CONTRIBUTION OF FIMBRIA AND FILAMENTOUS HEMAGGLUTININ IN *BORDETELLA* ADHERENCE AND IMMUNE SUPPRESSION

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ABSTRACT

Erich Vaughn Scheller: Contribution of fimbria and filamentous hemagglutinin in *Bordetella* adherence and immune suppression
(Under the direction of Peggy A. Cotter)

Pertussis, or whooping cough, is a highly contagious respiratory disease caused by *Bordetella pertussis.* Recent changes to the vaccine against *B. pertussis* have led to the re-emergence of this disease. Given the re-emergence of pertussis, it is critical to understand *Bordetella* pathogenesis. Fimbria and filamentous hemagglutinin (FHA), two proposed bacterial adhesins and components of the current vaccine, have been shown to be important virulence factors required for bacterial colonization of the lower respiratory tract of rats. *Bordetella* fimbria (Type I pili) are generally considered to function as adhesins despite a lack of supporting experimental evidence. FHA is a large exoprotein that has been shown critical for *Bordetella* adherence. We developed a novel *in vivo* adherence assay that confirmed FHA functions as an adhesin and we demonstrated a role for fimbria in adherence to ciliated respiratory epithelium. We further established that fimbria are required for bacterial persistence in the lower respiratory tract of mice and that fimbria contribute to suppression of the immune response. We also observed that wild-type and FHA-deficient bacteria induced cellular infiltrate around the major airways of the lungs and that FIM-deficient bacteria induced cellular infiltrate in the alveoli. A bacterial localization assay established that WT and FHA-deficient bacteria localized to the major airways and that FIM-deficient bacteria localized to the alveoli. Together, these findings suggest that fimbria mediate the first critical step of localizing the bacteria to the ciliated

epithelium of the major airways, and that FHA may mediate tight adherence to these cells. We also demonstrated that SCID mice, which contain functional neutrophils and macrophages but lack B and T-cells, are unable to clear FIM-deficient bacteria from the lungs. B and T-cells adoptively transferred in to SCID mice prior to inoculation partially restored clearance of FIM-deficient bacteria from the lungs. Collectively, our data suggest that fimbria and FHA mediate critical adherence steps necessary for *Bordetella* to colonize and to modulate the immune system and that B and/or T-cells are required to mediate the initial host response to control *Bordetella* infection.

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CHAPTER 1. INTRODUCTION

Reemergence of Pertussis

Pertussis, or whooping cough, is a highly contagious respiratory disease that is transmitted by human to human contact (1). The causative agent is the Gram-negative bacterium *Bordetella pertussis,* which accounts for the majority of cases of whooping cough. *Bordetella parapertussis* and *Bordetella bronchiseptica*, two closely related species of *B. pertussis,* account for the minority of cases (2, 3). Typically, pertussis affects young children and infants, though affects older individuals as well (4).

Pertussis disease is characterized by three phases: the catarrhal phase, the paroxysmal phase and the convalescent phase (5) . During the catarrhal phase, the bacteria presumably adhere to the respiratory epithelium of the upper respiratory tract and survive innate host responses (6, 7). During this phase, symptoms are similar to those of the common cold. After 1-2 weeks, the disease progresses to the paroxysmal phase. During this phase, which can last for up to 10 weeks, the characteristic 'whoops' occur, as well as severe spasmodic coughing episodes. During the convalescent phase, the adaptive immune response leads to bacterial clearance, however symptoms often persist for a month or longer (5).

In young children (1-9 years old), this disease, while distressing and uncomfortable, is not typically fatal. For infants, however, pertussis can often be life threatening, as the bacteria may disseminate into the lungs and cause pneumonia (7). Once in the lungs, the infection may lead to necrotizing bronchiolitis, intra-alveolar hemorrhage and fibrinous exudate.

Lymphocytosis may also occur, which is an influx of lymphocytes in to the bloodstream (7). This influx has been shown to correlate positively with pulmonary hypertension, respiratory failure and death (7).

Given the highly contagious nature of pertussis, as well as the severe threat to infants, a whole-cell pertussis (wP) vaccine was introduced in the 1940s. This vaccine quickly reduced the incidence of pertussis cases per year in the United States. While the wP vaccine was successful, there were unfounded concerns regarding its safety, and public pressure lead to the replacement of the wP vaccine with an acellular component vaccine (aP) in the United States and other developed nations in the 1990s (8). The aP vaccine contains secreted *Bordetella* virulence factors, such as pertussis toxin, filamentous hemagglutinin, pertactin and fimbrial subunits (9, 10). Since the introduction of the aP vaccine, pertussis cases have been increasing, with reported cases reaching numbers similar to what was seen in the 1950s (11). Recent studies indicate that the incidence of pertussis is significantly higher in children vaccinated with only the aP vaccine compared to those that receive even just one dose of the wP vaccine (12). Given the re-emergence of pertussis, it is important to further the understanding of *Bordetella* pathogenesis in order to develop better vaccines and treatment strategies to reduce morbidity and mortality.

Bordetella **phylogenomics**

The 'classic' *Bordetellae* (*B. pertussis, B. parapertussis* and *B. bronchiseptica*) are the three species known to cause disease in mammals (13). Phylogenetic studies indicate limited genetic diversity between these three species and suggest that *B. pertussis, B. bronchiseptica* and *B. parapertussis* are subspecies that have independently evolved from a *B. bronchiseptica*-like ancestor (2, 3). These data are intriguing, given the differences in host range of these subspecies:

B. bronchiseptica causes chronic colonization in a wide-range of mammalian hosts, while *B. pertussis* and *B. parapertussis* are strict human pathogens that cause acute respiratory disease (14). Whole genome sequence comparisons indicate that both *B. pertussis* and *B. parapertussis* underwent gene loss and accumulated insertion sequence elements, events that may have limited the host range of *B. pertussis* and *B. parapertussis* (2, 15, 16). Importantly, many of the known *Bordetella* virulence factors of *B. bronchiseptica, B. pertussis* and *B. parapertussis* are almost identical and functionally interchangeable (17). Given these similarities, and the fact that *B. bronchiseptica* infects a broad mammalian host range, *B. bronchiseptica* is often used as a model organism as it allows *in vivo* studies using laboratory animal hosts (17–20).

Animal Models

Given that *B. pertussis* is a human-restricted pathogen, one of the challenges of studying the pathogenesis of *B. pertussis* is developing models that reflect human disease. Using rodent models, such as mice, rabbits, rats and guinea pigs, none of which are natural hosts of *B. pertussis,* high numbers of *B. pertussis* must be delivered directly to the lungs in order to establish infection (13). Once the lungs are colonized, the bacteria will multiply for a week post-inoculation but are eventually cleared. During this time, the host animal will not mount a robust pertussis-specific immune response (13). Furthermore, these animals do not present classical whooping cough symptoms, and attempts to test transmission have not been fruitful. Taken together, these data suggest that rodent models using *B. pertussis* are not an ideal model system.

The most recent advance in modelling human pertussis has been the development of a baboon (*Papio Anubis*) model (21). When *B. pertussis* is inoculated into the nasopharynx of weanling baboons, the baboons will develop low-grade fever, paroxysmal coughing,

lymphocytosis and produce pertussis-specific protective antibodies (21). Additionally, the baboon model allows for study of transmission, as infected baboons have been shown to transmit to uninfected baboons by both contact and aerosols (22). Most importantly, this model has provided insights into failings of the current aP vaccine, as data suggest that the aP vaccine can protect from disease but not colonization or transmission (23).

While the baboon model provides a promising model for *B. pertussis* colonization, pathogenesis and transmission, using baboons is prohibitively expensive and requires animal facilities capable of housing and caring for the animals. As such, rodent models still provide a useful model system, and our lab and others have utilized *B. bronchiseptica*, rather than *B. pertussis*, as our pathogen in this model. As mentioned previously, *B. bronchiseptica* and *B. pertussis* share similar and functionally interchangeable virulence factors, but *B. bronchiseptica* will naturally colonize rodents (17, 24). While rodents do not present pertussis disease symptoms, they are natural hosts of *Bordetella*, providing insight to the contribution of virulence factors for colonization and persistence during a 'natural' infection. This dissertation will focus on using *B. bronchiseptica* in a mouse model of colonization and persistence, which allows for understanding the contribution of virulence factors to bacterial survival, as well as tools to understand the host response to infection.

Bordetella adhesins: Fimbria

B. pertussis, B. bronchiseptica and *B. parapertussis* all produce type I pili, also known as fimbria (FIM). Pili are non-flagellar, multi-subunit surface structures typically involved in adherence to other bacteria, host cells or environment surfaces (25, 26). Pili are characterized by their assembly pathways, with Type I pili (fimbria) being assembled by the usher-chaperone

pathway. Other pili groups include Type IV pili, curli pili, and 'alternative chaperone-usher pathway' (27). The Type I pili systems have been the most extensively characterized, with *Enterobacteriaceae* and uropathogenic *Escheria coli* (UPEC) being the model organisms for Type I pili (28). Type I pili consist of a rigid rod composed of pilin subunits extending from the bacterial surface with a flexible, adhesive tip (27). These pilin subunits are translocated across the cytoplasmic membrane by the sec translocation machinery (28). Once in the periplasm, these subunits will complex with the chaperone proteins (29). The chaperone-subunit complex is then targeted to the outer membrane usher protein (31, 32), where the complex then dissociates and the subunits are added to the base of the pilus by donor-strand exchange, a process by which the Ig-like folds of one pilin subunit interacts with the N-terminal extension of a second pilin subunit, non-covalently assembling the pilus (33–35). The usher protein also provides an anchor in the outer membrane for the growing pilus as the pilus is exported to the surface of the bacterium (36).

Pathogenic bacteria utilize pili to mediate adherence to mucosal surfaces (26). The tip adhesins often mediate this interaction (37), and this protein contains two domains, 1) the C-terminal Ig-like fold required for donor-stand exchange to assemble the pilus (34, 35) and 2) an N-terminal ligand binding domain (37). For example, the FimH adhesin of *E. coli* has been shown necessary for pathogenesis (38). FimH will bind to mannosylated receptors found on bladder epithelium (39). This binding then allows the bacteria to bind, invade and form intracellular communities and these interactions are necessary for the bacteria to survive during the acute stage of infection (40, 41). The roles of pili in bacterial pathogenesis of Gram-negative bacteria are broad, as these structures have been shown necessary for adherence, invasion, biofilm and inducing host cell signaling (Table 1). However, the common theme surrounding the

role of pili is that these structures mediate initial interactions during the early stages of infection that allow the bacteria to colonize and proliferate.

For the *Bordetella* spp., the fimbrial proteins are encoded by the *fimBCD* operon, with *fimB* encoding the chaperone, *fimC* encoding the usher, and *fimD* the putative adhesive tip (42). The major fimbrial subunits are encoded by *fim2* and *fim3* (43). These genes are located outside of the *fimBCD* operon, are capable of undergoing phase variation (43), and the respective proteins, Fim2 and Fim3, are components in the aP vaccine. There are additional, alternative major fimbrial subunits identified on the chromosome, such as *fimA, fimN* and *fimX,* however the expression of these alternative subunits is poorly understood (44–46). While there are no reported data showing that the fimbrial components are interchangeable between *B. pertussis* and *B. bronchiseptica*¸ the amino acid sequences are very similar. When comparing *B. pertussis* (Tohama I) and *B. bronchiseptica* (RB50), the FimD proteins are 95% identical, while Fim2 and Fim3 are 73% and 94% identical, respectively, and it is likely that the fimbria of *B. bronchiseptica* and *B. pertussis* play similar roles during infection (42, 47, 48).

Type I pili systems (and fimbria) of different bacterial species have been shown to be necessary components in mediating bacterial adherence (27), however *in vitro* studies using *Bordetella* lacking fimbria have produced varied results. Typically, to study adherence *in vitro*, bacteria will be centrifuged down on to various cell lines of interest (49). These cells will then be washed and the number of bacteria remaining will be determined. Using this method, a *B. pertussis* strain containing a TnphoA disruption of *fimD* was shown defective for adherence to monocytes (50). This mutant, however, was also defective for FHA production, and so the contribution of fimbria alone in this strain is unknown. A Δ*fimBCD* strain of *B. bronchiseptica*, which does not produce fimbria and is unaltered for FHA production, will still adhere to any cell

type of interest, including epithelial cells and macrophages, suggesting that in these *in vitro* assays, fimbria are not necessary for adherence (49). Assays using tracheal explants, however, suggest that fimbria may be required for adherence specifically to ciliated epithelial cells, as a *B. pertussis* strain defective for both FIM and FHA had an adherence defect to baboon tracheal explants (51), and *B. bronchiseptica* defective for FIM had decreased adherence to rabbit tracheal explants (6). Attempts to identify a receptor for FIM-mediated adherence showed that purified FimD protein adhered to monocytes (52), and purified Fim2 and Fim3 adhered to sulfated sugars (53). However, because these experiments used non-ciliated cell lines and purified proteins, the receptor for full length, native fimbria is still unclear as purified protein interactions may not be reflective of bacterial-associated fimbria.

While the role of fimbria in bacterial adherence is unclear, various animal models indicate an important role for these structures in bacterial colonization and persistence. To determine the role of virulence factors in colonization, a rat model, where a small volume inoculum is administered into the tip of the nose, has been used. In this model, WT *B. bronchiseptica* will colonize and persist in the nasal cavity and trachea indefinitely (49). When rats were inoculated with a *B. bronchiseptica* stain defective only for fimbria, the FIM-deficient bacteria colonized and persisted in the nasal cavity, but were unable to colonize the trachea (49). To determine if fimbria were required for the bacteria to migrate from the nasal cavity to the trachea, WT or FIM-deficient bacteria were inoculated directly into the trachea. The WT bacteria again were able to colonize and persist in the trachea and nasal cavity, however the FIM-deficient bacteria could not colonize or persist in the trachea, despite colonizing the nasal cavity (54). Together, these data suggest that fimbria are required to adhere to tracheal tissue, resist mucociliary clearance, and possibly to avoid innate immune effectors.

While the rat model of infection is useful to determine the role of virulence factors in colonization, our lab and others have utilized a mouse model to investigate the host response to *Bordetella* infection. This model uses a large volume, high number of CFUs inoculated intranasally. Using this method, bacteria are presumably deposited through the entirety of the respiratory tract. While this model has not been utilized to investigate the host response to bacteria lacking only fimbria, a study using a *B. pertussis* strain defective for both fimbria and FHA indicated that both fimbria and FHA are required for bacterial persistence and suggested that fimbria may contribute to immune suppression (55).

Bordetella adhesins: Filamentous Hemagglutinin

Filamentous hemagglutinin (FHA), one of the primary components of the aP vaccine, is a large exoprotein secreted by the two-partner secretion (TPS) pathway (56). TPS is a protein secretion pathway for Gram-negative bacteria which facilitates secretion of large proteins through the outer membrane and to the surface. Many TPS systems have been shown to contribute to bacterial virulence, with roles including adherence, iron acquisition, immune evasion and cytotoxicity (19, 57–61). TPS systems have a large exoprotein (generically known as TpsA) which is translocated through a cognate outer membrane β-barrel pore protein (generically known as TpsB). The secretion mechanism is complex, as secretion occurs in the absence of chemical energy and, despite an absence of mechanisms to ensure correct folding, results in translocation and proper folding of massive proteins. For *Bordetella*, one TpsA protein is FhaB, which is synthesized as a ~370 kDa pre-pro-protein, that, when processed, is a ~250 kDa protein known as FHA (62). The cognate TpsB protein, FhaC, is required to translocate FhaB across the outer membrane (56). The C-terminal prodomain is processed by SphB1 and other unidentified factors (56, 63). Once ‘mature’, FHA is oriented with the C-terminus distal to

the bacterial surface (56, 62). Mature FHA can also be released, as substantial amounts are found in culture supernatant *in vitro*, though the physiological relevance of this release is unclear (56). As for many processed proteins, this 'mature' molecule is typically considered to be the functional form of the protein.

FHA has been shown both necessary and sufficient to mediate bacterial adherence to a broad range of eukaryotic cell lines (64). Additionally, FHA was shown to contribute modestly to adherence to ciliated explants, much like fimbria (6), suggesting that FHA alone may not be the sole adhesin during infection. Furthermore, studies have shown that FHA molecules produced by *B. pertussis* and *B. bronchiseptica* are functionally interchangeable regarding adherence (17, 20). Studies using non-ciliated cell lines suggest that FHA binds to CR3, VLA-5, and leukocyte response integrin-integrin-associated protein (LRI-IAP) complexes (65, 66). However, the importance of these interactions has not been shown *in vivo*.

FHA has been shown necessary for *B. bronchiseptica* to colonize the lower respiratory tract in a rat colonization model (58), similar to results seen with bacteria lacking fimbria. The host response to FHA has been well characterized in the mouse model. FHA-deficient bacteria are defective for persistence compared to WT bacteria and induce a more robust inflammatory response, characterized by increased pro-inflammatory cytokine and chemokine production, such as IL-1β, IL-17, MCP-1 and KC (17, 19, 64). Furthermore, Hemotoxylin and Eosin (H&E) stained lung sections indicate increased cellular infiltrate around the major airways when mice were inoculated with FHA-deficient bacteria, compared to lung sections from mice inoculated with WT bacteria (19). This influx appears to be primarily neutrophilic (19). Together, these data suggest that FHA allows *B. bronchiseptica* to modulate the immune response and establish a persistent infection. Interestingly, experiments using *B. pertussis* lacking FHA in a mouse model

indicated that FHA is not necessary for colonization or persistence. However, because *B. pertussis* does not naturally infect mice, the implications of this finding are unclear.

Research objectives

Even though fimbrial subunits are included in the aP vaccine, very little is understood regarding the contribution of fimbria to *Bordetella* adherence, colonization and persistence. Within the rat model, FIM-deficient and FHA-deficient bacteria appear to have identical colonization defects (19, 49, 58), suggesting that FIM and FHA may have similar roles during infection. However, the *in vitro* adherence data suggest that fimbria do not contribute to adherence (54), despite fimbria typically acting as adhesins for other organisms.

The goals of this thesis project were to: 1) Develop an *in vivo* adherence assay to determine if fimbria contribute to bacterial adherence, as we hypothesize that fimbria mediate adherence specifically to ciliated epithelium, 2) Characterize the persistence defect of FIM-deficient bacteria and the host response of mice to FIM-deficient bacteria, as we hypothesize fimbria may also contribute to immune modulation.

TABLE

Table 1. Type I pili adhesin proteins and host cell targets

Organism	Adhesin	Host Cell	Receptor	Reference
E. coli	FimH	Bladder, kidney epithelial cells, neutrophils, macrophages	Uroplakin UP1a, CD48	(37, 67, 68)
E. coli	SfaS	Bladder, kidney epithelial cells, erythrocytes, endothelial cells	Sialic acid residues, plasminogen	(69)
Uropathogenic *E. coli*	PapG	Kidney epithelial cells, erythrocytes	GbO3, GbO4, GbO5	(70)
Uropathogenic *E. coli*	DraE	Bladder and kidney epithelial cells	CD55/DAF, CEACAMs	(71)
Uropathogenic *E. coli*	AfaE	Uroepithelium, erythrocytes	CD55/DAF, CEACAMs	(71)
P. mirabilis	PmfF	Bladder and kidney epithelial cells	Unknown	(72)
B. bronchiseptica	FimD	Unknown	Unknown	(49)

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CHAPTER 2. COOPERATIVE ROLES FOR FIMBRIA AND FILAMENTOUS HEMAGGLUTININ IN *BORDETELLA* ADHERENCE AND IMMUNE MODULATION[1]

Importance

Although fimbria have been shown to be important mediators of adherence for many bacterial pathogens, there is surprisingly little experimental evidence supporting this role for *Bordetella* fimbria. Our results provide the first demonstration that *Bordetella* fimbria function as adhesins *in vivo*, specifically to airway epithelium. Furthermore, our results suggest that fimbria mediate initial interactions with airway epithelial cells that are followed by tight FHA-mediated binding and that together, fimbria and FHA allow *Bordetella* to suppress inflammation, leading to prolonged colonization. Given the shortcoming of the current aP vaccine in preventing colonization, these findings suggest that generation of antibodies capable of blocking fimbria-mediated adherence could potentially prevent *Bordetella* colonization.

Introduction

The "classic" or mammalian *Bordetella* species, which include *Bordetella pertussis, Bordetella parapertussis* and *Bordetella bronchiseptica*, are Gram-negative bacteria that cause respiratory infections in mammals (1). *B. bronchiseptica* colonizes the nasopharynx and trachea in a broad range of hosts, including rabbits, rats, mice and occasionally humans, often resulting

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in persistent, asymptomatic infections (2). Phylogenetic analyses indicate that *Bordetella pertussis*, the causative agent of whooping cough (pertussis), evolved from a *B. bronchiseptica*-like ancestor, narrowing its host range to humans exclusively and typically causing acute respiratory disease, particularly in infants and young children (3, 4). Widespread use of a whole-cell pertussis (wP) vaccine in the 1950s led to a rapid decrease of pertussis morbidity and mortality. Safety concerns, however, led to the replacement of the wP vaccine with acellular component pertussis vaccines (aP) (5). aP vaccines contain pertussis toxin (PTX) and one or more of the putative adhesins: filamentous hemagglutinin (FHA), fimbria (FIM) or pertactin (PRN). Coinciding with the switch to using only aP vaccines, cases of pertussis in the United States and other countries have increased steadily since the 1990s (6–8). Given the reemergence of this disease, it is important to better understand the mechanisms utilized by *Bordetella* to colonize and persist in the respiratory tract.

Despite differences in host range and disease-causing propensity, *B. bronchiseptica* and *B. pertussis* are extremely similar and produce a nearly identical set of virulence factors. One such virulence factor is a type I pili system, typically called fimbria in *Bordetella*. The putative chaperone, usher, and tip adhesin are encoded by the *fimBCD* genes, respectively, and are required for fimbrial biogenesis (9). Most *Bordetella* strains characterized produce fimbria composed of either Fim2 or Fim3 as the major fimbrial subunit (10). The structural genes, *fim2* and *fim3*, are unlinked from each other and from the *fimBCD* operon (10). Additional major fimbrial subunit-encoding genes have been identified, including *fimX, fimN* and *fimA* (11–13). The *fimA* gene, located immediately 5' to the *fimBCD* operon, is a pseudogene in *B. pertussis* (13). Although most aP vaccines contain the major fimbrial subunits, Fim2 and Fim3, whether

antibodies against these proteins contribute to protection against colonization or disease is unknown.

Because *B. pertussis* is a human-specific pathogen that does not readily infect laboratory animals, we have been using *B. bronchiseptica* with its natural hosts to understand the contribution of specific virulence factors to *Bordetella* infection (14–16). The amino acid (aa) sequences of the FimD proteins produced by *B. pertussis* (Tohama I) and *B. bronchiseptica* (RB50) are 95% identical and the major fimbrial subunits, Fim 2 and Fim3, are 73% and 94% identical, respectively (9, 17, 18). It is likely that the fimbria produced by *B. bronchiseptica* and *B. pertussis* play similar, if not identical, roles during infection and we hypothesize that information gleaned from studies using *B. bronchiseptica* and natural-host animal models will be applicable to *B. pertussis*.

Although fimbria have been shown to be important mediators of adherence for many bacterial pathogens, there is surprisingly little experimental evidence supporting this role for *Bordetella* fimbria. A *B. pertussis* strain containing an insertion mutation in *fimD* was defective for adherence to adherent monocytes *in vitro* (19). However, as this strain is also defective for FHA production, the contribution of fimbria alone could not be determined (9, 19). We previously constructed a Δ*fimBCD* strain of *B. bronchiseptica* that does not produce fimbria of any type and is unaltered for FHA production. Unexpectedly, this strain did not differ from WT bacteria in its ability to adhere to various epithelial and macrophage cell lines *in vitro* (20). However, a *B. pertussis* strain defective for both FIM and FHA had reduced adherence to baboon trachea explants and FIM-defective *B. bronchiseptica* had reduced adherence to rabbit trachea explants (21, 22), suggesting that fimbria may be important for adherence specifically to ciliated respiratory epithelial cells. Although studies have been conducted to identify host cell receptors

for fimbria (23–25), these experiments used purified fimbrial subunits and non-ciliated cell lines and whether the interactions identified reflect those which occur with native fimbria *in vivo* is unknown.

Using a colonization model in which rats are inoculated with a small volume inoculum into the tip of the nose, we showed that fimbria and FHA are necessary for *B. bronchiseptica* to colonize the lower respiratory tract, specifically the trachea (20, 26). When inoculated directly into the tracheas of rats, FIM-deficient bacteria were unable to persist in the trachea, but colonized and persisted in the nasal cavity (20). These data suggest that fimbria are required to adhere to tracheal tissue, to resist mucociliary clearance and/or to avoid clearance by the innate immune system. Our lab and others have used a large volume, intranasal-inoculation mouse model to investigate the host response to *Bordetella* infection. This inoculation method presumably deposits bacteria throughout the nose, trachea and lungs of the animal. Using this model, our lab has shown that FHA is necessary for bacterial persistence in the lower respiratory tract and that FHA-deficient bacteria induce a more robust inflammatory response compared to WT bacteria (27–29). These data suggest that FHA is involved in suppressing the host immune response to aid bacterial persistence. Based on the similar tracheal colonization defect of Δ*fhaB* and Δ*fimBCD* mutants in rats, we hypothesized that fimbria may also contribute to immune modulation and bacterial persistence in the lower respiratory tract and we set out to test this hypothesis.

Results

Fimbria are required for adherence *in vivo*

To investigate the contribution of fimbria to adherence to respiratory epithelium in the context of natural infection, we developed an "*in vivo* adherence assay". We inoculated mice intranasally with 50µl PBS containing $7.5x10^4$ CFU of bacteria, euthanized the mice 30 minutes later, cannulated the tracheas, performed bronchoalveolar lavage (BAL) with 1ml PBS, and determined the number of CFU recovered. When BAL was not performed, we recovered equivalent CFU for all strains tested, approximately $3.0x10^4$ CFU, indicating that all mice received similar inocula (Fig 1A). This number also represents the maximum number of CFU recoverable from the lungs using this inoculation protocol. When we inoculated mice with wild-type (WT) bacteria and then performed BAL, we recovered approximately $7.5x10^2$ CFU in the lavage fluid (Fig 1A), corresponding to ~1% of the recoverable CFU, which we calculated as the mean CFU recovered by BAL divided by the mean CFU recovered from the lungs when BAL was not performed (Fig 1B). We homogenized and plated the post-lavage lungs and recovered approximately $5x10^4$ CFU (Fig 1A), corresponding to ~75% of the recoverable CFU (Fig 1B). Therefore, for WT bacteria, almost all of the recoverable bacteria remained in the lungs following BAL, presumably because they adhered tightly to respiratory epithelium.

When we inoculated mice with a strain defective for production of all known protein virulence factors (Δ*bvgS*), we recovered approximately $3x10^4$ CFU in the lavage fluid, corresponding to ~55% of the recoverable CFU, while we recovered $7x10^3$ CFU in the post-BAL lung homogenate, corresponding to ~15% of the recoverable CFU (Figs 1A, 1B). Maximum recovery of avirulent bacteria by BAL, therefore, is about 55% of the recoverable CFU in this assay (compared with 1% for WT bacteria). When we inoculated mice with a strain

deficient for production of fimbria (Δ*fimBCD*), the CFU recovered by BAL was ~55% of the inoculum and ~15% of the inoculum was recovered in the post-BAL lung homogenate, indicating that FIM-deficient bacteria are as defective for adherence as Δ*bvgS* bacteria (Fig 1A,1B). We conclude from these results that fimbria contribute substantially to adherence to mouse respiratory tissue. Moreover, as FIM-deficient *B. bronchiseptica* were not defective for adherence to a variety of cell lines *in vitro* (20), these results suggest that FIM are required specifically for adherence to respiratory epithelium and perhaps to ciliated respiratory epithelium.

We also inoculated mice with strains deficient in production of FHA (Δ*fhaB)*, pertactin (Δ*prn)*, both FHA and FIM (Δ*fimBCD*Δ*fhaB)*, adenylate cyclase toxin (Δ*cyaA)*, or the type 3 secretion system (Δ*bscN)* and measured adherence. We recovered ~$3x10^4$ CFU in BALF from mice inoculated with either Δ*fhaB* or Δ*fimBCD*Δ*fhaB* bacteria, which is nearly identical to the number recovered from FIM-deficient bacteria. By contrast, Δ*prn* mutants adhered similarly to WT bacteria, supporting *in vitro* evidence that pertactin is not necessary for adherence (30), and, as expected, bacteria defective for adenylate cyclase toxin and the type 3 secretion system also adhered similarly to WT bacteria (data not shown). These results indicate that both FIM and FHA are required for bacterial adherence to mouse respiratory epithelium within the first hour of infection. The fact that the number of CFU of Δ*fimBCD* and Δ*fhaB* mutant bacteria recovered from BAL was similar to that of the Δ*bvgS* strain suggests that FIM and FHA are the two main, if not only, factors that mediate adherence to respiratory tissue and that they function interdependently, i.e., both are required.

Fimbria are required for persistence in the lower respiratory tract

We inoculated 6 week old BALB/c mice intranasally with $7.5x10^4$ CFU of either wild-type (WT), Δ*fimBCD*, or Δ*fimBCD*Δ*fhaB B. bronchiseptica.* For mice inoculated with WT or Δ*fimBCD* mutant bacteria, we determined bacterial burden in the nasal cavity, trachea and right lung lobes at various times post-inoculation (p.i). For mice inoculated with the Δ*fimBCD*Δ*fhaB* mutant, we determined bacterial burden only in the lungs. CFU recovered from tissues harvested one hour p.i. (Day 0) were similar among all animals, indicating that consistent inoculation between bacterial strains and replicates occurred (Fig 2 A, B, C). There was no difference in the number of CFU recovered from the nasal cavities of animals inoculated with WT or Δ*fimBCD* bacteria at any time p.i. (Fig 2A). We also recovered similar numbers of CFU of WT and Δ*fimBCD* bacteria from the tracheas one and three days p.i. However, 14 days p.i., no Δ*fimBCD* mutants were recovered from the tracheas, while the number of WT bacteria in the trachea remained high at this time point (Fig 2B). Similar to what we have observed in rats (20), therefore, fimbria are required for persistence in the tracheas of mice.

We recovered approximately one log more CFU of Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* bacteria than WT bacteria from the lungs one day p.i. (Fig 2C). At three days p.i., mice inoculated with Δ*fimBCD* bacteria split into two distinct groups: one group had significantly higher bacterial burden compared to the burden in mice inoculated with WT bacteria, and were moribund. The second group had a similar bacterial burden as mice inoculated with WT bacteria and these mice showed no signs of respiratory distress. This "bimodal" phenotype at three days p.i is similar to what has been observed in mice inoculated with Δ*fhaB* bacteria (27–29). For mice inoculated with Δ*fimBCD*Δ*fhaB* mutants, the bacterial burden was not clearly bimodal, but spread between 10^5 and 10^7 bacteria at three days p.i. By seven days p.i., the burdens of both the Δ*fimBCD*

bacteria and Δ*fimBCD*Δ*fhaB* bacteria were significantly lower than the burden of WT bacteria and the mutants were undetectable by 14 days p.i.

The persistence defect of Δ*fimBCD* and Δ*fimBCD*Δ*fhaB* bacteria was similar to that of Δ*fhaB* bacteria, indicating that fimbria, like FHA, are required for bacterial persistence in the lower respiratory tract (27–29). These data suggest that, like FHA, fimbria may be involved in suppressing inflammation.

Fimbria are required to modulate the innate immune response in mice

We examined hematoxylin and eosin (H+E) stained lung sections to evaluate inflammation in both the major airways and the alveoli during infection (Fig 3A). Lungs from mice inoculated with only PBS appeared healthy, with little, if any, evidence of inflammation at any time point p.i. Lungs from mice inoculated with WT bacteria contained cellular infiltrates around the major airways (black arrows) but the alveoli and alveolar spaces were free of any signs of inflammation at three days p.i. The cellular infiltrates around major airways persisted to seven days p.i. in mice inoculated with WT bacteria (black arrow), coinciding with the high bacterial burden at this time point. Lungs from mice inoculated with Δ*fimBCD* bacteria displayed some cellular infiltration around the major airways, but also showed substantial visually distinct cellular infiltrate within the alveolar spaces at three days p.i (black arrows). This histopathology pattern, which was present in the lungs of mice with either high or low bacterial burdens at day 3 p.i., differed dramatically from that of the lungs of mice inoculated with FHA-deficient bacteria, which showed increased cellular infiltrate primarily around the major airways and no patches of cellular infiltration in alveoli (27). By seven days p.i., the patches of inflammatory cell recruitment in alveoli of Δ*fimBCD* mutant-inoculated mice was absent and

there was decreased cellular infiltrate around the major airways, corresponding with the decreased bacteria burden at this time point. Lungs of mice inoculated with Δ*fimBCD*Δ*fhaB* bacteria appeared similar to those of mice inoculated with Δ*fimBCD* bacteria at both time points p.i., with cellular infiltration evident around the major airways as well as distinct cell recruitment within the alveolar spaces (black arrows) at three days p.i. At seven days p.i. there were fewer inflammatory cells present, coinciding with decreased bacterial burden.

We also measured cytokine and chemokine levels in right lung homogenates by ELISA (Fig 3B). Interleukin-1β (IL-1β) levels were significantly increased in the lungs of mice inoculated with Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* bacteria compared to WT bacteria one and three days p.i. These differences did not correlate with burden, as IL-1β levels were increased in all animals inoculated with the mutant bacteria, even those with lower burdens at day 3 p.i.. Moreover Monocyte Chemotactic Protein-1 (MCP-1) and neutrophil chemokine CXCL1 (KC) levels were significantly increased in lungs of mice inoculated with Δ*fimBCD* bacteria compared to WT bacteria three days p.i. MCP-1 and KC levels in mice inoculated with Δ*fimBCD*Δ*fhaB* mutant bacteria appeared higher than in mice inoculated with WT bacteria, but the differences were not statistically significant. Other cytokines, such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), Interleukins-10, 12p70, 22, and 23 (IL-10, IL-12p70, IL-22 and IL-23) were measured and no significant difference was found between mice inoculated with Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* bacteria compared to those inoculated with WT bacteria at any time point.

Taken together, these data indicate that fimbria, like FHA, are required to suppress inflammation during infection. The dramatically different histopathology, however, suggests that FIM and FHA may play different roles in pathogenesis and the fact that the Δ*fimBCD*Δ*fhaB*

double mutant induced a histopathology pattern similar to that induced by the Δ*fimBCD* mutant indicates that the Δ*fimBCD* mutation is epistatic to the Δ*fhaB* mutation.

Fimbria do not complement "in trans" during co-inoculation

Our data here and from previous studies suggest that WT *B. bronchiseptica* is able to suppress the initial inflammatory response to infection, contributing to decreased pathology and increased bacterial persistence (27–29). We have previously shown that when mice are co-inoculated with WT and FHA-deficient bacteria, the level of inflammation in the lungs is decreased compared to that induced by inoculation with the Δ*fhaB* mutant alone and the Δ*fhaB* mutant persists longer than when inoculated into mice in the absence of the WT strain, suggesting that FHA-producing WT bacteria are able to complement "*in trans*"(29). We hypothesized that WT *B. bronchiseptica* would similarly be able to rescue FIM-deficient bacteria from inflammation-mediated clearance. To test this hypothesis, we inoculated 6 week old mice with $1.5x10^5$ CFU of WT, Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* bacteria alone, or a mixture of $7.5x10^4$ CFU of each WT and Δ*fimBCD* bacteria or WT and Δ*fimBCD*Δ*fhaB* bacteria (so that the total number of CFU in each inoculum was $1.5x10^5$) and determined bacterial burden.

Strikingly, the mutants and WT bacteria were recovered from the trachea and lungs at similar numbers from co-inoculated animals as from animals inoculated with WT or mutant bacteria alone (Fig 4A); the presence of WT bacteria did not improve the persistence of the mutant bacteria, and the presence of mutant bacteria did not lead to increased clearance of WT bacteria. These data indicate that, unlike the case with FHA, WT bacteria cannot complement Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* bacteria *in trans*, further supporting the hypothesis that FIM and FHA contribute differently to infection.

Lung sections from mice co-inoculated with WT and either mutant strain appeared similar to lungs of mice inoculated with Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* bacteria alone: there was cell recruitment around the major airways as well as in the alveolar spaces (black arrows), indicating that the presence of WT bacteria cannot prevent the histopathology seen in lungs inoculated with FIM-deficient bacteria (Fig 4B). In general, levels of IL-1β, KC and MCP-1 were higher in co-inoculated mice compared to mice inoculated with WT alone, although most differences were not statistically significant (Fig 4C).

These data indicate that the presence of WT bacteria in the lungs did not increase the survival of FIM-deficient bacteria and did not prevent cellular infiltrate into the alveolar spaces. A possible explanation for the inability of WT bacteria to complement FIM-deficient bacteria *in trans* is that WT and FIM-deficient bacteria localize differently in this model.

FIM-deficient bacteria localize differently than WT and FHA-deficient bacteria in the lower respiratory tract

To determine the location of WT and mutant bacteria in the lungs, we inoculated mice with $7.5x10^4$ CFU of WT or mutant bacteria then sacrificed the mice three days p.i. and prepared the left lung lobe for sectioning. We then performed immunohistochemistry using serum from a rabbit chronically infected with WT *B. bronchiseptica* as the primary antibody, goat anti-rabbit conjugated to alkaline phosphatase (AP) as the secondary antibody, and Naphthol Red as the AP substrate. The lung sections were then counterstained with hematoxylin which stains nuclei dark purple. Lungs from mice inoculated with WT bacteria showed red staining around the ciliated epithelium of the major airways (black arrows) while control lungs, which were not incubated with rabbit serum, did not have any red staining (Fig 4A). This staining pattern indicates that WT bacteria localize to the major airways during infection.

Lungs from mice inoculated with FHA-deficient bacteria appeared similar to lungs of mice inoculated with WT bacteria, with noticeable red staining around the major airways, both on the ciliated epithelium as well as in the cellular infiltrate beneath the epithelial cells (black arrows), but very little red staining in the alveolar spaces, suggesting that FHA-deficient bacteria localize similarly to WT bacteria. By contrast, lungs from mice inoculated with FIM-deficient bacteria showed a dramatically different staining pattern. These lungs had red staining throughout the alveolar spaces (black arrow). Lungs from mice inoculated with Δ*fimBCD*Δ*fhaB* double mutant bacteria appeared similar to lungs from mice inoculated with FIM-deficient bacteria. These results indicate that FIM-producing bacteria localize to major airways and bronchioles while FIM-deficient bacteria localize predominantly to alveoli, suggesting that FIM mediate attachment specifically to ciliated epithelia, which line bronchi and bronchioles. Without fimbria, many, if not most, bacterial bypass the ciliated epithelium and are deposited in alveoli.

Lungs from mice co-inoculated with WT and FHA-deficient bacteria had red staining around the major airways (black arrows), but very little red staining in the alveolar spaces, similar to lungs from mice inoculated with WT or FHA-deficient bacteria alone. By contrast, lungs from mice co-inoculated with WT and either Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* bacteria had red staining in the major airways as well as distinct staining in the alveolar spaces (black arrows). These data suggest that even during co-inoculation, WT bacteria and FHA-deficient bacteria localize primarily to major airways, while FIM-deficient bacteria are delivered primarily to alveoli. Furthermore, these results provide a possible explanation for why co-inoculation with WT bacteria can rescue FHA-deficient bacteria but not FIM-deficient bacteria: the WT-mediated immune suppression occurs locally in the major airways and does not affect bacteria in the alveolar space because the WT bacteria do not gain access to this location.

We conducted an *in vivo* adherence assay using an equal mixture of WT and FIM-deficient bacteria (Data not shown). The numbers of CFU recovered were similar to those from mice inoculated with WT or FIM-deficient bacteria alone. This result provides further evidence that WT and FIM-deficient bacteria function independently in the respiratory tract.

Discussion

Bordetella fimbria are generally considered to function as adhesins despite there being no reports of adherence studies using *B. pertussis* strains defective only for production of fimbria. Studies with *B. bronchiseptica* also failed to provide convincing evidence that *Bordetella* fimbria function as adhesins as FIM-deficient bacteria showed no defect in adherence assays using a variety of non-ciliated cell lines (20) and this strain was only modestly defective in adherence to ciliated tracheal explants (22). Our results therefore provide the first demonstration that *Bordetella* fimbria are important adhesins and they indicate that fimbria mediate adherence specifically to airway epithelium. Our results also show that fimbria and FHA work together, playing equally important roles in allowing *Bordetella* to suppress inflammation, leading to prolonged colonization.

A murine model in which large numbers of bacteria are delivered intranasally in a large volume has been used by our group and others to study respiratory infection by *Bordetella* (16, 27–29, 31–34). It has been presumed that this inoculation method deposits bacteria evenly throughout the respiratory tract. Within the trachea, bronchi and bronchioles, bacteria must overcome mucociliary clearance through tight adherence. The bacteria also interact with or stimulate sentinel innate immune cells, such as alveolar macrophages and dendritic cells within the lower respiratory tract, which in turn stimulate the initial inflammatory response characterized by the recruitment of phagocytic cells, predominately neutrophils, to the site of infection. In this model both bacterial burden and cellular infiltrate peak at about seven days post-inoculation. Bacterial load then decreases, with clearance from the lungs occurring over the next two to three weeks and requiring adaptive immunity (16). For *B. pertussis*, the bacteria are cleared from the entire respiratory tract by about 30-40 days post-inoculation while for *B.*

bronchiseptica the bacteria are cleared from lower respiratory tract but persist in the nasal cavity indefinitely (16).

Our newly-developed *in vivo* adherence assay and bacterial localization analyses showed that WT *B. bronchiseptica* are not distributed evenly throughout the lungs following high-dose, large volume inoculation. Instead they localize predominately to airway epithelium. This localization requires fimbria, as FIM-deficient mutants bypassed the ciliated epithelium and localized to the alveoli. In the alveoli, FIM-deficient bacteria did not adhere tightly enough to resist bronchoalveolar lavage, despite producing wild-type levels of FHA. It is possible that FHA receptors are not present on alveolar pneumocytes. Alternatively, tight adherence may require fimbria-mediated interactions that induce changes in either bacterial or host cells. FHA-deficient bacteria, by contrast, localized to the airways, presumably due to fimbrial attachment to ciliated epithelium, but these fimbria-mediated interactions alone were insufficient to resist bronchoalveolar lavage. Our data therefore suggest a model in which adherence of *Bordetella* is a two-step process requiring both fimbria and FHA. In this model, fimbria mediate initial interactions to ciliated epithelia and this critical first step then allows FHA to mediate tighter adherence to these cells.

Following adherence, the ability of *Bordetella* to influence the innate immune response is evident in the first three to four days post-inoculation. We have previously shown a role for FHA in modulating the innate immune response as FHA-deficient *B. bronchiseptica* were hyper-inflammatory compared to WT bacteria (27, 29). Here, we showed that FIM-deficient mutants were similar to FHA-deficient mutants in bacterial burden and in inducing high levels of pro-inflammatory cytokines and chemokines during the first three days of infection, suggesting that fimbria also contribute to suppression of inflammation (Fig 2). Lungs from mice inoculated with

FHA- or FIM-deficient bacteria, however, displayed strikingly different histopathology. Unlike FHA-deficient bacteria, which caused increased cellular infiltrate around the bronchioles, FIM-deficient bacteria caused increased cellular infiltrate in the alveoli. This difference correlates with the different localization of these strains in the lungs. It also indicates that FHA alone is insufficient to modulate the inflammatory response in the alveoli, since FIM-deficient bacteria that localize to this site produce FHA. Furthermore, cytokine levels and histopathology from mice inoculated with Δ*fimBCD*Δ*fhaB* bacteria were similar to those of mice inoculated with Δ*fimBCD* bacteria. These data indicate that lack of fimbria is epistatic to lack of FHA; without fimbria, the presence or absence of FHA did not change the outcome of infection, underscoring the importance of fimbria for FHA-mediated interactions.

In this murine model, bacterial burden and inflammatory infiltrate in the lungs both peak at about seven days post-inoculation. Subsequent to this time point, FHA- and FIM-deficient bacteria are cleared rapidly while WT bacteria persist for approximately three more weeks (16, 29). Rapid clearance of the FIM- and FHA-mutant bacteria may reflect a decreased ability of these mutants to resist killing by inflammatory cells. However, because these mutants induce a hyper-inflammatory environment, it is also possible that their rapid clearance is due primarily, if not solely, to the increased numbers and/or activation of recruited inflammatory cells. Our lab has shown that mice co-inoculated with WT and FHA-deficient bacteria induce less inflammation compared to mice inoculated with FHA-deficient bacteria alone. Furthermore, in co-inoculated animals, there was increased persistence of FHA-deficient bacteria compared to animals inoculated with only the Δ*fhaB* mutant (29). We hypothesized that the increased persistence of Δ*fhaB* bacteria in co-inoculated animals resulted from FHA-producing WT bacteria suppressing inflammation. We also hypothesized that the decreased ability of FHA-

deficient mutants, compared with WT bacteria, to persist in co-inoculated animals indicated a decreased ability of FHA-deficient bacteria to resist phagocytic cells. Our new results show that WT and FHA-deficient bacteria co-localize in the major airways, supporting the hypothesis that mutant bacteria benefit from local immune suppression mediated by WT bacteria and further supporting the hypothesis that FHA-deficient bacteria are unable to resist clearance by inflammatory cells, even if the activation state of these cells is suppressed by the presence of WT bacteria.

By contrast, WT bacteria did not improve the survival of FIM-deficient bacteria, which were cleared as rapidly from mice co-inoculated with WT bacteria as from mice inoculated with FIM-deficient bacteria alone (Fig 3). The inability of WT bacteria to "trans complement" the Δ*fimBCD* mutant (and the Δ*fimBCD*Δ*fhaB* mutant) may be due simply to differences in localization, which would suggest that FHA-mediated suppression of inflammation occurs locally within the major airways. Rapid clearance of the Δ*fimBCD* and Δ*fimBCD*Δ*fhaB* mutant bacteria likely reflects both an inability to suppress inflammation locally and an inability to resist the recruited phagocytic cells. Together, our data indicate that once FIM-mediated interactions localize WT bacteria to the ciliated epithelium, FHA-mediated interactions provide both localized immune suppression and protection against immune-mediated clearance.

