on days 28, 49 and 108 post-inoculation and were ~2,500 on day 77 post-inoculation (Fig. 6.4). IgG2a titers of serum antibodies from both C57BL/6 and IL-6−/− mice were low or below the limit of detection at all the time points tested (data not shown). IgG1 titers of serum antibodies from C57BL/6 and IL-6−/− mice were comparable. IgG2b titers of serum antibodies from C57BL/6 mice were >3,000 by day 28 post-inoculation, and increased to >6,000 by day 108 post-inoculation. In contrast, *B. pertussis*-specific IgG2b titers of serum antibodies from IL-6−/− mice were below the limit of detection on day 28 post-inoculation and were approximately 1/10 those from C57BL/6 mice on days 49 and 108 post-
inoculation (Fig. 6.4). Although IL-6−/− mice had higher and more sustained numbers of *B. pertussis* (Fig. 6.2), they generated much lower antibody titers, indicating that IL-6 contributes to the generation of *B. pertussis*-specific IgG2b antibodies.

To examine whether the lower serum antibody titers in IL-6−/− mice (Fig. 6.4) could contribute to the defect in efficient *B. pertussis* clearance, we adoptively transferred naïve serum from C57BL/6 mice or immune serum from *B. pertussis*-inoculated C57BL/6 or IL-6−/− mice into µMT (B cell deficient) mice right before *B. pertussis* inoculation, and bacterial numbers in the lungs were determined 14 days post-inoculation. µMT mice treated with immune serum from C57BL/6 mice reduced *B. pertussis* numbers in the lungs by >99% relative to those given naïve serum (Fig. 6.5). Treatment with immune serum generated in IL-6−/− mice resulted in similar reduction of bacterial numbers in the lungs (Fig. 6.5), indicating that despite having lower antibody titers, antibodies from IL-6−/− mice are sufficient to efficiently reduce *B. pertussis* numbers in the lungs.

**Supplementing antibodies is not sufficient to abrogate higher *B. pertussis* numbers in IL-6−/− mice.**

![Figure 6.6: Immune functions not corrected by supplementing antibodies are improperly regulated in IL-6−/− mice.](image)

To test whether antibody functions are impaired in the absence of IL-6, naïve or immune serum from C57BL/6 mice was adoptively transferred into groups of C57BL/6 or IL-6−/− mice right before *B. pertussis* inoculation. These mice, along with control mice not treated with any serum, were sacrificed 14 or 21 days post-inoculation to enumerate bacterial numbers in the lungs. Compared to the control mice, naïve serum treatment did not have any effect on *B. pertussis* numbers in the lungs of either type of mouse (Fig. 6.6). Consistent with previous experiments (Fig. 6.2), more *B. pertussis* was recovered from the lungs of control or naïve serum-treated IL-6−/− mice than similarly treated C57BL/6 mice on days 14 and 21.
post-inoculation (Fig. 6.6). On both time points, immune serum treatment reduced *B. pertussis* numbers in the lungs of both C57BL/6 and IL-6−/− mice by more than 98% as compared to control mice (Fig. 6.6). IL-6 was undetectable in the immune serum from wild-type mice used for adoptive transfer into IL-6−/− mice (data not shown), suggesting that the antibody-mediated clearance in IL-6−/− mice was not due to transferred IL-6 in the serum. In IL-6−/− mice, adoptively-transferred antibodies reduced *B. pertussis* numbers proportionately, but the overall bacterial loads were much higher than in C57BL/6 mice, indicating that some immune functions not ameliorated by the addition of antibodies are improperly regulated in these mice.

**IL-6 contributes to leukocyte recruitment during *B. pertussis* infection.**

![Figure 6.7: IL-6 contributes to the recruitment of leukocytes into *B. pertussis*-infected lungs.](image)

Cellular responses mediated by CD4+ T cells, but not CD8+ T cells, are critical for protective immunity against *B. pertussis* (32, 44). Neutrophils infiltrate into the site of infection (26, 42) and can take up and kill *B. pertussis* (33, 49, 55, 63, 64). Thus, we hypothesized that IL-6−/− mice are defective in recruiting these leukocytes to the site of infection. To test this, groups of C57BL/6 and IL-6−/− mice were inoculated with *B. pertussis* and total leukocytes, CD4+ T cells and Ly6G+ neutrophils in the lungs were quantified. On day 28 post-inoculation, ~9×10^5 leukocytes were recovered from lungs of wild-type mice, most of which were CD4+ T cells (Fig. 6.7). Interestingly, despite harboring about 100-fold more *B. pertussis* in their lungs at this time point (Fig. 6.2), the lungs of IL-6−/− mice contained about 13%, 4%, and 23% as many total leukocytes, CD4+ T cells, and neutrophils, respectively, compared to wild-type mice. The mean number of leukocytes recruited into lungs of IL-6−/− mice was lower than that of wild-type mice on days 3, 7, 14 and 49, despite much higher bacterial numbers at the later time points, though these differences did not reach statistical significance (data not shown). These data suggest that IL-6 is required for efficient recruitment of leukocytes to the lungs during *B. pertussis* infection.

**Dampened systemic cytokine responses in *B. pertussis*-infected IL-6−/− mice.**
To examine whether IL-6 contributes to the generation of *B. pertussis*-specific T cell responses, splenocytes were collected from C57BL/6 and IL-6−/− mice on various days post-*B. pertussis*-inoculation and cultured with media or media containing heat-killed *B. pertussis*. Incubation in media alone resulted in little cytokine production on all time points tested (data not shown). Stimulation with heat-killed *B. pertussis* induced similar levels of TNF-α, IL-2, IL-4, IL-5 and IL-12 production by splenocytes from both C57BL/6 and IL-6−/− (data not shown). Compared to the *B. pertussis*-stimulated wild-type splenocytes, splenocytes from IL-6−/− mice produced significantly less IL-10 on day 14 and less IFN-γ and GM-CSF on day 28 post-inoculation (Fig. 6.8A). IL-17 production by IL-6−/− mice splenocytes was significantly lower on days 3, 28 and 49 post-inoculation (Fig. 6.8B). The affected cytokines, IFN-γ, IL-10, GM-CSF, IL-17, are representatives of Th1, Th2, Th1/Th2, and Th17 cytokine respectively, indicating a generally dampened T cell response against *B. pertussis* infection in IL-6−/− mice despite higher bacterial loads in these mice.

Figure 6.8: Splenic cytokine production is dampened in *B. pertussis*-inoculated IL-6−/− mice. Splenocytes from groups of 3-4 C57BL/6 (black) and IL-6−/− (white) mice sacrificed on the indicated days post-*B. pertussis*-inoculation were exposed to heat-killed *B. pertussis* for 3 days. The (A) IFN-γ, IL-10, GM-CSF, or (B) IL-17 concentrations in the culture supernatant are expressed as mean ± standard error. *: p ≤ 0.05. **: p ≤ 0.01. Tania Goel performed experiments shown in Figure 6.8A.
Discussion

Cytokine regulation has been proposed as pivotal to the immunological switch from innate to adaptive immunity (22). Since its discovery as a factor promoting antibody production, more and more evidence has implicated IL-6 as a regulator of adaptive immune responses, with the exact mechanisms undefined (13). One approach to understand how exactly IL-6 impacts different arms of the adaptive immune responses is to examine the role of this cytokine in the generation of efficient immune responses against various pathogens. Immunity against the human respiratory pathogen, *B. pertussis*, requires several adaptive immune factors, which makes *B. pertussis* infection a suitable model to further explore the regulation of adaptive immunity by IL-6. We identified a role for IL-6 in the later stages of *B. pertussis* infection (after 7 days) (Fig. 6.2) as well as in generating efficient vaccine-induced immunity (Fig. 6.3), suggesting that IL-6 is critical to the generation of effective adaptive immunity against this pathogen.

*B. pertussis*-specific antibodies are detectable around two week post-inoculation (X. Zhang, S.E. Hester, M.J. Kennett, E.T. Harvill, submitted for publication) and are required for the control of *B. pertussis* numbers during later stages of infection (28, 38). Increased bacterial loads in IL-6−/− mice by two weeks post-inoculation (Fig. 6.2) led us to hypothesize a decreased antibody response in these mice. Indeed, IL-6 showed decreased *B. pertussis*-specific antibody generation (Fig. 6.4). Adoptively transferred immune sera from IL-6−/− mice were efficient in clearing *B. pertussis* in B-cell deficient mice (Fig. 6.5), suggesting that these antibodies are sufficient for antibody-mediated clearance and that some other aspects of adaptive immunity might be affected in IL-6−/− mice. Further supporting this, immune sera from wild-type mice supplied in addition to endogenous antibodies generated in response to *B. pertussis* infection failed to correct the increased bacterial burdens in the IL-6−/− mice. Together, these results suggest that the inefficient control of *B. pertussis* numbers in IL-6−/− mice is not due solely to decreased antibody generation.

Leukocyte accumulation in the lungs and systemic T cell cytokine responses were decreased in IL-6−/− mice following *B. pertussis* infection. It is known that cellular immunity mediated by CD4+ T cells is crucial to protective immunity against *B. pertussis* (32, 44). Furthermore, in vitro assays have indicated efficient killing of phagocytosed *B. pertussis* by human neutrophils (33). These previously published observations led us to test whether leukocyte accumulation in the lungs of *B. pertussis*-infected IL-6−/− mice was decreased.
We observed decreased neutrophil and CD4+ T cell accumulation in the lungs four weeks post-inoculation (Fig. 6.7), when the bacterial load was significantly higher in IL-6−/− mice than in wild-type mice (Fig. 6.2). Not only was the recruitment of T cells decreased, but we also observed a decreased splenic cytokine response (GM-CSF, IFN-γ, IL-10 and IL-17 production) in these mice (Fig. 6.8), indicating an overall dampening of T cell responses. It has been previously shown that neutrophils and CD4+ T cells are required for clearance of *B. pertussis* (4, 27, 32). Thus the decreased leukocyte recruitment may contribute to the higher bacterial numbers observed in *B. pertussis*-infected IL-6−/− mice. The observed decrease in CD4+ T cell accumulation, IFN-γ production and neutrophil recruitment in *B. pertussis*-infected IL-6−/− mice could be due to a direct impact of IL-6 deficiency on each of these responses. Alternatively, since there is a CD4+ T cell-dependent increase in IFN-γ levels during *B. pertussis* infection (4) and IFN-γ is known to contributes to neutrophil recruitment during microbial infections (6, 15, 58), the decreased IFN-γ production and/or neutrophil accumulation may be indirectly affected by IL-6 deficiency.

IL-6 contributes to the generation of *B. pertussis*-specific IL-17 responses. IL-17 producing T_{h}17 cells are distinct from T_{h}1 or T_{h}2 cells and have been shown to play pathogenic roles in autoimmune diseases (18, 31, 35, 47). There have been studies establishing a role for IL-17 in host defenses against bacterial pathogens, including *K. pneumonia*, *Bacteroides fragilis* and *M. tuberculosis* (7, 17, 24). Several reports have suggested that *B. pertussis* infection skews the host immune response towards the expansion of T_{h}17 cells (2, 14). IL-17 has been shown to promote macrophage killing of *B. pertussis* and depletion of IL-17 reduced the efficacy of a *B. pertussis* whole cell vaccine (20). Andreasen et al. have recently established the role of a *B. pertussis* virulence factor, pertussis toxin, in IL-17 induction and the involvement of IL-17 in controlling *B. pertussis* numbers in the lungs of mice (2). IL-6 has been shown to influence the production of IL-17 (5). Here we establish that IL-6 is required for efficient induction of systemic IL-17 response during *B. pertussis* infection (Fig. 6.8B).

IL-6 contributes to host defenses against various pathogens including *S. pneumoniae*, *E. coli*, *L. monocytogenes*, *B. burgdorferi*, and *C. trachomatis* (3, 10, 11, 60, 65). Here, we show that efficient clearance of *B. pertussis* and vaccine-induced immunity against this pathogen are IL-6 dependent (Fig. 6.2 and 6.3). Blocking IL-6 or its receptor may increase susceptibility to various pathogens, including *B. pertussis*, which
could be a concern for patients with Castleman’s disease and rheumatoid arthritis undergoing the trials of a new anti-IL-6 therapy (53, 69).
Authors and Contributions:

Xuqing Zhang\textsuperscript{1,2,\dagger} Tania Goel\textsuperscript{1,3,4,\dagger}, and Eric T. Harvill\textsuperscript{1}

\textsuperscript{1}Department of Veterinary and Biomedical Sciences, the Pennsylvania State University, 115 Henning Building, University Park, Pennsylvania 16802

\textsuperscript{2}Graduate Program in Genetics, The Pennsylvania State University, University Park

\textsuperscript{3}Department of Biochemistry, Microbiology, and Molecular Biology, The Pennsylvania State University, University Park

\textsuperscript{4}Graduate Program in Biochemistry, Microbiology and Molecular Biology, The Pennsylvania State University, University Park

\dagger XZ and TG contributed equally to this work.

Conceived and designed the experiments: XZ, TG, ETH.

Performed the experiments: XZ, TG.

Analyzed the data: XZ, TG.

Wrote the paper: XZ, TG, ETH.
References


Chapter 7

SigE Facilitate the Adaptation of *B. bronchiseptica* to Stress Conditions and Sepsis in Mice

Abstract

The extracytoplasmic function (ECF) sigma factor, σ^E^, is involved in stress response and virulence in a variety of bacteria species, but its role in the respiratory pathogen, *Bordetella bronchiseptica*, has not been investigated. An in-frame deletion mutant of *sigE* in the *B. bronchiseptica* strain RB50 is defective in growth under heat or ethanol stress, thermotolerance, and resistance to cell envelope perturbations posed by detergent or β-lactam antibiotics. Although a *sigE*-deficient strain colonizes immunocompetent mice and causes lethal infection in TLR4^def^ or TNF-α^−/−^ mice as efficiently as its wild-type counterpart, it is defective in causing systemic and lethal infection in RAG^−/−^ mice (lacking B-cells and T-cells). Deletion of *sigE* did not affect sensitivity to complement-mediated killing, but it did lead to decreased expression of some type three secretion system (TTSS) genes and decreased cytotoxicity to macrophages. This study extends the knowledge of how a transcription regulatory system other than the BvgAS system affects *B. bronchiseptica* physiology and pathogenesis.
Introduction

The cell envelope of gram-negative bacteria is a dynamic, strictly regulated and multifunctional cellular compartment. It keeps the cell intact, acts as a barrier against extracellular hazards and is involved in processes such as nutrient transport, energy production and biosynthesis of cell surface molecules. Changing environmental conditions are sensed at the cell envelope, and signals are transduced across the envelope via intercompartmental signaling mechanisms, so that gene expression can be altered accordingly. One such signaling system is the two-component signal transduction system, composed of a transmembrane sensor kinase and a DNA-binding response regulator. Another signaling system is the extracytoplasmic function (ECF) family of alternative sigma factors, which regulate gene expression in response to extracytoplasmic stress by binding to core RNA polymerase and conferring different promoter specificities.