We have been using *B. bronchiseptica* and rabbits, rats, and mice to investigate how specific virulence factors and the regulation of virulence factor-encoding genes contribute to pathogenesis (20, 27, 29, 30). Because these animals are natural hosts for *B. bronchiseptica,* the results obtained from these studies are biologically relevant, i.e., we are confident in our conclusions that FHA and fimbria are required for lower respiratory tract colonization of these hosts by *B. bronchiseptica.* However, *B. bronchiseptica* infection of these rodents does not result

in the same course of infection or disease characteristics as *B. pertussis* infection of humans and therefore *B. bronchiseptica* infection of rabbits, rats, or mice should not be considered models of human pertussis. Nonetheless, we have demonstrated previously that the genes encoding FHA, adenylate cyclase, and BvgAS from *B. pertussis* can substitute for the corresponding genes in *B. bronchiseptica* during infection (15, 28, 30), suggesting that these virulence factors and regulatory system perform the same function for these two bacterial species during infection of their respective hosts. In our current study, we did not determine if the genes encoding fimbria from *B. pertussis* could substitute for their homologs in *B. bronchiseptica*. However, our results are consistent with those of a report showing that a *B. pertussis* strain defective for both FHA and fimbria was defective for persistence and caused increased cellular infiltrate in the alveoli of mice in which the authors concluded that fimbria were important for modulating the immune response (35). We hypothesize, therefore, that *B. pertussis* fimbria are required for adherence to airway epithelium and for FHA-mediated immunomodulation during human infection. Testing this hypothesis and determining if and how these factors contribute to disease characteristics unique to human pertussis may be achievable with the recently developed baboon model (36).

Given the reemergence of pertussis and the increasing reports of shortcomings of the acellular vaccine (36–38), it is important to reevaluate vaccine design and immunization route. While FIM2 and FIM3 are components of the acellular vaccine and induce an antibody response, the contribution and method of action of this response to bacterial clearance is unclear. Our results suggest that antibodies that block FIM-mediated attachment may prevent bacterial attachment and colonization in the lower respiratory tract, but whether the current aP vaccine or natural immune response generates antibodies capable of performing this function is uncertain. As *Bordetella* is primarily an extracellular respiratory pathogen, generation of mucosal IgA

antibodies capable of blocking bacterial adherence could potentially prevent *Bordetella* colonization. Most studies focus primarily on serum IgG responses following vaccination, so further characterization of the antibody response may be warranted.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Nation Institutes of Health. Our protocol was approved by the University of North Carolina IACUC (10-134, 12-307, 13-238). All animals were properly anesthetized for inoculations, monitored regularly, euthanized when moribund and efforts were made to minimize suffering

Growth media and Bacterial Strains

Escherichia coli were grown in lysogeny broth (LB) or on LB agar (1.5%) at 37°C. Wild-type (WT) *Bordetella bronchiseptica* RB50 and mutant derivatives were grown at 37°C on Bordet-Gengou (BG) (Becton Dickinson Microbiology Systems) supplemented with 7.5% defibrinated sheep blood (Colorado Serum Co, Denver) or in Stainer-Scholte (SS) broth with 100 mg/ml (2,6-*O*-dimethyl)-b-cyclodextrin. When necessary, media were supplemented with streptomycin (Sm, 20 µg/ml), gentamicin (Gm, 30 µg/ml), Kanamycin (50 µg/ml or diaminopimelic acid (DAP; 300 µg/ml).

Construction and cloning of plasmids was accomplished in *E. coli* DH5α. Plasmids were introduced into *B. bronchiseptica* via mating with *E. coli* RHO3. In-frame markerless deletion mutations were made using the pSS4245 allelic exchange system. pUC18-based plasmids were utilized to deliver genes encoding gentamicin resistance (*aacC1*) and kanamycin resistance (*nptII*) to the *att*Tn7 site via transposase-mediated insertion. Detailed descriptions of each strain are given in Table 1.

Intranasal mouse inoculation

Bacteria were grown overnight Stainer-Scholte (SS) broth with 100 mg/ml (2,6-*O*-dimethyl)-b-cyclodextrin. When necessary, media were supplemented with streptomycin (Sm, 20 µg/ml), gentamicin (Gm, 30 µg/ml) or Kanamycin (50 µg/ml). Six-week-old BALB/c mice from Jackson Laboratories (Bar Harbor, ME) were inoculated intranasally with 7.5 x 10^4 or 1.5 x 10^5 CFU of *B. bronchiseptica* in 50 µl PBS. Mice were inoculated with strains RB50, RB63, RBX9F, RB54, SP5, AS16 or a mix of RB50/RB63 or RB50/AS16. Right lung lobes, trachea and nasal cavity were harvested from mice at specific time points post-inoculation (p.i.) in 1 ml PBS. Tissue was homogenized, serial dilutions were plated on BG agar, and CFU were determined.

Cytokine and histological analysis

Using lung homogenates, the cytokine and chemokine responses to infection were measured using ELISA kits (R&D Systems). Homogenates were diluted 1:10 and then cytokines and chemokines measured were IL-1β, KC, MCP-1 and IL-17 following manufacturer's instructions, and concentrations were calculated using standard curve data for each cytokine. Absorbance was determined using a Molecular Devices plate reader and analyzed by Softmax Pro software (Molecular Devices). To prepare histology slides, left lung lobes were harvested at indicated time points and inflated with 10% formalin. The Animal Histopathology Core Lab then embedded the tissue in paraffin, sectioned the tissue at 5 µm, and then stained the tissue with hemotoxylin and eosin (H&E). Lung sections were examined at the Microscopy Services Laboratory using Brightfield imaging on an Olympus BX61 microscope at 10x and 40x magnification.

In vivo adherence

Bacteria were grown as mentioned above, and 6 week old BALB/c mice were inoculated with 7.5 x 10^4 CFU of bacteria. 30 minutes p.i., mice were euthanized, the trachea was cannulated and bronchoalveolar lavage was performed with 1 ml PBS to determine adherence. The right and left lung lobes were then excised and homogenized in 1 ml PBS to determine CFU remaining post-BAL. Serial dilutions of both BAL and homogenate were plated on BG agar to determine CFU recovered.

Bacterial Localization

6 week old BALB/c mice were inoculated as described above. Lung tissue was harvested three days p.i. and the left lobe was inflated with 10% formalin. The Animal Histopathology Core Lab then embedded the tissue in paraffin and provided unstained 5 µm lung sections. Sections were incubated with serum from a rabbit chronically infected with RB50 bacteria, then incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Promega). Sections were then incubated with alkaline phosphatase substrate Naphthol Red (SIGMA), counterstained with hemotoxylin (SIGMA) and examined on an Olympus Bx61 microscope at 10x and 40x magnification.

Statistical Analysis

Statistical analyses were performed using Prism 5.0 software from Graphpad Software, inc. Statistical significance was determined using unpaired Student's T-test or analysis of variance (ANOVA). Figures were generated using Adobe Illustrator CS6 (Adobe Systems, Inc).

Supporting Information

Figure 1. Fimbria are required for adherence *in vivo*

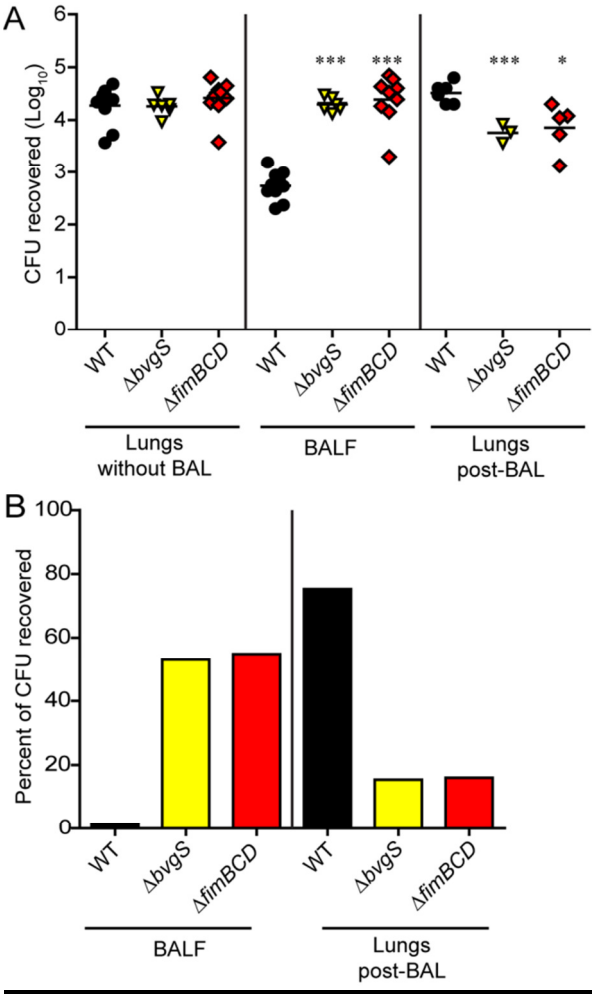

Fig 1. (A) Mice were inoculated intranasally with approximately 7.5×10^4 CFU of WT or mutant bacteria. The numbers of CFU recovered from lungs for which bronchoalveolar lavage (BAL) was not performed, from BAL fluid (BALF), and from lungs post-BAL, are shown. (B) Data shown as the percent of the recoverable CFU, which is calculated as mean CFU recovered from either BALF or lungs post-BAL divided by mean CFU recovered from lungs for which BAL was

not performed for each strain, respectively. Data are mean for two independent experiments. Significance when comparing WT to mutant bacteria * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Figure 2. Fimbriae are required for persistence in the lower respiratory tract

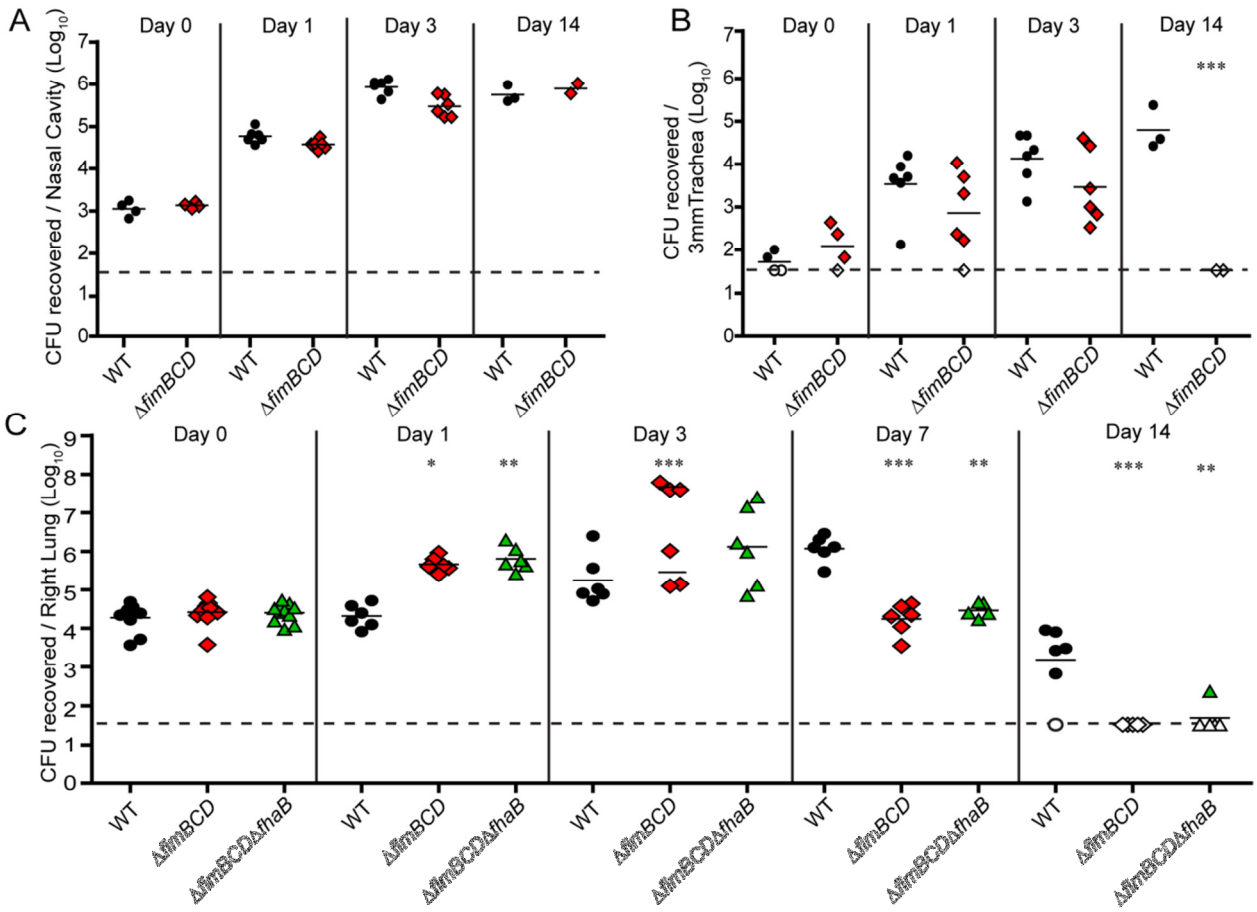

Fig 2. (A,B,C) Bacterial burden in respiratory tissues from mice inoculated with WT or mutant bacteria. Each symbol represents an individual animal, the horizontal dashed line represents the lower limit of detection and the black bar represents the mean. Data are mean (A,B,C) for at least two independent experiments. Significance when comparing WT to mutant bacteria * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Figure 3. Fimbriae are required to modulate the innate immune response in mice

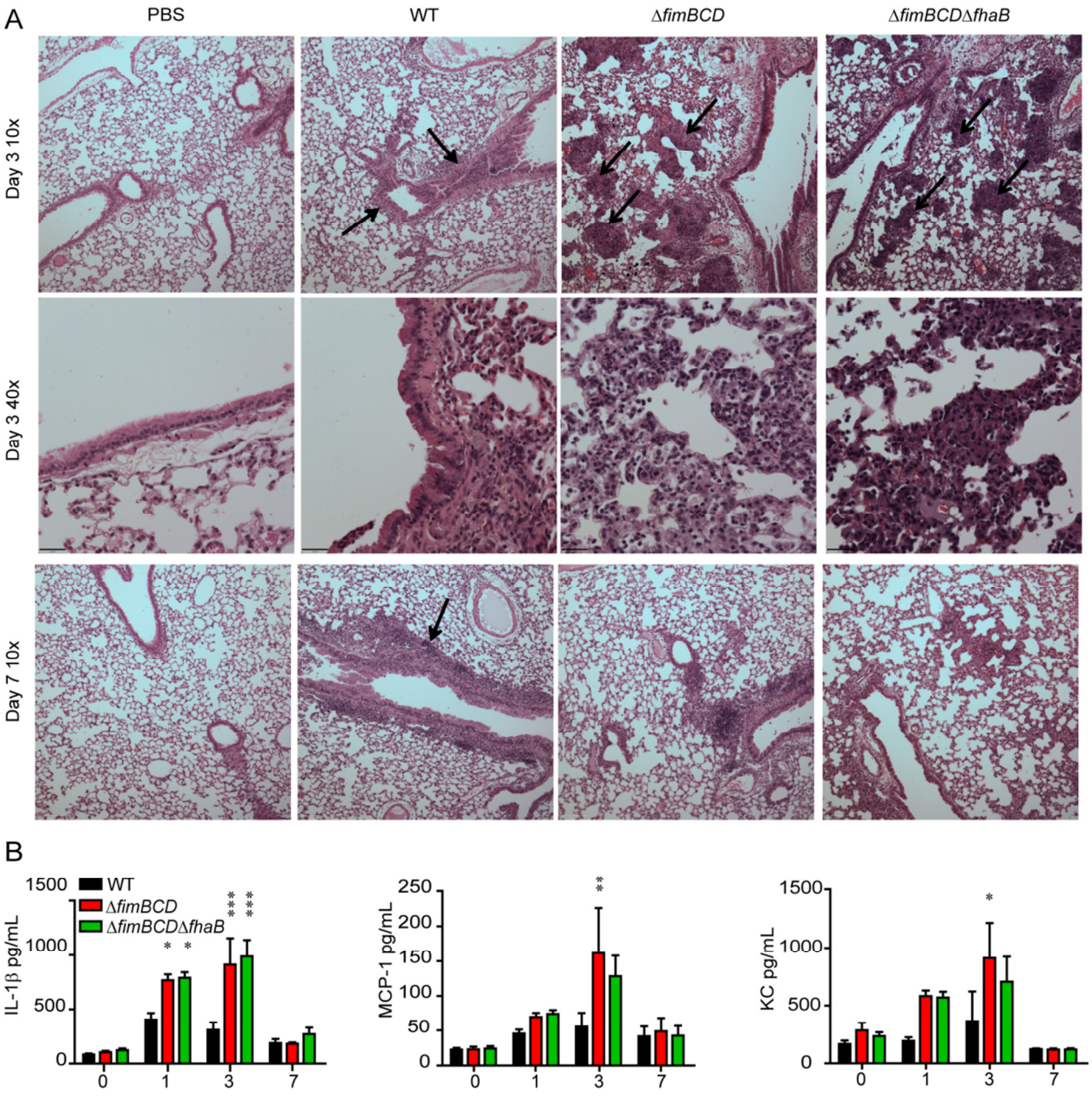

Fig 3. (A) Hematoxylin and eosin stained 5 µm lung sections at 10x magnification and 40x magnification. Black arrows indicate areas of cellular infiltrate. (B) Cytokine and chemokine levels in lung homogenates of WT, Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* mutant-inoculated animals.

Data are mean ±SE (B) for at least two independent experiments. Significance when comparing WT to mutant bacteria * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Figure 4. Fimbriae do not complement " in *trans* " during coinoculation

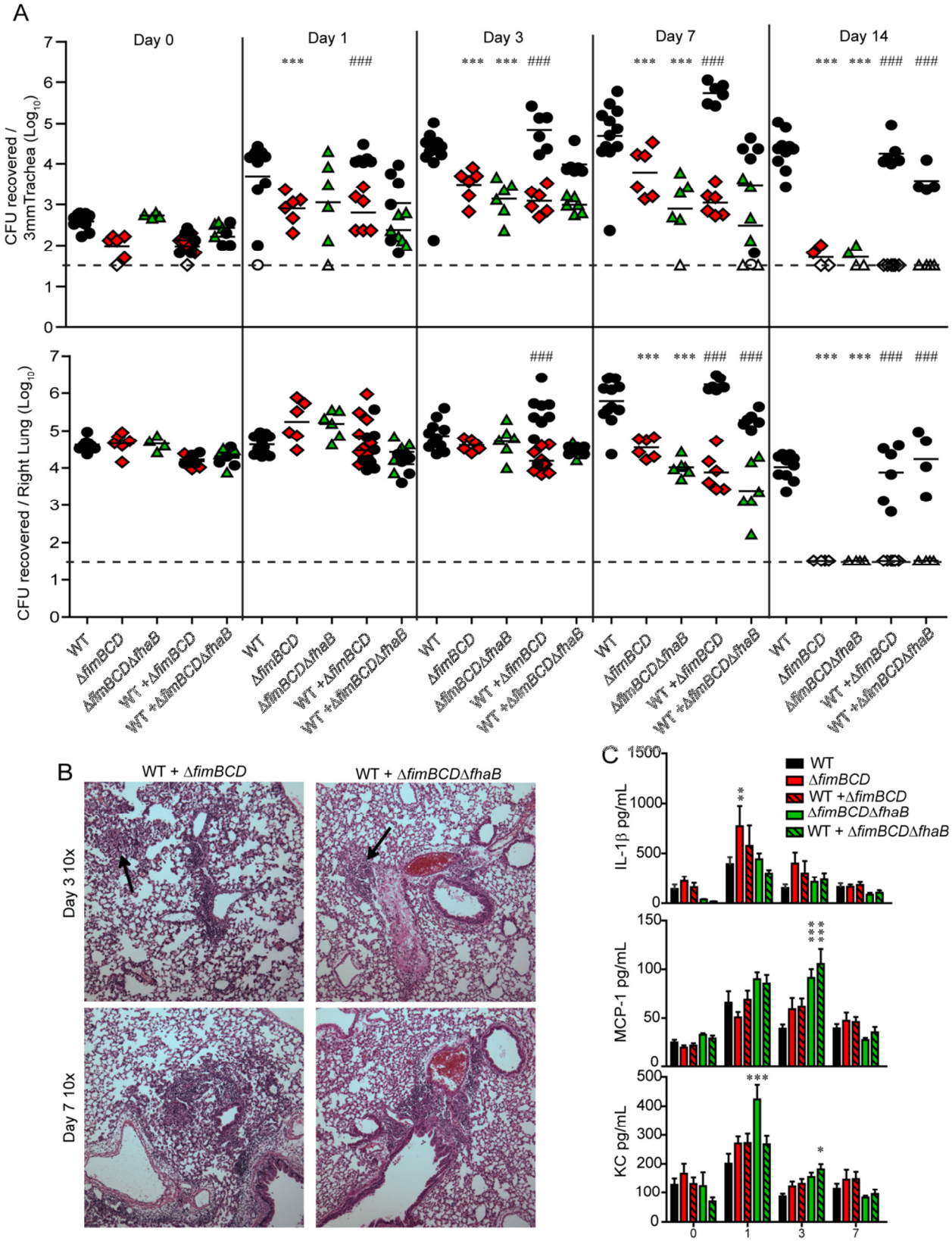

Fig 4. (A) Bacterial burden in respiratory tissue from mice inoculated with WT or mutant bacteria alone, or co-inoculations of WT and mutant bacteria. Each symbol represents an individual animal, the horizontal dashed line represents the lower limit of detection and the black bar represents the mean. (B) Hematoxylin and eosin stained 5 μm lung sections at 10x magnification. Arrows indicate areas of cellular infiltration. (C) Cytokine and chemokine levels in lung homogenates of WT, Δ*fimBCD* or Δ*fimBCD*Δ*fhaB* mutant-inoculated animals. Data are mean (A, B) or mean ±SE (C) for at least two independent experiments. Comparing single strain mutants to WT bacteria: * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ Comparing WT and mutant bacteria within co-inoculations # = $p<0.05$, ## = $p<0.01$, ### = $p<0.001$

Figure 5. FIM-deficient bacteria localize differently than WT and FHA-deficient bacteria in the lower respiratory tract

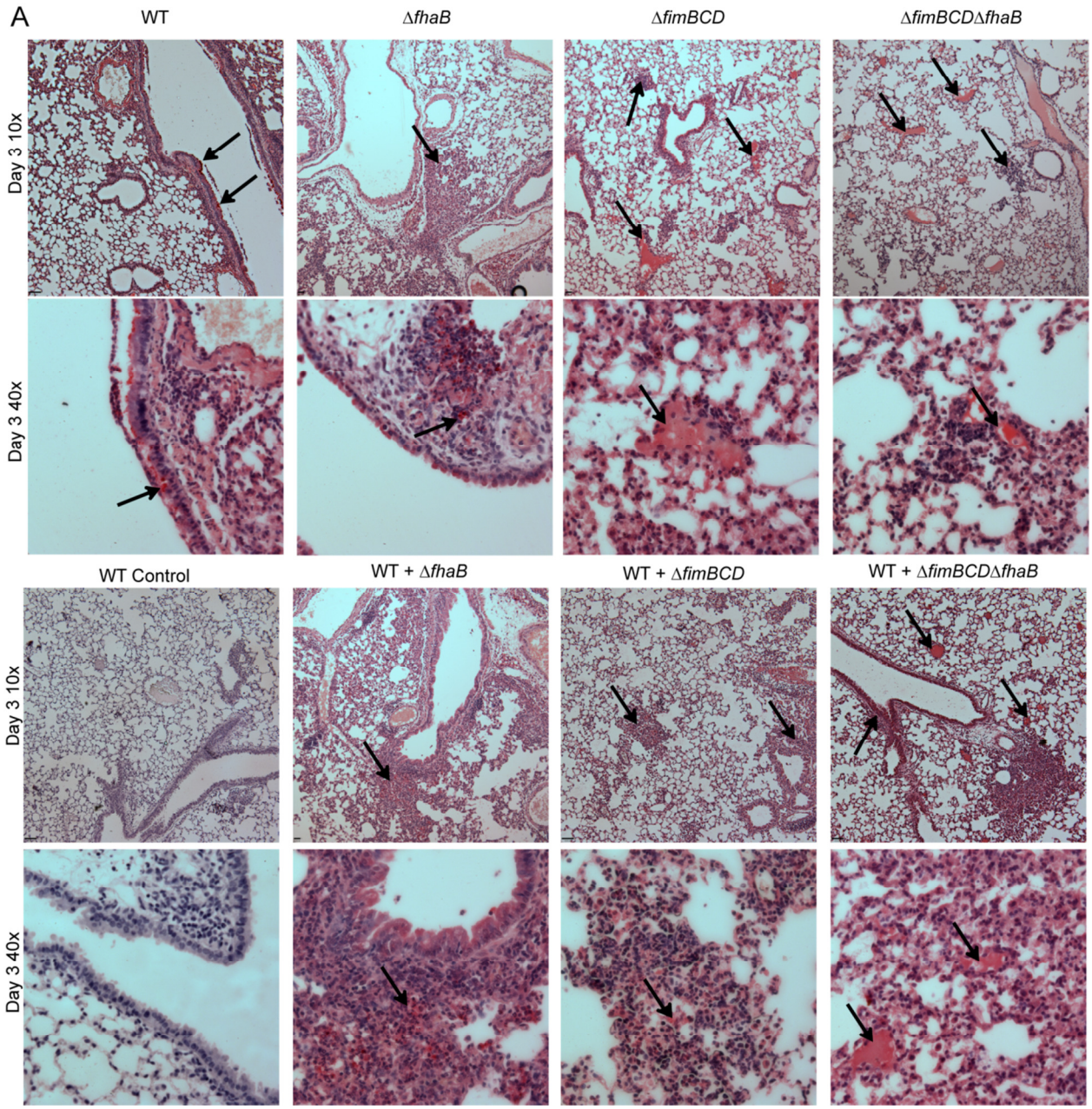

Fig 5. (A) Lung sections three days p.i. from mice inoculated with WT, mutant or co-inoculated with WT and mutant bacteria. Immunohistochemistry was performed using convalescent rabbit serum from a WT-infected rabbit, goat anti-rabbit secondary antibody conjugated to alkaline phosphatase, incubated with Naphthal Red substrate. Red staining (black arrows) indicate

alkaline phosphatase activity. Tissue was counterstained with hematoxylin. Pictures were taken at 10x and 40x magnification.

Tables

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description	Reference
E. coli strains		
DH5α	Molecular cloning strain	30
RH03	Conjugation strain; Km^s; DAP auxotroph	39
B. bronchiseptica strains		
RB50	"Wild-type" *Bordetella bronchiseptica* complex I strain	14
RB50 Km^r	RB50 containing constitutively expressed *nptII* inserted via a pUC18-based plasmid at the *att*Tn*7* site	This study
RB63	RB50 containing deletion of the *fimBCD* operon	20
RB63 Gm^r	RB63 containing constitutively expressed *aacC1* inserted via a pUC18-based plasmid at the *att*Tn*7* site	This study
SP5	RB50 containing an in-frame deletion of codons 227 to 756 of *prn*	22
RBX9f	RBX9 with a deletion mutation of the *fimA-fhaB* intergenic region	31
RB515	RB50 containing a deletion in *cyaA*	27
WD3	RB50 with an in-frame deletion of *bscN*	40
RB54	RB50 Bvg^- phase-locked variant with an in-frame deletion of *bvgS*	14
AS16	RB63 with a mutation of the *fhaB*-MCD	This study
AS16 Gm^r	AS16 containing constitutively expressed *aacC1* inserted via a pUC18-based plasmid at the *att*Tn*7* site	This study
Plasmids		
pSS4245	pBR322-based allelic exchange plasmid; Ap^r Km^r	30
pTsS3	Tn*7* transposase expression vector containing *tnsABCD*; Ap^r	41
pUC18-miniTn7	Transposition vector; Ap^r Km^r	41

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CHAPTER 3. NEW INSIGHT INTO FILAMENTOUS HEMAGGLUTININ SECRETION REVEALS A ROLE FOR PRE-PROCESSED FHAB IN *BORDETELLA* VIRULENCE[1]

Introduction

Two-partner secretion (TPS) is a widespread protein secretion pathway for Gram-negative bacteria in which a large exoprotein (generically called TpsA) is translocated through a cognate outer membrane β-barrel pore protein (TpsB) to the bacterial surface, where the exoprotein is then able to interact with its environment. The mechanism by which this occurs is complex, with fundamental aspects of the process, such as how unidirectional protein translocation across a membrane is achieved in the absence of chemical energy and how these massive proteins are able to fold correctly in the absence of typical quality control mechanisms, remaining unclear. Virulence functions have been attributed to most TPS systems and include adherence to host tissues[1,2], iron acquisition[3], cytotoxicity[4,5], and immune evasion[6], which contribute to bacterial colonization and persistence.

Whooping cough, or pertussis, is a currently reemerging disease in the United States and other developed countries. Increased incidence in recent years has coincided with a switch from whole-cell pertussis (wP) vaccines to acellular pertussis (aP) vaccines that display reduced reactogenicity[7]. Pertussis is primarily caused by the human-restricted Gram-negative pathogen *Bordetella pertussis*, and aP vaccines typically consist of three to five proteins that it secretes:

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[1]This chapter is currently in review to be published at mBio.

filamentous hemagglutinin (FHA), pertussis toxin (Ptx), pertactin (Prn), and often fimbrial subunits (Fim2 and Fim3). While the underlying reasons are not completely understood, the recent surge in pertussis incidence is likely largely due to deficiencies in aP vaccine efficacy, including induction of relatively short-lived immunity and inability to prevent colonization and transmission[8-10]. Despite decades of research, our understanding of the physiological properties of aP vaccine components remains incomplete, a critical shortfall in attempts to decide how best to prevent pertussis in the future.

In accordance with its role as a virulence factor, *in vivo* studies have shown that FHA is required for colonization and persistence in the lower respiratory tracts of mice, rats, and pigs by *Bordetella bronchiseptica*[2,6,11-14], a close relative of *B. pertussis* that naturally infects a broad range of mammalian hosts. Many *Bordetella* virulence factors, including FHA, are highly conserved and have been shown to be functionally interchangeable between *B. pertussis* and *B. bronchiseptica* in animal models of disease[12,15,16]. For these reasons, we use *B. bronchiseptica* infection of common laboratory animals to assess the contribution of *Bordetella* virulence factors to pathogenesis. In addition to its postulated role as an adhesin[17], FHA appears to perform immunomodulatory functions that contribute to colonization and/or persistence[6,11], though whether these effects are a direct result of some undefined FHA activity[18-22] or an indirect result of FHA-mediated adherence to specific host cells[6,23,24] is unclear.

FHA serves as a paradigm for TPS. FHA (defined as the ~250 kDa protein that is both surface-associated and released from the bacterial surface) is initially translated as a preproprotein called FhaB (~370 kDa), which contains an N-terminal signal peptide and a large C-terminal 'prodomain' that are removed during the secretion process. As for most processed proteins, the 'mature' molecule (~250 kDa FHA, in this case) has been assumed to be the

functional form of the protein. According to the current model of FHA secretion[25], the signal peptide directs FhaB across the inner membrane via the Sec translocation machinery[26] and is then removed by leader peptidase. The ~250 aa region of FhaB immediately C-terminal to the signal peptide, referred to as the TPS domain and which is highly conserved among TpsA proteins, is bound by chaperones that maintain the protein in a non-folded state as it transits through the periplasm[27,28]. FhaB then transits through FhaC in an N- to C-terminal direction, with the N-terminus remaining anchored to FhaC at the cell membrane[13,25]. The TPS domain initiates folding of FhaB into a rigid β-helix on the surface of the bacterium[29,30], and progressive folding results in formation of a ~50 nm long shaft. C-terminal to the β-helical shaft, a ~500 aa globular domain begins to fold into the mature C-terminal domain (MCD) at the distal end of the molecule[13,31], which mediates adherence of the bacteria to host cells *in vitro* and to respiratory epithelium *in vivo*[12,25]. Upon translocation of the residues composing the MCD, the proximal region of the FhaB prodomain (called the prodomain N-terminus or PNT), which is conserved among FhaB-like TpsA proteins, prohibits further translocation and retains the prodomain in an intracellular compartment[25]. We hypothesize that anchoring of the C-terminus of the MCD near the membrane by the PNT acts as a sort of intramolecular chaperone that restricts the conformations that the MCD can sample during folding[25]. Deletion of the prodomain abrogates the ability of *B. bronchiseptica* to adhere to host cells *in vitro* and to colonize the lower respiratory tracts of mice and rats *in vivo*[13,25], presumably due to misfolding of the MCD.

Subsequent to translocation and folding of the MCD, proteolysis of the FhaB C-terminal prodomain occurs by an as yet unidentified protease(s)[13,25]. Degradation is rapid and complete[13,25,32], making it unlikely that the prodomain performs an independent function. In ΔPNT strains, in which the prodomain is aberrantly translocated to the surface, the prodomain is

readily detected[25], indicating that the prodomain is not intrinsically unstable, but rather subject to regulated degradation inside the cell. Production of FhaB molecules lacking the C-terminal half of the MCD results in detectable, stable intracellular prodomain polypeptides[25], further supporting the hypothesis of regulated degradation and indicating a role for the extracellular MCD in this regulation. Additional processing to form the C-terminus of 'mature' FHA occurs and is dependent on the surface-localized serine protease autotransporter SphB1[33]. Although the primary function attributed to FHA is adherence to respiratory epithelium, FHA is ultimately released from the cell surface, liberating FhaC to secrete another FhaB molecule.

Our previous observations suggest that portions of the FhaB prodomain contribute to virulence activities that FHA performs in addition to mediating adherence. For example, small deletions near the C-terminus of the prodomain abrogated persistent tracheal colonization of rats by *B. bronchiseptica*, though adherence capabilities *in vitro* were preserved[13]. Here, we set out to determine the contribution of two C-terminal FhaB subdomains, a proline-rich region and a conserved extreme C-terminus, to FhaB/FHA function. We also investigated how prodomain degradation is controlled and what role its regulation plays in the maturation and function of FHA.

Results

The FhaB prodomain localizes to the periplasm

Previous studies demonstrated that the PNT is necessary for retention of the FhaB prodomain in an intracellular compartment, which is essential for proper folding and function of FHA[25]. To determine if the C-terminus of the prodomain enters the periplasm or if it remains in the cytoplasm, we created a *B. bronchiseptica* strain containing a fusion of *phoA* (lacking the codons for its natural signal peptide) to the 3' end of *fhaB*. (All mutations described in this study were made in a derivative of *B. bronchiseptica* strain RB50 lacking *fhaS* (RBX11) to facilitate genetic manipulation of *fhaB* and interpretation of FhaB maturation data[34]. Deletion of *fhaS* does not produce any detectable effects on *B. bronchiseptica* pathogenicity in animal models[34], and we will henceforth refer to RBX11 as the wild-type (WT) strain used in this study.) PhoA activity, assayed by conversion of the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (X-P) to a blue product, is observed only when PhoA is present in the periplasm[35]. Western blot analysis of whole-cell lysates (WCL) and culture supernatants of the WT strain producing the fusion protein displayed no changes in the amount of FhaB/FHA produced, processed, or released (Fig. 1a), indicating that fusion of PhoA to the C-terminus of FhaB did not alter FhaB secretion. We also performed a dot blot on the strain producing FhaB with the C-terminal PhoA fusion, probing the blots with an α-MCD antibody (and secondary antibody with 'green' fluorescence) and an α-PhoA antibody (and secondary antibody with 'red' fluorescence). As seen previously[25], the MCD was detected on the surface of intact bacteria and in disrupted cells (Fig. 1b). In contrast, PhoA was only detected in disrupted cells, indicating that the prodomain remained intracellular. Culture of this strain on plates containing X-P resulted in growth of blue colonies (Fig. 1c), which, combined with the fact that proteins are secreted

through the Sec translocon in an N- to C-terminal fashion[36], indicates that the entire FhaB prodomain is transported to the periplasm during secretion and therefore that the periplasm is the compartment in which prodomain functions occur.

The ECT of FhaB negatively regulates prodomain degradation, while the PRR plays no apparent role in FhaB/FHA processing

Western blot analysis of WT *B. bronchiseptica* reveals the ~370 kDa FhaB preproprotein and multiple processed FHA proteins of ~250 kDa in whole-cell lysates (WCL) and only processed ~250 kDa FHA proteins in culture supernatants (Fig. 2). The largest processed polypeptide is referred to as FHA' in *B. bronchiseptica* (FHA* in *B. pertussis*) and is produced via degradation of the prodomain by an unidentified protease(s)[13,33]. The most abundant processed polypeptide is referred to as FHA and is produced in an SphB1-dependent manner via cleavage of FhaB or FHA'[13,33]. Two smaller processed polypeptides, which are similar in size and often co-migrate on SDS-PAGE gels, are referred to as FHA_1 and FHA_2 and are also produced in an SphB1-dependent manner. The SphB1-dependent FHA, FHA_1, and FHA_2 proteins are the predominant forms released from the bacterial surface, while FHA'/FHA* is primarily only released in strains lacking SphB1, although a slight amount can sometimes be detected in culture supernatants of WT cells[13,33].

The FhaB C-terminus includes a proline-rich region (PRR)[25,37] that is composed of 27% proline residues (as opposed to 2% across the remainder of FhaB) and contains two conspicuous repeat motifs (Supplementary Fig. 1b). Excluding minor differences in the length/number of repeats, the predicted PRR is 87% identical and 92% similar among the strains of *Bordetella* spp. that cause disease in mammals for which genome sequence information is available. C-terminal

to the PRR are 98 aa (Supplementary Fig. 1c) that are 100% identical among all predicted FhaB proteins, which we will henceforth refer to as the FhaB extreme C-terminus (ECT). Western blot analysis of a strain producing FhaB that lacks both subdomains (ΔPRR-ECT) revealed the absence of full-length FhaB in WCL (Fig. 2), but lack of these subdomains had no discernable effect on the amount of FHA produced or released into the culture medium. Deletion of *sphB1* in the ΔPRR-ECT strain resulted in detection of a doublet including FHA' and a slightly smaller polypeptide that we had not observed previously, but which was also present in culture supernatants of the WT strain (Fig. 2).

To investigate the contribution of the individual C-terminal subdomains in FhaB processing, we constructed *B. bronchiseptica* strains that produced FhaB proteins lacking either the PRR or the ECT. Similar to the ΔPRR-ECT strain, no full-length FhaB was detected by western blot analysis of the ΔECT strain (Fig. 2). Deletion of *sphB1* in the strain lacking the ECT resulted in detection of only FHA' and the slightly smaller polypeptide (Fig. 2). Western blot analysis of a strain containing an HA epitope insertion 7 aa N-terminal to the FhaB C-terminus also abolished detection of full-length FhaB (Supplementary Fig. 2)[25], suggesting that the reason for the strict conservation of the ECT in *Bordetella* is that this functional element is unable to tolerate mutation. Additionally, SphB1-dependent processing still occurred in the ΔECT strain (Fig. 2), indicating that FhaB reaches the surface during secretion. These results indicate that the ECT is a negative regulator of prodomain degradation; without it, the prodomain is degraded aberrantly quickly such that full-length FhaB cannot be detected.

Western blot analysis of WCL and culture supernatants of the ΔPRR strain, by contrast, displayed no difference in the amount of FhaB/FHA produced, processed, or released compared with WT bacteria (Fig. 2). This finding suggests that the PRR does not play a role in FhaB

processing. Additionally, fusion of PhoA to the C-terminus of FhaB in the strain lacking the PRR resulted in blue colonies (Fig. 1c), while western blot analysis of WCL and culture supernatants of the ΔPRR strain producing the fusion protein displayed no changes in the amount of FhaB/FHA produced, processed, or released (Fig. 1a). These results indicate that the PRR does not influence prodomain transport to the periplasm. To test whether the PRR is involved in retention of the prodomain in the periplasm, we performed a dot blot. Similar to the WT strain, the MCD was detected on the surface of intact bacteria and in disrupted cells and PhoA was only detected in disrupted cells (Fig. 1b), indicating that the prodomain remained intracellular and that the PRR does not contribute to intracellular retention of the prodomain. Furthermore, deletion of the PRR did not result in increased proportions of full-length FhaB in WCL samples or release of full-length FhaB into culture supernatants in a Δ*sphB1* strain (Fig. 2), as is seen with strains lacking the PNT[25]. SphB1-dependent processing still occurs in the ΔPRR strain (Fig. 2), suggesting that FhaB reaches the surface during secretion. Together, these results suggest that the PRR does not play a role in production of 'mature' FHA.

'Mature' FHA is sufficient to mediate adherence to respiratory epithelium

Since the ΔECT strain essentially produces only 'mature' FHA, this strain provided a tool to investigate whether 'mature' FHA is indeed the active form of the protein *in vivo*. Because *in vitro* adherence assays are typically performed with non-ciliated, non-polarized cell lines and *Bordetella* adhere primarily to ciliated respiratory epithelium *in vivo*[38,39], we developed an *in vivo* assay to assess the contribution of FHA to adherence to the respiratory tract (Scheller). Using this protocol, ~1% of the inoculum of WT bacteria was recovered in bronchoalveolar lavage fluid (BALF), indicating that ~99% of the bacteria were retained in the respiratory tract.

In contrast, ~27% of the inoculum was recovered for an avirulent Bvg$^-$ phase-locked strain that is completely non-adherent *in vitro* (Fig. 3)[40]. Thus, ~30% recovery appears to be the upper limit of detection in this assay for non-adherent strains. Inoculation with a FHA-null strain resulted in recovery of ~30% of the inoculum in the BALF (Fig. 3), indicating that FHA is an essential adhesin for *B. bronchiseptica* in the murine respiratory tract.

To determine whether the accelerated degradation of the prodomain in strains lacking the C-terminal subdomains of FhaB alters adherence of *B. bronchiseptica in vivo*, we inoculated mice with strains lacking the PRR, the ECT, or both. Approximately two percent of the inoculum of the ΔPRR-ECT strain was recovered in the BALF (Fig. 3), indicating that the C-terminal subdomains are not required for FHA-mediated adherence *in vivo*. Accordingly, ~2% of the inoculum of the ΔECT strain and ~4% of the inoculum of the ΔPRR strain were recovered in the BALF (Fig. 3). These findings reveal that 'mature' FHA is sufficient to mediate adherence to the respiratory tract. Additionally, co-inoculation of WT *B. bronchiseptica* with the ΔPRR strain, the ΔECT strain, or the FHA-null strain did not alter recovery of either strain compared to inoculation with each strain alone (Fig. 3). These results indicate that *in vivo* adherence is determined on a per bacterium basis and that FHA proteins secreted by WT bacteria are unable to complement adherence defects.

Deletion of a large portion of the FhaB prodomain (including most of the PNT) was previously demonstrated to abrogate *B. bronchiseptica* adherence to rat lung epithelial L2 cells and *B. pertussis* adherence to human lung epithelial A549 cells *in vitro*[13,25]. This mutation also resulted in aberrant folding of the FHA MCD[25]. Furthermore, α-MCD antibodies were able to abrogate adherence of *B. pertussis* and *B. bronchiseptica* to both rat lung epithelial L2 cells and mouse macrophage-like J774A.1 cells *in vitro*[12]. Together, these findings suggest that the MCD

facilitates FHA-mediated adherence and that the FhaB prodomain is required for correct folding of the MCD. In agreement with these findings, ~24% of the inoculum of a *B. bronchiseptica* ΔProdomain strain was recovered in the BALF (Fig. 3). When considered with the result that deletion of the C-terminal subdomains did not abrogate FHA-mediated adherence to the respiratory tract, these findings support the requirement of an intact PNT to mediate retention of the prodomain, which facilitates folding of the MCD, and to produce an FHA molecule capable of mediating adherence to the respiratory tract. Combined, these data further suggest that FHA is folded correctly and is able to confer adherence capabilities to *B. bronchiseptica* in strains lacking the ECT. Since the PRR does not influence FhaB/FHA processing (Fig. 2) or FhaB prodomain localization (Fig. 1), these data also strongly suggest that the PRR does not play a role in production of functional 'mature' FHA. The 'mature' FHA molecules produced in the ΔPRR and ΔECT strains are thus indistinguishable from those produced by WT bacteria, and therefore the only difference between the WT and mutant strains is the 'premature' FhaB molecules they produce.

'Mature' FHA is not sufficient for **B. bronchiseptica** *persistence in the lower respiratory tract*

Deletion of both the PRR and ECT subdomains (ΔPRR-ECT) was previously shown to reduce persistence in the tracheas of rats[13], even though this strain is capable of adhering both *in vivo* (Fig. 3) and *in vitro*[13]. To determine which of the individual C-terminal subdomains is involved in FhaB/FHA-mediated virulence activities, we inoculated mice intranasally with various *B. bronchiseptica* strains and monitored bacterial burden in the respiratory tract over time. As has been observed previously[6,11,12], WT bacteria persisted at high levels in the trachea and lungs beyond 11 days post-inoculation, and FHA-null bacteria were mostly cleared from the

trachea and lungs by 11 days post-inoculation (Fig. 4a). The FHA-null strain displayed an increase in burden at 1 day post-inoculation compared to WT bacteria, a bimodal distribution of burden at 3 days post-inoculation, and a dramatic reduction in burden by day 11 (Fig. 4a). Additionally, as has been previously shown[2,11-13], the number of CFU recovered from the nasal cavity for the FHA-null strain was similar to that for WT bacteria (Supplementary Fig. 3), indicating that FHA is not required for colonization and persistence in the upper respiratory tract of rodents.