$\sigma^E$ is an ECF sigma factor present in many gram-negative bacteria that may play critical and distinct roles in different species (39, 46). In Escherichia coli, the gene $rpoE$ encodes $\sigma^E$, which is regulated in response to misfolded outer membrane proteins; it is essential for cell envelope maintenance and bacterial survival (21, 38). Pathogenic bacteria that colonize hosts also contend with the additional stress imposed by a host immune system, and $\sigma^E$ is often important in response to such stress. For example, Mycobacterium tuberculosis lacking $\sigma^E$ is more sensitive to heat shock, detergent, oxidative stress and macrophage-mediated killing than wild-type (33). $\sigma^E$ of Salmonella typhimurium is required for growth and survival in the presence of antimicrobial peptides, oxidative and acid stress, as well as survival and proliferation in epithelial and macrophage cell lines (23, 40). $\sigma^E$ is important for growth of Vibrio cholerae under ethanol stress and contributes to host colonization for both V. cholerae and S. typhimurium (23, 29). Expression of $\sigma^E$ in nontypeable Haemophilus influenzae (NTHi) is increased following phagocytosis by macrophages and $\sigma^E$ is required for intracellular survival of this bacterium (10). Together these studies suggest that $\sigma^E$ is responsive to various signals in different organisms.

Bordetella bronchiseptica is a pathogen that is closely-related to B. pertussis and B. parapertussis, the causative agents of whooping cough in humans (3, 43). B. bronchiseptica causes a range of diseases from asymptomatic infection to fatal pneumonia in a wide range of mammals (17, 37, 41). It is the etiological agent of atrophic rhinitis in swine, kennel cough in dogs and snuffles in rabbits (17, 37). In addition,
infections established by *B. bronchiseptica* are typically chronic, asymptomatic, and difficult to completely eradicate (17). *B. bronchiseptica* is capable of surviving harsh conditions, suggesting that it may exist outside mammalian hosts (15, 44). Therefore, it is likely that *B. bronchiseptica* flexibly regulates expression of sets of genes in response to various conditions. Relative to its genome size, *B. bronchiseptica* has a large number of putative transcription factors (32). One well-studied example of this is BvgAS, a two-component system that regulates virulence factor expression. In contrast to BvgAS, the role of other transcription factors in stress responses and pathogenesis has not been fully examined.

*B. bronchiseptica* genome encodes an *E. coli* σ^E^ ortholog, SigE. In fact, we have shown that *B. bronchiseptica* SigE actively directs transcription of the *E. coli* σ^E^-dependent promoter *rpoH*P3 in *vitro* and in *E. coli*, indicating that SigE_BB can function as a σ^E^-like sigma factor. To investigate the role of SigE in *B. bronchiseptica*, an in-frame sigE deletion mutant was constructed using a *Bordetella*-specific allelic exchange strategy. The resulting strain was viable with no growth defect under non-stress conditions compared to the wild-type strain. Growth of the sigE mutant strain in various stress conditions also showed that SigE contributes to the growth of *B. bronchiseptica* under ethanol and heat stress and resistance to agents that affect cell envelope integrity such as detergent and β-lactam antibiotics. Although *B. bronchiseptica* SigE was not required for colonization in immunocompetent hosts and causing lethal diseases in hosts deficient in TLR4 or TNF-α, SigE was required for lethal infection and systemic spread of *B. bronchiseptica* in RAG^-/-_mice. Together, these data indicate that SigE contributes to the ability of *B. bronchiseptica* to cope with certain stressful conditions and to colonize systemic organs in hosts lacking adaptive immunity.
Material and Methods

Bacterial strains and growth. Experiments were performed using the previously described *B. bronchiseptica* strain RB50 (9), an isogenic mutant lacking O-antigen, RB50Δwbm (45), a TTSS mutant of RB50 lacking *bcsN* (WD3) (58), *B. bronchiseptica* strain AVS lacking TTSS and adenylate cyclase toxin (34) and an isogenic mutant lacking *sigE*, RB50Δ*sigE*, which was constructed in this study and is described below. Bacteria were maintained on Bordet-Gengou agar (Difco) containing 10% defibrinated sheep blood (Hema Resources) and 20μg/mL streptomycin. For growth under ethanol stress, mid-log-phase cultures were sub-cultured into fresh Stainer-Scholte broth (51) with or without 3% ethanol and incubated at 37°C in a gyratory water bath unless otherwise noted. Unless otherwise noted, culture density was monitored by measuring OD$_{600}$ and viability was monitored by measuring CFU/mL.

Protein purification. The plasmid pXQZ001 was constructed by PCR amplifying *sigE* from RB50 genomic DNA (5’GGCCTGGGCATATGaGCGAACGCGATGTCGA3’, 5’GGCCTAGGATCCTTACCAGCGACGCTCGGCAT3’) and cloning it into the expression vector pET-15b (Novagen). N-terminally His-tagged *B. bronchiseptica* SigE and *E. coli* σ$^E$ were purified from strain BL21(DE3) slyD::kan pLysS pXQZ001, and BL21(DE3) slyD::kan pLysS pSEA5036, respectively, as previously described (8). Briefly, cells were grown at 25°C to an OD$_{600}$ of 0.5, at which point IPTG was added to induce protein production. Following 1.5-3 hours of induction, cells were harvested by centrifugation and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, 2.5 mM β-mercaptoethanol, 1 mM PMSF). Resuspended cells were then lysed by sonication and the lysate was cleared by centrifugation. The supernatant containing soluble His-SigE was loaded onto a Ni-NTA column (Qiagen). Bound proteins were eluted with a stepwise gradient of 20, 60, 100, and 200 mM imidazole in column buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2.5 mM β-mercaptoethanol). Fractions containing SigE were pooled and dialyzed into 20 mM Tris-HCl pH 8.0, 50 mM NaCl, and 2.5 mM β-mercaptoethanol.

Run-off *in vitro* transcription. 100 nM *E. coli* core RNA polymerase (RNAP) (Epicentre) was incubated with 500 nM His-SigE (His-SigE+RNAP), His-σ$^E$ (His-σ$^E$+RNAP), or the appropriate volume of transcription buffer (40 mM Tris-HCl pH 8.0, 10 mM MgCl$_2$, 50 mM NaCl, 1 mM DTT, 0.1 mg ml$^{-1}$ BSA) for 10 minutes.
at 30°C to form holoenzyme. Multi-round transcription reactions were initiated by addition of His-SigE alone, core RNAP alone, His-SigE+RNAP or His-σE+RNAP, to a final concentration of 50 nM SigE or σE and 10 nM core RNAP, to pre-warmed (30 °C) transcription mix containing 5.0 nM supercoiled plasmid template pSEB015(8), 5% glycerol, 200 μM ATP, 200 μM CTP, 200 μM GTP, 10 μM UTP, and 2.5 μCi [α-32P]UTP in transcription buffer. After 10 minutes at 30 °C, reactions were stopped by the addition of stop solution (80% formamide, 20 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). Samples were electrophoresed on 6% polyacrylamide gels containing 7.5 M urea, and visualized by phosphorimaging.

**Construction of RB50ΔsigE strain.** A left-flanking PCR product (5’GGGAATTCAAGATCGAGATCGGCCTGTCGAAT3’, 5’AGGGATCCGAAGGCTTTCTTGTCGCCACGTTGTA3’) with 637bp proximal to the sigE gene and a non-overlapping 534bp right-flanking product (5’AGGGATCCTGGTAAGGAGTGGCAGTCATGCAA3’, 5’GCGAATTCAAAGCAACCGGTGTCACTCAACGTCC3’) were amplified from *B. bronchiseptica* RB50 genomic DNA with EcoRI and BamHI sites overhanging on the 5’, 3’ and 3’, 5’, respectively. The two flanking fragments were digested with BamHI and ligated. The resulting construct was PCR-amplified (5’GGGAATTCAAGATCGAGATCGGCCTGTCGAAT 3’, 5’GCGAATTCAAAGCAACCGGTGTCACTCAACGTCC3’), cloned into TopoTA vector (Invitrogen) and verified by sequencing. The deletion construct was then cloned into the EcoRI site of pSS3962 allelic exchange vector (Stibitz S. unpublished data), resulting in the deletion of the 528bp central region of sigE with only 66bp of the 5’end and 6bp of the 3’end of the sigE gene remaining in place. Tri-parental mating with wild-type *B. bronchiseptica* strain RB50, *E. coli* strain DH5α harboring the pSS3962ΔsigE vector, and DH5α harboring the helper plasmid pSS1827 (53) was performed under modulating conditions (with 50mM MgSO4) and incubated on modulating plates containing 100μg/mL kanamycin and 60μg/mL streptomycin to select for the integration of pSS3962ΔsigE into RB50 chromosome. *Bordetella* colonies on modulating plates were streaked onto plates without MgSO4 to activate the pertussis toxin (Ptx) promoter on the vector which drives the expression of the endonuclease SceI. This resulted in the cutting of the SceI site on the vector and approximately 50% of the resultant colonies on these plates had the wild-type sigE gene deleted from the chromosome by homologous recombination. The deletion strain was verified by colony PCR
Cloning of sigE for complementation. Cholramphenicol resistance gene, _cat_, was PCR amplified (5’GCGGCGGGATCCTGTGTAAGGCTGGAGCTGCTTC3’, 5’GCCGCCGGATCCCATTAGATATCCCTCCCTA3’) from pKD3 (12) with BamHI sites overhanging on both 5’ and 3’ sites. This amplicon was blunt cloned into the BamHI cite of pJS72 [a kind gift from Dr. Kenneth Keiler, a pJS71 (50) derivative with _tac_ promoter] to replace the omega spectinomycin cassette, a plasmid with _tac_ promoter, resulting in cholramphenicol resistant pSEB025 (pEV). The _sigE_ gene was PCR amplified (5’GCGCGGTCTAGAAGGAGGAGGCGTCATGAGCGAACGCGATG3’, 5’GCCCGGCTCGAGTTACCAGCGACGCTCGGCAT3’) from RB50 genomic DNA with XbaI and XhoI site overhanging on the 5’ and 3’, respectively. The amplicon was cloned into the XbaI and XhoI site of pEV, resulting in pSEB026 (pSigE). In this construct, the _sigE_ gene was expressed from the _tac_ promoter of pSigE. Tri-parental mating with RB50ΔsigE, _E. coli_ strain DH5α harboring the pEV or pSigE, and DH5α harboring the helper plasmid pSS1827 (53) was performed on Bordet-Gengou plates, and incubated on Bordet-Gengou plates containing 20µg/mL cholramphenicol and 20µg/mL streptomycin to select for RB50ΔsigE that maintained pEV or pSigE. The resulting strains were designated RB50ΔsigE[pEV] and RB50ΔsigE[pSigE].

Disk diffusion assay. Mid-log-phase _B. bronchiseptica_ cultures were diluted to 6×10⁸ CFU/mL to generate a lawn of bacteria on Stainer-Scholte agar plates. Disks containing 300 iu Polymyxin B, 10 µg ampicillin (BD Diagnostics), 100 µg mecillinam (Leo Pharmaceutical Products) or 75 mg/mL sodium dodecyl sulfate (SDS) and 1 mM EDTA were applied to the plates and the zone of inhibition was measured after overnight incubation at 37°C.

Complement killing assay. Complement killing assays were performed as previously described (16). Briefly, blood freshly collected from C57BL/6 mice was pooled, incubated at 4°C for 1 hour and centrifuged at 2000g for 10min. The serum was collected and diluted 1:5 in phosphate buffered saline (PBS). Complement-inactive serum was prepared by heating an aliquot of the diluted sample at 56°C for 30 min. From mid-log-phase cultures, approximately 500 CFU of RB50, RB50ΔsigE and RB50Δwbm in 5 µl of PBS
were incubated with 45 µl of diluted serum or PBS for 1 hour at 37°C. Bacterial numbers before and after incubation were determined by plating and CFU counts. Each strain was run in triplicate.

**qRT-PCR.** For RNA isolation, 3 independent biological replicates of RB50 and RB50ΔsigE were grown in Stainer-Scholte media containing 1.5% ethanol at 37°C while shaking until the OD of *B. bronchiseptica* cultures reached 0.5. Another set of 3 independent biological replicates of RB50ΔsigE were cultured until they reached OD 0.5 (at later time). Bacteria were harvested and total RNA was extracted using RNeasy columns (Qiagen) as per the manufacturers’ instructions with the treatment with RNase-free DNase I (Invitrogen). Quantitative real-time PCR (qRT-PCR) was completed as previously described (42). 1 µg RNA from each biological replicate was reverse transcribed using 500 ng of random oligonucleotide hexamers (Invitrogen) and ImProm-II™ Reverse Transcriptase (Promega). The resulting cDNA was diluted 1:100 and 1 µL was used in qRT-PCRs containing 800 nM primers designed with Primer Express software (Applied Biosystems) and 2×SYBR Green PCR master mix (Invitrogen). Primer sequences are listed in the supplemental material (Appendix A). To confirm the lack of DNA contamination, reactions without reverse transcriptase were performed. Dissociation curve analysis was performed for verification of product homogeneity. Threshold fluorescence was established within the geometric phase of exponential amplification, and the cycle threshold (Ct) was determined for each reaction. The Ct from all biological replicates for each strain under the same treatment was compiled, and the 16S RNA amplicon was used as internal control for data normalization. The fold change in transcript level was determined using the relative quantitative method (ΔΔCt) (31).

**Cytotoxicity assay.** Cytotoxicity assays were performed as previously described (19). Briefly, J774 murine macrophage cells, were cultured in Dulbecco modified Eagle medium (DMEM) (HyClone) with 10% fetal bovine serum (FBS) (HyClone). The cells were grown to 80% confluency in 96-well plates at 37°C in 5% CO₂. Cells were washed with RPMI (Mediatech) containing 5% FBS and bacteria were added in 50 µL RPMI at a multiplicity of infection (MOI) of 15. After a 5 minute centrifugation at 250g, the J774 cells were incubated for the indicated time. The percent lactate dehydrogenase (LDH) release, a measure of cytotoxicity, was determined by using Cytotox96 Kit (Promega) according to the manufacturer’s protocol.
Animal experiments. C57BL/6, TNF-α−/−, RAG1−/− (RAG−/−), C3H/HEN (TLRsurf) and C3H/HEJ (TLRdef) mice were obtained from Jackson laboratories. All mice were bred in our Bordetella-free, specific pathogen-free breeding rooms at The Pennsylvania State University. For inoculation, mice were sedated with 5% isoflurane (Abbott laboratory) in oxygen and 50 μL of PBS containing 1×10^5 or 5×10^5 CFU of the indicated bacteria was pipeted onto the external nares (27). This method reliably distributes the bacteria throughout the respiratory tract (20). Survival curves were generated by inoculating TLRdef, TNF-α−/− and RAG−/− mice with either RB50 or RB50ΔsigE. Mice suffering from lethal bordetellosis as determined by severe hunched posture, ruffled fur, extremely labored breathing and apathy were euthanized to prevent unnecessary suffering (36). For quantifying bacterial load, mice were euthanized via CO₂ inhalation and lung, trachea, nasal cavity, spleen, liver and/or kidneys were excised. Tissues were homogenized in PBS, aliquots were serially diluted, plated, incubated at 37°C for 2 to 3 days, and CFU was determined. All protocols were reviewed by the university IACUC and all animals were handled in accordance with institutional guidelines.