Similar to the FHA-null strain, the ΔECT strain was unable to persist in the murine lower respiratory tract, as most mice had completely cleared this strain from their trachea and lungs by 11 days post-inoculation (Fig. 4a). However, the course of infection of the ΔECT strain differed from that of the FHA-null strain; there was no increase in burden at 1 day post-inoculation or bimodal burden distribution at 3 days post-inoculation in the lungs (Fig. 4a). The ΔPRR strain also displayed increased clearance from the mouse trachea and lungs compared to WT bacteria (Fig. 4a), while, similar to FHA-null bacteria, colonization and persistence in the nasal cavity were not compromised (Supplementary Fig. 3). We also created the identical ΔPRR mutation in WT strain RB50, which contains an intact *fhaS* gene, and deletion of the PRR in this background resulted in the same persistence defect in the lower respiratory tract (Supplementary Fig. 4), demonstrating that the decreased persistence in the lower respiratory tract is due to the lack of the FhaB PRR rather than lack of *fhaS* (in addition to the ΔPRR mutation) or an undetected additional mutation elsewhere on the chromosome. Since the 'mature' FHA molecules produced by the ΔPRR strain and the ΔECT strain are indistinguishable from those produced by WT bacteria, these results indicate that 'mature' FHA is not sufficient to mediate *B. bronchiseptica*

persistence, revealing an active role for the full-length FhaB polypeptide *in vivo* that is dependent on both the PRR and the ECT.

'Mature' FHA is sufficient for immunomodulation

Previous studies have suggested that FHA contributes to *Bordetella* persistence in mice via suppression of inflammation[6,11]. To ascertain whether the increased clearance observed with our ΔPRR and ΔECT strains is due to an increased induction of inflammation compared to WT bacteria, we measured global cytokine and chemokine levels in the mouse lung during infection. As previously reported [6], the FHA-null strain induced higher production of pro-inflammatory cytokines, such as interleukin (IL)-1β, and chemokines, such as the neutrophil chemoattractant KC and the monocyte chemoattractant MCP-1, than WT bacteria (Fig. 4b). There was no difference in global levels of gamma interferon (IFNγ), tumor necrosis factor α (TNFα), IL-10, IL-12p70, IL-17, IL-22, or IL-23 produced in the lungs of mice infected with WT or FHA-null bacteria (Supplementary Fig. 5), suggesting that the primary difference in the innate immune response to infection with WT or FHA-null bacteria is the intensity of the initial IL-1β-mediated inflammation.

In contrast to FHA-null bacteria, neither the ΔECT strain nor the ΔPRR strain stimulated higher inflammatory cytokine or chemokine production than the WT strain during infection (Fig. 4b). These data suggest that the increased clearance observed with these strains is <u>not</u> due to a lack of FHA-mediated immunosuppression and that 'mature' FHA is sufficient to suppress the initial inflammatory response.

'Premature' FhaB is required for resistance to innate immune response-mediated clearance

We have previously demonstrated that co-inoculation of mice with WT and FHA-null bacteria partially 'rescues' persistence of FHA-null bacteria in the lungs compared to inoculation with only the FHA-null strain, while persistence of WT bacteria after co-inoculation was unaltered compared to inoculation with only the WT strain[11]. Inflammation was suppressed after co-inoculation compared to inoculation with only FHA-null bacteria; however, FHA-null bacteria were still not able to persist as well as WT bacteria[11], suggesting that FhaB/FHA may mediate resistance to clearance even under less inflammatory conditions. Similar to these previous findings, the number of CFU of WT bacteria recovered after co-inoculation with the FHA-null strain was unchanged compared to inoculation with only WT bacteria, while the number of CFU of the FHA-null bacteria was increased compared to inoculation with only FHA-null bacteria at 11 days post-inoculation (Fig. 5a). Additionally, production of pro-inflammatory cytokines and chemokines after co-inoculation was identical to that seen in mice inoculated with only WT bacteria (Fig. 5b). However, the number of CFU of the FHA-null strain at 11 days post-inoculation in the co-inoculation experiment was still lower than that of WT bacteria, supporting the hypothesis that FhaB/FHA plays another role in persistence that is distinct from its role in suppressing the intensity of the inflammatory response.

In contrast to co-inoculation of mice with WT and FHA-null bacteria, co-inoculation of WT bacteria with either the ΔECT or ΔPRR strain did not alter clearance of the mutant bacteria, they were cleared as rapidly as when inoculated in the absence of WT bacteria (Fig. 5a). Production of cytokines and chemokines was similarly unaltered during co-inoculation compared to inoculation with only WT bacteria (Fig. 5b). Since WT bacteria failed to 'rescue' either the ΔECT or ΔPRR strain in the presence of an unaltered inflammatory response, these results

support the hypothesis that the virulence defect of the ΔECT and ΔPRR strains that leads to the decreased persistence is a lack of resistance to clearance by the innate immune response. Moreover, these results strongly argue that 'mature' FHA is not sufficient for this activity, revealing a functional role for 'premature' FhaB in resistance to innate immunity-mediated clearance and indicating that the PRR is essential for that activity.

Discussion

Our previous studies revealed that FhaB secretion and maturation is a highly regulated activity. The conserved N-terminal region of the prodomain is required for proper MCD folding and subsequent adherence capabilities[25]. Additionally, we observed production of stable intracellular prodomain fragments in strains lacking the C-terminal portion of the MCD[25], suggesting a role for the MCD in regulating prodomain degradation. Together, these findings indicated that information is relayed bidirectionally across the outer membrane via the FhaB primary sequence (i.e. regulation of MCD folding on the bacterial surface and initiation of prodomain degradation in the periplasm). Here, we determined that the prodomain resides in the periplasm during FhaB secretion (Fig. 1) and demonstrated that deletion of the FhaB ECT resulted in the inability to detect full-length FhaB molecules (Fig. 2), indicating that the ECT is a negative regulator of prodomain degradation in the periplasm. A model of FhaB secretion taking these findings into account is shown in Supplementary Fig. 6.

Taking advantage of the fact that the ΔECT strain essentially produces only 'mature' FHA, we examined whether FHA was in fact capable of performing all virulence functions that have been attributed to this molecule. Using our recently developed *in vivo* adherence assay, we found that FHA is both necessary and sufficient to mediate adherence to the murine respiratory tract (Fig. 3). Additionally, the ΔECT strain was capable of suppressing the acute inflammatory response in the murine lower respiratory tract (Fig. 4b), indicating that 'mature' FHA is also both necessary and sufficient to perform this function. Surprisingly, however, the ΔECT strain was unable to persist in the murine lower respiratory tract (Fig. 4a), and co-infection with WT bacteria did not rescue the persistence defect or alter the inflammatory response (Fig. 5). These data reveal three previously unappreciated aspects of FHA physiology: 1) the persistence defect

of FHA-mutant strains is not solely due to lack of adherence or suppression of inflammation, 2) 'mature' FHA is not sufficient to facilitate all *fhaB*-mediated virulence activities, and 3) full-length 'premature' FhaB is therefore required for resistance to innate immune effectors. In support of these conclusions, deletion of the FhaB PRR, which did not result in any FhaB processing abnormalities (Fig. 1, Fig. 2) or affect production of functional 'mature' FHA (Fig. 3, Fig. 4b), produced almost identical phenotypes as seen for the ΔECT strain, indicating that the defect for both these strains is the lack of functional 'premature' FhaB molecules and that the PRR is essential for FhaB activity.

We hypothesize that transmission of information is the basis for the activity of full-length FhaB, acting as a transmembrane sensor, and that the PRR is integral for relaying information *in vivo*. Due to the fact that it contains repeat motifs (Supplementary Fig. 1b) and the proclivity of polyproline peptides to adopt an extended conformation[41], we postulate that the PRR acts as a docking site for protein interactions in the periplasm and that modulation of interactions are required for resisting the innate immune response. These interactions likely direct the action of a short-range effector(s), as the defects observed with the ΔPRR and ΔECT strains appeared to operate on a per bacterium basis (Fig. 3, Fig. 5). Discriminating between potential mechanisms by which this occurs will be the subject of future investigations. We expect that the defect in *B. bronchiseptica* lower respiratory tract persistence observed for the ΔPRR strain is due to decreased resistance to clearance by phagocytes. While decreased resistance to other aspects of the innate immune response is possible, it is unlikely that full-length FhaB is necessary for resistance to mechanical clearance, as the ΔPRR and ΔECT strains display normal adherence to the respiratory tract (Fig. 3), or to secreted molecular immune effectors. By contrast, FhaB/FHA has been reported to mediate interactions with phagocytes[42-44].

Our results suggest that once FhaB is processed to 'mature' FHA, *B. bronchiseptica* is no longer able to resist clearance by the innate immune response. This finding perhaps elucidates a paradox in FHA physiology; namely, that FHA, a critical adhesin, is released from the bacterial surface in large quantities (Fig. 2). While our experiments are unable to determine whether released FHA plays a role in suppression of the immune response, as has been suggested[18-22], this work provides an alternative role for release of FHA. FHA release may predominantly serve the purpose of liberating FhaC to secrete a new FhaB molecule, which can then mediate persistence in the lower respiratory tract. If true, this hypothesis suggests that the multiple processing events that FhaB undergoes serve several purposes. Initiation of prodomain degradation may serve as the periplasmic signal that FhaB has sensed environmental changes, while SphB1-dependent cleavage, which results in increased release of FHA from the surface (Fig. 2)[13,33], may facilitate FhaB turnover.

Integral to understanding the physiological function of proteins is elucidation of the means by which they achieve their functional forms. For processed proteins, it is typically assumed that the final polypeptide observed *in vitro* is the functional form of the protein. This work suggests that precursor molecules are important to the physiological function of some processed proteins. The results presented here demonstrate the presence of multiple intramolecular determinants of FhaB prodomain degradation, in addition to multiple known intermolecular determinants, indicating that 'maturation' of FhaB is a tightly regulated process. While dysregulation of prodomain degradation does not affect the ability of 'mature' FHA to mediate adherence or immunosuppression during infection (Fig. 3, Fig. 4b), these studies yielded the unexpected finding that 'premature' FhaB plays an active role in pathogenesis, specifically in resisting clearance by innate immune effectors. Our results highlight the advantage of studying

protein secretion and function simultaneously as a role for the FhaB PRR was only evident from *in vivo* infection studies and not from studies on the production or maturation of FHA or even from virulence-associated *in vitro* assays[13]. Our results also provide a reminder that while culturing bacteria in the laboratory is extremely useful and often essential for understanding molecular mechanisms underlying microbiological processes such as protein secretion, *in vitro* growth conditions may not accurately mimic the conditions under which the microbiological processes under study function in nature.

Materials and Methods

Bioinformatics

Protein sequences were obtained from the NCBI Protein Database. Sequence alignments were conducted using Clustal Omega[45] and visualized using Jalview Version 2[46]. Searches for similar sequences were conducted with blastp.

Ethics statements

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. Our protocols were approved by the University of North Carolina IACUC (10-134, 12-307, and 13-238). All animals were properly anesthetized for inoculations, monitored regularly, and euthanized when moribund, and efforts were made to minimize suffering.

Growth media and bacterial strains

B. bronchiseptica strains were grown at 37°C in Stainer-Scholte (SS) broth or on Difco Bordet-Gengou (BG) agar (BD) supplemented with 5.5% defibrinated sheep blood (Colorado Serum Co., Denver, CO). *Escherichia coli* strains were grown at 37°C in Luria broth (LB) or on LB agar. A detailed description of bacterial strains, their construction, and the rationale for their use is included in Supplementary Methods. Where appropriate, media were supplemented with gentamicin (30 μg/mL), streptomycin (20 μg/mL), kanamycin (50 μg/mL), $MgSO_4$ (50 mM), diaminopimelic acid (200 μg/mL), or 5-bromo-4-chloro-3-indolyl phosphate (XP, 40 μg/mL).

Immunoblotting

To evaluate FHA production and processing, proteins were prepared from *B. bronchiseptica* cultures grown in SS broth and normalized based on optical density. For cell-associated proteins, whole-cell lysates (WCL) of bacteria were prepared by boiling in sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For released proteins, culture supernatants precipitated with 10% trichloroacetic acid (TCA, Fisher Scientific) were mixed with SDS-PAGE sample buffer and boiled. Proteins were separated by SDS-PAGE on 5% polyacrylamide gels, transferred to nitrocellulose (GE Healthcare), and probed with a rabbit polyclonal antibody generated against the FHA mature C-terminal domain (MCD)[12], a mouse monoclonal antibody generated against PhoA (Covance), or a mouse monoclonal antibody generated against an HA epitope (Covance). Corresponding α-rabbit and α-mouse IRDye secondary antibodies were used to detect proteins (Li-Cor Biosciences). Membranes were imaged on a Li-Cor Odyssey Classic Infrared Imager (Li-Cor Biosciences).

To assess the surface accessibility of proteins, *B. bronchiseptica* grown in SS broth were washed and resuspended in phosphate-buffered saline (PBS, Life Technologies), and half of each sample was boiled to disrupt the cells. Intact and disrupted cells were then spotted on nitrocellulose and probed with a rabbit polyclonal α-MCD antibody and a mouse monoclonal α-PhoA antibody. Corresponding α-rabbit and α-mouse IRDye secondary antibodies were used to detect proteins, and membranes were imaged on a Li-Cor Odyssey Classic Infrared Imager.

Bacterial adherence to the respiratory tract

Bacterial adherence was measured using a recently developed *in vivo* adherence assay (Scheller). Briefly, six-week old female BALB/cJ mice from Jackson Laboratories (Bar Harbor,

ME) were anesthetized with isofluorane and inoculated intranasally with 7.5×10^4 colony forming units (CFU) of *B. bronchiseptica* in 50 μL PBS. For co-inoculation experiments, WT and mutant strains were marked with different antibiotic resistance genes as described in Supplementary Methods. Mice were euthanized 30-60 minutes after inoculation. Bronchoalveolar lavage fluid (BALF) was collected by inserting a cannula into the trachea, ligating with suture, and injecting and retracting 1 mL PBS. Serial dilutions of BALF and the inoculum were plated on BG agar to quantitate recovery of bacterial CFU.

Bacterial persistence and characterization of the immune response during infection of mice

Six-week old female BALB/cJ mice were inoculated and euthanized as above. For co-inoculation experiments, WT and mutant strains were marked with different antibiotic resistance genes as described in the Supplementary Methods. Lungs, tracheas, and nasal cavities were harvested and homogenized in PBS. Serial dilutions of homogenized tissues were plated on BG agar (with appropriate antibiotics) to quantitate recovery of bacterial CFU. Cytokine and chemokine production were analyzed by conducting enzyme-linked immunosorbent assays (ELISA) on lung homogenates diluted in PBS (R&D Systems).

Statistical analysis

Data were plotted and statistical analyses were performed using Prism, version 5.0 software (GraphPad Software, Inc.). For all conditions analyzed, $n\geq6$ (two or more separate experiments on different days with $n\geq3$), and statistical significance was determined using analysis of variance (ANOVA).

Supporting Information

Figure 1. The FhaB prodomain transits to the periplasm, where it is retained during secretion

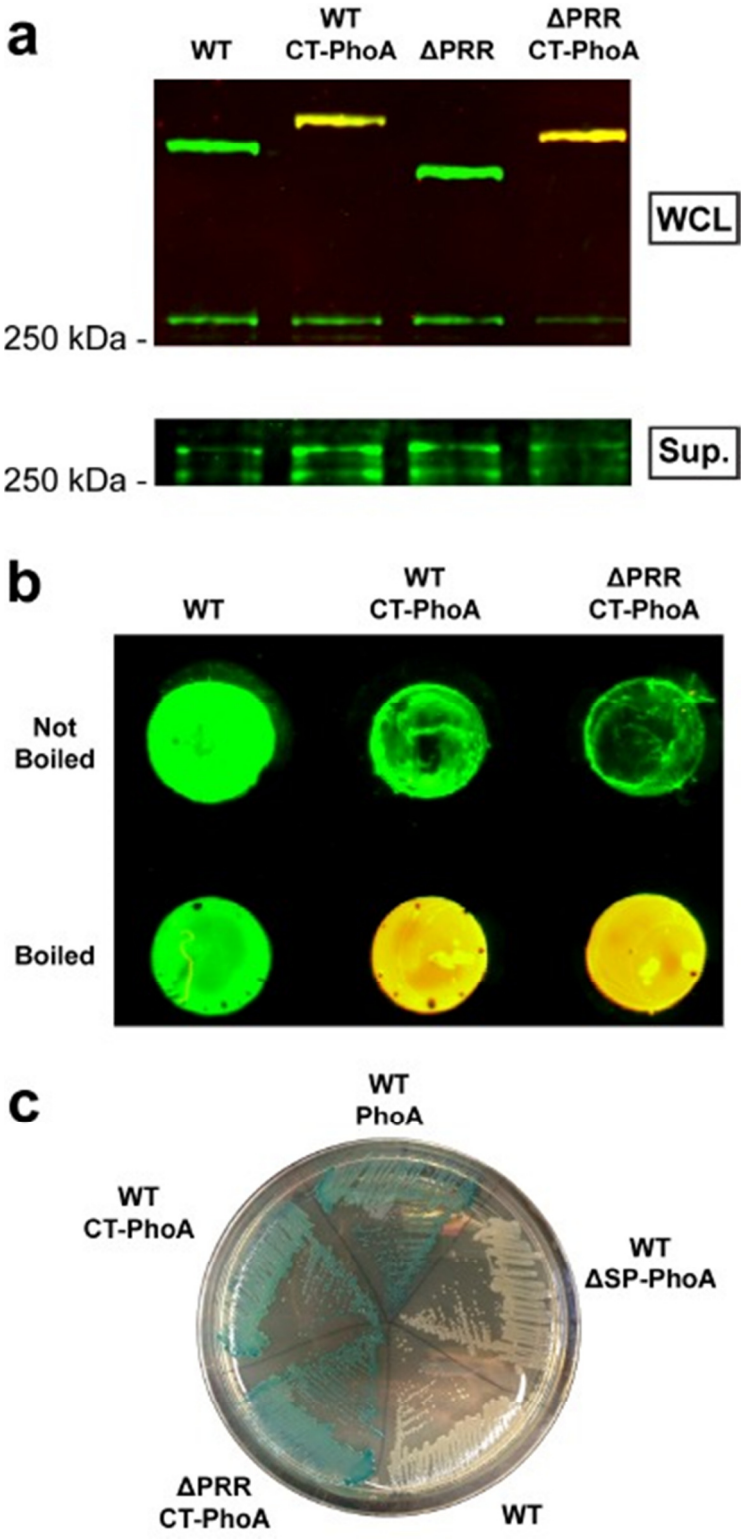

Figure 1. The FhaB prodomain transits to the periplasm, where it is retained during secretion. (a) Culture of strains containing various *phoA* insertions on an agar plate containing a chromogenic PhoA substrate. Fusion of PhoA (lacking its native signal peptide) to the C-terminus of FhaB (CT-PhoA strains) resulted in production of blue colonies similar to those produced by a strain constitutively producing PhoA (WT PhoA), while a strain constitutively producing PhoA lacking its native signal peptide (WT ΔSP-PhoA) or not producing PhoA (WT) did not produce blue colonies. (b) Dot blot analysis of intact (not boiled) and disrupted (boiled) bacteria for strains not producing PhoA (WT) or with PhoA fused to the C-terminus of FhaB (CT-PhoA strains). Membranes were probed with α-MCD (green) and α-PhoA (red) antibodies, and the green and red channels are overlaid. PhoA was only accessible to antibodies after lysis of the cells by boiling. (c) Western blot analysis of whole-cell lysates (WCL) and culture supernatants (Sup.) from strains containing 3' fusions of *phoA* to *fhaB*. C-terminal PhoA fusion (CT-PhoA strains) did not alter production or processing of FhaB or release of FHA. Membranes were probed as in (b).

Figure 2. The extreme C-terminus (ECT) of the FhaB prodomain inhibits prodomain degradation.

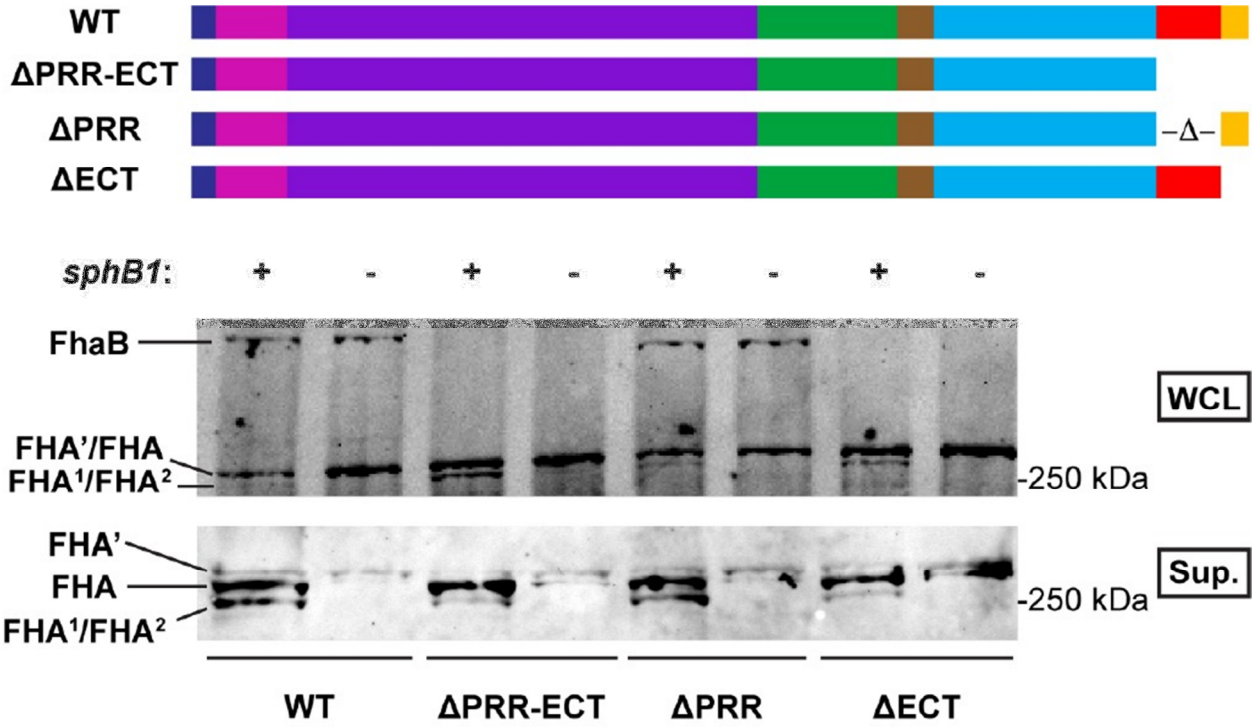

Figure 2. The extreme C-terminus (ECT) of the FhaB prodomain inhibits prodomain degradation. Western blot analysis of whole-cell lysates (WCL) and culture supernatants (Sup.) from strains lacking the FhaB PRR (ΔPRR), the FhaB ECT (ΔECT), both (ΔPRR-ECT), or neither (WT), as well as each strain lacking *sphB1*. Strains lacking the FhaB ECT do not contain any observable FhaB in WCL or Sup. fractions. Membranes were probed with α-MCD antibodies. The FhaB molecules translated by each strain are diagrammed above (dark blue – signal peptide, pink – TPS domain, purple – β-helical domain, green – MCD, brown – PNT, light blue – uncharacterized prodomain, red – PRR, yellow – ECT).

Figure 3. Properly folded FHA is necessary and sufficient for adherence of *B. bronchiseptica* to the respiratory tract.

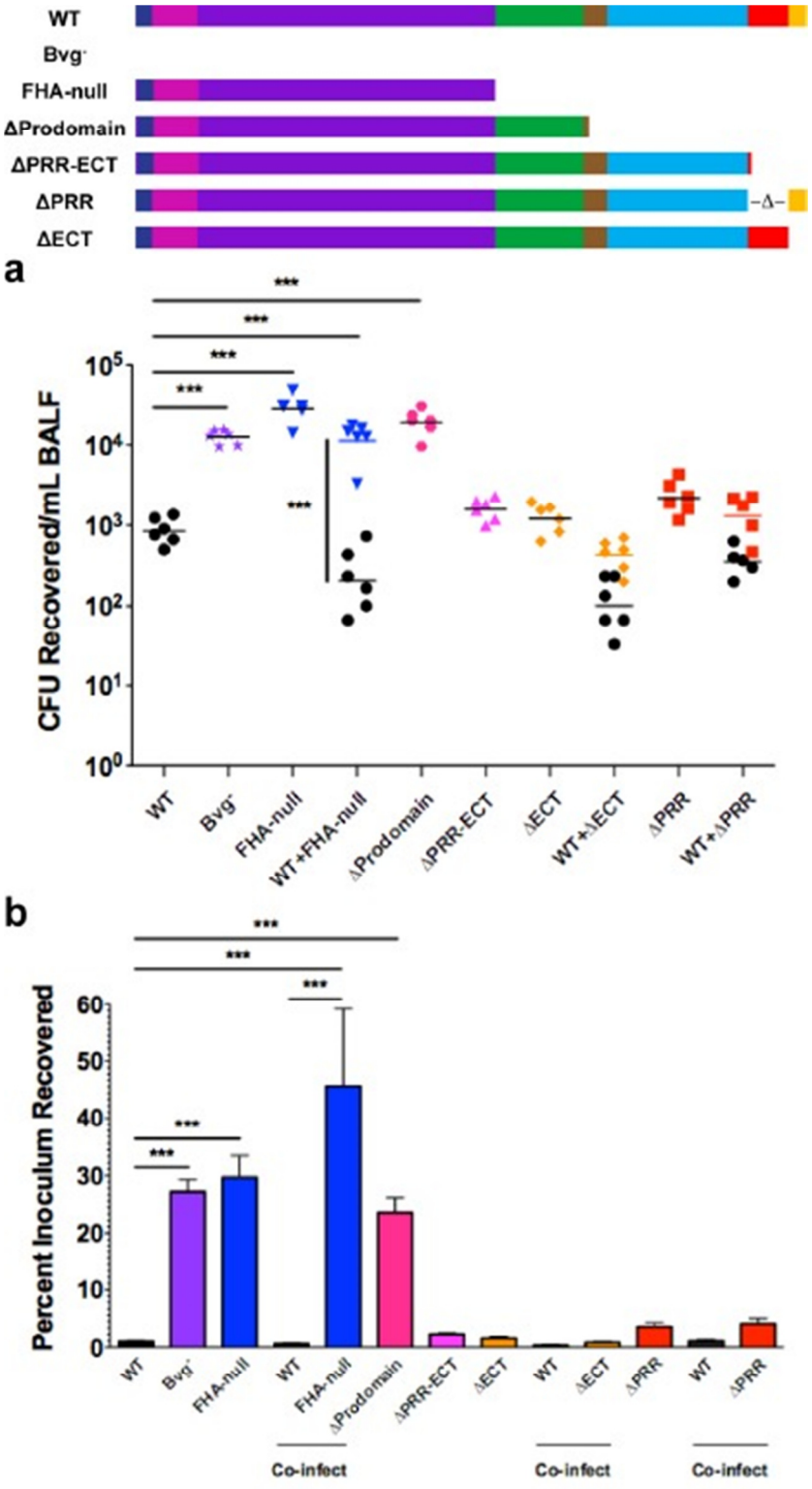

Figure 3. Properly folded FHA is necessary and sufficient for adherence of *B. bronchiseptica* to the respiratory tract. (a) CFU recovered in BALF 30-60 min after intranasal inoculation of mice. Each point represents the number of CFU recovered from a single animal. (b) Recovery of bacteria in BALF represented as percent of inoculum for each strain. Only strains lacking a properly folded MCD displayed decreased retention in the murine respiratory tract. The FhaB molecules translated by each strain are diagrammed above (dark blue – signal peptide, pink – TPS domain, purple – β-helical domain, green – MCD, brown – PNT, light blue – uncharacterized prodomain, red – PRR, yellow – ECT). Data are pooled from 2 separate experiments conducted on different days. Percent of inoculum data are represented as mean ± SEM. *** = $p<0.001$

Figure 4. The FhaB proline-rich region and extreme C-terminus are necessary for *B. bronchiseptica* persistence in the lower respiratory tract in a manner that is distinct from suppression of inflammation

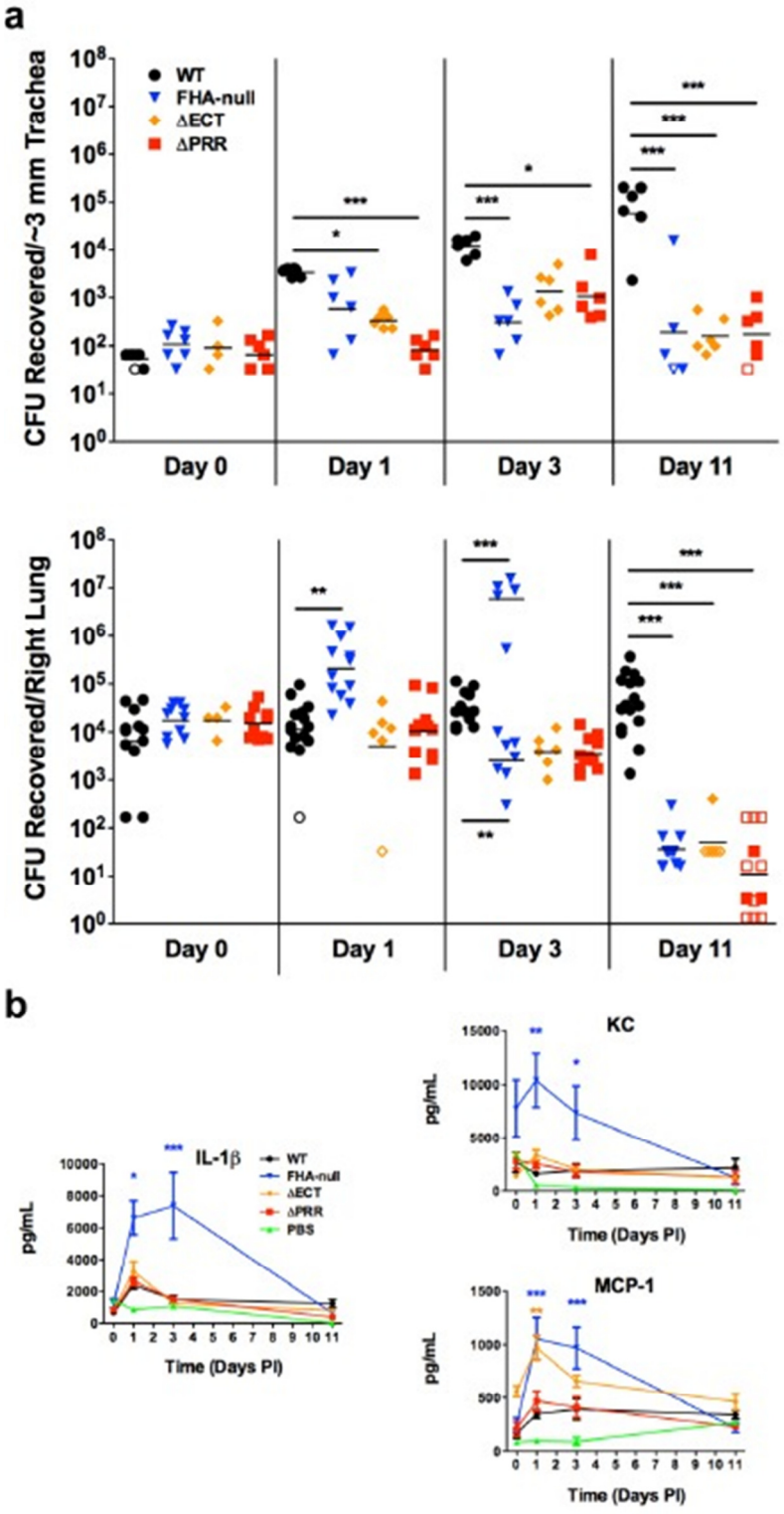

Figure 4. The FhaB proline-rich region and extreme C-terminus are necessary for *B. bronchiseptica* persistence in the lower respiratory tract in a manner that is distinct from suppression of inflammation. (a) Bacterial burden in the murine lower respiratory tract after intranasal inoculation. Each point represents the number of CFU recovered from a single animal. Open symbols represent the lower limit of detection for that particular experiment as no CFU were recovered. (b) Production of pro-inflammatory cytokines (IL-1β) and chemokines (KC, MCP-1) in the murine lung during infection. Cytokine and chemokine levels were determined from lung homogenates by ELISA. Data represent mean ± SEM for all samples, which were collected from all animals for which CFU are shown in Fig. 6a. Data are pooled from ≥2 separate experiments conducted on different days. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

Figure 5. The persistence defect of the ΔPRR and ΔECT strains cannot be rescued by WT bacteria.

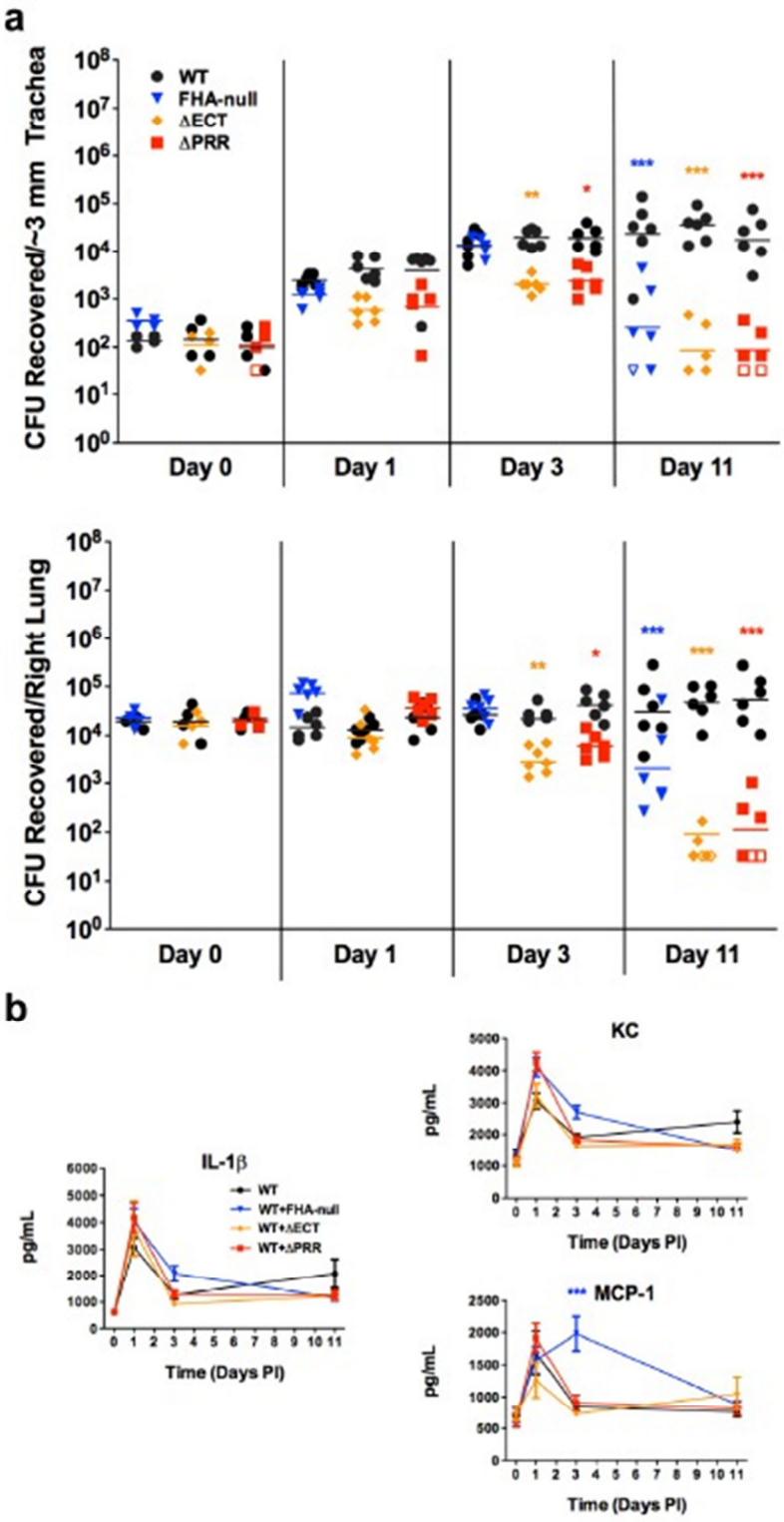

Figure 5. The persistence defect of the ΔPRR and ΔECT strains cannot be rescued by WT bacteria. (a) Bacterial burden in the murine lower respiratory tract after co-inoculation with WT and FHA mutant strains marked with different antibiotic resistance genes. Each point represents the number of CFU recovered from a single animal. Open symbols represent the lower limit of detection for that particular experiment as no CFU were recovered. (b) Production of pro-inflammatory cytokines (IL-1β) and chemokines (KC, MCP-1) in the murine lung during co-infection. Cytokine and chemokine levels were determined from lung homogenates by ELISA. Data represent mean ± SEM for all samples, which were collected from all animals for which CFU are shown in Fig. 7a. Data are pooled from 2 separate experiments conducted on different days. Statistical significance is indicated for mutant bacteria compared to WT from the same co-inoculation group. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.0011$

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CHAPTER 4. CLEARANCE OF FIM-DEFICIENT BACTERIA REQUIRES A COMPONENT OF THE ADAPTIVE IMMUNE RESPONSE

Rationale

As we have demonstrated in Chapter 2, FIM-deficient bacteria are defective for persistence in the lower respiratory tract of mice, as FIM-deficient bacteria were unrecoverable from the lungs by 14 days post-inoculation (1) (Chapter 2). This persistence defect coincides with increased pro-inflammatory cytokines, such as IL-1β, and increased production of the chemokines KC and MCP-1 (1) (Chapter 2). KC is a chemokine involved in the recruitment of neutrophils (2), and MCP-1 is a chemokine involved in the recruitment and activation of macrophages (3). H&E stained lung sections from mice inoculated with FIM-deficient bacteria indicated increased cellular infiltrate in the lungs (1) (Chapter 2). Taken together, these data suggest that FIM-deficient bacteria cause a hyper-inflammatory environment in the lower respiratory tract, leading to increased recruitment and activation of macrophages and neutrophils. We hypothesized that the innate immune response, specifically neutrophils and macrophages, would be sufficient to clear FIM-deficient bacteria from the lower respiratory tract of mice. To test this hypothesis, we used severe combined immunodeficiency (SCID) mice. We have previously shown that SCID mice, which do not have functional T or B-cells but do have macrophages and neutrophils (4) will not clear *B. bronchiseptica* and will succumb to infection approximately 60 days post-inoculation (5), suggesting the adaptive immune response is required for clearing WT *Bordetella* infection. Additionally, we have shown that neutrophils are required

to control the initial *Bordetella* infection (5). Given that SCID mice possess functional macrophages and neutrophils, that neutrophils are required to control the initial *Bordetella* infection, and that Chapter 2 suggests that neutrophils and macrophages are sufficient to clear FIM-deficient bacteria, we hypothesized that FIM-deficient bacteria would be defective for persistence in the lower respiratory tract of SCID mice.

Results

B and/or T-cells are required for clearance of FIM-deficient bacteria.

BALB/c SCID mice were inoculated intranasally with 7.5 x 10^4 CFUs of either WT or Δ*fimBCD B. bronchiseptica*. Mice were euthanized three, 14 and 45 days post-inoculation, and bacterial burdens were determined from the right lung lobes. SCID mice inoculated with WT bacteria had high bacterial burden at three, 14 and 45 days post-inoculation (Fig 1A), consistent with previous published data of high bacterial burden of WT bacteria in SCID mice (5). These data also differed, as expected, from results from immune-competent BALB/c mice inoculated with WT bacteria, where the bacteria are cleared from the lower respiratory tract approximately 30 days post-inoculation (5). Surprisingly, there was no significant difference between SCID mice inoculated with FIM-deficient bacteria and SCID mice inoculated with WT bacteria at any time point observed, indicating that the FIM-deficient bacteria were not defective for persistence in the SCID mice. These data suggest that SCID mice lack a cellular component that contributes to the early clearance of FIM-deficient bacteria that we had previously observed in immune-competent BALB/c mice.

In Chapter 2, we observed that H&E stained lung sections from mice inoculated with FIM-deficient bacteria had increased cellular infiltrate, presumably neutrophils and macrophages. To determine if SCID mice inoculated with WT or FIM-deficient bacteria had increased cellular infiltrate in the lungs, the left lung lobes were prepared for histology, stained with hemotoxylin and eosin and observed by microscopy. Despite high bacterial burdens in the lungs of mice at 14 and 45 days post-inoculation, we observed very little cellular infiltrate in lungs from mice inoculated with either WT or FIM-deficient bacteria (Fig 1B). The absence of

cellular infiltrate was startling; we have previously shown that mice with high bacterial burden have robust cellular infiltrate around the major airways (1) (Chapter 2). The lung sections from the SCID mice inoculated with WT or FIM-deficient bacteria appeared similar to H&E stained lung sections of PBS mock inoculated mice. These data suggested that cellular components absent in SCID mice were critical in recruiting phagocytes to the lungs during *Bordetella* infection. We hypothesized that adoptive transfer of bulk splenocytes from immune-competent mice, which would contain B and T-cells absent in SCID mice, would provide immune components necessary for clearance of the FIM-deficient bacteria and recruitment of cellular infiltrate to the lungs.

To test this hypothesis, bulk splenocytes were purified from the spleens of immune-competent BALB/c mice, and were adoptively transferred intravenously to SCID mice one day prior to inoculation. Mice were then inoculated intranasally with 7.5×10^4 CFU of either WT or *ΔfimBCD* bacteria, and lungs were harvested to determine CFUs. At seven days post-inoculation, there was no significant difference in bacterial burden in the lungs from mice inoculated with WT or FIM-deficient bacteria (Fig 2A). At 14 days post-inoculation, however, we recovered significantly fewer bacteria from the mice inoculated with FIM-deficient bacteria compared to those inoculated with WT bacteria. At 21 days post-inoculation there was no significant difference in the number of CFUs recovered from of mice inoculated with either strain. These data indicate that adoptive transfers of bulk splenocytes can partially restore the persistence defect of the FIM-deficient bacteria.

To determine if adoptive transfer of bulk splenocytes restored cellular infiltrate to the lungs during infection, the left lung lobe was sectioned and stained with hemotoxylin and eosin. Seven and 14 days post-inoculation, lung sections from mice inoculated with WT bacteria had

cellular infiltrate around the major airways (Fig 2B). The amount of cellular infiltrate, qualitatively, appeared similar to the cellular infiltrate observed from H&E stained lung sections from immune-competent mice inoculated with WT bacteria at similar time points. When mice were inoculated with FIM-deficient bacteria, we observed cellular infiltrate around the major airways, as well as in the alveolar spaces. The amount of cellular infiltrate did not appear as robust, however, as we had previously observed in lung sections from immune competent mice inoculated with FIM-deficient bacteria. Together, these data indicate that bulk splenocytes are capable of restoring innate immune cell recruitment in SCID mice. The qualitative differences in the amount of cellular infiltrate observed from H&E stained lung sections, and the partial restoration of FIM-deficient bacterial clearance, suggests that adoptive transfer of bulk splenocytes does not completely reconstitute the immune environment of an immune-competent mouse.

Discussion

Given that FIM-deficient bacteria are cleared from the lungs of mice by 14 days post-inoculation (1) (Chapter 2), that there is an increased influx of cellular infiltrate to the lungs of mice inoculated with FIM-deficient bacteria (1) (Chapter 2), and that neutrophils have been shown necessary to control *Bordetella* infection (5, 6), we hypothesized that the innate immune response alone would be sufficient to clear FIM-deficient bacteria. Hence, we tested this hypothesis using SCID mice that lack B and T cells and therefore rely on innate immunity for protection. Surprisingly, SCID mice were unable to clear FIM-deficient bacteria from the lungs, and H&E stained lung sections indicated very little cellular recruitment to the lungs of mice inoculated with either WT or FIM-deficient bacteria. Adoptive transfer of bulk splenocytes partially restored clearance of FIM-deficient bacteria, and restored recruitment of immune cells to the lungs. The histology pattern and timing of bacterial clearance, however, were not identical to previously published data in immune-competent mice inoculated with FIM-deficient bacteria (1) (Chapter 2). Together, these data suggest B and/or T-cells, which are absent in SCID mice, are required for the early immune response to *Bordetella* infection.

SCID mice have a defect in the enzyme Prkdc, an enzyme responsible for DNA repair and one that is integral for V(D)J recombination (4). V(D)J recombination is an important step in developing the broad diversity seen in the antigen binding regions of B and T-cell receptors (7, 8). In the absence of V(D)J recombination, B-cells and T-cells will not fully mature and will not recognize antigens (7, 8). SCID mice, then, do not have functional components of adaptive immunity, and we have shown that the adaptive immune response is necessary for clearance of *Bordetella* in mice (5). However, we did not expect SCID mice to be unable to clear FIM-

deficient bacteria, as our data suggested that the innate immune response, not a B or T-cell response, was responsible for clearing FIM-deficient bacteria.

There are many cells types beyond the traditional B and T-helper cells that require V(D)J recombination to function (9, 10). For example, γδT cells are cells with a TCR. These cells, which undergo maturation in the thymus similar to traditional T-cells, will migrate and reside in the mucosal epithelium of the stomach and lungs (11, 12). Within the epithelium, γδT cells will respond to pathogen associate molecular patterns (PAMPs) and respond to cytokines from dendritic cells (9). Following stimulation, γδT cells can kill infected cells directly, secrete defensins to kill bacteria, or indirectly activate macrophages and neutrophils by secreting cytokines (9). γδT cells have been shown necessary for promoting innate immune responses to clear *E. coli* from the mucosal gut epithelium of mice (13) and innate immune responses to clear RSV from the lungs of mice (14). However, a role for γδT cells has not yet been shown for clearance of *Bordetella*. We hypothesize, given the observations from this chapter, that γδT cells may play a role in promoting innate cell recruitment to the lungs of mice in response to *Bordetella* infection. Additionally, since γδT cells reside primarily at mucosal surfaces (15), it is unsurprising that bulk splenocytes would not contain many, if any, γδT cells, explaining why bulk splenocytes did not fully restore clearance of the FIM-deficient bacteria. By exploring this question further, we may gain insight in to the host immune mechanisms necessary to control *Bordetella* early during infection.