Statistical analysis. The mean +/- standard error (SE) of the geometric mean was determined for all appropriate data and expressed as error bars. Two-tailed, unpaired Student’s T-tests were used to determine statistical significance between groups. All experiments were performed at least twice with similar results.
Results

sigE encodes a $\sigma^E$-like sigma factor

$B. bronchiseptica$ SigE and $E. coli$ $\sigma^E$ share 54% amino acid sequence identity and 73% similarity when aligned using NEEDLE (EMBL-EBI), suggesting that these two proteins may share a similar function. For comparison, $P. aeruginosa$ AlgU and $E. coli$ $\sigma^E$, which are functionally homologous (57), share 65% identity and 80% similarity when aligned using this algorithm. To determine whether SigE is a $\sigma^E$-like sigma factor, we purified N-terminally His-tagged SigE, and assayed it for activity at the $E. coli$ $\sigma^E$-dependent promoter, $rpoH_P3$, using run-off in vitro transcription assays. $B. bronchiseptica$ His-SigE can direct transcription of $rpoH_P3$ similarly to $E. coli$ His-$\sigma^E$ (Fig. 7.1).

In $E. coli$, the gene encoding $\sigma^E$, $rpoE$, is essential. However, plasmid-encoded $B. bronchiseptica$ SigE allows the cells to live in the absence of $rpoE$ (data not shown). This further supports that sigE encodes a sigma factor functionally homologous to $E. coli$ $\sigma^E$.

Deletion of sigE from $B. bronchiseptica$

To investigate the role of $B. bronchiseptica$ SigE, we first constructed an in-frame sigE deletion strain of $B. bronchiseptica$ strain RB50 (RB50ΔsigE). 176 out of 200 codons were deleted in-frame leaving 22 and 2 codons at the 5' and 3' ends of the gene, respectively (Fig. 7.2 and Materials and Methods). Unlike in $E. coli$ or $Yersinia enterocolitica$, but the same as in many other bacterial pathogens, deletion of sigE was not lethal in $B. bronchiseptica$ (10, 14, 22, 29, 33, 54). RB50ΔsigE has no growth defect compared to RB50 under optimal, non-stress growth conditions: 37°C in Stainer-Scholte broth (Fig. 7.3B).
**SigE contributes to *B. bronchiseptica* stress response**

**Heat shock**

In *E. coli*, the P3 promoter of *rpoH* (*rpoHP3*), encoding σ^{32}, the heat shock sigma factor, is regulated by σ^E, when the cells are exposed to particularly high temperatures (1, 7). We have shown that *B. bronchiseptica* SigE directs transcription from *E. coli* *rpoHP3* (Fig. 7.1B). The promoter region of the gene encoding *B. bronchiseptica* σ^{32}, *fam*, is highly similar to the known *E. coli* σ^E-dependent promoters and *B. bronchiseptica* SigE can direct transcription of *fam* (data not shown) (46). We therefore predicted a role for SigE in response to high temperature. To examine this, RB50 and RB50ΔsigE were grown in a shaking water bath at 37°C, and then shifted to 50°C, a lethal temperature for *B. bronchiseptica*. Cell viability was measured for three hours after shifting to 50°C. RB50ΔsigE died more quickly than RB50 (Fig. 7.3A), suggesting that SigE contributes to heat shock response.

Inducing a heat shock response at an elevated, sublethal temperature, such as 40°C, allows cells to accumulate heat shock proteins, and prepares them to better survive a lethal temperature (47). To investigate the role of SigE in this phenomenon, known as thermotolerance, RB50 and RB50ΔsigE were diluted from overnight cultures into fresh media, grown to an OD_{600} of 0.1 at 37°C, shifted to 40°C for 90 minutes, then
shifted to 50°C, and cell viability was measured. Wild-type RB50 became thermotolerant after this treatment and CFU numbers had not been reduced by 2h incubation at 50°C (Fig. 7.3A). Similarly to the previous experiment, RB50ΔsigE died more quickly than RB50 after the shift to 50°C, providing further evidence that sigE is required for survival at a lethal temperature and that SigE contributes to thermotolerance. While overall survival at 50°C was lower in cells lacking sigE, RB50ΔsigE adapted at 40°C survived better than RB50ΔsigE shifted directly from 37°C to 50°C, indicating that RB50ΔsigE does still exhibit some thermotolerance.

Figure 7.3: Role of SigE in various environmental stress. (A) RB50 (square) and RB50ΔsigE (triangle) were subcultured to 10^5 CFU/mL in fresh Stainer-Scholte media. All cultures were incubated at 37°C to an OD_600 of 0.1. Half of the cultures were shifted to 40°C (dashed line), while the other half remained at 37°C (solid line). After 90 minutes, all cultures were shifted to 50°C, and cell viability was measured by CFU/mL after further culturing for 0, 1, 2, or 3 hours. The mean CFU ± SE of three independent experiments was shown. (B) RB50 (square), RB50ΔsigE (triangle) were sub-cultured to an OD_600 around 0.01 in fresh Stainer-Scholte media with (dashed line) or without (solid line) 3% ethanol. RB50ΔsigEpEV (circle) or RB50ΔsigEpSigE (open diamond) were sub-cultured to an OD_600 around 0.01 in fresh Stainer-Scholte media with 3% ethanol. 1mM IPTG was added to a separate culture of RB50ΔsigEpSigE (closed diamond) at the time of sub-culturing. Cell growth was measured by OD_600. A representative dataset of three independent experiments was shown. (C) RB50 (black) or RB50ΔsigE (white) was diluted to 6×10^8 CFU/mL to generate a lawn of bacteria on Stainer-Scholte agar plates with disks containing 300 iu Polymyxin B, 10 µg ampicillin, 100 µg mecillinam or 75 mg/mL SDS and 1mM EDTA placed on them and incubated overnight at 37°C. Average of diameters of zone of inhibition from 3-7 independent experiments was shown. *: P≤0.05; **: P≤0.01. Sarah E. Barchinger performed this experiment.

Ethanol Stress

Ethanol stress is also known to induce expression of heat shock proteins, and the V. cholerae σ^E ortholog is required for survival during ethanol stress (29). To test the role of SigE in response to ethanol stress, RB50 and RB50ΔsigE were sub-cultured from mid-log-phase overnight cultures into fresh Stainer-Scholte broth with or without 3% ethanol. Both strains grew similarly in the media without ethanol, as noted above. RB50 grew significantly slower in media containing 3% ethanol than in media without ethanol, but its
cell density continued to increase throughout the course of the growth curve, ultimately reaching a final
density similar to that of cells grown in media without ethanol (Fig. 7.3B, data not shown). The cell density
of RB50ΔsigE, however, never surpassed an OD$_{600}$ of about 0.1, even after 24 hours, and the number of viable cells decreased consistently after 5h of incubation, indicating that sigE is required for survival during ethanol stress (Fig. 7.3B, data not shown). Adding back a copy of sigE on a plasmid under the control of an IPTG-inducible promoter (RB50ΔsigEpSigE) restores cell growth in 3% ethanol to wild-type levels only in the presence of IPTG, indicating that the original phenotype is indeed specifically due to loss of SigE activity.

Specific Cell Envelope Stress

σE contributes to cell envelope stress responses in many bacterial species (21, 48). To test whether SigE of B. bronchiseptica aids in combating stress in the presence of agents affecting cell envelope integrity, we performed disk diffusion assays comparing the sensitivity of RB50 and RB50ΔsigE to the β-lactam antibiotic ampicillin, which inhibits proper formation of the peptidoglycan layer (25), mecillinam, which binds specifically penicillin binding protein 2 and blocks cell elongation (24), 7.5% SDS + 0.1mM EDTA, which affects outer membrane integrity (30, 55), or the cationic antimicrobial peptide polymyxin B, which specifically attacks the cytoplasmic membrane (18, 23, 49), using either 0.1mM EDTA or water as a control. Neither strain was susceptible to water or EDTA alone. Unlike in S. typhimurium, where σE is required for resistance to polymyxin B, both RB50 and RB50ΔsigE exhibited similar sensitivity to this treatment (Fig. 7.3D). However, RB50ΔsigE was significantly more sensitive than RB50 to ampicillin, mecillinam and SDS+0.1mM EDTA (Fig. 7.3C).

Both RB50 and RB50ΔsigE were equally sensitive to osmotic stress, another indicator of membrane integrity (28). Both strains also showed similar sensitivity to non-β-lactam antibiotics, and grew similarly when cultured in the presence of agents that induce oxidative stress, such as hydrogen peroxide or paraquat (Appendix B). All together, these data suggest a role for SigE in combating cell envelope stress.

Growth in the murine respiratory tract is not affected by the lack of SigE

Bacteria encounter different sets of microbicidal hazards inside of a host. It has been shown that σE-like sigma factors of various pathogens contribute to virulence in mice (2, 23, 29). To determine whether SigE contributes to colonization and persistence in the hosts, groups of C57BL/6 mice were inoculated with
RB50 or RB50ΔsigE, and colonization was measured in the nasal cavity, trachea and lung on days 0, 3, 7, 14, 28 and 63 post-inoculation. Both strains showed similar colonization kinetics in the lung and trachea of C57BL/6 mice, peaking in numbers on days 3 and 7 post-inoculation in trachea and lung, respectively, and declining thereafter with complete clearance in both organs being observed by day 63 post-inoculation (Fig. 7.4). Both wild-type and sigE-deficient RB50 also colonized the nasal cavity at comparable levels, peaking on day 3 post-inoculation, and stabilizing at about 10^{4.5} CFU by 2 weeks post-inoculation. These data indicate that *B. bronchiseptica* SigE is not required for colonization or persistence in the murine respiratory tracts.

**SigE contributes to systemic spread and lethal *B. bronchiseptica* infection in RAG^−/− mice**

More severe infections in various immunodeficient animals can involve bacterial growth in different locations and under different conditions.

For example, more inflamed and damaged respiratory organs, or even systemic organs that the bacteria do not normally colonize, can present the bacteria with different stresses. To examine the role of SigE in coping with these stresses, we tested if the strain lacking sigE is defective in causing lethal diseases in some immunodeficient mouse. TLR4^{def} and TNF-α^{−/−} mice were challenged with RB50 or RB50ΔsigE and signs of severe disease were monitored. Consistent with published studies, TLR4^{def} and TNF-α^{−/−} mice inoculated with 10^5 CFU wild-type *B. bronchiseptica* quickly developed signs of bordetellosis with ruffled fur, hunched backs, difficulty breathing, and succumbed 2 to 5 days post-inoculation (35, 36). Mice challenged with

**Figure 7.4: Colonization of the respiratory tract of C57BL/6 by RB50 and RB50ΔsigE over time.** Groups of three 4-6 week-old C57BL/6 mice were inoculated with 5×10^5 CFU of RB50 (black) or RB50ΔsigE (white). The bacterial load is expressed as Log_{10}CFU ± SE. The limit of detection is indicated as the dashed line.
RB50ΔsigE also showed similar signs of diseases and time to death (Fig. 7.5A, 7.5C). In a separate experiment, similarly challenged TLR4\(^{\text{def}}\) and TNF-\(\alpha\)^{−/−} mice were dissected on day 3 post-inoculation for bacterial enumeration in the respiratory as well as systemic organs. Both wild-type and sigE-deficient RB50 colonized the lungs of TLR4\(^{\text{def}}\) mice at similar levels, \(10^{7.8}\) CFU, which was almost 1000 fold higher than in the lungs of TLR4\(^{\text{sur}}\) mice. Moreover, both strains colonized the systemic organs in TLR4\(^{\text{def}}\), but not TLR4\(^{\text{sur}}\)
mice (Fig. 7.5B). Similarly, both strains colonized at higher levels in the lungs of TNF-α−/− mice than in the lungs of C57BL/6 mice and were recovered from systemic organs only in TNF-α−/− mice (Fig. 7.5D). These data indicate that SigE is not required for *B. bronchiseptica* to cause lethal infection or colonize systemic organs in TLR4 or TNF-α deficient host.

We then determined if SigE is required for virulence in mice deficient in adaptive immunity. Previously, we have observed that B and T cell deficient RAG−/− mice succumb to *B. bronchiseptica* infection, and death is associated with systemic spread of the infection. Groups of RAG−/− mice were intranasally inoculated with 5 × 10⁵ CFU of RB50 or RB50ΔsigE to assess the role of SigE in the systemic spread of *B. bronchiseptica* in the hosts lacking B and T cells. RB50-inoculated RAG−/− mice showed symptoms of lethal bordetellosis on day 13 post-inoculation and succumbed between days 14-35 post-inoculation (Fig.7.5A). However, RAG−/− mice inoculated with RB50ΔsigE survived 122 days post-inoculation without any overt signs of diseases and were euthanized on day 122 post-inoculation. Nasal cavity, trachea, lung, spleen, liver and kidneys of these mice were excised to enumerate the bacterial loads. Although 10⁵-⁷ CFU of RB50ΔsigE was recovered from the respiratory tract, this strain failed to colonize spleen or kidney; and only 300 CFU was recovered from liver (Fig. 7.5F, dashed bars). In a separate experiment, RB50- and RB50ΔsigE-inoculated RAG−/− mice were sacrificed on day 28 post-inoculation, when RB50 challenged mice were still alive. The bacterial loads of RB50 and RB50ΔsigE in the respiratory tract on day 28 post-inoculation were similar, about 10⁵-⁷ CFU. At this time point, 10⁵-⁶ CFU of RB50 was recovered from systemic organs. RB50ΔsigE, however, failed to colonize spleen, kidney or liver (Fig. 7.5F, black and white bars). These observations led us to conclude that SigE is required for *B. bronchiseptica* to cause sepsis and lethal infection in RAG−/− mice.

**RB50ΔsigE is not susceptible to complement-mediated killing**

The failure of RB50ΔsigE to colonize distal organs of RAG−/− mice suggests that this mutant is defective in either getting into or survival in their bloodstream. Since we have previously shown that the ability of *B. bronchiseptica* to cause systemic infection is dependent on its resistance to complement-mediated killing (6), we hypothesized that RB50ΔsigE might be susceptible to complement-mediated killing. To test this, 500 CFU of RB50 or RB50ΔsigE was incubated with 20% of complement-active or complement-
inactive serum from naïve mice at 37°C for 1 hour. Since it has previously been shown that RB50Δwbm is susceptible to complement-mediated killing; this strain was included in the assay as a positive control for complement activity (6) (Fig. 7.6). RB50Δwbm survived the complement-inactive serum treatment, but was almost completely killed when treated with complement-active serum, indicating that in this assay, complement is active. The percent survival of RB50 and RB50ΔsigE in PBS, PBS containing complement active or inactive serum were comparable, suggesting that SigE is not required for resistance to complement. RB50ΔsigE survives in the presence of serum without B. bronchiseptica-specific antibodies, indicating that its defect in causing systemic infection in mice lacking B and T cells is not due to failure to survive the antimicrobial components in serum, including complements.