Supporting Information

Figure 4.1 SCID mice cannot clear FIM-deficient bacteria from the lungs

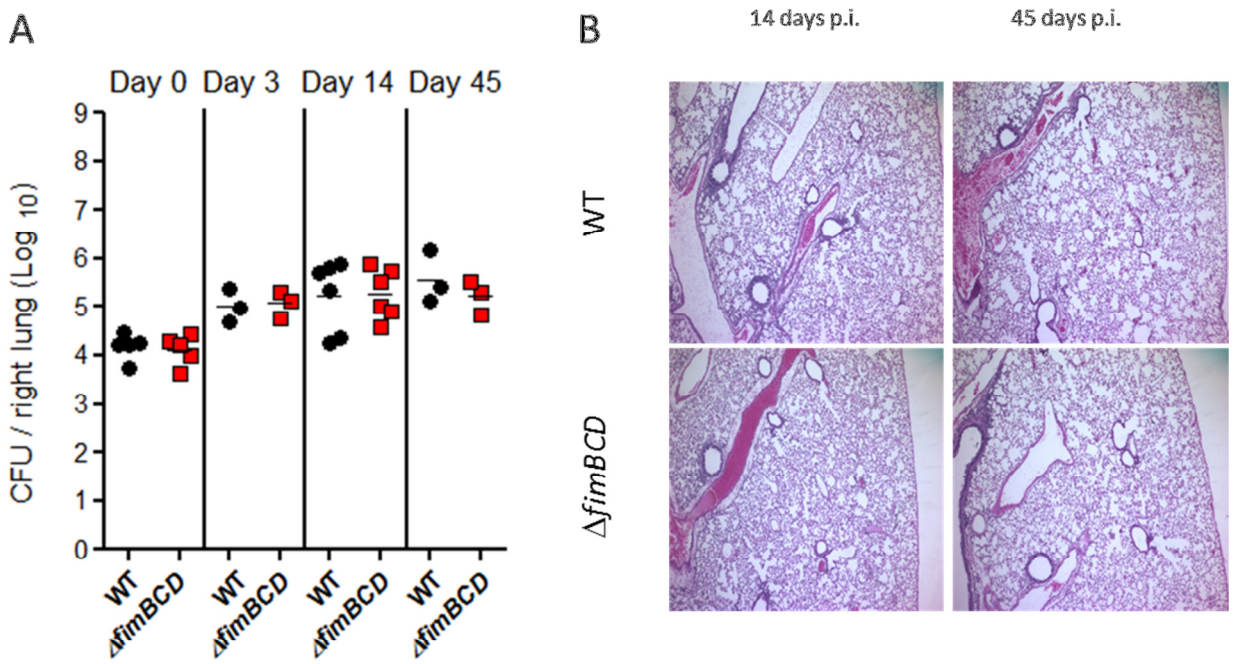

Figure 4.1 SCID mice cannot clear FIM-deficient bacteria (A) Bacterial burden in lung tissue from SCID mice inoculated with WT or FIM-deficient bacteria. Each symbol represents a value for an individual animal, and the black bar represents the mean for the group. Values are means from at least two independent experiments. (B) Hematoxylin-and-eosin stained 5 µm lungs sections at 10x magnification.

Figure 4.2 Adoptive transfer of bulk splenocytes restored partial clearence of FIM-deficient bacteria

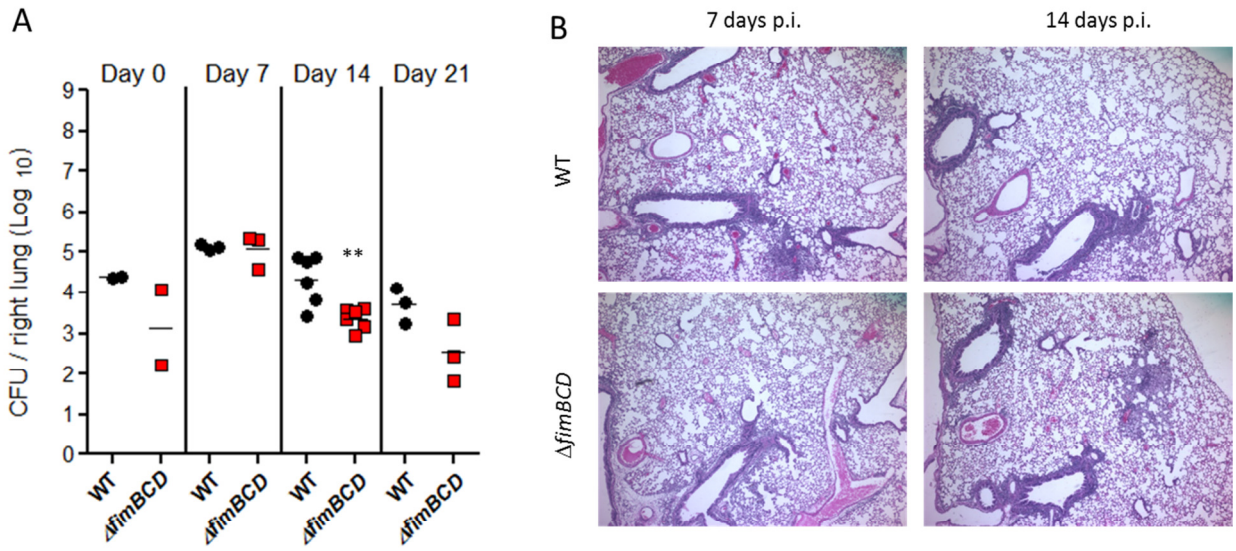

Figure 4.2. Adoptive transfer of bulk splenocytes restored partial clearance of FIM-deficient bacteria. (A) Bacterial burden in lung tissue from SCID mice that received adoptive transfer of bulk splenocytes inoculated with WT or FIM-deficient bacteria. Each symbol represents a value for an individual animal, and the black bar represents the mean for the group. Values are means from at least two independent experiments. Mean values for mutant bacteria that are significantly different from the mean value for WT bacteria are indicated by asterisks as follows: **, $P<0.01$. (B) Hematoxylin-and-eosin stained 5 µm lungs sections at 10x magnification.

Materials and Methods

Animal model

5-6 week old BALB/c SCID (Severe Combined Immunodeficiency) mice were inoculated with 7.5 x 10^4 CFUs of either WT or Δ*fimBCD B. bronchiseptica*. The right lobes of the lungs were harvested, homogenized in 1 ml PBS and plated on BG agar to enumerate CFUs. The left lobe of the lungs was inflated with formalin, embedded in paraffin, cut to 5 µm thickness and stained with hemotoxylin and eosin (H&E).

Adoptive transfer

Donor BALB/c mice were euthanized and the spleens were removed and placed in a tube of PBS. Lymphocytes were prepared as a cell suspension by pressing the spleen through a plastic strainer; then, 10 ml of PBS was added to pass cells through the strainer. The spleen cell suspension was then depleted of red blood cells (RBCs) using RBCs lysis buffer. The suspension was then washed three times in 0.1% BSA in PBS and centrifuged at 1600 RPM at 4° C for 5 minutes. The cells were counted and divided in to 2 parts, one for injection and the second for flow cytometry analysis. The cells to be injected were resuspended and injected intravenously one day prior to inoculation.

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CHAPTER 5. CONCLUSIONS

Summary of research accomplishments

Prior to this research project, evidence suggested that fimbria were not required for *Bordetella* adherence. Furthermore, while fimbria had been shown necessary for *Bordetella* colonization, it was unclear how fimbria contributed to *Bordetella* colonization and persistence. The original goals of this project were to: 1) Develop an *in vivo* adherence assay to determine if fimbria contribute to bacterial adherence, as we hypothesize that fimbria mediate adherence specifically to ciliated epithelium, 2) Characterize the persistence defect of FIM-deficient bacteria and the host response of mice to FIM-deficient bacteria, as we hypothesize fimbria may also contribute to immune modulation.

From this work, we successfully developed a novel *in vivo* adherence assay capable of determining the contribution of virulence factors to adherence. We showed that both fimbria and FHA are necessary for bacterial adherence to mouse epithelium in the lower respiratory tract, contrasting the existing *in vitro* data suggesting that fimbria are not required for adherence.

Additionally, this work was the first to show that fimbria are necessary for *B. bronchiseptica* to persist in the lungs of mice, and that inoculation with FIM-deficient *Bordetella* causes increased pro-inflammatory cytokine production and distinct histological patterns of cellular infiltrate. This distinctive histological pattern, in which cellular infiltrate appeared in the alveolar spaces of mice inoculated with FIM-deficient bacteria, led us to develop a bacterial

localization assay, as we hypothesized that fimbria were required to localize the bacteria to the ciliated epithelium of the major airways.

Results from the localization assay indicated that *Bordetella* containing fimbria localize to the ciliated epithelium of the major airways, and FIM-deficient bacteria localize to the alveoli. FHA was shown to be unnecessary for initial localization to the ciliated epithelium of the major airways, though our data suggest that FHA mediates tight adherence to the epithelium. Together, this work provides a model for the initial colonization of the respiratory epithelium by *Bordetella*. Upon inoculation, fimbria mediate initial attachment of the bacteria to the ciliated epithelium of the major airways in the respiratory system. Once this interaction occurs, FHA will then mediate tight interactions to the epithelium. This FHA-mediated interaction is important as it allows the bacteria to persist and suppress the innate immune response. We hypothesize that FHA may directly affect host cells by altering signal transduction or by facilitating the delivery of other virulence factors, such as adenylate cyclase toxin.

Future Directions

1. Modulation of the Immune System

B. pertussis and *B. bronchiseptica* are highly contagious respiratory pathogens that can successfully colonize their hosts (1). The *Bordetella* genome encodes a variety of virulence factors that allow the bacteria to subvert the host immune response (2–5), and we have shown that both fimbria and filamentous hemagglutinin are necessary factors to suppress pro-inflammatory cytokine production and recruitment of innate immune cells. In experiments described in Chapters 2 and 3, we showed that FIM-mediated localization of the bacteria to the major airways is the first key step in colonization. FHA-mediated interactions following this

initial adherence then likely contribute to immune suppression. However, there are many questions that still remain regarding the mechanism of immune suppression.

The first question, with regards to fimbria, is whether fimbria directly modulate the immune system through specific receptor interactions, or if FIM-mediated adherence is required for other virulence factors to modulate the immune system. FimH of UPEC has been shown to bind to mannose-containing receptors (6) and influence cell signaling pathways (7), and these data would suggest that the adhesive tip of *Bordetella* fimbria may also affect cell signaling. Purified FimD has been shown to adhere to monocytes (8) and possibly VLA-5 (9) and purified Fim2 and Fim3 have been shown to adhere to sulfated sugars (10), however because these experiments used purified protein, the binding partner of full length, native fimbria is still unclear. Because the binding partners of native fimbria have not been identified, a strain containing mutations in the putative ligand binding region of FimD can be tested to determine the effects on host response and bacterial persistence. Alternatively, laser capture microdissection can be used to isolate epithelial cells colonized with *Bordetella* producing fimbria but lacking other virulence factors, such as FHA. We could then perform qRT-PCR to investigate changes in cell signaling to determine if FIM-mediated adherence can directly modulate the immune response.

A second question pertaining to FIM and FHA is the respective contribution of these factors to avoiding phagocytosis by macrophages and neutrophils. Co-inoculation assays with a mix of WT and FHA-deficient bacteria indicated that WT bacteria can partially improve the persistence defect of FHA-deficient bacteria (11), presumably by suppressing the local immune environment. However, the persistence defect of the FHA-deficient bacteria was only partially improved, indicating that FHA may be required for another facet of immune evasion. H&E

stained lung sections indicated recruitment of immune cells seven days post-inoculation in mice are inoculated with WT (2, 12), yet the bacterial burden of WT bacteria does not decrease until 14 days post-inoculation and this decrease requires the adaptive immune system (13). Together, these data suggest that FHA, and possibly fimbria, may contribute to avoidance of phagocytic killing. One possibility would be that FIM and FHA allow the bacteria to actively kill the phagocytic cells. Alternatively, these structures may contribute to inactivation of these cell types to prevent phagocytosis. To address this question, we can perform gentamycin protection assays using neutrophils or macrophages inoculated with FIM-deficient or FHA-deficient bacteria to determine if FIM or FHA can prevent phagocytic uptake. Additionally, we can perform LDH (lactate dehydrogenase) assays to determine eukaryotic cell death in the presence of bacteria with or without FIM or FHA. Together, these experiments would begin to answer the question as to how *Bordetella* avoids the innate immune response.

2. Immune effectors necessary for *Bordetella* clearance

While Chapters 2 and 3 addressed how *Bordetella* modulates and avoids the host immune response, Chapter 4 raised the question of what components of the host immune response are important for clearance of *Bordetella*. We have previously shown that SCID mice inoculated with WT *B. bronchiseptica* will die 60 days post-inoculate, suggesting that B-cells and/or T-cells are necessary for bacterial clearance (13). We have also shown that neutropenic mice inoculated with *B. bronchiseptica* will die three days post-inoculation, suggesting that neutrophils are required to control early *Bordetella* infection (13). Given these previous data and the data from Chapter 2 suggesting that the innate immune response was responsible for clearing FIM-deficient bacteria, we hypothesized that SCID mice, which do not contain cell types with B or T-cell receptors, but do contain macrophages and neutrophils, would be capable of clearing FIM-

deficient *B. bronchiseptica*. Surprisingly, SCID mice were unable to clear FIM-deficient bacteria, suggesting that the neutrophils and macrophages of SCID mice are insufficient to clear FIM-deficient bacteria. Interestingly, H&E stained lung sections indicated that, despite high bacterial numbers, there was very little cellular infiltrate in the lungs of SCID animals inoculated with either strain. SCID animals that received adoptive transfer of bulk splenocytes and were then inoculated with WT or FIM-deficient bacteria were partially able to clear the FIM-deficient bacteria. Together, these data suggest that cell types with a B or T-cell receptor, which are absent in SCID mice, are required for recruitment of macrophages and neutrophils to the lungs early during *Bordetella* infection.

To determine what components of the immune system are required for the early response to *Bordetella* infection, we could first perform adoptive transfer of specific components of the adaptive immune response. To do this, we could isolate T and B cells from bulk splenocytes, and adoptively transfer those to SCID mice to be inoculated with WT or FIM-deficient bacteria. We could then determine the contribution of only cells possessing B or T-cell receptors to controlling bacterial burden and cellular infiltrate to the lungs. Given that T-cells can contribute to innate immunity, we would expect T-cells to contribute to the early immune response to *Bordetella*. The T-cell populations are broad, however, as there are many T-cell subsets: T-helper cells, T-regulator cells as well as NKT and γδT cells (14). γδT cells are particularly interesting, as these cells are primarily located in the epithelium, can response to pathogen associated molecular patterns (PAMPs) and can activate macrophages and neutrophils (15, 16). Given these cells are located in the epithelium, the role of γδT cells clearing gut pathogens (16) and promoting inflammation in the lungs (17), one hypothesis would be that γδT cells are responsible for activation and recruitment of macrophages and neutrophils to the lung during early time points

following *Bordetella* inoculation. One study has indicated that γδT cells may play a regulatory role in the lungs of mice inoculated with *B. pertussis* (18). We could more rigorously test this hypothesis in a natural host model by isolating and adoptively transferring γδT cells to SCID mice, or alternatively depleting γδT cells in immune-competent mice, and then determine if γδT cells contribute to control of *B. bronchiseptica* infection and recruitment of phagocytic cells early in infection. Together, these experiments would further the understanding of what host cell components are necessary for the control of early *Bordetella* infection. Knowledge gained from these experiments may contribute to developing better therapeutics and vaccines.

3. Vaccine Implications

Bordetella pertussis is a reemerging pathogen: The CDC and others report that the cases of pertussis are reaching numbers that have not been seen since the 1950s (19). This reemergence coincides with the change from a whole cell vaccine (wP) to an acellular component vaccine (20, 21). Data from the baboon model indicate that the aP vaccine, while protective against pertussis disease, does not protect from colonization (22). Furthermore, studies indicate that the wP and aP vaccine elicit different immune responses, with the aP vaccine causing a more Th2 driven immune response, and the wP vaccine driving a Th1/Th17 immune response (23), suggesting that the type of immune response is important for protective immunity. This different T-cell response is believed to be due to the alum adjuvant in the aP vaccine (24, 25).

The evidence is compelling that the vaccine strategy against pertussis needs to change: the aP vaccine is not sufficient to protect against colonization in those that have been vaccinated or prevent transmission of bacteria to those that cannot be vaccinated. The best form of

protection against colonization, at least in the baboon model, has been shown to be natural infection (22). One possibility to explore, then, is that the aP components themselves may not elicit the optimal immune response, either because the antibody response against those specific virulence factor cannot prevent colonization or due to the antigens in the vaccine themselves. As we have shown, adherence early during colonization is critical for *Bordetella* to establish infection (12) (Chapter 2). One hypothesis would be that antibodies capable of blocking adherence would prevent *Bordetella* from successfully colonizing. To test this, we could pre-incubate WT *Bordetella* with anti-FIM or anti-FHA antibodies and determine if antibody-antigen interactions are capable of blocking bacterial adherence. Additionally, it would be interesting to investigate the different antibody types for the ability to protect against colonization. Current vaccine administration is intramuscular and leads to anti-*Bordetella* IgG antibodies (23, 26). Within the mucosa, however, IgA has been shown to be critical for mucosal immunity (27). It is possible that anti-*Bordetella* IgA, not IgG, would be a crucial in preventing *Bordetella* colonization, and focusing vaccine development efforts to explore this possibility may lead to improved vaccine efficacy.

Another possibility is that the current aP vaccine components are not optimal for generating an antibody response capable of preventing *Bordetella* colonization. For example, Fim2 and Fim3, the major fimbrial subunits, are included in the aP vaccine. For most Type I pili, however, the adhesive tip is the important end of the molecule, responsible for mediating ligand binding (28, 29). Additionally, other major fimbrial subunits are encoded within the *Bordetella* genome, so it is unclear whether antibodies against Fim2 and Fim3 would be protective against colonization and the addition of FimD as a vaccine component could be considered. Another antigen of interest would be FHA. To prepare FHA in the acellular vaccine, the secreted form is

processed and prepared to remain stable within the vaccine formulation. Chapter 3, however, indicated that surface bound, pre-mature FhaB is an important contributor to virulence. It is possible that the antibodies formed against the FHA found in acellular vaccine would not protect against the physiological relevant FHA utilized by the bacteria. Beyond considering FIM and FHA, there are a variety of other surface and secreted factors that would be present in the wP vaccine that would be absent from the aP vaccine, and should be considered as viable vaccine components.

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APPENDIX A. *BORDETELLA PERTUSSIS* PATHOGENESIS: CURRENT AND FUTURE CHALLENGES[1]

Introduction

Pertussis is a highly contagious respiratory disease that is transmitted directly from human to human[1], most likely via aerosolized respiratory droplets. The primary causative agent, *Bordetella pertussis,* is a Gram-negative bacterium that was first described by Bordet and Gengou in 1906 [2]. The closely related bacterium *Bordetella parapertussis*$_{Hu}$ is responsible for a minority of cases (approximately 14%) and is less capable of causing severe disease[3]. Both *B. pertussis* and *B. parapertussis*$_{Hu}$ are human-specific, and phylogenetic analyses indicate that they evolved from *Bordetella bronchiseptica* or a *B. bronchiseptica*-like ancestor[4, 5] (Box 1). *B. bronchiseptica* infects a broad range of mammals, including humans, and although it can cause overt disease such as kennel cough in dogs and atrophic rhinitis in pigs, it typically colonizes its hosts chronically and asymptomatically[6]. Despite differences in host range and disease-causing propensity, *B. pertussis*, *B. parapertussis*$_{Hu}$ and *B. bronchiseptica* are so closely related that they are now considered subspecies. Together, these organisms provide a paradigm for understanding bacterial adaptation to humans and the dichotomy between acute disease and chronic asymptomatic infection[4, 5]. Although other *Bordetella* species have been isolated from humans, they seem to be primarily opportunistic human pathogens.

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In the pre-vaccine era, pertussis was widespread and mainly affected young children (1-9 years old)[7]. The classical manifestation of the disease, which typically occurs in this age group, is characterized by three phases: catarrhal, paroxysmal, and convalescent[8]. Clinical observations combined with results from studies using animal models (Box 2) suggest that classical pertussis is initiated by adherence of bacteria to ciliated respiratory epithelium in the nasopharynx and trachea[9, 10]. Adherent bacteria survive innate host defences, such as mucociliary clearance and the action of antimicrobial peptides, multiply locally and resist elimination by inflammatory cells. Symptoms during this catarrhal phase are similar to those of many upper respiratory infections, including the common cold. After one to two weeks, the disease progresses to the paroxysmal phase, which can persist from one to ten weeks and is characterized by periods of normal airway function interspersed with multiple severe spasmodic coughing fits, followed by characteristic inspiratory whoops and often emesis. The onset of adaptive immunity correlates with bacterial clearance but not with the cessation of symptoms, which typically decline gradually over another month but can persist for much longer (the convalescent phase)[8]. In infants (< 1 year old), pertussis can take a more serious course with bacteria disseminating into the lungs causing necrotizing bronchiolitis, intra-alveolar hemorrhage and fibrinous edema[10]. In severe cases, extreme lymphocytosis occurs, which is positively correlated with intractable pulmonary hypertension, respiratory failure and death[10].

Introduction of whole-cell pertussis (wP) vaccines in the late 1940s resulted in a rapid reduction in both the incidence of pertussis and death caused by the infection. However, the success of these vaccines was undermined by concerns over their safety (Box 3); thus, they were replaced with acellular pertussis (aP) vaccines in the late 1990s in many developed countries[11]. Since then, pertussis cases have increased and dramatic epidemic cycles have returned. In 2012,

48,277 cases of pertussis and 18 deaths were reported to the Centers for Disease Control and Prevention (CDC), which represents the greatest burden of pertussis in the United States in 60 years and similar outbreaks are occurring in other countries[12-14]. However, the epidemiology of contemporary pertussis does not replicate that of the pre-vaccine era. Disease is now more common in infants and older children (ages 9-19) and, strikingly, older children who develop pertussis are often fully vaccinated according to current recommendations[15, 16]. Ominously, studies that have analyzed pertussis incidence among children that were born and vaccinated during the transition to aP vaccines have found that the rate of infection is significantly higher among children vaccinated with only aP vaccines compared to those vaccinated with even a single dose of wP vaccine[17]. To combat the rise of infections in this group, regulatory agencies have called for boosters to be administered earlier[18]. However, the benefit of boosting with aP vaccines is unclear because it is unknown whether the re-emergence of pertussis is due simply to waning immunity or to fundamental differences in the nature of the immune response induced by aP vaccines compared with wP vaccines or with natural infection.

The increased incidence of disease among older children and adults is especially worrisome because of the corresponding risk of transmission to non- or incompletely-immunized infants[1]. Compounding the problem, antibiotic treatment has minimal efficacy by the time most diagnoses are made and severe cases can be unresponsive to standard therapies for respiratory distress (such as mechanical ventilation)[10]. Therefore, the re-emergence of pertussis as a global public health problem presents two challenges: first, the development of vaccines that have an acceptable safety profile, provide long-lasting immunity, reduce infection burden and prevent transmission; and second, the development of therapeutic agents and treatment strategies that reduce morbidity and mortality in vulnerable populations. Both goals require a better

understanding of the etiological agents of pertussis and the mechanisms by which they cause disease.

In this Review, we discuss our current understanding of the mechanisms used by *Bordetella* spp. to cause respiratory disease, focusing on the roles and functions of virulence factors in pathogenesis. For the interested reader, more specialized recent reviews on pertussis toxin biology[19, 20], virulence gene regulation[21], immunity[22, 23] and vaccines[24] are available, as well as an earlier comprehensive review on *Bordetella* spp. pathogenesis[25].

***Bordetella* spp. virulence regulation**

Several *Bordetella* spp. virulence factors were identified and characterized biochemically before genetic tools became available, including pertussis toxin (PT), adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT), filamentous hemagglutinin (FHA) and fimbriae (Fim). The first transposon mutagenesis screen of *B. pertussis* identified the genes encoding these factors as well as a locus, now known as *bvgAS*, encoding a two-component regulatory system required for their expression[26]. Reasoning that BvgAS also activates expression of genes encoding additional unknown *Bordetella* spp. virulence factors, mutagenesis screens using Tn*5lac* and Tn*5phoA* were conducted[27, 28]. These, together with subsequent genome-wide analyses, revealed that BvgAS controls hundreds of genes in response to changing environmental conditions, including those encoding surface structures and secreted proteins involved in pathogenesis, factors required for survival outside the mammalian host, enzymes involved in cellular metabolism and physiology and additional regulatory systems[29, 30].

The BvgAS phosphorelay

BvgA is a typical response regulator protein with a receiver domain at its N-terminus and a DNA-binding helix-turn-helix domain at its C-terminus[21] (Fig. 1A). BvgS is a polydomain sensor kinase containing two N-terminal venus flytrap (VFT) domains, which are located in the periplasm[31]. C-terminal to the VFT domains is a membrane-spanning region, followed by a cytoplasmically-located PAS domain, a histidine kinase (HK) domain, a receiver domain and a histidine phosphotransferase (Hpt) domain. During growth in standard medium at 37°C, BvgAS is active and uses ATP to phosphorylate a conserved histidine within the HK domain[32]. The phosphoryl group is subsequently relayed to an aspartate in the receiver domain, then to a histidine in the Hpt domain and finally to an aspartate in the receiver domain of the response regulator BvgA[32]. Phosphorylated BvgA is competent for dimerization and binds to specific DNA sequences to either activate or repress transcription[33, 34]. Although the signal(s) to which BvgS responds in nature are unknown, growth at low temperature (~25°C) or in the presence of $MgSO_4$ or nicotinic acid (so-called"chemical modulators" of BvgS) inactivates BvgS; thus, BvgA remains unphosphorylated and is unable to regulate transcription.

BvgAS controls multiple phenotypic phases

The genes regulated by the BvgAS phosphorelay fall into four classes and their differential regulation results in at least three distinct phenotypic phases (Fig. 1B). Class 1 genes include the *ptx-ptl* operon (which encodes PT and its transport system), *cyaA-E* (which encodes ACT) and the *bsc* operon (which encodes a Type III Secretion System (T3SS)). These genes are maximally expressed when BvgAS is fully active (the so-called Bvg^+ phase). Class 2 genes are expressed maximally in both the Bvg-intermediate (Bvg^i) and Bvg^+ phase. The Bvg^i phase occurs when bacteria are grown in the presence of low concentrations of chemical modulators or within the first few hours following a switch from Bvg^- phase conditions to Bvg^+ phase

conditions. Class 2 genes include *fhaB* (encoding filamentous hemagglutinin (FHA)), *fim* (encoding fimbriae) and *bvgAS* itself; thus *bvgAS* is positively autoregulated. Class 3 genes, of which only one (*bipA*, encoding an outer membrane protein of unknown function) has been characterized so far[35, 36], are expressed maximally in the Bvg^{i} phase. Class 4 genes, which are also known as *vrg*s (virulence repressed genes), are expressed maximally in the Bvg^{-} phase and include genes required for flagella synthesis and motility in *B. bronchiseptica*.

Role of BvgAS-mediated gene regulation

The conservation of BvgAS among *Bordetella* spp. and its ability to control multiple phenotypic phases in response to environmental cues suggests that it has an important and conserved role in the infectious cycle. Because *B. pertussis* and *B. parapertussis*$_{Hu}$ strains are unable to survive extended periods of time outside of the human host (unpublished observations from various research groups), it was hypothesized that BvgAS-mediated gene regulation must occur within the mammalian respiratory tract. However, experiments with mutants that were locked in either the Bvg^{+} or Bvg^{-} phase, or that expressed Bvg^{-} phase factors ectopically in the Bvg^{+} phase, showed that the Bvg^{+} phase is necessary and sufficient for respiratory infection; that the Bvg^{-} phase is unable to survive *in vivo*; and that failure to repress Bvg^{-} phase factors (such as flagella) is detrimental to the development of infection[37-40]. Moreover, recent studies with sensitive reporter systems have provided strong evidence that switching to the Bvg^{-} phase does not occur *in vivo*[41, 42]. In *B. bronchiseptica*, the Bvg^{-} phase is required for survival under nutrient-limiting conditions, such as those that might be encountered in an external environment[43]. It has been hypothesized that the Bvg^{i} phase is important for transmission, and

with the development of the baboon model (Box 2), this hypothesis is now testable. Although additional regulatory systems are undoubtedly important during the *Bordetella* spp. infectious cycle, their precise roles have not yet been determined.

Toxins

Pertussis toxin

One of the first identified and most extensively characterized *B. pertussis* virulence factors, pertussis toxin (PT), sometimes referred to as lymphocytosis-promoting factor for its ability to induce lymphocytosis in mammals[44]. The presumed requirement of PT for the development of infection and the observed positive correlation between PT-specific immunity and bacterial clearance led to the hypothesis that pertussis, like cholera and diphtheria, is a toxin-mediated disease[45]. However, although PT is important for pathogenesis, it is now clear that pertussis results from the coordinated function of many different bacterial factors[46].

PT is an ADP-ribosylating AB_5-type toxin[47] (Fig. 2a). The holotoxin is composed of one catalytic subunit (A) and five membrane-binding/transport subunits (B), which are assembled in the periplasm and then exported by the type IV secretion system encoded by the *ptl* locus[48]. PT holotoxin can bind nearly any sialic acid-containing glycoprotein[49] and thus multiple receptors have been identified and characterized in a broad range of cell types *in vitro*[20]; however, the specific cell types targeted by PT *in vivo* are unknown. After binding, PT enters the host cell by receptor-mediated endocytosis and follows a retrograde transport pathway to the Golgi apparatus and then the endoplasmic reticulum (ER) (Fig. 2b)[50]. The A subunit exits the ER, possibly by hijacking the ER-associated degradation pathway that normally expels misfolded proteins[51]. In the cytoplasm, the A subunit catalyzes the transfer of ADP-ribose from NAD^+ to a cysteine

residue near the C-terminus of the alpha subunit of heterotrimeric G-proteins, some of which are inhibitory G -proteins. Amongst other downstream effects, this modification eliminates the ability of these inhibitory G proteins to inhibit adenylate cyclase activity and blocks other G protein regulated enzymes and pathways[20, 52], leading to dysregulation of the immune response**.**

PT has an extraordinarily broad range of pharmacological effects in cell culture and animal models, which has confounded efforts aimed at identifying its precise role(s) during human infection. PT inhibits the migration of cells that express G-protein coupled chemokine receptors *in vitro*, such as neutrophils, monocytes and lymphocytes[53]. In mouse models, production of PT by *B. pertussis* correlates with decreased proinflammatory chemokine and cytokine production, decreased recruitment of neutrophils to the lungs and increased bacterial burdens early in infection[54, 55]. Experiments in which alveolar macrophages are depleted with clodronate suggest that PT initially targets these cells[56]. PT production at the peak of infection correlates with exacerbated inflammation and pathology in the airways[57]. While these and other observations in animal models suggest that PT contributes to the establishment of infection by suppressing early inflammation and inhibiting the microbicidal action of inflammatory cells, in addition to contributing to inflammatory pathology at the peak of infection, it is unknown whether PT produces these effects during human infection. However, it has been shown that PT production positively correlates with the extreme lymphocytosis that occurs in primary human pertussis cases[58], and antibodies against PT protect against severe disease[59].

Adenylate cyclase toxin

Adenylate cyclase toxin (ACT, Fig. 2c, d), which is a member of the RTX (repeats in toxin) toxin family, is encoded by *cyaA* and produced by all *Bordetella* subspecies that infect mammals[19]. ACT is secreted by the *cyaBDE*-encoded Type I secretion system and is palmitoylated by the product of *cyaC*[60, 61]. The toxin contains two distinct functional modules: the C-terminal domain, which contains the RTX repeats, mediates binding to target cells and forms cation-selective pores in plasma membranes[62, 63]; and the N-terminal domain is a calmodulin-dependent adenylate cyclase that converts ATP to cyclic AMP (cAMP)[64, 65]. Recent studies indicate that ACT can adopt multiple conformations and that these forms are distinct in their ability to effect pore formation or adenylate cyclase translocation into the host cell[66]. Thus, the observed effects of ACT on different cell types are the result of a combination of ion permeability, increased levels of cAMP (leading to perturbation of downstream signalling events) and possibly the depletion of intracellular ATP.

Although ACT can intoxicate many cell types, it binds with high affinity to CR3 (CD11b/CD18, Mac-1), which is present on neutrophils, macrophages and dendritic cells[67], and early work correlated ACT-dependent cAMP production in human neutrophils with inhibition of phagocytosis and oxidative burst[68]. More recent studies have shown that ACT blocks complement-dependent phagocytosis by macrophages[69]. In addition, this toxin also suppresses activation and chemotaxis of T-cells[70]. The significance of these *in vitro* observations is unclear; however, a recent study using the baboon model and clinical samples from humans showed that the concentrations of ACT in *B. pertussis*-infected respiratory tissues are significantly lower than the amount of purified protein used in most *in vitro* studies[71]. In mouse models, ACT-deficient bacteria are cleared faster than wild-type bacteria, and studies with immunodeficient and neutropenic mice suggest that ACT has a crucial role in enabling bacteria to resist neutrophil-

mediated clearance[72, 73]. These data, in addition to the fact that ACT is one of the few virulence factors that is conserved and produced by all pathogenic *Bordetella* species[5], suggest that ACT has the potential to be an effective antigen in future vaccine formulations[74].

Type III Secretion

For reasons of experimental tractability, the *Bordetella* spp. Bsc type III secretion system (T3SS) is most extensively studied in *B. bronchiseptica* and induces caspase-independent necrotic death in a diverse array of cell types *in vitro*[75]. Mutations that eliminate T3SS activity decrease bacterial persistence in the lower respiratory tract following intranasal inoculation of rats and mice[76, 77]. Infection of mice with T3SS-defective *B. bronchiseptica* mutants also results in a more robust antibody response and re-stimulated splenocytes from animals infected with these mutants show increased production of pro-inflammatory IFN-γ and decreased production of anti-inflammatory IL-10[78]. Consistently, IFN-γ facilitates clearance of *B. bronchiseptica* from the lower respiratory tract, whereas IL-10 delays it [78]. Together, these observations suggest that the Bsc T3SS has an immunomodulatory role that promotes persistence in the lower respiratory tract but the mechanistic basis of this phenomenon remains to be determined.

Remarkably, and despite concerted efforts by several research teams, only a single effector protein, BteA, has been definitively identified as a translocated substrate of the Bsc T3SS[79, 80]. BopN, a homolog of YopN (which regulates type III secretion in pathogenic *Yersinia* spp.) has been proposed as a second effector[81] but thus far, evidence that BopN is translocated by the Bsc system is lacking. BteA is both necessary and sufficient for cytotoxicity *in vitro*, and mutations in *bteA* recapitulate the phenotypes associated with eliminating T3SS activity *in vitro*

and *in vivo*[77, 79]. Following translocation into host cells, the N-terminal targeting domain results in BteA localization to ezrin-rich lipid rafts that underlie sites of bacterial attachment[82]. However, the mechanisms responsible for the potent cytotoxicity of BteA remain unclear.

Type III secretion is tightly regulated in *Bordetella* spp. The *bteA* and *bsc* genes are transcriptionally activated by the alternative sigma factor BtrS, which is activated by BvgAS[83]. Expression of the *bcs* genes is also up-regulated by iron starvation[84]. In addition to these regulatory mechanisms, the partner-switching proteins BtrU, BtrV and BtrW mediate a cycle of serine phosphorylation and dephosphorylation events that regulate secretion activity[83, 85].

Perhaps the most pressing question regarding the Bsc T3SS relates to its potential role during human infection. A requirement of T3SS activity for *B. pertussis* cytotoxicity has not been documented, despite the fact that T3SS genes are intact, highly conserved, transcribed and regulated, in addition to the observation that *bteA* alleles are functionally interchangeable between subspecies[82, 83]. Fortunately, recent studies are beginning to shed light on this paradox. Although Bsc activity is not generally observed with laboratory-adapted *B. pertussis* strains, the tip complex of the T3SS, Bsp22, is secreted by clinical isolates *in vitro*, and mutations in the ATPase gene, *bscN*, result in elevated production of pro-inflammatory cytokines and accelerated clearance of *B. pertussis* from the lungs of aerosol-infected mice[86]. Furthermore, T3SS activity seems to be lost following laboratory passage of *B. pertussis* and regained after passage in mice[86, 87].

Tracheal cytotoxin

Tracheal cytotoxin (TCT) is a disaccharide-tetrapeptide monomer of peptidoglycan that is produced during cell wall remodelling[88]. Although most Gram-negative bacteria recycle this molecule[89, 90], *B. pertussis* does so inefficiently and releases a large amount of TCT into the extracellular environment. TCT is the only known *B. pertussis* virulence factor that is not regulated by BvgAS. In hamster tracheal rings, TCT functions synergistically with lipooligosaccharide to stimulate the production of pro-inflammatory cytokines (TNF-α, IL-1α, IL-1β, and IL-6) and iNOS, resulting in destruction and extrusion of ciliated cells from the epithelial surface[91, 92]. The biological activity of TCT depends on NOD1, a cytosolic pattern recognition receptor that senses bacterial peptidoglycan and induces the production of pro-inflammatory mediators[93]. NOD1-dependent detection of TCT seems to be host specific, as human NOD1 poorly detects TCT whereas mouse NOD1 does so efficiently[93]. Although it has been postulated that TCT-mediated cytopathology contributes to the characteristic cough in pertussis, the lack of relevant animal models has prevented testing of this hypothesis. Thus, the contribution of TCT to pertussis pathogenesis in humans remains unclear.

Dermonecrotic toxin

Subcutaneous injection of *B. pertussis* or *B. bronchiseptica* cells into mice results in the formation of necrotic lesions due to the activity of dermonecrotic toxin (DNT)[94]. Consistent with a role in infection, DNT production is positively regulated by BvgAS [26, 29] and there is evidence that DNT contributes to the ability of *B. bronchiseptica* to induce turbinate atrophy and lung pathology in swine[95]. DNT has transglutaminase activity, can activate Rho GTPases[96, 97] and inhibits osteogenic cell differentiation *in vitro*, suggesting that the toxin acts directly on host cells[98, 99]. However, as DNT lacks a signal sequence for export and is not secreted from bacterial cells grown in culture[94, 100] ,it may actually function within the bacterial cytoplasm during

infection, possibly by facilitating bacterial survival within a specific host niche and hence functioning indirectly in pathogenesis.

Surface Structures

Filamentous hemagglutinin

Filamentous hemagglutinin (FHA, Fig. 3a) is a large rod-shaped protein and, together with FhaC, serves as a prototypical member of the Two-Partner Secretion (TPS) pathway [101]. It is initially synthesized as an ~370 kDa preproprotein (FhaB) that undergoes processing to produce the mature ~250 kDa FHA as it is translocated across the cytoplasmic membrane by the Sec translocation system and across the outer membrane by FhaC[102]. The N-terminal signal peptide is likely removed by leader peptidase and the C-terminal prodomain is processed by SphB1 and other as yet unidentified factors[103, 104]. Mature FHA is oriented with its mature C-terminus (the MCD) distal to the bacterial surface, and a substantial amount of FHA is also released into culture supernatants when the bacteria are grown *in vitro*[104].

FHA is both necessary and sufficient to mediate bacterial adherence to several eukaryotic cell types *in vitro*[105, 106]. However, FHA is only one of several factors contributing to bacterial adherence to tracheal explants[9, 107], suggesting that additional adhesins are important for adherence *in vivo*. Studies using cultured, non-ciliated cells have reported that FHA binds to CR3, Very Late Antigen V (VLA-5) and Leukocyte Response Integrin/Integrin Associated Protein (LRI/IAP) complexes, and an RGD motif located in the centre of the FHA molecule is implicated in this process[108-110]. More recent studies that have examined *B. bronchiseptica* infection of animal and cell culture models have shown that the FHA molecules produced by *B. pertussis* and *B. bronchiseptica* are functionally interchangeable. These studies have also

demonstrated that production of an FHA protein containing an RAE motif instead of RGD results in no observable differences and that the MCD is required for function[111]. Whether FHA interacts with CR3, VLA-5, LRI/IAP or other mammalian receptors during infection has yet to be determined.

Experiments in which *B. bronchiseptica* is delivered in a small volume to the nasal cavities of rats and pigs have revealed FHA is essential for progression of the infection from the upper to the lower respiratory tract[111, 112]. In mouse models, in which large numbers of bacteria are delivered directly into the lungs, FHA-deficient *B. bronchiseptica* strains induce a more robust inflammatory response than wild-type bacteria[73, 111]. This response is characterized by increased production of proinflammatory cytokines and chemokines in lung tissue (such as TNF-α, KC, MCP-1 and IL-17) and increased recruitment of neutrophils to the lungs during the first four days post-inoculation[73]. Animals that do not succumb to inflammation-mediated pulmonary damage clear the FHA-deficient bacteria from their lungs much faster than animals inoculated with wild-type bacteria[73, 111]. These data suggest that FHA enables *B. bronchiseptica* to modulate inflammation during the establishment of infection, thereby facilitating bacterial persistence. It is currently unknown whether FHA exerts these effects by binding directly to host receptors while attached to the bacterial cell surface or after release from the bacterial cell. Furthermore, it has been suggested that FHA serves as a scaffold to direct the delivery of other virulence factors (such as ACT[113]); however, the *in vivo* relevance of this activity has not been determined.

Fimbriae

Bordetella spp. produce type 1 pili, which are also known as fimbriae (Fig. 3b). The putative chaperone (FimB), usher (FimC) and tip adhesin (FimD) proteins are encoded by the *fimBCD* operon, which is located between the *fhaB* and *fhaC* genes[114]. The genes encoding the two primary major fimbrial subunits, *fim2* and *fim3*, are located elsewhere on the chromosome and can undergo phase variation[115]. Alternative major fimbrial subunit genes (*fimA*, *fimN* and *fimX*) have also been identified[116-118]. Although *in vitro* adherence assays using cultured cells have yielded variable results[119, 120], studies with tracheal explants indicate a role for fimbriae in mediating adherence to ciliated respiratory epithelium[9, 107]. Studies with both *B. pertussis* and *B. bronchiseptica* have demonstrated a requirement for fimbriae during colonization of the lower respiratory tract in rodents[120, 121], and mice inoculated with Fim-deficient *B. pertussis* display a more robust inflammatory response than mice inoculated with wild-type bacteria[122]. Similarly to FHA, fimbriae seem to be involved in adherence and/or suppression of the initial inflammatory response to infection, potentially contributing to persistence.

Pertactin

Pertactin (PRN) is a member of the classical autotransporter family of outer membrane proteins (Fig. 3c)[123]. The surface-localized 'passenger' domain forms a β-helix with β-strands connected by short turns or, in a few cases, large extrahelical loops[124]. Similarly to fimbriae, studies using non-ciliated mammalian cells to investigate a role for PRN in adherence or invasion have yielded equivocal results[112, 125]. Studies using ciliated rabbit tracheal explant cultures suggest that PRN contributes to *B. pertussis* adherence to ciliated respiratory epithelium[9], although experiments with mice failed to identify a role for PRN *in vivo*[126]. However, in the case of *B. bronchiseptica*, studies indicate that PRN is involved in mediating resistance to neutrophil-mediated clearance and promoting persistence in the lower respiratory

tract[112, 125]. In recent years, *B. pertussis* strains that do not produce PRN have been isolated from pertussis patients[127], raising the concern that such strains have been selected due to the presence of anti-PRN antibodies generated in response to immunization with PRN-containing aP vaccines. Whether vaccine driven evolution of *B. pertussis* strains is actually occurring is currently under investigation, as it has decisive implications for the development of new and improved vaccines.

Lipopolysaccharide

B. pertussis, *B. parapertussis*$_{Hu}$ and *B. bronchiseptica* produce different forms of lipopolysaccharide (LPS). *B. pertussis* produces a penta-acylated lipid A linked to a complex core trisaccharide, *B. bronchiseptica* produces hexa-acylated lipid A linked to a similar, if not identical, complex core trisaccharide and O-antigen repeats and *B. parapertussis*$_{Hu}$ produces a hexa-acylated lipid A linked to an altered core structure and O-antigen repeats[128-130]. Because it lacks O-antigen, *B. pertussis* LPS is often referred to as lipooligosaccharide (LOS)[131]. The genes required for synthesis of O-antigen in *B. bronchiseptica* and *B. parapertussis*$_{Hu}$ are repressed by BvgAS[132]; however, some O-antigen is produced under Bvg$^+$ phase conditions and mutants unable to produce O-antigen display defective virulence in mouse models[132, 133].

In mice, *B. bronchiseptica* LPS is sensed by TLR4, resulting in an early TNF-α response and recruitment of neutrophils to the lungs[134, 135]. Although *B. parapertussis*$_{Hu}$ LPS and *B. pertussis* LOS can stimulate murine TLR4, they do so less efficiently and TLR4$^{-/-}$ mice are only modestly impaired in their ability to control infection by these organisms[136-138]. In addition, it has been reported that *B. pertussis* LOS stimulation of murine dendritic cells results in the development of anti-inflammatory regulatory T cells[136]. On the basis of these observations it has been suggested that *B. pertussis* and *B. parapertussis*$_{Hu}$ have evolved to be less inflammatory

than *B. bronchiseptica* and that diminished inflammation might facilitate persistence during human infection[136, 137]. However, subsequent studies have demonstrated that human and murine TLR4-MD-2-CD14 complexes differ in their ability to recognize different forms of lipid A. Although murine TLR4-MD-2-CD14 responds similarly to both penta- and hexa-acylated lipid A, human TLR4-MD-2-CD14 responds robustly to hexa-acylated lipid A but only weakly to penta-acylated lipid A[139]. Furthermore, as opposed to murine TLR4-MD-2-CD14, which responds to *B. pertussis* lipid A regardless of whether the phosphate groups are modified or not, human TLR4-MD-2-CD14 responds more robustly to lipid A containing glucosamine (GlcN)-modified phosphate groups than to lipid A with unmodified phosphates[140]. Although it seems that the majority of *B. pertussis* LOS contains GlcN-modified phosphate groups[141], the fact that it is penta-acylated suggests that its ability to stimulate TLR4 in humans is even weaker than its ability to stimulate TLR4 in mice. These data provide additional support for the hypothesis that *B. pertussis* and *B. parapertussis*$_{Hu}$ strains have evolved to be relatively non-inflammatory in humans. However, they also raise concerns about extrapolating conclusions drawn from murine studies to humans, as the TLR4-MD-2-CD14-dependent immune responses clearly differ in these hosts.

Additional surface proteins

Many additional BvgAS-activated genes encode known or predicted surface-localized or secreted proteins and are suspected to have roles in pathogenesis[29, 142]. BrkA, TcfA, BapC, BatB, Vag8, SphB1 and Phg are BvgAS-activated classical autotransporter proteins, and their putative roles in pathogenesis include mediating adherence, serum resistance, evasion of antibody-

mediated clearance and proteolytic processing of other surface proteins[103, 143-148]. BipA and BcfA are BvgAS-regulated members of the intimin/invasin family, and although their roles in pathogenesis are unknown[35, 149], immunization of mice with BcfA can accelerate clearance of *B. bronchiseptica* following intranasal challenge[150], suggesting that these poorly characterized surface molecules should be considered for the development of new vaccines containing different or additional antigens.

Metabolic proteins

Many BvgAS-regulated genes encode proteins that are probably involved in metabolism, respiration and other physiological processes[29, 142], presumably reflecting the diversity of environmental conditions encountered by *Bordetella* spp. as they travel within and outside the mammalian respiratory tract. Among these factors, those involved in the acquisition and use of iron have been the focus of most studies. In addition to producing and using the siderophore alcaligin[151], *B. pertussis* and *B. bronchiseptica* can use a variety of xenosiderophores (including enterobactin[152]) and haem iron sources such as hemoglobin[153]. Most, if not all, of these iron acquisition mechanisms are required during murine respiratory infection[154, 155], demonstrating the necessity of iron for bacterial survival, the variety of mechanisms used by the host to sequester iron and the reciprocal array of mechanisms used by the bacteria to acquire this essential element.

In addition, accumulating evidence suggests that biofilm production by pathogenic *Bordetella* spp. *in vitro* and during infection may contribute to colonization of the respiratory tract. This process is regulated by a complex program of both Bvg-dependent and Bvg-independent gene expression[156-159], with genes that promote biofilm formation being maximally

expressed in the Bvg^{i} phase[156]. Bvg-independent production of an exopolysaccharide via expression of the *bps* locus and the presence of extracellular DNA are also required for biofilm production[157, 160, 161]. Recent evidence suggests that the second messenger cyclic-di-GMP is also crucial for the regulation of biofilm formation[162].

Current and future challenges

Despite high rates of immunization with aP vaccines, epidemics of pertussis have recently occurred in the US, Europe, Australia and Japan (CDC, Australian Government Department of Health and Aging, Japanese National Institute of Infectious Diseases)[163, 164] and similar outbreaks seem imminent in developed countries throughout the world. Moreover, irrespective of socioeconomic status, the highest rates of mortality are in infants, who are also the most difficult population to treat and protect. In considering these challenges and looking ahead, we suggest three priorities for future studies.

The first priority is to improve the robustness and duration of protection conferred by vaccination, which will require further study of the immunological responses to infection and vaccination (Box 4). The deficiencies of current aP vaccines are well documented, including the striking observation that aP vaccination of baboons only protects against disease symptoms but not colonization or transmission[165]. Numerous efforts are in progress to overcome these deficiencies[24], such as the inclusion of additional antigens in aP vaccines, reformulation with adjuvants that favour Th1/Th17 responses as opposed to the Th2-type immunity generated by alum-adjuvanted vaccines as well as the development of live, attenuated *B. pertussis* vaccines[166]. The latter approach has significant advantages, including the ability to generate mucosal

immunity, but the issue of public acceptance looms large. In a similar vein, it is interesting to note that outside of North America, Europe and parts of Asia, wP vaccines remain in widespread use and approaches to decrease their reactogenicity while retaining immunogenicity should be considered[167]. The known efficacy of these vaccines combined with the cost effectiveness of this approach might be of more benefit to people than the development of improved but more costly vaccines composed of purified proteins. It is important to remember that the development and approval of novel vaccines will be a prolonged process. In light of recent findings concerning the lack of protection against colonization or transmission by aP vaccination[165], maximizing the efficacy of current vaccines through prenatal vaccination, additional boosting and additional strategies is imperative.

A second priority is to mitigate infant mortality. Nearly 90% of all deaths due to pertussis occur in infants that are less than four months of age[168], and the most frequent cause is intractable pulmonary hypertension associated with marked lymphocytosis and bronchopneumonia. Currently, the only efficacious therapy for severe cases is rapid leukodepletion, which is only available at advanced critical care centres[169, 170]. Respiratory samples obtained during autopsies show luminal aggregates of leukocytes occluding small pulmonary arteries, along with an abundance of *B. pertussis*[10, 171]. The pathology of fatal pertussis pneumonia appears to be largely caused by pertussis toxin. Thus, in addition to protecting susceptible infants by maternal vaccination or by vaccination at birth, it is also imperative to pursue approaches for limiting PT activity during infection. Potential therapeutic modalities include humanized monoclonal antibodies and small molecules to target PT interactions with host cell receptors or the enzymatic activity of PT, as well as regulatory factors such as the BvgAS system.

Finally, although animal models have proven useful, we need to enhance our understanding of human disease. Decades of research on *B. pertussis* virulence determinants have primarily been based on tissue culture models and murine infections. These studies have shown what adhesins, toxins and other virulence factors *can do* under laboratory conditions, but very little, if anything, is known about what they *really do* during human disease. Specificity is the rule for human adapted pathogens and it can manifest at several levels including gene expression, virulence factor delivery, binding specificity and activity. Perhaps the most vivid illustration of our lack of understanding of *B. pertussis* is that we still don't know why infection makes people cough!

Box 1- *Bordetella* phylogenomics

The *Bordetella* genus includes nine species and the "classical" or "mammalian" bordetellae (*B. pertussis*, *B. parapertussis*$_{Hu}$ and *B. bronchiseptica*) are the most important species in the context of mammalian infection. Comparative analyses of their genomes have been informative on at least two levels. The first involves comparisons between subspecies, which has revealed intriguing clues regarding mechanisms of host adaptation and virulence evolution; and the second involves studies of the population dynamics of *B. pertussis* in the context of recent outbreaks and the possibility of vaccine-driven evolution.

Early studies revealed limited genetic diversity between isolates, arguing that the classical bordetellae should be regarded as closely related subspecies and that *B. pertussis* and *B. parapertussis*$_{Hu}$ evolved independently from different *B. bronchiseptica*-like ancestors[172, 173]. This hypothesis suggested that *B. bronchiseptica*, a zoonotic generalist, has the propensity to

give rise to host-restricted specialists that cause acute disease. Whole genome sequence comparisons support this idea and show that the transition by *B. pertussis* and *B. parapertussis*$_{Hu}$ to the human-restricted niche was accompanied by large-scale gene loss, an accumulation of pseudogenes and an expansion of IS elements[174, 175]. Based on this framework, a seminal study characterized a set of 132 *Bordetella* isolates from diverse mammalian hosts using a combination of multilocus sequence typing, comparative whole-genome microarray analysis and IS typing[4]. Four distinct complexes comprised of related strains were resolved, representing *B. pertussis* (complex II), *B. parapertussis*$_{Hu}$ (complex III) and two distinct *B. bronchiseptica* complexes (I, IV) (see the figure). Surprisingly, although *B. bronchiseptica* complex I isolates were primarily of animal origin (68%), 80% of *B. bronchiseptica* complex IV strains were from humans that had whooping cough-like symptoms. Notwithstanding the fact that human isolates were overrepresented in the collection, the clustering of *B. bronchiseptica* strains isolated from humans into a genetically related group was intriguing. A follow-up study based on whole-genome sequencing suggested that *B. pertussis* evolved from a *B. bronchiseptica* complex IV-like ancestor[5]. These observations illustrate the evolutionary dynamics of *Bordetella* subspecies and raise the question of whether complex IV *B. bronchiseptica* are on a path towards human adaptation.

At the other end of the evolutionary spectrum, the resurgence of pertussis has prompted numerous efforts to characterize polymorphisms in *B. pertussis* populations in a search for correlations with the introduction of aP vaccines. Although numerous shifts in allelic frequencies have been documented in different countries since the vaccines were introduced[176, 177], a comprehensive analysis of isolates from the United States shows that the majority of these polymorphisms predate the wP to aP transition and are not the result of aP-driven selection[178].

Nonetheless, currently circulating strains in the Americas, Asia, Australia and Europe carry a single nucleotide substitution in the PT promoter (the *ptxP3* allele) that confers a slight (ca. 1.6 fold) increase in PT production compared to the previously dominant allele[179]. *ptxP3* strains were present before aP vaccines, but given the roles of PT in immunosuppression it has been suggested that their expansion and apparent fixation reflects a selective advantage for establishing infection in partially immune hosts, regardless of the source of immunity[180]. Cause and effect is unclear, as is the likelihood that such a small difference in PT expression could have such a major effect. One of the difficulties in interpreting these studies is their reliance on allelic variability in small subsets of genes, raising the possibility that "hitchhiker" mutations located elsewhere in the genome are responsible for apparent clonal shifts[177]. It is essential that these analyses continue, but they need to be based on whole genome sequences and appropriate sampling of circulating and reference strains.

Box 2: Animal models

One of the greatest challenges in studying the pathogenesis of pertussis has been the development of animal models that accurately reflect human disease as the most commonly used laboratory animals do not cough and none of them are natural hosts for *B. pertussis*. Nonetheless, the availability of wild-type, knock-out and transgenic mouse strains, as well as a plethora of murine-specific reagents, have made mice attractive and commonly used model organisms. To establish infection in mice, high numbers of *B. pertussis* must be delivered directly to the lungs, where they multiply for the first week or so post-inoculation and are eventually cleared. Although murine models do not mimic classical whooping cough, they have provided insight

into the importance of several virulence factors, the roles of various host immune responses in controlling infection and the potential efficacy of vaccines (as outlined in the main text).

Suckling pigs have been used to model infant pertussis and intrapulmonary inoculation with *B. pertussis* results in low-grade fever, mild cough, hypoglycemia, lymphocytosis, weight loss and pneumonia[181]. In this model, passively transferred immunity provides protection for newborn piglets upon challenge[182], supporting the recently instituted policy of maternal immunization during pregnancy[183, 184]. Studies with infant and adult pigs have also revealed that *B. pertussis* is susceptible to host antimicrobial peptides, whereas *B. bronchiseptica* and *B. parapertussis*$_{Hu}$ are resistant [185].

The most recent and exciting advance in modelling human pertussis is the development of a baboon (*Papio anubis*) model[186]. Delivery of *B. pertussis* to the nasopharynx of weanling baboons results in low-grade fever, paroxysmal coughing, lymphocytosis, robust production of anti-PT antibodies and protection from subsequent challenge, all of which are manifestations of pertussis in humans[186]. A unique advantage of this model is the ability to study transmission, which was demonstrated to occur by contact and via aerosols[187]. Importantly, a recent study showed that aP vaccination provided protection against the development of disease symptoms but not against colonization or transmission[165], which suggests that nonsymptomatic individuals might be capable of transmitting the infection to unprotected infants. Use of this model to investigate mechanisms of transmission and disease will be crucial for the development of new vaccines and therapeutics.

Rodents, rabbits, and swine are not natural hosts for *B. pertussis* or *B. parapertussis*$_{Hu}$; however, they are commonly infected with *B. bronchiseptica* in nature. This fact, in combination

with the close phylogenetic relationships between these subspecies (Box 1) and the conservation of many known virulence factors among them, has prompted several groups to use *B. bronchiseptica* infection of natural hosts to study features of pathogenesis that are common to *B. pertussis* and *B. parapertussis*$_{Hu}$. Studying *B. bronchiseptica* pathogenesis is also of veterinary and clinical importance, as *B. bronchiseptica* is a common pathogen of domestic animals[188, 189] and occasionally causes disease in humans[190]. These models have revealed roles for several conserved virulence determinants and regulatory factors (see main text), and have also shown that several factors are functionally interchangeable between *B. pertussis* and *B. bronchiseptica*[111, 125, 191], thereby validating the use of *B. bronchiseptica* as model for studying *B. pertussis* virulence and regulatory function. Thus far, the results of these studies suggest that host specificity is determined by differences other than sequence polymorphisms in highly conserved virulence genes.

Box 3: Vaccination against pertussis

The introduction of whole cell vaccines (wP) in the 1940s resulted in dramatic decreases in morbidity and mortality caused by pertussis, and by the early 1970s pertussis was nearly eradicated in the developed world[7]. Attention then turned to the side-effects associated with immunization. The National Childhood Encephalopathy Study (NCES), a prospective case-controlled study that was carried out in the U.K. in the late-1970s to evaluate acute neurological illnesses in children aged between 2 and 26 months, concluded that pertussis vaccination was associated with brain damage[192] – although this correlation was later demonstrated to be unfounded. Lack of circulating disease was misinterpreted as being equivalent to a lack of risk in

contracting the disease, and the NCES report (among others) led to a precipitous decline in vaccine coverage in many countries worldwide. The reported association with brain damage also effected a change in manufacturing policies; decreased vaccination compliance combined with a rise in legal action associated with previous vaccinations led many pharmaceutical companies to abandon the production of wP vaccines. However, the decrease in vaccine coverage almost immediately sparked the largest pertussis epidemics since the pre-vaccine era in many countries, which resulted in a substantial rise in infant mortality, a much more severe outcome than the alleged dangers caused by the wP vaccine. Eventually, public concern over the increase in pertussis disease and the number of infant deaths rose and vaccine coverage returned to high levels. Efforts to develop improved pertussis vaccines were rekindled, with the subsequent development, testing and deployment of aP vaccines. Although supposedly safer and undoubtedly less reactogenic than the wP vaccine, we now know that aP vaccines are also less effective. The experience with vaccination against pertussis clearly highlights the effect that public perception and misperception can have on the implementation of public vaccination programs.

Box 4: Immunity to pertussis

Clinical studies suggest that both humoral and cell-mediated immunity are important for controlling human pertussis. Measurements of *B. pertussis*-specific T cell proliferation, cytokine production and titers of different IgG subclasses from human samples suggest that naive infection causes primarily a Th1 response, resulting in pro-inflammatory cytokine and opsonizing antibody production, combined with the stimulation of antigen presenting cells[193, 194].

wP vaccines also stimulate a Th1 response[195, 196], whereas aP vaccines seem to produce a mixed Th1/Th2 response[196-198]. Many studies have demonstrated that vaccination with aP vaccines produces antibody responses that are equal to or exceed those produced by vaccination with wP vaccines[194, 196, 198], yet the immunity induced by aP vaccines is inefficient, which underscores the importance of investigating and evaluating cell-mediated immune responses induced by vaccination and infection. Studies that have evaluated human infection or vaccination have not comprehensively addressed the role of Th17 responses, which result in pro-inflammatory cytokine production and stimulation of professional phagocytes. However, studies with the baboon infection model show that infection causes a mixed Th1/Th17 response that is long-lived and protective against colonization following subsequent challenge[186, 199]. Immunological data from animal models mostly agree with these findings[23, 165, 199]. Regardless of the natural immune response to infection, efficacious vaccines need to be long lasting, prevent transmission and reduce disease burden. As *B. pertussis* is primarily an extracellular respiratory pathogen, it is likely that an effective immune response will require the induction of a mixed Th1/Th17 response that stimulates the production of opsonizing, toxin-neutralizing and mucosal antibodies, along with memory T cells that produce cytokines to recruit and activate professional phagocytes at the site of infection. As such, there is a pressing need to re-evaluate antigens, adjuvants and immunization routes to achieve these goals.

Figure 1. The BvgAS master regulatory system.

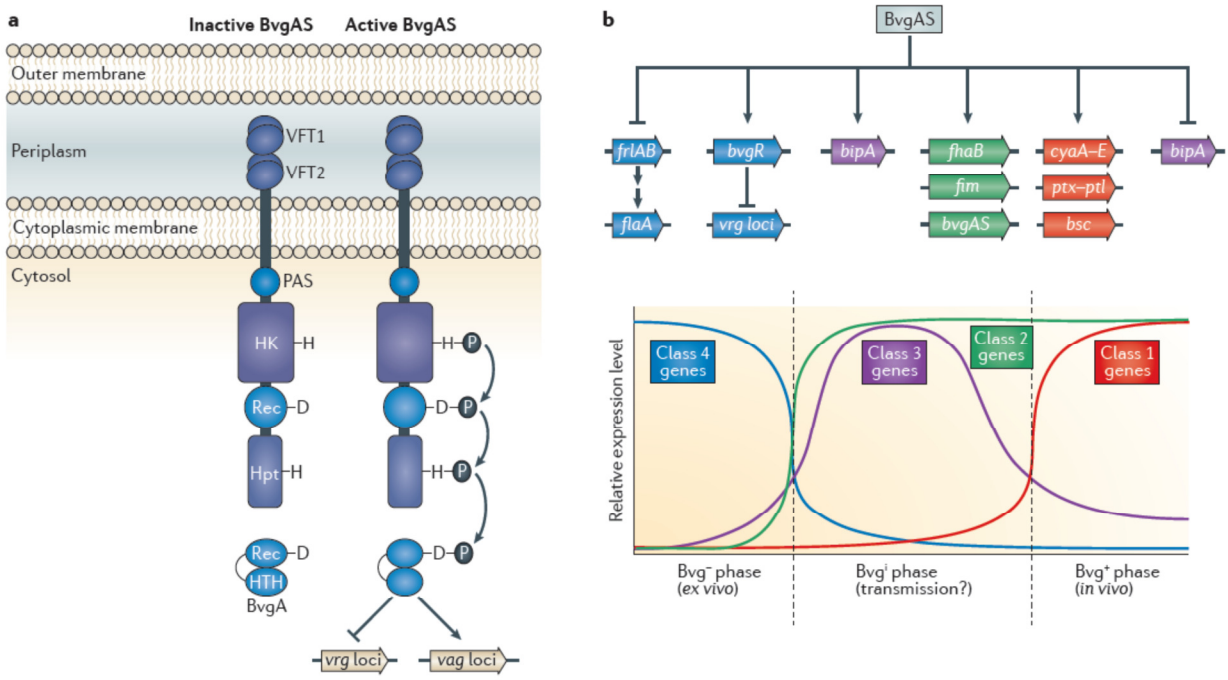

Figure 1. The BvgAS master regulatory system. (a) BvgS is a polydomain histidine sensor kinase containing (from the N- to the C-terminus) two periplasmically-located venus flytrap domains (VFT1 & VFT2), a transmembrane domain, a PAS domain (PAS), a histidine kinase domain (HK), a receiver domain (Rec) and a histidine phosphoryl transfer domain (Hpt). BvgA is a response regulator protein with an N-terminal receiver domain (Rec) and a C-terminal helix-turn-helix domain (HTH). BvgS is activate at 37°C, autophosphorylates at a conserved histidine in the HK domain, and transfers the phosphoryl group to the Rec, the Hpt and then to the Rec domain of BvgA. Phosphorylated BvgA (BvgA-P) activates expression of virulence-associated genes (*vag*s; which are subdivided into class 1 and 2 genes) and represses expression of virulence-repressed genes (*vrg*s; known as class 4 genes). BvgS is inactive and remains unphosphorylated when bacteria are grown at a low temperature (~25°C) or at 37°C in the presence of chemical modulators (such as $MgSO_4$ or nicotinic acid). (OM-outer membrane, CM-

cytoplasmic membrane) **(b)** BvgAS controls four classes of genes and three distinct phenotypic phases. The Bvg^+ phase occurs when BvgAS is fully active and is characterized by maximal expression of genes encoding adhesins (class 2 genes, such as *fhaB, fim2* and *fim3,* expression levels indicated by an orange line) and toxins (class 1 genes, such as *cyaA-E, ptx-ptl* and *bsc* genes, expression levels indicated by a red line), and minimal expression of class 3 and class 4 genes (expression levels indicated by purple and blue lines, respectively). The Bvg^+ phase is necessary and sufficient to cause respiratory infection (i.e., *in vivo*). The Bvg^- phase occurs when BvgAS is inactive and is characterized by maximal expression of class 4 genes and minimal expression of class 1, 2, and 3 genes. (Note that regulation of some *vrg*s is indirect; when BvgAS is inactive, it does not repress *frlAB,* a positive regulator at the top of the motility regulon, and it does not activate *bvgR*, a negative regulator of *vrg* loci.) The Bvg^- phase is required for growth under nutrient limiting conditions, such as may be encountered in the environment (i.e., *ex vivo*). The Bvg^i phase occurs when BvgAS is partially active and is characterized by maximal expression of class 3 genes and minimal expression of class 1, 2, and 4 genes. The only class 3 gene characterized so far is *bipA*, which is activated by BvgA under Bvg^i phase conditions and repressed by BvgA under Bvg^+ phase conditions. The Bvg^i phase may be important for transmission between hosts, but this has not been fully elucidated.

Figure 2. Toxin-mediated virulence of *Bordetella* spp.

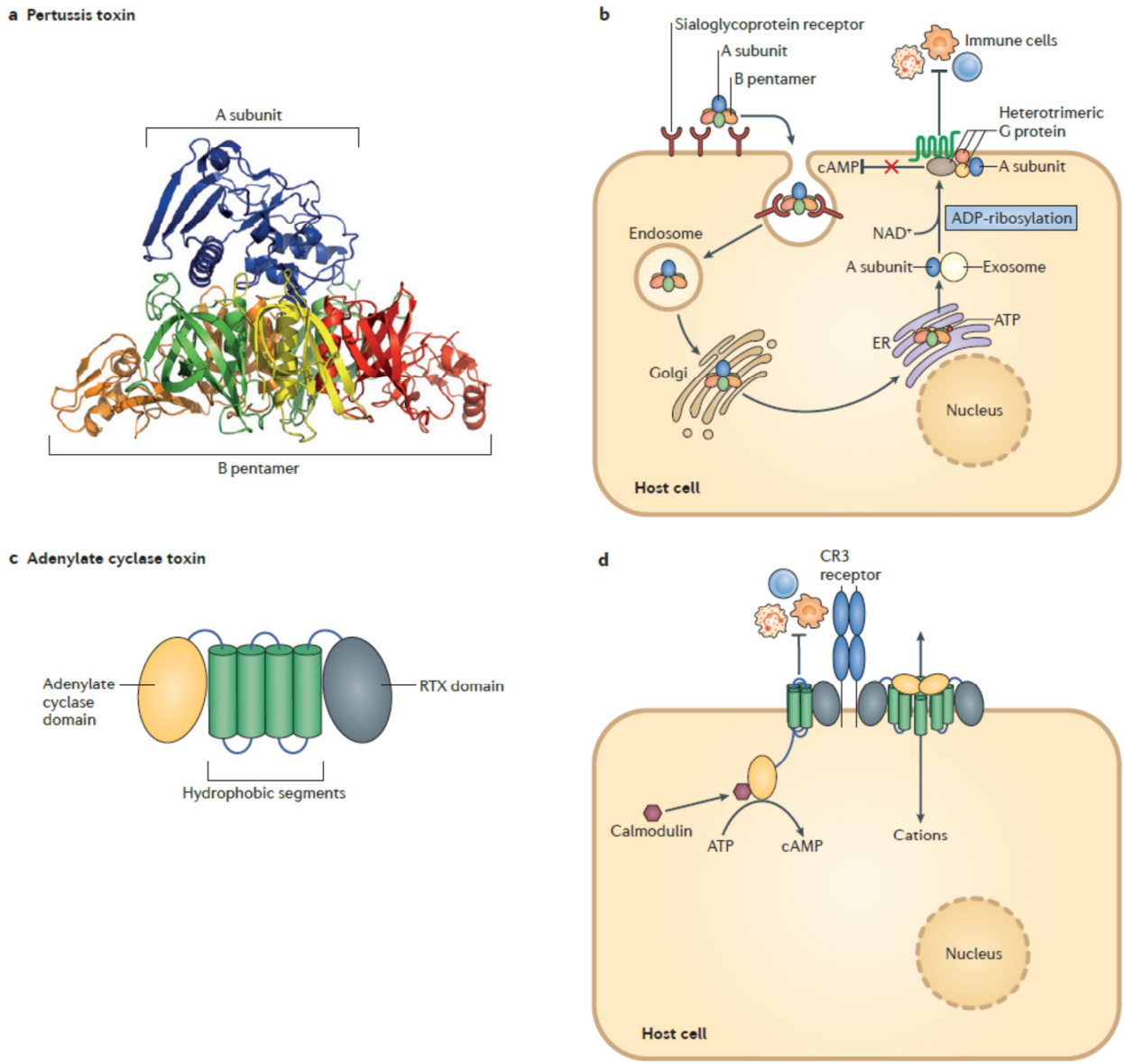

Figure 2. Toxin-mediated virulence of *Bordetella* spp.

(a) Pertussis toxin (PT, PDB ID 1PRT), is an AB_5-type toxin composed of one catalytic subunit (A subunit) and five membrane-binding/transport subunits (B subunits)[47]. PT is assembled in the bacterial periplasm and exported by a type IV secretion system. **(b)** On binding to a sialoglycoprotein host cell receptor, PT is endocytosed and trafficked through the Golgi to the

endoplasmic reticulum. In the endoplasmic reticulum, the B_5 complex binds to ATP and dissociates from the A subunit. The A subunit is then transported into the cytoplasm and traffics on exosomes to the cytoplasmic membrane, where it ADP-ribosylates the α subunit of heterotrimeric G proteins. This modification alters the ability of G proteins to regulate multiple enzymes and pathways, including their ability to inhibit cyclic AMP (cAMP) formation. The overall result of these modifications is an initial suppression of inflammatory cytokine production and inhibition of immune cell recruitment to the site of infection. **(c)** *Bordetella* spp. adenylate cyclase toxin (ACT) is composed of two primary domains, a calmodulin-responsive adenylate cyclase enzymatic domain (yellow) and an RTX domain (black), which are connected by hydrophobic segments (green). **(d)** The RTX domain of ACT interacts with CR3 receptors that are expressed on host cell membranes from a wide range of cell types. The hydrophobic segments of the linker region (green) form pores in the membrane that enable the passage of ions and translocation of the adenylate cyclase domain into the cytoplasm. Adenylate cyclase activity is stimulated by binding to calmodulin in the host cell. The combined effects of ACT intoxication and pore formation result in inhibition of complement-dependent phagocytosis, induction of anti-inflammatory cytokines, suppression of pro-inflammatory cytokines and inhibition of immune cell recruitment**.**

Figure 3. Presentation of filamentous hemagglutinin, fimbriae and pertactin on the *Bordetella* cell surface.

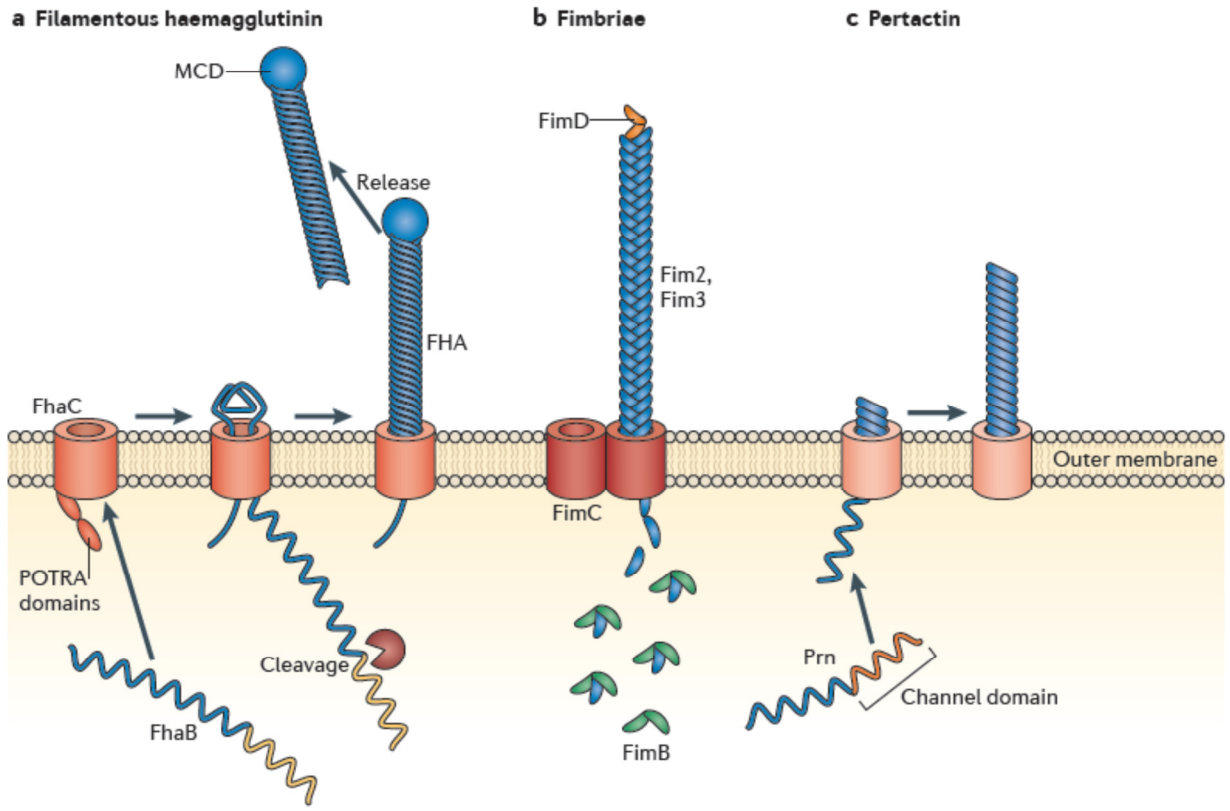

Figure 3. Presentation of filamentous hemagglutinin, fimbriae and pertactin on the *Bordetella* cell surface. **(a)** Filamentous hemagglutinin (FHA) is a TpsA exoprotein (blue) that is translocated across the outer membrane through its cognate TpsB pore protein (red), FhaC. This translocation occurs via the two-partner secretion pathway. Processing during translocation removes the C-terminal prodomain (yellow) from the full-length FhaB protein to produce the mature ~250 kDa FHA protein. FHA is required for adherence to ciliated epithelial cells and for persistence during infection, possibly by directly or indirectly modulating the host immune system. **(b)** *Bordetella* spp. fimbriae are type 1 pili. FimB is similar to chaperone proteins that

traffic major fimbrial subunits (Fim2 and Fim3, in this case) to the membrane usher FimC**.** FimB and FimC are necessary for fimbrial secretion and FimD (the tip subunit) is necessary for fimbrial assembly. Fimbriae are required for persistence during infection, possibly by functioning similarly to FHA by directly or indirectly modulating the immune system. Furthermore, studies have suggested that fimbriae are necessary for adherence to ciliated epithelial cells. **(c)** Pertactin is a classical autotransporter. The C-terminal ~30 kDa region (red) forms a channel in the outer membrane (om) that is required for translocation of the ~70 kDa β-helical passenger domain (blue) to the cell surface. Although the precise role of pertactin is unclear, data suggests that pertactin may contribute to virulence by resisting neutrophil-mediated clearance.

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APPENDIX B. EVIDENCE FOR PHENOTYPIC BISTABILITY RESULTING FROM TRANSCRIPTIONAL INTERFERENCE OF BVGAS IN *BORDETELLA BRONCHISEPTICA*[1]

Summary:

Members of the *Bordetella* genus cause respiratory infections in mammals. The *Bordetella* master regulatory system BvgAS controls expression of at least three distinct phenotypic phases in response to environmental cues. Studies with BvgAS phase-locked and ectopic expression mutants have shown that the Bvg^+ phase is necessary and sufficient for respiratory infection, the Bvg^- phase is required for survival *ex vivo*, and that alterations in BvgAS-dependent gene regulation can be detrimental to the infectious process. We obtained large colony variants (LCVs) from the lungs of mice infected with *B. bronchiseptica* strain RBX9, which contains a large in-frame deletion mutation in the gene encoding filamentous hemagglutinin. RBX9 also yielded LCVs when switched from Bvg^- phase conditions to Bvg^+ phase conditions *in vitro*. We determined that LCVs are composed of both Bvg^+ and Bvg^- phase bacteria and that they result from defective *bvgAS* positive autoregulation that "traps" a small proportion of bacteria in the Bvg^- phase. The LCV phenotype was linked to the presence of a divergent promoter 5′ to *bvgAS*, suggesting a previously undescribed mechanism of transcriptional interference that, in this case, leads to feedback-based bistability (FBM)

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by decreasing *bvgAS* expression in the Bvg$^-$ phase. Our results also indicate that a small proportion of RBX9 bacteria modulates to the Bvg$^-$ phase *in vivo*, but this level of modulation had no impact on the overall outcome of infection. In addition to providing insight into transcriptional interference and FBM, our data provide an example of an in-frame deletion mutation exerting a 'polar' effect on nearby genes.

Introduction

The genus *Bordetella* includes Gram-negative bacteria that cause respiratory infections. *Bordetella pertussis* and *Bordetella parapertussis*$_{hu}$ are strictly human-specific pathogens that cause whooping cough, an acute disease that has experienced a recent resurgence despite widespread vaccination (1–3). Phylogenetic analyses indicate that *B. pertussis* and *B. parapertussis*$_{hu}$ have recently evolved from *Bordetella bronchiseptica,* which infects a wide range of mammals and can also survive naturally for long periods of time outside the host (4–6). Although the factors that determine host specificity remain unknown, the presence and regulation of virulence factor-encoding genes is highly conserved between these three sub-species (7, 8).

Filamentous hemagglutinin (FHA), encoded by the *fhaB* gene, is a well-characterized virulence factor of *Bordetella* and is a primary component of acellular pertussis vaccines (1, 9, 10). A prototypical member of the Two Partner Secretion family of proteins, FHA is a large, surface-exposed protein that is produced and secreted at a high levels during growth *in vitro* (10–12). In *B. bronchiseptica,* FHA mediates adherence to a wide range of cell lines and is required for colonization of the lower respiratory tract in both rats and mice (13–15). Although FHA was first characterized as an adhesin, it has subsequently been reported to perform several other important functions. For example, exposure of lipopolysaccharide and IFN-γ-stimulated macrophages to purified *B. pertussis* FHA suppresses interleukin-12 (IL-12) production, and macrophages treated with FHA exhibit higher rates of apoptosis compared to untreated controls (16, 17). FHA-deficient *B. bronchiseptica* causes an infection that is hyperinflammatory compared to infection caused by wild-type bacteria and is characterized by increased influx of interleukin-17 (IL-17)-positive neutrophils, macrophages, and $CD4^+$ Tcells, suggesting that FHA

plays an immunomodulatory role *in vivo* (15, 18). Additionally, there is strong evidence that FHA interacts with another important virulence factor, adenylate cyclase toxin ACT (19, 20).

In *Bordetella,* the master regulator that controls the expression of all known virulence factor-encoding genes is called BvgAS (21). A two-component sensory transduction system, BvgAS controls at least three distinct phenotypic phases (Bvg^+, Bvg^i, and Bvg^-) by altering gene expression patterns in response to environmental stimuli (Figure 1A) (22). The Bvg^+ phase is induced during standard laboratory growth conditions at 37ºC on Bordet Gengou (BG) blood agar or in Stainer-Scholte broth. Bvg^+ phase bacteria are non-motile and form small, dome-shaped, hemolytic colonies on BG blood agar. Bacteria can be induced (or *modulated*) to the Bvg^- phase in the laboratory by growth at room temperature or by supplementing media with millimolar concentrations of chemical modulators such as $MgSO_4$ or nicotinic acid. Bacteria in the Bvg^- phase are motile (*B. bronchiseptica* only) and form large, flat, non-hemolytic colonies. The Bvg^i phase can be induced in the laboratory with intermediate concentrations of chemical modulators and these bacteria form colonies that appear phenotypically intermediate compared to Bvg^- and Bvg^+ phase colonies. Each phenotypic phase is defined by a unique pattern of gene expression (Figure 1B) (7, 22, 23). For example, bacteria in the Bvg^+ phase are characterized by maximal expression of virulence-activated genes (*vags)* such as *fhaB*, *cyaA-E* (encoding adenylate cyclase toxin ACT), *ptxA-E* (encoding pertussis toxin in *B. pertussis*), and *bvgAS* itself (which is positively autoregulated). In contrast, Bvg^- phase bacteria maximally express virulence-repressed genes (*vrgs*) including those required for motility (i.e., *flaA,* encoding flagellin and *frlAB*, the *Bordetella flhDC* homolog) and chemotaxis (*B. bronchiseptica* only) but do not express *vags*. The *vags* fall into two classes: those expressed in the Bvg^i phase *and* the Bvg^+ phase, and those expressed maximally only in the Bvg^+ phase. Additionally, some genes,

such as *bipA*, (encoding *B*vg-*i*ntermediate phase *p*rotein A) are expressed maximally only in the Bvg^i phase (24, 25). Thus, Bvg^i phase bacteria are characterized by maximal expression of *bipA*, *bvgAS*, and *fhaB*, and minimal expression of *vrgs*, *cyaA*, and *ptxA* (*B. pertussis* only) (Figure 1B) (7, 23–26).

Upon activation of the BvgAS phosphorelay in response to environmental signals, BvgS (the sensor kinase) autophosphorylates, becoming the substrate for BvgA (the response regulator). BvgA-phosphate (BvgA~P) binds DNA and activates or represses transcription (22, 25, 27–30). *In vitro* transcription and DNA binding experiments have identified both high and low affinity BvgA binding sites located at various positions relative to the transcription start sites of BvgAS-regulated genes (22, 25, 27, 30, 31). These data, together with a recent report describing a direct assessment of BvgA~P levels in *B. pertussis* cultures (32), support a model in which BvgA~P levels are extremely low under Bvg^- phase conditions, moderate under Bvg^i phase conditions, and high under Bvg^+ phase conditions (22, 32). In addition to controlling distinct phenotypic phases in response to steady-state conditions, BvgAS can regulate gene expression in a temporal manner (27, 33–35). Because *bvgAS* is positively autoregulated, both the concentration of BvgA and the proportion that is phosphorylated increase when bacteria sense activating signals. Therefore, gene expression patterns change temporally as the total concentration of BvgA~P gradually increases when bacteria are switched from Bvg^- to Bvg^+ phase conditions (27, 33–35).

The *bvgAS* and *fhaB* genes are adjacent and transcribed divergently. Experiments with *B. pertussis* indicate that the 426 bp intergenic region contains at least three promoters (with at least two that control *bvgAS*, called P_2 and P_1) and multiple high-affinity BvgA binding sites (29, 30, 36, 37) (Figure 1C). In the Bvg^- phase, *bvgAS* transcription is driven by the BvgAS-independent

promoter P_2 that is responsible for basal levels of BvgA (which likely remain unphosphorylated) (36, 37). When switched to the Bvg^+ phase, BvgA becomes phosphorylated and activates *fhaB* and *bvgAS* via binding to high-affinity sites near P_{fhaB} and P_1 (36, 38). Once a relatively high concentration of BvgA~P is achieved, genes with low-affinity BvgA binding sites at their promoters, such as *cyaA* and *ptxA* in *B. pertussis*, are activated and the bacteria transition into the Bvg^+ phase (27, 35, 39). The Bvg^+ phase, and therefore high levels of BvgA~P, is maintained as long as bacteria sense a Bvg^+ phase environment. Without *bvgAS* positive autoregulation, the ability of *B. bronchiseptica* to transition between and maintain each phenotypic phase is compromised (35).

Data obtained thus far indicate that the Bvg^+ phase is necessary and sufficient to cause respiratory infection, the Bvg^- phase facilitates survival outside of the host, and BvgAS modulation to the Bvg^i or Bvg^- phase does not occur during infection (40–45). For example, several groups have shown that Bvg^+ phase-locked bacteria behave identically to wild-type bacteria in colonization, persistence, and contribution to lung pathology (40, 42–44). In contrast, Bvg^- phase-locked bacteria cannot establish an infection and Bvg^i phase-locked bacteria are severely limited in colonization and persistence (23, 40–43). Additionally, we recently demonstrated that *flaA* is not expressed at a detectable level when mice are infected with the *B. bronchiseptica* wild-type strain RB50 (46) and Akerley *et. al.* showed that production of flagella in the Bvg^+ phase is detrimental to infection (44). Although the natural signals that affect BvgAS activity and the role of modulation in nature remain unknown, all of these data suggest that wild-type *Bordetella* do not modulate to the Bvg^- phase within the mammalian host.

B. bronchiseptica strain RBX9, which contains an in-frame deletion mutation of *fhaB* (Figure 1C), has been used extensively to characterize the function of FHA *in vitro* and *in vivo*

(11, 13, 15, 18, 47, 48). RBX9 is defective in adherence to multiple cell lines, is unable to autoaggregate in liquid culture, and causes hyperinflammation in the murine lung infection model (13–15, 18, 48). We isolated large colony variants (LCVs) from mice infected with RBX9 and also by modulating RBX9 to the Bvg$^-$ phase *in vitro*. We determined that the LCVs were a product of transcriptional interference that influenced *bvgAS* and produced an unusual bistable phenotype. Despite evidence suggesting that *Bordetella* do not modulate during infection, the discovery of LCVs indicates that a subpopulation of RBX9 bacteria modulates *in vivo*.

Results

Isolation and characterization of LCVs

While comparing wild-type *B. bronchiseptica* strain RB50 with its Δ*fhaB* derivative RBX9 in a murine lung infection model, we noticed that at early time points post-inoculation (12 and 24 h), a small proportion (~1%) of cfus recovered from the lungs of RBX9-inoculated mice formed colonies on BG blood agar (Bvg^+ phase conditions) that were larger, flatter, and less hemolytic than colonies typically formed by RBX9 and RB50 (Table 1) (Figure 2D). These Large Colony Variants (LCVs) were not recovered from RB50-infected mice. When LCVs were picked, diluted, and replated on BG blood agar, approximately 95% of the resulting colonies were phenotypically Bvg^+ phase, and approximately 5% were LCVs (Table 1) (Figure 2H). When replated again, LCVs continued to yield 95% Bvg^+ phase colonies and 5% LCVs. All phenotypically Bvg^+ phase colonies yielded only phenotypically Bvg^+ phase colonies after replating onto BG agar.

We found serendipitously that LCVs were also induced *in vitro* under certain conditions. Specifically, when RBX9 was grown on BG blood agar + 50mM $MgSO_4$ (Bvg^- phase conditions) and then replated onto BG blood agar—effectively switching the bacteria from Bvg^- to Bvg^+ phase conditions—approximately 95% of the colonies were phenotypically Bvg^+ phase and 5% were LCVs (Table 1) (Figure 2C). When these LCVs were picked, diluted, and replated onto BG blood agar, approximately 95% of colonies displayed the Bvg^+ phase morphology and approximately 5% of colonies were LCVs (Table 1) (Figure 2G). Again, all phenotypically Bvg^+ phase colonies yielded only phenotypically Bvg^+ phase colonies after replating onto BG agar. When RBX9 was streaked onto BG agar supplemented with 50mM $MgSO_4$, or passaged

continuously under Bvg$^-$ phase conditions, all colonies displayed typical Bvg$^-$ phase morphology.

To determine if the generation of LCVs in RBX9 was due to the Δ*fhaB* mutation and not an unknown secondary mutation, we reconstructed strain RBX9 by allelic exchange. The newly constructed strain behaved identically to RBX9, producing LCVs following BvgAS modulation and generating a similar proportion of LCVs upon restreaking an LCV onto BG blood agar. Although LCVs appear morphologically similar to Bvg^i phase colonies (Figure 2), the fact that restreaking LCVs yielded a heterogeneous population of morphologically different colonies indicates that the Δ*fhaB* mutation does not lock bacteria into one particular phenotypic phase (such as the Bvg^i phase.)

LCVs are composed of both Bvg$^-$ and Bvg$^+$ phase bacteria

To better understand the properties of LCVs, we investigated specific gene expression patterns within the bacteria that composed them. The fact that LCVs are hemolytic suggests that *cyaA*, a Bvg$^+$ phase gene, is expressed because ACT is responsible for hemolysis on BG blood agar (49). Additionally, electron micrographs of negatively stained LCVs revealed the presence of numerous flagella, which are only produced in the Bvg$^-$ phase (data not shown). To determine if bacteria within LCVs were motile, we grew bacteria on Stainer-Scholte plates with 0.3% agar (Bvg$^+$ phase conditions). LCVs stab-inoculated into this agar produced a zone of migration that was smaller than that produced by Bvg$^-$ phase-locked bacteria, but larger than Bvg$^+$ and Bvg^i phase bacteria, which do not produce a zone of migration (data not shown), suggesting that at least some bacteria within LCVs are motile. Together, our observations indicate that both *cyaA*

and *flaA* are expressed within each LCV. However, previous studies have shown that expression of *vags*, such as *cya*, and *vrgs*, such as *flaA*, is mutually exclusive (7). Therefore, we hypothesized that LCVs are composed of at least two phenotypically distinct populations of bacteria: a population in the Bvg^+ phase and a population in the Bvg^- phase.

To determine the phenotypes of individual bacteria present in each LCV, we created RBX9BatB-HA*fla-gfp*, a strain that contains two unique tags that permit the distinction between Bvg^+ and Bvg^- phase bacteria. RBX9BatB-HA*flaA*-gfp contains an HA epitope-encoding sequence in *batB* (encoding the Bvg^+ phase surface-exposed protein BatB) as well as *gfp* driven by the *flaA* promoter at a neutral site in the chromosome. The *batB* gene is expressed maximally in the Bvg^+ phase and minimally in the Bvg^i and Bvg^- phases (50). The *flaA* gene, as described previously, is a typical *vrg* and contains a strong promoter that is active only in the Bvg^- phase (22, 51). Therefore, Bvg^+ phase bacteria should produce a surface-exposed HA-tagged BatB protein and be GFP^- and Bvg^- phase bacteria should be GFP^+ and lack a surface-exposed HA epitope.