**SigE contributes to cytotoxicity to macrophages**

![Figure 7.6: SigE is not required for serum resistance.](image)

Figure 7.6: **SigE is not required for serum resistance.** RB50 (black), RB50ΔsigE (white) and RB50Δwbm (dashed) were exposed to PBS (none) or PBS containing complement (C') active or complement inactive serum for 1 hour. The average percent survival of three replicate ± SE is shown.

![Figure 7.7: RB50ΔsigE is less cytotoxic to macrophage.](image)

Figure 7.7: **RB50ΔsigE is less cytotoxic to macrophage.** (A) J774 cells were incubated with media only (white diamond) or media containing RB50 (black square), RB50ΔsigE (white triangle), TTSS deficient RB50 stain WD3 (black diamond), or ACT and TTSS deficient RB50 stain AVS (black circle) for indicated time. The average of percent cytotoxicity of four wells treated similarly as measured by (LDH release from a well/LDH release from positive control well) ×100 ± SE is shown. (B) Quantitative real-time PCR analysis was performed to measure mRNA levels in three independent biological replicates of RB50 (black), RB50ΔsigE, which was harvested at the same time as RB50 (white) and RB50ΔsigE, which was harvested at similar culture density to RB50 (hatched) growing in the Stainer-Scholte containing 1.5% ethanol. Differences in mRNA levels are expressed as mean fold-changes compared to RB50 ± SE. *:P≤0.05 as compared to wild-type strain. Sara E. Hester performed the experiment shown in Fig. 7.7 A

RB50ΔsigE interacts with complement similarly to RB50, but does not colonize systemic organs of RAG−/− mice. We further tested whether
RB50ΔsigE interacts with another major bactericidal component in the bloodstream, phagocytes, differently from RB50. *B. bronchiseptica* is cytotoxic to one type of phagocytes, macrophages. We thus hypothesized that SigE might contribute to the cytotoxicity of *B. bronchiseptica* to macrophages. To test this, J774 murine macrophages were incubated with RB50, RB50ΔsigE, RB50 lacking type three secretion system (TTSS) (WD3), or RB50 lacking both TTSS and adenylate cyclase toxin (AVS) at a MOI of 15 for up to 4 hours. Cytotoxicity was measured according to LDH release. Since the TTSS contributes to cytotoxicity (58), strains WD3 and AVS were included as negative controls and these two stains had induced little cytotoxicity by 4 hours of incubation, similar to media only treated group. Wild-type RB50 has caused 24% and 85% cytotoxicity by 3 and 4 hours of incubation. However, RB50ΔsigE had caused significantly less cytotoxicity: 7% and 45% by 3 and 4 hours of incubation (Fig. 7.7A). RB50ΔsigE shows intermediate cytotoxicity between that caused by wild-type and TTSS deficient RB50, suggesting that SigE only partially contributes to the TTSS-dependent cytotoxicity of *B. bronchiseptica* to macrophages. This is consistent with preliminary microarray data indicating that SigE may be involved in regulating gene expression of some TTSS genes under ethanol stress. To determine whether SigE regulates some TTSS genes, qRT-PCR was performed on RNA extracted from cells under ethanol stress, conditions that requires SigE for survival (Fig. 7.3B). Since RB50ΔsigE grows slower in the presence of ethanol, RNA were extracted from this strain exposed to ethanol either for the same period of time or at the similar culture density compared to the wild-type strain to rule out the impact of different growth phase on TTSS gene expression. Under both circumstances, the expression of some TTSS genes, *bsp22, bcrHI, bopD* and *bopB*, was lower in RB50ΔsigE compared to wild-type RB50 and *bopD* expression is significantly lower in RB50ΔsigE, extracted at the similar culture density to RB50 (Fig. 7.7B).
Discussion

The ability of *B. bronchiseptica* to cause diseases in a wide range of hosts and transmit between them suggests that it can survive various stress imposed both inside and outside a host. Although the BvgAS two-component system is known to regulate virulence factor expression, this system alone is not likely to sense all the *in vitro* and *in vivo* conditions, and mount the optimum responses. ECF sigma factors, such as $\sigma^E$, have been increasingly implicated as contributing to both stress responses and virulence in many bacteria, including *Salmonella enterica*, *M. tuberculosis*, *V. cholerae*, and *Pseudomonas aeruginosa* (29, 33, 48, 56). In this work, we show that SigE, a $\sigma^E$-like sigma factor in the respiratory pathogen *B. bronchiseptica*, is involved in both stress responses and pathogenesis.

*B. bronchiseptica* SigE shares 54% amino acid sequence identity and 73% similarity with the well-characterized ECF sigma factor, *E. coli* $\sigma^E$. *B. bronchiseptica* SigE can direct transcription of an *E. coli* $\sigma^E$-dependent promoter, *rpoH*P3, indicating that SigE is a $\sigma^E$-like sigma factor. Moreover, the predicted SigE operon structure, including potential co-transcribed negative regulators, shows some similarities to the $\sigma^E$ systems of *E. coli* and *P. aeruginosa*, and studies on the regulation of the *B. bronchiseptica* SigE system are currently underway. In *E. coli*, the gene encoding $\sigma^F$, *rpoE*, is essential, however, in many other organisms the gene encoding a $\sigma^E$-like sigma factor can be deleted (10, 13, 23, 29, 33, 56). Using an allelic exchange strategy, we generated a *sigE* in-frame deletion strain of *B. bronchiseptica* and the growth kinetics of SigE deficient strain is similar to its isogenic parent under optimal growth conditions, indicating that SigE is not essential in *B. bronchiseptica*.

Changes in osmolarity, detergents, $\beta$-lactam antibiotics, high temperature, and ethanol, among others, can induce damage to the cell envelope (48). $\sigma^E$ senses alterations in the composition of the cell envelope in many bacterial species. For example, *E. coli* $\sigma^E$ is required for response to extreme heat shock, under which circumstances $\sigma^{32}$ is activated through its $\sigma^E$-dependent promoter, P3 (1). We have demonstrated that *B. bronchiseptica* SigE can also transcribe this *E. coli* $\sigma^E$-dependent promoter (Fig. 7.1B). SigE can also transcribe a putative promoter upstream of the gene encoding $\sigma^{32}$ in *B. bronchiseptica*, fam (data not shown). Consistent with a role in regulating the heat shock response, we have demonstrated a role for SigE in survival at 50°C, growth in the presence of 3% ethanol, and in thermotolerance. *E. coli* $\sigma^E$ is involved in maintenance
of the cell envelope (21), similarly SigE deficient *B. bronchiseptica* is more sensitive to agents that act at the cell envelope, including SDS and some β-lactam antibiotics (Fig. 7.3D). However, this response is specific to certain types of damage, as SigE is not important in response to antimicrobial peptides that also affect the envelope, such as polymyxin B (Fig. 7.3D). Neither *E. coli* nor *B. bronchiseptica* requires σ^E^ to cope with osmotic or oxidative stress (S2) (47).

Pathogens encounter a spectrum of stresses in different compartments inside of mammalian hosts imposed by numerous effectors of the immune system. We took an approach of using mouse strains that are deficient in specific aspects of the immune responses which exposed *B. bronchiseptica* to a variety of different conditions in different compartments of the host. SigE is not required for colonization and persistence of RB50 in an immunocompetent host. Deletion of *sigE* also does not seem to affect the virulence of RB50 in TLR4- or TNF-α-deficient mice. However, in mice lacking B and T cells, RB50Δ*sigE* fails to cause systemic lethal infections, indicating that the SigE-deficient strain fails to either get into or survive in the blood stream of these mice. Complement is a major bactericidal factor in the blood stream but RB50Δ*sigE* survives incubation with complement active serum, indicating that this mutant is resistant to complement-mediated killing and other serum anti-microbial components. RB50Δ*sigE* is less cytotoxic to macrophages as compared to RB50. The TTSS is required for full cytotoxicity, suggesting that RB50Δ*sigE* may have lower levels of TTSS. This is consistent with preliminary microarray data showing that expression of some TTSS genes is decreased in cells lacking SigE. Although cytotoxicity is decreased, it is not completely abolished; the remaining cytotoxicity may be enough to help establish systemic infection in TLR4- and TNF-α-deficient mice, but not in RAG^{-/-} mice. In a TLR4- or TNF-α-deficient host, there is uncontrolled bacterial growth until the bacterial number is so high that even ineffective cytotoxicity is enough to kill phagocytic cells. In comparison, bacterial numbers are kept lower in RAG^{-/-} mice and low cytotoxicity has a more limited effect on killing phagocytic cells, which may prevent systemic colonization. Members of the σ^E^ regulon of *S. typhimurium* are more important for systemic than enteric infection (23, 30, 48). Our data suggest that members of the *B. bronchiseptica* SigE regulon might be required for systemic infection in certain immunocompromised hosts. Studies on how SigE is activated *in vivo* and the members of its regulon will shed more light on its role during infection.
σE has different functions in various pathogens. For instance, σE mutant of *V. vulnificus* exhibits increased sensitivity to the membrane perturbing agents, ethanol, peroxide and SDS, and to heat (5); whereas a σE mutant of the closely-related species *V. cholerae* displays different patterns of stress sensitivity (29). σE of *V. cholerae* is required in the presence of ethanol, but is not required for heat shock or for resistance to peroxide or antimicrobial peptides (29). σE of *Salmonella enterica* serovar Typhimurium contributes to resistance to oxidative stress and stationary-phase survival, but not heat shock (11, 23, 26, 54). In *P. aeruginosa*, σE facilitates persistence in the airway of cystic fibrosis patients (4). It is likely that the discrepancies in the functions of σE are due to differences in environmental niches and specific insults bacteria encounter *in vivo*. Since *B. bronchiseptica* is predicted to have multiple other ECF sigma factors (52), their complimentary and redundant functions might in part account for the dispensability of *B. bronchiseptica* SigE in coping with stresses which other organisms require σE to contend. Future studies on what conditions activate other ECF sigma factors and their roles in *B. bronchiseptica* pathogenesis will provide a more comprehensive understanding of how *B. bronchiseptica* copes with extracytoplasmic stresses.
Authors and Contributions:

Xuqing Zhang¹,²,†, Sarah E. Barchinger³,⁴,†, Sara E. Hester¹,³,⁴, Sarah E. Ades³,⁴, and Eric T. Harvill¹

¹Department of Veterinary and Biomedical Sciences, Pennsylvania State University, 115 Henning Building, University Park, Pennsylvania 16802

²Graduate Program in Genetics, the Pennsylvania State University, University Park

³Department of Biochemistry, Microbiology, and Molecular Biology, The Pennsylvania State University, University Park

⁴Graduate Program in Biochemistry, Microbiology and Molecular Biology, The Pennsylvania State University, University Park

†XZ and SEB contributed equally to this work.

Conceived and designed the experiments: XZ, SEB, SEA, ETH.

Performed the experiments: XZ, SEB, SEH.

Analyzed the data: XZ, SEB.

Wrote the paper: XZ, SEB, SEA, ETH.
References:


Chapter 8

Constitutively Active SigE in *B. bronchiseptica* Leads to Decreased Virulence in Murine Hosts.

Abstract:

The extracytoplasmic function (ECF) sigma factor activity is usually tightly regulated by its negative regulators. Although ECF sigma factors have been shown to be involved in stress responses and virulence in many bacterial species, the role of their regulators during infection are less defined. A *Bordetella bronchiseptica* strain lacking *rseA* and *rseB*, encoding negative regulators of an ECF sigma factor, SigE, is constructed to examine the effects of constitutive SigE activity on pathogenesis. RB50Δ*rseAB* less efficiently colonizes and fails to persist in the lower respiratory tracts of C57BL/6 mice. Adoptively-transferred antibodies against this strain are efficient in reducing RB50Δ*rseAB* numbers *in vivo*. This mutant is as resistant to complement as wild-type bacteria but induces lower cytotoxicity to macrophage. RB50Δ*rseAB* does not cause lethal diseases in mice lacking B and T cells or in mice lacking TLR4. These data suggest that B and T cells- or TLR4-mediated host defense mechanisms alone might not be responsible for the decreased colonization of RB50Δ*rseAB*. Taken together, these results indicate that constitutively active SigE disrupts some as yet unknown virulence mechanisms required for efficient colonization and persistence, highlighting the importance of tight regulation of SigE activity during infection.
Introduction:

The ability to modify gene expression according to changes in the environment or the state of the cell is critical to bacterial pathogens that need to defend themselves against stress originating from the external environment between hosts as well as within the infected hosts. The extracytoplasmic stress responses (ESR) of bacteria have thus received much attention to understand bacterial pathogenesis. The best characterized ESR are those mediated by the two-component systems and the alternative extracytoplasmic function (ECF) sigma factors (39, 41). Sigma factor is the dissociable subunit of RNA polymerase that confers promoter specificity (13). The interaction between sigma factor and promoter region provides a means of regulating gene expression in response to various environmental conditions (34). By having multiple alternative sigma factors, bacteria can rapidly redirect transcription to a specific subset of promoters according to a particular stimulus. A subgroup of sigma factor in the ECF family regulates functions related to sensing and responding to changes in the bacterial cell envelope and extracellular environment, and in some cases genes involved in virulence (21, 41).

Many conditions, such as temperature changes, oxidative stress, and osmotic stress are commonly encountered by bacteria within various locations in the host. $\sigma^E$, an ECF sigma factor, has been shown to play diverse roles in responding to these conditions in various bacterial species. In *E. coli*, $\sigma^E$ directs transcription of many genes important for cell envelope maintenance and during heat shock (19, 32). In *Pseudomonas aeruginosa*, a major cause of respiratory infection in cystic fibrosis patients, *algU*, an ortholog of *E. coli rpoE*, is important for the production of alginate, a substance that is often found in excess in cystic fibrosis patients and plays several roles in pathogenesis (12, 15, 47). In *Salmonella typhimurium*, $\sigma^E$ is important for surviving oxidative stress and resistance to antimicrobial peptides (35, Humphreys, 1999 #8). In *Haemophilus influenzae*, $\sigma^E$ helps the bacteria survive inside macrophages (8). $\sigma^E$ is important for growth of *Vibrio cholerae* under ethanol stress as well as its colonization of the intestine (24).

The best characterized $\sigma^E$ regulation system is in *E. coli* (1). In this organism, $\sigma^E$ is regulated by RseA, an anti-sigma factor located in the inner membrane whose cytoplasmic domain binds tightly to $\sigma^E$, preventing its interaction with RNA polymerase (9, 34). $\sigma^E$ is also regulated by RseB, a periplasmic protein that binds to RseA and protects it from degradation (6, 16). During certain stress responses, accumulation of
outer membrane proteins activates the sequential degradation of the periplasmic domain of RseA by two inner
membrane proteases, DegS and RseP (YaeL) (2, 3, 46). The ClpXP protease degrades the cytoplasmic RseA-
N-terminus (11), freeing σE to associate with core RNA polymerase and direct transcription of its regulon.
Some other bacteria, especially many beta and gamma proteobacteria, have been found to utilize ECF sigma
factors and their regulatory modules related to E. coli σE system, supported by homologous sequences,
conserved gene contexts, and, in some cases, overlapping regulatory mechanisms (4, 20, 29).