We used Alexa-Fluor 594-conjugated antibodies to indirectly recognize HA epitopes so that BatB-producing bacteria displayed red fluorescence. Bacteria expressing *fla-gfp* produced GFP and displayed green fluorescence. When Bvg^+ phase colonies of RBX9BatB-HA*fla*-gfp were stained with anti-HA and an Alexa-Fluor 594-conjugated secondary antibody, only red-fluorescing bacteria were observed and no green fluorescence was detected (Figure 3). When Bvg^- phase colonies were stained, only green-fluorescing bacteria were observed and no red fluorescence was detected. When Bvg^i phase colonies were stained, a small proportion of cells displayed red or green fluorescence, but the majority of cells were not fluorescent and no bacteria displayed both red and green fluorescence (Figure 3). In contrast, when LCVs from

RBX9BatB-HA*fla*-gfp were stained, approximately half of the bacteria fluoresced red and approximately half fluoresced green (Figure 3). No co-localization of red and green fluorescence from either LCVs or Bvg^i phase colonies was observed, confirming that the expression of *vags* and *vrgs* is mutually exclusive under these conditions. These data demonstrate that LCVs are composed of both Bvg^+ and Bvg^- phase bacteria and are not a homogeneous population of Bvg^i phase bacteria.

*The Δ*fhaB* mutation in RBX9, but not lack of FHA protein, is responsible for the LCV phenotype*

To determine if the generation of LCVs was due to lack of FHA protein production or the specific genetic architecture created by the Δ*fhaB* mutation in RBX9, we first determined if other *fhaB* mutants yielded LCVs. Strain RB50ΔP_{fhaB} contains a deletion mutation of the *fhaB* promoter, strain RB50ΔSP_{fhaB} contains a deletion mutation in *fhaB* such that the extended signal peptide of FHA is missing, and strain RB50$\Delta\beta helix_{fhaB}$ contains a large deletion mutation in the region of *fhaB* encoding the β-helix structure (Figure S1). These strains were analyzed for FHA production by western blot, and either produced no FHA protein (RB50ΔP_{fhaB} and RB50ΔSP_{fhaB}) or a severely truncated FHA protein (RB50Δβ-helix) (data not shown). We grew these strains under Bvg^- phase conditions and plated single colonies onto Bvg^+ phase conditions to determine if they would produce LCVs similar to RBX9. No LCVs were observed, suggesting that a lack of wild-type FHA protein is not sufficient to produce the LCV phenotype.

To investigate the contribution of the genetic architecture created by the *fhaB* deletion to the LCV phenotype, we created RB50::pBam, a strain that produces FHA and contains an altered *fhaB-bvgAS* locus (Figure S1). RB50::pBam was created by integrating pBam, a suicide plasmid containing the *fhaB-bvgAS* intergenic region, into the RB50 chromosome. When integrated in the

chromosome, the genetic architecture 5' to *bvgAS* is similar in that *fhaB* coding sequences are replaced with non-native sequences (those from the suicide plasmid, in this case). By contrast with RBX9, however, the complete, intact *fhaB* gene, including its promoter region, is present in RB50::pBam – it is located 5' (relative to *bvgAS*) to the integrated plasmid sequences. After modulation, RB50::pBam produced LCVs similar to RBX9. These data suggest that the LCV phenotype can be produced by altering the genetic architecture 5′ to the *fhaB-bvgAS* intergenic region and is independent of FHA production.

The LCV phenotype results from a defect in bvgAS positive autoregulation

In addition to activating all of the known virulence factor-encoding genes in *Bordetella*, BvgAS activates *bvgAS* expression through positive autoregulation. Williams *et al.* demonstrated that positive autoregulation is required for the precise transition between and maintenance of the Bvg^+, Bvg^i, and Bvg^- phases (35). Three observations suggested that LCVs resulted from defective *bvgAS* autoregulation in RBX9. First, the mutations that cause LCVs are genetically linked (immediately 5′) to *bvgAS*; second, LCVs consist of bacteria in least two separate BvgAS-controlled phenotypic phases; and third, LCVs were induced *in vitro* following a switch from Bvg^- to Bvg^+ phase growth conditions.

We hypothesized that when RBX9 (or RB50::pBam) bacteria are switched from Bvg^- phase conditions to Bvg^+ phase conditions, most, like all wild-type bacteria, are able to activate transcription at the *bvgAS* P_1 promoter, leading to increased BvgAS levels and resulting in the transition to and maintenance of the Bvg^+ phase. According to our hypothesis, however, a small subset of RBX9 and RB50::pBam bacteria are unable to activate *bvgAS* transcription, possibly

due to insufficient levels of BvgA and/or BvgS, and these bacteria are therefore "trapped" in the Bvg$^-$ phase. We hypothesized further that although some descendants of these "Bvg$^-$ phase-trapped" bacteria will be able to activate *bvgAS* transcription and hence "escape" to the Bvg$^+$ phase, many will remain Bvg$^-$ phase-trapped and thus a substantial Bvg$^-$ phase population will be maintained in the LCV. An alternate hypothesis is that LCVs arise from spontaneous or transient shifting of bacteria between the Bvg$^+$ and Bvg$^-$ phases, which would also result in a mixture of Bvg$^+$ and Bvg$^-$ phase bacteria within a single colony.

To determine if LCVs contain Bvg$^-$ phase-trapped bacteria, we used the recombinase-based reporter system pGFLIP, which creates a permanent genetic change in response to gene activation (46). In this system, a promoter of interest drives expression of the site-specific recombinase-encoding gene *flp*, which when activated, results in recombination between Flp recombinase target (*FRT*) sites that flank *gfp* and the kanamycin (Km) resistance gene *nptII*. Therefore, any activation (even transient, low-level expression) of *flp* results in a permanent loss of Km resistance and GFP fluorescence. This system targets the reporter construct to the neutral *att*Tn7 site 3' to *glmS*. We created strain RBX9*cyaA*FLP by mating the plasmid pGFLIP-P_{cyaA}, in which the *B. bronchiseptica cyaA* promoter drives *flp* expression, into RBX9. The *cyaA* gene is exclusively controlled by BvgAS, is highly expressed in the Bvg$^+$ phase, and is expressed minimally in the Bvg$^-$ phase (22). In RBX9*cyaA*FLP, bacteria that have never expressed *cyaA* should remain GFP$^+$ and Km^r, whereas bacteria that have expressed *cyaA* should convert to GFP$^-$ and Km^s. If *gfp* is lost due to P_{cyaA} activation, all descendent cells will also be GFP$^-$ and Km^s.

Previously, we demonstrated that RB50*cyaA*FLP bacteria remain GFP$^+$ under Bvg$^-$ phase conditions with Km selection and that they reach 100% resolution (GFP$^-$ cfu/total cfu) when grown under Bvg$^+$ phase conditions (46). When RBX9*cyaA*FLP was plated under Bvg$^-$ phase

conditions with Km selection, each colony was morphologically identical and fluorescent, indicating that *cyaA* had not been activated to a level required for sufficient *flp* expression to lead to recombination between *FRT* sites (Figure 4A). In contrast, when a colony of RBX9*cyaA*FLP that was grown under Bvg^- phase conditions was plated and grown under Bvg^+ phase conditions, approximately 15% of the colonies were LCVs and approximately 80% of those LCVs were GFP^+ (Figure 4B). None of the Bvg^+ phase colonies were GFP^+. When a GFP^+ LCV was replated and grown under Bvg^+ phase conditions, approximately 5% of the colonies were LCVs and approximately 80% of those were GFP^+ (Figure 4C). We serially replated GFP^+ LCVs eight times and in all cases, additional GFP^+ LCVs were generated (data not shown). These data indicate that a significant proportion of bacteria within a GFP^+ LCV had never activated *cyaA* and had therefore failed to switch to Bvg^+ phase in response to a change in conditions; i.e., they were Bvg^- phase-trapped. Moreover, our data suggest that all LCVs arise from a Bvg^- phase-trapped bacterium and that upon subsequent multiplication, most descendants have "escaped" to the Bvg^+ phase but a small proportion remain trapped in the Bvg^- phase. These data do not support a model in which LCVs consist of bacteria that transiently fluctuate between Bvg^+ and Bvg^- phase, because if this was true, LCVs would not be GFP^+.

Approximately 20% of the LCV colonies were GFP^-, indicating that P_{cyaA}-*flp* expression was sufficient to mediate recombination in the bacterium that founded the LCV or in its early descendants. Although this result appears inconsistent with our model, we have observed previously that when RB50*cyaA*FLP is grown under Bvg^- phase conditions (when *cyaA* expression is minimal) and without Km selection, P_{cyaA}-*flp* is expressed sufficiently in approximately 15% of bacteria such that they convert to GFP^- and Km^S (46). These data indicate that the *cyaA* promoter activity under Bvg^- phase conditions is near the threshold level required

for *flp* expression and subsequent recombination. Therefore, GFP$^-$ LCVs are most likely due to the activity level of the *cyaA* promoter under Bvg$^-$ phase conditions and not due to bacteria switching to the Bvg$^+$ phase and then back to the Bvg$^-$ phase. Nonetheless, our data indicate that in approximately 80% of LCVs, there are a substantial number of bacteria that appear to have a defect in *bvgAS* positive autoregulation, leading to the observed Bvg$^-$ phase-trapped population.

Sequences upstream of the fhaB-bvgAS intergenic region affect the efficiency of bvgAS activation in the Bvg$^-$ phase.

Our data indicate that *bvgAS* autoregulation is defective in RBX9 and specifically, that LCVs are composed of a subpopulation of Bvg$^-$ phase-trapped bacteria. As stated in the introduction, *bvgAS* expression is controlled primarily by two promoters. Studies with *B. pertussis* have shown that under Bvg$^-$ phase conditions, P_2 is transcribed at a low basal level (36, 37). This level of transcription results in BvgS levels that are sufficient to respond to Bvg$^+$ phase conditions by autophosphorylating and mediating phosphorylation of BvgA. The resulting BvgA~P levels are sufficient to bind at the *bvgAS* P_1 promoter, recruit RNAP, and activate transcription (38). (Although similar transcriptional analyses have not been conducted with *B. bronchiseptica*, the nucleotide sequence of the *fhaB-bvgAS* intergenic region in *B. bronchiseptica* is %91.1% identical and most of those differences are located in regions that, based on *B. pertussis* studies, are not bound by either BvgAS or RNAP.) We considered two hypotheses: 1) the level of transcription from P_2 in RBX9 is lower than in RB50 such that in some bacteria the levels of BvgAS are too low to activate transcription at P_1 in response to Bvg$^+$ phase conditions, and 2) transcription activation at P_1 by BvgA~P is somehow defective in RBX9 compared to RB50. Our data suggest that defective autoregulation in RBX9 is due to the lack of native sequences or presence of non-native sequences 5′ to the *fhaB-bvgAS* intergenic region.

To test our hypotheses, we constructed RB50P$_{short}$*bvgAFLP* and RB50P$_{long}$*bvgAFLP*, where RB50P$_{short}$*bvgAFLP* contains only the *fhaB-bvgAS* intergenic region driving *flp* and RB50P$_{long}$*bvgAFLP* contains this region plus an additional 1200 bp of *fhaB* sequences driving *flp* (Figure 5A). These strains were constructed under Bvg$^-$ phase conditions in the presence of Km. To investigate P_2 expression, we determined the percent resolution (the percentage of cfu that had activated *flp*) of each strain grown under Bvg$^-$ phase conditions by counting the ratio of GFP$^-$ cfu to total cfu when one colony was plated from Bvg$^-$ phase conditions with Km selection to Bvg$^-$ phase conditions without Km selection. The average resolution under Bvg$^-$ phase conditions in RB50P$_{short}$*bvgAFLP* was 68% whereas the average resolution in RB50P$_{long}$*bvgAFLP* was 97%. Plates are shown from one representative experiment (Figure 5B, C). Our data indicate that the per-cell activation of RB50P$_{short}$*bvgAFLP* is lower than the per-cell activation of RB50P$_{long}$*bvgA*FLP under Bvg$^-$ phase conditions, and demonstrate that either a lack of native *fhaB* sequences or the presence of non-native sequences has a direct effect on the efficiency of *bvgAS* transcription from the P_2 promoter in the Bvg$^-$ phase.

When a colony from each strain was grown on Bvg$^-$ phase conditions with Km selection and then plated onto Bvg$^+$ phase conditions without Km selection, maximum resolution was achieved and there were no GFP$^+$ colonies in either strain (Figure 5D, E). These data suggest that RBX9 does not have a defect in transcription activation at P_1.

The LCV phenotype is caused by a (divergent) promoter in proximity to bvgAS

Based on our results, we hypothesized that sequences within *fhaB* (1-1200 nucleotides of the coding region) 5′ to the *fhaB-bvgAS* intergenic region effect the low level of transcription from P_2 that occurs under Bvg$^-$ phase conditions. To test this hypothesis, we constructed a strain

containing a deletion mutation from nt 8 to 1256 of *fhaB* (Figure S1, C). This mutant did not produce LCVs when switched from the Bvg^- to the Bvg^+ phase. We conclude that the lack of specific sequences within the first 1200bp of *fhaB* does not cause the LCV phenotype.

These data led us to closely reexamine the genetic architecture of each strain that produced LCVs (RBX9 and RB50::pBam) as well as the strains that showed a difference in *bvgAS-flp* reporter activation (RB50P_{short}*bvgAFLP* and RB50P_{long}*bvgAFLP*). A comparison of these strains revealed the presence of a divergent promoter 5′ to the *fhaB-bvgAS* intergenic region in RBX9, RB50::pBam, and RB50P_{short}*bvgAFLP* (Figure 6). In RBX9, the *fimA* promoter is very close to the *fhaB-bvgAS* intergenic region, in contrast to RB50 in which it is separated by the entire (>12kb) *fhaB* gene. In RB50::pBam, the *bla* promoter (driving expression of the ampicillin resistance gene on the plasmid) is adjacent to the *fhaB-bvgAS* intergenic region. In the strain RB50P*bvgA*$_{short}$*FLP*, the *npt* promoter (driving expression of the kanamycin resistance gene on the plasmid) is proximal to P_{bvgA}-*flp*, whereas the RB50P*bvgA*$_{long}$*FLP* reporter is "buffered" from the same *npt* promoter by an additional 1200bp of *fhaB* (Figure 6). We hypothesized that the presence of a promoter 5′ to the *bvgAS-fhaB* intergenic region was interfering with *bvgAS* P_2 transcription, possibly by sequestering RNA polymerase away from P_2. To test the hypothesis that a nearby promoter could affect P_2 transcription, we reversed the orientation of the insert in the plasmid pBam. In the resulting plasmid, pBamR, the *bla* promoter is no longer proximal to the *bvgAS* homology region. Instead, the closest promoter 5' to *bvgAS* on the plasmid is the *aaC1* promoter (driving expression of the gentamicin resistance gene), which is more than 1.5kb away (Figure 6). We created RB50::pBamR by integrating the pBamR plasmid into the RB50 chromosome. Modulating RB50::pBamR and plating bacteria onto Bvg^+ phase conditions resulted in all colonies having the typical Bvg^+ phase morphology, and no

LCVs were observed. The LCV phenotype was therefore abolished by changing the sequences upstream of the *bvgAS-fhaB* region, presumably by increasing the distance between a promoter and *bvgAS*. Additionally, we deleted the intergenic region between *fhaB* and *fimA* (which includes the *fimA* promoter) in RBX9. The resulting strain RBX9F did not produce LCVs after modulation. These data strongly support a model in which a promoter upstream of *bvgAS* interferes with normal P_2 transcription efficiency, resulting in some cells having an insufficient quantity of BvgAS to activate transcription at P_1.

Each Bvg⁻ phase-trapped bacterium within an LCV initiates the formation of a new LCV and the proportion of Bvg⁻ phase-trapped bacteria within an LCV decreases over time

Our data indicate that LCVs are founded by a single Bvg⁻ phase-trapped bacterium and that each LCV harbors Bvg⁻ phase-trapped bacteria that can propagate additional LCVs. However, it is unclear whether all Bvg⁻ phase-trapped bacteria, or only a subset of these cells, yield LCVs upon replating. To address this question, we needed two pieces of information: the proportion of Bvg⁻ phase-trapped bacteria within one LCV and the frequency of new LCV formation from the same parent colony when replated. We used RBX9*cyaA*FLP to evaluate the composition and LCV-forming capacity of single LCVs.

Plating a GFP⁺ LCV onto Bvg⁻ phase conditions, in which *cyaA* expression is minimal, will minimize further Flp-mediated recombination due to *cyaA* activation during colony formation, permitting us to determine the proportion of GFP⁺ bacteria (and hence Bvg⁻ phase-trapped) that existed within the original LCV at that time. Plating the same GFP⁺ LCV onto Bvg⁺ phase conditions allows us to determine the number of new LCVs generated from the subpopulation of

Bvg$^-$ phase-trapped bacteria in the parent LCV. Comparing the frequency of newly generated LCVs under Bvg$^+$ phase conditions to the frequency of GFP$^+$ cfu under Bvg$^-$ phase conditions will reveal the proportion of Bvg$^-$ phase bacteria that form LCVs when replated.

We first grew RBX9*cyaA*FLP on Bvg$^-$ phase conditions with Km selection to maintain the *gfp* and Km^r markers (Figure 7A I). Then we took single GFP$^+$ (Bvg$^-$ phase) colonies and plated them onto Bvg$^+$ and Bvg$^-$ phase conditions (Figure 7A II). GFP$^+$ LCVs that were recovered from Bvg$^+$ phase plates were then plated again onto Bvg$^+$ and Bvg$^-$ phase conditions (Figure 7A III). The frequencies of GFP$^+$ cfu, LCVs, and Bvg$^+$ phase colonies from each plate were recorded, and the results from one representative experiment are shown in Figure 7A. When the GFP$^+$ LCVs were plated onto Bvg$^+$ and Bvg$^-$ phase conditions, there was no significant difference in the average number of GFP$^+$ cfu under Bvg$^-$ phase conditions compared to the average number of GFP$^+$ LCVs on the corresponding Bvg$^+$ phase BG blood agar plate (Figure 7B). These data suggest that each Bvg$^-$ phase bacterium within an LCV is Bvg$^-$ phase-trapped and forms an LCV when replated onto Bvg$^+$ phase conditions.

Additionally, we asked if the composition of LCVs (i.e., the ratio of Bvg$^-$ to Bvg$^+$ phase bacteria) changed over time. We hypothesized that this ratio would change due to bacterial division as well as the rate of conversion of Bvg$^-$ phase-trapped bacteria to Bvg$^+$ phase bacteria. Because we expected a unidirectional conversion of phenotypes (Bvg$^-$ to Bvg$^+$ phase only) under Bvg$^+$ phase conditions, we predicted that the ratio of Bvg$^-$ to Bvg$^+$ phase bacteria would decrease as the bacterial population increased. To determine if the compositions changed after an additional day of growth, we compared GFP$^+$ LCVs plated after our standard incubation time (48h) (Figure 7A III) to GFP$^+$ LCVs plated after 72 h (Figure 7A IV). When GFP$^+$ LCVs were plated after 48h of incubation, we obtained an average of 12 ± 1.2% GFP$^+$ cfu under Bvg$^-$ phase

conditions, whereas after 72h, we obtained an average of 5.75 ± 1% GFP^+ cfu under the same conditions (P=0.005) (Figure 7C). These data indicate that the frequency of GFP^+ (and therefore Bvg^- phase-trapped) bacteria in an LCV decreases over time.

These data also strongly suggest (as discussed in a previous result) that GFP^- LCVs are a result of the background activation of *cyaA* in RBX9P*cyaA*flp, as the background *cyaA-flp* activation under conditions of inactivity (Bvg^- phase conditions) was the same as the frequency of GFP^- LCVs (to total LCVs) under Bvg^+ phase conditions (Figure 7A II).

Modulation of RBX9 in vivo *occurs at a very low frequency*

All data published thus far strongly suggest that wild-type *Bordetella* do not modulate to the Bvg^i or Bvg^- phase *in vivo* and that the Bvg^+ phase is necessary and sufficient for infection (40–45). The recovery of LCVs from mouse lung homogenates and the fact that LCVs were recovered *in vitro* only following modulation, however, supports the hypothesis that RBX9 modulates during infection. To test this hypothesis, we constructed strain RBX9*flaA*FLP, a strain containing the pGFLIP cassette in which the *flaA* promoter drives expression of *flp* (46). Previously, using the same P_{flaA}-*flp*-containing cassette in wild-type RB50, we showed that *flaA* was not activated to a detectable level in RB50 during murine infection (46). RB50*flaA*FLP and RBX9*flaA*FLP bacteria were grown under Bvg^+ phase conditions with Km selection to minimize background resolution prior to inoculation. Mice were inoculated intranasally with $7.5x10^4$ – $1x10^5$ cfu and lungs were harvested at 3, 24, 30 and 72 hours post-inoculation. We conducted this experiment several times. In all experiments, a low proportion (≤1%) of GFP^- bacteria was recovered from the lungs of both RB50*flaA*FLP- and RBX9*flaA*FLP-inoculated animals (data not

shown). This low proportion was not significantly different, however, from the proportion of GFP^- bacteria present in the samples used for inoculation (plated after inoculating the animals). Data from previous work with RB50*flaA*FLP (46) and our experiments with RBX9*flaA*FLP indicate that resolution of the P_{flaA}-*flp* cassette is BvgAS-dependent, and GFP^- bacteria were not recovered from strains containing the *bvgS-C3* mutation, which locks the bacteria in the Bvg^+ phase (data not shown). For the RBX9*flaA*FLP-inoculated animals, most of the GFP^- colonies recovered from the mouse lungs were LCVs and no GFP^+ LCVs were recovered, indicating that formation of LCVs *in vivo* correlates with, and is most likely caused by, BvgAS modulation. Together, these data suggest that a very small proportion of RBX9 modulates to the Bvg^- phase during infection. However, our data neither support nor refute the possibility that a small number of RB50 bacteria modulates as well.

To determine if bacteria modulate to the Bvg^i phase during infection, we attempted to construct strains with Bvg^i phase promoters, including the *bipA* promoter, driving *flp*. However, we were unable to construct these strains, presumably because the level of expression of these genes in Bvg^+ phase conditions was above the threshold of *flp* activation required for recombination and loss of GFP and Km^R.

If BvgAS modulation occurs in vivo*, it does not alter the outcome of infection*

Our data suggest that a small proportion RBX9 (and possibly RB50) bacteria may modulate to the Bvg^- phase during infection. Although several previous experiments have shown that wild-type and Bvg^+ phase-locked *B. bronchiseptica* strains are indistinguishable in animal models (40–43, 45), we considered the possibility that the proportion of RBX9 bacteria that

modulate *in vivo* could actually be greater than that of RB50, but not apparent from the P_{flaA}-*flp* data because modulated RBX9 bacteria are killed in the host (i.e., that modulated bacteria, and perhaps specifically modulated RBX9 bacteria, are more susceptible to host-mediated clearance than modulated RB50 bacteria). To test this hypothesis, we compared RBX9, RBX9c (the Bvg^+ phase-locked derivative of RBX9), RBX9F (the ΔP_{fimA} derivative of RBX9 which is not defective for *bvgAS* autoregulation), and RBX9cF (a Bvg^+ phase-locked derivative of RBX9F) in mice. The results of three independent experiments are shown in Figure 8. In no case did a statistically significant difference in bacterial burden occur amongst the various strains. These data negate our hypothesis and provide strong evidence that the low level of BvgAS modulation that occurs *in vivo* (based on the recovery of LCVs) does not impact the outcome of infection.

Discussion

The discovery and characterization of LCVs in *B. bronchiseptica* yielded several interesting findings, the most significant being evidence that transcriptional interference can result from activity at a promoter located several hundred nucleotides 5′ to the affected promoter. Given this relatively large distance between promoters, the mechanism of interference likely does not involve direct blocking of transcription; therefore, we suggest the name "passive transcriptional interference" for this phenomenon. Our data indicate that the *bvgAS* P_2 promoter is sensitive to passive transcriptional interference and that it results in the emergence of a bistable phenotype, apparent as LCVs, when bacteria are switched from Bvg^- phase conditions to Bvg^+ phase conditions. The fact that LCVs, which contain Bvg^- phase-trapped bacteria, were recovered from the lungs of infected mice, provided evidence that BvgAS modulation occurs *in vivo*. Our experiments indicate, however, that although a small proportion of bacteria apparently do modulate during infection, this level of modulation does not alter the outcome of infection.

Bacterial populations often exhibit phenotypic heterogeneity. A common mechanism by which bacteria can generate this heterogeneity is phase variation, a reversible and heritable change in phenotype (due to either genetic or epigenetic modifications) often manifested as different colony morphologies (52, 53). Phase variation frequently alters the production of surface-exposed epitopes such as pili, capsule, flagella, lipopolysaccharide (LPS), and adhesins (52). Coincidently, phase variation is often associated with virulence and is an important strategy used by pathogens to avoid immune selection. Some well-characterized examples of phase variation include the *opa* operon encoding adhesin proteins in *Neisseria* species, the *pap* operon encoding fimbrae in *E. coli*, and the flagella subunits encoded by *fljBA* and *fliC* in *Salmonella*

enterica serotype Typhimurium (54–56). In *Bordetella*, phase variation in both *fim3* and *bvgAS* has been described (57, 58).

More recently, a phenomenon that generates bistable populations at the single-cell level has been discovered, called feedback-based multistability (FBM) (59). FBM is distinct from phase variation in that it is not based on genetic mutations but is instead based on feedback loops of regulatory networks (59). In isogenic populations, these feedback networks can result in bistability, which occurs when individuals in a population exhibit either one of two alternative stable steady-states (but not intermediate states) (60). A well-characterized example of FBM is in *Bacillius subtilis*, in the regulation of competence orchestrated by the transcription factor ComK (61). Competence is a cellular state induced by nutrient depletion, but only occurs in a fraction of the *B. subtilis* population due to oscillating levels of ComK at the single-cell level (62, 63). In one study, Smits *et al*. removed the external regulation of *comK*, leaving only positive autoregulation, and showed that ComK levels continued to exhibit bistability. Therefore, the authors argue that ComK bistability can be reduced to a positive autoregulatory loop in concert with random transcriptional and translational fluctuations or "noise" (64). This claim is supported by other examples, in which feedback regulation and a non-linear input are the only required components for a bistable system (60, 65).

We discovered LCVs of *B. bronchiseptica* after plating lung homogenates of mice infected with strain RBX9 and found that they yielded a heterogeneous population upon restreaking onto BG blood agar. We did not find evidence of classical phase variation in RBX9. Instead, the mechanism by which LCVs are generated appears more similar to FBM, in which the concentration of BvgA under Bvg^- phase conditions varies in the population and results in some, but not all, bacteria committing to a positive feedback loop when switched to Bvg^+ phase

conditions. In support of this hypothesis, we were able to label bacteria within LCVs with tags unique to the Bvg$^+$ and Bvg$^-$ phases and demonstrate the existence of two phenotypically distinct populations within LCVs (Figure 3). The dual-tagged RBX9 strain also provided the first direct evidence that Bvg^i phase cultures are not simply a mixture of Bvg$^+$ and Bvg$^-$ phase bacteria.

Use of the recombinase-based reporter system pGFLIP (46) showed that the Δ*fhaB* mutation in RBX9 causes a decrease in the efficiency of *bvgAS* positive autoregulation and results in Bvg$^-$ phase-trapped bacteria that decline in proportion over time and can initiate the formation of new LCVs (Figures 4, 5, and 7). Based on these results and previous data, we postulate a model of LCV formation and propagation (Figure 9). According to this model, the concentration of BvgA varies in a population and also in individual cells as they grow and divide. In RB50, the average concentration of BvgA under Bvg$^-$ phase conditions is such that 100% of the bacteria are able to respond to Bvg$^+$ phase conditions and transition to the Bvg$^+$ phase phenotype (Figure 9A, B). In RBX9 however, the average concentration of BvgA is decreased under Bvg$^-$ phase conditions compared to wild-type bacteria (curve shifted to the left in Figure 9A), such that a subpopulation is below the threshold level required to respond to Bvg$^+$ phase conditions (Figure 9B). These bacteria are thus Bvg$^-$ phase trapped and remain phenotypically Bvg$^-$ phase even under Bvg$^+$ phase conditions. Under Bvg$^+$ phase conditions, these bacteria form LCVs, which continue to harbor Bvg$^-$ phase-trapped bacteria. The Bvg$^-$ phase-trapped bacteria within LCVs occasionally escape to become Bvg$^+$ phase descendants, possibly through unequal distribution of BvgA upon cell division or by stochastic accumulation of BvgA (Figure 9C). Therefore, in this system, a mutation that decreases the basal concentration of the positively autoregulated factor (BvgA) results in an FBM-like phenotype, whereas in other systems, FBM is the natural mechanism by which bacteria reach a bistable state.

Our pGFLIP data indicated that the *bvgAS* positive autoregulation defect is due to decreased activity of P_2. This result explains why RBX9 has lower levels of BvgA: it has decreased transcription of *bvgAS*. Our data suggest that the reason for decreased transcription is the presence of a promoter located 5′ to the P_2 promoter. This upstream divergent promoter exerts its negative effects on *bvgAS* from relatively far away (~800 bp) and this phenomenon appears to represent a previously undescribed form of transcriptional interference (the suppressive influence of one transcriptional process on another) (66). It is unclear whether this promoter must be highly active or divergently transcribed. However, as with other examples of transcription interference, we predict that increasing this promoter's strength would also increase the degree of interference (67). Additionally, we predict that the orientation of the promoter may not be important and that reversing its orientation would not abolish interference if transcription read through was prevented. We do not understand mechanistically how this "passive" transcriptional interference occurs. One possibility is that the divergent promoter sequesters RNA polymerase away from the sensitive promoter (P_2) or that transcription at this site influences DNA topology in a way that is prohibitive to P_2 activation. These data suggest that the level of expression from the *bvgAS* P_2 promoter is poised at the threshold of that required for all cells in the population to respond to Bvg^+ phase conditions when they are encountered. Although the LCV phenotype appeared as an artifact of genetic manipulation, our results are important as they demonstrate a form of transcriptional interference that, to our knowledge, has not been described previously, and also because they reveal a mechanism by which in-frame deletion mutations can have unanticipated polar effects on neighboring genes. Furthermore, RBX9 and its derivatives constitute a genetically tractable system for studying additional mechanisms of transcriptional interference and details of FBM.

The LCVs also provided insight into the behavior of BvgAS during infection. The role of BvgAS-dependent modulation in the *Bordetella* life cycle is not completely understood and remains an important area of investigation. Several studies have attempted to determine if Bvg^i or Bvg^- phase bacteria exist at any point during *Bordetella* infection, and so far none have yielded positive results (40–43, 45). These data, together with those demonstrating that Bvg^- phase bacteria transition rapidly to the Bvg^+ phase following intranasal inoculation (46, 68), have led to the conclusion that not only is the Bvg^+ phase necessary and sufficient for infection, but that bacteria switch to and remain in the Bvg^+ phase *in vivo*. The Bvg^i and Bvg^- phases are hypothesized to be important for transmission and survival *ex vivo*, however, no role for these phenotypic phases in a natural setting has been demonstrated. For *B. pertussis* particularly, which appears to survive outside the host only briefly during transmission to a new host, the role of BvgAS modulation remains mysterious. The isolation of LCVs from mouse lungs provides strong evidence that at least some RBX9 bacteria modulate during infection. However, the proportion of bacteria that modulated and that could be recovered from the animals was very low. Because the P_{flaA}-*flp* system was unable to reliably distinguish this low proportion of modulated bacteria from background resolution, we could not determine if wild type bacteria modulate *in vivo*. If they do not, our data would suggest that only FHA-deficient bacteria modulate *in vivo*, which would suggest that FHA functions to prevent the bacteria from experiencing a Bvg^- phase environment during infection. In pilot experiments, we also recovered LCVs from mice infected with Δ*fhaB*, Δ*cyaA* double mutants – in higher proportions, in some cases, than in mice infected with RBX9. These preliminary data suggest the intriguing possibility that FHA and ACT function together to prevent *Bordetella* from creating or entering a

modulating environment in the host. Our future experiments will be aimed at testing this hypothesis.

Experimental Procedures

Strains and growth conditions

Escherichia coli were grown in lysogeny broth (LB; 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 2.5 g l^{-1} NaCl) or on LB with agar (1.5%) at 37°C. *Bordetella* were grown in Stainer-Scholte (SS) broth (25) or on Bordet-Gengou (BG) agar (1.5%) (BD Biosciences, San Jose, CA) supplemented with 7.5% defibrinated sheep's blood (Colorado Serum Company, Denver, CO) at 37°C (16). As required, culture media were supplemented with kanamycin (Km; 50 μg ml^{-1}), ampicillin (Ap; 100 μg ml^{-1}), streptomycin (Sm; 25 μg ml^{-1}), magnesium sulfate ($MgSO_4$; 50 mM in plates and 20 mM in liquid), and diaminopimelic acid (DAP; 400 μg ml^{-1}) for the *E. coli* mobilizer strain RHO3 (Δ*asd* Δ*aphA*) (19). Unless otherwise noted, all restriction enzymes and T4 DNA ligase was purchased from New England Biolabs.

Construction of bacterial strains

Allelic exchange and Campbell-type integrations were done by matings using parental *Bordetella* and *E. coli* strain RHO3 harboring the appropriate suicide plasmid. The pGFLIP plasmid was delivered to the *att*Tn7 site using tri-parental mating with the above strains and with RHO3 cells harboring pTNS3, which encodes the transposase genes *tnsABCD*. Integration at the *att*Tn7 locus was confirmed via PCR. For details on specific strain constructions see the Supplemental text.

Immunofluorescence

HA epitopes on the surface of RBX9BatB-HA*flaA-gfp* bacteria were stained and visualized using indirect immunofluorescence. After 72h of growth at 37˚C, five to twenty colonies of each morphology including Bvg^+, Bvg^-, Bvg^i, and LCV, were scraped off of BG blood agar plates and resuspended into 1ml of 4% paraformaldehyde and were allowed to fix on ice for 30 min. Cells were pelleted and washed with 1% BSA in PBS in a microcentrifuge tube. Primary antibody (mouse monoclonal anti-HA IgG diluted 1:2000 in 1% BSA in PBS) was used to resuspend the pellet and this mixture was incubated for 1h at room temperature (RT). The pellets were washed twice in 1% BSA for 5 min. Secondary antibody (Alexa Fluor 594-conjugated goat anti-mouse IgG, diluted 1:250 in 1% BSA in PBS; Invitrogen) was used to resuspend the pellet and this mixture was then incubated for 30 min at RT in the dark. The pellets were washed twice with 1% BSA for 5 min. Four microliters of the leftover pellet and liquid was pipetted onto a slide for visualization.

Confocal Microscopy

Immunofluorescent RBX9BatB-HA*flaA-gfp* bacteria were visualized using a Zeiss LSM 710 confocal microscope. Secondary antibody (Alexa Fluor 594-conjugated goat anti-mouse IgG) was detected using a 594 nm laser and GFP was detected at 488 nm. We used the 63× oil objective with 3× digital zoom. Images were viewed and saved with the Zen software from Carl Zeiss Microscopy.

pGFLIP assays

For RBX9*cyaA*FLP, RB50P*bvgA*$_{short}$*FLP* and RB50P*bvgA*$_{long}$*FLP*, strains were grown on BG blood agar plates under promoter-inactive conditions (Bvg$^-$ phase) with Km for 48h at 37°C and were determined to be GFP positive (GFP$^+$) using a G:BOX Chemi imaging system with an UltraBright-LED blue transilluminator and an SW06 short-pass filter (495 to 600 nm; Syngene, Frederick, MD). Single GFP$^+$ colonies were then resuspended in PBS, diluted, and plated onto Bvg$^+$ or Bvg$^-$ phase conditions (promoter-active and promoter-inactive conditions, respectively) in the absence of Km selection. For RBX9*cyaA*FLP, GFP$^+$ LCVs were picked, diluted, and plated onto Bvg$^+$ and Bvg$^-$ phase conditions in the absence of Km selection. Percent resolution was determined by averaging the ratio of GFP$^-$ cfu/total cfu for at least three plates.

Intranasal inoculation of mice

Four- to eight-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were inoculated intranasally with 1×10^5 cfu of *B. bronchiseptica* in 50 µl of PBS. For all infections, bacteria were grown overnight in SS medium. Lungs were harvested at 1h and 72h p.i. Right lungs were homogenized in 1 ml of PBS, diluted in PBS, and plated in at least duplicate on BG agar. Figure 8 represents data from three independent experiments performed with at least two mice per strain per time point.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our protocol

was approved by the University of North Carolina IACUC (10-134, 12-307). All animals were properly anesthetized for inoculations, monitored regularly, euthanized when moribund, and efforts were made to minimize suffering.

Statistical analyses

Statistical analyses were performed using Prism 5.0 (GraphPad Software, Inc.). Statistical significance was determined using the unpaired Student's t-test or analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Images were formatted using Adobe Photoshop CS5 and figures were generated using Adobe Illustrator CS5 (Adobe Systems, Inc.).

Figures

Figure 1. The *Bordetella* BvgAS system controls at least four different classes of genes and three different phenotypic phases in response to environmental stimuli

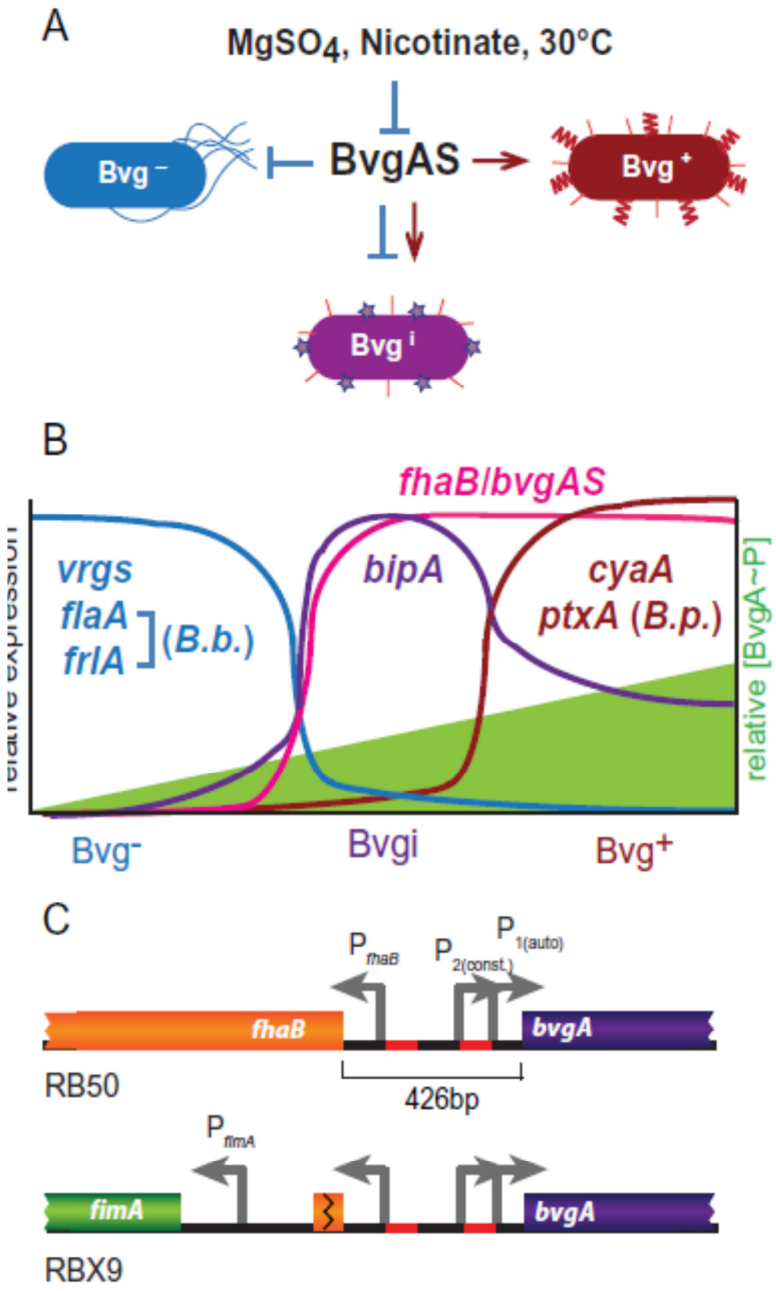

Figure 1: The *Bordetella* BvgAS system controls at least four different classes of genes and three different phenotypic phases in response to environmental stimuli. A, BvgAS is responsible

for the Bvg^{+}, Bvg^{i}, and Bvg^{-} phases and is repressed by chemical modulators or low temperature. B, The three phenotypic phases are defined by unique patterns of gene expression as indicated, and rely on the intracellular concentration of BvgA~P. C, The chromosomal organization of the *fhaB* and *bvgAS* loci in RB50 (top) and RBX9 (Δ*fhaB*, bottom).

Figure 2: RB50 and RBX9 colony morphology.

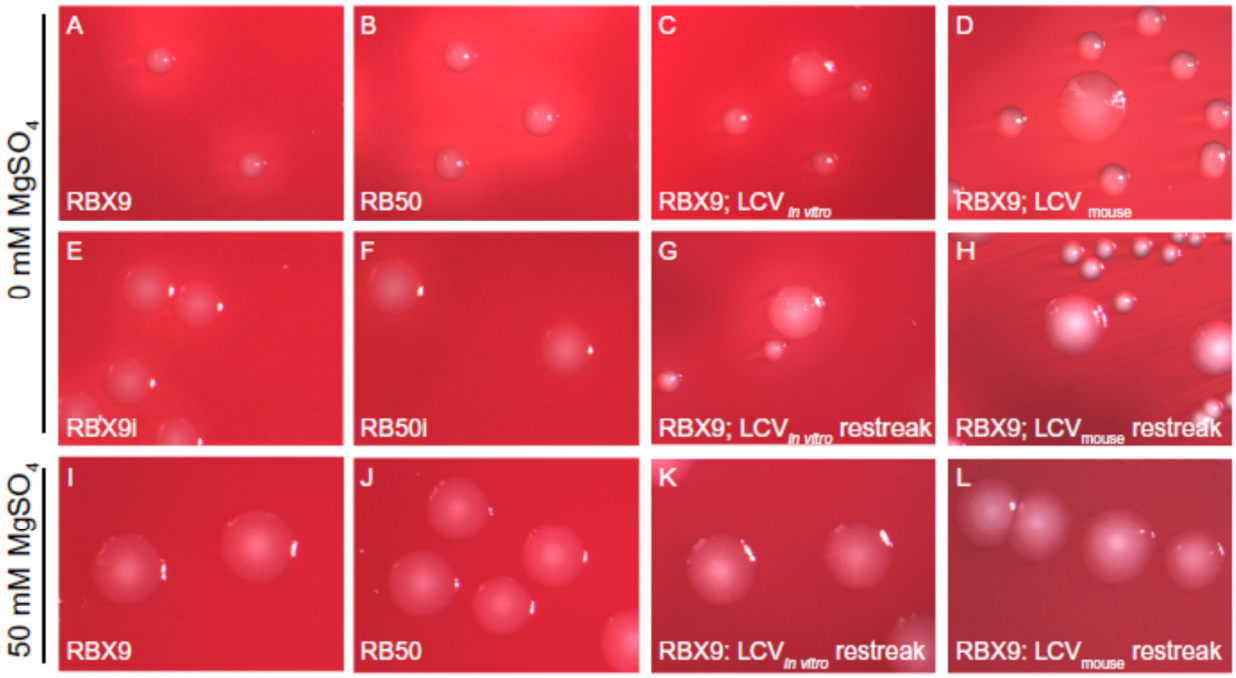

Figure 2: RB50 and RBX9 colony morphology. Bacteria were plated on either BG agar or BG agar + 50mM $MgSO_4$ and were imaged after 48h. A, RB50; B, RBX9; C, RBX9 LCV produced after *in vitro* modulation: D, RBX9 LCV recovered from mouse lung homogenate; E, RB50i (a Bvg-intermediate phase-locked strain in the RB50 background); F, RBX9i (a Bvg-intermediate phase-locked strain in the RBX9 background); G, RBX9 restreak of an LCV produced after modulation; H, RBX9 restreak of an LCV recovered from the mouse lung; I, RB50; J, RBX9; K, RBX9 restreak of an LCV produced after modulation; L, RBX9 restreak of an LCV recovered from the mouse lung.

Figure 3. Detection of Bvg$^+$ and Bvg$^-$ phase bacteria in typical RBX9 colonies and LCVs.

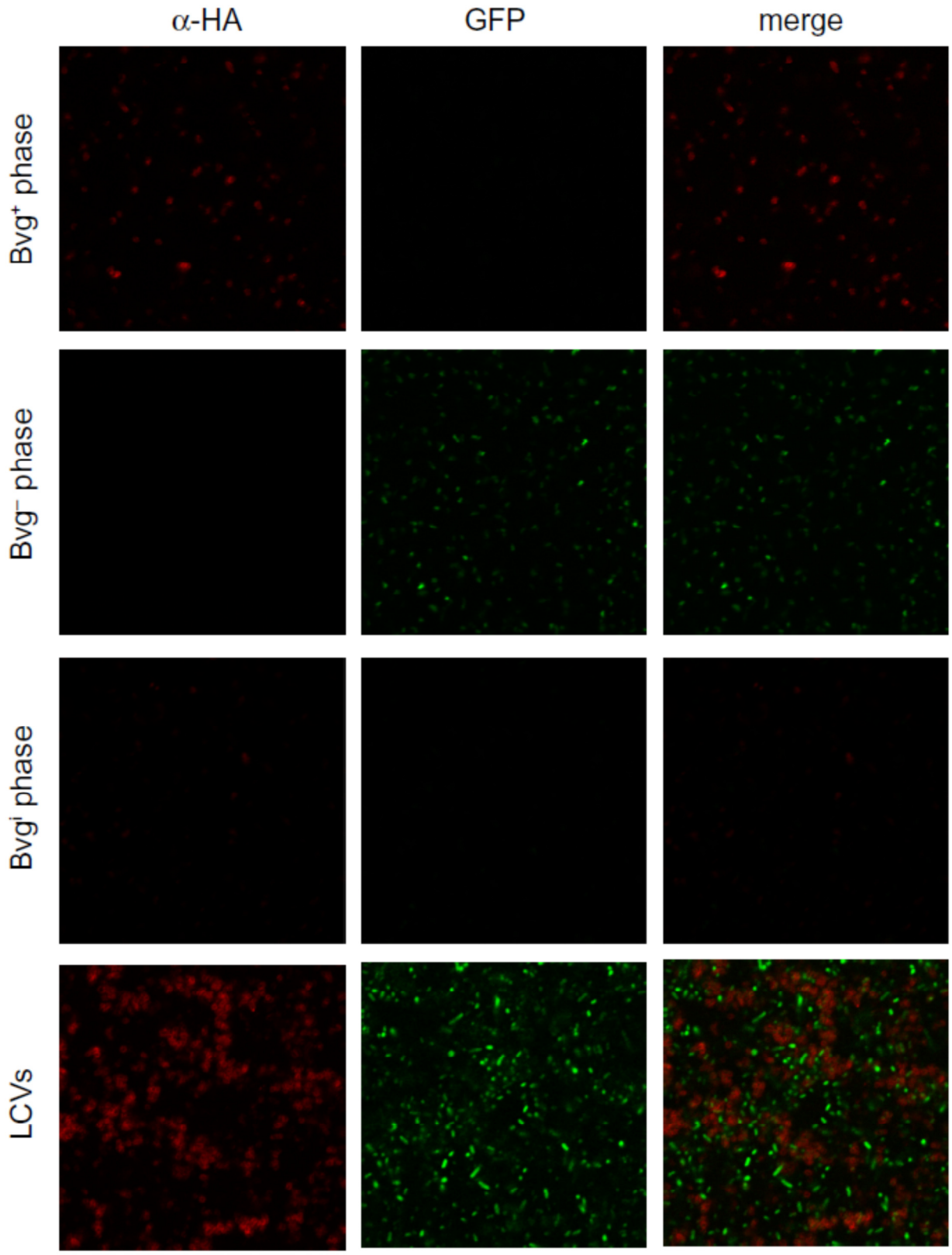

Figure 3: Detection of Bvg^+ (α-HA, red) and Bvg^- phase (*flaA-gfp*, green) bacteria in typical RBX9 colonies and LCVs. RBX9BatBN-HA*flaA-gfp* was grown on BG blood agar (Bvg^+ phase conditions), BG blood agar + 50 mM $MgSO_4$ (Bvg^- phase conditions), or BG blood agar + 6 mM $MgSO_4$ (Bvg^i phase conditions). Several colonies of each phenotype were combined and stained with mouse monoclonal α-HA IgG followed by an Alexa Fluor 594-conjugated goat anti-mouse IgG secondary antibody. Fluorescence was detected using a Zeiss LSM 710 confocal microscope.

Figure 4. LCVs from the strain RBX9P*cyaA*FLP are GFP^{+}, indicating that *cyaA* has not been activated in a substantial proportion of these colonies

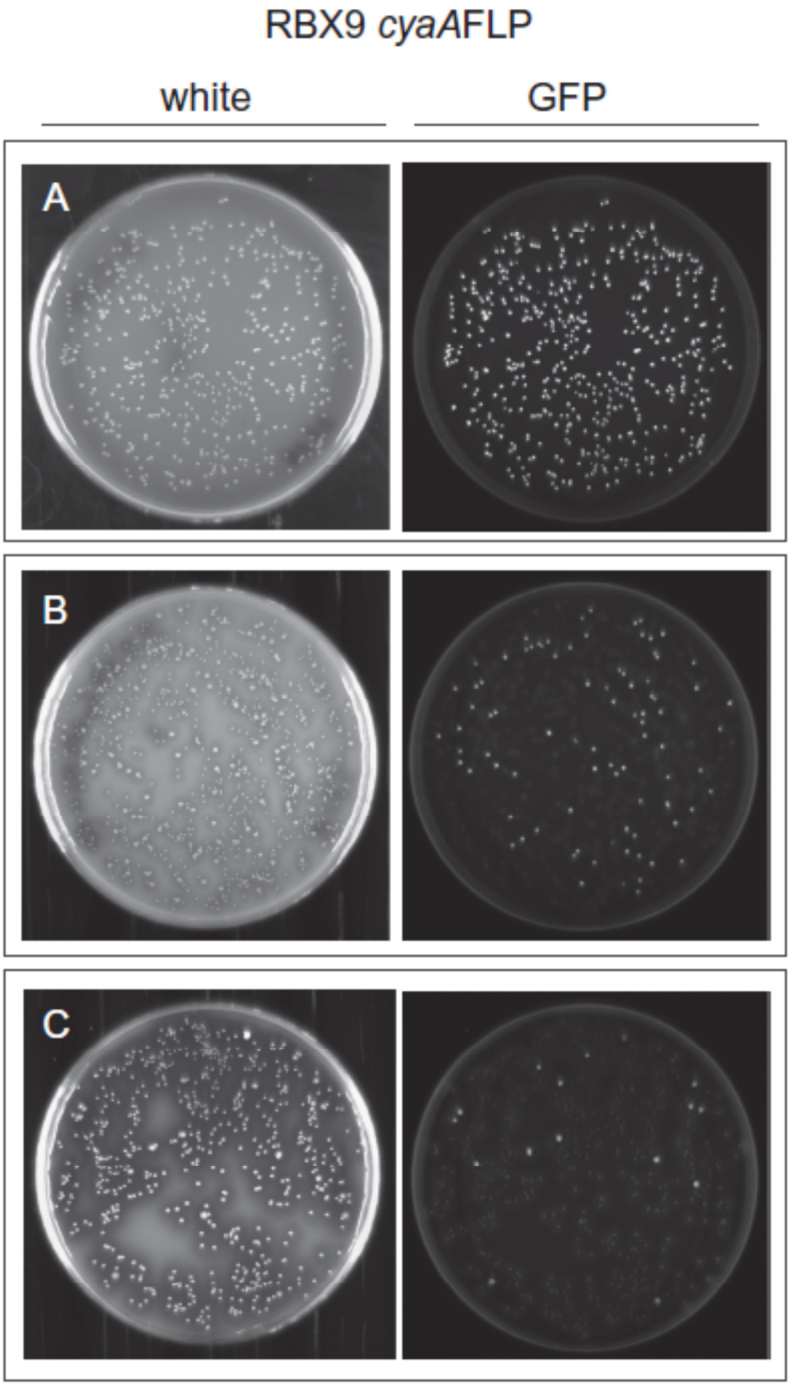

Figure 4: LCVs from the strain RBX9P*cyaA*FLP are GFP^+, indicating that *cyaA* has not been activated in a substantial proportion of these colonies. A, RBX9P*cyaA*FLP on BG blood agar + 50mM $MgsO_4$ (Bvg^- phase conditions); B, a GFP^+ colony from A plated onto BG blood agar (Bvg^+ phase conditions); C, a GFP^+ LCV from B plated onto BG blood agar (Bvg^+ phase conditions). Colonies were visualized after 48h of growth.

Figure 5: Sequences upstream of *bvgAS* affect transcription efficiency under Bvg⁻ phase conditions.

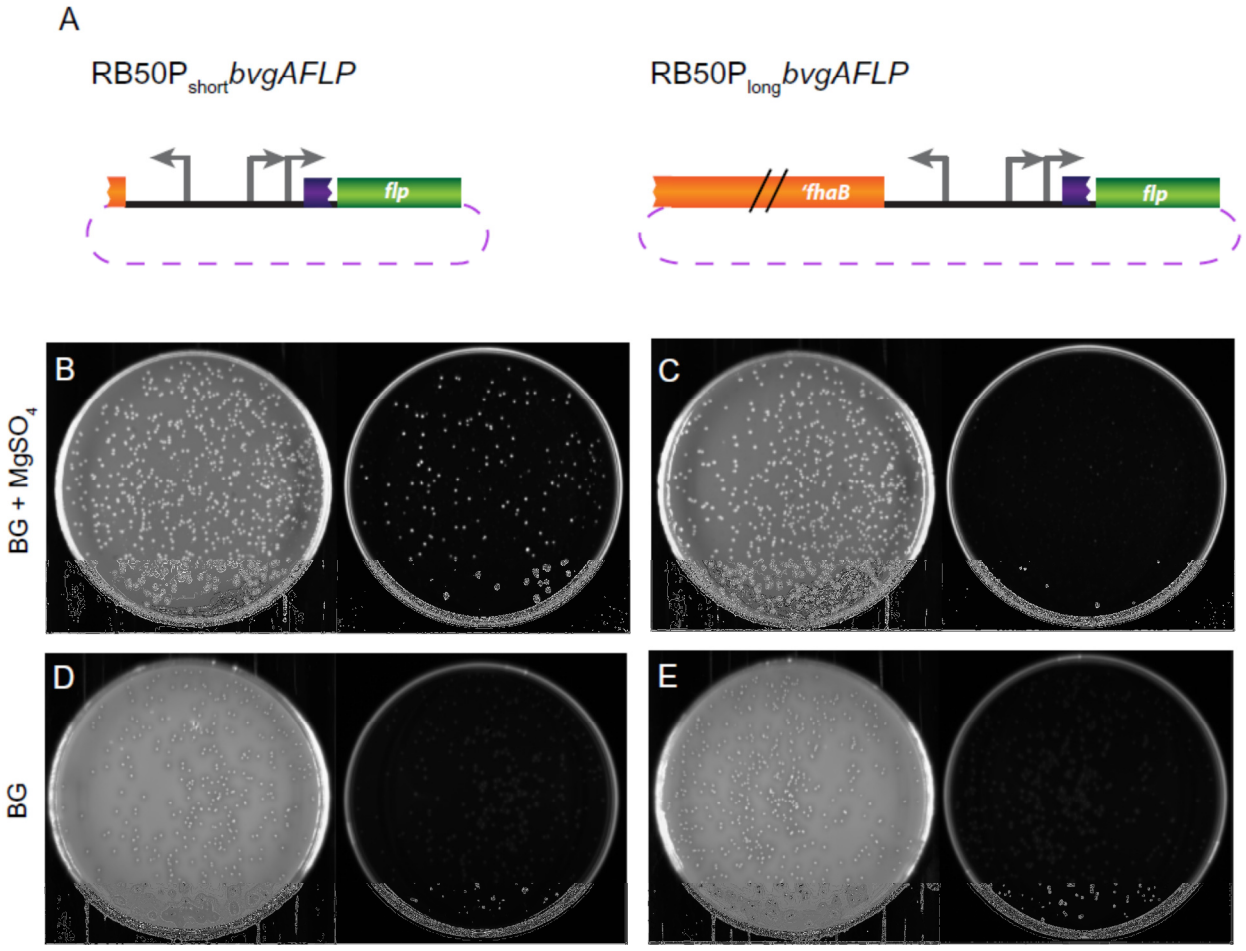

Figure 5: Sequences upstream of *bvgAS* affect transcription efficiency under Bvg⁻ phase conditions. A, Schematics of RB50P$_{short}$*bvgAFLP* and RB50P$_{long}$*bvgAFLP* showing the sequences 5′ to *flp* (not drawn to scale); B and C, strains were first grown on BG blood agar + 50 mM $MgSO_4$ + Km and one colony was plated onto BG blood agar + 50 mM $MgSO_4$ (Bvg⁻ phase conditions) without Km selection; D and E, strains were grown on BG blood agar + 50 mM $MgSO_4$ + Km selection and then one colony of each was plated onto BG blood agar (Bvg$^+$ phase

conditions) without selection. Representative white light (left) and fluorescent (right) images are shown for panels B, C, D, and E.

Figure 6: The genetic architecture of the *bvgAS-fhaB* intergenic region of strains that do and do not produce LCVs.

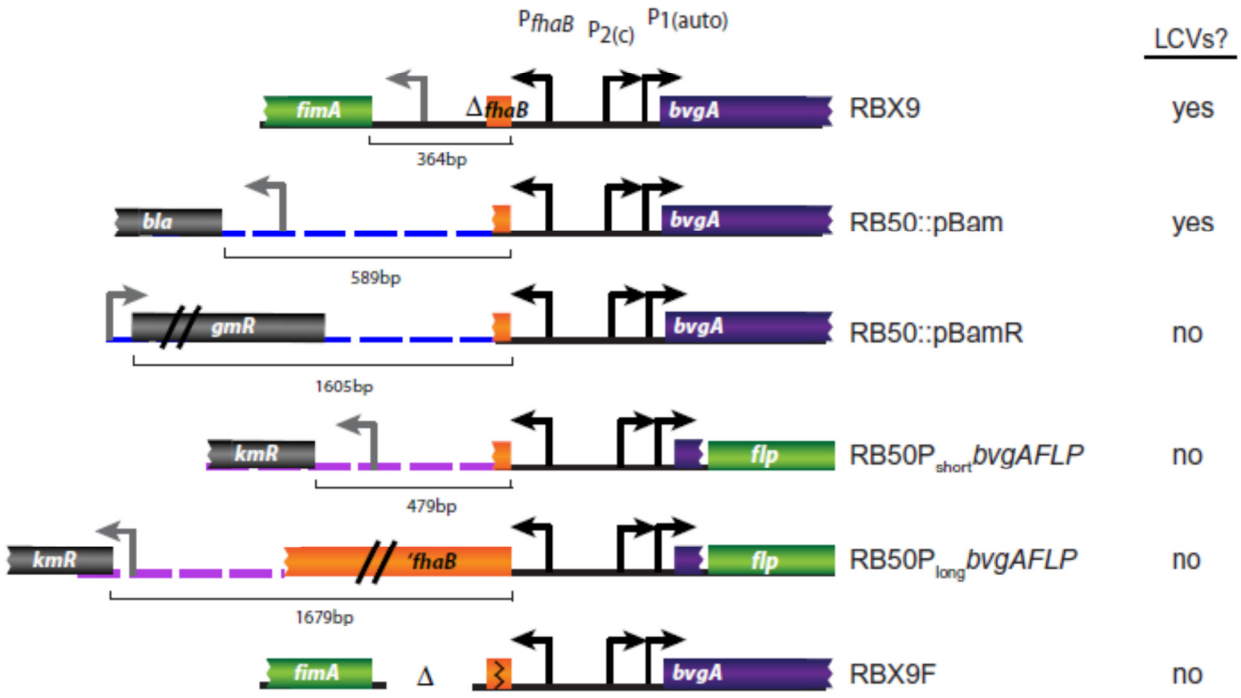

Figure 6: The genetic architecture of the *bvgAS-fhaB* intergenic region of strains that do and do not produce LCVs. Strains that produce LCVs or demonstrate a defect in *bvgAS-flp* activation have divergent promoters 5′ to the *bvgAS-fhaB* intergenic region. Schematics for RB50P$_{short}$*bvgAFLP* and RB50P$_{long}$*bvgAFLP* represent sequences inserted at the *att*Tn7 site. Dotted lines represent non-coding plasmid DNA. Sequence lengths from the ATG of *fhaB* to the nearest 5' ATG are indicated.

Figure 7: Schematic of RBX9*cyaA*FLP experimental design

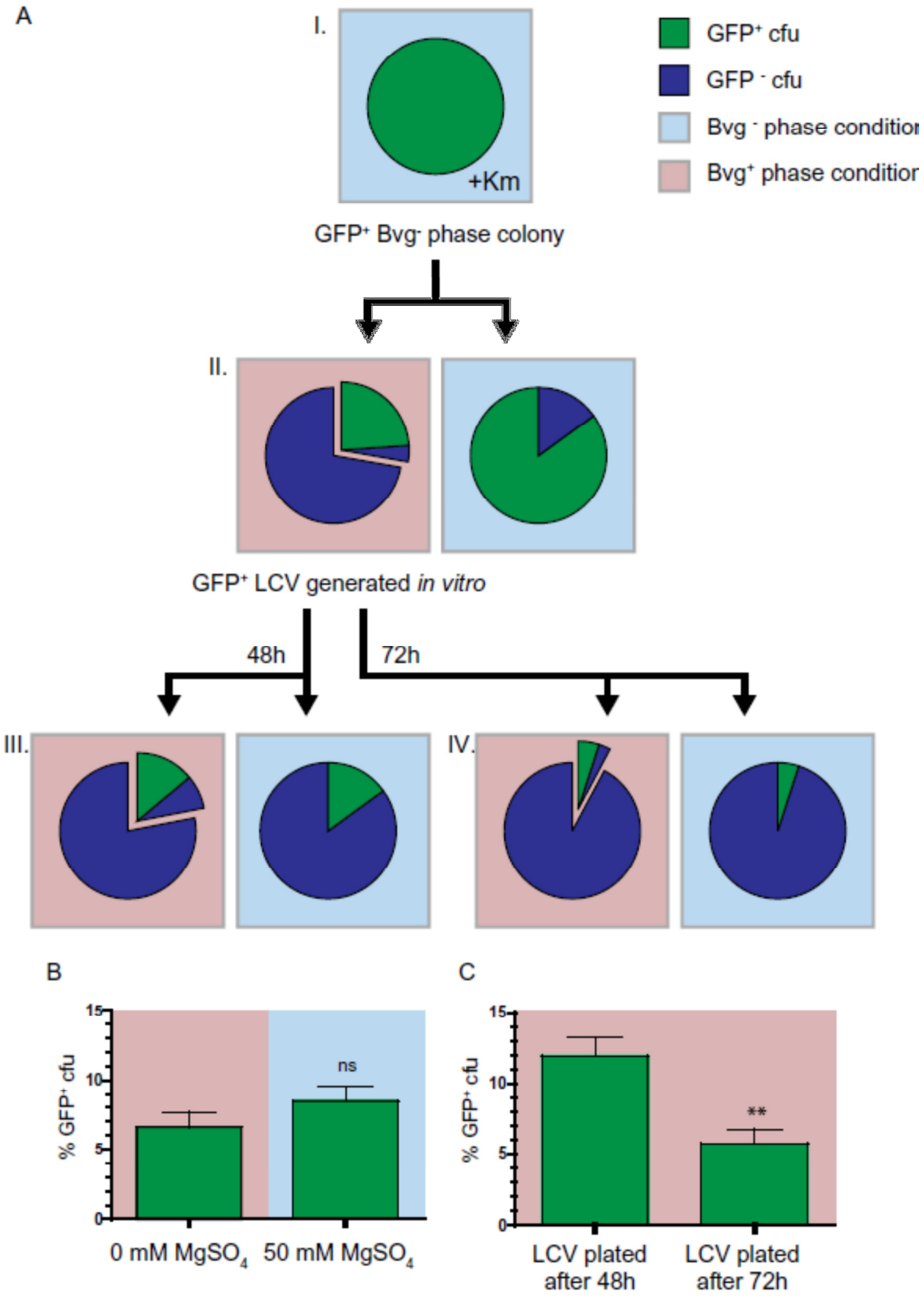

Figure 7: A, Schematic of RBX9*cyaA*FLP experimental design, including a data set from one replicate. Each pie chart represents the population obtained from plating a single GFP^+ colony from the previous plate (see text for details). Blue sectors in pie charts represent the frequency of GFP^- cfu; Green sectors in pie charts represent the frequency of GFP^+ cfu; offset regions of pie charts represent the frequency of LCVs; Frequencies were determined by counting at least 500 cfu per condition. B, Comparison of GFP^+ cfu frequencies obtained from plating a single GFP^+ LCV onto Bvg^+ and Bvg^- phase conditions. C, Comparison of GFP^+ cfu frequencies obtained from plating a GFP^+ LCV grown after 48h and 72h. Background color represents BvgAS conditions, where red is Bvg^+ phase conditions and blue is Bvg^- phase conditions. **, P = 0.005 by Student's Unpaired T-test.

Figure 8: Comparison of RBX9, RBX9c, RBX9F, and RBX9cF burdens in the mouse lung

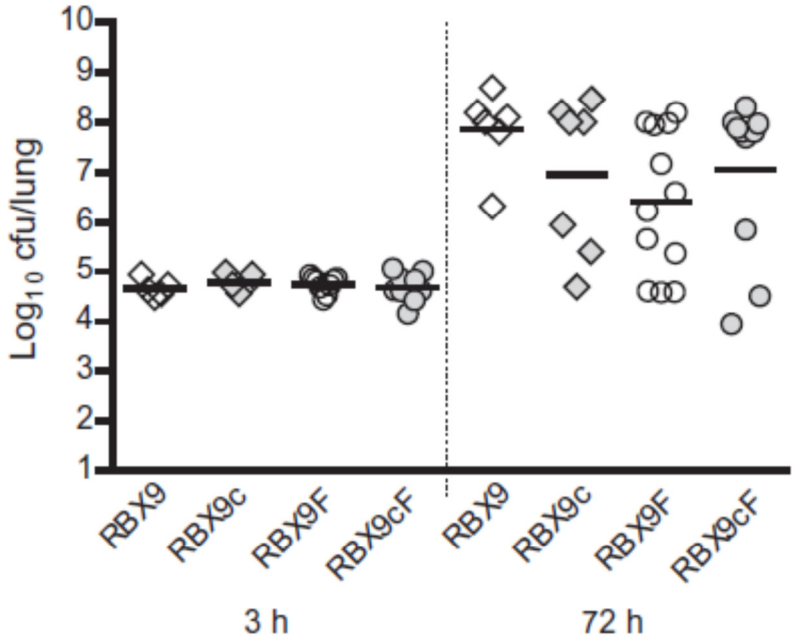

Figure 8: Comparison of RBX9, RBX9c, RBX9F, and RBX9cF burdens in the mouse lung after 3h and 72h p.i. RBX9c and RBX9cF are Bvg^+ phase-locked derivatives of RBX9 and RBX9F, respectively; four-to eight-week-old BALB/C mice were intranasally infected with 1×10^5 cfu in 50µl and lungs were harvested at each time point; each diamond or circle indicates the number of cfu recovered from a single animal and each horizontal line indicates the geometric mean for each group; these data represent three independent experiments with at least two mice per strain per time point.

Figure 9. Proposed distribution of BvgA concentration within populations of RBX9 and RB50.

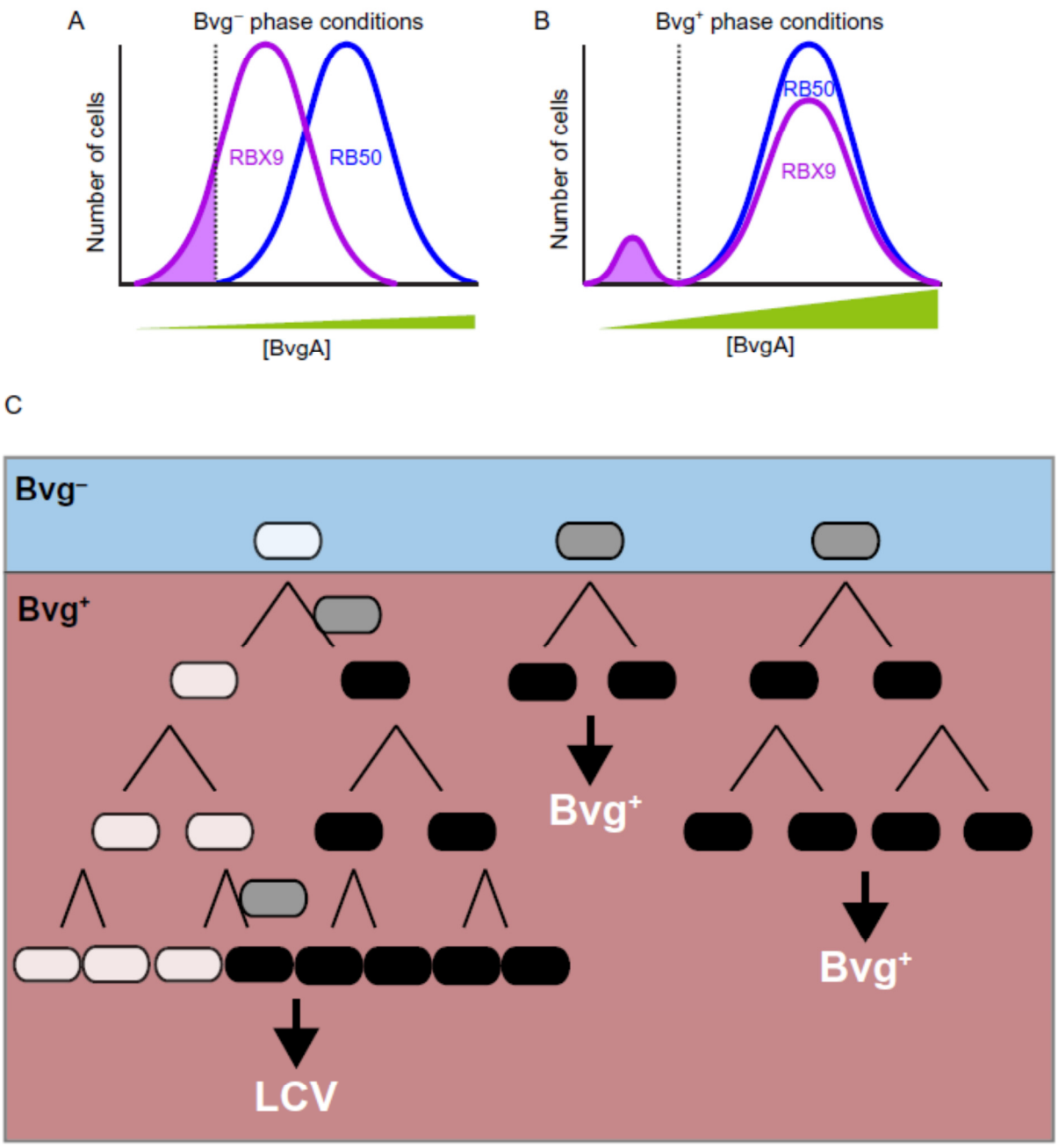

Figure 9. Proposed distribution of BvgA concentration within populations of RBX9 and RB50. A, In the Bvg⁻ phase, a proportion of RBX9 cells (shaded region) are Bvg⁻ phase-trapped (i.e.,

have a concentration of BvgA below the threshold [dotted line] necessary to stimulate positive autoregulation upon transition to Bvg^+ phase conditions). By contrast, all RB50 cells have a level of BvgA sufficient to initiate positive autoregulation upon transition to Bvg^+ phase conditions. B, In the Bvg^+ phase, the RBX9 cells that were below the threshold BvgA concentration in the Bvg^- phase (shaded region as in A) maintain their low concentration of BvgA and are thus unable to switch to the Bvg^+ phase. These cells are able to initiate LCV formation as described in C. Consistent with our *in vitro* data, all RB50 cells are able to switch to the Bvg^+ phase. C, Model of LCV formation and propagation illustrated as a lineage diagram (see text for details). RBX9 bacteria exist as a heterogeneous population under Bvg^- phase conditions, with some bacteria (white) being below the threshold of BvgA required to initiate positive autoregulation and others above this threshold (gray). When bacteria are switched to the Bvg^+ phase, the Bvg^- phase trapped bacteria form LCVs, whereas the other bacteria transition into the Bvg^+ phase (black) and form Bvg^+ phase colonies. Occasionally, Bvg^- phase-trapped bacteria "escape" and can transition into the Bvg^+ phase (indicated by gray cells between white and black cells), resulting in LCV formation after 48h.

Table 1: LCV recovery frequency

Condition	%[a] LCVs recovered on BG blood agar
Plating murine lung homogenate after 30 hours	0.39 ± 0.13
Replating any RBX9 Bvg$^-$ phase colony	5.43 ± 0.98
Replating an LCV from BG agar (derived *in vitro*)	3.90 ± 0.80
Replating an LCV from BG agar (derived *in vivo*)	6.03 ± 1.26
Replating any RBX9 Bvg$^+$ phase colony	0

[a]Values are means ± standard errors for experiments performed at least in triplicate

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APPENDIX C. AN IMPROVED RECOMBINATION-BASED *IN VIVO* EXPRESSION TECHNOLOGY-LIKE REPORTER SYSTEM REVEALS DIFFERENTIAL *CYAA* GENE ACTIVATION IN *BORDETELLA* SPECIES[1]

Introduction

Bordetella species are Gram-negative bacterial respiratory pathogens. The genus includes *Bordetella pertussis*, an obligate human pathogen and the causative agent of whooping cough, and the closely related *Bordetella bronchiseptica*, which can infect a wide range of mammals including several species that are commonly studied in the laboratory (1-3). These bacteria rely on the global two-component regulatory system BvgAS for virulence (1-3). The BvgAS phosphorelay regulates gene expression patterns according to environmental cues and controls at least three distinct phenotypic phases: Bvg minus (Bvg^-), Bvg plus (Bvg^+), and Bvg intermediate (Bvg^i) (4, 5). All evidence thus far suggests that the Bvg^+ phase is necessary and sufficient for infection, and that modulation to the Bvg^- or Bvg^i phase does not occur *in vivo* (6-8). Although it has been hypothesized that the Bvg^i phase and/or the Bvg^- phase is required for transmission or survival outside of a host (7, 9, 10), a recent study provided evidence that neither of these phenotypic phases is required for *B. bronchiseptica* transmission in swine (8).

Genes that define these three phenotypic phases have been divided into four classes based on their expression profile. Class 1 (late Bvg^+ phase) genes include *cyaA-E* (encoding the

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bifunctional hemolysin/adenylate cyclase toxin ACT) and *ptxA-E* (encoding the AB_5-type pertussis toxin) (4). Class 2 (early Bvg^+ phase) genes include those encoding filamentous hemagglutinin (*fhaB*), fimbriae (*fim2* and *fim3*), and *bvgAS* itself (4). *bipA* is the only class 3 (Bvg^i phase) gene that has been characterized. Class 4 (Bvg^- phase) genes include those encoding proteins involved in motility (*frlAB*) and chemotaxis in *B. bronchiseptica* (4).

Expression of genes that define the various Bvg-dependent phenotypic phases is determined mechanistically by the location and affinity of BvgA binding sites near the transcription start site (4). Class 1 genes contain multiple low affinity BvgA binding sites 5′ distal to the start of transcription (4, 11), while class 2 genes contain high-affinity BvgA binding sites proximal to the transcription start site (12-14). The promoter region of the class 3 gene *bipA* contains high affinity BvgA binding sites 5′ proximal to the transcription start site and low affinity sites 3′ to the transcription start site (10, 15). Although it has been hypothesized that BvgAS directly represses transcription of *frlAB* in *B. bronchiseptica* (16), BvgA binding to the *frl* promoter has not been demonstrated and BvgAS-mediated repression of at least some genes in *B. pertussis* is indirect (17).

In vitro transcription and DNA binding studies indicate that the phosphorylated form of BvgA (BvgA~P) is required to activate transcription of Bvg^+ phase genes and that a higher concentration of BvgA~P is necessary to bind "late" Bvg^+ phase promoters than "early" Bvg^+ phase promoters (11, 18). Although the natural signals that activate the BvgAS system are unknown, it is possible to modulate *Bordetella* spp. to the Bvg^- phase in the laboratory by adding a chemical modulator ($MgSO_4$ or nicotinic acid) to the growth medium, or by growing bacteria at 25°C (15, 19-21). When grown under Bvg^- phase conditions, class 4 genes are expressed while class 1–3 genes are not expressed. When switched from Bvg^- phase to Bvg^+ phase conditions,

transcription of class 4 genes ceases and transcription of class 2 genes, along with the sole class 3 gene *bipA*, is immediately activated. After several hours, class 1 genes are expressed and class 3 (*bipA*) genes are repressed. These data are consistent with the model in which the concentration of BvgA~P within the cell is nearly zero in the Bvg^- phase, low in the Bvg^i phase, and high in the Bvg^+ phase (4, 5, 11, 15, 18, 22, 23).

By contrast with the extensive *in vitro* characterization of the steady-state expression patterns of BvgAS-regulated genes in both *B. pertussis* and *B. bronchiseptica*, as well as kinetic analyses of gene expression upon switching from modulating to non-modulating conditions and vice versa (5, 10, 11, 15, 22-24), only one study has investigated BvgAS-dependent gene regulation *in vivo* (24). Veal-Carr et al. utilized recombination-based *in vivo* expression technology (RIVET) to analyze the kinetics of BvgAS-activated gene expression in *B. pertussis* both *in vitro* and following intranasal infection of mice (24, 25). They showed that the *in vivo* activation of Bvg^+ phase genes, including *ptxA*, *cyaA*, *fhaB*, and *prn*, temporally recapitulated the activation pattern of these genes upon switching *B. pertussis* from Bvg^- to Bvg^+ phase conditions *in vitro*; i.e., *fhaB* was activated early post-inoculation (~1 h), followed by *prn* (~4 h), then later by *cyaA* (~12 h) (24). Significantly, the fact that the pattern of gene activation was nearly identical in bacteria switched from Bvg^- to Bvg^+ phase conditions *in vitro* and post-inoculation of mice indicates that the mouse lung is a Bvg^+ phase environment (24).

We constructed a plasmid, pGFLIP, that encodes a Flp recombinase-based fluorescent reporter system to assess the activation kinetics of genes *in vivo* (26). The region of pGFLIP delivered to the chromosome contains *gfp* and *nptII* genes, encoding green fluorescent protein and neomycin phosphotransferase (conferring kanamycin resistance [Km^r]), respectively, flanked by *F*lp *r*ecombinase *t*arget (*FRT*) sites. The plasmid also contains a promoterless *flp* recombinase

gene with a multiple cloning site (MCS) immediately 5′ to the start codon. Upon expression of *flp* under the control of a promoter of interest, the *gfp* and *nptII* genes are permanently excised from the bacterial chromosome.

To test our system, we cloned the promoter regions of several BvgAS-controlled genes, including the Bvg-activated genes *cyaA*, *fhaB*, and *ptxA* and the Bvg-repressed gene *flaA* (encoding flagellin) into pGFLIP and evaluated transcription activation *in vitro* and *in vivo* in *B. bronchiseptica*. Amongst other results, we found unexpectedly that the *cyaA* gene is expressed differently in *B. pertussis* and *B. bronchiseptica*.

Materials and Methods

Strains, reagents, and growth conditions. *Escherichia coli* were grown in lysogeny broth (LB; 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) or on LB plates with 1.5% agar at 37°C. *Bordetella* were grown in Stainer-Scholte (SS) broth (27) or on Bordet-Gengou (BG) plates with 1.5% agar (BD Biosciences, San Jose, CA) supplemented with 7.5% (*B. bronchiseptica*) or 15.0% (*B. pertussis*) defibrinated sheep's blood (Colorado Serum Company, Denver, CO) at 37°C (28). As required, culture media were supplemented with kanamycin (Km; 50 or 100 μg/ml), ampicillin (100 μg/ml), streptomycin (25 μg/ml), $MgSO_4$ (20 mM or 50 mM), heptakis (1 mg/ml, Sigma), and for the Δ*asd* Δ*aphA* mobilizer strain RHO3 (29), diaminopimelic acid (DAP; 400 μg/ml). Unless otherwise noted, all restriction enzymes and T4 DNA ligase was purchased from New England Biolabs.

Construction of pGFLIP and derivatives containing *Bordetella* promoters. The Tn7 transposition plasmid pUC18T-mini-Tn7T-Km-*FRT* (30) was digested with BamHI, resulting in fragments of 3636 and 1299 bp in length containing the plasmid backbone and the *nptII* (Km^r)

gene, respectively. Separately, a 797-bp fragment containing *gfp* driven by the constitutive *Burkholderia pseudomallei rpsL* promoter P_{S12} was amplified by PCR from mini-Tn7-*kan-gfp* (31) using Pfu Ultra II (Agilent) and primers GFP_UP and GFP_DN. This fragment was blunt-end ligated into the cloning vector pJET1.2/blunt (Fermentas) and was transformed into *E. coli* DH5α according to the manufacturer's instructions. Using restriction sites introduced by PCR, the P_{S12}-*gfp* fragment was digested from pJET using BamHI and was ligated together with the BamHI-digested pUC18T-mini-Tn7T-Km-*FRT* backbone and *nptII* fragment. As the *flp* gene would be sensitive to transcription read-through from either the P_{S12} or the *nptII* promoter, primers specific to *gfp* (gfpseqR) and *nptII* (kanseqR) were used to confirm that both genes would be transcribed opposite the promoter of interest and would therefore not drive *flp* expression. Once the orientation of *gfp* and *nptII* was verified, the plasmid was digested with KpnI and StuI; a fragment containing promoterless *flp* amplified by PCR from pFLPe4 (30) using primers FLP_UP and FLP_DN was likewise digested with KpnI and StuI and was ligated into the digested vector. The resulting plasmid, pGFLIP, thus contained an MCS 5′ to *flp*, the *flp* recombinase gene, and constitutively expressed *nptII* and *gfp* genes flanked by *FRT* sites. pGFLIP was fully sequenced using a primer-walking approach with the primers listed in Table 2.

Promoters for five *Bordetella* genes (*cyaA*, $cyaA_{Bp}$, *fhaB*, *ptxA*, and *flaA*) were cloned into the MCS of pGFLIP as follows. For *cyaA* and $cyaA_{Bp}$, 605 and 604-bp fragments containing the *cyaA* promoter were amplified by PCR from *B. bronchiseptica* RB50 and *B. pertussis* BPSM, respectively, using primers cyaA_F and cyaA_R. These fragments were digested with SacI and KpnI and ligated into pGFLIP. For *fhaB*, a 426-bp fragment containing the *fhaB* promoter was amplified by PCR from RB50 using primers fhaprF2 and fhaprR2, digested with SacI and KpnI, and ligated into pGFLIP. For *ptxA*, a 454-bp fragment containing the *ptxA* promoter was

amplified by PCR from BPSM using primers ptxprF and ptxprR, digested with SacI and ApaI, and ligated into pGFLIP. For *flaA*, a 514-bp fragment containing the *flaA* promoter was amplified by PCR from RB50 using primers flaA_F and flaA_R, digested with SacI and KpnI, and ligated into pGFLIP. These constructs were delivered to the chromosome by transposase-mediated insertion as described below.

Transposase-mediated delivery of pGFLIP to the *B. bronchiseptica att*Tn7 site. The pGFLIP plasmid was delivered to *Bordetella* by tri-parental mating using a procedure modified from (28). *B. bronchiseptica* strains RB50 and RB52 were grown on BG agar for 48 h, and a portion of the cells was co-incubated with conjugation-competent *E. coli* RHO3 cells (29) harboring pGFLIP and RHO3 cells containing the helper plasmid pTNS3 (30), which expresses *tnsABCD* from a constitutive promoter, on BG agar supplemented with DAP for 6 h at 37°C. Following incubation, cells were restreaked onto BG/Km agar containing 20 mM $MgSO_4$ (to maintain strains containing pGFLIP with Bvg^+ phase promoters in the Bvg^- phase) or without $MgSO_4$ (to maintain RB50*flaA*FLP in the Bvg^+ phase) and were incubated an additional 48 h at 37°C. Delivery of pGFLIP to BPSM followed the same procedure except that incubations required four days at 37°C and plates were supplemented with 50 mM $MgSO_4$. pGFLIP without a promoter driving *flp* was used as a positive control for GFP production and as a negative control for *flp* activation. The delivery of all constructs to the *att*Tn7 site was confirmed by PCR using primers glmSF and gfpseqR (data not shown).

Evaluation of pGFLIP *in vitro*. We determined the functionality of pGFLIP using a plate-based assay in which RB50*cyaA*FLP and RB50*flaA*FLP were modulated between promoter-inactive and promoter-active conditions. BG agar containing 20 mM $MgSO_4$ was used to de-repress P_{flaA} and to deactivate P_{cyaA}, while BG agar without $MgSO_4$ was used to activate

P_{cyaA} and to repress P_{flaA} (7). The pGFLIP strains were grown under promoter-inactive conditions for 48 h at 37°C and were determined to be GFP^+ using a G:BOX Chemi imaging system with an UltraBright-LED Blue transilluminator and a SW06 short-pass filter (495–600 nm; Syngene, Frederick, MD). A single GFP^+ colony was resuspended in PBS and was diluted and plated on BG agar under promoter-active and promoter-inactive conditions. To demonstrate the loss of Km^r upon promoter activation, Km (100 μg/ml) was added to some plates. Plates were incubated for 48 h at 37°C, and white-light and fluorescent images were obtained using the G:BOX Chemi system.

For kinetic assays, strains containing pGFLIP were grown overnight with Km selection in SS under promoter-inactive conditions (20 mM $MgSO_4$ for RB50*cyaA*FLP, RB50$cyaA_{Bp}$FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP; no added $MgSO_4$ for RB50*flaA*FLP). Cells were washed twice in PBS with or without 5 mM $MgSO_4$ to prevent the premature activation of Bvg^+ phase and *flaA* promoters, respectively, and were added to fresh SS medium under promoter-active conditions (20 mM $MgSO_4$ for RB50*flaA*FLP; no added $MgSO_4$ for RB50*cyaA*FLP, RB50$cyaA_{Bp}$FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP) at the OD_{600} that was equivalent to 1×10^9 cfu/ml. Tubes were incubated on a roller at 37°C for 8 h. At each time point, an aliquot of cells was removed, diluted in PBS under promoter-inactive conditions, and was plated on BG agar under promoter-inactive conditions. Plates were incubated for 48 h at 37°C, and total cfu were enumerated. For kinetic assays with *B. pertussis*, cultures were inoculated at the cfu/ml corresponding to an OD_{600} of ~ 0.1, and cells were grown overnight in SS containing 50 mM $MgSO_4$, 100 μg/ml Km, and 1 mg/ml heptakis. Cells were washed in PBS containing 25 mM $MgSO_4$ to prevent premature *flp* activation. All other aspects of the assay were the same as for *B. bronchiseptica*, except that dilutions were plated on BG agar containing 50 mM $MgSO_4$ and

plates were incubated four days at 37°C. GFP fluorescence was quantified using the G:BOX Chemi imaging system as described. The percent resolution (the ratio of GFP^- colonies to the total number of colonies × 100%) was calculated for each strain at each time point.

Evaluation of pGFLIP in a murine model of infection.

Five- to seven-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were inoculated intranasally with 1×10^5 cfu of *B. bronchiseptica* pGFLIP strains in 50 µl of PBS. For infections with RB50*cyaA*FLP, RB50$cyaA_{Bp}$FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP, bacteria were grown overnight in SS medium containing Km (100 µg/ml) and 20 mM $MgSO_4$ to maintain cells in the Bvg^- phase. For infections with RB50*flaA*FLP, bacteria were grown overnight in SS media containing Km (100 µg/ml) to maintain cells in the Bvg^+ phase. Lungs were harvested from infected mice at 1 h and 30 h post-inoculation (p.i.). For RB50*flaA*FLP, right lungs were homogenized in 1 mL of PBS, diluted in PBS, and plated in duplicate on BG agar. For strains containing Bvg^+ phase promoters in pGFLIP, homogenization, dilution, and plating were carried out in the presence of 20 mM $MgSO_4$. Percent resolution was calculated for each strain at each time point. This study was done in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Our protocol was approved by the University of North Carolina IACUC (12-307.0). All animals were properly anesthetized for inoculations, monitored regularly, euthanized when moribund, and efforts were made to minimize suffering.

Statistical analyses.

Statistical analyses were performed using Prism 5 (GraphPad Software, Inc.). Statistical significance was determined using the unpaired Student's *t*-test or analysis of variance

(ANOVA). Images were formatted using Adobe Photoshop CS5 and figures were generated using Adobe Illustrator CS5 (Adobe Systems, Inc.).

Results

Design and Construction of pGFLIP.

To study transcription activation of genes both *in vitro* and *in vivo*, we engineered a reporter system, pGFLIP, that provides both fluorescent and selectable markers. We designed our system for simplicity and ease of use. Therefore, all of the components necessary for pGFLIP function are contained on a single plasmid. The region of pGFLIP delivered to the chromosome includes *gfp* and *nptII*, each under the control of a strong constitutive promoter and together flanked by *FRT* sequences (Fig. 1A). The Flp recombinase gene *flp* is present in the opposite orientation from *gfp* and *nptII* with an immediate 5′ MCS to facilitate the insertion of a promoter of interest. In the absence of promoter activity *flp* is not transcribed and cells remain GFP^+ and Km^r. When the promoter of interest is activated, *flp* is transcribed and the gene product mediates recombination between *FRT* sites, permanently excising *gfp* and *nptII*.

To test the system, we cloned promoter regions from *B. bronchiseptica* and *B. pertussis* into the MCS to generate the strains described in Table 1. The region of pGFLIP between Tn7L and Tn7R ends was delivered to the *att*Tn7 site 3′ to the *glmS* gene in *Bordetella* spp. as described in Materials and Methods. Transcription terminators are present near the 5′ end of the transposon to prevent transcription read-through from *glmS*. Delivery of promoterless pGFLIP to *B. bronchiseptica* RB50 and *B. pertussis* BPSM resulted in strains that were stably GFP^+ and Km^r. These strains did not lose GFP fluorescence or Km^r when passaged multiple times *in vitro* in the absence of selection (data not shown).

Functional evaluation of pGFLIP *in vitro*.

To evaluate the functionality of pGFLIP, we used two *B. bronchiseptica* promoters for which activity can readily be induced *in vitro*: P_{cyaA} and P_{flaA}. In *B. bronchiseptica*, the *flaA* promoter is highly transcribed in the Bvg^- phase, while the *cyaA* promoter is transcribed strongly in the Bvg^+ phase (9, 10, 15, 22, 32). Therefore, RB50*flaA*FLP was constructed under Bvg^+ phase conditions to prevent expression of *flaA*, while RB50*cyaA*FLP was maintained under Bvg^- phase conditions to prevent expression of *cyaA*. Km was added to the media during construction of these strains to select against any bacteria that had activated *flp*, thus ensuring that the population only contained GFP^+ and Km^r cells. To test these strains for pGFLIP functionality, we suspended a single GFP^+ colony of each strain in PBS and plated on BG, BG/Km, BG/$MgSO_4$, and BG/Km/$MgSO_4$ agar and observed colony formation and GFP fluorescence after two (*B. bronchiseptica*) or four (*B. pertussis*) days incubation at 37°C.