We recently identified a genetic module similar to the E. coli rpoE-rseA-rseB system in Bordetella
bronchiseptica. B. bronchiseptica is closely-related to B. pertussis and B. parapertussis (36), the causative
agents of whooping cough in humans (31). This bacterium causes a wide range of diseases in various
mammals, including kennel cough in dogs, atrophic rhinitis in swine, snuffles in rabbits and asymptomatic
colonization in the upper respiratory tracts of many mammals (31). The rseA homologue of B.
bronchiseptica, BB3751, has some homology to E. coli rseA (amino acid sequence similarity 33.3%); while
the rseB homologue, annotated as mucB, is more conserved (amino acid sequence similarity 40.3%). It is
worth-noting that most of the homology between BB3751 and E. coli rseA is in the region important for
binding σE (Barchinger S.E. unpublished data). B. bronchiseptica SigE and E. coli σE share ~75% amino acid
sequence similarity. In fact, SigE can initiate transcription from an E. coli σE-dependent promoter,
demonstrated by an in vitro multi-round transcription assay and an in vivo reporter assay (Chapter 7,
Barchinger S.E. unpublished data). SigE can complement a deletion of the E. coli rpoE gene (Barchinger
S.E. unpublished data), which is essential for E. coli viability (10), further supporting SigE being a σE-like
sigma factor. The regulatory relationships appear to be conserved for the B. bronchiseptica proteins,
demonstrated by cloning the B. bronchiseptica putative regulators, BB3751 and mucB, along with sigE under
the control of an IPTG-inducible promoter on a plasmid, in a strain with a chromosomally-encoded lacZ
reporter of E. coli σE activity. Overexpression of BB3751 with sigE reduces reporter activity compared to
overexpression of sigE alone. Overexpression of sigE, BB3751 and mucB reduces the reporter activity to
basal levels (Barchinger S.E. unpublished data). The cytoplasmic domain of BB3751 is sufficient to inhibit B.
bronchiseptica SigE, demonstrated by an in vivo reporter assay and an in vitro multi-round transcription assay
(Barchinger S.E. unpublished data). Taken together, these data indicate that BB3751 and mucB encode
negative regulators of SigE in *B. bronchiseptica*; and we have designated these genes *rseA* and *rseB*, respectively.

Previous work has identified roles for SigE in *B. bronchiseptica* stress responses and during infection (Chapter 7). Unlike in *E. coli* strain K-12 (10), *sigE* is not essential in RB50. However, *B. bronchiseptica* lacking *sigE* are more sensitive to heat and ethanol stress, and agents that affect the integrity of the cell envelope, such as SDS and some β-lactam antibiotics. This is consistent with a predicted role for SigE in cell envelope maintenance, similar to the *E. coli* system. We have also deleted the genes encoding both of the negative regulators, *rseA* and *rseB*, in *B. bronchiseptica* strain RB50, to constitutively activate SigE. RB50Δ*rseAB* is more resistant to detergents (SDS) and β-lactam antibiotics (ampicillin, mecillinam, meropenem) than RB50 (Barchinger S.E. unpublished data). Survival after 18h of growth in liquid culture at 45°C was increased ~100-fold for RB50Δ*rseAB* compared to RB50, indicating a role of the SigE system in response to heat shock. This provides further evidence that SigE is important for promoting survival during cell envelope stress. Moreover, SigE is required for causing lethal systemic infection in mice lacking B and T cells (Chapter 7), indicating that it might be important for coping with certain stress during infection.

Compared to σE, there are fewer reports on the roles of the negative regulators during infection. In *P. aeruginosa*, mutations in *rseA* homologue, *mucA*, or *rseB* homologue, *mucB*, lead to the alginate-overproducing mucoid phenotype, associated with the establishment of lethal disease in CF patients (29, 30). This is opposite the situation for *Vibrio vulnificus*, in which the *rseB* mutation causes decreased expression of extracellular polysaccharides and decreased virulence (4). Similarly, in *Actinobacillus pleuropneumoniae*, a strict respiratory pathogen of swine, inactivation of *rseA* (*mlcA*) attenuates the virulence in porcine lungs (43). The roles of *rseA/rseB* during *B. bronchiseptica* infection have not been investigated prior to this study.

In a murine model of respiratory infection, RB50Δ*rseAB* colonized the lower respiratory tract (LRT) of C57BL/6 mice less efficiently than did RB50. Adoptively-transferred antibodies generated from RB50Δ*rseAB*-infected mice were similar to antibodies against RB50 in efficiently reducing RB50Δ*rseAB* numbers. This strain still colonized mice lacking B and T cells at lower levels, suggesting that constitutive SigE activity may disrupt virulence mechanisms targeting some innate immune factors. RB50Δ*rseAB* was as resistant as RB50 to complement-mediated killing, but was less cytotoxic to macrophages. In addition,
RB50ΔrseAB failed to cause lethal diseases in mice lacking TLR4. Although large numbers of bacteria colonized the LRTs of these mice, unlike RB50 causing severe pathology, RB50ΔrseAB appeared to cause little pathology in TLR4\textsuperscript{def} mice. These data established the importance of proper control of SigE activity during \textit{B. bronchiseptica} infection.
Materials and Methods

Bacterial strains and growth. Experiments were performed using the previously described \(B. bronchiseptica\) strain RB50 (7), an isogenic mutant lacking O-antigen, RB50\(\Delta\)wbm (38), a TTSS mutant of RB50 lacking \(bscN\) (WD3) (50), \(B. bronchiseptica\) strain AVS lacking TTSS and adenylate cyclase toxin (25), RB50 lacking \(sigE\) (Chapter 7), and an isogenic mutant lacking \(BB3751\) (\(rseA\)) and \(mucB\) (\(rseB\)), RB50\(\Delta\)rse\(AB\), which was constructed in this study and is described below. Bacteria were maintained on Bordet-Gengou agar (Difco) containing 10% defibrinated sheep blood (Hema Resources) and 20\(\mu\)g/mL streptomycin.

Construction of RB50\(\Delta\)rse\(AB\) strain. A left-flanking PCR product (5’AGTGAATTCCCCCTTCTTCAGC\(G\)TCTTTG\(G\)3’, 5’GATGGGATCCCAACAATAAGCCCGCAAAG\(G\)3’) with 636bp proximal to the \(sigE\) gene and a non-overlapping 767bp right-flanking product (5’ATTGGATC\(C\)CTTG\(C\)AGC\(A\)CTGC\(A\)CTC\(G\)3’, 5’GGAGAATCCG\(G\)GT\(G\)GTTAC\(A\)ATAGC\(G\)3’) were amplified from \(B. bronchiseptica\) RB50 genomic DNA with EcoRI and BamHI sites overhanging on the 5’, 3’ and 3’, 5’, respectively. The two flanking fragments were digested with BamHI and ligated. The resulting construct was PCR-amplified (5’AGTGAATTCCCCCTTCTTCAGC\(G\)TCTTTG\(G\)3’, 5’GGAGAATCCG\(G\)GT\(G\)GTTAC\(A\)ATAGC\(G\)3’), cloned into TopoTA vector (Invitrogen) and verified by sequencing. The deletion construct was then cloned into the EcoRI site of pSS4245 allelic exchange vector (Stibitz S. unpublished data), resulting in the deletion of the 1544bp central region of \(rseA\) and \(rseB\) with only 6bp of the 5’end of \(rseA\) and 9bp of the 3’end of the \(rseB\) gene remaining in place. Tri-parental mating with wild-type \(B. bronchiseptica\) strain RB50, \(E. coli\) strain DH5\(\alpha\) harboring the pSS4245\(\Delta\)rse\(AB\) vector, and DH5\(\alpha\) harboring the helper plasmid pSS1827 (45) was performed under modulating conditions (with 50mM MgSO\(_4\)) and incubated on modulating plates containing 100\(\mu\)g/mL kanamycin and 60\(\mu\)g/mL streptomycin to select for the integration of pSS4245\(\Delta\)rse\(AB\) into the \(B. bronchiseptica\) chromosome. \(Bordetella\) colonies on modulating plates were streaked onto plates without MgSO\(_4\) to activate the pertussis toxin promoter on the vector which drives the expression of the endonuclease SceI. This resulted in the cutting of the SceI site on the vector and approximately 50% of the resultant colonies on these plates had the wild-type \(rseA\) and \(rseB\) gene deleted from the chromosome by homologous recombination. The deletion
strain was verified by colony PCR (5’AGTGAATTCCCCCTTCTTCAGCGTCTTG3’, 5’GGAGAATTCGGGTCTCGGGTTACAAATAGC3’) and Southern blot analysis.

**Complement killing assay.** Complement killing assays were performed as previously described (14). Briefly, blood freshly collected from C57BL/6 mice was pooled, incubated at 4°C for 1 hour and centrifuged at 2000g for 10min. The serum was collected and diluted 1:5 in phosphate buffered saline (PBS). Complement-inactive serum was prepared by heating an aliquot of the diluted sample at 56°C for 30 min. From mid-log-phase cultures, approximately 500 CFU of RB50, RB50ΔrseAB or RB50Δwbm in 5 µl of PBS were incubated with 45µl of diluted serum or PBS for 1 hour at 37°C. Bacterial numbers before and after incubation were determined by plating and CFU counts. Each strain was run in triplicate.

**Cytotoxicity assay.** Cytotoxicity assays were performed as previously described (17). Briefly, J774 murine macrophage cells, were cultured in Dulbecco modified Eagle medium (DMEM) (HyClone) with 10% fetal bovine serum (FBS) (HyClone). The cells were grown to 80% confluency in 96-well tissue culture plates (Falcon) at 37°C in 5% CO₂. Cells were washed with RPMI (Mediatech) containing 5% FBS and bacteria were added in 50µL RPMI at a multiplicity of infection (MOI) of 15. After a 5 minute centrifugation at 250g, cells were incubated for the indicated time. The percent lactate dehydrogenase (LDH) release, a measure of cytotoxicity, was determined by using Cytotox96 Kit (Promega) according to the manufacturer’s protocol.

**Animal experiments.** C57BL/6, RAG1<sup>−/−</sup> (RAG<sup>−/−</sup>), C3H/HEN (TLR4<sup>surf</sup>) and C3H/HEJ (TLR4<sup>def</sup>) mice were obtained from Jackson laboratories. All mice were bred in our Bordetella-free, specific pathogen-free breeding rooms at The Pennsylvania State University. For inoculation, mice were sedated with 5% isoflurane (Abbott laboratory) in oxygen and 50µL of PBS containing 5×10<sup>5</sup> CFU of the indicated bacteria was pipeted onto the external nares (22). This method reliably distributes the bacteria throughout the respiratory tract (18). Survival curves were generated by inoculating TLR4<sup>def</sup> or RAG<sup>−/−</sup> mice with RB50 or RB50ΔrseAB. Mice suffering from lethal bordetellosis as determined by severe hunched posture, ruffled fur, extremely labored breathing and apathy were euthanized to prevent unnecessary suffering (28). For quantifying bacterial loads, mice were euthanized via CO₂ inhalation and lung, trachea, nasal cavity, spleen, liver and/or kidneys were excised. Tissues were homogenized in PBS, aliquots were serially diluted, plated, incubated at 37°C for 2 to 3 days, and CFU was determined. For immune serum collection, C57BL/6 mice were
vaccinated with RB50 or RB50ΔrseAB by intraperitoneally injecting 10^8 CFU heat-killed bacteria in 200µL PBS on days 0 and 14 and euthanized on day 28 to collect serum. For adoptive transfer, 200µL of pooled immune serum or serum from naïve animals was intraperitoneally injected before inoculation (23). Bacterial numbers in the lungs were quantified 12h post-inoculation. All protocols were reviewed by the university IACUC and all animals were handled in accordance with institutional guidelines.

**Lung pathology.** For analysis of lung pathology, mice were intranasally inoculated and euthanized as described above. The tracheas and lungs were excised and inflated with approximately 2 mL of 10% formaldehyde. The tissues were then sectioned and stained with haemolysin and eosin (H&E) at the Animal Diagnostic Laboratories Facility of The Pennsylvania State University. Sections were examined and scored by a veterinarian with training and experience in rodent pathology (M.J.K.) who was blinded to experimental treatment (25, 37). A score of 0 indicates no noticeable inflammation or lesions; a score of 1 indicates few or scattered foci affecting <10% of the tissue; a score of 2 indicates frequent mild perivascular and/or peribronchial lymphoid aggregates, with overall inflammation affecting no more than 10 to 20% of the tissue; a score of 3 indicates moderate lesions, typically with abundant perivascular and peribronchial lymphoid infiltrates, with inflammation affecting ~20-30% of the tissue; and a score of 4 indicates extensive pneumonia and marked inflammation affecting >30% of the tissue; a score of 5 indicates extensive lesions with >50% of the tissue affected. If a severity falls between categories, 0.5 was added to the pathology score of the lower category.

**Statistical analysis.** The mean +/- standard error (SE) of the geometric mean was determined for all appropriate data and expressed as error bars. Two-tailed, unpaired Student’s T-tests were used to determine statistical significance between groups.
Results:

RB50ΔrseAB does not efficiently colonize or persist in the lower respiratory tracts of C57BL/6 mice.

rseA and rseB, except for 6bp on the 5’ end of rseA and 9bp on the 3’ end of rseB, were deleted from the RB50 chromosome using an allelic exchange strategy, resulting in the isogenic rseA and rseB-deficient strain of RB50, RB50ΔrseAB. The generation time of RB50ΔrseAB (73.14±0.17min) is longer than its parental strain RB50 (63.26±0.08min) under optimal, non-stress growth conditions: 37°C in Stainer-Scholte broth. To evaluate the impact of constitutive SigE activity on infection, C57BL/6 mice were inoculated with 5×10⁵ CFU RB50 or RB50ΔrseAB in 50μL PBS and euthanized on the indicated days post-inoculation to evaluate bacterial loads in the lungs, tracheas and nasal cavities. RB50 colonized all respiratory organs efficiently and grew rapidly to millions of CFUs in the nasal cavities and lungs during the first week of infection and bacterial numbers declined thereafter (Fig. 8.1, squares). RB50 was cleared from the tracheas and lungs around day 60, although the nasal cavity remained colonized (Chapter 7). Although the rseA and rseB double deletion strain retained its ability to efficiently colonize and persist in the nasal cavity, it was observed at lower numbers than RB50 as early as day 3 post-inoculation and was cleared earlier from the lower respiratory tracts (LRT) (Fig. 8.1). These data indicate the importance of proper regulation of SigE activity in combating some immune mediated clearance mechanisms, perhaps unique in the LRT.
Serum antibodies from RB50ΔrseAB-vaccinated animals are efficient in reducing bacterial numbers in vivo.

Deletion of rseA and rseB appears to change the profile of outer membrane proteins in B. bronchiseptica (Barchinger S.E. unpublished data), which may result in altered antigen profile. These observations led us to hypothesize that the antigens exposed on the surface of RB50ΔrseAB might be more immunogenic and thus antibodies generated against them might be more efficient in clearing this bacterium.

To test this, immune serum was collected from RB50- or RB50ΔrseAB-vaccinated C57BL/6 mice. These sera or naïve sera were adoptively transferred to C57BL/6 mice at the time of RB50ΔrseAB inoculation. Mice were sacrificed 12h later to enumerate bacterial loads in the respiratory tract. Compared to the naïve serum, both types of immune serum had no effect in the nasal cavity (Fig. 8.2). Immune serum from either strain rapidly reduced RB50ΔrseAB numbers in the LRT to a similar extent (Fig. 8.2). These data indicate that antibodies generated in RB50ΔrseAB-vaccinated animals are similarly effective in vivo compared to RB50-specific antibodies, and suggest that the quicker clearance of RB50ΔrseAB may not be attributable to differential antibody functions.

**RB50ΔrseAB failed to cause lethal infection in RAG^- mice.**

The defect of RB50ΔrseAB in colonization of the LRTs of C57BL/6 mice was more pronounced during the later stages of infection (Fig. 8.1). We thus hypothesized that virulence mechanisms disrupted in RB50ΔrseAB might target host adaptive immune responses. If RB50ΔrseAB were particularly defective in resisting the adaptive immunity, then deleting adaptive immunity would affect the phenotypes of RB50ΔrseAB relative to that of RB50. To test this hypothesis, RAG^- mice, lacking B and T cells, were inoculated with 5×10^5 CFU of RB50 or RB50ΔrseAB and monitored for survival. As we have shown
previously (Chapter 7), RAG<sup>−/−</sup> mice succumbed to RB50 infection by day 35 post-inoculation (Fig. 8.3A, squares). In contrast, the rseAB deficient strain failed to cause lethal infection in these animals, which remained healthy until the end of the experiment (day 100 post-inoculation) (Fig. 8.3A, triangle). To explore why RB50<sub>ΔrseAB</sub> failed to cause lethal infection in RAG<sup>−/−</sup> mice, in a separate experiment, RAG<sup>−/−</sup> mice were inoculated with RB50 or RB50<sub>ΔrseAB</sub> and euthanized on day 21 post-inoculation to enumerate bacterial loads in various organs. Although RB50<sub>ΔrseAB</sub> colonized nasal cavities similarly to RB50, about two orders of magnitude more RB50 was recovered from lungs and tracheas of RAG<sup>−/−</sup> mice than RB50<sub>ΔrseAB</sub> (Fig. 8.3B), indicating that RB50<sub>ΔrseAB</sub> is defective in colonization of the LRTs of mice lacking adaptive immunity. These data suggest that innate immune factors are responsible for the defect of this mutant in colonization of the LRTs.

**RB50ΔrseAB is not susceptible to complement-mediated killing.**

It has been previously determined that the decreased virulence of the *V. vulnificus* rseB mutant is associated with its sensitivity to complement due to the apparent lost of extracellular polysaccharides (4). Among the innate immune defense mechanisms, we first hypothesized that RB50<sub>ΔrseAB</sub> might show decreased resistance to complement-mediated killing. To test this, 500 CFU of RB50 or RB50<sub>ΔrseAB</sub> were incubated with 20% of complement-active or complement-inactive serum from a naïve mouse at 37°C for 1 h. Since RB50<sub>Δwbm</sub> is susceptible to complement-mediated killing; this strain was incubated with the same serum as a positive control for complement activity (5). RB50<sub>Δwbm</sub> survived the complement-inactive serum treatment but was almost completely killed when treated with complement-active serum, indicating that in
this assay, complement is active (Fig. 8.4, dashed bars). The percent survival of RB50 or RB50ΔrseAB in PBS, PBS containing complement active or inactive serum were comparable, indicating that RB50ΔrseAB is as resistant to complement as wild-type bacteria. These data indicate that constitutive active SigE does not alter serum resistance of*B. bronchiseptica* and suggest that complement-mediated killing might not be responsible for the quick reduction of RB50ΔrseAB numbers.

**RB50ΔrseAB** is less cytotoxic to murine macrophages.

*B. bronchiseptica* is cytotoxic to macrophages and the Type Three Secretion System (TTSS) contributes to this cytotoxicity (50). Since previous study indicates that SigE might directly or indirectly regulate expression of some TTSS related genes (Chapter 7), we hypothesized that constitutive SigE activity might affect the cytotoxicity of *B. bronchiseptica* to macrophages. To test this, J774 murine macrophages were incubated with media or media containing RB50, RB50ΔrseAB, RB50 lacking TTSS (WD3), or RB50 lacking both TTSS and adenylate cyclase toxin (AVS) at a MOI of 15 for up to 4h. Cytotoxicity was measured according to LDH release. Similar to the media-treated group (Fig. 8.5, cross), the negative control strains, WD3 and AVS, had caused little cytotoxicity even by 4h incubation (Fig. 8.5, diamond and circle). Wild-type RB50 had caused ~20% and ~85% cytotoxicity by 3h and 4h incubation, respectively (Fig. 8.5, square). However,
RB50ΔrseAB caused significantly less cytotoxicity: ~20% by 4 hours incubation (Fig. 8.5, triangle). These results indicate that RB50ΔrseAB is less cytotoxic to macrophages.

RB50ΔrseAB failed to cause lethal infection in TLR4 deficient mice.

Since the decreased colonization of RB50ΔrseAB was observed as early as day 3 post-inoculation (Fig. 8.1), and since this mutant was still defective in colonizing hosts lacking adaptive immune responses (Fig. 8.3), we hypothesized that the proper regulation of SigE activity might be important for B. bronchiseptica to resist these innate immune factors.

To test this, we challenged mice lacking a key component of the innate immunity, TLR4, with RB50 or RB50ΔrseAB, and monitored these mice for survival. Consistent with previous findings, RB50-challenged TLR4-def mice developed severe bordetellosis and succumbed 2 to 5 days post-inoculation (26, 28). In contrast, RB50ΔrseAB-challenged TLR4-def mice did not display any sign of diseases during the 100-day period, and were euthanized at the end of the experiment (Fig. 8.6A). Since RB50-induced bordetellosis is associated with overwhelming bacterial loads and severe inflammatory pathology (26), we sought to determine whether these disease parameters are absent in RB50ΔrseAB-challenged TLR4-def mice. Groups of TLR4-def or TLR4-def mice were challenged with RB50 or RB50ΔrseAB and euthanized 3 days later to enumerate bacterial loads in the respiratory organs and to evaluate histopathology damage in the LRT. RB50 and

Figure 8.6: RB50ΔrseAB does not cause lethal infection in TLR4-def mice. (A) TLR4-def mice were inoculated with 5×10^5 CFU of RB50 (square) or RB50ΔrseAB (triangle) and monitored for survival. TLR4^+^ (+) or TLR4^−^ (-) mice were inoculated with 5×10^5 CFU of RB50 (black bar) or RB50ΔrseAB (white bar) and sacrificed on day 3 post-inoculation. (B) Bacterial numbers in the indicated organs are expressed as mean Log_{10}CFU ± SE. The limit of detection is indicated by the y axis. (C) Histology pathology scores of H&E-stained lung sections are shown. The line represents the mean pathology score. ** indicates P≤0.01. Mary J. Kennett contributed to the experiment shown in Fig. 8.6 C.
RB50ΔrseAB colonized at similar levels in the nasal cavities of both TLR4<sup>surf</sup> and TLR4<sup>def</sup> mice (Fig. 8.6B). In the LRT of TLR4<sup>surf</sup> mice, the average loads of RB50ΔrseAB, as well as the histopathology damage caused by this mutant, appeared to be lower than those of RB50, but these comparisons did not reach statistical significance (Fig. 8.6B, C). In the TLR4<sup>def</sup> mice, significantly less RB50ΔrseAB was recovered from lungs, spleens and kidneys compared to RB50 (Fig. 8.6B). Interestingly, although large numbers of RB50ΔrseAB (~10<sup>7</sup> CFU) colonized the LRT of these mice, unlike wild-type RB50, these bacteria did not appear to cause much histopathology damage (Fig. 8.6C). Taken together, these data indicate that RB50ΔrseAB fails to cause lethal disease in TLR4 deficient mice, which was associated with decreased lung colonization, decreased systemic colonization and less histopathology damage.
Discussion:

ECF sigma factors, such as $\sigma^E$, have been increasingly implicated in stress responses and virulence in many bacteria, including *Salmonella enterica*, *V. cholerae* and *P. aeruginosa* (24, Rowley, 2006 #6, 49). The activity of $\sigma^E$ is usually tightly regulated by its negative regulators, for example, the well-characterized *rseA-rseB* system in *E. coli* (1). Whether similar regulatory mechanisms are shared by other bacterial species and whether these regulators play a role during infection are less understood. We identified a similar genetic module to *E. coli rpoE-rseA-rseB* system in the respiratory pathogen, *B. bronchiseptica*. Moreover, we demonstrated that *rseA* (*BB3751*) and *rseB* (*mucB*) encode negative regulators of SigE, and are required for efficient colonization and persistence of *B. bronchiseptica* in murine hosts.

By binding tightly to the periplasmic domain of RseA, *E. coli* RseB inhibits the protease cleavage of RseA (6). Deletion of *rseB* in *E. coli* results in a two-fold increase in transcription from $\sigma^E$–dependent promoters, whereas deletion of *rseA* leads to a nine-fold increase (9, 33). Conversely, inactivation of either MucA (RseA) or MucB (RseB) in *P. aeruginosa* results in comparably large increases in AlgU ($\sigma^E$) activity (40, 42). These findings suggest that the relative contribution of RseB to regulating SigE activity varies between species. Reporter assays have shed some light on the relative regulatory roles for RseA and RseB in *B. bronchiseptica* SigE activity. A strain overexpressing *sigE* and *rseA* has decreased reporter activity compared to a strain overexpressing *sigE* alone. In the strain overexpressing *sigE, rseA* and *rseB*, beta-galactosidase activity is reduced to the same levels as a vector control, suggesting that RseB contributes substantially to the negative regulation of SigE in *B. bronchiseptica*. This study measured the combined effects of deletion of *rseA* and *rseB* on *B. bronchiseptica* virulence. Although we expect similar phenotypes of *rseA* single and *rseA/rseB* double mutant, future studies determining the role of RseB in SigE activity regulation in *B. bronchiseptica* and during infection can be accomplished by constructing and testing a *rseB* single deletion mutant.

It is intriguing that a strain lacking the negative regulators of SigE is more resistant to a lot of stressful conditions measured *in vitro*, including heat stress, perturbations by detergents and $\beta$-lactam antibiotics (Barchinger S.E. unpublished data), but is less fit during infection (Fig. 8.1). It is possible that constitutive SigE activity is beneficial when bacteria encounter the specific environmental hazards tested in
our *in vitro* experiments, while failure to appropriately shut off SigE activity during infection is instead deleterious to bacteria. When constitutively active, SigE might occupy the RNA core polymerase to prevent its engagement with other alternative sigma factors, preventing their regulon from being expressed at appropriate time and locations, and thus decreasing the fitness of *B. bronchiseptica* during infection. The presence of 12 genes encoding ECF sigma factors in *B. bronchiseptica* strain RB50 (44) is an indication of the need of this organism to express distinct groups of genes to adapt to different extracytoplasmic stress conditions, although their roles in *B. bronchiseptica* physiology and pathogenesis have not been investigated. It is also not understood what *in vitro* or *in vivo* conditions will induce SigE activity in *B. bronchiseptica*. Future studies using a SigE reporter system might address these questions.

There are two main possibilities to explain the attenuated virulence of the RB50Δ*rseAB* mutant. Because different profiles of outer membrane proteins were observed for RB50 and RB50Δ*rseAB* (Barchinger S.E. unpublished data), it is plausible that attenuation is due to a different profile of outer membrane proteins that may have been directly or indirectly affected by the *rseA* and *rseB* mutation. The second possibility is that the attenuation is due solely to the deleterious effects of overexpression of SigE and/or some member(s) of its regulon. Future experiments using isogenic RB50Δ*rseAB* strain lacking *sigE* might distinguish between these two possibilities.

RB50Δ*rseAB* grows slower than RB50 in optimal *in vitro* growth conditions for some undefined reasons. This raises the concern that this slower growth might lead to the observed defects of this mutant. Although we cannot rule out this possibility, several observations suggest that the defects may not be explained solely by slower growth. If decreased growth rate was the only reason why RB50Δ*rseAB* colonizes the host less efficiently, the bacterial numbers recovered throughout the respiratory tract would be decreased. However, RB50Δ*rseAB* colonizes efficiently in the nasal cavities of C57BL/6 (Fig. 8.1, 8.2), RAG−/− (Fig. 8.3) and TLR4<sup>−/−</sup> (Fig. 8.6) mice. Future experiments are needed to rule out the possibility that conditions in the nasal cavities favor the growth of RB50Δ*rseAB*. It is also plausible that some immune defense mechanisms unique to the LRT, instead of the slower growth rate, is responsible for the reduced numbers recovered from the LRT but not from the nasal cavities.
Although the lower colonization by RB50ΔrseAB compared to RB50 is more pronounced at the later stages of infection, adaptive immune factors might not be the targets of virulence determinants disrupted in RB50ΔrseAB. This is supported by efficient bacteria number reduction mediated by antibodies raised against RB50ΔrseAB (Fig. 8.2). This strain fails to cause lethal diseases in mice lacking B and T cells (Fig. 8.3), further indicating that some innate immune mechanisms might be the primary targets of virulence determinants lacking in RB50ΔrseAB.

RB50ΔrseAB survives incubation with complement active serum (Fig. 8.4), indicating that rseA and rseB are not required for resistance to complement-mediated killing or other serum anti-microbial components. RB50ΔrseAB is less cytotoxic to macrophages compared to RB50. This is contrary to our previous findings that RB50ΔsigE, the strain lacking SigE, also causes less cytotoxicity to macrophages, possibly due to decreased expression of TTSS related genes (Fig. 7.7). Although we cannot rule out the possibility that the lower cytotoxicity is due to slower growth of the RB50ΔrseAB mutant, it is also plausible that SigE activity negatively impacts cytotoxicity through TTSS dependent mechanisms. In line with this hypothesis is the previous finding that AlgU has negative effect on expression of TTSS in *P. aeruginosa* (48). qRT-PCR experiments measuring relative expression of genes contributing to cytotoxicity or experiments determining bacterial growth after exposure to macrophages may distinguish between these possibilities.