When plated under promoter-active conditions (20 mM $MgSO_4$ for RB50*flaA*FLP; no added $MgSO_4$ for RB50*cyaA*FLP) in the absence of Km, all RB50*flaA*FLP and RB50*cyaA*FLP colonies examined had lost fluorescence, and when tested subsequently for growth on agar containing Km, all colonies had lost Km^r (Fig. 2A and D). As expected, plating either strain under promoter-active conditions and in the presence of Km resulted in a lack of growth due to loss of *nptII* from the chromosome (Fig. 2E and H). Conversely, when plated under promoter-inactive conditions (no added $MgSO_4$ for RB50*flaA*FLP; 20 mM $MgSO_4$ for RB50*cyaA*FLP) *in the presence of Km,* all RB50*flaA*FLP and RB50*cyaA*FLP colonies maintained fluorescence (Fig. 2F and G). Likewise, under promoter-inactive conditions in the absence of Km, all RB50*flaA*FLP colonies maintained fluorescence (Fig. 2B), indicating that *flaA* is not expressed under these conditions. Unexpectedly, however, when RB50*cyaA*FLP was plated under

promoter-inactive conditions *in the absence of Km selection*, approximately 14% of the colonies had lost fluorescence at the time of imaging (Fig. 2C). These colonies did not grow when restreaked onto BG/Km/$MgSO_4$, indicating that cells in these colonies were not *gfp* mutants but had lost fluorescence and Km^r due to Flp-mediated recombination. These results suggest that the transcription activity of P_{cyaA} under Bvg^- phase conditions *in vitro* is close to the threshold level of *flp* transcription required for Flp-mediated recombination between *FRT* sites. These plate-based assays for fluorescence and Km^r demonstrated that pGFLIP is indeed functional and that both markers (*gfp* and *nptII*) can be used to document promoter activation under active and inactive conditions.

Kinetic analysis of *Bordetella* gene activation using pGFLIP.

We next assessed the ability of this system to monitor gene activation over time in bacteria grown in liquid culture. Strains containing pGFLIP plasmids with *cyaA*, *fhaB*, *ptxA*, and *flaA* promoters were grown overnight under promoter-inactive conditions, switched to promoter-active conditions, and the percent resolution (i.e., loss of GFP fluorescence and Km^r) was calculated over 8 h. For the first 4 h after switching from Bvg^+ phase to Bvg^- phase conditions, the percentage of RB50*flaA*FLP colonies that had lost fluorescence remained below 20% (Fig. 3A), indicating that less than 20% of the bacteria had reached or surpassed the threshold of *flp* expression to result in recombination during this time. Approximately 75% of cells had lost fluorescence at 6 h, and this proportion was maintained at 8 h (Fig. 3A). When grown under Bvg^- phase conditions for 24 h, greater than 85% of cells demonstrated a loss of fluorescence and Km^r (data not shown). The fact that resolution did not reach 100% suggests that the

maximum level of *flaA* expression under *in vitro* Bvg^- phase conditions (SS medium containing 20 mM $MgSO_4$) is just at or above the threshold of *flp* expression needed for Flp-mediated recombination between *FRT* sites. The relatively long amount of time required for the majority of RB50*flaA*FLP cells to cross the threshold of *flp* expression is in agreement with a previous study that examined the expression kinetics of the BvgAS-regulated operon *frlAB*, which encodes the *E. coli* FlhDC homologs FrlAB that are at the top of the flagella transcription cascade in *B. bronchiseptica* (16). Upon shifting from Bvg^+ phase to Bvg^- phase conditions, a *frlAB-lacZ* fusion strain exhibited a gradual increase in β-galactosidase activity over time, reaching approximately 40% of the maximum overnight expression level by 7.5 h (15).

In contrast, when RB50*fhaB*FLP were switched from Bvg^- phase to Bvg^+ phase conditions, the *fhaB* promoter was apparently activated as early as 1 h, at which time approximately 75% of cells had lost fluorescence (Fig. 3A). At 2 h and later time points, nearly 100% of RB50*fhaB*FLP cells had lost fluorescence, consistent with the classification of *fhaB* as a class 2 gene requiring a relatively low level of BvgA~P for activation. As a canonical class 1 (or "late") gene, *ptxA* had only been activated in approximately 8% of cells at 1 h (Fig. 3A). Approximately 20% of cells had activated *ptxA* by 4 h, increasing to 90% of cells by 8 h. Surprisingly, *cyaA*, which is also considered to be a class 1 gene, was activated in over 85% of cells at 1 h, and in greater than 95% of cells at 2 h and later time points (Fig. 3A). These data suggest that at early time points after switching to promoter-active conditions, the *cyaA* promoter is activated to a level sufficient to drive *flp*-mediated recombination. This result was unexpected based on *cyaA* transcription results in studies of *B. pertussis* (5, 11, 18, 23, 24).

At the initiation of each kinetic experiment, we observed a consistent background resolution (i.e., the percent resolution at 0 h) for each Bvg^+ phase promoter in pGFLIP.

RB50*cyaA*FLP displayed the highest background resolution at 8.9%, while the background resolution for *fhaB* and *ptxA* was significantly lower at 1.54% and 1.99%, respectively ($p < 0.01$). The background resolution for RB50*flaA*FLP was the lowest of the pGFLIP constructs at 0.23% (Fig. 3A). The fact that a portion of RB50*cyaA*FLP cells had lost fluorescence at 0 h suggests that low-level expression of *cyaA* might be occurring in a BvgAS-independent manner, which could interfere with measuring BvgAS-dependent P_{cyaA} activation over time. Therefore, to test the sensitivity of pGFLIP to background resolution we grew RB50*cyaA*FLP, RB50*fhaB*FLP, and RB50*ptxA*FLP strains overnight as in Fig. 3A, washed the cells to remove Km, and maintained the cultures under Bvg^- conditions in the absence of selection for 8 h. At 0, 1, 4, and 8 h, the percent resolution was determined for each strain. We hypothesized that if P_{cyaA} was indeed active under Bvg^- conditions we would observe a steady increase in resolution over time for RB50*cyaA*FLP but not for RB50*fhaB*FLP or RB50*ptxA*FLP. As expected, RB50*cyaA*FLP displayed the greatest background resolution at 11.9%, increasing to 18.1% at 8 h (Fig. 3B). Loss of fluorescence for RB50*fhaB*FLP and RB50*ptxA*FLP remained essentially unchanged over 8 h, averaging 0.22% and 5.4%, respectively (Fig. 3B). Although *cyaA* had been activated in approximately one-fifth of the population at 8 h, these data do not account for the >85% resolution observed for RB50*cyaA*FLP as early as 1 h following the switch to Bvg^+ phase conditions.

Neither the *B. bronchiseptica bvgAS* allele nor P_{cyaA} accounts for the unexpectedly early activation of *cyaA*.

As *cyaA* expression in RB50 was activated unexpectedly early compared to what has been observed for *B. pertussis* (5, 11, 18, 23, 24), we sought to determine whether differences in the *cyaA* promoter or in the *bvgAS* allele between *B. bronchiseptica* and *B. pertussis* accounted

for this difference. There are five single nucleotide changes and one nucleotide insertion in the sequence 5′ to *cyaA* in *B. bronchiseptica* RB50 compared to *B. pertussis* BPSM. We hypothesized that replacing the *cyaA* promoter of RB50 with that of BPSM would delay the activation of P_{cyaA} relative to P_{fhaB}, similar to what was observed by Veal-Carr et al. (24). We cloned the *cyaA* promoter from *B. pertussis* BPSM into pGFLIP and introduced this plasmid into RB50, generating strain RB50*cyaA*$_{Bp}$FLP. When evaluated in the kinetic assay, there was no difference in the rate of resolution between RB50*cyaA*FLP and RB50*cyaA*$_{Bp}$FLP, suggesting that differences in the *cyaA* promoter do not account for the rapid resolution observed in *B. bronchiseptica* (Fig. 4).

Some strains of *B. pertussis*, such as BP338 and BPSM, exhibit decreased sensitivity to chemical modulation compared to *B. bronchiseptica* RB50 due to amino acid differences in BvgS, which causes these strains to remain in the Bvg^+ phase at a lower concentration of modulator compared to RB50 (22). We hypothesized, therefore, that the *bvgAS* allele from BP338 would permit activation of *cyaA* more quickly and in a greater percentage of cells compared to the *B. bronchiseptica* RB50 *bvgAS* allele. To assess this, we utilized strain RB52, which contains the entire *bvgAS* locus and *bvgA* promoter from *B. pertussis* BP338 in place of *bvgAS* in RB50 (22). RB52 recapitulates the decreased sensitivity to modulation characteristic of both BP338 and BPSM, requiring ≥ 40 mM $MgSO_4$ to modulate to the Bvg^- phase, in contrast to RB50, which requires ≥ 10 mM $MgSO_4$ to fully modulate (15, 22). We introduced pGFLIP containing the RB50 *cyaA* promoter driving *flp* expression into RB52, producing strain RB52*cyaA*$_{Bb}$FLP. We evaluated the kinetics of *cyaA* activation in RB52*cyaA*$_{Bb}$FLP compared to RB50*cyaA*FLP and did not observe any difference. Likewise, when we evaluated an RB52 derivative containing the BPSM *cyaA* promoter driving *flp* expression (RB52*cyaA*$_{Bp}$FLP) in the

kinetic assay, there was no impact on *cyaA* activation compared to RB50*cyaA*FLP. Together, these data suggest that neither the *cyaA* promoter nor the *bvgAS* allele significantly affects the kinetics of *cyaA* activation in *B. bronchiseptica* as reported by the P_{cyaA}-*flp* promoter fusion in pGFLIP (Fig. 4).

Evaluation of *Bordetella* promoter activation *in vivo*.

Although the kinetics of Bvg^+ phase gene activation *in vivo* have been examined for *B. pertussis* (24), these experiments have not been done in *B. bronchiseptica* or in the context of a natural bacteria-host interaction. To address BvgAS-regulated gene activation in *B. bronchiseptica in vivo*, we infected BALB/c mice intranasally with 1×10^5 cfu of the RB50 pGFLIP strains shown in Fig. 3A, grown under promoter-inactive conditions (100 μg/ml Km, 20 mM $MgSO_4$). Mice were sacrificed and lungs were harvested at 1 h and 30 h p.i. Lungs were homogenized and dilutions were plated on BG agar containing 20 mM $MgSO_4$ to prevent further recombination. Total cfu were enumerated and the percentage of colonies that had lost fluorescence (% resolution) was calculated for each strain and time point. The percentage of RB50*fhaB*FLP that had lost fluorescence at 1 h was 95.3 ± 0.60%, while only 5.4 ± 0.69% of RB50*ptxA*FLP had lost fluorescence at this time (Fig. 5). Similar to the *in vitro* kinetic assay (Fig. 3A), the majority (85.3 ± 3.3%) of RB50*cyaA*FLP cells had lost fluorescence at 1 h. At 30 h p.i., essentially all cells had lost fluorescence, indicating that the environment in the mouse induces the expression of Bvg^+ phase genes to a level at or above that required to activate *flp* expression (Fig. 5). The fact that the pGFLIP system functions *in vivo* much the same as *in vitro* when switched from Bvg^- to Bvg^+ phase conditions suggests that the pattern of Bvg^+ phase gene

activation is similar between these two conditions and that the unexpectedly early expression from the *cyaA* promoter *in vitro* was not due to an artifact of that assay.

Although all evidence thus far suggests that the Bvg^+ phase is necessary and sufficient for *Bordetella* spp. to cause respiratory infection in rats and mice (6, 7, 22, 33), it is possible that rare *in vivo* environments exist that induce modulation to the Bvg^- phase. Therefore, using the activation of the *flaA* promoter as an indicator of the Bvg^- phase, we infected mice as described above and evaluated the loss of fluorescence at 1 h and 30 h p.i. We did not observe activation of *flaA* in any RB50*flaA*FLP cells at 1 h, 30 h, 3 d, 5d, or 7 d (Fig. 5). These data suggest that *B. bronchiseptica* do not enter the Bvg^- phase in the mouse during the time period that we examined.

Kinetic analysis of *Bordetella* gene activation in *B. pertussis* reveals delayed activation of *cyaA* compared to *B. bronchiseptica.*

Due to the observation that neither the *cyaA* promoter nor the *bvgAS* allele affects the kinetics of *cyaA* activation in *B. bronchiseptica*, we hypothesized that additional, species-specific factors account for the differences in the activation of *cyaA*. To evaluate this possibility, we delivered the same pGFLIP plasmids as those used in *B. bronchiseptica* to the chromosome of *B. pertussis* BPSM and evaluated loss of fluorescence in the kinetic assay. We reasoned that if the activation of *cyaA*, *fhaB*, and *ptxA* in *B. pertussis* resembled the pattern seen in *B. bronchiseptica*, then the discrepancy with was likely due to differences in resolvase/recombinase expression sensitivity between pGFLIP and RIVET (i.e., Flp recombinase is activated at a lower threshold of P_{cyaA} activity than TnpR, making *cyaA* appear to be activated sooner in the Flp-*FRT*

system). Alternatively, if activation of *cyaA*, *fhaB*, and *ptxA* was canonical as in Veal-Carr et al. (24), then the difference in *cyaA* activation would be attributed to species-specific gene regulation.

In contrast to what we observed in *B. bronchiseptica*, the proportion of BPSM*cyaA*FLP that had lost fluorescence at 1 h was less than 10%, essentially equal to the level of background resolution (Fig. 6). There was a steady loss of fluorescence in BPSM*cyaA*FLP beginning at 2 h post-switch and continuing until 6 h, at which time greater than 90% of colonies had lost fluorescence. The pattern of P_{cyaA} activation in *B. pertussis* matched that of P_{ptxA}, which displayed similar but somewhat earlier activation in *B. pertussis* compared to *B. bronchiseptica* (Fig. 6). Interestingly, the loss of fluorescence in BPSM*fhaB*FLP occurred more slowly compared to RB50*fhaB*FLP, with approximately 55% of cells having lost fluorescence at 1 h. At 2 h post-switch the proportion of GFP^- BPSM*fhaB*FLP equaled that of RB50*fhaB*FLP at 1 h, indicating that the activation of P_{fhaB} may be delayed in *B. pertussis* compared to *B. bronchiseptica*; however, the background resolution for BPSM*fhaB*FLP (29.5 ± 11.3%) was substantially higher compared to RB50*fhaB*FLP. At 1.99%, the background resolution for BPSM*ptxA*FLP was identical to that seen for RB50*ptxA*FLP, while the background for BPSM*cyaA*FLP (4.8 ± 2.2%) was not significantly different compared to RB50*cyaA*FLP (Fig. 6).

Discussion

Evaluating bacterial gene expression within the host is critical for understanding the complex host-pathogen interactions that result in disease, clearance, or asymptomatic colonization. One approach that has been developed to evaluate gene expression in the host is *in vivo* expression technology (IVET) (34). In IVET, promoter sequences are cloned 5′ to a gene that either confers resistance to an antibiotic or complements a specific auxotrophy (34-36). Under conditions in which the promoter is active, these genes are expressed and permit the survival of bacteria in hosts that have either been dosed with the relevant antibiotic (for antibiotic resistance-based selection) or naturally lack the ability to complement the auxotrophy (for auxotrophy-based selection) (34). A drawback of using IVET is that promoters that are activated transiently or at a low level may not be identified due to insufficient production of the missing survival factor (34). In RIVET, a modification of IVET, a promoter of interest drives the expression of a site-specific recombinase that irreversibly excises a genetic marker, often an antibiotic resistance gene (24, 34, 37). By selective plating it is possible to determine if the promoter of interest was active at some time during infection. Unlike IVET, RIVET permits the detection of transiently or weakly expressed genes because the recombinase-mediated loss of a marker need only occur once to document promoter activation (34).

pGFLIP is a pUC18-based plasmid that, while conceptually similar to RIVET, possesses several advantages over this well-characterized genetic tool. The Tn7 transposon system specifically delivers sequences to the *att*Tn7 site located 3′ to the highly conserved *glmS* gene, which provides pGFLIP with an especially broad host range that includes many Gram-negative bacteria (38). As a result of this specific recombination at the *att*Tn7 site, only one integration event is required for all components of pGFLIP to be delivered in single copy to the bacterial

chromosome. Single-copy delivery eliminates potential gene dosage issues inherent to multi-copy plasmid systems, and integration at the *att*Tn7 site does not disturb the native locus of the gene to be tested. Once delivered, a Tn7 transposon is stable in the absence of selection, unlike suicide plasmids that have been integrated into the chromosome via single-crossover homologous recombination, which can spontaneously resolve in the absence of selection. In contrast with other published systems, pGFLIP also possesses two markers—*gfp* and *nptII*—allowing either the loss of fluorescence or Km^r to indicate promoter activation.

In this study, we used pGFLIP to analyze the transcription activation of Bvg-regulated genes in both *B. bronchiseptica* RB50 and *B. pertussis* BPSM. Our results showed that, *in vitro*, *fhaB* and *ptxA* promoters were activated early and late, respectively, following a switch from Bvg^- to Bvg^+ phase conditions, which is in agreement with previous reports for both *Bordetella* species (15, 23, 24). However, the *cyaA* promoter was activated unexpectedly early in our assay; these results appear to stand in contradiction to the established view that *cyaA* is transcribed solely as a late gene. Using *B. bronchiseptica* RB50 in a BALB/c mouse model, we found that the pattern of gene activation for *cyaA*, *fhaB*, and *ptxA* was nearly identical to that observed *in vitro* at early time points. Veal-Carr et al. reported similar agreement between *in vitro* and *in vivo* gene activation patterns using RIVET (24), although in that study, maximal activation of *fhaB*, *cyaA*, and *prn* occurred over a much greater time scale (approximately 24 h to full activation), likely a result of differences in sensitivity between the TnpR-*res* and Flp-*FRT* systems. Our use of pGFLIP to evaluate gene activation in *B. bronchiseptica* is both the first kinetic analysis of P_{cyaA} and the first *in vivo* kinetic analysis of any promoter to be reported for this organism.

Although we expected P_{cyaA} to behave like a class 1 promoter in *B. bronchiseptica*, based on gene activation and expression data obtained by us and others for *B. pertussis* (5, 11, 18, 23,

24), our data nevertheless suggest that P_{cyaA} acts more like a class 2 promoter in this organism. However, it is not necessarily the case that *cyaA* reaches maximal expression at 1–2 h following a switch from Bvg^- phase to Bvg^+ phase; it is possible that *cyaA* activation occurs in a stepwise manner, i.e., P_{cyaA} may rapidly reach a level of expression necessary to activate *flp* transcription in our system, but may not reach maximal expression until much later. This scenario would account for the apparently rapid activation of P_{cyaA} without requiring a bacterium to be producing and secreting a significant amount of ACT immediately upon switching to non-modulating conditions. Evidence exists to support *cyaA* activation and ACT production, albeit at a reduced level, in the Bvg^i phase, as strains RB50i and RB53i are slightly hemolytic on BG agar (a consequence of the hemolysin activity of ACT), and RB53i produces measurable levels of *cyaA* transcript (9). We were able to determine that neither differences in the *cyaA* promoter nor in the *bvgAS* allele between the two species accounted for the difference in *cyaA* activation (see Fig. 4), suggesting that other, potentially Bvg-independent, factors may be influencing *cyaA* activation in *B. bronchiseptica* compared to *B. pertussis*.

In *B. pertussis* BPSM, P_{cyaA} demonstrated an activation pattern consistent with both indirect (RIVET) and direct (RNA hybridization) assessments, reinforcing the conclusion that *cyaA* activation is indeed different between RB50 and BPSM (23, 24). Given the differences in host range and the ability of *B. bronchiseptica* to survive outside of a host (7, 9, 10, 33), it is possible that the relatively early activation of *cyaA* in *B. bronchiseptica* is advantageous during the establishment of infection in a mammalian host or in transmission between hosts. Previous studies have shown that RB50 *cyaA* deletion mutants are more susceptible to clearance from the mouse respiratory tract, presumably as a result of neutrophil-mediated killing, and that ACT may interact with FHA (an "early" Bvg^+ gene) on the cell surface and modulate cytokine production

by the host (39, 40). All strains of *B. bronchiseptica* that have been tested show the same modulation characteristics *in vitro* (i.e., the same relatively low concentration of modulator is required for inducing the Bvg$^-$ phase), while *B. pertussis* isolates exhibit variable resistance to modulation at concentrations of modulator equal to or greater than for *B. bronchiseptica* (22). These observations suggest that early expression of *cyaA* may not be detrimental to human-adapted *B. pertussis* but may be necessary for *B. bronchiseptica* to establish an infection.

For both *B. bronchiseptica* and *B. pertussis* it has been shown that the Bvg$^+$ phase is necessary and sufficient for infection (6, 7, 9, 16, 22). Our results do not contradict these observations for *B. bronchiseptica*, but demonstrate that once inside the host, bacteria begin to transcribe Bvg$^+$ phase genes within 1 h p.i., with essentially every cell having activated *fhaB*, *cyaA*, and *ptxA* by 30 h p.i. The transition of *B. bronchiseptica* from Bvg$^-$ phase to Bvg$^+$ phase upon inoculation also indicates that the mouse lung is a Bvg$^+$ phase environment. We likewise provided evidence that *B. bronchiseptica* does not modulate to the Bvg$^-$ phase *in vivo*, supporting studies conducted using Bvg$^-$ phase-locked mutants that were unable to establish an infection and were quickly cleared from the respiratory tract and those using Bvg$^+$ phase-locked mutants that displayed no colonization defect (7, 8). Moreover, ectopic expression of flagellin in Bvg$^+$ phase *B. bronchiseptica* results in impaired persistence in the rat respiratory tract, possibly due to an immune response to this antigen (16). Therefore, modulation to the Bvg$^-$ phase likely does not occur *in vivo* as it would be disadvantageous to bacterial survival. We cannot rule out the possibility, however, that some Bvg$^+$ RB50*flaA*FLP bacteria did modulate to the Bvg$^-$ phase and were quickly eliminated by the immune response, or were present in a niche other than the lung (such as the trachea or nasopharnx) and were not represented in the lung homogenates that we analyzed. It is also conceivable that *B. bronchiseptica* is able to partially modulate, perhaps to

the Bvg^{i} phase, which would not be documented using RB50*flaA*FLP. We are currently constructing additional strains to test this possibility.

The pGFLIP plasmid has proven to be useful in understanding the regulation of gene activation in *Bordetella*. However, there remain caveats for the use of this system in other applications. As with other IVET and RIVET systems, pGFLIP requires that strains be manipulated under strict promoter-inactive conditions to prevent unwanted Flp-mediated resolution. This requirement poses problems for studying genes for which conditions of repression or lack of activation are unknown, genes that are essential for growth, and for genes that may not be fully transcriptionally inactive *in vitro*. And, like other systems that have been developed to monitor transcription, pGFLIP cannot provide information about post-transcriptional or post-translational regulation of target genes. Finally, as was shown by the variable background resolution of Bvg-regulated promoters in our study, pGFLIP appears to be sensitive to low-level promoter activation even under "repressed" conditions for certain genes. Lee et al. were able to modulate the sensitivity of RIVET by mutating the ribosome-binding site (RBS) of *tnpR*, effectively raising the threshold of promoter activity required for resolution (41). pGFLIP does not possess an RBS 5′ to *flp*, instead relying on the RBS of the promoter of interest, though it would be feasible to develop a modified pGFLIP plasmid that contains an RBS with reduced sensitivity to permit the study of genes that are not fully inactive or are constitutively active at a low level.

In this study we used pGFLIP to detect the activation of BvgAS-regulated genes in *Bordetella*, but there are additional uses for this system to measure transcription activation at the population or single cell level. Using pGFLIP, fluorescence can be used to quickly differentiate cells that have activated the promoter of interest from those that have not. Over time, stochastic

and/or transient promoter activation can result in sectoring of fluorescent colonies, permitting spatiotemporal observation of gene activation within a single colony (M. S. Byrd and E. Mason, unpublished observation). The addition of a second, non-*gfp* fluorescent label (e.g., a constitutively expressed fluorescent protein or a fluorescently labeled antibody) to cells already containing pGFLIP would allow cells that had activated the promoter of interest to be differentiated from cells that had not. Cells labeled using such an approach could be visualized using fluorescence-activated cell sorting or by microscopy. We are currently developing an improved pGFLIP plasmid that contains a constitutively expressed fluorescent protein gene not flanked by *FRT* sites that will provide a two-color to one-color readout upon activation of *flp* by the promoter of interest. The development of pGFLIP has resulted in a sensitive genetic tool that can be used to document promoter activation in a broad range of Gram-negative bacteria both *in vitro* and *in vivo*. Our use of pGFLIP to document the activation of Bvg-regulated promoters revealed unexpectedly early activation of *cyaA* in *B. bronchiseptica*, suggesting a possible explanation for the less restrictive host range of this organism compared to *B. pertussis*, and is the first *in vivo* use of a recombination-based genetic reporter of *B. bronchiseptica* gene activation.

Table 1. Strains and plasmids used in this study.

Strain or Plasmid	Description	Source or reference
Strains		
E. coli		
DH5α	Molecular cloning strain	(28)
RHO3	Conjugation strain; Km^s, Δ*asd*, Δ*aphA*	(29)
Bordetella		
RB50	Wild-type *B. bronchiseptica* strain; Sm^r	(7)
RB52	RB50 containing *bvgAS* from BP338; Sm^r	(22)
BPSM	Sm^r Tohama I derivative	(29, 42)
RB50FLP	RB50 with promoterless pGFLIP integrated at *att*Tn7	This study
RB50*cyaA*FLP	RB50 with *flp* recombinase driven by P_{cyaA} integrated at *att*Tn7	This study
RB50$cyaA_{Bp}$FLP	RB50 with *flp* recombinase driven by P_{cyaA} from Tohama I integrated at *att*Tn7	This study

RB52*cyaA*$_{Bb}$FLP	RB52 with *flp* recombinase driven by P$_{cyaA}$ from RB50 integrated at *att*Tn7	This study
RB52*cyaA*$_{Bp}$FLP	RB52 with *flp* recombinase driven by P$_{cyaA}$ from Tohama I integrated at *att*Tn7	This study
RB50*fhaB*FLP	RB50 with *flp* recombinase driven by P$_{fhaB}$ from RB50 integrated at *att*Tn7	This study
RB50*ptxA*FLP	RB50 with *flp* recombinase driven by P$_{ptxA}$ from Tohama I integrated at *att*Tn7	This study
RB50*flaA*FLP	RB50 with *flp* recombinase driven by P$_{flaA}$ integrated at *att*Tn7	This study
BPSMFLP	BPSM with promoterless pGFLIP integrated at *att*Tn7	This study
BPSM*cyaA*FLP	BPSM with *flp* recombinase driven by P$_{cyaA}$ from RB50 integrated at *att*Tn7	This study
BPSM*fhaB*FLP	BPSM with *flp* recombinase driven by P$_{fhaB}$ from RB50 integrated at *att*Tn7	This study
BPSM*ptxA*FLP	BPSM with *flp* recombinase driven by P$_{ptxA}$ integrated at *att*Tn7	This study
Plasmids		

pUC18T-mini-Tn7T-Km-*FRT*	Mobilizable transposition vector; Ap^r, Km^r	(30)
pFLPe4	Site-specific excision vector, source of Flp recombinase; Ap^r, Km^r	(30)
mini-Tn7-*kan-gfp*	Mobilizable transposition vector, source of *gfp* driven by P_{S12}; Km^r	(31)
pTNS3	Tn7 transposase expression vector containing *tnsABCD*; Ap^r	(30)
pGFLIP	pUC18-based vector with P_{S12}-*gfp* and *nptII* flanked by *FRT* sequences and *flp* 3′ to the MCS; Ap^r, Km^r	This study
pGFLIP-P_{cyaA}	pGFLIP with *flp* recombinase driven by the RB50 *cyaA* promoter, Ap^r, Km^{ra}	This study
pGFLIP-P_{cyaABp}	pGFLIP with *flp* recombinase driven by the BPSM *cyaA* promoter, Ap^r, Km^{ra}	This study
pGFLIP-P_{fhaB}	pGFLIP with *flp* recombinase driven by the RB50 *fhaB* promoter, Ap^r, Km^{ra}	This study
pGFLIP-P_{ptxA}	pGFLIP with *flp* recombinase driven by the BPSM *ptxA* promoter, Ap^r, Km^{ra}	This study
pGFLIP-P_{flaA}	pGFLIP with *flp* recombinase driven by the RB50 *flaA*	This

	promoter, Ap^r, Km^{ra}	study

[a]Km^r only under promoter-inactive conditions

Table 2. Primers used in this study.

Primer	Sequence (5′-3′)[a]	Description
FLP_UP	ATCTACGGTACCATGAGCCAGTTCGATATCC	Forward and reverse primers to amplify *flp* from pFLPe4
FLP_DN	AGGTCCAGGCCTCTATATGCGTCTATTTATG	
GFP_UP	ATATATGGATCCCAGCTGTTGACTCGCTTG	Forward and reverse primers to amplify *gfp* from mini-Tn7-*kan-gfp*
GFP_DN	ACCTGGGGATCCTTATTTGTATAGTTCATCC	
Tn7seqF	GAGCGCTTTTGAAGCTGATGTGCT	Forward sequencing primer annealing within Tn7R
gfpseqR	GATGACGGGAACTACAAGACACGT	Reverse sequencing primer annealing within *gfp*
kanseqR	ATCGCCTTCTATCGCCTTCTTGAC	Reverse sequencing

		primer annealing within *nptII*
GFLseq1	ACGGTGAAAACCTCTGACACATGC	Forward sequencing primers for sequencing pGFLIP by primer-walking
GFLseq2	CTGAAATCAGTCCAGTTATGCTGTG	
GFLseq3	AAATCCGCCGCTAGGAGCTT	
GFLseq4	GTCTGCCATGATGTATACATTGTGTG	
GFLseq5	GGGACAACTCCAGTGAAAAGTTCTTC	
GFLseq6	CTGATGCTCTTCGTCCAGATCATC	
GFLseq7	CCTGCGTGCAATCCATCTTGTTCA	
GFLseq8	CAGGGGATCTTGAAGTTCCTATTCCG	
GFLseq9	GCAACAATTCTGGAAGCCTCATT	
GFLseq10	TCTTTAGCGCAAGGGGTAGGATCG	
GFLseq11	TCCAATTGAGGAGTGGCAGCAT	
GFLseq12	TATCAGAGCTTATCGGCCAGCCT	
GFLseq13	ATAAAGATACCAGGCGTTTCCCCC	
GFLseq14	AAACAAACCACCGCTGGTAGC	
GFLseq15	CGCAGAAGTGGTCCTGCAACTTTA	

GFLseq16	CCGCGCCACATAGCAGAACTTTAA	
gflpmcsF	GTTGACAAAGGGAATCAGGGGATC	Forward sequencing primer for inserts in the MCS
gflpmcsR	GAACTGGGTGTAGCGTCGTAAGCT	Reverse sequencing primer for inserts in the MCS
glmSF	CAGCTGCTGTCGTACCACACGG	Forward primer to confirm Tn7 insertion at *glmS* by PCR
cyaA_F	ATTATAGAGCTCTGCGAGCAGATGCAC	Forward and reverse primers to amplify P_{cyaA} from RB50 and BPSM
cyaA_R	TATAATGGTACCGTGGATCTGTCGATAAGTAG	
ptxprF	AGCTTCGAGCTCCAAGATAATCGTCCTGCTC	Forward and reverse primers to amplify P_{ptxA} from BPSM
ptxprR	ATATATGGGCCCTCCCGTCTTCCCCTCT	
fhaprF2	AGGCCTGAGCTCGATAAGAAGAATATGCTT	Forward and reverse primers to amplify P_{fhaB} from RB50
fhaprR2	ATATTCGGTACCATTCCGACCAGCGAAGTG	

flaA_F	AATGAGCTCGCCGTGCTCAACGTCA	Forward and reverse primers to amplify P_{flaA} from RB50
flaA_R	ATTATAGGTACCAGGCTCCCAAGAGAGAAA	

Figure 1. Design and Mechanism of pGFLIP

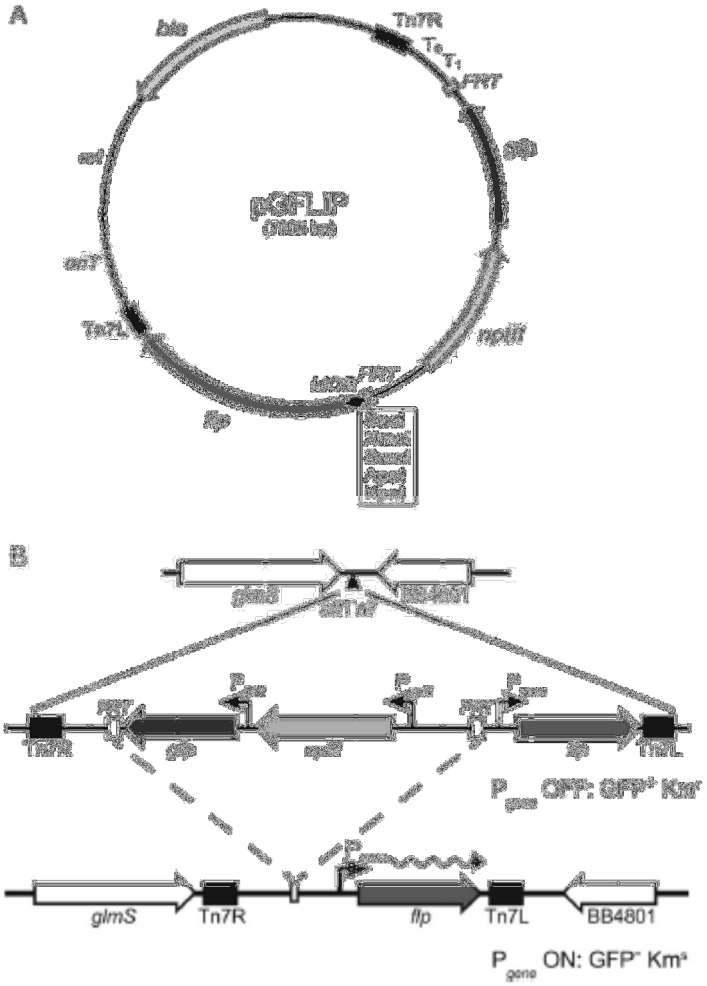

FIG 1 Design and mechanism of pGFLIP. (A) Diagram of pGFLIP. Tn*7R* and Tn*7L*, left and right ends of the Tn*7* transposon, respectively; T_0 and T_1, bacteriophage λ and *E. coli rrnB* transcriptional terminators, respectively; *FRT*, Flp recombinase target; *gfp*, green fluorescent protein gene; *nptII*, neomycin phosphotransferase gene; MCS, multiple cloning site with restriction sites indicated; *flp*, Flp recombinase gene; *oriT*, origin of conjugative transfer; *ori*, ColE1 origin of replication; and *bla*, β-lactamase gene. (B) Schematic illustration of Tn*7*-mediated delivery and Flp-mediated excision of pGFLIP in RB50. The region of pGFLIP flanked by Tn*7L* and Tn*7R* sequences is delivered to the *att*Tn*7* site located between *glmS* and BB4801. While the promoter driving expression of the gene of interest (P_{gene}) remains inactive, *gfp* and *nptII* are expressed constitutively, resulting in fluorescent and Km^r bacteria. When P_{gene} is activated, *flp* is expressed and Flp recombinase mediates site-specific recombination between *FRT* sites, permanently excising *gfp* and *nptII* and yielding bacteria that are nonfluorescent and sensitive to Km.

Figure 2. Plate-based validation of pGFLIP

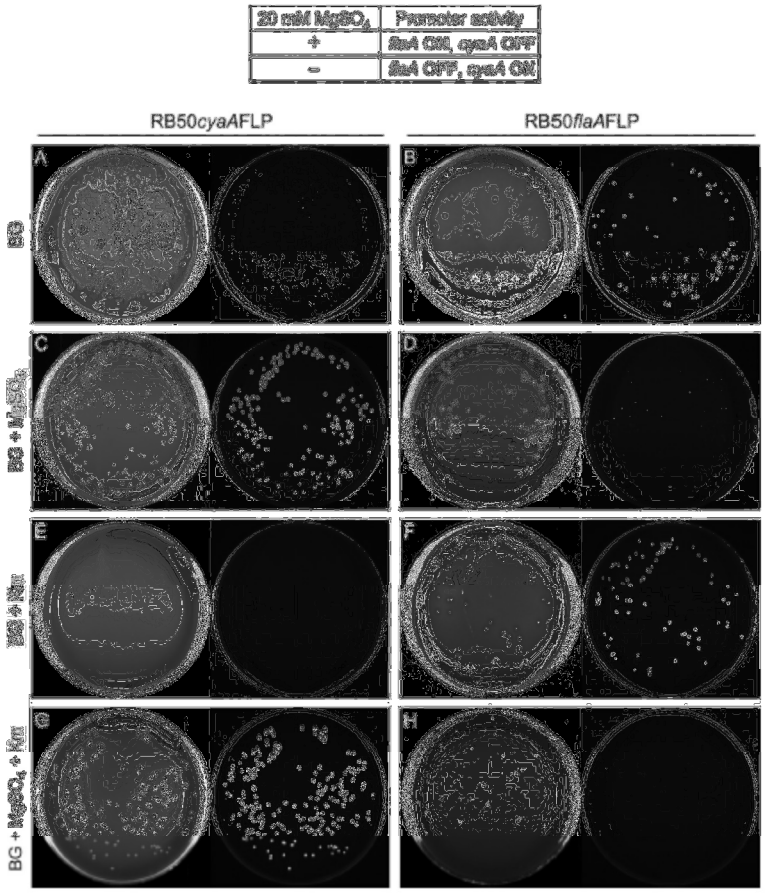

FIG 2 Plate-based validation of the pGFLIP reporter system using P_{cyaA} and P_{flaA}. Left column, images of RB50*cyaA*FLP plated under Bvg^+ phase (A) and Bvg^- (C) phase conditions (achieved by supplementing BG agar with 20 mM $MgSO_4$) in the presence (E) or absence (G) of 100 μg/ml Km. Right column, images of RB50*flaA*FLP plated under Bvg^+ phase (B) and Bvg^- (D) phase conditions in the presence (F) or absence (H) of 100 μg/ml Km. White light photographs are on the left and fluorescent images are on the right in each pair of images.

Figure 3. Kinetic analysis of *Bordetella* gene activation *in vitro*

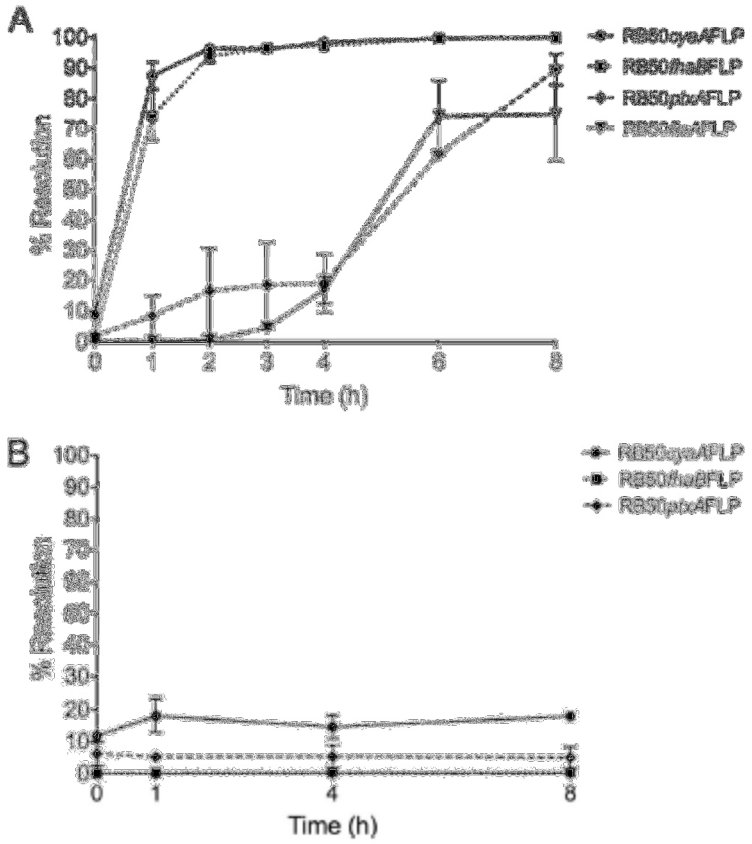

FIG 3 Kinetic analysis of *Bordetella* gene activation *in vitro*. *B. bronchiseptica* strains grown under promoter-off conditions and in the presence of 100 μg/ml Km were washed and placed in fresh SS medium under promoter-on conditions (A) or promoter-off conditions (B). Colonies arising from aliquots plated over 8 h were monitored for loss of fluorescence, and the percent resolution was calculated. Results are the means ± standard errors of the means (SEM) for experiments performed in duplicate or triplicate.

Figure 4. Effect of P_{cyaA} and *bvgAS* alleles on *cyaA* activation in *B. bronchiseptica*

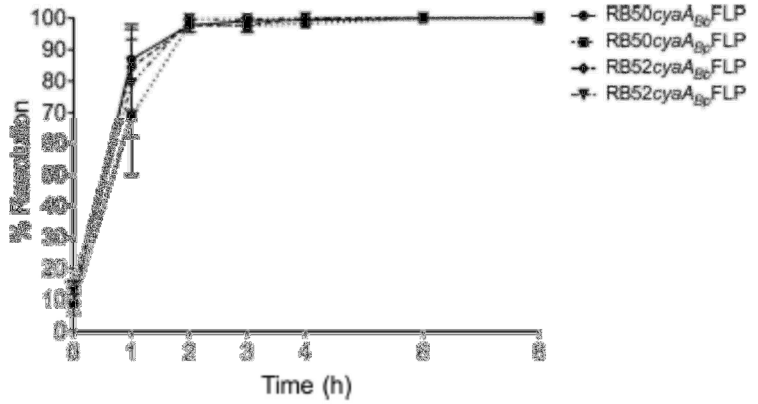

FIG 4 Effect of P_{cyaA} and *bvgAS* alleles on *cyaA* activation in *B. bronchiseptica*. RB50 and RB52, an RB50 derivative carrying the *bvgAS* allele from *B. pertussis* BP338 in place of the native *bvgAS* allele, each with pGFLIP containing RB50 and BPSM P_{cyaA}, were grown as shown in Fig. 3 and were switched to promoter-on conditions for 8 h. Loss of fluorescence was calculated for each strain as described in Materials and Methods. Results are the means ± SEM for experiments performed in duplicate.

Figure 5. Analysis of *Bordetella* gene activation *in vivo* using pGFLIP

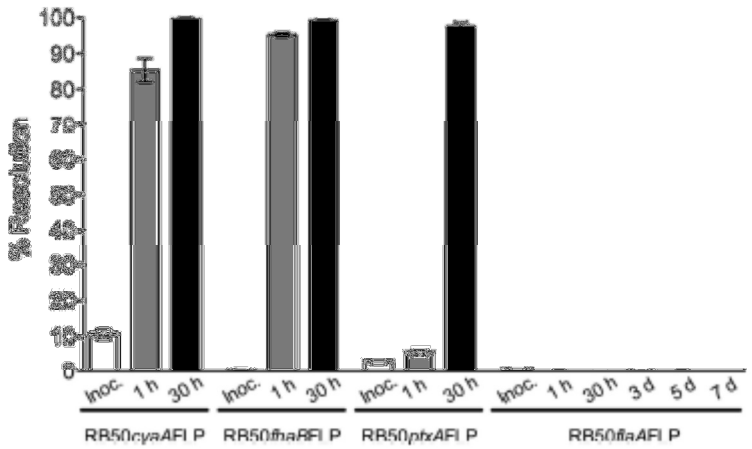

FIG 5 Analysis of *Bordetella* gene activation *in vivo* using pGFLIP. RB50*cya*AFLP, RB50*fhaB*FLP, RB50*ptx*AFLP, and RB50*fla*AFLP were grown as shown in Fig. 3, and 1×10^5 CFU was inoculated intranasally into mice in a total volume of 50 μl. Mice were sacrificed at 0, 1, and 30 h p.i., with additional time points at 3, 5, and 7 days for RB50*fla*AFLP, and lungs were homogenized and plated on BG agar supplemented with 20 mM $MgSO_4$ and Sm (for RB50*cya*AFLP, RB50*fhaB*FLP, and RB50*ptx*AFLP) or on BG agar supplemented only with Sm (for RB50*fla*AFLP). Loss of fluorescence was calculated for each strain as described in Materials and Methods. Results are the means ± SEM for experiments performed in duplicate with three mice per time point.

Figure 6. Kinetic analysis of *Bordetella* gene activation *in vitro* in *B. pertussis*

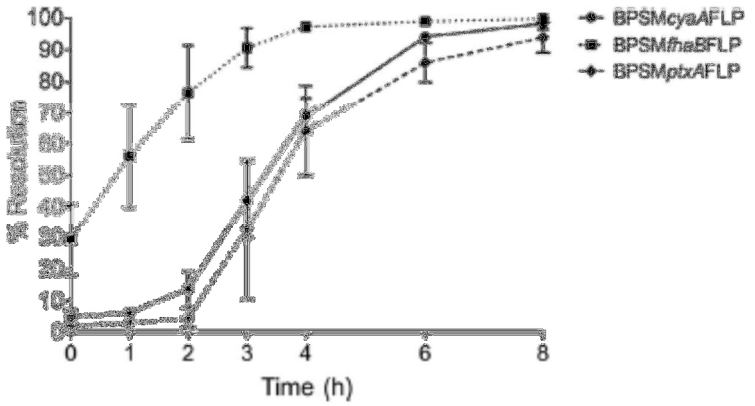

FIG 6 Kinetic analysis of *Bordetella* gene activation *in vitro* in *B. pertussis*. Strains grown under promoter-off conditions and in the presence of 100 μg/ml Km were washed and placed in fresh SS medium under promoter-on conditions. Colonies arising from aliquots plated over 8 h were monitored for loss of fluorescence, and the percent resolution was calculated. Results are the means ± SEM for experiments performed in triplicate.

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