TLR4 and downstream signaling events are critical innate immune defense mechanisms against gram-negative bacteria including *B. bronchiseptica* (26, 27). TLR4\textsuperscript{def} mice challenged with 5×10\textsuperscript{5} CFU RB50 develop lethal diseases 2-5 days post-inoculation; whereas RB50ΔrseAB fails to cause lethal diseases in these animals (Fig. 8.6 A). This correlates with decreased systemic spread of RB50ΔrseAB as well as less histopathology damage caused by this bacterium (Fig. 8.6 B, C). These data suggest that TLR4 is not the primary target of virulence mechanisms disrupted in RB50ΔrseAB. In the respiratory tract of TLR4\textsuperscript{def} mice, RB50ΔrseAB grows to large numbers, which are higher than the loads of RB50 and RB50ΔrseAB in TLR4\textsuperscript{suf} mice (Fig. 8.6 B). However, RB50ΔrseAB induces less inflammatory pathology in TLR4\textsuperscript{def} mice compared to the pathology observed in TLR4\textsuperscript{suf} mice following infection by either the wild-type or mutant bacteria (Fig.
Thus some virulence mechanisms that induce inflammatory pathology appear to be disrupted in RB50ΔrseAB.

It has been observed in several bacterial pathogens that $\sigma^E$ is dispensable for virulence during infection but deletion of the negative regulators results in attenuated virulence. For instance, *V. vulnificus* rpoE deletion mutant is not significantly attenuated for virulence in immunocompetent mice (4). The negative regulator mutant of *V. vulnificus* lacking rseB, however, is attenuated in virulence (4). Similarly, in *A. pleuropneumoniae*, a strict respiratory pathogen of swine, inactivation of rseA (mlcA), but not rpoE, results in attenuated virulence in porcine lungs (43). RB50 lacking sigE colonizes wild-type mice as efficiently as RB50 (Fig. 7.4); whereas the mutant lacking the negative regulators, rseA and rseB, is attenuated in these mice (Fig. 8.1). These findings suggest that ECF sigma factors of RB50 may have overlapping functions and the others sigma factors might compensate for the lack of SigE. However, it is critical to ensure the tight regulation of these sigma factors activities during infection.
Authors and Contributions:

Xuqing Zhang\textsuperscript{1,2}, Sara E. Hester\textsuperscript{1,3,4}, Mary J. Kennett\textsuperscript{1}, and Eric T. Harvill\textsuperscript{1}

\textsuperscript{1}Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, 115 Henning Building, University Park, Pennsylvania 16802

\textsuperscript{2}Graduate Program in Genetics, The Pennsylvania State University, University Park

\textsuperscript{3}Department of Biochemistry, Microbiology, and Molecular Biology, The Pennsylvania State University, University Park

\textsuperscript{4}Graduate Program in Biochemistry, Microbiology and Molecular Biology, The Pennsylvania State University, University Park

Conceived and designed the experiments: XZ, SEH, ETH.

Performed the experiments: XZ, SHE, MJK.

Analyzed the data: XZ, SEH, MJK.

Wrote the paper: XZ, ETH.
References:


Chapter 9

Summary and Significance

*B. pertussis*, *B. parapertussis*, *B. holmesii*, and *B. bronchiseptica* are endemic respiratory pathogens in humans or other mammals. *B. pertussis* and *B. parapertussis* are both etiologic agents of whooping cough (36, 50), a resurging disease in developed countries despite high vaccine coverage (1, 14, 18, 66, 73). *B. holmesii*, a recently recognized *Bordetella* species, also causes whooping-cough like diseases in humans (75, 79). *B. bronchiseptica* is prevalent in companion and agricultural mammals (28), and is very closely-related to these human-adapted bordetellae (19, 56). This dissertation presented several aspects of interactions between these endemic bordetellae with the host, as well as cross-protection mediated by vaccines, aiming to not only address some basic scientific questions but also to provide implications for future vaccine design.

Implications

O-antigen and the emergence of *B. parapertussis* as a human pathogen

O-antigen is costly to produce in terms of energy and is a protective antigen of some bacterial species (41, 57, 58). Our research determined that O-antigen is a dominant surface antigen of *B. parapertussis*, efficiently inducing immunity that is protective (83) (Chapter 2). O-antigen must therefore confer some advantages to bacteria in order to be maintained. For example, the O-antigen of *Klebsiella pneumoniae* decreases macrophage activation, conveys resistance to neutrophil killing, and promotes persistent infection after the onset of bacteremia (44). O-antigen also protects *B. bronchiseptica* and *B. parapertussis* from complement-mediated killing in naïve animals (26, 59).

Our study suggested that O-antigen may favor the emergence of *B. parapertussis* as a human pathogen (Chapter 3). *B. parapertussis* diverged from a *B. bronchiseptica*-like progenitor some time later than *B. pertussis* (56). *B. parapertussis* likely invaded a population in which *B. pertussis* was endemic. Thus, this pathogen had to avoid *B. pertussis*-induced immunity to exploit the human hosts (8). Therefore, blocking binding and functions of *B. pertussis* infection- or vaccination-induced antibodies via O-antigen could have allowed *B parapertussis* to invade the human population and prosper in it (77, 84) (Chapter 3). Immune-
mediated competition with *B. pertussis* may thus be one selective pressure that causes *B. parapertussis* to maintain its O-antigen.

**Cross-protection by *B. pertussis* immunity against endemic bordetellae**

Cross-protection by closely-related pathogens is common. One well-known example is the cross-protection of cow pox immunity against small pox. However, pathogens may evolve to avoid cross-protection. For instance, evolution of the hemagglutinin and neuraminidase genes allows multiple influenza strains to co-circulate in one population (24). While protection by closely-related species is observed for a range of pathogens, this is not always the case for the bordetellae. The great majority of human populations have immunity to *B. pertussis* due to vaccination and/or natural infection. *B. bronchiseptica* is susceptible to *B. pertussis* immunity, possibly via antibody responses against two shared antigens, pertactin and filamentous hemagglutinin (27). Moreover, current *B. bronchiseptica* isolates from humans are sensitive to *B. pertussis*-induced immunity (27), suggesting that *B. bronchiseptica* may not be antigenically distinct enough to avoid *B. pertussis*-induced immunity. Although *B. parapertussis/B. holmesii* and *B. pertussis* do share cross-reactive antigens, *B. pertussis*-specific antibodies do not efficiently bind to *B. parapertussis* or *B. holmesii* (77, 83) (Chapter 3, 4). Therefore, *B. parapertussis* and *B. holmesii* are resistant to *B. pertussis*-induced immunity (77, 83) (Chapter 3, 4).

**Human health and vaccine design**

Long term epidemiology studies of *B. pertussis*, *B. parapertussis* and *B. holmesii* are essential to understand the relative roles of these pathogens in the resurgence of whooping cough. Current vaccines, consisting of only *B. pertussis* antigens, are effective against *B. pertussis*, but are ineffective against *B. parapertussis* and *B. holmesii* (17, 32, 33, 42, 49, 84) (Chapter 3, 4). This may confer a selective, immune-mediated advantage to these two pathogens. Indeed, *B. parapertussis* has been reported to cause a higher proportion of disease in vaccinated versus unvaccinated individuals (42), and in the age-group that is most recently vaccinated against *B. pertussis* (6, 40, 76). *B. holmesii* has been consistently isolated from patients in Massachusetts where the vaccine coverage is quite high (Chapter 4). These data may warrant efforts to design vaccines against these two human pathogens.
The immune response induced by *B. parapertussis* or *B. holmesii* does protect against subsequent challenge against itself (77, 83) (Chapter 2, 4). This raises the question: what antigens of *B. parapertussis* or *B. holmesii* induce a protective immune response? Our data indicate that O-antigen is a key protective antigen of *B. parapertussis* (83) (Chapter 2). Further research to identify other protective antigens of *B. parapertussis* or *B. holmesii* and inclusion of them in the vaccine would likely decrease the incidence of these two endemic bordetellae. *B. parapertussis* and *B. holmesii* can account for a substantial portion of whooping cough cases (9, 46, 61, 74, 79). Thus, introducing vaccines that are effective against these two pathogens would likely decrease the overall whooping cough incidence.

**Indispensable and distinct roles played by IL-1 and IL-6 signaling following *B. pertussis*-infection**

One of the key innate immune defense mechanisms is the recognition of pathogen-associated-molecular-patterns by pattern-recognition-receptors, such as Toll-like receptors. For gram-negative bacteria, the engagement of LPS with TLR4 leads to activation of signaling events that induce various cellular responses including secretion of cytokines. These cytokines thus engage with their receptors and mount downstream signaling events affecting various aspects of host defenses. Elucidating functions of these cytokine-mediated signaling pathways is critical to understanding the innate immune response. Since innate immune effectors also impact adaptive immune functions, studies on these innate immune cytokines may also reveal critical events regulating the switch from innate to adaptive immunity. Following *B. pertussis* infection, TLR4-deficient mice harbor more bacteria later during infection, which is associated with elevated cellular infiltration and pathology (35). *B. pertussis*-induced TNF-α, IL-1 and IL-6 production are dependent on TLR4 (35, 48). TNF-α deficient mice have increased *B. pertussis* loads and elevated inflammation in their lungs (78). We showed indispensible and distinct roles of IL-1 and IL-6 in immune responses to *B. pertussis*, as discussed below.

Mortality or morbidity associated with infectious diseases often occurs as a result of dysregulated inflammation. The mucosal surface of the respiratory tract is normally maintained sterile by the generation of strong immune responses. These responses must be strong enough to eliminate pathogens. They must also be resolved appropriately to limit any damage. This underscores a critical balance between host and pathogen that dictate the outcome of an infection. Intranasal inoculation of wild-type C57BL/6 mice with
approximately $5 \times 10^5$ CFU of \textit{B. pertussis} has no apparent effect on the health of the animal, despite the ability of this bacterium to efficiently colonize the respiratory tract and persist in the lungs for more than 30 days. However, a similar dose of \textit{B. pertussis} challenge in IL-1R$^{-/-}$ mice results in lethal infection, likely due to uncontrolled bacterial numbers in the respiratory tract, massive cellular infiltration, overwhelming pathology, as well as disseminated spread of the infection (Chapter 5). Compared to the wild-type cells, IL-1R-deficient dendritic cells or macrophages produce less IL-10 in response to \textit{B. pertussis}. Moreover, IL-1R$^{-/-}$ mice fail to increase antigen-specific systemic IL-10 responses in the later stages of infection (Chapter 5). Several clinical and experimental studies have shown that IL-10 depresses \textit{B. pertussis}-specific IFN-$\gamma$ production (23, 52). Higgins et al. have shown that TLR4-mediated innate IL-10 responses inhibit Th1 responses during \textit{B. pertussis} infection (35). Since IL-1R and TLR4 signaling share the adaptor molecule, MyD88, IL-1R signaling may be involved in inducing the IL-10 responses via similar signaling events. These data suggest that the decreased IL-10 responses in IL-1R$^{-/-}$ mice may be partially responsible for their elevated pro-inflammatory cytokine responses and uncontrolled inflammatory responses.

Although IL-6$^{-/-}$ mice harbor similar numbers of bacteria compared to wild-type mice during the first week following \textit{B. pertussis} inoculation, they harbor more bacteria later during infection, and show a delayed clearance (Chapter 6). These data implicate a role for IL-6, an innate immune effector, in adaptive immune responses against \textit{B. pertussis}. Indeed, we observed decreased \textit{B. pertussis}-specific antibody generation, decreased leukocyte accumulation in the lungs, as well as decreased T cell cytokine responses following \textit{B. pertussis} infection in IL-6$^{-/-}$ mice. These are consistent with increasing evidence showing the modulating role of IL-6 in adaptive immune functions (20, 37).

TNF-$\alpha$, IL-1 and IL-6 are all effector cytokines of TLR4 signaling (35, 48). Although each plays indispensable roles for efficient immune responses against \textit{B. pertussis}, they impact different aspects of the host immunity (78) (Chapter 5, 6). This highlights the complexity of immunity against \textit{B. pertussis}. TNF-$\alpha$ or IL-1R deficient mice survive the challenge of \textit{B. parapertussis} or a \textit{B. pertussis} strain lacking Ptx (78) (Chapter 5), suggesting that TNF-$\alpha$ and IL-1R are required to overcome the effects of this toxin. What these Ptx-associated effects are may warrant further investigation.

\textbf{Regulation of \textit{B. pertussis}-induced IL-17 responses by IL-6 and IL-1R}
IL-17-producing Th17 cells are distinct from Th1 or Th2 cells and have been shown to play pathogenic roles in autoimmune diseases (31, 39, 43, 55). IL-17 also contributes to host defenses against bacterial infections, including those induced by *Klebsiella pneumonia* (30), *Bacteroides fragilis* (15), *Streptococcus pneumonia* (85) and *Mycobacterium tuberculosis* (38). Some recent studies suggest that *Bordetella* infection may induce a Th17-polarized immune response. *B. bronchiseptica*-infected murine macrophages induce IL-17 production by T cells *in vitro* and a strong IL-17 response is detected by re-stimulated lung tissue from *B. bronchiseptica*-infected mice (64). Nasso et al. have recently shown that the genetically detoxified pertussis toxin induces the secretion of IL-17 by purified T cells, through crucial roles played by MAPK and IL-10 (54). Pertussis toxin contributes to the induction of IL-17 during *B. pertussis* infection in mice (5). IL-17 has been shown to promote macrophage killing of *B. pertussis* and the efficacy of a *B. pertussis* whole cell vaccine (34). Studies using the neutralizing antibodies have suggested the involvement of IL-17 in controlling *B. pertussis* numbers in the lungs (5).

Several cytokines have been implicated in Th17 cells development. IL-23 can differentiate T cells into Th17 cells (31, 39, 55). Transforming growth factor β (TGF-β) upregulates IL-23R expression and is critical for the commitment to Th17 development (47). IL-6 and TGF-β together induce the differentiation of Th17 cells from naïve T cells (7). IL-1β has been found to be essential for the differentiation of IL-17-producing human Th cells (2). Mouse models of arthritis and encephalomyelitis have also revealed the involvement of IL-1 in the generation of IL-17-producing cells (53, 70). The role for IL-6 or IL-1R in inducing IL-17 production during bacterial infection has not been previously described. In this dissertation, IL-6 is determined to be required for the efficient induction of antigen-specific splenic IL-17 response during *B. pertussis* infection (Chapter 6). Our data also showed that IL-1R is necessary for the local and systemic IL-17 responses during *B. pertussis* infection (Chapter 5). Although the roles for Th17 cells in immune responses against *B. pertussis* are not fully understood, this work demonstrated the involvement of IL-6 and IL-1R in inducing IL-17 responses following *B. pertussis* infection.

**Potential microbial infection following anti-cytokine therapies**

IL-1R and IL-6 are required for efficient control of *B. pertussis* infection, which raises the concern of exacerbating *B. pertussis* infection for patients undergoing anti-cytokine therapies. Blocking IL-1 secretion or
IL-1R mediated signaling is a common treatment for chronic inflammatory diseases, such as rheumatoid arthritis and Crohn’s disease (10, 29). Anti-IL-6 treatment has been proposed for patients with Castleman’s disease or rheumatoid arthritis (67, 80). These treatments are associated with increased incidence of respiratory infections (25). Based on our data (Chapter 5, 6), similar effects may be observed for B. pertussis infection.

**SigE system in B. bronchiseptica physiology and pathogenesis, regulation other than BvgAS**

The ability to communicate information about the changing environment and the state of the cell is critical to pathogenic bacteria, and is usually through signal transduction systems. One example of such systems is the BvgAS two-component system. BvgAS regulates the expression of many virulence-associated genes in classical bordetellae and is both necessary and sufficient for respiratory tract infections (16). Another well-established class of signal transduction systems is the extracytoplasmic (ECF) sigma factor. B. bronchiseptica strain RB50 is predicted to encode 12 ECF sigma factors (68). Although members of the iron starvation subfamily of ECF sigma factors have been reported to regulate expression of heme iron transport genes (60, 72), the functions of other ECF sigma factors in B. bronchiseptica physiology and pathogenesis have not been previously determined.

We focused on a genetic module in RB50, which encodes the E. coli σ^E-like sigma factor, SigE, and the putative negative regulators, RseA and RseB. By constructing the RB50ΔsigE and RB50ΔrseAB mutants and exposing them to various stress conditions, we determined that the B. bronchiseptica SigE system contributes to resistance to heat and ethanol stress, as well as cell envelope stresses posed by SDS and some β-lactam antibiotics. RB50ΔsigE colonizes and persists in wild-type mice similarly to wild-type bacteria, whereas RB50ΔrseAB colonizes the LRTs of these mice less efficiently and is cleared from the LRTs faster than the wild-type bacteria (Chapter 7, 8). In hosts lacking B and T cells (RAG^- mice), wild-type B. bronchiseptica establishes systemic colonization and survives additional stresses in the systemic organs. RB50ΔsigE, however, does not cause systemic and lethal infection in these mice (Chapter 7). Together, these findings indicate the role of SigE and the proper regulation of its activity in both physiology and pathogenesis of B. bronchiseptica.
Future Directions

Remaining questions on the roles of IL-1R or IL-6 in immunity against bordetellae

Disseminated colonization of \(B. pertussis\) outside of the respiratory tract is only observed in mice lacking certain immune functions. For example, systemic colonization of \(B. pertussis\) has been observed in IFN-\(\gamma\)R\(^{-/-}\) mice (45). Depletion of NK cells results in a marked reduction of IFN-\(\gamma\) in the lung that leads to disseminated spread of \(B. pertussis\) (12). We observed systemic spread of \(B. pertussis\) into blood, spleens and livers of the IL-1R\(^{-/-}\) mice (Chapter 5). Small numbers of \(B. pertussis\) are recovered from the livers of both wild-type and IL-1R\(^{-/-}\) mice on day 3 post-inoculation. However, the bacterium is consistently recovered from the livers of mice lacking IL-1R but not wild-type mice later during infection. This indicates some IL-1R-dependent clearance mechanisms in the liver. Future studies trying to understand this phenomenon and the broader questions related to the mechanisms of systemic spread of bordetellae may improve our understanding of \textit{Bordetella} pathogenesis.

IL-1R\(^{-/-}\) mice are not responsive to either IL-1\(\alpha\) or IL-1\(\beta\). We determined that these mice are more susceptible to \(B. pertussis\) infection. Future experiments using depletion antibodies specific for IL-1\(\alpha\) or IL-1\(\beta\), or using IL-1\(\alpha\)\(^{-/-}\) or IL-1\(\beta\)\(^{-/-}\) mice, may address which IL-1 is critical to host defenses against \(B. pertussis\). If IL-1\(\beta\) is required, we can further explore the role of caspase-1 in immune responses against \(B. pertussis\) since caspase-1 is required for the secretion and activation of IL-1\(\beta\) (21). IL-18, a critical cytokine for Th1 cell activation, is structurally similar to IL-1R ligands (71). Moreover, IL-18R is in the same TIR superfamily as IL-1R, sharing MyD88 as the downstream adaptor molecule (22). Future experiments using IL-18/IL-18R and/or MyD88 deficient mice will shed more light on the distinct roles of TIR superfamily receptor pathways during \(B. pertussis\) infection.

We have preliminary results indicating a role of IL-1R and IL-6 in host defenses against \(B. bronchiseptica\). Since \(B. bronchiseptica\) has an overlapping but distinct repertoire of virulence determinants compared to \(B. pertussis\), the interactions between IL-1R or IL-6 signaling pathway and this bacterium might be different from how it interacts with \(B. pertussis\). It will be interesting to determine what \(B. bronchiseptica\) factors induce IL-1 and IL-6 production during infection, what aspects of the immune responses are impaired
in IL-1R−/− or IL-6−/− mice following *B. bronchiseptica* infection, and how these differ from the situations during *B. pertussis* infection.

**Determine the conditions inducing SigE activity via reporter assay**

RB50ΔsigE behaves similarly to RB50 in wild-type mice (Chapter 7). This observation may indicate that SigE is not important in colonizing the immunocompetent hosts. Alternatively, redundant roles played by other ECF sigma factors may compensate for SigE functions. To distinguish between these two possibilities, we can develop a reporter assay through fusing SigE-dependent promoter sequence with a reporter gene. SigE-dependent promoter sequence can be identified by *in silico* approaches or by comparing 5’ RACE (Rapid Amplification of cDNA Ends) products from RNA generated in the presence and absence of SigE. Once the reporter assay is developed, we can examine whether SigE activity is induced by various stresses, and determine when and where SigE activity is induced during infection.

**Define SigE regulon in RB50**

Another approach to better understand the function of SigE is to determine its regulon. SigE has similar activity to σE, indicating that SigE-dependent promoters are similar to *E. coli* σE-dependent promoters (Chapter 7). We took the advantage of this observation and constructed a position-weight matrix using known *E. coli* σE-regulated promoter and scanned the *B. bronchiseptica* RB50 genome with this matrix (63) (Barchinger S.E., Nixon B.T., unpublished data). Using *in vitro* transcription assays, we have identified five sequences that are transcribed by holoenzyme reconstituted with purified SigE and *E. coli* core RNA polymerase *in vitro* (Barchinger S.E., unpublished data). These sequences are in the promoter regions of *fam*, which encodes the heat shock sigma factor, σ32, *hfq*, which encodes a small RNA-binding protein, *rseA*, *mucD*, a serine protease, and *BB3108*, encoding a hypothetical protein (Barchinger S.E., unpublished data). A second approach to identify SigE-dependent promoters is run-off transcription coupled to microarray analysis (ROMA) (13). A third approach is to identify the genes with increased expression in the strain lacking the negative regulators, RB50ΔrseAB, by microarray analysis. Once more SigE-dependent promoters are verified, a new PWM specific for SigE can be created to better predict the SigE regulon by *in silico* analyses. It will also be informative to compare these methods and discuss the advantages and disadvantages of each.
approach. By focusing on the genes identified by multiple approaches, we will also minimize the number of false positives.

**Determine functions of other ECF sigma factors in RB50**

*B. bronchiseptica* strain RB50 has twelve predicted sigma factors, six of which are conserved in other bacterial species [BB1837, BB2661, BB1302, BB3268, SigE (BB3752), BB3268] (Barchinger S.E., Ades S.E., unpublished data) (68). The genomic context of these sigma factors is also conserved, suggesting that their adjacent genes might be regulators of the sigma factor. Future studies on the roles of these sigma factors and their putative regulators in *B. bronchiseptica* will not only improve our understanding of physiology and pathogenesis of *B. bronchiseptica* but also shed light on the functions of their homologues in other bacterial species. Although three of the remaining sigma factors in RB50 do not share significant sequence conservation with other species, the other three are FecI-like sigma factors, which are predicted to be involved in iron scavenging (11, 60). Since iron scavenging is a critical aspect of bacterial physiology, these FecI-like sigma factors might be important for *B. bronchiseptica*.

**ECF sigma factors in other bordetellae**

The ECF sigma factors have an interesting pattern of conservation in *B. parapertussis* and *B. pertussis*. Future studies can explore questions on the evolution of these proteins and possibly relate these to functional changes during host adaptation. *B. parapertussis* retained all six conserved sigma factors, whereas *BB2661* was deleted from *B. pertussis* genome and homologue of *BB3268* in *B. pertussis* is a pseudogene. It is likely that the sigma factors lost or not functional in *B. pertussis* are required for interactions with the hosts specific for *B. bronchiseptica*. Sequences are available for several *Bordetella* genomes. With these and the knowledge of SigE regulon members in RB50, comparisons can be made to determine how many SigE regulon members are present in other *Bordetella* species and whether SigE-dependent promoter sequence can be found for these genes. In addition, the PWM can be used to search for regulon members in the other *Bordetella* genomes to determine if any additional genes have been added to the regulon.

**Proteolytic degradation of RseA in RB50**

In *E. coli*, the membrane proteases, DegS and RseP, initiate the proteolytic cascade to degrade RseA and thus release $\sigma^E$ (3, 4). Putative homologues of these membrane proteases in *B. bronchiseptica* are
identified (Barchinger S.E., Ades S.E., unpublished data), suggesting similar regulatory mechanism. In *E. coli*, an inducing stress, such as exposure to ethanol or high temperature, triggers the degradation of RseA (4). Future studies comparing RseA stability before and after exposure to these stresses will determine if this mechanism is conserved in *B. bronchiseptica*. Mutants of homologues of these proteases in *B. bronchiseptica* can be constructed to determine their roles in stress responses and during infection.

**Potential roles for SigE in regulating TTSS**

*B. bronchiseptica*, like several other gram-negative pathogens, expresses a TTSS (82). *In vitro* studies have implicated the TTSS in causing necrotic cell death or cytotoxicity to mammalian cells (69). The ability of *B. bronchiseptica* to establish persistent infection is dependent on TTSS, presumably due to modulation of cellular signaling pathways and subsequent cellular functions (62, 65, 81). We observed the impact of SigE system on cytotoxicity and the expression of some TTSS genes (Chapter 7, 8). We will be able to determine if this effect is direct or indirect once we determine if SigE regulon members include TTSS genes. BvgAS two-component system regulates TTSS secretion in *B. bronchiseptica* (82). *btrS*, encoding an ECF sigma factor, is necessary and sufficient for transcription of all *bsc* TTSS genes (51). BvgAS exerts control over the entire TTSS by regulating *btrS* (51). Future experiments are needed to determine the role, if any, SigE plays in the TTSS regulatory cascade.
References


# Appendix

## Appendix A: qRT-PCR primers for chapter 7

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<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>16S</td>
<td>TCAGCATGTCGCGGTGAAT</td>
<td>TGTGACGCGCGGTGTGTA</td>
</tr>
<tr>
<td><em>bsp22</em> (BB1617)</td>
<td>CGGCACGGGCGTCAT</td>
<td>GGTGTAAGGCACTTTCGAGTTCC</td>
</tr>
<tr>
<td><em>bcrH1</em> (BB1619)</td>
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<td>CAGCAGATAACGCGCTTCCA</td>
</tr>
<tr>
<td><em>bopD</em> (BB1620)</td>
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</tr>
<tr>
<td><em>bopB</em> (BB1621)</td>
<td>GCTCAATTCCGACGAGGCTAT</td>
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### Appendix B: Comparison of RB50ΔsigE to RB50 under various stress conditions

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<th>RB50ΔsigE</th>
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</thead>
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<tr>
<td>Ampicillin</td>
<td>10µg</td>
<td>Disk</td>
<td>more sensitive</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>10U</td>
<td>Disk</td>
<td>more sensitive</td>
</tr>
<tr>
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<tr>
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<td>Disk</td>
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<td>Deoxycholate</td>
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<td>Ethanol</td>
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<td>Culture</td>
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<td>Kanamycin</td>
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</tr>
<tr>
<td>Mecillinam</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Mecillinam</td>
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</tr>
<tr>
<td>Meropenem</td>
<td>10µg</td>
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<td>=</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>30µg</td>
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</tr>
<tr>
<td>Paraquat</td>
<td>0.01%</td>
<td>Culture</td>
<td>=</td>
</tr>
<tr>
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<td>0.10%</td>
<td>Culture</td>
<td>=</td>
</tr>
<tr>
<td>Paraquat</td>
<td>2%</td>
<td>Disk</td>
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</tr>
<tr>
<td>Polymyxin B</td>
<td>0.36%</td>
<td>Culture</td>
<td>=</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>300iu/ie/ui</td>
<td>Disk</td>
<td>=</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>150µg</td>
<td>Disk</td>
<td>=</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>0.06M</td>
<td>Plates</td>
<td>=</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>0.1M</td>
<td>Plates</td>
<td>=</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>0.3M</td>
<td>Plates, Culture</td>
<td>=</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>0.6M</td>
<td>Plates, culture</td>
<td>=</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>2.0M</td>
<td>Plates</td>
<td>=</td>
</tr>
<tr>
<td>SDS</td>
<td>5%</td>
<td>Disk</td>
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</tr>
<tr>
<td>SDS</td>
<td>7.50%</td>
<td>Disk</td>
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<td>Disk</td>
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</tr>
<tr>
<td>SDS</td>
<td>15%</td>
<td>Disk</td>
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</tr>
<tr>
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<td>Disk</td>
<td>more sensitive</td>
</tr>
<tr>
<td>Sulfamethoxazole/Trimethoprim</td>
<td>23.75/1.25µg</td>
<td>Disk</td>
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</tr>
<tr>
<td>Tetracycline</td>
<td>30µg</td>
<td>Disk</td>
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</tr>
<tr>
<td>TritonX</td>
<td>175µg/µL</td>
<td>Disk</td>
<td>=</td>
</tr>
</tbody>
</table>
Vita
Xuqing Zhang

Education and Professional Positions:

The Pennsylvania State University  Ph.D. in Genetics, GPA 3.96  Fall 2005 – Spring 2010
• Graduate Research Assistant  (Advisor: Dr. Eric T. Harvill)
Fudan University, China  B.S. in Biological Sciences, GPA 3.55  Fall 2001 – Spring 2005
• Undergraduate Research Assistant  (Advisor: Dr. Long Yu)

Awards and Honors:

• University Graduate Fellowship ($18,000)
The Pennsylvania State University, Graduate School  Fall 2005 – Spring 2006
• Interdisciplinary Seed Grant Award ($5000)
The Huck Institute/Center for Network Analysis  Summer 2007
• Travel Grant
  College of Agricultural Sciences ($500)  Fall 2006
  Genetics Program ($500)  Fall 2006
  8th International Bordetella Symposium ($750)  Fall 2006
  College of Agricultural Sciences ($500)  Fall 2007

Selected Presentations at Professional Meetings:

• Eighth International Symposium of the Genus Bordetella  November 2006
  Institut Pasteur, Paris, France
  Poster: SigE in B. bronchiseptica gene expression and in vitro stress
• Cambridge Bordetella workshop  July 2008
  University of Cambridge, Cambridge, UK
  Oral presentation: O-antigen and B. parapertussis evasion of B. pertussis vaccines
• Bordetella Group reception for 99th general ASM meeting  May 2009
  Philadelphia, USA
  Poster: O-antigen allows Bordetella parapertussis to evade a B. pertussis vaccine via blocking binding of cross-reactive antibodies.

Publications: